# IDENTIFICATION AND EXPRESSION PROFILING OF *Banana bract mosaic virus* (BBrMV*)* RESPONSIVE microRNAs IN BANANA CULTIVAR NENDRAN (*Musa* AAB)

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*by*

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**(2018-11-100)**

**THESIS**

**Submitted in partial fulfilment of**

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**VELLAYANI, THIRUVANANTHAPURAM – 695 522**

**KERALA, INDIA**

**2020**

## DECLARATION

I, hereby declare that this thesis entitled **"Identification and expression profiling of *Banana bract mosaic virus* (BBrMV) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)"** is a bonafide record of the 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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## CERTIFICATE

Certified that this thesis, entitled **"Identification and expression profiling of *Banana bract mosaic virus* (BBrMV) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)"** is a record of research work done independently by **Ms. Athira Vijayan (2018-11-100)** 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.

Vellayani
Date: 7.1.2021

**Dr. K. B. Soni**
(Chairman, Advisory Committee)

# CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Athira Vijayan (2018-11-100)**, a candidate for the degree of **Master of Science in Agriculture** with major in Plant Biotechnology, agree that the thesis entitled **"Identification and expression profiling of *Banana bract mosaic virus* (BBrMV) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)"** may be submitted by **Ms. Athira Vijayan (2018-11-100)**, in partial fulfilment of the requirement for the degree.

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***Athira Vijayan***

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## LIST OF ABBREVIATIONS AND SYMBOLS USED

| | |
|---|---|
| A genome | *Musa acuminata* genome |
| A260 | Absorbance at 260 nm wave length |
| A280 | Absorbance at 280 nm wave length |
| amiRNA | Artificial microRNA |
| B genome | *Musa balbisiana* genome |
| BBrMV | *Banana bract mosaic virus* |
| BBTV | *Banana bunchy top virus* |
| BLAST | Basic local alignment search tool |
| bp | Base pairs |
| BSMysV | *Banana streak mysore virus* |
| BSV | *Banana streak virus* |
| CaMV | *Cauliflower mosaic virus* |
| cDNA | Complementary DNA |
| CMV | *Cucumber mosaic virus* |
| Cq/Ct | Cycle quantification/ cycle threshold |
| Cv | Cultivar |
| DEPC | Diethyl pyrocarbonate |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribo nucleotide triphosphates |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immune sorbent assay |
| EST | Expressed sequence tags |
| EtBr | Ethidium bromide |
| GA | Gibberellic acid |
| Gen Bank | Nucleotide sequence database |
| Gm | Gram |
| GSS | Genomic survey sequences |

| | |
|---|---|
| ha | Hectare |
| IC-PCR | Immuno capture PCR |
| ihpRNA | Intron – hairpin RNA |
| Kb | Kilo bases |
| LAF | Laminar air flow chamber |
| LiCl | Lithium chloride |
| *M. acuminata* | *Musa acuminate* |
| *M. balbisiana* | *Musa balbisiana* |
| Mbp | Mega base pairs |
| MFE | Minimal folding free energy |
| Min | Minute |
| miRNA | microRNA |
| ml | Mililitre |
| mM | Milimolar |
| mRNA | Messenger RNA |
| mt/yr | Million tonnes per year |
| NCBI | National center for biotechnology information |
| NGS | Next generation sequencing |
| Nm | Nanometer |
| nt | Nucleotides |
| PCR | Polymerase chain reactions |
| PLPs | Patatin like phospholipase protein |
| PMRD | Plant MicroRNA Database |
| PR proteins | Pathogenesis related proteins |
| Pre-miRNA | Precursor microRNA |
| Pri-miRNA | Primary microRNA |
| PTGS | Post transcriptional gene silencing |
| RDV | *Rice dwarf virus* |
| RFU | Raw fluorescence unit |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |

| | |
|---|---|
| RNAi | RNA interference |
| RSV | *Rice stripe virus* |
| RT-PCR | Reverse-transcriptase PCR |
| RT-qPCR | Reverse-transcription quantitative PCR |
| RTV | *Rice tungro virus* |
| S genome | *Musa schizocarpa* genome |
| SAR | Systemic acquired resistance |
| sec | Seconds |
| siRNA | Small interfering RNA |
| spp. | Species (plural) |
| ssRNA | Single sranded RNA |
| T genome | *Australimusa* species |
| TBE | Tris borate EDTA |
| Tm | Melting temperature |
| tRNA | Transfer RNA |
| TSA | Transcript sequence assemblies |
| U | Unit |
| V | Volt |
| v/v | Volume per volume |
| VIGS | Virus induced gene silencing |
| VSRs | Viral suppressors of RNA silencing |
| XTHs | Xyloglucan endotransglycosylase/hydrolases |
| µl | Microliter |
| % | Percentage |

## LIST OF APPENDICES

# *Introduction*

# 1. INTRODUCTION

Banana (*Musa* spp.) belonging to the family Musaceae is an important food crop all over the world. It is the fourth most important crop after the major cereal crops with a global production of 116 million tonnes in 2017-2019 (FAOSTAT, 2019). Also, bananas are the fifth largest agricultural commodity in world trade after cereal, sugar, coffee, and cocoa. It is a tropical fruit crop grown over 120 countries across the globe and an important food security crop. In some countries, bananas contribute 25 per cent of the daily calorie intake.

Major banana producing countries in the world include India (29 million tonnes per year), China (11mt/yr), Philippines (7.5mt/yr), Ecuador (~7mt/yr) and Brazil (7mt/yr). In India, bananas are grown under an area of 830.5 thousand ha and contribute 29.19 per cent of world production. The Indian subcontinent has about 50 different cultivars of banana. The commercially cultivated ones include Robusta, Monthan, Poovan, Dwarf Cavendish, Nendran, Red banana, Basrai, Ardhapuri, Nyali, Safed Velchi Rasthali, Karpuravalli etc. In India, Tamilnadu, Gujarat, Maharashtra, Andhra Pradesh and Karnataka are the top banana producing states. In Kerala, the major cultivated varieties include Nendran (Plantain), Palayankodan (Poovan), Rasthali, Monthan, Red banana, and Robusta.

Although India is the leading producer of bananas in the world, the production is highly affected by various biotic and abiotic stress factors. The major abiotic factors include extreme temperature, salinity, water stress, strong winds, storms etc. and the biotic stress factors include various pests and diseases which hampers banana production.

Diseases in banana are mainly caused by fungi and viruses. Viral diseases that contribute to considerable yield loss include Bract mosaic (*Banana bract mosaic virus* BBrMV), Bunchy top (*Banana bunchy top virus*), *Banana Streak Virus* (BSV), Infectious chlorosis (*Cucumber mosaic virus*, CMV) etc.

Viral diseases are comparatively difficult to manage compared to others. Banana bract mosaic is an important viral disease of banana which is caused by a ssRNA virus belonging to the family *Potyviridae* causing a yield loss of up to 40 per

cent (Rodoni *et al*., 1997, Selvarajan and Jeyabaskaran, 2006). In Kerala and Tamilnadu, the yield loss is 30-70 per cent in Nendran and Robusta (Cherian *et al*., 2002; Selvarajan *et al*., 2017). The virus is transmitted mainly by aphids belonging to three different sp. *Pentalonia nigronervosa*, *Aphis gossypii*, and *Rhopalosiphum maidis* and also by infected planting material. The prominent symptoms include dark reddish-brown spindle shaped discontinuous streaks on the bract, pseudostem, midrib, peduncle and fingers, and severe infection produces malformed bunches and underdeveloped fingers (Selvarajan *et al*., 2020).

Most of the commercially cultivated varieties are highly susceptible to bract mosaic virus infection and there is no source of natural resistance, hence conventional breeding program is not efficient to develop any resistant varieties. Since there are no effective management strategies, different biotechnological approaches are being tried. The role of small RNAs responsive to biotic and abiotic stress in plants has gained importance in recent years. The development of RNA interference (RNAi) mechanism by small interfering RNAs (siRNAs) or microRNA (miRNAs) provides resistance towards various biotic and abiotic stresses in plants and animals. In recent years microRNA mediated gene silencing approaches have been gaining popularity in imparting stress response in plants.

MicroRNAs (miRNAs) are defined as 21-25 nucleotide single-stranded RNAs which are produced from hairpin shaped precursors (Ghag *et al*., 2015). They are a class of endogenous non- coding short RNAs which are abundantly found in mammalian cell types and are regulators of gene expression in eukaryotes. They involve in RNA silencing and post transcriptional regulation of gene expression. Different studies show the presence of certain miRNAs during biotic and abiotic stresses in plants which are differentially expressed under these conditions.

In banana, limited studies have been conducted to understand the role of miRNAs in biotic stress and host-pathogen interaction. In a study conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2016-18, 52 mature miRNAs have been predicted using computational tools in the banana genome and their possible target genes were identified (Mathew, 2018). The objective of the present study is to validate computationally predicted selected microRNAs

(miRNAs) in banana cultivar Nendran (*Musa* AAB) and to study their expression in *Banana bract mosaic virus* (BBrMV) infection.

# *Review of Literature*

# 2. REVIEW OF LITERATURE

## 2.1 BANANA: ORIGIN AND GENOME CONSTITUTION

Banana (*Musa* spp.) is a monocotyledonous herb that belongs to the order *Zingiberales* and family Musaceae. They are originally evolved in Southeast Asia and Africa is the secondary centre of diversity. The important cultivars include *Musa acuminata* (A genome, $2n = 2x = 22$; *n* represents the haploid chromosome number) and *Musa balbisiana* (B genome, $2n = 2x = 22$), *Musa schizocarpa* (S genome, $2n = 2x = 22$) and the *Australimusa* species (T genome, $2n = 2x = 20$). The modern cultivars are either inter or intra specific hybrids of *M. acuminata* and *M. balbisiana*. There are diploid (AA, BB and AB) triploid (AAA, AAB and ABB) and synthetic tetraploid (AAAA, AAAB, AABB and ABBB) varieties of which triploids form the major cultivated group. Most of the edible cultivars are allopolyploid triploids with a genome constitution of AAA (dessert banana), AAB (plantains) and ABB (cooking bananas). It is the most important crop of tropical and subtropical regions and a major staple food for millions of people.

In the world, banana is the second largest produced fruit after citrus (16 per cent of total production). India is the largest producer contributing 27 per cent of world production. In India, Tamilnadu, Gujarat and Maharashtra are the leading producers (Mohapatra *et al*., 2010). The common varieties include Grand Naine, Dwarf Cavendish, Robusta, Red Banana, Nendran, Poovan, Monthan etc. The species include both dessert and cooking varieties. The common dessert varieties are Cavendish, Robusta, Nendran, Rasthali etc. and the culinary varieties include Monthan, Nendran etc. Bananas are one of the first crops to be domesticated. They are propagated by vegetative means by using suckers.

The genome sequencing of DH-Pahang, a doubled-haploid *M. acuminata* genotype (2n = 22) has been done and the total length is 472.2 Mb, which represented 90 per cent of the estimated DH-Pahang genome size (D'Hont *et al*., 2012). Also, the sequence of wild *M. balbisiana* diploid variety 'Pisang Klutuk Wulung' ('PKW', B-genome) is available which has a genome size of 402.5 Mbp (Davey *et al*., 2013). Both

the sequences are available in banana genome hub (https://banana-genome-hub.southgreen.fr/organism/Musa/acuminata and http://banana-genome.cirad.fr/content/musa-balbisiana-pisang-klutuk--wulung). Banana genome hub is a next-generation information system for *Musa* genomics (http://banana-genome.cirad.fr/). It is based on Tripal—a construction tool kit for online genome databases and is supported by the South Green Bioinformatics Platform (http://southgreen. cirad.fr/) (Droc *et al.*, 2013).

The cultivar used in this study is Nendran which is a dual -purpose variety, i.e. used both for dessert and culinary purposes. It is the most common variety in Kerala. The fruits are long with a distinct neck, thick green skin turning buff yellow on ripening, and are starchy.

## 2.2 DISEASES IN BANANA

The growth, reproduction and yield of bananas are highly affected by many abiotic and biotic stress factors of which the major contributors are diseases. The diseases in bananas are mainly caused by fungus and viruses, also some bacteria and nematode causing diseases are also known. The diseases which cause considerable damage include Panama disease or Fusarium wilt, Sigatoka leaf streak, Bunchy top viral disease and Bract mosaic viral disease.

### 2.2.1 Viral Diseases in Banana

The majority of banana cultivated worldwide are highly susceptible to viral diseases. Viral diseases spread easily and are very difficult to manage compared to bacterial or fungal diseases. There are four different viral diseases infecting banana plants all over the world, they are Banana bunchy top disease (BBTD) caused by *Banana bunchy top virus* (BBTV), bract mosaic disease (BBrMD) caused by *Banana bract mosaic virus* (BBrMV), banana streak disease (BSD) caused by different species of *Banana streak virus* (BSV), and banana mosaic or infectious chlorosis caused by *Cucumber mosaic virus* (CMV). Viral diseases are transmitted by vectors, mainly

insects and also through infected planting material. As banana is a vegetatively propagated crop, the virus spreads easily.

#### *2.2.1.1 BBrMV*

*Banana bract mosaic virus* was first reported in 1979 in the Philippines at Davao on the island of Mindanao (Thomas and Magnaye, 1996). It is widespread throughout the Philippines, India, Srilanka, Vietnam and Western Samoa, Costa Rica, Uganda, Ghana, Zanzibar and South Africa (Rodoni *et al*., 1999). In India, it was first reported in 1966 in Kerala in cv. Nendran, it is common in the southern states of Tamil Nadu and Kerala where it is commonly referred to as Kookan. In Kerala, BBrMV causes 52.5 per cent yield loss in cv. Nendran (Cherian *et al.,* 2002).

##### *2.2.1.1.1 Symptomology*

The characteristic symptom of the infection includes spindle-shaped pinkish to reddish streaks on the pseudostem, midrib and peduncle, similar type lesions are also observed on bract and fingers. In some varieties like Nendran, a change in leaf orientation is observed which is referred to as 'Travelers Palm' symptom. In severe cases, yellowish streaks opposite to the midrib towards the edge of the leaf is observed. The plants have stunted growth and the fruits are malformed (Selvarajan and Jeyabaskaran, 2006).

##### *2.2.1.1.2 Pathogen*

The disease is caused by the virus *Banana bract mosaic virus* (BBrMV) which is a ssRNA virus belonging to the family *Potyviridae* and has a size of 10kb. Studies show that the virus titre is more in bract and midrib than in leaf sheath (Selvarajan *et al.,* 2009).

#### *2.2.1.1.3 Vector*

The virus is transmitted by banana aphids. There are mainly three types of banana aphids: *Rhopalosiphum maidis, Aphis gossypii* and *Pentalonia nigronervosa*. These aphids are common in semiarid tropical conditions. The cowpea aphid *Aphis craccivora* is also known to transmit the virus (Kiranmai *et al.,* 2005).

#### *2.2.1.1.4 Detection of Viruses*

Reverse transcriptase PCR (RT-PCR) is a sensitive and efficient method for the detection of viruses than ELISA (Enzyme Linked Immuno Sorbent Assay) and IC-PCR (Immunocapture-PCR) (Rodoni *et al*., 1999). Amplification of viral RNA from crude plant sap is done through RT-PCR (Selvarajan *et al*., 2009).

### *2.2.1.2 Virus Vector Interaction*

Plant viruses are dependent on vectors for their transmission. Mainly sap-feeding insects are vectors of viruses. There are specific interactions between viruses and their vectors. Three different interactions result in three different modes of vector transmission they are non-persistent, semi-persistent and persistent. In the non-persistent mode of transmission, the viruses are retained in the insect stylet for a few seconds eg: *cauliflower mosaic virus* (CaMV). In semi-persistent mode the virus is retained for few minutes to hours by the vector as they bind to the chitin lining the gut eg: *Rice TungroVirus* (RTV) and in persistent mode the virus is retained for several hours and are characterized by invading the salivary glands eg: *Rice dwarf virus* (RDV) (Dietzgen *et al*., 2016). *Banana bract mosaic virus* infection is transmitted in a non-persistent manner by different plant aphids (Ng and Falk, 2006).

### *2.2.1.3 Viral Disease Management*

The growth and development of plants are highly affected by various stress factors both biotic and abiotic. Biotic factors like pests and pathogens contribute a

major share. Among these viral pathogens are a major threat and the economic loss caused by these viral pathogens is more compared to others (Simon-Mateo and Garcia, 2011). This is mainly because the viral pathogens are difficult to manage compared to other pathogens. Hence, they are extensively studied and almost 13000 plant viruses are identified according to the ninth international committee report on the taxonomy of viruses (King *et al*., 2012).

Various strategies are adopted for developing resistance against viral diseases which includes conventional breeding techniques that utilizes the natural source of resistance and cross-protection, and nonconventional techniques like genetic engineering to develop transgenic plants. Thorough knowledge about the pathogenic strains is essential for developing management strategies.

The entry of viruses into the plant cell is done by interrupting the plasma membrane (Rodrigues *et al.,* 2009). These viral particles then replicate inside the cell and spread throughout the host with the help of different connective tissues (Taliansky *et al*., 2008).

The common methods adopted to eliminate virus infection includes: - Use of virus-free planting materials (seeds, suckers etc.), adopting meristem culture techniques, control of vectors and breeding for resistance and cross-protection (Stevens, 1983).

## 2.3 APPROACHES FOR DEVELOPING VIRUS RESISTANCE

### 2.3.1 Cross Protection

Cross protection (mild strain cross protection) is a phenomenon by which the plant is infected with a mild strain of virus that prevents or delays infection by a severe strain (Ziebell and Carr, 2010). Cross protected plants show improved resistance to secondary viral infections (Zhang and Qu, 2016).

Other than these conventional approaches, biotechnology plays an important role in suppressing the viral diseases in plants. Since most of the traditional plant breeding methods are time-consuming and there is only limited availability of genetic

resources, biotechnological approaches are gaining importance (Prins *et al.,* 2008). This includes various methods like recombinant DNA technology.

### 2.3.2 Recombinant DNA Technology

Recombinant DNA Technology is defined by the Encyclopedia Britannica as "the joining together of DNA molecules from different organisms and inserting it into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture and industry (Nambisan, 2017). Here the plants are engineered for resistance against diseases. In the case of virus resistance, it is achieved by the transfer and expression of the coat protein genes of the virus to the plant which suppresses the infection.

#### *2.3.2.1 Virus Resistant Transgenic Plants*

It is one of the very successful approaches in developing virus resistance in plants. The resistance genes are sourced from the plants, usually their wild relatives and are used for imparting resistance in commercial varieties. The transgenes which are commonly used to confer resistance include pathogen derived transgenes (coat protein mediated resistance, non-coat protein mediated resistance, replicase mediated resistance), plant derived transgenes (genes encoding pathogenesis related proteins, plant genes encoding anti-viral proteins, and natural host resistance genes) (Kavanagh and Spillane, 1995).

For developing transgenic plants, virus resistance genes (*R* genes) are taken from the wild relatives of crop plants and they are tagged using molecular markers like RFLP, RAPD, AFLP etc. The first successful evidence of the use of transgenic plants against viral infection comes from Hawaii against *Papaya Ring Spot Virus* (PRSV). The coat protein gene resistant to the PRSV was transferred to plants using a plasmid containing neomycin phosphotranferase II (*npt* II gene) (Azad *et al*., 2013). The *Bdv* 1 allele of wheat is linked to the *R* gene *Lr* 34 and *Yr* 18 which is resistant to *Barley yellow dwarf virus* (BYDV) (Kumar *et al*., 2014).

## 2.4 RNA SILENCING MECHANISMS

A recent approach in developing resistance against pathogens in plants involves RNA mediated silencing strategies. It was initially considered as a defense mechanism of plants against RNA virus infection (Waterhouse *et al*., 2001; Baulcombe 2004). The discovery of RNA interference (RNAi) by Andrew Fire and Craig C. Mello (1998) in the nematode *Caenorhabditis elegans* was a major breakthrough in RNA mediated silencing mechanisms, for which they shared the 2006 Nobel Prize for physiology or medicine (Waterhouse *et al*., 2001).

RNA interference or RNAi is a biological process by which RNA molecules inhibit the gene expression or translation by the destruction of their mRNA intermediaries. The silencing can occur both during transcription and translation. The gene silencing, also known as gene knockdown, is done by two small RNAs, they are siRNAs and miRNAs. The exogenous RNAi pathway works through siRNAs, whereas the endogenous RNAi pathway works through miRNAs (Novina and Sharp, 2004). RNAi is also called as quelling in fungi and post transcriptional gene silencing (PTGS) in plants (Hamilton *et al*., 2002).

### 2.4.1 Post Transcriptional Gene Silencing

Post transcriptional gene silencing (PTGS) was first reported in petunia hybrid expressing the chalcone synthase transgene by Napoli *et al*. (1990). It is a basic mechanism that regulates the level of gene expression through small RNAs which are complementary to the target mRNA sequence of a particular gene (Bivalkar-Mehla *et al*., 2011). In PTGS the RNA-dependent RNA polymerase helps in converting target ssRNA into dsRNA which are then processed by DICER to generate siRNAs (Wesley *et al*., 2004). Correlation of elevated siRNA levels with the reduced viral titre during infection suggests that PTGS acts as RNA mediated defense response against plant viral infections (Filipowicz, *et al*., 2005).

There are mainly three RNA silencing pathways in plants which include miRNA mediated gene silencing, siRNA directed RNA degradation pathway, and siRNA directed DNA methylation. The miRNA mediated gene silencing pathway is triggered

by the entry of a dsRNA into the cell which is made into small fragments. These fragments can either be siRNAs or miRNAs (Hutvagner and Zamore, 2002).

## 2.5 miRNA

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that functions in RNA silencing and post transcriptional regulation of gene expression which is achieved either by targeting mRNA for cleavage or inhibiting translation. They are usually 21-25 nucleotide ssRNAs which are produced from hairpin shaped precursors. Plant miRNAs are known to be involved in various functions such as biotic and abiotic stress responses as well as morphological and developmental functions (Kidner and Martienssen, 2005). Also, miRNAs have role in hormonal pathways in plants. A number of genes in auxin signalling pathways are targets for miRNAs eg: *TIR*1 auxin receptor is a predicted target of miR393 (Chen, 2005).

In plants, miRNA formation occurs in the nucleus through a two- step process involving the processing of pri-miRNA into precursor miRNA involving a single RNase III enzyme DICER like I (DCL I). The pri-miRNA is formed by the transcription of MIR gene by RNA polymerase II enzyme. The precursor miRNAs are then moved to the cytoplasm by a plant orthologue of exportin V protein called HASTY (Park *et al*., 2005). In cytoplasm, the pre-miRNAs are converted into miRNAs and they integrate with the multi subunit protein complex the RNA induced silencing complex (RISC), which has argonaute proteins. The argonaute protein has an RNA guide strand which leads the miRNA to bind to mRNA. One strand helps in binding and the other act as a passenger strand. When the double strand is degraded by helicase, the guide strand helps in gene silencing.

In animals, miRNAs are formed from primary miRNAs (pri-miRNAs) in two stages involving two RNase III type proteins Drosha in the nucleus and Dicer in the cytoplasm (Wahid *et al.,* 2010). The pri-miRNA is converted into pre-miRNAs by Drosha a class 2 RNase III enzyme in the nucleus. The pre-miRNAs are transported into cytoplasm by exportin 5 (EXP-5). In cytoplasm, the pre-miRNAs are converted into mature miRNAs by Dicer, a RNase III type protein and are loaded on to argonaute proteins in RISC.

### 2.5.1 Plant miRNA Vs Animal miRNA

Plant miRNAs are generally conserved in the evolutionary process ranging from mosses to flowering plants. Unlike animal miRNAs which are arranged in clusters, most of the plant miRNAs are annotated to intergenic regions. The precursors of plant miRNAs are diverse and have longer stem-loops than animal pri-miRNA. Most of the plant miRNAs have 5'cap and poly-adenylated tails (Wahid *et al*., 2010).

### 2.5.2 Function of miRNAs in Plants

In plants, miRNAs perform crucial roles in various developmental stages. Plant miRNAs are highly complementary to conserved target mRNAs which allows fast identification of plant miRNA targets using bioinformatic tools. miRNA target genes mainly belong to a class of transcription factors and F box proteins which constitute plant developmental and regulatory networks (Nazarov *et al*., 2013). miRNAs are involved in diverse functions in plants like cell proliferation, cell death, fat metabolism, cell signalling, hormonal activities, leaf and flower development, pathogen interactions, response to abiotic stress factors etc (Ghag *et al.*, 2015).

### 2.5.3 Role of miRNA in Abiotic Stress in Plants

As the climatic conditions are changing due to global warming and greenhouse effects, the plants are continuously being exposed to varying climatic conditions. Thus, the plants are regularly exposed to various forms of abiotic stresses like high temperature, salinity, water deficit, alternating light conditions etc. Studies show that microRNAs have specific roles in coping with these stresses. Jones-Rhoades and Bartel (2004) showed that in Arabidopsis the expression of miR395 was increased upon sulfate starvation. miR393 was upregulated during cold, dehydration and salinity in Arabidopsis (Khraiwesh *et al*., 2012). Drought stress inhibited miR169 in Arabidopsis (Li *et al*., 2008) while it was induced by salinity (Xu *et al*., 2014; Zhang, 2015).

### 2.5.4 Role of miRNA in Biotic Stress in Plants

MicroRNAs have a significant role in biotic stress response in plants. In plants, miRNAs perform post transcriptional gene silencing to deal with the stress. Due to the continuous and fast evolution of biotic agents, this area is a major concern of research (Chauhan *et al.*, 2017). The levels of miRNAs are either up-regulated or down-regulated during biotic stress. In Arabidopsis, miR398 was down regulated in response to *Pseudomonas syringe* infection (Jagadeeswaran *et al*., 2009). Navarro *et al*. (2006) reported that the overexpression of miR393 in Arabidopsis inhibited bacterial growth. A study by Liu *et al*. (2020) showed that miR168 and miR398 were up-regulated in a variety of plants during infection with different viruses.

### 2.5.5 Role Of miRNAs During Virus Infection

The plant viruses enter the plants and cause infection by utilizing the biosynthetic pathway of the host cells. In plants, during virus infection, the response takes place either by directly attacking the viral genes by endogenous miRNA or by cleavage of the miRNA responsible for virus multiplication.

Du *et al*. (2011) reported that two distinct viruses, *Rice dwarf virus* (RDV) and Rice stripe virus (RSV) had a distinct impact on small RNA metabolism in rice. During RDV infection miR167a, miR171 and miR1863 were down regulated and only miR393 was induced. In RSV infected rice plant miR1425, miR160 and miR171 were up-regulated.

In sugarcane, during *Sugarcane streak mosaic virus* infection the infected plants only showed the presence of miR16 and further analysis showed that miR16 is specific to SCSMV and had 19 different target genes in sugarcane. miR16 helps to create a favourable environment for the virus life cycle and helps in infection thus, targeting miR16 helps in developing resistance against SCSMV infection (Viswanathan *et al.*, 2014).

In potato, expression of miRNAs belonging to miR482, miR6023, miR6024 and miR6027 families were found increased during *Potato virus Y* infection (Kriznik *et al*., 2017). In soybean during *Mung bean yellow mosaic India virus* (MYMIV) infection

they showed an increased production of AGO due to gma-miR5787 which resulted in reduced symptoms (Ramesh *et al*., 2017).

In maize, 154 known miRNAs and 213 novel miRNAs were profiled by Zihao *et al*. (2018) and most of them were differentially expressed after *Sugarcane mosaic virus* infection. The miRNAs, miR168 and miR528 were upregulated and miR159, miR397, miR827 were down regulated. The target of miR168 is AGO 1 which is an important component in RNA silencing and has an antiviral response. The induction of miR168 negatively regulated AGO 1 thus imparting host defense (Xia *et al*., 2018).

Subramanian (2019) reported that during BBrMV infection in Nendran there was an upregulation of miR3900-5p, miR2172-5p, miR6928-5p and miR971-5p (1.2, 2.0, 1.27, 2.0 fold respectively) 24 h after infection and showed variation in expression during different time intervals.

Sweet orange (*Citrus sinensis*) when infected with two different isolates of *Citrus psorosis virus* (CPsV) showed an altered accumulation of a set of endogenous miRNAs. Analysis of the target expressions showed that there was an increase in the level of protein expression with the severity of the symptoms (Marmisolle *et al.*, 2020).

Sankaranarayanan *et al*. (2020) reported that among the predicted BBrMV miRNAs, miRNA2 is conserved and is present in all infected isolates and was absent in uninfected samples also it has multiple targets.

In barley, during *Barley yellow dwarf virus* (BYDV) infection there was a conserved upregulation of miRNA 10778 regardless of barley genotype. The novel miR 1724 whose target is a receptor like protein kinase also showed a change in its expression during infection (Jarosova *et al.*, 2020).

Cui *et al*. (2020) reported that in brassica during *Turnip mosaic virus* (TuMV) infection miR1885 level was lowered and miR1885 mediated the turnover of R genes, also they promoted plant growth through direct and *trans* acting RNA silencing. They suggested that miR1885 and its target can be used for breeding brassica for high yield and disease resistance.

Lang *et al*. (2011) and Kriznik *et al.* (2020) reported that a GAMYB- targeting miR159 (MYB transcription factor controlling gibberellin signalling) was involved in limiting the disease symptoms in *Arabidopsis* during *Cucumber mosaic virus* (CMV) infection.

In *Southern tomato virus* (STV) infected tomato plant the microRNAs miR-398-3p, miR-398-5p, miR-3627-3p, and miR-408b-5p are upregulated whereas miR-319-3p was downregulated (Elvira-Gonzalez *et al.*, 2020). During *Beet necrotic yellow vein virus* (BNYVV) infection in *Nicotiana benthamiana* there was up regulation of miR168, which may be associated with the virus-encoded viral suppressors of RNA silencing (VSRs). Also, the expression of miR398 was upregulated when infected with BN1234 strain and the expression of the target gene umecyanin was decreased. The downregulation of the target gene was due to virus–induced gene silencing (VIGS) and was associated with the primary defense response (Liu *et al.*, 2020).

## 2.6 ARTIFICIAL miRNA AND IT'S ROLE

In plants for studying their developmental and defense functions gene silencing approaches are found convenient. Thus, small RNA based silencing techniques are frequently employed in plant research. RNAi and antisense suppression are the main viral defense mechanisms. At present siRNA/miRNA mediated gene regulation is an active area of study. The development of high throughput sequencing technologies, computational and bioinformatics prediction tools enhances the research. Small RNA profiling is widely used in economically important crops like rice, maize, tomato, brassica, potato etc. amiRNA performs homology-dependent gene silencing that is, a complementary target mRNA is required for its action.

Artificial microRNAs (amiRNAs) are single stranded ~21nt long and is designed by replacing the mature miRNA sequences of duplex within pre-miRNAs. It is a robust tool and is more efficient than other gene silencing approaches. They were first used to develop resistance against *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV) in transgenic *Arabidopsis thaliana.* The co-expression of several amiRNAs reduces the chance of simultaneous mutation of the viral pathogen as well as the target site, thus enhancing the antiviral resistance (Cisneros and Carbonell, 2020).

In Arabidopsis, amiRNAs expression helps in down regulation of many circadian clock identifiers (Kim and Somers, 2010) and is employed successfully in understanding the molecular mechanisms of flowering and its associated genes

(Schwartz *et al*., 2009). Transgenic Arabidopsis lines resistant to plant viruses have been developed using amiRNA technology (Niu *et al.*, 2006).

amiRNA designed for monocot crops can efficiently trigger gene silencing, they can also modulate agronomically important traits. The amiRNAs are highly specific and also triggers the production of secondary siRNAs from the primary target gene (Warthmann *et al*., 2008).

Jiang *et al*. (2011) reported that in transgenic tobacco the amiRNA derived from Potato virus Y (PVY) coat protein - amiRcp-8 targeting the 3'end exhibited higher virus resistance of 64.69 percent, showing that the degree of resistance was related to amiRNA accumulation.

Transgenic banana resistant to *Banana bunchy top virus* was created using two intron-hairpin RNA (ihp-RNA), ihpRNA-Prorep and ihpRNA-Rep. The ihpRNA in the transformed banana plant represented the viral master replication initiation protein (Replicase) gene of BBTV (Shekhawat *et al*., 2012).

Transgenic tobacco plants (*N. benthamiana*) expressing an amiRNA which silences suppressor 2b (SS 2b) of Cucumber mosaic virus (CMV) showed various degree of response during infection ranging from resistance, recovery, delayed infection, susceptibility etc. (Tiwari *et al*., 2014).

Yogindran and Rajam (2016) constructed an amiRNA against *Helicoverpa armigera* (cotton bollworm) and cloned it by inserting into a bacterial vector. During the infestation, the amiRNA was expressed and caused a reduction in the target gene expression.

To combat Ugandan *cassava brown streak virus* causing cassava brown streak disease amiRNAs were constructed in *N. benthamiana* and they were successful in combating the disease (Wagaba *et al*., 2017).

amiRNA developed based on the sequence of Arabidopsis miRNA319a, targeting the ATP/GTP binding domain of AC1 gene of *Tomato leaf curl New Delhi virus* (ToLCNDV) regulated the expression of AC1 gene conferring disease resistance. Transgenic lines overexpressing AC1 amiR showed reduced disease symptoms and increased resistance when infected with the virus (Sharma and Prasad, 2020).

## 2.7 DETECTION of miRNAs

Detection of miRNAs is done by several methods like northern blot, hybridization between the target miRNA and a complementary strand nucleic acid probe, primer extension, invader assay, signal-amplifying ribozymes, splinted ligation, mirMASA bead-based technologies, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), microarray, and high-throughput sequencing. Northern blot is the oldest method and the disadvantage is that it requires large amounts of total RNA (Baykal 2020). Zeng and Cullen (2003) reported that primer extension is a feasible method for quantitative analysis of miRNA expression. In Arabidopsis primer extension method has been successfully utilised for miRNA detection. miR173 and miR828 were detected from total RNA samples and primer extension done by reverse transcription using radio labelled specific primers (Baykal *et al*., 2016).

The invader miRNA assay has the ability to detect and quantitate as few as 20,000 molecules of an individual miRNA. It distinguishes between miRNAs and their precursors, as well as between closely related miRNA isotypes. The assay is rapid and can be performed in detergent lysates of cells (Allawi *et al.,* 2004).

miRNAs are detected based on the hairpin ribozymes that cleave a short RNA substrate labelled with a fluorophor at the 3′- and a quencher at the 5′-end, as a function of the presence or absence of a miRNA effector. This design enables real-time monitoring of ribozyme activity *via* FRET read-out 4 (Hartig *et al.,* 2004).

Splinted ligation assay is specific and quantitative and does not require any specialized equipment and makes use of readily available enzymes. It is faster and more sensitive than Northern blotting while having the same applications such as expression analysis and validation of microarray studies (Maroney *et al*., 2007).

miRNAs can be directly detected using microarrays (Babak *et al*., 2004). It is a low cost and high throughput method and has lower specificity than qRT-PCR or RNA sequencing (Pritchard *et al*., 2012). Another method is by using stem-loop RT followed by TaqMan PCR analysis. Stem-loop RT primers are better in terms of RT efficiency and specificity. TaqMan miRNA assays are specific for mature miRNAs. Precise quantification can be achieved using a small quantity of total RNA (Chen *et al.,* 2005). qRT-PCR enables fast, sensitive and specific miRNA expression profiling and is

suitable for the facilitation of high-throughput detection and quantification of miRNA expression (Varkonyi-Gasic *et al*., 2007).

Next-generation sequencing techniques enable more efficient and faster identification and quantification of miRNAs. Different NGS platforms like Illumina Genome Analyzer, Roche 454 Genome Sequencer FLX, Applied Biosystem's SOLiD Sequencer are used. Also, various detection and analysis software are utilized. miRNA databases like miRBase, deepBase, microRNA.org, miRGen 2.0, miRNAMap, and PMRD and data analysis tools like miRanalyzer, miRCat miRDeep and miRExpress were used (Liu *et al.,* 2011).

## 2.8 COMPUTATIONAL PREDICTION OF miRNA AND THEIR TARGETS

The prediction of miRNA is possible because of the complementarity between miRNA and their targets, this is analysed by several computer-based algorithms to predict miRNAs. miRNAs due to its sequence homology targets multiple mRNAs and also transcription factors.

There are mainly two approaches for predicting miRNAs, experimental approaches such as direct cloning and deep sequencing methods and computational identification and structure prediction. Computational approaches include homology-based search and bioinformatics or computational prediction. Since homology based computational methods are rapid, accurate and affordable, they are widely used (Allmer, 2014). They are developed based on the reference genome for prediction of stemloop structure (Unver *et al.*, 2009).

miRNAs are conserved among organisms, which makes the homology-based analysis easy (Zhang *et al.*, 2006). Different databases like expressed sequence tags (EST), genomic survey sequences (GSS), and transcript sequence assemblies (TSA) are utilized for searching miRNA homologs.

### 2.8.1 Computational Method

#### *2.8.1.1 Homology Based Analysis*

Homology based methods predict miRNA based on their similarity to other known miRNA with respect to sequence, structure or target site. For non- model plants, their ESTs and GSSs are used for search against the model plants in homology-based analysis (Dehury *et al*., 2013). Search algorithms use the genome matching hits between the known miRNA sequence and the unknown sequence. There are separate search algorithms based on EST and GSS. The unique plant miRNAs downloaded from miRBase were analysed by BLAST tool against the EST or GSS sequence from EST databases (dbEST) or GENBANK. The blast result with 0-3 mismatches against known miRNA is analysed. These sequences were again blasted against protein database to remove non-protein coding sequences. The resulted sequences are accepted only if they fulfil certain criteria like 1) they should be 18nt long, 2) when compared to all other miRNA only 2-3 miss matches are allowed in the sequence (Pani and Mahapatra, 2013). The hairpin or secondary structure is predicted by using MFold 3.2, minimal folding free energy (MFE) of potential miRNAs were identified.

Prabhu and Mandal (2010) performed the computational prediction of miRNAs in Tea (*Camellia sinensis)*. They identified 299 EST sequence by BLAST using known mature miRNA sequence. The sequences were further analyzed in miRNA prediction software, mirEval.

In *Ocimum basilicum*, 9 miRNAs were predicted by Singh and Sharma (2014), for this 23,260 ESTs are analysed by using the tool C-mii version 1.11 (Numnark *et al*., 2012).

miRNAs in Cavendish banana were reported by Bi *et al*. (2015). Small RNA sequences of the banana were mapped against annotated miRNAs of miRbase. A total of 125 potential miRNAs belonging to 39 different families were found.

In banana 32 miRNAs belonging to 13 families were identified using ESTs and GSS (Chai *et al*., 2015). BLAST analysis done using known sequences from miRBase (Kozomara and Griffith-Jones, 2014) and 46,111 banana ESTs and 31,544 GSS obtained from NCBI GenBank (Benson *et al*., 2012).

### *2.8.1.2 Bioinformatics studies*

Computational or bioinformatics methods are widely used for the prediction of miRNAs as they are very accurate and efficient compared to homology-based analysis (Dong *et al*., 2012). The various criteria used by the bioinformatics tools in identifying novel miRNAs include similarity among species or conserved sequences in species, length, secondary structure formation and minimum fold energy (Li *et al*., 2010). The different tools available for miRNA prediction are NovoMIR, MiPred, MIR-PD and MIRFINDER.

**NovoMIR** is a program used for the identification of plant miRNAs. It uses a series of criteria followed by a statistical model for filtering the pre-miRNAs from other RNAs in the genome. The input is the genome sequence. The tool uses *Arabidopsis thaliana* miRNAs as positive controls and mRNA, tRNA, non-coding RNA, and genome sequence as negative control. The specificity and sensitivity of NovoMIR was ~0.83 and ~0.99 respectively for pre-miRNAs prediction. NovoMIR uses RNAfold and RNAshapes for prediction of RNA secondary structure (Teune and Steger, 2010).

In the Department of Plant biotechnology, College of Agriculture Vellayani, a study conducted during 2016-18 identified 52 mature miRNAs using NovoMIR and their 124 targets identified using psRNATarget tool in banana (*Musa* AAB genome) (Mathew, 2018).

**miRBase** is a centralised registry and earliest database for miRNA annotation for all studied species (http://www.mirbase.org/). It contains 38589 hairpin precursor miRNA entries, 48860 mature miRNA sequences from over 271 organisms (Kozomara *et al*., 2019). The primary purpose is to assign consistent nomenclature to miRNA loci and to allow easy access to all known miRNAs and their sequences (Axtell and Meyers, 2018). It was established in the year 2002 (then called microRNA registry) to provide researchers with stable and unique gene names for their novel microRNA discoveries and an archieve of all miRNA sequences (Kozomara and Jones, 2010). miRbase also provides links to popular miRNA target prediction tools and databases, such as microRNA.org (http://microrna.org), DIANA-microT (http://www.microrna.gr/microT-CDS), TargetScan (http://www.targetscan.org), miRecords (http://mirecords.biolead.org), DIANA-TarBase

(http://www.microrna.gr/tarbase) etc. miRbase have additional information about miRNA families and clusters (Oulas *et al.,* 2015).

## 2.9 EXPRESSION ANALYSIS OF PREDICTED miRNAs

The computational and bioinformatics tools which are used for the identification and prediction of miRNAs are essential for the prediction of novel microRNAs in plants and to confirm these predicted one's various web tools are used. Confirmation of the predicted miRNAs is done by two processes, the evaluation of the host miRNA target sequences and creation of amiRNA sequences (Watanabe *et al*., 2007). For experimental validation of miRNAs, gene specific primers for target genes and stem-loop primers for miRNAs need to be constructed. RNA from the plant samples are isolated and converted to cDNA. Expression profiling of miRNAs is done by Real Time PCR using cDNA samples and gene specific primers. Validation is done by analysing the correlation between miRNA and their corresponding target genes during viral infection.

### 2.9.1 Expression Profile Analysis

Chen *et al*. (2005) suggested that during different conditions of stress plants perform differently, mainly because the level of expression of genes differs during these conditions, hence studies about such variations in expression levels should be done. The differential expression of genes correlates with the difference in the levels of plant miRNAs. Thus, expression profiling of miRNAs helps to study the metabolism and regulation of gene expression. RT-qPCR is a most efficient method to confirm and quantify the expression levels of genes (Chen *et al*., 2005). The method is very sensitive and gives accurate results.

In Real Time PCR, relative or competitive analysis is done by using a stable gene like β-Actin (control-housekeeping gene) and a gene of our choice for normalization of expression. The results are demonstrated as fold change (increase or decrease). Dyes like SYBR – Green 1 or Ethidium Bromide is used for real-time detection of the amplicon. SYBR Green is a dsDNA binding dye that intercalates non-

specifically into dsDNA. As the amplification proceeds the fluorescence increases proportional to the amount of product accumulated. The dye has an absorption and emission maxima at 497 and 520nm (Ponchel *et al*., 2003).

For each RT-qPCR assay threshold cycle or quantification cycle values (Cq or $C_t$) values are determined. The values are based on amplification curves which are sensitive to background fluorescence. For each reaction a threshold is fixed which should be above the background fluorescence baseline, should be in the log phase of the amplification plot, should be at a position where the log phases of all amplification plots are parallel (Khan-Malek and Wang 2017). There are four approaches for estimation of RT-qPCR results first one is to derive ΔΔCt from interaction of gene and treatment effects, second approach is ANCOVA (analysis of covariance) model, next one involves calculation of ΔCt followed by a two-group *t*- test and non-parametric analogous Wilcoxon test (Yuan *et al*., 2006).

In cucumber, during *Pseudomonas syringe* infection the expression of miR159f which target Auxin response factor ARF16 was found to be up regulated during RT-qPCR analysis (Weibo and Wu, 2015).

In Bract mosaic infected banana cv. Nendran the positive correlation of miR-3900-5p, miR9112, and miR5417 with its corresponding targets Fbox/Kelch- repeat protein, Cytochrome oxidase subunit 5B protein and Glyceraldehyde-3- phosphate dehydrogenase respectively was done using RT-qPCR (Mathew, 2018). Also, the relation of miRNAs miR-3900-5p, miR-2172-5p, miR-5417, miR-6928-5p, and miR-971-5p with its corresponding targets plant viral response protein family gene/putative disease resistance protein gene, putative ethylene response transcription factor 1 gene, the stress associated endoplasmic reticulum protein 2 gene, FAD dependent oxidoreductase gene, Argonoute 1A protein gene/ transport inhibitor response 1 like protein gene respectively was established using RT-qPCR (Subramanian, 2019).

Moran *et al*. (2020) used RT-qPCR analysis for the detection and absolute quantification of *grapevine roditis leaf discoloration – associated virus* (GRLDaV), a grapevine virus included in the European and Mediterranean Plant Protection Organization (EPPO) alert list as a potential phytosanitary risk for the region. RT-qPCR technique was successfully employed in specific detection of *Tomato leaf curl New*

*Delhi virus* (ToLCNDV) which is a bipartite *begomovirus* affecting cucurbitaceous and solanaceous crops (Luigi *et al*., 2020).

## 2.10 SELECTION OF BIOTIC STRESS RESPONSIVE miRNAs

For the present study 'Identification and expression profiling of *Banana bract mosaic virus* (BBrMV) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)", 3 microRNAs were selected from the already predicted 52 miRNAs. The miRNAs were selected based on its responsiveness to biotic stress especially pathogen infection. The 3 selected miRNAs are: miR-5417 (target: F Box family protein), miR-1634 (target: Patatin like phospholipase family protein) and miR-67 (target: Probable xyloglucan endotransglucosylase/hydrolase protein 33).

### 2.10.1 Expression of Proteins During Biotic Stress

A pathogen when infecting a plant cell should overcome its morphological barriers, secondary metabolites produced by the plant cells etc. Once the connection is established the pathogens start utilising the host biosynthetic machinery which will trigger the plant's defense response system to produce defense related proteins (van Loon et *al.,* 2006), also other changes like the production of ethylene, change in hormonal levels, production of phytoalexins etc. will be induced.

### 2.10.2 F Box Family Protein

F box protein is an important member of SCF ubiquitin ligase complex which is involved in protein degradation pathway. Nearly 700 F Box proteins are encoded by *Arabidopsis* genome. They help in the activation of Auxin induced genes. They are found to be involved in both abiotic and biotic stress responses in plants (Maldonado-Calderon *et al.,* 2012). The protein is involved in signal transduction and regulation of cell cycle. Also, it regulates cell death and is involved in miRNA mediated gene silencing *via* RNAi. Kim and Delaney (2002) reported that in *Arabidopsis* the *son 1* mutant (suppressor of *nim1-1*) showed resistance against pathogen in a SAR (Systemic

Acquired Resistance) independent manner. Cloning of *son1* revealed that it encodes a novel protein containing a F box element. In *Arabidopsis* during *Polerovirus* infection (*Beet western yellows virus* BWYV and *Cucurbit aphid- borne yellows virus* CABYV*)* the pathogen interacts with the plant *via* a F box like domain and directs the host PTGS-based virus defense system to E3 Ubiquitination ligase machinery and suppresses the pathogenicity (Pazhouhandeh *et al*., 2006). Roshan *et al*. (2020) reported that the *AV2* protein of *Tomato leaf curl Palampur virus* (ToLCPalV) interacts with tomato F-box Kelch protein (KFB) and causes destabilization of KFB in *Nicotiana benthamiana,* this resulted in an increase in PAL (phenylalanine ammonia-lyase) activity which is related to the pathogenicity. During *Cucurbit chlorotic yellows virus* (CCYV) infection, the *CCYV RNA 1*-encoded *p22,* which is a weak suppressor of RNA silencing interacts with cucumber *SKP1LB1* for its silencing suppressor activity *via* an F-box-like motif possessed by *p22* (Salavert *et al*., 2020).

### 2.10.3 Patatin- Like Phospholipase Family Protein

In many plants, Patatin like phospholipases is induced by infection to help control the spread of disease. It is involved in cell signalling especially defense signalling in plants through jasmonic acid and oxylipin accumulation (Canonne *et al*., 2011). The protein is involved in the immune response. In pepper, during *Xanthomonas campestris* pv. *vesicatoria* (Xcv) infection *Capsicum annuum* patatin like phospholipase (CaPLP1) were induced transcriptionally. During *Pseudonomas syringae* pv. tomato (Pst) and *Hyaloperonospora arabidopsis* infection in *Arabidopsis* overexpression of CaPLP1 resulted in enhanced resistance (Kim *et al*., 2014). In *Arabidopsis, PLP* 2 promotes programmed cell death in tissues infected with *Cucumber mosaic virus.* In tobacco, during *Tobacco mosaic virus* infection Patatin like phospholipase strongly induce transcription of three PLPs (Wilson and Knoll, 2018). Here accumulation of 12-oxophytodienoic and jasmonic acids occur as a response to virus infection (D'hondt *et al*., 2000). In *Arabidopsis* during *Turnip crinkle virus* infection, Patatin-related phospholipase As- *pPLAIIIα* was overexpressed causing an

increased expression of the defense gene pathogenesis-related protein 1(PR1) causing increased resistance to the virus (Jang *et al*., 2020).

### 2.10.4 Probable xyloglucan endotransglucosylase/hydrolase protein 33

The xyloglucan endotransglucosylase/hydrolases (XTHs) are a family of enzymes that specifically use xyloglucan as a substrate to catalyze xyloglucan endotransglucosylase (XET) and/or xyloglucan endohydrolase activities. It is a hemicellulosic polysaccharide presented in the primary cell walls of land plants. They are involved in cellwall biogenesis and reconstruction also, important structural component of cell plates (Moore and Staehelin, 1988). The cellwall architecture is an important determinant of plants response to pathogen attack (Sasidharan *et al.,* 2011). They are thought to have an important role in construction and restructuring of xyloglucan cross links in plants. The cell wall strengthening and expansion depends upon the concentration of protein in their cellwall (Rose *et al*., 2002). In Arabidopsis, 33 open reading frames (ORFs) encoding XTH proteins have been identified (Yokoyama and Nishitani, 2001). Their expression patterns are tissue specific and respond differentially to hormonal stimulus (Xu *et al.,* 1996; Akamatsu *et al;* 1999). Each of the XTH proteins perform a wide range of physiological processes (Yokoyama *et al*., 2004). Gibberellic acid (GAs) upregulates the expression of *OsXTH8* (Fu *et al.,* 2019). The proteins are known to have an important role in coping with environmental stresses like heat stress, chilling stress, alkali and salinity stress and hormonal stress. They show multifunctional activity in relation to biotic stress tolerance (Wang *et al*., 2018).

# *Materials and Methods*

# 3. MATERIALS AND METHODS

The study entitled “Identification and expression profiling of *Banana bract mosaic virus* (BBrMV) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)” was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthpuram during 2018-2020. The detailed description of the methodologies followed for this research is mentioned below;

## 3.1. COLLECTION OF SAMPLES

*Banana bract mosaic virus* (BBrMV) infected plants of *cv*. Nendran were identified from the Instructional Farm of College of Agriculture, Vellayani based on the visual symptoms like chlorotic streaks on leaves, reddish-brown streaks on pseudostem and bract (Plate 1). Uninfected control plants were also identified. The infection was then reconfirmed by RT-PCR using primers specific to the coat protein gene of the virus (Jadhav, 2019).

## 3.2. CONFIRMATION OF BBrMV INFECTION IN SAMPLES

### 3.2.1 RNA Isolation

RNA was isolated from the leaf samples of both healthy and infected plants using the modified Rodrigues-Garcia protocol (Ekatpure *et al*., 2019). The modifications followed was increasing the incubation time from 10 min to 30 min at 65ºC, chloroform -isoamyl wash was given two times to remove all cell debris and phenolic contamination and overnight precipitation of RNA was done with 3M LiCl at 4ºC instead of -20ºC.

Leaf tissue (0.5g) was ground into a fine powder using liquid nitrogen in a chilled pestle and mortar. The extraction buffer (3ml) (Appendix I) was added to homogenate and incubated in a hot water bath at 65ºC for 30min. From this homogenate, 750µl was transferred to a sterile 2ml Eppendorf tube using a wide bore tip to avoid damage to RNA. To this 5mM Potassium acetate (66µl) and 150µl absolute

ethanol was added for precipitation. Tubes were then vortexed for 1min and an equal volume of chloroform: isoamyl alcohol (49:1 v/v) was added. It was vortexed briefly and centrifuged at 16000g for 20min at room temperature. The supernatant was carefully transferred to fresh tubes and the above step was repeated. The supernatant from this step was collected in a fresh tube and equal volume (850 µl) phenol: chloroform: isoamyl alcohol (25:24:1) was added and vortexed for 10 sec and centrifuged at 16000g for 15min at room temperature. The supernatant collected in a fresh tube and 850 µl of chloroform: isoamyl alcohol was added and vortexed for 10 sec and centrifuged at 16000g for 15 min at 4ºC. The supernatant from this step was collected and 3M LiCl is added and mixed by inversion, and kept overnight at 4ºC. The content was then centrifuged at 16000g for 20min at 4ºC. The pellet was recovered at this stage was washed twice with 70 per cent ethanol. It was airdried at room temperature under sterile Laminar Air Flow chamber (LAF) and resuspended in 30 µl Diethyl pyro carbonate (DEPC) treated sterile distilled water and stored in -80ºC. All the chemicals used were of analytical reagent grade.

#### *3.2.1.1 Qualitative Analysis of RNA*

The quality of RNA was determined by using Gel electrophoresis in a horizontal electrophoresis unit. The concentration of agarose gel used is 1.2 percent which is prepared by melting 1.2g of agarose powder in 1X TBE electrophoresis buffer (Tris base 10.8g, Boric acid 5.5g, 0.5M EDTA 4ml, pH-8 for 1L). The solution was then cooled at room temperature and ethidium bromide (EtBr) was added to make a final concentration of (0.5 µg /ml) and poured into the casting tray and comb inserted. After solidification of the gel (20-30 min), it was placed in the buffer tank containing 1X TBE buffer and the comb was gently removed. RNA samples (5µl) were loaded by mixing with (1µl) 6X gel loading dye (Bromophenol blue 0.25 per cent, and Glycerol 30 per cent) to increase the density of the RNA sample. In one of the wells, a 100bp ladder was loaded as a marker for identifying the size of the bands. The electrophoresis unit was closed and the samples were allowed to run at a voltage of 75V till the dye front reaches three-fourth of the gel. The samples run from cathode to anode as the RNA is

negatively charged and the final results were viewed in Gel Doc$^{TM}$ XR+ System (BIO-RAD) using ImageLab software.

### *3.2.1.2 RNA Quantification*

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

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

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

### 3.2.2. cDNA Conversion

The RNAs of both control and infected plants were converted to their complementary DNA using Verso cDNA synthesis kit (Thermo Fisher Scientific). The kit consists of cDNA synthesis buffer, dNTP mix, random hexamers, oligo dT, RT enhancer, and verso enzyme mix. The enzyme is an RNA-dependent DNA polymerase that prevents RNA degradation and the RT enhancer removes DNA contamination if any. The RNA primer consists of random hexamers and anchored oligo-dT in 3:1 (v/v). A 10 µl reaction mix was prepared for reverse transcription of RNA. Following components were added to a sterile microfuge tube in the following order:

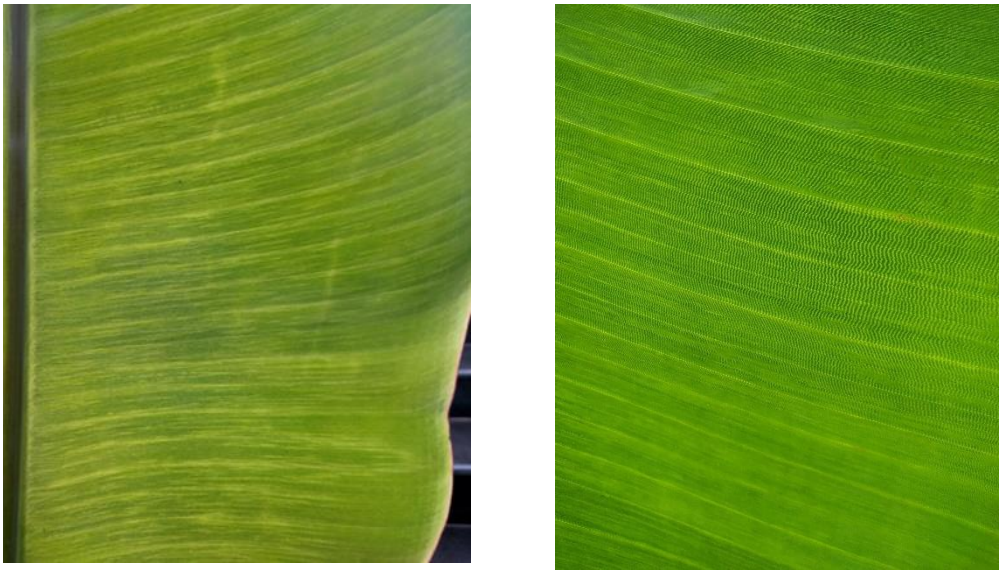
| **Components** | **Volume (µl)** |
|---|---|
| cDNA synthesis buffer (5X) | 2 µl |
| dNTP (500 µM) | 1 µl |
| RNA primer (2 µM) | 0.5 µl |
| RT enhancer | 0.5 µl |
| RNA (1µg/µl) | 3 µl |
| Reverse transcriptase (1 U/µl) | 0.5 µl |
| Water | 2.5 µl |
| Total volume | 10 µl |

The contents were mixed well and after a flash spin, PCR was carried out in Thermal cycler (T100$^{TM}$ BIO-RAD). The thermal profile for the reverse transcriptase reaction of the target gene was:

42$^{o}$C for 30 min

92$^{o}$C for 2 min

For the reverse transcription of miRNA, stem-loop primers specific to the selected miRNAs were used. A 10 µl reaction mix was prepared using the following components:



(a)

(b)

**Plate 1. Symptoms of BBrMV infection in samples collected**

**a. Chlorotic streaks on leaf samples of the infected plants**

**b. Reddish-brown streaks on pseudostem of the infected plants**

| Components | Volume (µl) |
|---|---|
| cDNA synthesis buffer (5X) | 2 µl |
| dNTP (500 µM) | 1 µl |
| Stem-loop primers (10 µM) | 0.5 µl |
| RNA samples (1µg/ µl) | 2.5 µl |
| Reverse transcriptase enzyme (1U/ µl) | 0.5 µl |
| Water | 3.5 µl |
| Total volume | 10 µl |

The contents were mixed well and after a short spin, the PCR was carried out in Thermal cycler (T 100$^{TM}$ BIO-RAD). The thermal profile used for this reaction was:

16$^{o}$C incubation: 30 min

Step 1: 30$^{o}$C for 30 sec

Step 2: 42$^{o}$C for 30 sec

Step 3: 50$^{o}$C for 1sec

Steps 1 to 3 were repeated for 60 cycles.

Step 4: 85$^{o}$C for 5min

The cDNA samples were maintained at -20$^{o}$C

#### *3.2.2.1 Confirmation of cDNA Synthesis*

The cDNA synthesized for target genes was tested for quality by RT-qPCR. cDNA was amplified with forward and reverse primers specific to the housekeeping gene i.e. β-actin gene to confirm the successful conversion of RNA to cDNA. The sequence of β-actin gene specific primer is given below:

**Forward Primer: 5'ACATTGTTCTCAGCGGTGGAT 3'**

**Reverse Primer: 5'CCACCTTAATCTTCATGCTGCTT3'**

A 20 µl standard PCR mix was prepared, which contains the following components:

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

The thermal profile used for the reaction was as follows

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

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

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

Step 4: 60$^{o}$C for 45sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 60$^{o}$C for 5 min

The results of the PCR reaction were analysed by running the PCR products on 1.5 per cent agarose gel in a horizontal gel electrophoresis unit. The products were loaded into wells after mixing with the tracking dye. A 50 bp ladder was used as the marker. The electrophoresis was carried out 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+ System (BIO-RAD).

### 3.2.3 Confirmation of BBrMV Infection

In the samples collected from the field (2 BBrMV infected and 2 control samples) the *Banana bract mosaic virus* infection was confirmed by checking the presence of BBrMV coat protein gene. The cDNA from healthy and infected plants were amplified with coat protein gene-specific primers of BBrMV by RT-PCR (Jadhav, 2019). The sequences of the coat protein gene-specific primers are given below (Table 1):

**Table 1. Sequence of coat protein gene-specific primers of BBrMV**

| | PRIMER SEQUENCE (5'-3') | LENGTH | GC (%) |
|---|---|---|---|
| Forward primer | ATGTCAGCTCCATCTTCATC | 20 bp | 45 |
| Reverse primer | TATCACGCTTCACATCTTCA | 20 bp | 40 |

PCR mix of 20µl volume was prepared, which included:

| 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 |
| Template cDNA (200ng/ µl) | 1 µl |
| Nuclease free water | 13.5 µl |
| Total volume | 20 µl |

For the amplification of cDNA samples of both healthy and infected plants, the thermal profile used was:

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

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

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

Step 4: 72$^{o}$C for 45 sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 72$^{o}$C for 5 min.

The PCR product was analysed by gel electrophoresis in agarose gel (1.5 per cent) in a horizontal gel electrophoresis unit. 100 bp ladder (2µl) was loaded to one of the wells as a marker. Electrophoresis was carried out at 75 V until the loading dye reached three fourth of the gel and the gel was documented by using Gel Doc$^{TM}$ XR+ System (BIO-RAD), using ImageLab Software.

## 3.3. SELECTION OF miRNAs FOR VALIDATION

In a previous study conducted in the Department of Plant Biotechnology, 52 mature miRNAs were predicted using NOVOIMR software and over 142 targets were identified for these miRNAs using psRNATarget in the banana genome. From these computationally predicted miRNAs, three miRNAs were selected for this study, based on the function of their target genes with a possible role in stress conditions especially biotic stress (Table 2).

## 3.4. PRIMER DESIGNING

### 3.4.1. Designing of Stem-Loop Primer For preparing cDNA

miRNAs are smaller in size (18-22nt) and hence are difficult to extract from the total RNA. Thus, for the easy extraction of miRNAs, stem-loop primers are used. Chen *et al*. (2005) designed a stable stem-loop sequence of 44 nt in order to lengthen the target cDNA. The miRNA specific stem-loop primers were designed by combining the stem-loop sequence (44 nt) (5’ GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG ACG AC-3’) with the reverse complement of the six 3’ nucleotides of the mature miRNA sequence (Chen *et al*., 2005). For extraction of miRNAs, cDNAs were synthesised from total RNA using stem-loop primers and then these cDNAs were amplified using miRNA-specific forward primer and universal reverse primer in a Real-Time PCR reaction using SYBR Green assay.

### 3.4.2. Designing of Primers for The Amplification Of miRNA

For amplification of the cDNA of the miRNAs, specific forward and reverse primers were designed. miRNA specific forward primers were designed by adjusting the Tm to 60ºC. For this, the first 12 to 17 nts of the 5' end of the mature miRNA were taken and 5 to 7 additional nucleotides (rich in G+C% and Tm of 60 ± 1ºC) were added at 5'nt. The reverse primers were designed with the 44nt stem-loop sequence proposed by Chen *et al*., (2005). The reverse primer is universal for all miRNAs (5' CCAGTGCAGGGTCCGAGGTA-3') (Kramer, 2011). Eurofins genomics oligo analysis tool software (https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/) was used for verifying the compatibility and secondary structure formation of forward and reverse primers (Fig. 1).

### 3.4.3. Primer Design for Target Gene

Primer3plus software (http://www.bioinformatics.nl/primer3plus) was used for designing target gene-specific primers (Untergasser *et al.,* 2007). The software comprises an input box for sequence information and they are pasted in FASTA or EMBL format in the space provided. The region required for amplification was selected using '[ ]' indicated below the dialogue box. Primers were designed by adjusting the default parameters like length: 20-22nt, GC per cent: 50-60 per cent, melting temperature: 55-56ºC etc. (Fig. 2).

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

**Table 2. List of miRNAs and their target genes selected for the study**

| Sl. No | miRNA family | Target ID | miRNA Aligned Fragment | Target Aligned Fragment | Target Description | Target Function |
|---|---|---|---|---|---|---|
| 1 | miR-5417 | GSMUA_Achr9T26610_001 | AGGGGAGAAATGGGGATG | CATCCCCATTGCTCCTCA | F BOX family protein | Cleavage |
| 2 | miR-1634 | GSMUA_Achr6T21820_001 | AAGAGAGGACGATGCATCGA | CCGATCTGTCGTCCTCTTTT | Patatin like phospholipase family protein | Cleavage |
| 3 | miR-67 | GSMUA_Achr3T10480_ 0012.5 20.2 | CCUCUCUGUCUGG GAGGUUG | CAAUCUCCCAGUU AGAGAGG | Probable xyloglucan endotransglucosylase/hydrolase protein 33 | Translation |

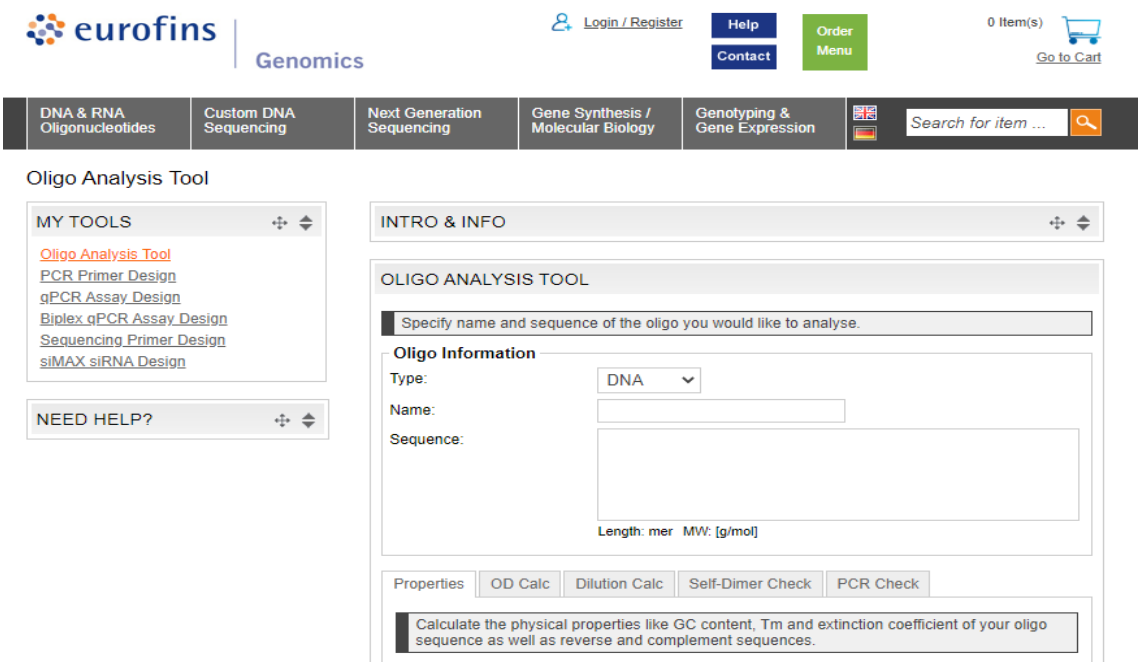



**Fig. 1. Home Page of Eurofins Genomics Oligo Analysis Tool Software**

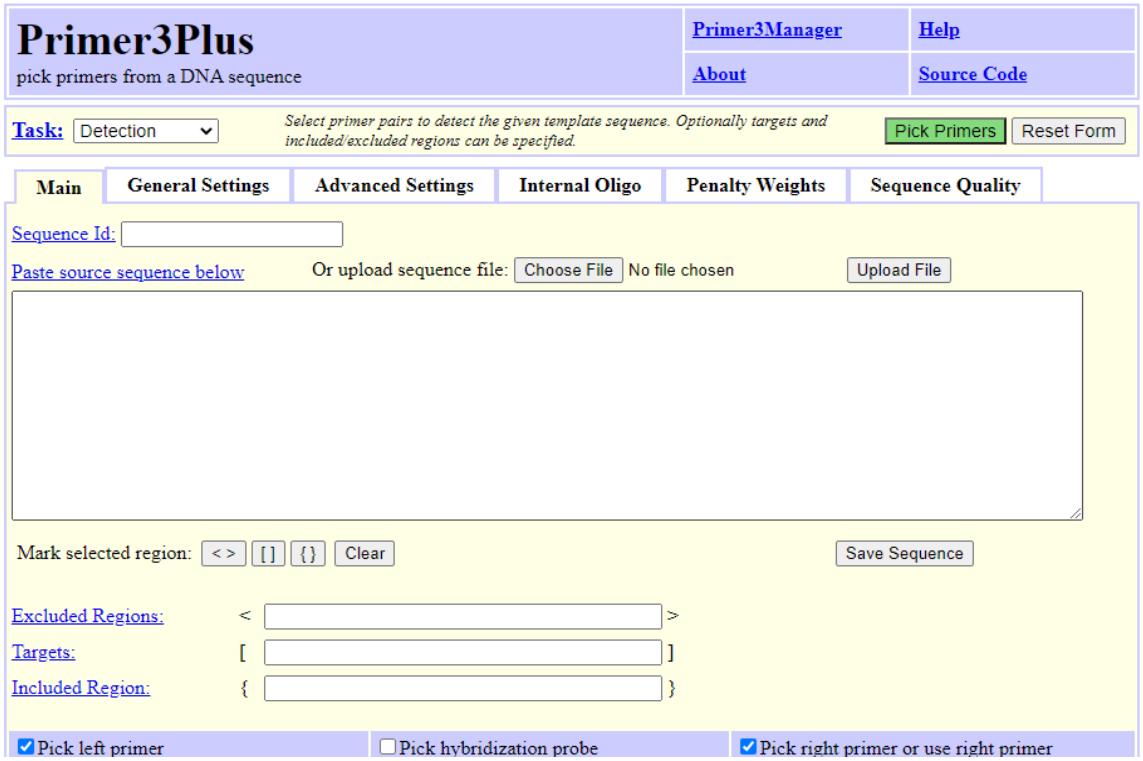



**Fig. 2. Home Page of Primer3Plus**

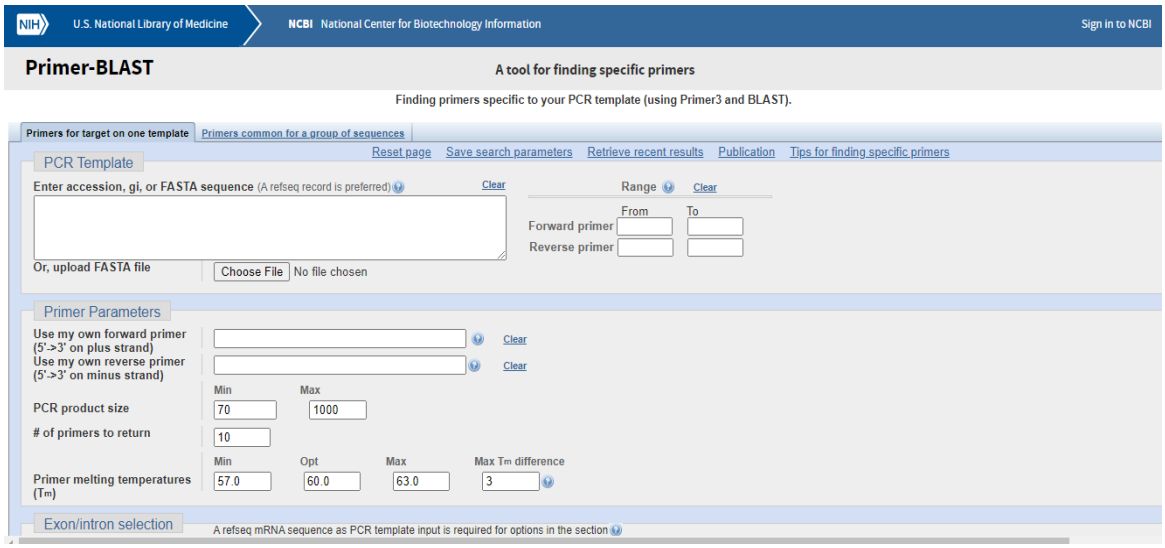



**Fig. 3. Home Page of NCBI Primer BLAST Software**

## 3.5. ASSAY FOR CHECKING SPECIFICITY OF PRIMERS FOR miRNAs

The expression of miRNA in the BBrMV infected plant samples and control plant samples were checked by doing PCR. The miRNA-specific forward primer and universal reverse primer were used for the amplification of miRNAs. A 20 µl standard reaction mix was prepared, which includes the following components:

| **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 (500ng/ µl) | 1 µl |
| Nuclease free water | 13.5 µl |
| Total volume | 20 µl |

The thermal profile used was:

Step 1: 95$^{o}$C for 5 min

Step 2: 95$^{o}$C for 5sec

Step 3: 60$^{o}$C for 10 sec

Step 4: 72$^{o}$C for 8 sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 72$^{o}$C for 5 min

After completion of the reaction, the PCR products were separated on 1.5 per cent agarose gel in a horizontal gel electrophoresis unit. A 50 bp ladder was used as the marker. The voltage was set to 75 V till the gel loading dye reached three fourth of the gel. The final gel was viewed in Gel Doc$^{TM}$ XR+ System (BIO-RAD).

## 3.6 ASSAY FOR CHECKING TARGET GENE AND REFERENCE GENE AMPLIFICATION

The expression of target genes in the infected and control samples was checked for amplification using target gene-specific primers. A 20 µl standard reaction mix was prepared, which includes the following components:

| **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 (500ng/ µl) | 1 µl |
| Nuclease free water | 13.5 µl |
| Total volume | 20 µl |

The thermal profile followed for the reaction was:

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

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

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

Step 4: 72$^{o}$C for 45sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 72$^{o}$C for 5 min

After completion of the reaction, the PCR products were gel checked using 1.5 per cent agarose gel in a horizontal gel electrophoresis unit. 50 bp ladder was used as the marker. The electrophoresis was conducted at a voltage of 75 V till the tracking dye reached three-fourth of the gel. The final results were viewed in Gel Doc$^{TM}$ XR+ System (BIO-RAD).

## 3.7. EXPRESSION PROFILING OF miRNAs AND THEIR TARGET GENES

RT-qPCR is one of the most efficient methods used for gene expression analysis. It monitors the amplification of the genes during the PCR and not at the end as in conventional PCR. This technique was used to analyse the expression levels of miRNAs and their corresponding target genes as well as the expression of the reference gene. The analysis was done in CFX96 Touch fluorescence quantitative PCR detection system (BIO-RAD).

Two technical replicates were kept for each sample and a non -template control for each reaction. The fluorescent dye used for the assay was SYBR Green (Chai *et al.,* 2015). A 20 µl standard reaction mix was prepared, which includes the following components:

| **Components** | **Volume (µl)** |
|---|---|
| cDNA (50ng/ 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 |

The following thermal profile was used for the amplification of cDNA of miRNAs from both uninfected and infected plants:

Step 1: 95ºCfor 5 min

Step 2: 95ºC for 5 sec,

Step 3: 60ºC for 10 sec,

Step 4: 72ºC for 8 sec. Steps 2 to 4 were repeated for 45 cycles

Step 5: 72ºC for 5min

The thermal profile followed for target genes and reference gene assay:

Step 1: 95ºC for 2 min,

Step 2: 95ºC for 15 sec,

Step 3: 55ºC for 15 sec,

Step 4: 72ºC for 30 sec. Steps 2 to 4 were repeated for 40 cycles

Step 5: 72ºC for 5 min

For expression profiling of miRNAs and target gene, relative expression values were analysed using qBase plus software. Using this software, the relative expression of miRNAs and their target genes in healthy samples were compared to the expression of miRNAs and target genes in infected samples.

A melt curve analysis was done following the PCR reaction. For determining the product specificity, fluorescence signals at 530nm wavelength were monitored from 65ºC to 95ºC at every 0.2ºC. Raw fluorescence data (RFU) was generated by CFX96 software. To generate the threshold cycle (Cq) values, a threshold fluorescence passing through the exponential phase of log RFU plot of all the reactions was set manually for all the assays.

The results of the RT-qPCR reactions were expressed as fold change (increase or decrease) in expression. For normalization of RT-qPCR data in qBase plus software, the housekeeping gene β-Actin was used as a reference gene. The threshold cycle (Cq) value was determined for all the reactions and fold changes were calculated by using ΔΔCq method (Rao *et al.*, 2013) in the software. The threshold cycle (Cq) is defined as the number of cycles required for the fluorescent signals to exceed the background fluorescence. The difference between Cq values of control and infected samples and reference genes was considered as ΔCq value.

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

The difference between $\Delta C_q$ values of control and infected samples were considered as $\Delta\Delta C_q$ value. The fold change in expression of miRNAs is calculated by using $2^{\wedge(-\Delta\Delta C_q)}$.

$$\Delta\Delta C_q = \Delta C_q\ (\text{Infected Sample}) - \Delta C_q\ (\text{Healthy Sample})$$

# *Results*

# 4. RESULTS

The study entitled “Identification and expression profiling of *Banana bract mosaic virus* (BBrMV) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)” was conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2018-2020. The results of the above study are presented in this chapter.

## 4.1 COLLECTION OF SAMPLES

For this study, samples of *Banana bract mosaic virus* infected banana plants of cv. Nendran were collected from the field. The collected plants showed characteristic reddish-brown streaks on the pseudostem, leaf and bract. Leaf samples were collected from these plants for the study. Leaf samples collected from symptomless plants served as control (Plate 1).

## 4.2 DESIGNING AND SYNTHESIS OF PRIMERS FOR miRNAS AND THEIR TARGET GENES

### 4.2.1 miRNAs And Their Target Genes Selected

List of miRNAs selected for the study and their corresponding targets are given in Table 2.

### 4.2.2 Primers Designed For miRNAs And Target Genes

For miRNAs, stem-loop primers were designed and synthesized based on the reports by Chen *et al*. (2005). Also, a forward primer for the amplification of each miRNA was designed. The sequences of the primers designed for miRNAs are given in Tables 3 and 4. The primers designed for the target genes are given in Table 5.

**Table 3. Sequence of stem-loop primers used for amplifying the miRNAs**

| Sl. No. | miRNA (Name) | miRNA sequence (5'..3') | miRNA specific stem-loop sequence (5'..3') |
|---|---|---|---|
| 1 | miR-5417 | AGGGGAGAAATG GGGATG | GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACCATC CC |
| 2 | miR-1634 | AAGAGAGGACGA TGCATCGA | GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACTCGA TG |
| 3 | miR-67 | CCTCTCTGTCTGG GAGGTTG | GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACCAAC CT |

**Table 4. Sequence of primers designed for miRNAs**

| Sl. No | miRNA (Name) | miRNA sequence (5'..3') | Forward primer (5'..3') | Universal reverse primer (5'..3') |
|---|---|---|---|---|
| 1 | miR-5417 | AGGGGAGAAA TGGGGATG | GCTGGCGAGGG GAGAAATG | CCAGTGCAGGGTCC GAGGTA |
| 2 | miR-1634 | AAGAGAGGAC GATGCATCGA | GCACTGGAAGA GAGGACGATG | CCAGTGCAGGGTCC GAGGTA |
| 3 | miR-67 | CCTCTCTGTCT GGGAGGTTG | CGTCGCCCTCTC TGTCTGGG | CCAGTGCAGGGTCC GAGGTA |

**Table 5. Sequence of primers designed for target genes**

| Sl. No. | Target name | Forward primer (5'…3') | Reverse primer (5'…3') | Expected amplicon size (bp) |
|---|---|---|---|---|
| 1 | F-box family protein | ACGCTTGGTAC TTTGCAACC | CCAGCATTAGA ACCGACACA | 178 |
| 2 | Patatin like phospholipase family protein | CAGCCCAGTTC GACTACTCC | TGGGGTTAGGG TTTGTGGTA | 171 |
| 3 | Probable xyloglucan endotransglucosyla se/hydrolase protein 33 | TCGACCCCACT GCAGATTTC | GGGCTTGGAGG GATAAGCTC | 216 |

4.3. EXPRESSION PROFILING OF miRNAs AND TARGETS DURING BBrMV INFECTION

**4.3**.1. **RNA Isolation**

RNA was isolated from the infected as well as control samples using the protocol of Rodriguez-Garcia modified by Ekatpure *et al.* (2019). The RNA profile (Plate 2 a) on 1.2 percent agarose showed the presence of intact bands representing the 28SrRNAs and 18 SrRNA. Quantity and quality of RNA isolated measured using the NanoDrop spectrophotometer are shown in Table 6. The purity of RNA ($A_{260}/A_{280}$) ranged from 1.85 - 2.00.

**Table 6. Quantity of RNA measured by NanoDrop spectrophotometer**

| SAMPLE | CONCENTRATION ($ng\mu l^{-1}$) | $A_{260}/A_{280}$ |
|---|---|---|
| Control 1 | 214.67 | 2.08 |
| Control 2 | 310.77 | 2.01 |
| BBrMV Infected 1 (Br1) | 492.46 | 2.12 |
| BBrMV Infected 2 (Br2) | 542.45 | 2.10 |

### 4.3.2 cDNA Synthesis

cDNA was synthesized from both healthy as well as BBrMV infected field grown plants using Verso cDNA synthesis kit (Thermo Fisher Scientific). The qualitative analysis of cDNA was done by Polymerase Chain Reaction (PCR) with primers specific to the housekeeping gene β-Actin. The amplicons of the β Actin gene obtained were of the expected size (102 bp) (Plate 2 b).

### 4.3.3 Confirmation of BBrMV in the Selected Samples

Both infected and control samples collected from the field based on the symptoms were checked for the confirmation of BBrMV infection by conducting PCR using coat protein gene-specific primers. The samples collected from plants showing symptoms of BBrMV yielded amplicons of the expected size (745 bp). No amplicons were obtained in control plants that showed no symptoms (Plate 2 c).

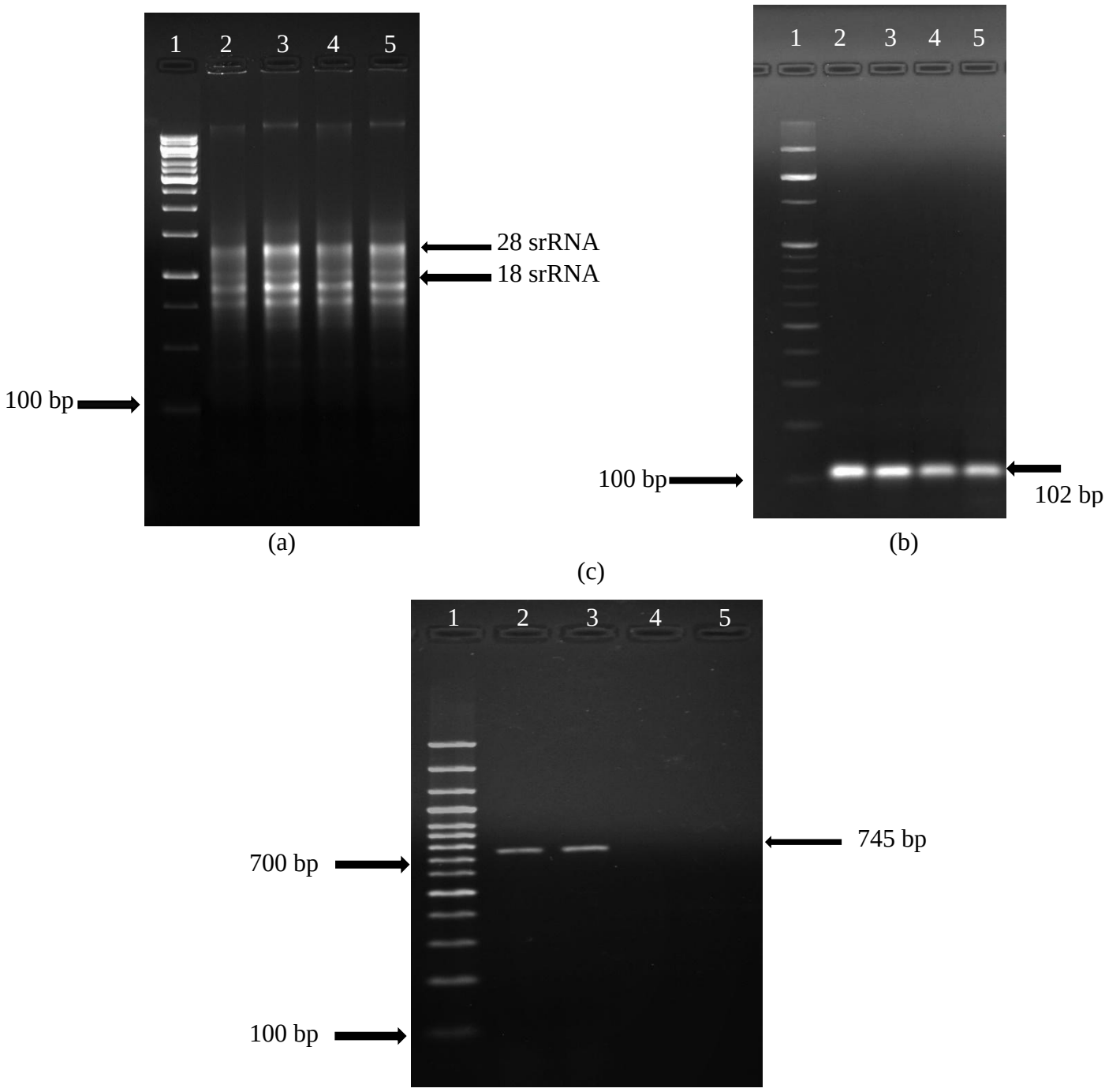



**Plate 2: Confirmation of BBrMV infection in the samples collected by PCR using coat protein gene specific primers.**

**a: Gel profile of RNA isolated from infected and control plants. b: β-Actin gene specific fragments amplified from cDNA. c: Amplicons specific to coat protein gene**

**Lane 1: 100bp ladder, Lane 2 and 3: BBrMV Infected plant samples, Lane 4 and 5: Uninfected Control plant samples**

### 4.3.4 Assay of Primers Designed For miRNAs And Target Genes

The selected miRNAs, were converted to cDNA using stem-loop primers (Table 3) and amplified using the miRNA specific primers (Table 4), they were designed and synthesized based on the reports by Chen *et al*. (2005). The specificity of the primers was analysed by PCR. The products separated on 1.5 per cent agarose gel showed amplicons of expected size for miR-5417, miR- 1634 and miR- 67 in both control and infected plants. The size of the amplicons was 62 bp, 64 bp and 64 bp respectively (Plate 3).

The primers specific to the target genes were designed using Primer3Plus software. The complementarity of the primers to the banana genome was used as a criterion for synthesis and validation of primers. The list of primers designed is given in Table 5. The amplification efficiency of the primers designed for the target genes was checked using PCR. The gel profile of the PCR products separated on 1.5 percent agarose gel showed amplification in both control and infected plants, amplicons were of size 178 bp for F Box family protein (target of miR-5417), 171 bp for Patatin like phospholipase family protein (target of miR- 1634) and 216 bp for Probable xyloglucan endotransglucosylase/hydrolase protein 33 (target of miR-67) respectively (Plate 4).

### 4.3.5 RT-qPCR Analysis of miRNAs and Target Genes in BBrMV Infected samples

The expression of miRNAs and their corresponding target genes in BBrMV infected and control plants were analysed by RT-qPCR and the expression patterns were compared. Two biological replicates were kept for the assay.

#### *4.3.5.1 Raw Expression Data and Cq Values Of miRNAs and Target Genes*

The RT-qPCR reactions were carried out with two technical replicates for each assay. Amplification plot showing the raw expression data of miRNAs and target genes were developed. The amplification point represented by the Cq values ranged from 22

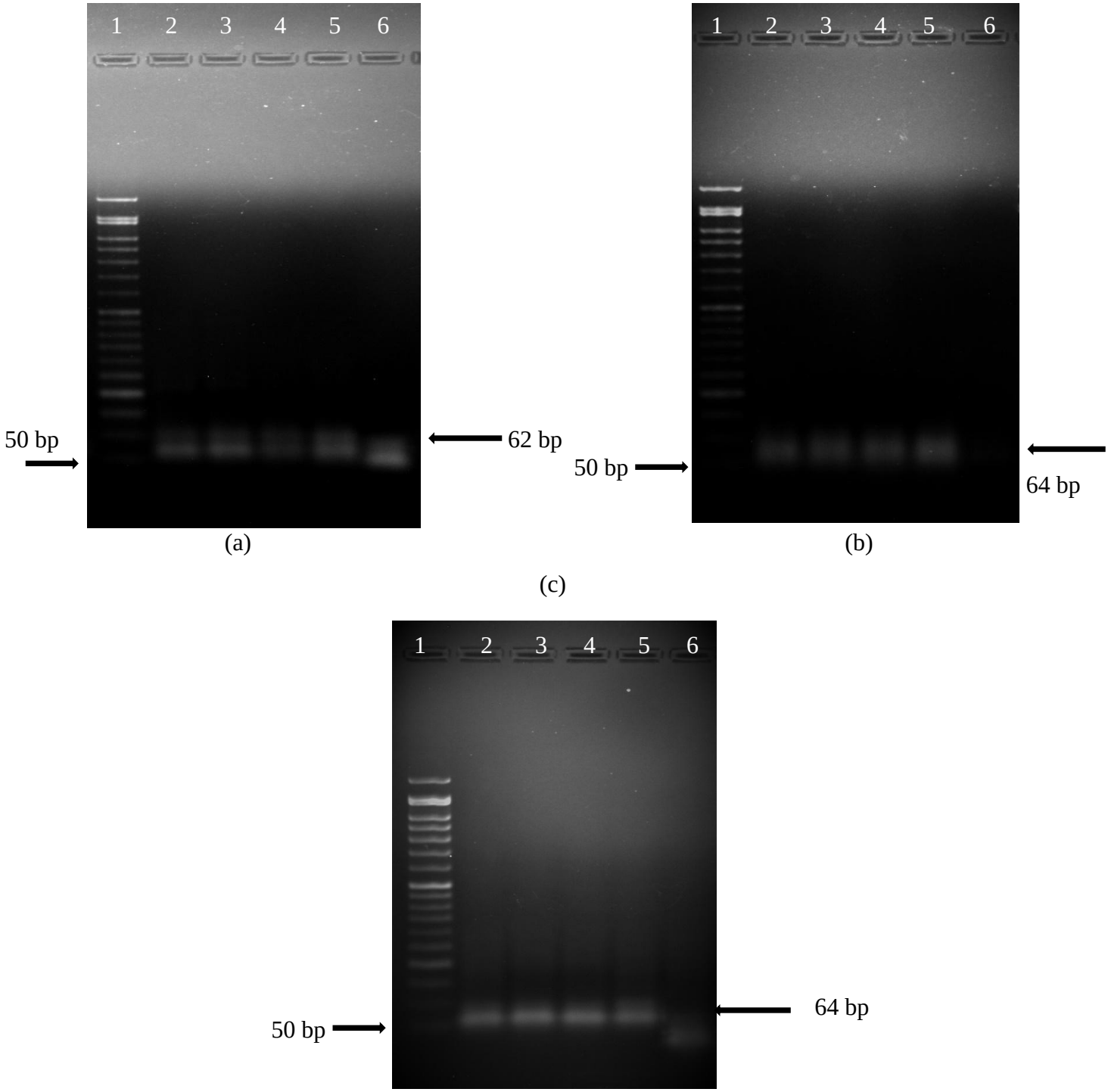



**Plate 3. Amplification of miRNAs**

**a. Amplification of miR-5417, b. Amplification of miR-1634, c. Amplification of miR-67**

**Lane 1: 50bp ladder, Lane 2 and 3: uninfected control samples, Lane 4 and 5: BBrMV infected samples, Lane 6: Non-template control**

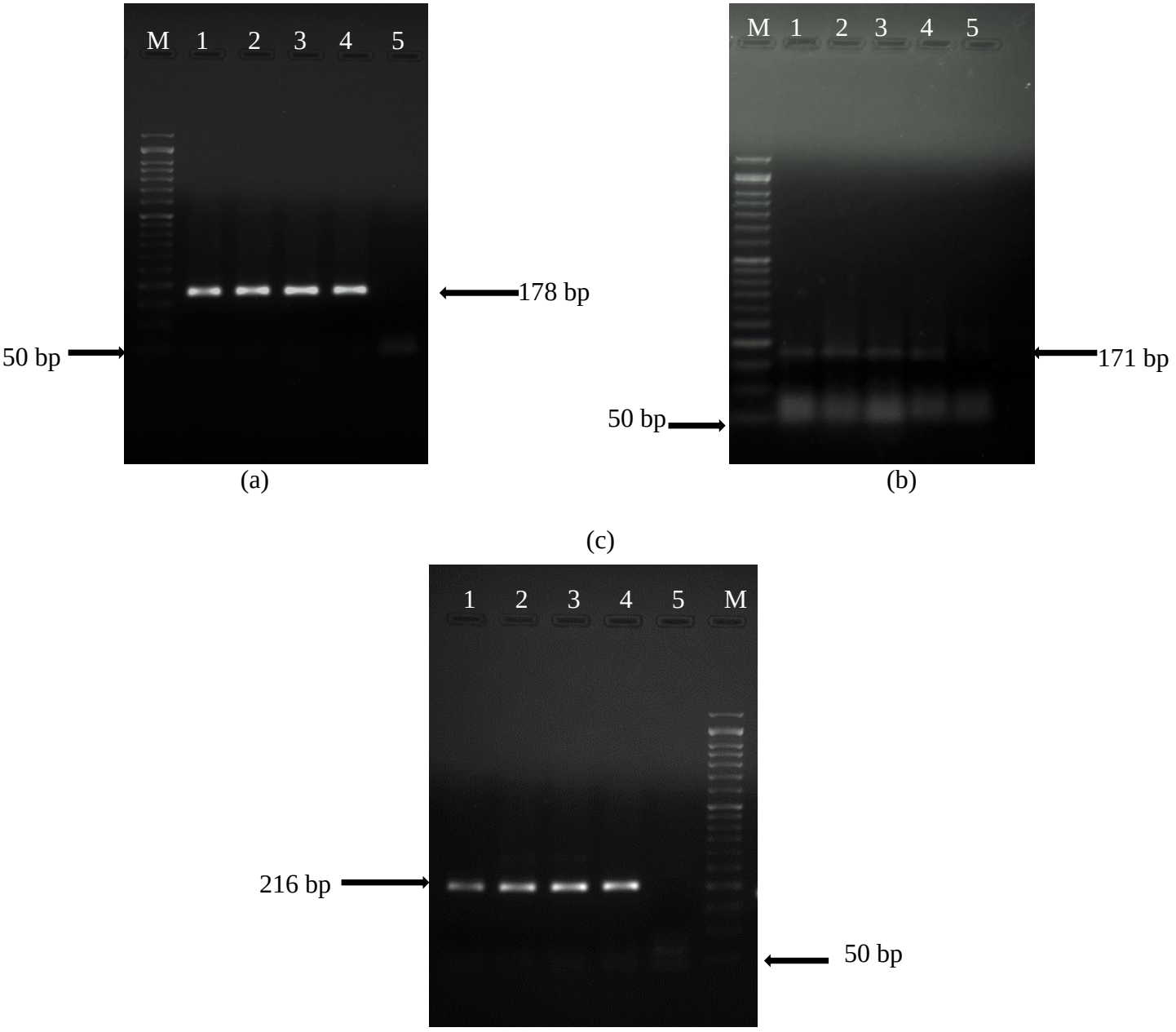
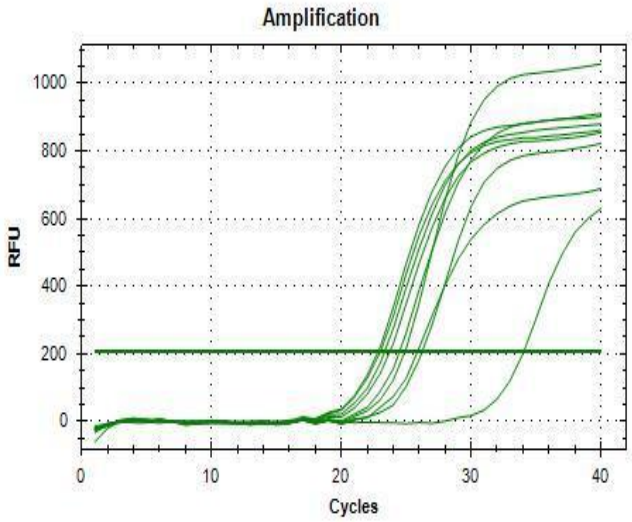


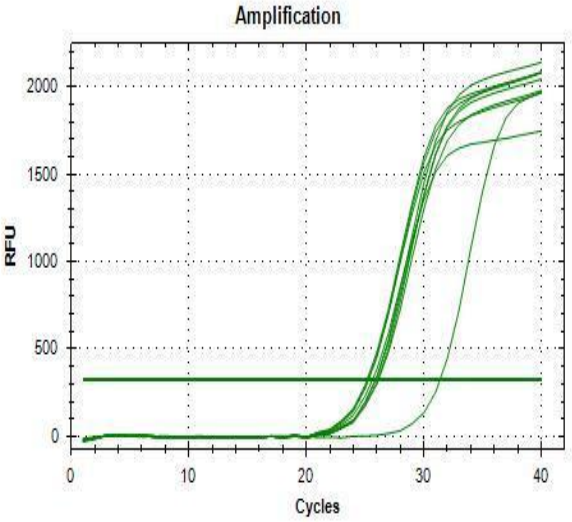

**Plate 4. Amplification of target genes of miRNAs**

a. **F Box Family Protein (target of miR-5417)**
b. **Patatin like Phospholipase Family Protein (target of miR-1634**
c. **Probable xyloglucan endotransglucosylase/hydrolase protein 33 (target of miR-67)**

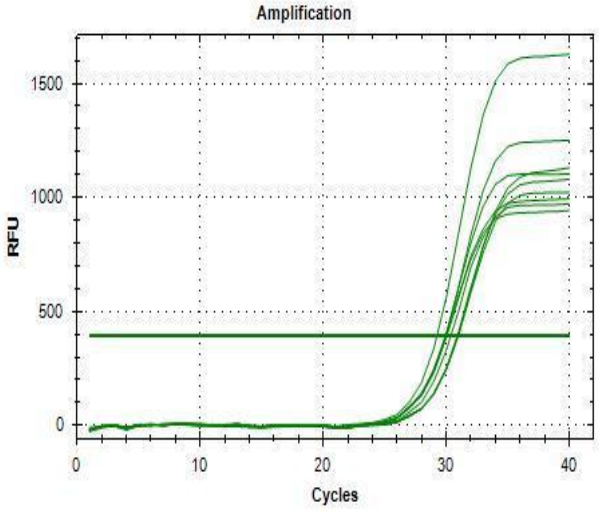
**Lane M: 50bp ladder, Lane 1, 2: uninfected control samples, Lane 3, 4: BBrMV infected samples, Lane 5: Non-template control**




(a) miR-5417

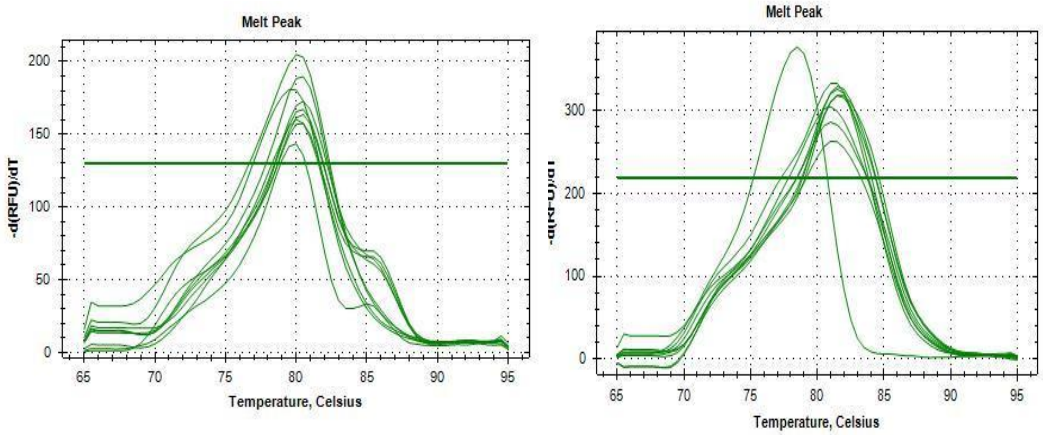



(b) miR-1634

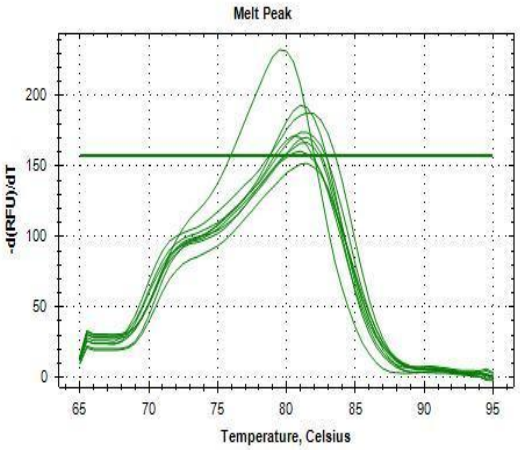



(c) miR-67

**Fig 4. Raw expression data of miRNAs**

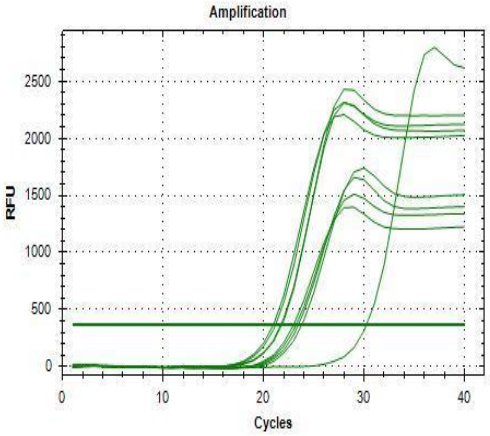



(a) miR-5417 (b) miR-1634

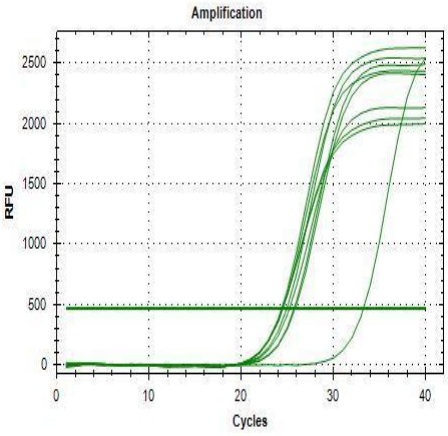



(c) miR-67

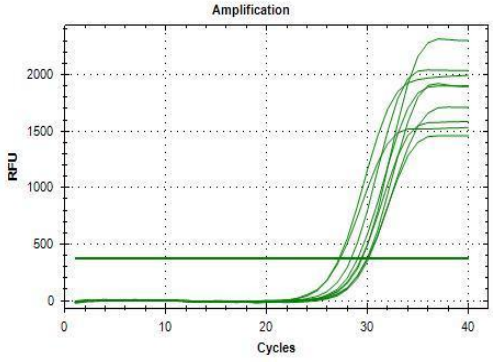
**Fig 5. Melt peak of miRNAs**



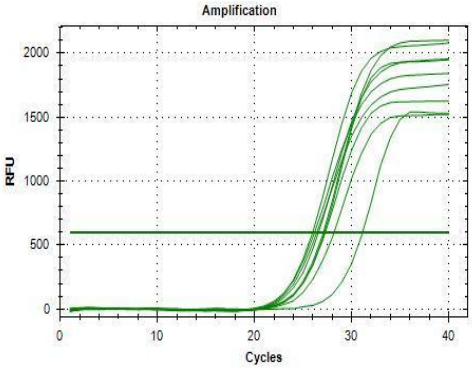

(a) β-actin




(b) F Box Family Protein genes

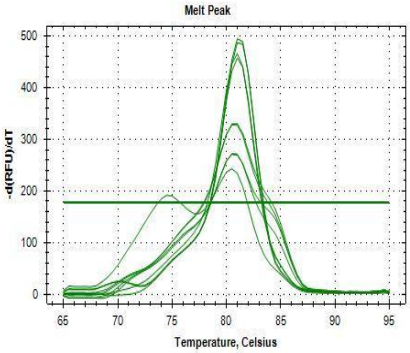



(c) Patatin Like Phospholipase Family Protein genes

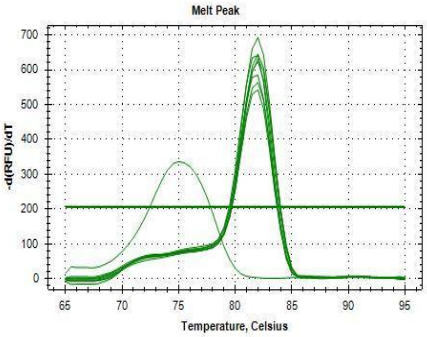



(d) Probable xyloglucan endotransglucosylase/hydrolase protein 33 genes

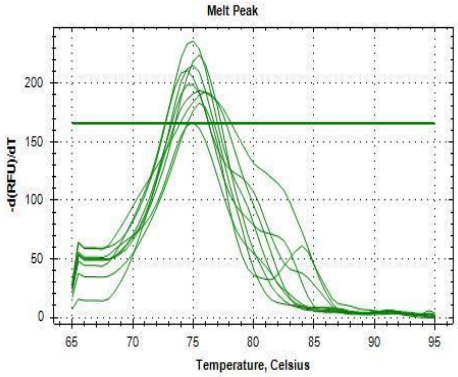
**Fig 6. Raw expression data of target genes**



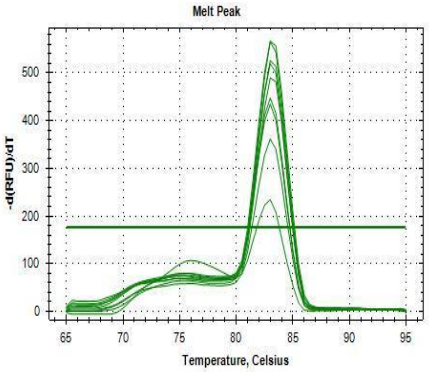

(a) β-actin




(b) F Box Family Protein genes




(c) Patatin Like Phospholipase Family Protein genes




(d) Probable xyloglucan endotransglucosylase/hydrolase protein 33 genes

**Fig 7. Melt peak of target genes**

**Table 7. Cq values of miRNAs**

| SAMPLE | miR-5417 | miR-1634 | miR-67 |
|---|---|---|---|
| Control (C1) | 23.51 | 26.11 | 30.11 |
| Control (C2) | 22.89 | 25.39 | 29.13 |
| Infected (Br 1) | 26.02 | 25.89 | 29.30 |
| Infected (Br 2) | 24.66 | 25.26 | 29.64 |

**Table 8. Cq values of target genes**

| SAMPLE | β-actin | F Box | PAT | HP |
|---|---|---|---|---|
| Control (C1) | 21.77 | 25.75 | 33.04 | 26.72 |
| Control (C2) | 21.08 | 25.06 | 31.78 | 26.22 |
| Infected (Br 1) | 23.11 | 24.45 | 32.80 | 25.27 |
| Infected (Br 2) | 23.63 | 24.41 | 29.71 | 25.34 |

#### *4.3.5.3 Relative Expression of miRNAs and Their Target Genes*

The Cq values of all the RT-qPCR assays for both control and infected samples were analysed by qBase plus software. The software generated comparative expression values of miRNAs and their target genes with respect to the control plants. The Cq values of the target genes were normalized using the Cq values of β-actin (housekeeping gene). The relative expression values ranging from 0.4-1.6 was observed for miR-5417, 2.9-6.5 was observed for miR-1634, and 4.4-5.04 was observed for miR-67, which are shown in Table 9 and Table 10. The values are represented in graphs and the comparative analysis is shown in (Fig. 8, Fig. 9, and Fig. 10) respectively.

**Table 9. NRQ values of miRNAs**

| SAMPLE | miR-5417 | miR-1634 | miR-67 |
|---|---|---|---|
| Control (C1) | 1.00 | 1.00 | 1.00 |
| Control (C2) | 0.955921 | 1.022848 | 1.23 |
| Infected (Br 1) | 0.445681 | 2.950107 | 4.46 |
| Infected (Br 2) | 1.632415 | 6.524631 | 5.04 |

**Table 10. NRQ values of target genes**

| SAMPLE | β-actin | F Box | PAT | HP |
|---|---|---|---|---|
| Control (C1) | 1.00 | 1.00 | 1.00 | 1.00 |
| Control (C2) | 1.00 | 1.008 | 0.881372 | 0.88 |
| Infected (Br 1) | 1.00 | 6.275159 | 5.074211 | 6.95 |
| Infected (Br 2) | 1.00 | 9.247948 | 10.87555 | 9.45 |

The three miRNAs selected for the study, miR-5417, miR-1634, and miR-67 and their corresponding target genes F-box family protein, Patatin like phospholipase family protein and Probable xyloglucan endotransglucosylase/hydrolase protein 33 showed expression in both uninfected and infected samples. The infected plants showed an increased expression of miR-1634 (2-6 fold) and miR-67 (4-5 fold) compared to uninfected control. However, miR-5417 did not show much difference in its expression in these samples.

F-box family protein (target of miR-5417), Patatin like phospholipase family protein (target of miR-1634) and Probable xyloglucan endotransglucosylase/hydrolase protein 33 (target of miR-67) were found upregulated in all the *bract mosaic virus* infected samples. The maximum upregulation was shown by Probable xyloglucan endotransglucosylase/hydrolase protein 33 (6.9-9.4 fold). Patatin like phospholipase

family protein gene expression was increased by 5.0-10.8 fold and a 6.3 to 9.2 fold increase was noticed in F-box family protein gene compared to the control sample. F-Box family protein showed an inverse coreation with miR-5417, by showing an upregulation with decreased miRNA expression.

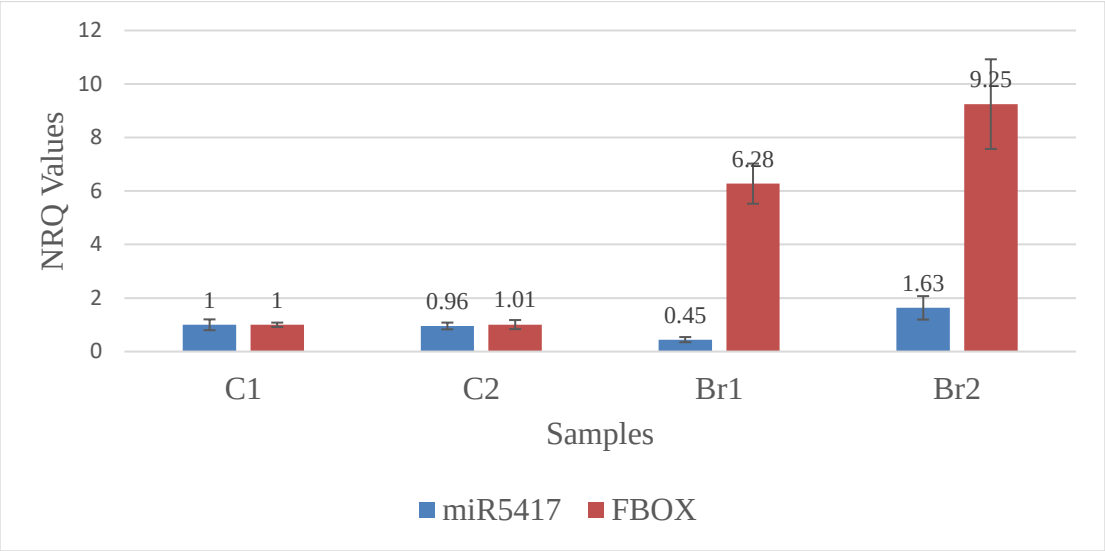



**Fig. 8. Relative expression of miR-5417 and its target F Box family protein gene**

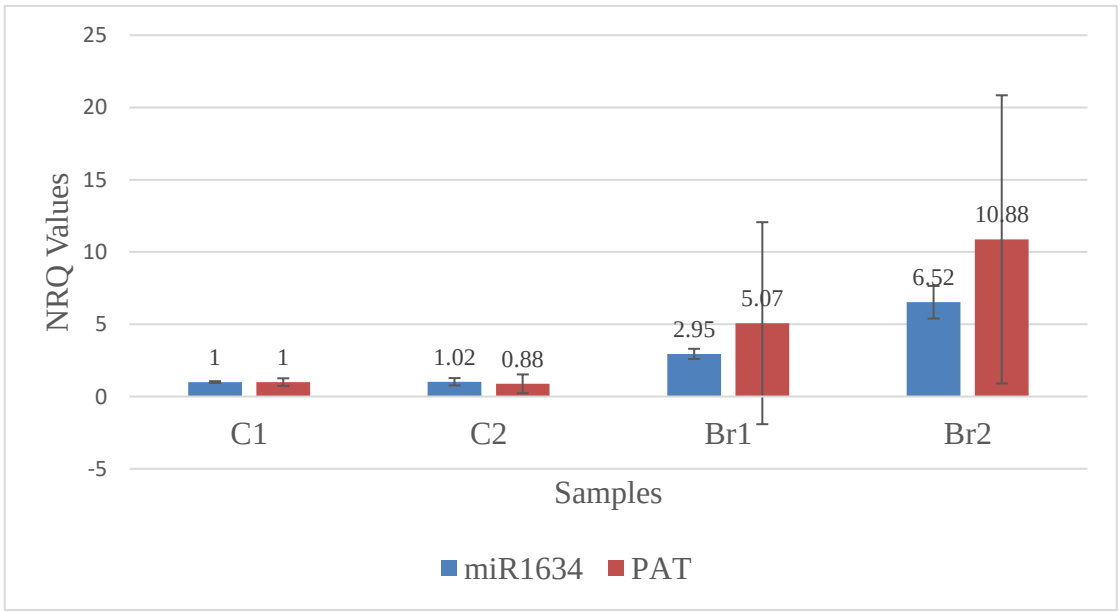



**Fig. 9. Relative expression of miR-1634 and its target Patatin Like Phospholipase Family Protein genes (PAT)**

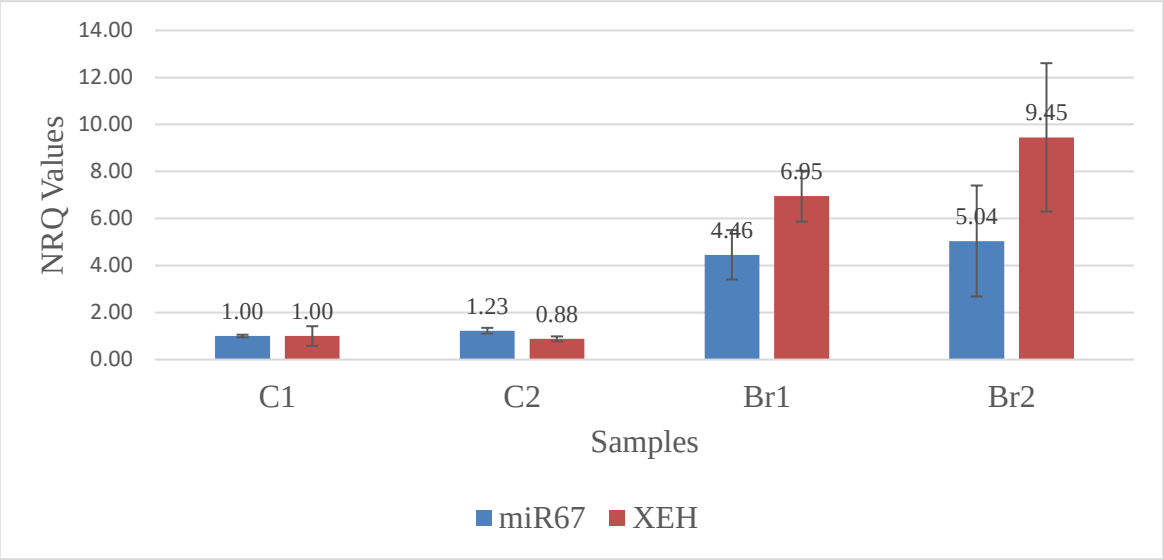



**Fig. 10. Relative expression of miR-67 and its target Probable xyloglucan endotransglucosylase/hydrolase protein 33 gene (XEH)**

# *Discussion*

# 5. DISCUSSION

Banana is one of the most important food crops all over the world. It is a tropical fruit crop grown over 120 countries and is an important food security crop. The global production was 116 million tonnes in 2018 and the revenue of the global banana industry was estimated to be USD 8 billion per year. In the least developed and low-income food-deficit countries bananas provide up to 25 per cent of the daily calorie intake. The global harvested area has been increased from 3.6 million ha in 1993 to 4.6 million ha in 2000 to 5.6 million ha in 2017. India and China are the leading producers contributing 28 per cent of global production (FAOSTAT, 2019).

The major constraints that affect the production and productivity of banana include both biotic and abiotic factors. The major abiotic factors include water stress, salinity, drought and other environmental and climatic factors. Among them, viruses pose a major threat and cause considerable economic loss. *Banana bract mosaic virus* is an important pathogen causing bract mosaic disease (Kokkan disease) in bananas. In India, the disease was first reported in Thrissur district of Kerala in cultivar Nendran and later it spreads to all other cultivars. All the cultivars are highly susceptible to the pathogen. A yield loss of 40 per cent is reported in some cultivars (Thomas and Magnaye, 1996). In Kerala, 52 per cent yield loss is reported in cv. Nendran (Cherian *et al.,* 2002). Since there is no source of natural resistance the conventional breeding methods are not suitable for developing disease resistance.

Plants have evolved various mechanisms to compact the viral diseases. Recent studies have shown the change in the expression pattern of endogenous microRNAs when the plant encounters any biotic stress. The miRNAs play important role in defense response as well as the normal biological processes in plants and miRNA mediated gene silencing approaches have been widely studied, as it is a novel strategy involved in post transcriptional gene regulation (Sunkar *et al.,* 2007).

The microRNA mediated gene silencing is a novel approach gaining popularity for imparting virus resistance in crops. MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that functions in RNA silencing and post transcriptional regulation of gene expression which is achieved either by targeting mRNA for cleavage or inhibiting translation (Brodersen *et al.,* 2008). They are usually 21-25 nucleotide

ssRNAs which are produced from hairpin shaped precursors. They are known to regulate gene expression during abiotic and biotic stresses in plants.

In banana, very few studies have been conducted to understand the role of miRNAs in biotic stress. During *Banana streak mysore virus* (BSMysV) infection in banana the expression of miR156, miR159 and miR166 were found to be upregulated (Sheeba *et al.,* 2013). Ghag *et al*. (2015) analyzed the small RNA expression profiles of two banana cultivars Grand Naine and Rasthali using high-throughput sequencing technology and identified a total of 170 and 244 miRNAs in their libraries. Several other cultivar specific microRNAs along with their putative target transcripts were also detected. Sankaranarayanan *et al.* (2020) reported that miRNA2 is 100 per cent conserved during BBrMV infection and it has many targets related to virus-specific functions. Identification of miRNAs and their target genes which are differentially expressed during virus infection will serve to develop biotechnological approaches to impart virus resistance in banana.

Mathew (2018) predicted 52 mature miRNAs from the banana genome using NOVOMIR and their target genes were identified using psRNATarget software. From them, 5 miRNAs and their target genes were validated in tissue culture banana plants to identify virus responsive ones. In the present study, 3 miRNAs and their target genes were analysed for their expression in BBrMV infected field banana cv. Nendran. The *bract mosaic virus* infected plants were selected for the study based on the visual symptoms such as spindle shaped reddish-brown streaks on the pseudostem, mosaic patterns on leaves etc. Plants not showing any such symptoms served as control. The infection was further confirmed by RT-PCR using coat protein gene specific primers. All the plants showing BBrMV symptoms showed coat protein gene specific amplicons of size 745 bp. The miRNAs and their target genes selected for the study were: miR5417 (target: F Box family protein), miR-1634 (target: Patatin like phospholipase family protein) and miR-67 (target: Probable xyloglucan endotransglucosylase/hydrolase protein 33).

The RNA was isolated using the modified Rodrigues-Garcia protocol (Ekatpure *et al*., 2019). Quality of RNA isolated was found to be good from the gelprofile and

spectrophotometric measurements. They were then converted to cDNA using gene specific primers for target genes and stem-loop primers for miRNAs. miRNAs are smaller in size and are difficult to isolate, but stem-loop primers lengthen then for its successful conversion to cDNA. All the primres designed for the three selected mirnas and their target genes showed single prominent peaks in melt curve analysis indicating the efficiency of primers for amplification of the genes. They could amplify products of expected size.

The Relative expression of the 3 miRNAs and their target genes in BBrMV infection was studied by conducting RT-qPCR. The results of RT-qPCR were analysed with qBase plus software by normalizing with the housekeeping gene β-actin with reference to uninfected control plants. All the selected miRNAs and their target genes showed expression in both control and infected plants. Out of the three miRNAs studied, miR-67 and miR-1643 were found to be upregulated in all the BBrMV infected samples compared to uninfected control. The maximum increase was shown by miR-67 (4.46-5.04 fold) followed by miR-1634 (2.95-6.52 fold).

The target genes of the three miRNAs showed elevated expression in the BBrMV infected samples. At the annealing temperature of 55ºC, the target of miR-1634, Patatin like phospholipase family protein did not get amplified. By doing gradient PCR it was found to be amplified at 63ºC.

The target gene of miR-5417, F Box family protein showed an increased expression in the infected samples. miR-5417 showed a marginal increase (0.4 to 1.6 fold) in the infected samples compared to the control whereas its target gene F Box family protein showed 6.3 and 9.2 fold increased expression. Here a negative correlation was observed between the miRNA and its target. As the miRNA level decreased the corresponding increase in the target gene was observed when compared to control plants. The target gene F Box family protein is known to be involved in virus mediated gene silencing in plants. The genes which are closely related to F Box family protein regulates plant hormone signalling (Yu *et al.*, 2007). They contribute to defense response *via* methyl- jasmonate and abscisic acid responsive genes (Vandenburg *et al*., 2008). Both jasmonic acid and abscisic acid play a vital role in plant immune response

(Santner and Estelle, 2009; He *et al*., 2020). Lechner *et al.* (2006) reported that the Faba bean necrotic yellow virus protein CELL CYCLE LINK (CLINK) contains an F-box motif and binds to the suppressor of gene expression. In *Arabidopsis* the polerovirus P0 protein interacts with the *Arabidopsis* ASK1 through its F-box motif and leads to reduced suppressor activity and diminished virus pathogenicity. The gene silencing modulates through interaction with SCF complexes (Pazhouhandeh *et al*., 2006). Also, the protein P0 promotes the degradation of ARGONAUTE1 (AGO1), a key component of the RNA induced silencing complex (RISC) (Ho *et al.*, 2008). By maintaing a lower expression level, the miR-5417 may be reulating the expression of the F-box family protein, for initiating a defense mechanism by the plants towards the virus.

The Patatin like Phospholipase family protein gene, the target of miR-1634 showed an increased expression of 5.0 and 10.8 fold in the infected samples. Here, the level of miRNA showed an increase of 2.9 to 6.5 fold in both the infected samples compared to the uninfected control. Both the miRNA as well as the target gene increased proportionally, contradictory to the expected miRNA target relation. Patatin like Phospholipase family protein (PLPs) are known to be involved in plant stress pathways both during abiotic and biotic stresses. Overproduction of the protein is seen during virus infection (Xu and Huang, 2018). Subr *et al*. (2020) showed that during *Cucumber mosaic virus* (CMV) infection in two cucumber cultivars, sensitive cv. Vanda and resistant cv. Heliana, a 3-fold fall of expression of PLPs in cv. Heliana and 29-fold fall in the sensitive cv. Vanda was observed, which showing a possible involvement of patatin in plant stress pathways. The overexpression of pPLA-IIa enhanced plant cell death and resistance against *Cucumber mosaic virus* (Scherer *et al.*, 2010).

In the case of miR-67, both the target as well as the miRNA were found to be highly expressed in the infected plants. The gene encoding probable xyloglucan endotransglucosylase/hydrolase protein 33, the target of miR67 showed a drastic increase of 6.9 and 9.4 fold in the *bract mosaic virus* infected plants compared to uninfected control. Similarly, the miR-67 also showed an upregulation of 4.46-5.04 fold in the BBrMV infected samples. The xyloglucan endotransglucosylase/hydrolase

proteins are tissue-specific and respond differentially to hormonal signals like abscisic acid (Wu *et al.*, 1994), ethylene (Saab and Sachs, 1996), brassinosteroids (Zurek and Clouse, 1994), gibberellins (Genovesi *et al.*, 2008) and auxins (Potter and Fry, 1994). When the plant encounters a stress, the protein gets upregulated which affects the biomechanical properties of plant cell walls and improves the plant's response to stress (Saladie *et al.*, 2006). The upregulation of this gene in the infected samples may be the plants response to protect the damaging effect on the cellwall. But an expected negative correlation between the miRNA and the target could not be established.

This study was successful in validating the three computationally predicted miRNAs and finding out their response towards BBrMV infection in banana cv. Nendran. The miRNAs, miR-5417, miR-1643 and miR-67 showed differential expression in the plants infected with BBrMV. While miR- 5417 was downregulated, the others showed upregulation during infection. An expected inverse corelation could be observed between miR-5417 and its target indicating its possible role in biotic stress response. The exact role of miR-1643 and miR-67 can be found out by studying the other identified targets.

# *Summary*

# 6. SUMMARY

The study entitled “Identification and expression profiling of *Banana bract mosaic virus* (*BBrMV*) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)” was conducted in the Department of Plant Biotechnology, College of Agriculture Vellayani, Thiruvananthapuram during 2018-2020. The objective of the study was to validate computationally predicted selected microRNAs (miRNAs) in banana cultivar Nendran (*Musa* AAB) and to study their expression in *Banana bract mosaic virus* (BBrMV) infection.

Banana is the fourth most important crop after the major cereal crops. It is the most consumed fruit in the world. In India, it is the second most-consumed fruit after mango. India is the leading producer of banana contributing 25.7 per cent of global production. There are more than 50 different cultivars grown in India. It is grown in different parts of the world and is an important food security crop. Abiotic and biotic stresses are the major constraints in banana cultivation. Among them, viruses are the most damaging, which are comparatively difficult to manage than fungal or bacterial diseases. The major viral diseases of banana include *Banana bunchy top virus*, *Banana bract mosaic virus*, *Banana streak virus*, *Cucumber mosaic virus*, etc. Among them, BBrMV is a deadly virus transmitted by aphids causing a loss of up to 40 per cent. The majority of the cultivated varieties are highly susceptible to the virus infection and there is no source of natural resistance. Hence, conventional methods fail to develop resistance against these viruses. Therefore, different biotechnological approaches have been tried like transgenics approach in developing virus resistance (Collinge *et al.*, 2010; Simon -Mateo and Garcia, 2011). The major drawback of this technology is the cost involved, gene flow, change in vector specificity as well as continuous mutation of the virus making the transgenic plant ineffective in coping with the viral infection (Tepfer, 2002).

RNA interference technology (RNAi) is a very promising technology in this context (Noris *et al*., 2004). It is a biological process by which RNA molecules inhibit the gene expression or translation by the destruction of their mRNA intermediaries. Small RNA mediated post transcriptional gene silencing using siRNA and miRNA

proves to be effective in imparting resistance to stresses in plants (Tripathi *et al*., 2007). The miRNA mediated gene silencing is a novel strategy to develop resistance against various stresses. Studies have shown the presence of certain miRNAs during abiotic and biotic stresses in plants (Khraiwesh *et al*., 2012), which are differentially expressed under stress conditions. This study aimed to validate the expression of the computationaly predicted miRNAs and their target genes during BBrMV infection in banana cv. Nendran using RT-qPCR. The salient findings of the study are summarized below:

From a list of 52 computationally predicted mature miRNAs, 3 miRNAs and their corresponding target genes were selected based on their targets and their possible role in biotic stress conditions. The miRNAs selected for the study were: miR-5417 (target: F Box family protein), miR- 1634 (target: Patatin like phospholipase family protein) and miR- 67 (target: Probable xyloglucan endotransglucosylase/hydrolase protein 33).

For studying the expression analysis of miRNAs during *Bract mosaic virus* infection in banana, leaf samples were collected from the bract mosaic infected and uninfected plants selected from the field based on the visual symptoms. The presence of BBrMV was confirmed in the infected samples by performing RT-PCR using coat protein gene specif primers. Expression analysis of the moRNAs and their target genes studied by performimg RT-qPCR and the results were analysed by using qBase plus software. The miRNAs and their target genes were studied with reference to the uninfected control plants and the Raw Cq values obtained were normalized using a reference gene β-actin to study the relative expression.

RT-qPCR analysis confirmed the presence of all the three computationally predicted miRNAs in banana cv. Nendran. All the BBrMV infected samples showed an elevated level of miR-1634 and miR-67 compared to the uninfected control. No considerable change in the expression of miR-5417 was observed in the infected samples.

The F Box family protein (target of miR-5417) showed higher expression in the infected plants when the miRNA was downregulated, establishing an inverse correlation between the miRNA and its target. The Patatin like phospholipase family

protein (target of miR-1634) and Probable xyloglucan endotransglucosylase/hydrolase protein 33 (target of miR- 67) also showed higher expression in the infected plants compared to the control plants. But the miRNA and the target levels seemed to be increasing proportionately, without showing an expected negative correlation.

The study showed that all the three miRNAs (miR-5417, miR-67 and miR-1634) respond to BBrMV infection and have a role in stress response of the plants. All the target genes showed upregulation in the BBrMV infected samples, showing their role in biotic stress response. Only miR-5417 showed an inverse correlation with its target.

# *References*

## 7. REFERENCES


Akamatsu, T., Hanzawa, Y., Ohtake, Y., Takahashi, T., Nishitani, K., and Komeda, Y. 1999. Expression of endoxyloglucan transferase genes inacaulis mutants of Arabidopsis. *Plant Physiol*. 121(3): 715-722.

Allawi, H. T., Dahlberg, J. E., Olson, S., Lund, E., Olson, M., Ma, W. P., Takova, T., Neri, B. P., and Lyamichev, V. I. 2004. Quantitation of microRNAs using a modified Invader assay. *RNA* 10 (7): 1153–1161. pmid: 15208450.

Allmer, J. 2014. Computational and bioinformatics methods for microRNA gene prediction. In *miRNomics: MicroRNA Biology and Computational Analysis*. Clifton, N. J., Humana Press, Totowa. 157-175p.

Axtell, M. J., and Meyers, B. C. 2018. Revisiting criteria for plant microRNA annotation in the era of big data. *Plant Cell* 30(2): 272-284.

Azad, M., Kalam, A., Rabbani, M., Amin, L., and Sidik, N.M. 2013. Development of transgenic papaya through agrobacterium-mediated transformation. *Int. J. Genomics* 2013:1-6.

Babak, T., Zhang, W., Morris, Q., Blencowe, B. J., and Hughes, T. R. 2004. Probing microRNAs with microarrays: Tissue specificity and functional inference. *RNA* 10:1813–1819. pmid:15496526.

Baulcombe, D. 2004. RNA silencing in plants. *Nature* 431(7006): 356-363.

Baykal, U. 2020. Development of a sensitive primer extension method for direct detection and quantification of miRNAs from plants. *PLoS One* 5(3): 1-9. e0230251. https://doi.org/10.1371/journal.pone.0230251.

Baykal, U., Liu, H., Chen, X., Nguyen, H. T., and Zhang, Z. J. 2016. Novel constructs for efficient cloning of sRNA-encoding DNA and uniform silencing of plant genes employing artificial trans-acting small interfering RNA. *Plant Cell Rep*. 35: 2137–2150. pmid:27417696.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Sayers, E. W. 2012. *GenBank. Nucleic Acids Res.* 41(D1): D36–D42. doi:10.1093/nar/gks1195

Bi, F., Meng, X., Ma, C., and Yi, G. 2015. Identification of miRNAs involved in fruit ripening in Cavendish bananas by deep sequencing. *BMC Genomics* 16(1): 776p.

Bivalkar-Mehla, S., Vakharia, J., Mehla, R., Abreha, M., Kanwar, J. R., Tikoo, A., and Chauhan, A. 2011. Viral RNA silencing suppressors (RSS): novel strategy of viruses to ablate the host RNA interference (RNAi) defense system. *Virus Res*. 155(1): 1-9.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. *Science* 320 (5880): 1185-1190.

Canonne, J., Froidure-Nicolas, S., and Rivas, S. 2011. Phospholipases in action during plant defense signaling. *Plant Signal. Behav.* 6(1): 13-18.

Chai, J., Feng, R., Shi, H., Ren, M., Zhang, Y., and Wang, J. 2015. Bioinformatic identification and expression analysis of banana microRNAs and their targets. *PLoS One* 10(4). e0123083.

Chauhan, S., Yogindran, S., and Rajam, M. V. 2017. Role of miRNAs in biotic stress reactions in plants. *Indian J. Plant Physiol.* 22(4): 514-529.

Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., Barbisin, M., Xu, N. L., Mahuvakar, V. R., Andersen, M. R., and Lao, K. Q. 2005. Real-time quantification of microRNAs by stem–loop RT–PCR. *Nucleic Acids Res.* 33(20): e179 https://doi.org/10.1093/ nar/gni178.

Chen, X. 2005. MicroRNA biogenesis and function in plants. *FEBS lett.* 579(26): 5923-5931.

Cherian, K. A., Menon, R., Suma, A., Nair, S., and Sudeesh, M. V. 2002. Effect of banana bract mosaic disease on yield of commercial varieties in Kerala (*Abstr*.) In: *Global conference on banana and plantain*, Bangalore, 155p.

Cisneros, A. E., and Carbonell, A. 2020. Artificial small RNA-based silencing tools for antiviral resistance in plants. *Plants* 9(6): 669p.

Collinge, D. B., Jorgensen, H. J., Lund, O. S., and Lyngkjaer, M. F. 2010. Engineering pathogen resistance in crop plants: current trends and future prospects. *Annu. Rev. Phytopathol*. 48: 269-291.

Cui, C., Wang, J. J., Zhao, J. H., Fang, Y. Y., He, X. F., Guo, H. S., and Duan, C. G. 2020. A brassica miRNA regulates plant growth and immunity through distinct modes of action. *Mol. Plant*. 13(2): 231-245.

Davey, M. W., Gudimella, R., Harikrishna, J. A., Sin, L. W., Khalid, N. and Keulemans, J. 2013. A draft *Musa balbisiana* genome sequence for molecular genetics in polyploid, inter-and intra-specific *Musa* hybrids. *BMC Genomics* 14(1): 683p. https://doi.org/10.1186/1471-2164-14-683.

Dehury, B., Panda, D., Sahu, J., Sahu, M., Sarma, K., Barooah, M., Sen, P., and Modi, M. K. 2013. In silico 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): p.e26543.

D'hont, A., Denoeud, F., Aury, J. M., Baurens, F. C., Carreel, F., Garsmeur, O., Noel, B., Bocs, S., Droc, G., Rouard, M., and Da Silva, C. 2012. The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* 488(7410): 213-217.

D'hondt, S., Geoffroy, P., Stelmach, B. A., Legrand, M., and Heitz, T. 2000. Soluble phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves and is contributed by patatin-like enzymes. *The Plant J.* 23(4): 431-440.

Dietzgen, R. G., Mann, K. S., and Johnson, K. N. 2016. Plant virus–insect vector interactions: current and potential future research directions. *Viruses* 8(11): 303p.

Dong, Q. H., Han, J., Yu, H. P., Wang, C., Zhao, M. Z., Liu, H., Ge, A. J., and Fang, J. G. 2012. Computational identification of microRNAs in strawberry expressed sequence tags and validation of their precise sequences by miR-RACE. *J. Hered.* 103(2):268-277.

Droc, G., Lariviere, D., Guignon, V., Yahiaoui, N., This, D., Garsmeur, O., Dereeper, A., Hamelin, C., Argout, X., Dufayard, J.F., Lengelle, J., Baurens, F. C., Cenci, A., Pitollat, B., D'hont, A., Ruiz, Rouard, M., and Bocs, S. 2013. The banana genome hub. *Database* 2013: bat035 https://doi.org/10.1093/database/bat035.

Du, P., Wu, J., Zhang, J., Zhao, S., Zheng, H., Gao, G., Wel, L., and Li, Y. 2011. Viral infection induces expression of novel phased micro RNA from conserved cellular micro RNA precursors. *PLoS pathog*. 7(8): 1-14. http://doi.org/10.1371/journal. ppat. 1002176.

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. *Indian J. Agric. Sci*. 11(6): 8096-8099.

Elvira-Gonzalez, L., Medina, V., Rubio, L., and Galipienso, L. 2020. The persistent *southern tomato virus* modifies miRNA expression without inducing symptoms and cell ultra-structural changes. *Eur. J. Plant Pathol*. 156(2): 615-622.

FAOSTAT [Food and agriculture organization statistics]. 2019. FAOSTAT home page [online]. Available: http://www.fao.org/economic/est/est-commodities/bananas/en/.

Filipowicz, W., Jaskiewicz, L., Kolb, F. A., and Pillai, R. S. 2005. Post-transcriptional gene silencing by siRNAs and miRNAs. *Curr. Opin. Struct. Biol*. 15(3): 331-341.

Fu, M. M., Liu, C., and Wu, F. 2019. Genome-wide identification, characterization and expression analysis of xyloglucan endotransglucosylase/hydrolase genes family in barley (*Hordeum vulgare*). *Molecules* 24(10):1935p.

Genovesi, V., Fornale, S., Fry, S. C., Ruel, K., Ferrer, P., Encina, A., Sonbol, F. M., Bosch, J., Puigdomenech, P., Rigau, J., and Caparros-Ruiz, D. 2008. ZmXTH1, a new xyloglucan endotransglucosylase/hydrolase in maize, affects cell wall structure and composition in *Arabidopsis thaliana*. *J. Exp. Bot*. 59(4): 875-889.

Ghag, S. B., Shekhawat, U. K. S., 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): e0127179 https://doi.org/10.1371/journal.pone.0127179.

Hamilton, A., Voinnet, O., Chappell, L., and Baulcombe, D. 2002. Two classes of short interfering RNA in RNA silencing. *EMBO J.* 21(17): 4671-4679.

Hartig, J. S., Grune, I., Najafi-Shoushtari, S. H., and Famulok, M. 2004. Sequence-specific detection of MicroRNAs by signal-amplifying ribozymes. *J. Am. Chem. Soc*. 126: 722–723. pmid:14733539.

He, L., Chen, X., Yang, J., Zhang, T., Li, J., Zhang, S., Zhong, K., Zhang, H., Chen, J., and Yang, J. 2020. *Rice black-streaked dwarf virus*-encoded P5-1 regulates the ubiquitination activity of SCF E3 ligases and inhibits jasmonate signaling to benefit its infection in rice. *New Phytol*. 225(2): 896-912.

Ho, M. S., Ou, C., Chan, Y. R., Chien, C. T., and Pi, H. 2008. The utility F-box for protein destruction. *Cell. Mol. Life Sci.* 65(13): 1977–2000.

Hutvagner, G., and Zamore, P. D. 2002. A microRNA in a multiple turnover RNAi enzyme complex. *Science* 297:2056–2060.

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 Agriculture University, Thrissur 67-68p.

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

Jang, J. H., Nguyen, N. Q., Legeret, B., Beisson, F., Kim, Y. J., Sim, H. J., and Lee, O. R. 2020. Phospholipase pPLAIIIα increases germination rate and resistance to *turnip crinkle virus* when overexpressed. *Plant Physiol*. 53p. doi: https://doi.org/10.1104/pp.20.00630.

Jarosova, J., Singh, K., Chrpova, J., and Kundu, J. K. 2020. Analysis of small RNAs of barley genotypes associated with resistance to *barley yellow dwarf virus. Plants* 9(1): 60p.

Jiang, F., Song, Y., Han, Q., Zhu, C., and Wen, F. 2011. The choice of target site is crucial in artificial miRNA-mediated virus resistance in transgenic *Nicotiana tabacum*. *Physiol. Mol. Plant Path.* 76(1): 2-8.

Jones-Rhoades, M. W., and Bartel, D. P. 2004. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* 14(6): 787-799.

Kavanagh, T. A., and Spillane, C. 1995. Strategies for engineering virus resistance in transgenic plants. *Euphytica* 85(1-3): 149-158.

Khan-Malek, R., and Wang, Y. 2017. Statistical analysis of quantitative RT-PCR results. In *Drug Safety Evaluation.* Humana Press, New York, NY. 281-296p.

Khraiwesh, B., Zhu, J. K., and Zhu, J. 2012. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochim. Biophys. Acta. Gene. Regul. Mech.* 1819(2): 137-148.

Kidner, C. A., and Martienssen, R. A. 2005. The developmental role of microRNA in plants. *Curr. Opin. Plant Biol.* 8(1): 38-44.

Kim, D. S., Jeun, Y., and Hwang, B. K. 2014. The pepper patatin-like phospholipase *CaPLP1* functions in plant cell death and defense signaling. *Plant. Mol. Biol.* 84(3): 329-344.

Kim, H. S., and Delaney, T. P. 2002. Arabidopsis SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. *The Plant Cell*. 14(7): 1469-1482.

Kim, J., and Somers, D. E. 2010. Rapid assessment of gene function in the circadian clock using artificial microRNA in *Arabidopsis* mesophyll protoplasts. *Plant physiol.* 154(2): 611-621.

King, A. M., Adams, M. J., Carstens, E. B., and Lefkowitz, E. J. 2012. Virus taxonomy. *Ninth report of the International Committee on Taxonomy of Viruses* 486-487p.

Kiranmai, G., Kumar, P. L., Hema, M., Venkatramana, M., Kirshna-Prasadji, M. R., and Sreenivasalu, P. 2005. Partial characterization of a *potyvirus* causing bract mosaic of banana in Andhra Pradesh. *Indian J. Virol.* 16: 7-11.

Kozomara, A., Birgaoanu, M., and Griffiths-Jones, S. 2019. miRBase: from microRNA sequences to function. *Nucleic Acids Res.* 47(D1): D155-D162.

Kozomara, A., and Griffiths-Jones, S. 2010. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39(suppl_1): D152-D157.

Kozomara, A., and Griffiths-Jones, S. 2014. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42(D1): D68-D73.

Kramer, M. F. 2011. Stem-loop RT-qPCR for miRNAs. *Curr. Protoc. Mol. Biol*. 95(1): 15-10.

Kriznik, M., Baebler, S., and Gruden, K. 2020. Roles of small RNAs in the establishment of tolerant interaction between plants and viruses. *Curr. Opin. Virol.* 42: 25-31.

Kriznik, M., Petek, M., Dobnik, D., Ramsak, Z., Baebler, S., Pollmann, S., Kreuze, J. F., Zel, J., and Gruden, K. 2017. Salicylic acid perturbs sRNA gibberellin regulatory network in immune response of potato to *Potato virus Y* infection. *Front. Plant Sci*. 8: 2192p.

Kumar, D., Singh, D., Kanodia, P., Prabhu, K. V., Kumar, M., and Mukhopadhyay, K. 2014. Discovery of novel leaf rust responsive microRNAs in wheat and prediction of their target genes. *J. Nucleic Acids* 15:1154-1199.

Lang, Q., Jin, C., Lai, L., Feng, J., Chen, S., and Chen, J. 2011. Tobacco microRNAs prediction and their expression infected with *Cucumber mosaic virus* and *Potato virus X*. *Mol Biol Rep*. 38:1523-1531.

Lechner, E., Achard, P., Vansiri, A., Potuschak, T., and Genschik, P. 2006. F-box proteins everywhere. *Curr. Opin. Plant Biol.* 9(6): 631–638.

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. *The 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., Fan, H., Wang, Y., Han, C., Wang, X., Yu, J., Li, D., and Zhang, Y. 2020. Genome-wide microRNA profiling using oligonucleotide microarray reveals regulatory networks of microRNAs in *Nicotiana benthamiana* during *beet necrotic yellow vein virus infection*. *Viruses* 12(3): 310p.

Liu, J., Jennings, S. F., Tong, W., and Hong, H. 2011. Next generation sequencing for profiling expression of miRNAs: technical progress and applications in drug development. *J. Biomed. Eng.* 4(10): 666p.

Liu, X., Tan, C., Cheng, X., Zhao, X., Li, T., and Jiang, J. 2020. Identification of microRNAs regulated by miR-168a-mediated Argonaute1 in response to potassium deficiency stress in tomatoes. *Authorea Preprints* 17p.

Luigi, M., Manglli, A., Bertin, S., Donati, L., Tomassoli, L., Ferretti, L., and Faggioli, F. 2020. Development and validation of a specific real-time PCR protocol for the detection of *tomato leaf curl New Delhi virus*. *Eur. J. Plant Pathol.* 157(4): 969-974.

Maldonado-Calderon, M. T., Sepulveda-Garcia, E., and Rocha-Sosa, M. 2012. Characterization of novel F-box proteins in plants induced by biotic and abiotic stress. *Plant Sci.* 185: 208-217.

Marmisolle, F. E., Arizmendi, A., Ribone, A., Rivarola, M., Garcia, M. L., and Reyes, C. A. 2020. Up-regulation of microRNA targets correlates with symptom severity in *Citrus sinensis* plants infected with two different isolates of *citrus psorosis virus*. *Planta* 251(1): 7p.

Maroney, P. A., Chamnongpol, S., Souret, F., and Nilsen, T. W. 2007. A rapid, quantitative assay for direct detection of microRNAs and other small RNAs using splinted ligation. *RNA* 13(6):930–936. pmid:17456563.

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 Agriculture University, Thrissur, 120p.

Mohapatra, D., Mishra, S. and Sutar, N. 2010. Banana post-harvest practices: Current status and future prospects-A review. *Agric. Rev*. 31(1): 56-62.

Moore, P. J., and Staehelin, L. A. 1988. Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways. *Planta* 174(4): 433-445.

Moran, F., Sassalou, C. L., Canales, C., Maliogka, V. I., Olmos, A., and Ruiz-Garcia, A. B. 2020. Specific Real-Time PCR for the detection and absolute quantitation of *grapevine roditis leaf discoloration-associated virus,* an EPPO Alert Pathogen. *Plants* 9(9): 1151p.

Nambisan, P. 2017. An introduction to ethical, safety and intellectual property rights issues in biotechnology. Academic Press. 1-357p.

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.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J. D. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312(5772): 436-439.

Nazarov, P. V., Reinsbach, S. E., Muller, A., Nicot, N., Philippidou, D., Vallar, L., and Kreis, S. 2013. Interplay of microRNAs, transcription factors and target genes: linking dynamic expression changes to function. *Nucleic Acids Res.* 41(5): 2817-2831.

Ng, J. C. and Falk, B. W. 2006. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annu. Rev. Phytopathol.* 44: 183-212.

Niu, Q. W., Lin, S. S., Reyes, J. L., Chen, K. C., Wu, H. W., Yeh, S. D., and Chua, N. H. 2006. Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat. Biotechnol.* 24(11): 1420-1428.

Noris, E., Lucioli, A., Tavazza, R., Caciagli, P., Accotto, G. P., and Tavazza, M. 2004. *Tomato yellow leaf curl sardinia virus* can overcome transgene-mediated RNA silencing of two essential viral genes. *J. Gen. Virol.* 85(6): 1745-1749.

Novina, C. D., and Sharp, P. A. 2004. The rnai revolution. *Nature* 430(6996): 161-164.

Numnark, S., Mhuantong, W., Ingsriswang, S., and Wichadakul, D. 2012. C-mii: a tool for plant miRNA and target identification. In *BMC genomics*. 13: S7-S16. https://doi.org/10.1186/1471-2164-13-S7-S16.

Oulas, A., Karathanasis, N., Louloupi, A., Pavlopoulos, G. A., Poirazi, P., Kalantidis, K., and Iliopoulos, I. 2015. Prediction of miRNA targets. In *RNA Bioinformatics*. Humana Press, New York, NY. 207-229p.

Pani, A., and Mahapatra, R. K. 2013. Computational identification of microRNAs and their targets in *Catharanthus roseus* expressed sequence tags. *Genom. Data*, 1: 2-6.

Park, M. Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., and Poethig, R. S. 2005. Nuclear processing and export of microRNAs in Arabidopsis. *Proc. Natl. Acad. Sci.* 102(10): 3691-3696.

Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richards, K. E., Genschik, P., and Ziegler-Graff, V. 2006. F-box-like domain in the *polerovirus* protein P0 is required for silencing suppressor function. *Proc. Natl. Acad. Sci.* 103(6): 1994-1999.

Ponchel, F., Toomes, C., Bransfield, K., Leong, F. T., Douglas, S. H., Field, S. L., Bell, S. M., Combaret, V., Puisieux, A., Mighell, A. J., and Robinson, P. A. 2003. Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnol.* 3(1): 18p.

Potter, I., and Fry, S. 1994. Changes in xyloglucan endotransglycosylase (XET) activity during hormone-induced growth in lettuce and cucumber hypocotyls and spinach cell suspension cultures. *J. Exp. Bot*. 45(1): 1703-1710.

Prabhu, G. R., and Mandal, A. K. A. 2010. Computational identification of miRNAs and their target genes from expressed sequence tags of tea *(Camellia sinensis). Proc. Natl. Acad. Sci.* 8(2): 13-121.

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M., and Tepfer, M. 2008. Strategies for antiviral resistance in transgenic plants. *Mol. Plant Pathol.* 9(1): 73-83.

Pritchard, C. C., Cheng, H. H., and Tewari, M. 2012. MicroRNA profiling: Approaches and considerations. *Nat. Rev. Genet.* 13:358–369. pmid:22510765.

Ramesh, S. V., Chouhan, B. S., Kumar, G., Praveen, S., and Chand, S. 2017. Expression dynamics of *Glycine max* (L.) Merrill microRNAs (miRNAs) and their targets during *Mungbean yellow mosaic India virus* (MYMIV) infection. *Physiol. Mol. Plant Path.* 100: 13-22.

Rao, X., Huang, X., Zhou, Z., and Lin, X. 2013. An improvement of the 2^(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostat. Bioinforma. Biomath*. 3: 71-85.

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: 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.

Rodriguez-Negrete, E. A., Carrillo-Tripp, J., and Rivera-Bustamante, R. F. 2009. RNA silencing against *geminivirus:* complementary action of post transcriptional gene silencing and transcriptional gene silencing in host recovery. *J. Virol*. 83(3): 1332-1340.

Rose, J. K. C., Braam, J., Fry, S. C., and Nishitani, K. 2002. The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: Current perspectives and a new unifying nomenclature. *Plant Cell Physiol*. 43: 1421–1435.

Roshan, P., Kulshreshtha, A., Purohit, R., and Hallan, V. 2020. AV2 protein of *tomato leaf curl Palampur virus* interacts with F-box Kelch protein of tomato and enhances phenylalanine ammonia-lyase activity during virus infection. *Physiol. Mol. Plant Pathol.* 101479p.

Saab, I. N., and Sachs, M. M. 1996. A flooding-induced xyloglucan endo-transglycosylase homolog in maize is responsive to ethylene and associated with aerenchyma. *Plant Physiol*. 112(1): 385-391.

Saladie, M., Rose, J. K., Cosgrove, D. J., and Catala, C. 2006. Characterization of a new xyloglucan endotransglucosylase/hydrolase (XTH) from ripening tomato fruit and implications for the diverse modes of enzymic action. *The Plant J.* 47(2): 282-295.

Salavert, F., Navarro, J. A., Owen, C. A., Khechmar, S., Pallas, V., and Livieratos, I. C. 2020. *Cucurbit chlorotic yellows virus* p22 suppressor of RNA silencing binds single-, double-stranded long and short interfering RNA molecules in vitro. *Virus Res.* 279: 197887p.

Sankaranarayanan, R., Palani, S. N., Kumar, A., AS, P. S., and Tennyson, J. 2020. Prediction and experimental confirmation of *banana bract mosaic virus* encoding miRNAs and their targets. *ExRNA*. 2(1): 5p.

Santner, A., and Estelle, M. 2009. Recent advances and emerging trends in plant hormone signalling. *Nature* 459(7250): 1071-1078.

Sasidharan, R., Voesenek, L. A., and Pierik, R. 2011. Cell wall modifying proteins mediate plant acclimatization to biotic and abiotic stresses. *CRC Crit. Rev. Plant Sci.* 30(6): 548-562.

Scherer, G. F. E., Ryu, S. B., Wang, X., Matos, A. R., and Heitz, T. 2010. Patatin-related phospholipase A: nomenclature, subfamilies and functions in plants. *Trends Plant Sci.* 15(12): 693–700.

Schwartz, C., Balasubramanian, S., Warthmann, N., Michael, T. P., Lempe, J., Sureshkumar, S., Kobayashi, Y., Maloof, J. N., Borevitz, J. O., Chory, J., and Weigel, D. 2009. Cis-regulatory changes at FLOWERING LOCUS T mediate natural variation in flowering responses of *Arabidopsis thaliana*. *Genetics* 183(2): 723-732.

Selvarajan, R., Balasubramanian, V., Jeyabaskaran, K. J. and Mustaffa, M. 2017. Yield loss management through fertilizers in *banana bract mosaic virus* affected by French plantain cv. Nendran (AAB). *Indian J. Agric. Sci.* 87 (8): 1055–1061.

Selvarajan, R., Balasubramanian, V., Priyanka, P., Jebakumar, R. M., Selvam, K. P., and Uma, S. 2020. Evidence of seed transmission of *Banana bract mosaic virus* in *Musa* synthetic diploid H-201, a possible threat to banana breeding. *Eur. J. Plant Pathol*. 156: 1181–1187 https://doi.org/10.1007/ s10658-019-01924-7

Selvarajan, R., Balasubramanian, V., Sheeba, M. M., Raj Mohan, R., and Mustaffa, M. M. 2009. Virus-indexing technology for production of quality banana planting material: a boon to the tissue-culture industry and banana growers in India. In *V International Symposium on Banana: ISHS-ProMusa Symposium on Global Perspectives on Asian Challenges* 897: 463-469.

Selvarajan, R. and Jeyabaskaran, K. J. 2006. Effect of *Banana bract mosaic virus* (BBrMV) on growth and yield of cultivar Nendran (Plantain, AAB). *Indian Phytopath.* 59 (4): 496-500.

Sharma, N., and Prasad, M. 2020. Silencing AC1 of *Tomato leaf curl virus* using artificial microRNA confers resistance to leaf curl disease in transgenic tomato. *Plant Cell Rep.* 39(11): 1565-1579.

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.* 100p.

Shekhawat, U. K., Ganapathi, T. R., and Hadapad, A. B. 2012. Transgenic banana plants expressing small interfering RNAs targeted against viral replication initiation gene display high-level resistance to *banana bunchy top virus* infection. *J. Gen. Virol.* 93(8): 1804-1813.

Simon-Mateo, C., and Garcia, J. A. 2011. Antiviral strategies in plants based on RNA silencing. *Biochimica et Biophysica Acta (BBA)-Gene Regul. Mech.* 1809(11-12): 722-731.

Singh, N., and Sharma, A. 2014. In-silico identification of miRNAs and their regulating target functions in *Ocimum basilicum*. *Gene* 552(2): 277-282.

Stevens, W. A. 1983. Transmission of plant viruses. In *Virology of Flowering Plants*. Springer, Boston, MA. 41-68p.

Subr, Z., Predajna, L., Soltys, K., Bokor, B., Budis, J., and Glasa, M. 2020. Comparative transcriptome analysis of two cucumber cultivars with different sensitivity to *cucumber mosaic virus* infection. *PLoS Pathog*. 9(2): 145p.

Subramanian, A. 2019. Identification and characterisation of virus responsive miRNAs in Banana *Musa* (AAB) Nendran. M.Sc. (Ag.) Thesis, Kerala Agricultural University, Thrissur, 120p.

Sunkar, R., Chinnusamy, V., Zhu, J., and Zhu, J. K. 2007. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Trends Plant Sci*. 12(7): 301-309.

Taliansky, M., Torrance, L., and Kalinina, N. O. 2008. Role of plant virus movement proteins. In *Plant Virology Protocols*. Humana Press. 33-54p.

Tepfer, M. 2002. Risk assessment of virus-resistant transgenic plants. *Annu. Rev. Phytopathol*. 40(1): 467-491.

Teune, J. H., and Steger, G. 2010. NOVOMIR: de novo prediction of microRNA-coding regions in a single plant-genome. *J. Nucleic Acids* 2010: 10p.

Thomas, J. E., and Magnaye, L.V. 1996. Banana bract mosaic disease. *Musa diseases fact sheet No. 7*. *INIBAP*, Montpellier, France. 7p.

Tiwari, M., Sharma, D., and Trivedi, P. K. 2014. Artificial microRNA mediated gene silencing in plants: progress and perspectives. *Plant Mol. Biol.* 86(1-2): 1-18.

Tripathi, L., Tripathi, J. N., and Vroh-Bi, I. 2007. Bananas and plantains (Musa spp.): Transgenics and biotechnology. *Transgenic Plant J.* 1(1): 185-201.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J. A. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.* 35(suppl_2): W71-W74.

Unver, T., Namuth-Covert, D. M., and Budak, H. 2009. Review of current methodological approaches for characterizing microRNAs in plants. *Int. J. Plant Genomics* 2009: 11p.

Van den Burg, H. A., Tsitsigiannis, D. I., Rowland, O., Lo, J., Rallapalli, G., MacLean, D., Takken, F. L., and Jones, J. D. 2008. The F-box protein ACRE189/ACIF1

regulates cell death and defense responses activated during pathogen recognition in tobacco and tomato. *The Plant Cell*. 20(3): 697-719.

Van Loon, L. C., Rep, M., and Pieterse, C. M. 2006. Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44: 135-162.

Varkonyi-Gasic, E., Wu, R., Wood, M., Walton, E. F., and Hellens, R. P. 2007. Protocol: A highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant methods* 3(1): 1-12.

Viswanathan, C., Anburaj, J., and Prabhu, G. 2014. Identification and validation of *Sugarcane streak mosaic virus* encoded microRNAs and their targets in sugarcane. *Plant Cell Rep*. 33(2): 265-276.

Wagaba, H., Beyene, G., Aleu, J., Odipio, J., Okao-Okuja, G., Chauhan, R. D., Munga, T., Obiero, H., Halsey, M. E., Ilyas, M., and Raymond, P. 2017. Field level RNAi-mediated resistance to Cassava brown streak disease across multiple cropping cycles and diverse East African agro-ecological locations. *Front. Plant Sci.* 2060: 7p.

Wahid, F., Shehzad, A., Khan, T., and Kim, Y. Y. 2010. MicroRNAs: synthesis, mechanism, function, and recent clinical trials. *Biochimica et Biophysica Acta (BBA)-Mol. Cell Res.* 1803(11): 1231-1243.

Wang, M., Xu, Z., Ding, A., and Kong, Y. 2018. Genome-wide identification and expression profiling analysis of the xyloglucan endotransglucosylase/hydrolase gene family in tobacco (*Nicotiana tabacum* L.). *Genes* 9(6): 273p.

Warthmann, N., Chen, H., Ossowski, S., Weigel, D., and Herve, P. 2008. Highly specific gene silencing by artificial miRNAs in rice. *PloS one* 3(3): 1829p.

Watanabe, Y., Tomita, M., and Kanai, A. 2007. Computational methods for microRNA target prediction. *Methods Enzymol.* 427: 65-86.

Waterhouse, P. M., Wang, M. B., and Lough, T. 2001. Gene silencing as an adaptive defense against viruses. *Nature* 411(6839): 834-842.

Weibo, J., and Wu, F. 2015. Identification and characterization of cucumber miRNAs in response to *Pseudoperonospora cubensis* infection. *Gene* 569: 225-232.

Wesley, S. V., Helliwell, C., Wang, M. B., and Waterhouse, P. 2004. Posttranscriptional gene silencing in plants. In *RNA Interference, Editing, and Modification*. Humana Press 117-129p.

Wilson, S. K., and Knoll, L. J. 2018. Patatin-like phospholipases in microbial infections with emerging roles in fatty acid metabolism and immune regulation by Apicomplexa. *Mol. Microbiol.* 107(1): 34-46.

Wu, Y., Spollen, W. G., Sharp, R. E., Hetherington, P. R., and Fry, S. C. 1994. Root growth maintenance at low water potentials (increased activity of xyloglucan endotransglycosylase and its possible regulation by abscisic acid). *Plant Physiol*. 106(2): 607-615.

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

Xu, M. Y., Zhang, L., Li, W. W., Hu, X. L., Wang, M. B., Fan, Y. L., Zhang, C. Y., and Wang, L. 2014. Stress-induced early flowering is mediated by miR169 in *Arabidopsis thaliana*. *J. Exp. Bot.* 65(1): 89-101.

Xu, W., Campbell, P., Vargheese, A. K., and Braam, J. 1996. The Arabidopsis XET-related gene family: environmental and hormonal regulation of expression. *The Plant J.* 9(6): 879-889.

Xu, Y., and Huang, B. 2018. Comparative transcriptomic analysis reveals common molecular factors responsive to heat and drought stress in *Agrostis stolonifera*. *Sci. Rep*. 8: 15181.

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 Bioinformatics* 13(1): 134p.

Yogindran, S., and Rajam, M. V. 2016. Artificial miRNA-mediated silencing of ecdysone receptor (EcR) affects larval development and oogenesis in *Helicoverpa armigera*. *Insect Biochem. Mol. Biol*. 77: 21-30.

Yokoyama, R., and Nishitani, K. 2001. A comprehensive expression analysis of all members of a gene family encoding cell-wall enzyme allowed us to predict cis-

regulatory regions involved in cell-wall construction in specific organs of Arabidopsis. *Plant Cell Physiol*. 42(10): 1025-1033.

Yokoyama, R., Rose, J. K., and Nishitani, K. 2004. A surprising diversity and abundance of xyloglucan endotransglucosylase/hydrolases in rice. Classification and expression analysis. *Plant Physiol.* 134(3): 1088-1099.

Yuan, J. S., Reed, A., Chen, F., and Stewart, C. N. 2006. Statistical analysis of real-time PCR data. *BMC bioinformatics*. 7(1): 85p.

Yu, H., Wu, J., Xu, N., and Peng, M. 2007. Roles of F-box proteins in plant hormone responses. *Acta Biochimica et Biophysica Sinica*. 39(12): 915-922.

Zeng, Y., and Cullen B. R. 2003. Sequence requirements for micro RNA processing and function in human cells. *RNA* 9:112–123. pmid:12554881.

Zhang, B. 2015. MicroRNA: a new target for improving plant tolerance to abiotic stress. *J. Exp. Bot.* 66(7): 1749-1761.

Zhang, B., Pan, X., Cannon, C. H., Cobb, G. P., and Anderson, T. A. 2006. Conservation and divergence of plant microRNA genes. *The Plant J.* 46(2): 243-259.

Zhang, X. F., and Qu, F. 2016. Cross protection of plant viruses: recent developments and mechanistic implications. In *Current Research Topics in Plant Virology*. Springer, Cham. 241-250p.

Ziebell, H., and Carr, J. P. 2010. Cross-protection: a century of mystery. In *Advances in virus research*, Academic Press. 76: 211-264.

Zihao, X., Zhenxing, Z., Mingjun, L., Ling, C., Zhiyuan, J., Yuanhua, W., Tao, Z., Weichang, Y., and Zaifeng, F. 2018. Identification of miRNAs and their targets in maize in response to *Sugarcane mosaic virus* infection. *Plant Physiol. Biochem*. 125: 143-152.

Zurek, D. M., and Clouse, S. D. 1994. Molecular cloning and characterization of a brassinosteroid-regulated gene from elongating soybean (*Glycine max* L.) epicotyls. *Plant Physiol*. 104(1): 161-170.

# *Appendix*

# APPENDIX 1

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

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

# *Abstract*

# IDENTIFICATION AND EXPRESSION PROFILING OF *Banana bract mosaic virus* (BBrMV) RESPONSIVE microRNAs IN BANANA CULTIVAR NENDRAN (*Musa* AAB)

*by*

**ATHIRA VIJAYAN**

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## ABSTRACT

The study entitled "Identification and expression profiling of *Banana bract mosaic virus* (BBrMV) responsive microRNAs in banana cultivar Nendran (*Musa* AAB)" was conducted in the Department of Plant Biotechnology, College of Agriculture Vellayani, Thiruvananthapuram during 2018-2020. The objective of the study was to validate computationally predicted selected microRNAs (miRNAs) in banana cultivar Nendran (*Musa* AAB) and to study their expression in *Banana bract mosaic virus* (BBrMV) infection.

In a previous study conducted in the Department of Plant Biotechnology, College of Agriculture Vellayani, 52 mature miRNAs were computationally predicted in the banana genome using NOVOMIR tool and their target genes identified. From them, three miRNAs were selected to study their response to BBrMV infection. The selection was done based on the possible role of their target genes in biotic stress responses. The miRNAs selected were miR-5417 (target: F Box family protein), miR-1634 (target: Patatin like phospholipase family protein) and miR- 67 (target: Probable xyloglucan endotransglucosylase/hydrolase protein 33).

The study was carried out in the BBrMV infected field grown banana plants of cv. Nendran. BBrMV infection in the infected plants were confirmed by RT-PCR using viral coat protein gene specific primers and the expected amplicon of size 745 bp was obtained. Uninfected plants served as control.

For studying the expression of these miRNAs, RNA was isolated from the leaves of infected and control plants samples following the modified Rodrigues-Garcia protocol. Stem-loop primers were used for reverse transcribing miRNAs to cDNA. The primers for the amplification of miRNAs and their target genes were designed, synthesized and their specificity was analyzed.

The expression profiling of miRNAs and their target genes was done by RT-qPCR and the results were analyzed using qBase plus software. The Raw Cq values obtained were normalized using a reference gene β-actin. The miRNAs and their target genes were analysed with reference to the uninfected control plants.

RT-qPCR analysis confirmed the presence of all the three computationally predicted miRNAs in banana cv Nendran. All the BBrMV infected samples showed an

elevated level of miR-1634 (2.95 and 6.52 fold) and miR-67 (4.46 and 5.04 fold) compared to the uninfected control. Similarly, their target genes Patatin like phospholipase family protein (target of miR-1634) showed 5 to 10 fold increase and Probable xyloglucan endotransglucosylase/hydrolase protein 33 (target of miR- 67) showed 6.95 and 9.45 fold increase in the infected plants.

No considerable change in the expression of miR-5417 was observed in the infected samples. But its target gene (F Box family protein) was found upregulated by 6.3 to 9.2 folds in both BBrMV infected plants compared to the control. Hence an inverse correlation could be established between the miRNA and its target.

The study showed that all the three miRNAs (miR-5417, miR-67 and miR-1634) respond to BBrMV infection and have a role in stress response of the plants. All the target genes showed upregulation in the BBrMV infected samples, showing their role in biotic stress response. miR-5417 showed an inverse correlation with its target.

# സംഗ്രഹം

## നേന്ത്ര വാഴ (*Musa* AAB) ലെ ബനാന ബ്രാക്റ്റ് മൊസൈക് വൈറസ് (BBrMV) രോഗത്തോട് പ്രതികരിക്കുന്ന മൈക്രോ RNAകളുടെ തിരിച്ചറിയലും എക്സ്പ്രഷൻ പ്രൊഫൈലിംഗും

"നേന്ത്ര വാഴ (*Musa* AAB) ലെ കൊക്കൻ രോഗത്തോട് പ്രതികരിക്കുന്ന മൈക്രോ RNAകളുടെ തിരിച്ചറിയലും എക്സ്പ്രഷൻ പ്രൊഫൈലിംഗും" എന്ന തലക്കെട്ടിലുള്ള പഠനം 2018 മുതൽ 2020 വരെ വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യ ജൈവസാങ്കേതികവിദ്യ വിഭാഗത്തിൽ നടന്നു. നേന്ത്രനിൽ (മൂസ എഎബി) കമ്പ്യൂട്ടേഷൺ രീതി ഉപയോഗിച്ച് പ്രവചിച്ച തിരഞ്ഞെടുത്ത മൈക്രോ RNAകളുടെ (miRNA) സാന്നിദ്ധ്യം സാധൂകരിക്കുക, ബനാന ബ്രാക്റ്റ് മൊസൈക് വൈറസ് (BBrMV) അണുബാധയിൽ അവയുടെ സാന്നിദ്ധ്യം പഠിക്കുക എന്നിവയായിരുന്നു പഠനത്തിന്റെ ലക്ഷ്യം.

വെള്ളായണി കാർഷിക കോളേജിലെ സസ്യ ജൈവസാങ്കേതികവിദ്യ വിഭാഗത്തിൽ മുമ്പ് നടത്തിയ ഒരു പഠനത്തിൽ കമ്പ്യൂട്ടേഷൺ രീതിയിൽ നോവോമിർ എന്ന സോഫ്റ്റ്വെയർ ഉപയോഗിച്ച് വാഴയുടെ ജീനോമിൽ മെച്ചർ മൈക്രോ RNAകളെ (miRNA) പ്രവചിക്കുകയും അവയുടെ ടാർഗെറ്റ് ജീനുകളെ തിരിച്ചറിയുകയും ചെയ്തു. ഇവയിൽ നിന്നും, നിലവിലെ പഠനത്തിനായി മൂന്ന് മൈക്രോ RNAകളെ തിരഞ്ഞെടുത്തു. ബയോട്ടിക് സ്ട്രെസ് പ്രതികരണങ്ങളിൽ അവരുടെ ടാർഗെറ്റ് ജീനുകളുടെ സാധ്യമായ പങ്ക് അടിസ്ഥാനമാക്കിയാണ് തിരഞ്ഞെടുപ്പ് നടത്തിയത്. miR-5417 (ടാർഗെറ്റ്: F ബോക്സ് ഫാമിലി പ്രോട്ടീൻ), miR- 1634 (ടാർഗെറ്റ്: ഫോസ്ഫോലിപേസ് ഫാമിലി പ്രോട്ടീൻ പോലുള്ള പാറ്റാറ്റിൻ), miR-67 (ടാർഗെറ്റ്: പ്രോബബിൾ സൈലോഗ്ലുകാൻ എൻഡോട്രാൻസ്ഗ്ലൂക്കോസൈലേസ്/ ഹൈഡ്രോലേസ് പ്രോട്ടീൻ 33) എന്നിവയാണ് തിരഞ്ഞെടുത്ത മൈക്രോ RNAകൾ.

വയലിൽ വളരുന്ന കൊക്കൻ രോഗം ബാധിച്ച നേന്ത്ര വാഴച്ചെടികളിലാണ് പഠനം നടത്തിയത്. രോഗം ബാധിച്ച സസ്യങ്ങളെ വെള്ളായണി കാർഷിക കോളേജിലെ ഇൻസ്ട്രക്ഷണൽ ഫാമിൽ നിന്ന് രോഗലക്ഷണങ്ങളെ അടിസ്ഥാനമാക്കി തിരഞ്ഞെടുത്തു. ആരോഗ്യമുള്ള അണുബാധയില്ലാത്ത സസ്യങ്ങളെ കൺട്രോൾളായി തിരഞ്ഞെടുത്തു. വൈറസിന്റെ കോട്ട് പ്രോട്ടീൻ ജീൻ നിർദ്ദിഷ്ട പ്രൈമറുകൾ ഉപയോഗിച്ച് തിരഞ്ഞെടുത്ത വാഴകളിൽ RT-PCR വഴി വൈറസിന്റെ കോട്ട് പ്രോട്ടിനിന്റെ സാന്നിദ്ധ്യം സ്ഥിരീകരിക്കുകയും, പ്രതീക്ഷിച്ച വലുപ്പമുള്ള ആംപ്ലികോൺ (745bp) ലഭിക്കുകയും ചെയ്തു.

കൊക്കൻ രോഗം ബാധിച്ച വാഴകളിൽ തിരഞ്ഞെടുത്ത മൈക്രോ RNAകളുടെ സാന്നിദ്ധ്യം പഠിക്കുന്നതിനായി, രോഗബാധയുള്ളതും ഇല്ലാത്തതുമായ സസ്യങ്ങളിൽ നിന്ന് ഇല സാമ്പിളുകൾ ശേഖരിക്കുകയും, RNA വേർതിരിച്ചെടുക്കുകയും ചെയ്തു. മൈക്രോ RNAകളുടെ ആമ്പ്ലിഫിക്കേഷനായി സ്റ്റെം ലൂപ്പ് പ്രൈമറുകളും, അവയുടെ ടാർഗെറ്റ് ജീനുകളുടെ ആമ്പ്ലിഫിക്കേഷനായി പ്രൈമറുകളും രൂപകൽപ്പന ചെയ്തു.

മൈക്രോ RNA കളുടെയും അവയുടെ ടാർഗെറ്റ് ജീനുകളുടെയും എക്സ്പ്രഷൻ പ്രൊഫൈലിംഗ് RT-PCR മുഖേന കണ്ടെത്തുകയും, കൂടാതെ ഫലങ്ങൾ qBase പ്ലസ് സോഫ്റ്റ്വെയർ ഉപയോഗിച്ച് വിശകലനം ചെയ്യുകയും ചെയ്തു. മൈക്രോ RNAകളും അവയുടെ ടാർഗെറ്റ് ജീനുകളും കൺട്രോൾ ചെടികളെ ബന്ധപ്പെട്ടുത്തി പഠിക്കുകയും ലഭിച്ച റോ Cq മൂല്യങ്ങൾ റഫറൻസ് ജീൻ β- ആക്ടിൻ ഉപയോഗിച്ച് നോർമലൈസ്സ് ചെയ്യുകയും ചെയ്തു.

RT-qPCR വിശകലനത്തിൽ, തിരഞ്ഞെടുത്ത മൂന്ന് മൈക്രോ RNAകളുടെയും സാന്നിദ്ധ്യം സ്ഥിരീകരിച്ചു.

മൂന്ന് മൈക്രോ RNAകളും (miR-5417, miR-1634, miR-67) രോഗബാധയില്ലാത്തതും കൊക്കൻ രോഗം ബാധിച്ചതുമായ സസ്യങ്ങളിൽ സാന്നിദ്ധ്യം കാണിച്ചു. കൊക്കൻ രോഗം ബാധിച്ച സസ്യങ്ങൾ, ബാധിക്കാത്ത സസ്യങ്ങളെ അപേക്ഷിച്ച് miR-1634 (2.95, 6.52 മടങ്ങ്), miR-67 (4.46, 5.04 മടങ്ങ്) എന്നിവയുടെ ഉയർന്ന പ്രകടനം കാണിച്ചു. അതുപോലെ തന്നെ അവരുടെ ടാർഗെറ്റ് ജീനുകളായ ഫോസ്ഫോലിപേസ് ഫാമിലി പ്രോട്ടീൻ (miR -1634 ന്റെ ടാർഗെറ്റ്) 5, 10 മടങ്ങ് വരെയും, പ്രോബബിൾ സൈലോഗ്ലുകാൻ എൻഡോട്രാൻസ്ഗ്ലൂക്കോസൈലേസ് / ഹൈഡ്രോലേസ് പ്രോട്ടീൻ 33 (miR-67 ന്റെ ടാർഗെറ്റ്) 6.95, 9.45 മടങ്ങ് വർദ്ധനവും പ്രകടിപ്പിച്ചു.

രോഗം ബാധിച്ച സസ്യത്തിൽ miR-5417 ന്റെ പ്രകടനത്തിൽ വലിയ വ്യത്യാസം കാണിച്ചില്ല. എന്നാൽ അതിന്റെ ടാർഗെറ്റ് ജീൻ (F ബോക്സ് ഫാമിലി പ്രോട്ടീൻ) കൺട്രോളുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ കൊക്കൻ രോഗം ബാധിച്ച രണ്ട് ചെടികളിലും 6.3 മുതൽ 9.2 മടങ്ങ് വരെ വർധനവ് കാണിച്ചു. കൊക്കൻ രോഗം ബാധിച്ച വാഴകളിൽ miR-5417 നും അതിന്റെ ടാർഗെറ്റ് ജീനും തമ്മിൽ വിപരീത ബന്ധം ഉള്ളതായി തെളിഞ്ഞു.

തിരഞ്ഞടുത്ത മൂന്ന് miRNAകളും നേന്ത്ര വാഴയിൽ ബനാന ബ്രാക്ക്റ്റ് മൊസൈക് വൈറസ് അണുബാധയോട് പ്രതികരിക്കുന്നു എന്ന് പഠനം തെളിയിച്ചു. എല്ലാ ടാർഗെറ്റ് ജീനുകളും വൈറസ് അണുബാധയുള്ള നേന്ത്ര വാഴയിൽ ഉയർന്ന പ്രകടനം കാണിച്ചു. miR-5417 അതിന്റെ ടാർഗെറ്റ് ജീൻ (F ബോക്സ് ഫാമിലി പ്രോട്ടീൻ) മായി വിപരീത ബന്ധം പ്രകടിപ്പിച്ചു.