EVALUATION OF siRNA MEDIATED *Banana bract mosaic virus* (BBrMV) RESISTANCE IN BANANA PLANTS WITH ihpRNA CONSTRUCT FOR REPLICASE GENE

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Evaluation of siRNA mediated *Banana bract mosaic virus* (BBrMV) resistance in banana plants with ihpRNA construct for replicase gene"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Evaluation of siRNA mediated *Banana bract mosaic virus* (BBrMV) resistance in banana plants with ihpRNA construct for replicase gene"** is a record of research work done independently by **Ms. Harshitha C. K. (2015-11-086)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
BA	N^6- benzyl adenine
BBrMV	*Banana bract mosaic virus*
bp	Base pairs
$CaCl_2$	Calcium chloride
cm	Centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
EDTA	Ethylenediaminotetraacetic acid
et al.	and others
Fig.	Figure
g	gram
h	hour
HCL	Hydrochloric acid
IAA	Indole-3-acetic acid
M	molar
mg	milligram
ml	milliliter
min	minute
mM	millimolar
MS	Murashige and Skoog, 1962
miRNA	microRNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nt	nucleotide
µM	micromolar
pH	potential of hydrogen
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PVP	Polyvinylpyrrollidone
rpm	revolutions per minute
s	second
siRNA	Small interfering RNA
sp.	species
TE	Tris-EDTA buffer
Tris HCl	Tris (hydroxymethyl) aminomethanehydrochloride
V	Volt

viz.	namely
X	times

LIST OF SYMBOLS

°C	degree celsius
%	per cent
±	plus or minus

Introduction

1. INTRODUCTION

Banana (*Musa* sp.) is the second most important fruit crop in India next to mango. Among the five most consumed fruits in the world it holds first place by production volume (FAO, 2012). The crop serves as a staple food in many developing countries and shifted human uses, extending from the eatable bananas and plantains of the tropics to cool solid fiber and ornamental plants. Banana is of extraordinary nutritious esteem and it has a rich source of vitality esteem, tissue building components, proteins, vitamins and minerals. It is a good source of vitamin B_6, vitamin C, and potassium. It is the favorite fruit among all classes of people due to its year round availability, varietal range, taste, affordability, medicinal and nutritive value and also it has good export potential. It is generally grown in tropical and subtropical regions including Kerala, Karnataka, Tamil Nadu, Gujarat, Andhra Pradesh, and Maharashtra *etc.* and in wide range of agricultural systems such as from small, mixed, subsistence gardens, to large commercial cultivation.

The production and productivity of banana cultivation have adversely affected by many pests and diseases problems. Viral and Fungal diseases extensively hamper banana production in India. Export potential of banana germplasm has been increased in recent years. The presence of any virus represents a threat as novel viruses that might be disseminated in huge quantities to new places. Four major viruses affecting banana production are *Banana bunchy top virus* (BBTV) of genus *Nanovirus*, *Banana bract mosaic virus* (BBrMV) of genus *Potyvirus, Banana streak virus* (BSV) of genus *Badnavirus*; and *Cucumber mosaic virus* (CMV) of genus *Cucumovirus* (Diekmann and Putter 1996).

BBrMV is a recently described virus disease of banana classified as a potyvirus which is non-persistently transmitted through aphid species *Rhopalosipum maidis, Pentalonia nigronervosa* and *Aphis gossypii* and also transmitted through infected planting material (Selvarajan *et al.*, 2006). In states like Kerala, Tamil Nadu, Andhra Pradesh and Karnataka there is an incidence of

banana bract mosaic virus disease (Rodoni *et al.*, 1997, Thomas *et al.*, 1997, Selvarajan *et al.*, 1997, Thangavelu *et al.*, 2000, Singh, 2002, Anita Cherian *et al.*, 2002, Kiranmai *et al.*, 2005). In Kerala, Thrissur district the disease was first described in the Nendran variety, which is found to be highly susceptible. Later the disease was found to affect other varieties such as Chenkadali, Poovan (Rasthali), Palayankodan, Kodappanillakunnan, Kanchikela, Monthan, Karpooravally and. Incidence of the disease ranges from 5 to 36 per cent and more in cv. Nendran in Kerala (Selvarajan *et al.*, 2006).

The disease is identified by discontinuou streaks on the bract of the banana inflorescence. The infected plants produce smaller bunches bearing brittle fruits. In severe stage of infection plants may fail to flower and die by necrosis of the pseudostem. Besides yield reduction, the fingers become malformed and curved, which reduce the market acceptability of the fruits (Cherian *et al.*, 2002).

To reduce losses caused by viral infections control measures have been taken which are inadequate and exclusive. Use of planting material which are free from virus is one of the method for virus control (Kumar *et al.*, 2015) but, these preventive measures do not completely control the viruses. Population resistance is the most effective method of controlling plant viruses. Conventional methods like utilization of natural resistance in plants and cross-protection have been used to fight pathogen infections in banana. But the lack of natural sources of resistance, low female fertility and poor seed setting make the conventional breeding methods difficult in banana. Many of the popular varieties are triploid in nature. This demands alternate strategies for improvement in banana.

To decrease the losses caused by plant pathogens, biotechnological methods like genetic transformation have been adopted to engineer resistant plants. Both pathogen-derived resistance approach and non-pathogen derived strategies have been applied with differing efficiencies (Prins *et al.*, 2008). The majority of transgenic plants resistant to virus are the result of pathogen derived resistance (PDR), which is mediated either by proteins encoded by transgenes or

by the transcripts produced from the transgene. For controlling viral diseases one of the most desirable way is development of resistant varieties (Blackburn and Barker, 2001). Among them, RNA silencing-based resistance is one of the most promising approaches used to engineer resistant crops to control viruses (Cheng-Guo *et al.*, 2012).

RNA interference (RNAi) is a sequence- specific post-transcriptional gene-silencing mechanism that has been widely characterized in eukaryotic organisms induced by dsRNA (Baulcombe, 2004, Herr *et al.*, 2005, Voinnet, 2005). It is a conserved regulatory mechanism associated with regulatory endogenous gene expression, developmental processes and furthermore as a barrier against viruses, transposons and foreign nucleic acids. "short interfering RNA (siRNA)" is the key component of RNAi formed by the slicing of dsRNA by a double stranded RNA specific ribonuclease named "Dicer" which are 21 -25 bp in length (Schauer *et al.*,2002). Once produced, the siRNAs complexs with the RNA-induced silencing complex (RISC) and use as a controller for the recognition and degradation of complementary mRNAs (Nykanen *et al.*, 2001), bringing about post-transcriptional gene silencing (PTGS).

In the Department of Plant Biotechnology, RNAi technology has been attempted to develop resistance against BBrMV in banana (*Musa* spp. Var. Nendran). An ihpRNA cassette targeting the replicase gene of BBrMV was constructed and introduced into banana and shoots were developed through somatic embryogenesis. The present study envisaged evaluation of the transgenics developed for siRNA mediated resistance by artificial infection and to confirm the expression of siRNA products in infected plants.

Review of Literature

2. REVIEW OF LITERATURE

The important fruit crops in India are bananas and plantains which belongs to the genus *Musa* which are monocotyledonous plants in nature. It is one of the oldest cultivated plants. For most of the developing countries of tropics, banana is the staple food. Rainforests of South-East Asia is the center of origin of banana (Simmonds, 1962).

Banana is a native of India and is extensively grown in tropical, subtropical and coastal region of India. Banana and plantains have a prominent position in many parts of the world because of its high nutritional value. Banana in an area of 0.802 million ha with production of 29.7 million tonnes and productivity of 37 MT Ha^{-1} in India (NHB, 2014). Although India occupy area of 15.5 per cent, even though in world production India accounts for 25.58 per cent. Since banana is easily available to common man it has emerged as one of the important fruit crops, which is in the easy reach of common man. In 2050, the demand is expected to be 60 million tonnes of banana which meets the local demand. Export of banana has a demand with a considerable scope in its products also which further enhances the demand. In worldwide, bananas and plantains are showing a spectacular growth continuously. Banana has a good export potential due to its year-round availability, affordability, varietal range, taste, nutritive and medicinal value. World banana production is concentrated in Africa, Asia, the Caribbean and Latin America because of the climatic conditions.

In 2012, banana and plantain both constitute rank six after maize, rice, wheat, potato, and cassava with an area of 10.3 million ha and production 139 million tonnes in the world (FAO Stat, 2013). The area of banana cultivation during 2014-15 was 61,936 Ha. It occupied 19% of the category of fresh fruits and it has 3rd top position in this category. 0.5% area is decreased during 2014-15 in banana cultivation than 2013-14 and 22% increase from 2001-02. Palakkad, Wayand & Malappuram district stands first three positions with areas 30%, 17% and 11% respectively during 2014-15. Banana cultivation is least in Alappuzha district with

450 ha. On analysing the area of last 10 years, banana is maximum during the agricultural year 2013-14 and the area is 62,261 Ha. (GOK, 2016).

2.1 Nendran (AAB)

In Kerala Nendran is a popular variety where it is commonly used as a fruit as well as used for processing. In Tamil Nadu, commercial cultivation has rapidly grown in Nendran variety. Diversity has been shown in Nendran variety due to its plant stature, pseudostem colour, presence or absence of male axis, size of the bunch etc. Bunch has 5-6 hands weighing about 12-15 kg. On ripening the fruits with profuse green skin turning buff yellow in the neck region. Fruits remain as starchy even on ripening. Among all the varieties Nendran is more vulnerable to *Banana Bract Mosaic Virus* (BBrMV), nematodes and borers (Kumar, 2012).

2.2 VIRAL DISEASES OF BANANA

Banana and plantain are cultivated which are not dependent on the season. Suckers emerge out from the rhizome after the fruiting of the main shoot withers off and this flow continues indefinitely (Morton, 1987). However, the above process leads to many biotic and abiotic stress. Around the world planting materials is the major source of virus which causes outbreak of many pest and diseases when the young suckers are collected from the old banana plantations to establish new plantation.

The export Musa germplasm are mainly threatened by the viruses which in turn decreases the banana production. Because of the reduced production there is a proportional losses and inverse losses are accompanying to plant health along with virus-free planting material production. In worldwide banana and plantain are affected by 20 different kinds of virus species which belongs to five families. Which includes *Banana bunchy top virus* (BBTV), *Banana bract mosaic virus* (BBrMV, genus Potyvirus, family Potyviridae). *Cucumber mosaic virus* (CMV), *Banana streak virus* (BSV), *Banana mild mosaic virus* and *Banana virus X* (Diekmann and Putter 1996).

2.2.1 BANANA BRACT MOSAIC DISEASE (KOKKAN DISEASE)

About 40 per cent of yield loss in banana caused due to *Banana bract mosaic virus* (BBrMV) which is nonpersistently aphid transmitted virus (Bateson and Dale 1995). In Kerala Nendran variety has 5 to 36 per cent of disease. The states like Kerala, Tamil Nadu, Karnataka and Andhra Pradesh *banana bract mosaic virus* has been reported (Rodoni *et al.* 1997; Selvarajan *et al.*, 1997).

The infected planting material and aphid species viz., *Aphis gossypii, Pentalonia nigronervosa*, *Rhopalosiphum maidis*, (Magnaye & Espino, 1990; Munez, 1992) and *Aphis craccivora* (Selvarajan *et al.*, 2006) are the major vector for BBrMV transmission.

BBrMV has been termed as a potyvirus based on characteristics such as flexuous rods measuring 660 to 760 nm, coat protein reacts with potyvirus antisera, and (iii) sequence homology with other potyviruses (Bateson and Dale 1995).

The symptoms of the BBrMV infected plants shows irregular spindle-shaped streaks on the midribs, pseudostems, peduncles and even on fruits (*Rodoni et al.*, 1997; Selvarajan & Jeyabaskaran, 2006; Thomas *et al.*, 1997). The infected plants flowers but produce very small bunches with curved brittle fruits. Very severely affected plants may flop to flower and may die by stunted growth and necrosis of pseudostem. The male buds of the infected plants are dark purple in colour with mosaic patches. There are varietal changes in the symptomatology of the disease. BBrMV has been reported to cause significant damage in Cavendish banana plantations. The disease has a great impact on the yield of the crop with determined yield reduction in cv. Robusta (AAA) (70%), followed by cv. Nendran (AAB) (52%) (Cherian *et al.*, 2002). In the infected plants either long or stout peduncle are seen in bunches and "traveler's palm" appearance is seen in the leaves of Nendran (Balakrishnan *et al.*, 1996). In addition to yield reduction, the fingers become malformed and curved, which reduce the market acceptability of fruits (Cherian *et al.*, 2002).

2.3 MAJOR CONSTRAINTS IN BANANA PRODUCTION

Banana is the least investigated among all those major fruit crops, although it is one of the world's most important crops perhaps because of the biology of the banana plant. Most cultivated banana varieties are sterile triploids and this prevents elaboration of successful hybridization programmers. In addition, the long-life cycle of this crop makes even simple field testing experiments lasting for 2-3 years. These obstacles hamper banana breeding to deal with the attacks of a number of virulent diseases and pests whose protection costs may constitute greater than 40% of the total production cost (Sagi *et al.*, 1998).

Banana production and productivity have been extensively increased from the last few decades due to the growing alertness of banana in nutritional aspects, gross value and its trade. Musa wild species and its related genera form a major source of resistant genes against environmental factors, pest and diseases. The availability of germplasm has to be maintained from urbanization and erosion of wild bananas in their natural habitat.

Constraints in banana production varies from locality to locality and constant in nature. Productivity has to be maximized through basic, strategic and adaptive research. To achieve real impact in future recent biotechnological approaches, help in breaking the jinx in the breeding banana and plantain which has inherent complex problems. In 2050 to reach the estimated production of 60 million tonnes, there is a need to overcome barriers such as increase in cost of input like fertilizers, water source and management of crop protection (NRCB, 2015).

New inventions have to be taken up to give a stimulate in the areas like genetic engineering, molecular genetics, organic farming, integrated disease and pest management, bio-chemical, physiological and genetic basis for abiotic and biotic stress management, processing technology and value addition.

2.4 METHODS TO INDUCE RESISTANCE AGAINST VIRUS DISEASES

Virus diseases are major risks to modern agriculture and their management remains as a task to the cultivation practices of crops. Among the plant pathogens plant viruses are most significant and one of the major limitations in banana cultivation resulting in significant yield loss. Conventional methods using cross-protection and utilization of natural resistance in plant have been taken to reduce losses caused by viral infections. Mutation in the viral genome leads to emergence of new virus varieties (Mangrauthia *et al.*, 2008; Jones 2009).

2.4.1 CROP PROTECTION BASED ON NATURAL RESISTANCE

For maximizing plant productivity, the control of diseases caused by viruses is very important. But presently no direct control measures are available to farmers. A few conventional methods of control such as eradication of infected crop, avoidance of the source of infection or limiting spread of disease are practiced. But all these approaches have their drawbacks such as poor effectiveness, high cost and lack of reliability. Resistant or tolerant varieties developed through conventional breeding programmes are also expensive and time consuming. Besides, virus variability is able to circumvent the resistance. One more type of protective measure used, was the cross protection in which a crop is deliberately infected with a mild symptomless or attenuated strain of virus so that the former develops resistance against further infection with a related severe strain (Fultos 1986). Although this approach raised serious ecological concern about conversion of the mild strain to a pathogenic form or potential of the mild strain to cause serious damage to other crops, the approach was a promising one and investigators tried to find out the mechanism of this cross protection.

A superior comprehension of plant defense mechanisms would promote the development of novel strategies for successful plant protection. Extensive knowledge of virus infection is needed for management of disease. Instead of treating the infected plant which is commonly done for fungal and bacterial diseases, crop loss can also be reduction in the light of monitoring the pathogen

distribution (Ventura *et al*, 2004). To reduce the virus distribution all over the plantation different approaches have been used. Epidemiological studies help in understanding route followed by virus to reach the host and mechanism of inoculation (Gilligan and Bosch 2008, Rodrigues *et al*, 2009). Common cultivation practices and infected seeds act as mean for transmission of viral diseases (Fereres and Moreno 2009). Occurrence of certain viruses can reduce significantly by the use of certified seeds (Novy *et al*, 2007). "Hygienic" cultivation practices can significantly reduce the virus transmission (Fereresa and Moreno 2009; Castle *et al*, 2009). Virus replication can be reduced by set of plant resistance responses (Ascencio *et al*, 2008). In certain cases, weakened virus strains can be used to intensify the tolerance responce (Ichiki *et al*, 2005). Crop damage caused by virus can be met through breeding techniques by developing resistant cultivars (Ma *et al*, 2004). Advances in the understanding the biochemistry of virus infection, potential new methods such as RNA silencing, have resulted to boundary the viral diseases efficiently (Tenllado *et al*, 2004).

2.4.2 BIOTECHNOLOGICAL APPROACHES FOR PLANT VIRUSES RESISTANCE

The main virus resistance strategies are based on either natural resistance or genetically modified virus-resistant plants. For the development of novel strategies against virus infection, Characters of resistance genes has to be understood in the molecular level which will endorsed the progress. Expression of RNA mediated/ directed sequence specific silencing of nucleotides through genetic modification are winding up gradually significant which will possible lead to more viable and active approaches in imminent.

In order to regulate the damage caused by viral pathogens, biotechnological methods are being adopted by genetically modifying the plants to obtain resistance. Monitoring of viral infections in fiscal and ecofriendly manner is through development of new cultivars resistant to virus (Blackburn and Barker, 2001). From the last few years, RNA mediated silencing straturgie has been termed as a novel

approach to obtain crops by engineering crops at molecular level (Cheng-Guo *et al.*, 2012).

Investigations on cross protection line led to the development of the concept of pathogen-derived resistance (PDR) (Sanford & Johnston 1985). Transgenic plants resistant to virus are mainly due to the mechanism called PDR (Prins *et al*, 2008). In this concept, the excess amount of expression of any of the viral genes in inappropriate time would disrupt the normal balance between viral products, thereby viral life cycle could be disturbed. PDR are mainly due to resistance mediated by protein and nucleic acid. Coat protein, movement proteins, proteases, are the commonly used proteins of the virus to induce for PDR (Tepfer, 2002). Transgenic RNA was responsible for the observed resistance, rather than the expressed viral proteins created new opportunities based on resistance mediated by the RNAs (Tenllado *et al*, 2004). Application of PDR was first reported by Powell-Abel et al. (1986) who revealed that transgenic expression of TMV coat protein gene in tobacco produced resistance against TMV. Efficiency of this methodology was extended by the use of other viral genes like replicase gene, defective movement protein gene, viral helper component gene, viral protease gene, ameliorating satellite RNA sequences, interfering nucleic acids *etc*. Other approaches include expression of foreign gene which prevent virus multiplication and spread by interacting with the incoming viral genome or their expression products or by killing the host cell. These also include transfer of natural virus resistant gene from one species to another via plant transformation techniques.

RNA mediated silencing strategy is considered as new approach for developing resistance in plants against pathogens, it was initially considered as a defense mechanism of plant which secures against infection form the RNA viruses (Waterhouse *et al.*, 2001; Prins *et al.*, 2008). But the actual importance of RNAi in silencing was completely understood after discovery of miRNAs which results in silencing (Bartel, 2004; Naqvi *et al.*, 2009). Many miRNA works primarily by degrading the homologous complementary transcript and it is genetically conserved.

virus-induced gene silencing (VIGS) is the primary mechanism which will lead to the inactivation viral gene by the siRNA through RNAi. Other than the regulatory roles in plant development, the PDR can also be acquired by introducing the viral sequence in plant genome, resistance being achieved relies on the transcript called Nucleic acid mediated resistance (NMR). In plants, Resistance obtained from the RNAi is due to the silencing at the transcriptome level know as post transcriptional gene silencing (Prins *et al.*, 2008). Napoli *et al.* (1990) first testified this transcriptome silencing in the hybrids of Petunia, by introducing the chalcone synthase gene transgenically. And the result of their study was silencing of the gene present in the petunia and also the silencing of complementary mRNA. The process behind the gene silencing is known as RNA interference (RNAi) which is present in various organisms (Mlotshwa *et al.*, 2008). Short nucleotides of length 21-26nt RNAs are formed by splicing of a dsRNA by enzyme called ribonuclease. The spliced RNAs are called as microRNAs (miRNAs) and short interfering RNAs (siRNA). These two small RNAs will form a complex with the RNA-induced silencing complex (RISC) which will degrade the complementary transcript (Naqvi *et al.*, 2009). In the same way longer siRNAs of 24-26 nt long will leads to silencing of the complementary DNA by methylation resulting in modification of the chromatin structure and gene silencing in transcriptional level known as transcriptional gene silencing (TGS).

2.4.2.1 RNA INTERFERENCE (RNAi)

In plants to obtain resistance against viruses, RNA facilitated silencing technique has emerged as a powerful source. The important feature of this mechanism is mainly due to the occurrence of double stranded RNA (dsRNA), which will serve both as activates of RNAi and also the creation of RNA mediated silencing. In RNAi induction the dsRNAs are cleaved into short fragments of RNA known as small interfering RNAs (siRNAs), also called as symbols of RNAi. By exploiting the silencing mediated by RNAs, strong resistance can be achieved against viruses.

RNA silencing is referred as RNA interference (RNAi) in animals and gene quelling in case of fungi and normally conserved regulatory mechanism observed in higher organisms for expression of genes. RNA induced silencing is a sequence specific silencing of nucleotides by splicing the mRNA or inhibition by PTGS or in the transcriptional level by methylation known as RNA directed DNA methylation (RdDM). The RNA mediated silencing prosses is carried out by mechanisms such as; dsRNA, Dicer, siRNAs or miRNAs, and RISC (Peragine *et al.,* 2004). Engineered plants expressing dsRNA of the virus are known to induce resistance against by a process called as RNA interference.

The siRNAs guide RISC and degrades the complementary RNAs. Insertion of either hairpin RNA or inverted repeat will result in the silencing of the complementary viral gene by PTGS (Scorza *et al.*, 2001). Effectiveness of silencing can also be increased by placing an intron between 2 arms of the complementary regions to form intro-hairpin RNA (ihpRNA) (Wesley *et al.*, 2001). Interaction of the 2 complementary sequence will be more due to the presence of intron.

Other than the use of inverted repeats and ihpRNA, antisense RNA is also known to trigger RNA interference. RNA mediated resistance was discovered unexpectedly by Fire and Mello when they were trying to induce silencing in *Caenorhabditis elegans*. Silencing was increased ten times when sense and antisense strands were used to form dsRNA equated with antisense and sense solitarily (Fire *et al.*, 1998).

In tobacco plants, PDR against *tobacco mosaic virus* infection was established using coat protein gene from *tobacco mosaic virus* and resistance was successfully achieved in transformed tobacco plants by Powell-Abel *et al.* (1986). The mechanism behind the observed resistance against viruses mediated by RNAs was due to PTGS in plants and RNAi in animals (Hannon, 2002).

An approach for expressing antisense RNA in transgenic plants have been employed successfully against *tomato golden mosaic virus* (TGMV) by Day *et al.* (1991) by expressing an antisense AL1 transcript, a protein absolutely required for

TGMV DNA replication. the frequency of symptom development was very significantly reduced in transgenic lines after infection with TGMV, by agroinoculation.

Tomato yellow leaf curl virus (TYLCV) is one of the most severe diseases of tomato which is transmitted by the whitefly *Bemisia tabaci*. Bendahmane and Gronenborn (1997) used antisense RNAs to induce resistance against TYLCV by silencing C1 gene which codes for Rep protein. Engineered tobacco plants expressing rep protein gene showed resistance against TYLCV.

Wesley *et al.* (2001) used ihpRNA constructs containing sense and antisense sequence of around 98 to 853 nucleotides for inducing resoistance in a wide range of plant species, and inclusion of an intron in these constructs had a consistently enhancing effect. Intron-containing constructs (ihpRNA) gave 90-100percent silencing. They also constructed a generic vector, pHANNIBAL for developing ihpRNA silencing construct.

Missiou *et al.* (2004) engineered potato plants resistant to *potato virus Y* (PVY). In potatoes of the commercial variety 'Spunta', highly conserved sequence dsRNA encoding coat protein gene from PVY, which is amongst different PVY isolates, in transgenic. In order to detect siRNAs in transformed plants northern blotting was carried out before infection. Transgenic plants expressing siRNAs were resistant PVY. In addition, infection with *Potato virus X* (PVX) to the same plants before PVY infection as no relation with the resistance being shown against PVY.

A Gateway vector, pANDA was developed by Miki and Ko Shimamoto (2004) for RNA interference of rice genes using *Agrobacterium* transformation. In the construct, hairpin RNA is placed on either side intron along with constitutively expressing ubiquitin promoter. Engineered plants showed suppression of mRNA greater than 90%.

Antisense RNA technology has been employed for achieving virus resistance in cassava plants against cassava mosaic disease (CMD) caused by African cassava mosaic virus (ACMV). Zhang, *et al.* (2005) developed transgenic cassava plants by silencing the viral mRNAs of *Rep AC1 1* through RNAi. Analysis in detached leaves for Viral DNA replication demonstrated that replication of virus was strongly reduced in transgenic lines. In transgenic plants build up of viral DNA was decreased. Short sense and antisense RNAs specific to *AC1* were identified in transgenic lines expressing *AC1* antisense RNA, demonstrating that the siRNAs are involved in observed gene silencing.

Bonfim *et al.* (2007) developed PTGS against the AC1 gene to produced tolarent engineered common bean plants against *Bean golden mosaic virus* (BGMV). Out of eighteen transgenic lines obtained, one line presented high (93%) resistance upon inoculation with more than 300 viruliferous whiteflies. Transgene-siRNAs specific to the inserted sequence were noticed in both artificially infected and uninfected transformed plants.

Tyagi *et al.* (2008) applied the concept of RNAi to obtain resistance against *rice tungro bacilliform virus* (RTBV) infection and developed transgenic rice plants inserting sense and antisense DNA of RTBV resulted in the formation of dsRNA. The accumulation of siRNA, a hallmark for RNA-interference was detected by using RNA blot analysis in transgenic rice plants.

RNAi was builtup in tobacco plants by inserting construct comprising faulty interfering (DI) sequence of *Tomato bushy stunt virus* (TBSV) and also a preserved sense sequence from *Grapevine fanleaf virus* (GFLV) (Winterhagen *et al.*, 2009). Silencing was confirmed by *Agrobacterium*-mediated infiltration by using GFP-sensor specific to the insert.

Wu *et al.* (2009) engineered melon lines resistant to *Zucchini yellow mosaic virus* (ZYMY), a construct containing the coat protein (CP) sequence joined with the untranslateble region of the virus was generated and used to transform a cultivar of oriental melon (Silver light) via *Agrobacterium*. Gathering of viral mRNA was

observed using northern blotting technique and observed an inverse relation virus accumulation to the degrees of virus resistance, indicating RNA mediated resistance.

Xiuchun *et al.* (2011) achieved robust resistance in soybean plants to multiple viruses with a single dsRNA-expressing transgene. In the transgene construct, Inverted repeats *Alfalfa mosaic virus*, *Bean pod mottle virus*, and *Soybean mosaic virus* were gathered along with terminater and promoter from *Cauliflower mosaic virus*. Three independent transgenic lines obtained exhibited resistance to the spontaneous infection of 3 viruses.

Yu *et al.* (2011) developed transgenic watermelon resistant to *Papaya ring spot virus* type W (PRSV W) and *Zucchini yellow mosaic virus* (ZYMV) shortened CP gene of ZYMV and PRSV via *Agrobacterium*. siRNAs were readily detected transgenic plants representing the RNA mediated silencing at post transcriptional level.

Hernandez *et al.* (2012) proved that eIF4E (eukaryotic translation initiation factor) control the susceptibility of melon (*Cucumis melo* L.) to a wide range of viruses, by knocking down Cm- eIF4E. Transformed plants were resistant to *Cucumber vein yellowing virus* (CVYV), *Moroccan watermelon mosaic virus (*MWMV*), Melon necrotic spot virus* (MNSV) and *Zucchini yellow mosaic virus* (ZYMV).

RNAi construct was transformed through *Agrobacterium* in hill banana cultivar Virupakshi (AAB) for obtaining tolarece against *Banana bunchy top virus* (BBTV) was carried out by Elayabalan *et al.* (2013). They developed RNAi construct targeting the BBTV replicase gene, by cloning *rep* gene fragment in the vector, pSTARLING-A. The cloned construct was freed by *Not*I site. Then transformed embryogenic cells of banana via *Agrobacterium*.

Ntui, *et al.* (2014) attempted to develop plants resistant to Cucumber mosaic virus which is major constraints in tomato production. An RNAi vector was constructed with 1138 bp fragment inverted repeat which encodes partial replicase

gene of CMV-O. Transgenic tomato plants were inoculated with CMV-O showed resistance against CMV-O virus. Resistant lines were selected and tested CMV-Y which is a similar strain. Fascinatingly, the engineered plant lines either showed superior levels of resistance or remained immune to the strain. Virus were detected using RT-PCR and Dot immunobinding assay (DIBA) analyses. Viruses were not noticed in resistant plants and the observed resistance is due to siRNA mediated resistance.

Dutta *et al.* (2015) generated tomato transgenic lines against *M. incognita*-specific protease gene. Due to RNAi 60–80% reduction in infection was observer transgenic tomato plants. Evidence for silencing was confirmed using RT-PCR.

In plants, siRNA mediated gene silencing work at the transcriptome level or post transcriptome level in order to supress the target gene. PTGS or the RNAi approach has been achieved in a broad range of plant species for inhibiting the expression of target genes by generating double-stranded RNA (dsRNA). However, fruitful RNAi-application to knock-down endogenous genes has not been reported in the important staple friut crop banana.

2.5 EVALUATION OF TRANSGENICS FOR siRNA MEDIATED VIRUS RESISTANCE

Evaluation can be done by artificially infecting the transgenics plants under protected condition and detection for the presence of virus by reliable methods.

2.5.1 Transmission of virus using aphids

CMV transmission studies were carried out by Dheepa and Paranjothi (2010) using two aphid species *Aphis craccivora* and *A. gossypii*. Aphid were allowed to feed on infected plant for 20 min (acquisition period) and aphids were allowed to feed on healthy *Commelina sp, Nicotiana glutinosa, Vigna radiata, Vigna mungo and Chenopodium spp* for 10-15 min. After infection insects were killed by spraying insecticide. The plants were maintained in insect proof cage at 25-30°C for 30 days and transmission was confirmed through Direct antigen coating

enzyme linked immuno sorbent assay (DAC-ELISA) technique and concluded the efficient transmission of the virus in a non-persistent manner.

Krishna *et al.* (2013) transmitted BBTV to banana plants transformed with components of the BBTV viral genome. Banana aphids (*Pentalonia nigronervosa*) were collected from infected plants from a banana plantation and reared in the laboratory. Transgenic plants and healthy plants were caged individually and virus transmission was carried out by transferring ten infected viruliferous aphids for three days.

Study of the virus-vector relationship of BBrMV using *Pentalonia nigronervosa* was conducted by Herradura *et al.* (2003). Results of their study showed that the 30 min of optimum acquisition feeding time and 24 hr of infection feeding time could produce symptom expression on the plants and five aphids can transmit the virus at 15% efficiency.

2.5.2 RNA isolation in banana

In the current study CTAB method was used for RNA isolation from tissues containing high levels of phenolics and/or polysaccharides the protocol standardized by Tsai and Harding (2003) was followed.

2.6 CONFIRMATION OF RNAi MEDIATED RESISTANCE

2.6.1 Conformation at genomic level

Incorporation of the construct in the plant genomic DNA is mandatory to have a stable expression by RNAi mediated silencing of the virus gene. Several studies have reported the presence of the silencing cassette by using PCR and southern blotting.

PCR analysis was used by Tripathi *et al.* (2005) with GUS gene specific primers to confirm presence or absence of transgene in the plant genome. Amplified product of expected size showed the stable integration of the transgene in the transformed plants. Krubphachaya *et al.* (2007) developed Cantaloupe resistant

against *Papaya ring spot virus* type W by using RNAi. Coat protein gene was introduced using *Agrobacterium tumefaciens*. They used PCR analysis to confirm presence of trans gere using cp gene specific primers and *nptII* gene specific primers.

Zhang *et al.* (2011) examined soybean plants transformed with a transgene construct to confer resistance against *Alfalfa mosaic virus* (AMV), *Soybean mosaic virus* (SMV) and *Bean pod mottle virus* (BPMV) simultaneously. The transformation was confirmed by presence of the herbicide resistance (BAR) gene using PCR based genotyping and was found positive.

Kantor *et al.* (2013) engineered transgenic tomato plants expressing the PfCP-2.9 protein (a chimera of the antigens *MSP1* and *AMA1* of *Plasmodium falciparum*). Transgenic expression in the transformed plants was verified by extracting DNA from fruits and PCR analysis to test the presence of the gene of interest in the transgenic tomato plants generation. The result showed bands of expected size confirming the transformation indicating the stable integration of the *PfCP-2.9* gene in the tomato genome.

Ntui *et al.* (2014) induced RNAi-mediated resistance against *Cucumber mosaic virus* in tomato, the plantlets transformed with ihpRNA construction were screened by PCR using replicase gene specific primers. The result showed bands of expected size confirming the transformation.

Aragao *et al.* (2013) analysed the effect of siRNAs derived from an ihpRNA construct targeted to the rep (AC1) viral gene of bean golden mosaic virus (BGMV). Southern analyses revealed the presence of the transgene.

Gao *et al.* (2015) demonstrated *soybean mosaic virus* (SMV) resistance in transgenic soybean plants by transforming an insert containing inverted repeat-SMV-HC-Pro. PCR verification and southern hybridization confirmed insertion of the target gene into the plant genomic DNA.

Batuman *et al.* (2006) utilized PTGS to impart resistance against *citrus tristeza virus* (CTV) into citrus and *Nicotiana benthamiana*. PCR and southern analysis confirmed insertion of the T-DNA into the genomic DNA of transformed plants. Transgenic *N. benthamiana* were resistant to infection, as indicated by the absence of the virus from inoculated plants.

2.6.2 Conformation at transcriptome level

Ntui *et al.* (2014) generated an RNAi construct containing replicase gene of *Cucumber mosaic virus* to produce transgenic tomato plants. Analysis of transgenic plants was done by RT-PCR and Dot immunobinding assay (DIBA) analyses. Virus was not noticed in uninfected new leaves of the transgenic lines. siRNA mediated resistance was the key mechanism underlaying the observed resistance in the transformed plants.

Bonfim *et al.*, (2007) discovered the idea of using an RNAi construct to silence the *AC1* viral gene sequence and generated modified common bean plants resistant against *Bean golden mosaic virus* (BGMV). Transgene-specific siRNAs were detected in both infected and uninfected transgenic plants.

Borth *et al.* (2011) engineered resistance against Banana bunchy top virus (BBTV) by introducing replicase gene into banana. Transformed and control plants were infected with infectious banana aphids (*Pentalonia nigronervosa*). Plants transformed with replicase gene were found to be resistant to BBTV and showed no bunchy top symptoms whereas the control plants became infected with BBTV. DNA isolated from the transformed banana plants did not produce any amplicons in PCR analyses with primer specific to BBTV, confirming tolerant of BBTV infection.

Hernandez *et al.* (2012) generated Cm-eIF4E knockdown melon plants by transformation of explants with a construct that silences eukaryotic translation initiation factors (eIF) required for the multiplication of many different viruses. Transgenic melon plants were resistant to *cucumber vein yellowing virus* (CVYV), *melon necrotic spot virus* (MNSV), *Moroccan watermelon mosaic virus* (MWMV)

and *zucchini yellow mosaic virus* (ZYMV). Cm-eIF4E was specifically silenced by the decreased accumulation of Cm-eIF4E mRNA and the appearance of siRNAs derived from the transgene in transformed plants.

Aragao *et al.* (2013) analysed the effect of siRNAs derived from an ihpRNA construct targeted to the rep (AC1) viral gene of *bean golden mosaic virus* (BGMV). Southern analyses revealed the presence of the transgene. Transgene expression analyses was carried out by using northern hybridization was carried out to detect the siRNA in leaves of transgenic and non transgenic bean plants. This analysis showed siRNA bands of expected size range and conformed RNAi mediated resistance.

2.6.3 Conformation at proteome level

Gao *et al.* (2015) demonstrate siRNA mediated resistance against *Soybean mosaic virus* (SMV) in soybean plants by silencing inverted SMV-*HC-Pro* gene. PCR and southern blotting verification confirmed the presence of insert in transformed plants. Examination of level of virus infection in the plants was performed using qRT-PCR and ELISA assays; the results revealed no virus or a gradual reduction over time in the viral demonstrating PDR to SMV brought by inverted repeat-SMV-HC-Pro gene.

Fuentes *et al.* (2006) established transgenic tomato plants, transformed with an ihpRNA construct to silence *early tomato yellow leaf curl* virus (TYLCV) multiplication related protein gene. In extreme conditions of infection transgenic tomato plants showed immunity to TYLCV. Inoculated plants were tested for the presence of TYLCV to detect the viral CP protein using an ELISA assay. Transgenic plants resulted immune to TYLCV infection.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Evaluation of siRNA mediated *Banana bract mosaic virus* (BBrMV) resistance in banana plants with ihpRNA construct for replicase gene" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during September 2015 to September 2017. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 GERMINATION OF THE TRANSFORMED SOMATIC EMBRYOS

An ihpRNA cassette targeting the replicase gene of BBrMV was constructed and the components of ihpRNA construct contained ubiquitin promoter, ubiquitin intron, sense replicase strand, *cre* intron, antisense replicase strand and termination sequence in the order within the *Not*I restriction site. Somatic embryos transformed with ihpRNA construct targeting the replicase gene of BBrMV was used for the study.

3.1.1 Culture medium

3.1.1.1 *Chemicals*

For preparing culture medium all the chemicals used were of analytical grade and procured from Sisco research laboratories (SRL), India. The antibiotics and the plant growth regulators were purchased from Himedia Laboratories, India.

3.1.1.2 *Glassware, plastic ware and other materials*

Borosilicate glass wares and Tarsons (DNase, RNase and protease free) micro centrifuge tubes, PCR tubes and tips were used for the study. Scot Duran screw capped bottles were used for storing stock solutions of antibiotics, plant hormones and buffers. Bacterial membrane filters (Axiva) of diameter 25mm and pore size 0.2 µm were used in the sterilization of antibiotics and heat sensitive plant hormones.

3.1.2.3 *Composition of the medium*

Basal MS medium (Murashige and Skoog, 1962) supplemented with plant growth substances, benzyl adenine (BA), picloram, were used for plant tissue culture experiments (Appendix I).

3.1.2.4 *Preparation of medium*

Standard MS medium was used for the study. The pH of the medium was adjusted to 5.7 using 0.1N NaOH/ HCl. Gelrite were used at the rate of 4.5 g L^{-1} and the medium was heated to dissolve the solidifying agent. The medium was then dispensed into autoclaved culture bottles at the rate of 50 ml/ bottle. The culture bottles were then autoclaved at a pressure of 1.06 Kg cm^{-2} and a temperature of 121°C for 20 min (Dodds And Roberts, 1982). The medium was then stored at 25± 2°C.

3.1.2.5 *Germination of embryos and regeneration into plantlets*

The glassy somatic embryos transformed with LBA 4404 carrying the ihpRNA construct which was developed as part of Ph.D. thesis work at the Department of Plant Biotechnology against the replicase gene of BBrMV was used for the study (Lekshmi *et al.,* 2016). Embryos transformed were subcultured on to half strength semisolid (3 g L^{-1}gelrite) MS medium supplemented with BA and IAA. The cultures were incubated in dark and were later transferred to 14 h photoperiod 31.4µmol$^{-2}S^{1}$. For the development of complete plantlets the germinating somatic embryos were transferred to half strength semisolid MS medium with BA and NAA under14 hr photoperiod.

3.2.1 *Confirmation of plantlets for transformation*

The binary vector was having *nptII* gene conferring kanamycin resistance as plant selectable marker. So, the transformation was confirmed based on the presence of *nptII* gene. PCR reaction was taken out to notice the presence of *nptII* gene in the plantlet survived in the selection medium (kanamycin 100 mg L^{-1}). The presence of ihpRNA cassette in the vector was also confirmed by PCR

analysis of the extracted genomic DNA of the transformants using specific primers for *cre* intron.

3.2.2 Isolation of genomic DNA

Leaf tissues from the regenerated shoots were used for genomic DNA isolation. Total genomic DNA was isolated using modified procedure standardized by Chong-Perez *et al.* (2012). Leaf tissue (0.2 g) was taken, washed in distilled water and blot dried. It was ground into fine powder using liquid nitrogen with mortar and pestle. The powder was then transferred quickly to a 2 ml centrifuge tube and 2ml of pre warmed extraction buffer was added (Appendix II). The mixture was homogenized by vortexing for a few seconds. Then the samples were incubated for 30 min at 55°C with occasional mixing. At room temperature the mixture was cooled and centrifuged at 5000 rpm for 5 min. The supernatant was collected in a fresh tube and the samples were treated with RNaseI (200µg ml-1) for 15 min at 37°C for removing the RNA contamination. With an equal volume of chloroform: isoamyl alcohol (24:1) the extracts were mixed and was centrifuged at 5000 rpm for 5 min. To a new tube the aqueous phase was transferred and an equal volume of ice-cold 2-propanol was added. It was kept at –20° C for 1 hr and centrifuged for 15 min at 11,000 rpm, at 4°C. The pellet was washed twice using 70 per cent ethanol; air dried and was dissolved in 100 µl water.

3.2.3 Polymerase Chain Reaction (PCR)

PCR mix (25 µl) was prepared which contained 100 ng of template DNA, 200 µM dNTPs, 10 pM of each primers, 1 unit of Taq polymerase and 1XTaq polymerase buffer. The genomic DNA of the transformed and the non-transformed plants isolated were amplified with the specific primers for ihpRNA insert (i.e. sense- intron- antisense fragment) *nptII* gene and *cre* intron. The following conditions were provided for the amplification of the specific region in the genomic DNA in a Thermal cycler

Step	Stage		Gene specific primers for *npt*II, *cre* intron and, ihpRNA	
			Temperature (°C)	**Duration**
1.	Initial denaturation		94	2 min
2.	Denaturation		92	30 sec
3.	Annealing	Sense –For Antisense -Rev	57	40 sec
		*npt*II	55	30 sec
		cre intron	53	30 sec
4.	Extension		72	45 sec
5.	Final extension		72	5 min

The steps 2-4 were allowed to repeat 29 times.

3.2 HARDENING OF THE *IN VITRO* DEVELOPED PLANTLETS

After conformation, transformed plants were hardened under protected condition in the glass house. Rooted plantlets were removed from the culture bottle carefully and planted into seedling trays filled with coir pith compost for primary hardening (Plate 3a & 3b). After 2 week later they were transferred into 6 X 9 polythene bags containing sterilized soil and cow dung (Plate 3c). After one month the seedlings having four to five leaves are transplanting into pots filled with soil and cowdung (1:1).

3.3 BBrMV INFECTION OF THE REGENERATED PLANTS USING APHIDS

BBrMV infected banana plants showing symptoms (Plate 8a) were identified from the Instructional Farm, College of Agriculture, Vellayani and the infected suckers were collected (Plate 8b). These BBrMV source plants were maintained in the glasshouse (Deparment of Plant Pathology).

No. 2 camel hair paint-brush with water moistened was used for the banana aphids (*Pentalonia nigronervosa*) collection from a banana plantation

(Farm, College of Agriculture, Vellayani) and released on the BBrMV infected sucker.

BBrMV-infected sucker was transferred with 500 aphids were to for 30min for BBrMV acquisition-access. Infectious aphids were then collected and transferred to disease free untransformed and transformed banana plants having four to five well developed leaves for infection feeding for 24 hr (Herradura *et al.*, 2003). After infection period aphids were removed from the plants and new aphid were released in two days interval for 2 weeks. At 12 h light/dark photoperiod. both the acquisition and inoculation access steps were conducted. Two healthy control and transformed plants each of same age were taken as healthy control.

3.5 DETECTION OF BBrMV INFECTION USING RT-PCR

3.5.1 RNA isolation

The CTAB RNA extraction method standardized by Tsai *et al.* (2003) was followed to isolate RNA. The protocol included the following steps:

Spindle leaf of BBrMV infected banana cv. Nendran was used for RNA isolation. Using motar and pestle the leaf tissue (0.1 g) was homogenized with liquid nitrogen. Finely ground tissue was quickly transferred to 1.3 ml CTAB buffer (Appendix III) and 30 µl of 2, β-mercaptoethanol to a microcentrifuge tube and vortexed briefly and incubated at 65°C for 10min with occasional vortexing. Tubes were cooled and Chloroform 700 µL was added to the tube and vortexed many times and centrifuged for 5 minutes at 16,000 g (these steps were carried out in ice). The phase above the interface was transferred to a new tube and 1/3 volume of 8M LiCl was added. The RNA was precipitated at 4°C for 5 hours, and was pelleted by centrifugation at 16000 g for 20 min at 4°C. The pellet was resuspended in 300 µL DEPC treated water. To that 0.1 Vol 3 M NaOAc (pH 5.0) and 2.5 Vol 100% ethanol was added and mixed and stored at -80°C for 30 min to precipitate RNA and pelleted out by centrifugation at 15,000 g for 10 minutes at

4°C. The pellet was washed with 1ml of 70 per cent ethanol. The pellet was dried inside the laminar hood and was resuspended in 100 µL DEPC treated water.

3.5.2 Agarose gel electrophoresis

Using horizontal gel electrophoresis unit, agarose gel electrophoresis was carried. Agarose (1 per cent) was weighed out and melted in 1X TAE buffer (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0) prepared in (0.1 per cent) DEPC treated water. After cooling to about 50°C, ethidium bromide (10^{-3} mg ml^{-1}) was added. The mixture was then poured to a pre-set template with appropriate comb. After the gel was set, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank and was flooded with 1X TAE buffer, to about 1mm above the gel. Required volume of RNA sample and gel-loading dye (6 x RNA loading dye, M/s Merck Genei) were mixed. Electrophoresis was carried out at 50 volts until the gel loading dye reached three fourth of the gel. The gel was documented using a gel documenting system (BIO-RAD).

3.5.3 Quantification of RNA

Quantification of RNA was carried out using UV-visible spectrophotometer (Spectronic Genesys 5). The optical density of the RNA samples was recorded at both 260 and 280 nm wave lengths. The concentration of RNA was calculated using the following formula:

Amount of RNA (µg/µl) = A_{260}x 40 x dilution factor / 1000

Where A_{260}= absorbance at 260 nm

The quality of the RNA was judged from the ratio of the OD values recorded at 260 and 280 nm. The A_{260}/A_{280} values between 1.8 and 2 indicate the best quality of RNA.

3.5.4 Confirmation of infection by RT-PCR

3.5.4.1 cDNA synthesis

cDNA was synthesized from the isolated RNA using AMV RT-PCR kit according to the manufacturers' protocol. The kit contained *Avian Myeloblastosis Virus* Reverse Transcriptase enzyme and OligodT primers.

3.6.2 Confirmation of cDNA synthesis

PCR analysis was done to confirm the cDNA synthesis. cDNA was amplified with the specific primers for actin gene (house-keeping gene) and 18S rRNA to confirm successful conversion of mRNA to cDNA. A standard PCR mix was prepared for 20 µl total volume containing 100 ng of template cDNA, 200 µM dNTPs, 10 pM of each primers, 1 unit of Taq polymerase and 1x Taq polymerase buffer.

The following conditions were provided for the amplification of the specific region of cDNA in the Thermal cycler (PTC-150 mini cycler).

Step	**Stage**		**Gene specific primes for *Actin* and *18S rRNA***	
			Temperature (°C)	**Duration**
1.	Initial denaturation		94	1 min
2.	Denaturation		92	30 sec
3.	Annealing	*Actin*	55	30 sec
		18S rRNA	61	30 sec
4.	Extension		72	30 sec
5.	Final extension		72	5 min

The steps 2-4 were allowed to repeat 29 times.

The PCR product was electrophoretically analysed in agarose gel (one per cent) in a horizontal gel electrophoresis unit as explained above. 5 µl of the 100 bp molecular weight marker was loaded to one of the wells (Merck Genei) with

essential volume of gel loading dye. Electrophoresis was carried out at 50 volts until the loading dye reached three fourth length of the gel and the gel was documented.

3.2.6 checking the presence of replicase gene sequence of BBrMV

Two set of primers designed by Lekshmi *et al.* (2016) for BBrMV replicase gene, specific for two regions of replicase gene sequence was used in the study. The sequences of the primers for the amplification of the partial replicase gene is given below.

BBrMV Forward – 5' TTAGCTGAACATAAGGGTGC 3'
Reverse – 5' CTGCTGTGAACACGCGTGTT 3'
Rep Forward – 5' AGCAATGTACGCTGGGAAGA 3'
Reverse – 5' TCCGTTCCATATGCCTAAGTG 3'

A standard PCR mix was prepared for 25 µl total volume containing 100 ng of template cDNA, 200 µM dNTPs, 10 pM of each primers, 1 unit of Taq polymerase, 1x Taq polymerase buffer. The cDNA was amplified using 2 sets of primers in a Thermal cycler (PTC-150 mini cycler) using the program shown in the table below

Step	**Stage**		**BBrMV *replicase* specific primers**	
			Temperature (°C)	**Duration**
1.	Initial denaturation		94	1 min
2.	Denaturation		92	30 sec
3.	Annealing	BBrMV	55	30 sec
		Rep	58	30 sec
4.	Extension		72	45 sec
5.	Final extension		72	5 min

The steps 2-4 were allowed to repeat 29 times.

The PCR product was electrophoretically analysed in agarose gel (one per cent) in a horizontal gel electrophoresis unit (as explained above). 5 µl of 100 bp ladder loaded to one of the wells (Merck Genei) with essential quantity of gel loading dye Electrophoresis was carried out at 50 volts until the loading dye reached three fourth of the gel and the gel was documented.

3.7 DETECTION OF siRNAs USING NORTHERN HYBRIDIZATION

3.7.1 siRNA extraction

The siRNA was isolated using a RNASure® Fusion miRNA Mini Kit (Genetix Biotech Asia Pvt. Ltd New Delhi). The gel tanks, combs were soaked in 30% H_2O_2 for 15 minutes to remove RNases contamination before rinsing in DEPC treated water. The small RNA samples were loaded on 1.2 % RNA gel. Agarose was melted in 12.5 ml 10 x running MOPS buffer, 102.5 ml DEPC water, it was cooled to less than 60°C, then 6 ml 35% formaldehyde, Ethidium Bromide 10 mg/ml was added and gel was casted.

RNA samples were resuspended in 1X sample loading buffer (Running Buffer 10x, deionised formamide 250ul, formaldehyde 90 µl water (DEPC) 108 µl). Samples were heated at 65°C for 10 minutes then cooled on ice. 3µl of 10x RNA loading dye was added and loaded the gel. Electrophoresis was carried out at 50 volts until the loading dye reached three fourth length of the gel. The gel was documented using a gel documenting system (BIO-RAD).

3.7.2 RNA transfer and UV cross-linking

The siRNAs were then electroblotted onto positively charged nylon membrane using transblot apparatus (BIO-RAD) at 200 mA for 2 hrs by using 1X SSPE as transfer buffer. Blot was exposed to UV light for 30 seconds using a gel documenting system (BIO-RAD).

3.7.3 Hybridization using biotin labelled probe

Before hybridization, the blots were pre-hybridized at 40°C for 3 hrs to minimise background (Minimum 30 mins) in pre-hybridization buffer [7% SDS, 5 µg/ml salmon sperm DNA and 200 mM Na_2HPO_4 (pH 7.0)]. Pre-hybridization buffer was removed, and hybridization buffer comprising 50 pmol ml^{-1} biotin labeled probes was added. The membrane was hybridized for 12 hours at 40°C with gentle shaking. Membrane was washed 2 times, 5 min each, in 20 mL 1X washing buffer (1X PBS, 0.5% SDS). Membrane was washed again 2 times in 10 mL 1X blocking buffer 5 min each (1X PBS, 0.5% SDS, 0.1% I-Block reagent/ fat free milk powder). Membrane was washed once for 30 min in 20 mL 1X blocking buffer.

Membrane was incubated in conjugate solution containing 10 mL blocking buffer and 1 ul of streptavidin-peroxidase conjugate for 30 min. After incubation membrane was washed once in 20 mL 1X blocking buffer for 15 min. Again membrane was washed 3 times in 20 mL 1X washing buffer for 15 min each.

3.7.4 Staining

10mg of 3,3′-diaminobenzidine (DAB) was dissolved in 10 ml of 50mM Tris pH 7.6 and blot was incubated in DAB solution. 10µL H_2O_2 (30%) was added to the DAB solution and blot was incubated by shaking till the color develops. Once the color was developed blot was transferred to double distilled water and washed. The blots developed blots were stored dry and sealed in polypropylene bags and observed for signals.

Results

4. RESULTS

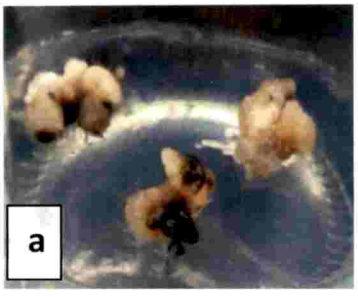
The results of the present study on "Evaluation of siRNA mediated *Banana bract mosaic virus* (BBrMV) resistance in banana plants with ihpRNA construct for replicase gene" carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during September 2015 to September 2017 are presented below. The study included evaluation of siRNA mediated resistance against *Banana bract mosaic virus* (BBrMV) in banana (*Musa* spp. Var. Nendran) plants harboring ihpRNA construct for replicase gene by artificial infection and to confirm the expression of siRNA products in infected plants.

In a previous study conducted at the Department of Plant Biotechnology, an ihpRNA cassette targeting the replicase gene of BBrMV was constructed and the components of ihpRNA construct contained ubiquitin promoter, ubiquitin intron, sense replicase strand, *cre* intron, antisense replicase strand and tmL terminator within the *Not*I restriction site. Somatic embryos transformed with ihpRNA construct targeting the replicase gene of BBrMV were used for the study (Plate 1a).

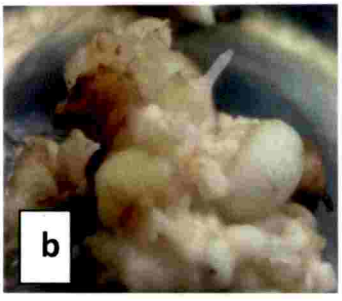
4.1 GERMINATION OF EMBRYOS

The glassy elongated monocot embryos developed in semisolid MS with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} showed 60-80 per cent germination in 3 weeks of inoculation in half strength semisolid MS supplemented with similar combination of growth regulators kept in the dark (Plate 1b). When transferred to 14 h photoperiod with light intensity of 31.4 $\mu mol^{-2}S^{-1}$ proper morphogenesis was observed (Plate 2b). Germinated embryos when transferred to MS supplemented medium with BA 2 mg L^{-1} and NAA 1 mg L^{-1} and incubated in light developed green shoots and showed proper root development (Plate 2b). Transformed plantlets were maintained by subculturing in MS medium containing BA 2 mg L^{-1}, NAA 0.5 mg L^{-1} and kanamycin 100 mg L^{-1} (Plate 3).

Plate 1. Germination of somatic embryos transformed with ihpRNA construct targetting BBrMV replicase gene

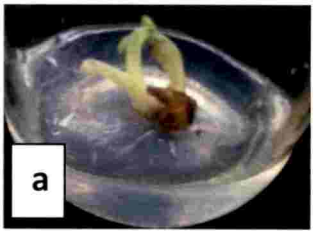


Transformed embryogenic calli in MS +Kan100 mg L^{-1}



Glassy elongated embryos in MS (semisolid) + BA 2 mg L^{-1}, IAA 0.5 mg L^{-1}, Kan100 mg L^{-1}

Plate 2. Development of germinated plantlets in MS + BA 2 mg L^{-1}, NAA 1 mg L^{-1}+ Kan100 mg L^{-1}



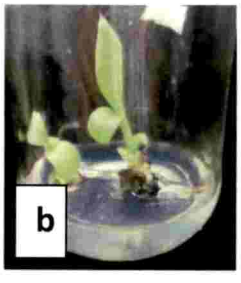


Plate 3. Transformed plantlets maintained by subculturing in MS medium containing BA 2 mg L^{-1}, NAA 0.5 mg L^{-1} and kan100 mg L^{-1}

4.1.1 Confirmation of transformation

The plants developed were checked for the presence of ihpRNA cassette using PCR. The DNA isolated from the leaves of transformed Nendran shoots (tissues selected on kanamycin 100 mg L^{-1}) was amplified with *npt II* and *cre intron* specific primers. Out of 8 transformed plants 6 plants showed the amplicon of 475 bp which was of expected size designed for *npt II* gene (Plate 4). Similarly, out of 8 transformed plants 6 plants showed the amplicon of 450 bp which was of expected size designed for *cre intron* gene (Plate 5). The results indicated successful insertion of the ihpRNA construct in the transgenic banana plantlets developed. The genomic DNA isolated from leaf tissues of the plantlets selected on kanamycin 100 mg L^{-1} when amplified using forward primer for sense strand and reverse primer for antisense strand of replicase gene, an amplicon of size approximately 1300bp was produced (Plate 6). This band matched with the size of the ihpRNA cassette inserted which covers sense and antisense strands of replicase gene connected through *cre* intron. So, the result obtained confirmed the presence of ihpRNA cassette in the transformed plants.

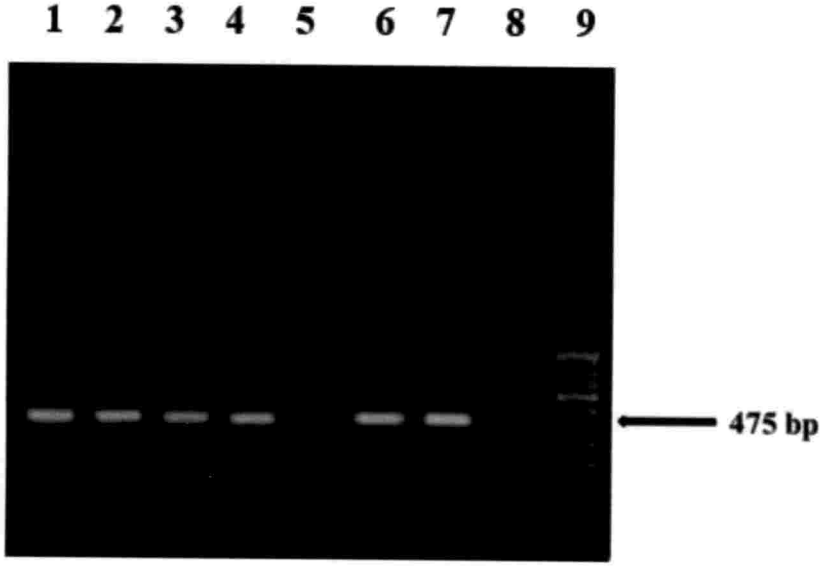
4.2 HARDENING OF THE *IN VITRO* RAISED PLANTS

The conformed plantlets were removed from the culture bottle carefully and planted into seedling trays filled with coir pith compost and kept in the glass house for primary hardening (Plate 7a & 7b). After 2 week they are transferred into 6 X 9 polythene bags containing sterilized soil and cow dung (Plate 7c). After one month the seedlings having four to five leaves were transplanted into pots filled with soil and cowdung (1:1). Survival rate of hardened plants (%) is shown in the table 2.

4.3 BBrMV INFECTION USING APHIDS (*Pentalonia nigronervosa*)

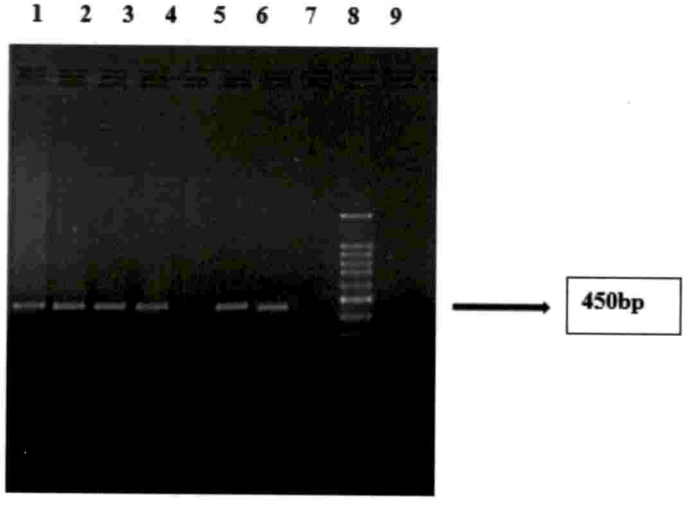
BBrMV infected banana plants showing symptoms (Plate 8a) were identified from the Instructional Farm, College of Agriculture, Vellayani and the infected suckers were collected (Plate 8b). For the experiment aphids collected from infected plants were released on suckers and reared in glass house. The hardened banana plants transformed with ihpRNA vector and having four to six well established

Plate 4. PCR analysis of transformed plants with *nptII* specific primers



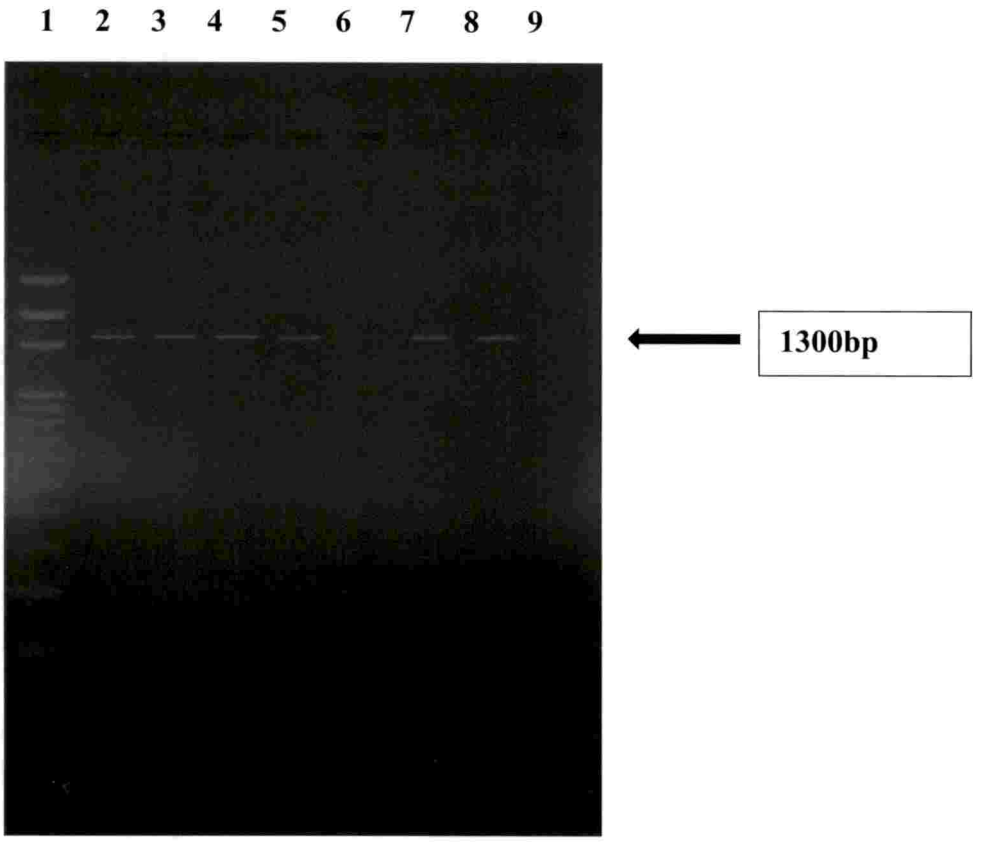
Lane 1-8– Transformed plants
Lane 9 – 100bp ladder

Plate 5. PCR analysis of transformed plants with *cre intron* specific primers



Lane 1-8 - Transformed plants
Lane 9 - 100bp ladder

Plate 6. PCR confirmation of presence of the ihpRNA cassette by PCR



Lane 1 - 100 - 2 kb DNA ladder
Lane 2-9 - Transformed plantlets

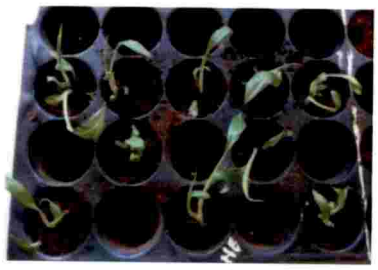
Table 1. Germinated embryos with ihpRNA construct (percentage)

No: of embryo inoculated	No: of embryo germinated	No: of embryod with ihpRNA construct	Percentage of embryos with ihpRNNA construct (%)
8	8	6	75%

Table 2. Survival of hardened plants (percentage)

Treatment	No: of plants hardened	No: of plants survived	Hardened plants (%)
Control plants	8	7	87
Transformed plants	6	6	100

Plate 7. Hardening of the transformed plants



A. Day 1

a. Primary hardening

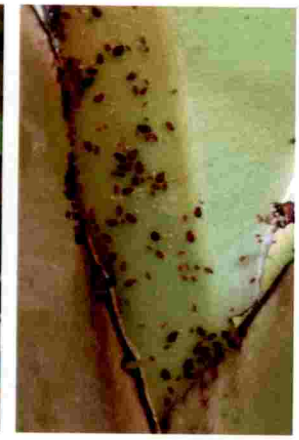
B. Day 14

c. After one month

Plate 8. BBrMV infection using aphids (*Pentalonia nigronervosa*)

a. BBrMV infected mother plant

b. BBrMV infected sucker



c. Aphids released for acquisition-access

leaves were challenged with 50 infectious infectious aphids (Plate 9a) immediately after acquisition-access to BBrMV-infected banana plant (Plate 8c) for 30min. After 24 h of infection period aphids were removed from the plants and 50 new aphids were released in two days interval for 2 weeks for 7 times. Out of 6 transformed plants 4 were infected and 2 plants were kept as control. Untransformed control plants developed through tissue cultured of the same developmental stage were also inoculated with a same number of viruliferous infectious aphids (Plate 9b) keeping 2 plants as positive control. During first two month no visual symptoms were noticed in any of the plants. After 3 months visual symptoms were noticed in the control plants.

4.4 DETECTION OF VIRUS INFECTION USING RT-PCR

The detection of virus infection was done with 2 sets of primers specific for replicase gene of BBrMV. RT-PCR analyses using primers specific to BBrMV Rep gene were performed. Plants were tested for the presence of viral RNA in young leaf by RT-PCR using total RNA extracted from the BBrMV-inoculated untransformed control and transgenic plants. The results are given below.

4.4.1 RNA isolation

Total RNA was isolated from BBrMV infected banana leaves according to the protocol reported by Tsai *et al.* (2003) using CTAB method. The RNA was electrophoretically separated on 1per cent agarose gel and was documented. The gel picture showed typical eukaryotic RNA profile with bands representing 23S, 18S and 4S rRNA (Plate 10). The crisp bands indicated intact nature of the nucleic acid isolated from all the samples.

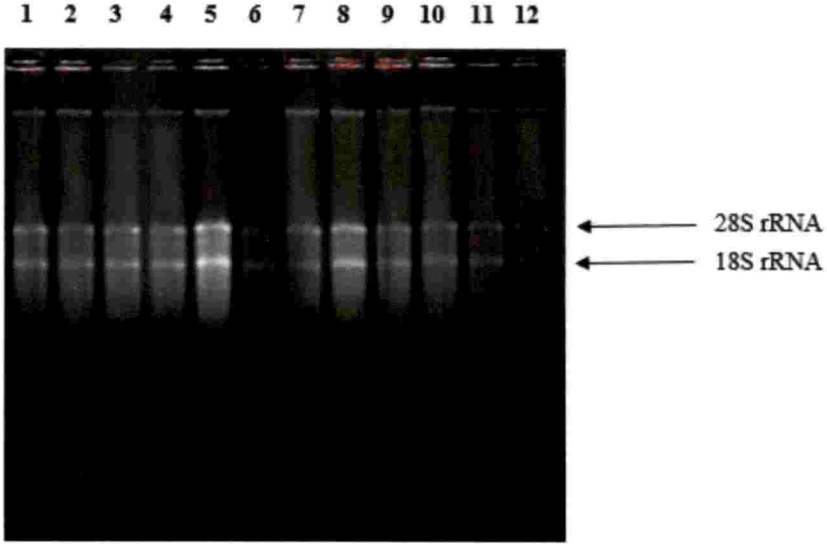
RNA isolated was quantified with UV- visible spectrophotometer. Absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) was recorded. The concentration of RNA and A_{260}/A_{280} value is shown the table 3 indicating good quality of RNA.

Plate 9. Banana plants challenged with infectious aphids

a. Transformed plant

b. Control plant

Plate 10.RNA isolated from the BBrMV infected Nendran plant after aphids infection



Lane 1 to 6 - Control plants
Lane 7 to 12 - Transformed plants

Table 3. Spectrophotometric observations of the RNA samples

Treatment	A_{260}	A_{280}	A_{260} / A_{280}	Concentration (µg/µl)
Control plant infected 1	1.130	0.593	1.905	27.12
Control plant infected 2	1.193	0.645	1.846	28.632
Control plant infected 3	1.226	0.682	1.797	29.424
Control plant infected 4	1.262	0.688	1.834	30.288
Control plant uninfected 5	1.780	0.652	1.800	28.272
Control plant uninfected 6	1.165	0.629	1.852	27.980
Transformed plant infected 1	1.142	0.630	1.810	27.408
Transformed plant infected 2	1.178	0.684	1.722	28.272
Transformed plant infected 3	1.197	0.691	1.732	28.728
Transformed plant infected 4	1.241	0.609	2.037	29.784
Transformed plant uninfected 5	1.194	0.663	1.800	28.656
Transformed plant uninfected 6	1.250	0.689	1.814	30.001

4.5.2 cDNA synthesis

cDNA synthesis using AMV RT-PCR kit was confirmed by doing PCR with *actin* (house-keeping gene) and 18S rRNA gene specific primer. The PCR product on separation on 1 per cent agarose gel showed a band corresponding to 340 bp which was the expected amplicon size of the *actin* gene (Plate 11). And band corresponding to 100 bp was obtained which was the expected amplicon size of 18S rRNA. The result confirmed (Plate 12) the successful conversion of mRNA to cDNA.

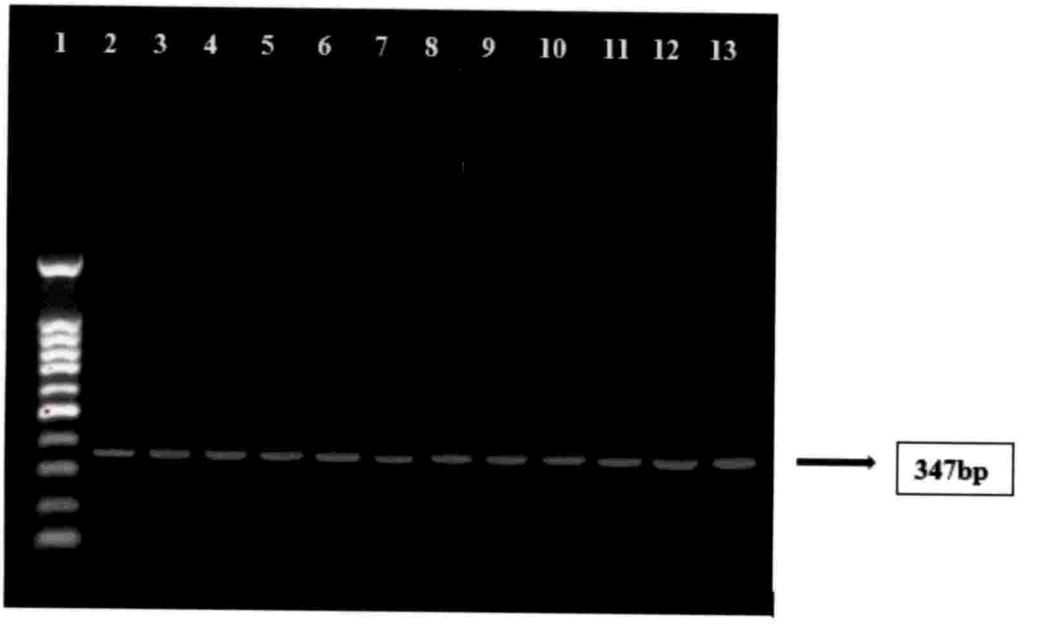
4.5.3 Detection of BBrMV infection using BBrMV specific primer

PCR analysis with BBrMV replicase gene specific primers designed for 2 different regions resulted in the amplification of fragment of size 733bp and 110bp in untransformed control plants inoculated with aphids (Plate 13&14). None of the transformed plants produced the amplicon specific to replicase gene.

4.6 CONFIRMATION OF siRNA SYNTHESIS

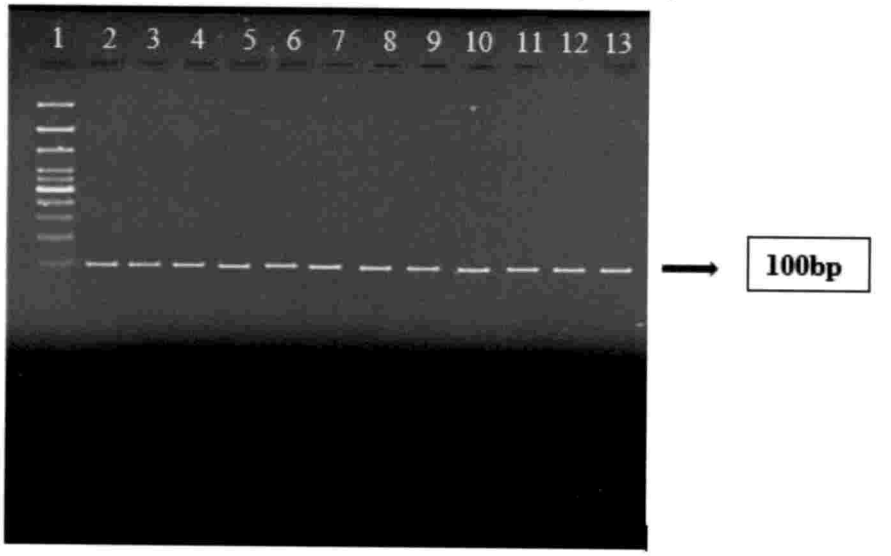
The siRNAs isolated using RNASure® Fusion miRNA Mini Kit (Genetix Biotech Asia Pvt. Ltd. New Delhi) were separated on 1.2% per cent formaldehyde agarose gel. Band corresponding to 21nt was obtained in all the transformed plants (both infected and uninfected) and band of around 22nt was obtained in the untransformed control plants (Plate 15a &15c). Northern hybridisation of the siRNA on positively charged nylon membrane hybridised with siRNA specific probe, when stained using DAB, showed strong signals corresponding to 21nt long siRNAs in samples from transformed plants (Plate15b & 15d).

Plate 11. cDNA amplified with actin specific primer



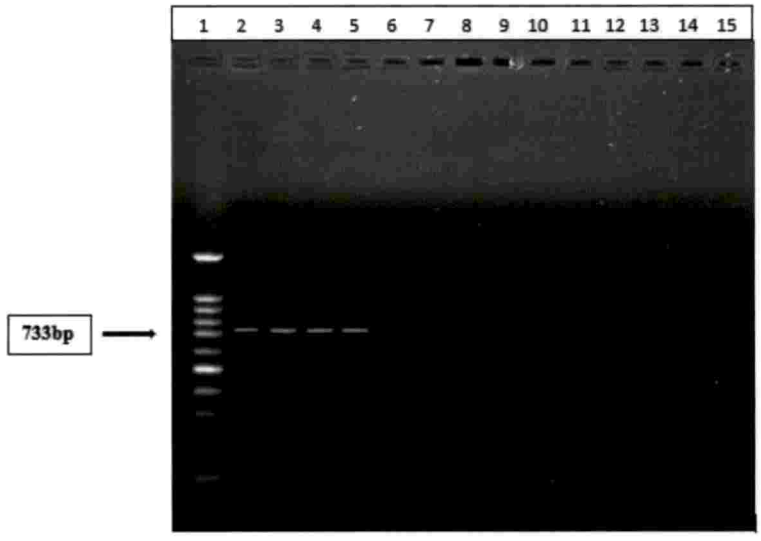
Lane 1 - 100bp ladder
Lane 2 to 7 -Transformed plants
Lane 8 to 13 - Controle plants

Plate 12. cDNA amplified with 18srRNA specific primers



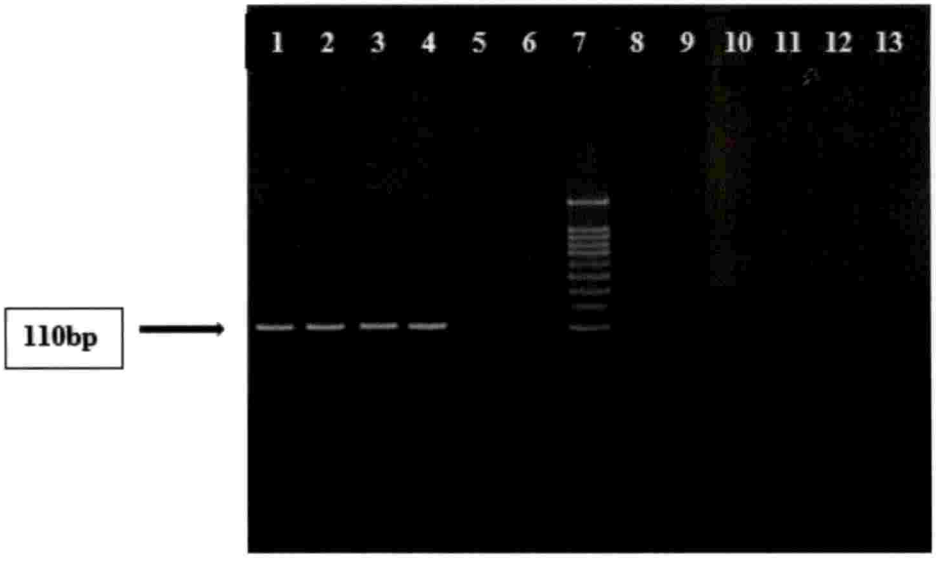
Lane 1 - 100bp ladder
Lane 2 to 7 -Transformed plants
Lane 8 to 13 - Controle plants

Plate 13. cDNA amplified with BBrMV specific primers



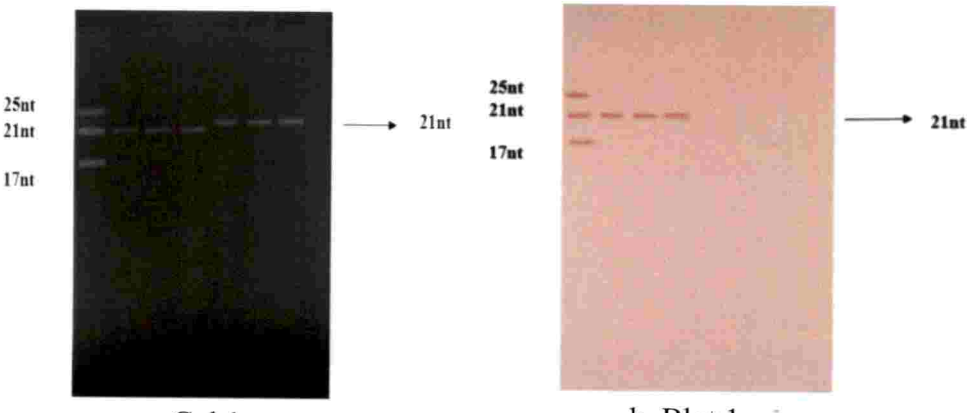
Lane 1 - 100bp DNA ladder
Lane 2-5 - Control plant infected
Lane 6-7 - Control plant uninfected
Lane 8-11 - Transformed plant infected
Lane 12-13 - Transformed plant uninfected

Plate 14. cDNA amplified with replicase gene specific primers



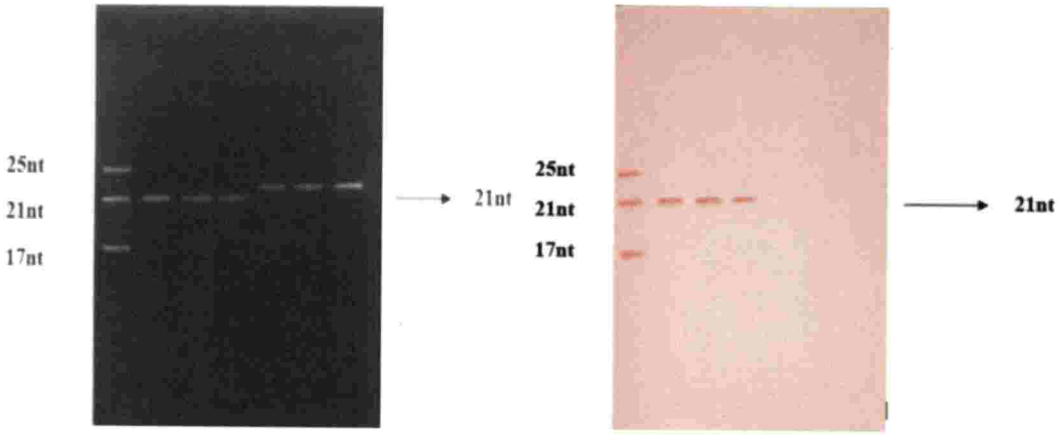
Lane 1-4 - Control plant infected
Lane 5-6 - Control plant uninfected
Lane 7 - 100bp DNA ladder
Lane 8-11 - Transformed plant infected
Lane 12-13 - Transformed plant uninfected

Plate 15. Confirmation of the synthesis of siRNA targeting replicase using Northern hybridization



a. Gel 1 b. Blot 1

Lane 1 - siRNA Marker
Lane 2-3 - Transformed plant infected
Lane 4 - Transformed plant uninfected
Lane 5-6 - Control plant infected
Lane 7 - Control plant uninfected



c. Gel 2 d. Blot 2

Lane 1 - siRNA Marker
Lane 2-3 - Transformed plant infected
Lane 4 - Transformed plant uninfected
Lane 5-6 - Control plant infected
Lane 7 - Control plant uninfected

Discussion

5. DISCUSSION

Banana is one of the major fruit crops of the tropical and sub-tropical regions of the world and consumed worldwide. Bananas and plantains belonging to the family Musaceae are perennial herbaceous monocots. Banana cultivation is prone to various biotic and abiotic stress, specially viruses which subsidize to significant yield reduction and also act as threats to the trade of germplasm. The viruses involved in the infection of banana leading to yield loss are *Banana bunchy top virus* (BBTV), *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV), *Banana streak virus* (BSV), *Banana mild mosaic virus* and *Banana virus X*. BBrMV is previously described virus disease of banana. In India, incidence of BBrMV has been reported in Kerala, Karnataka, Tamil Nadu, and Andhra Pradesh (Rodoni *et al.*, 1997, Thomas *et al.*, 1996, Selvarajan *et al.*, 1997, Thangavelu *et al.*, 2000, Cherian *et al.*, 2002, Kiranmani *et al.*, 2005). BBrMV was first reported in Thrissur district of Kerala in the Nendran variety, due to its susceptible. Later varieties like Palayankodan, Chenkadali, Kodappanillakunnan, Monthan, Poovan (Rasthali), Kanchikela, Karpooravally were also found to be infected. Incidence of the disease ranges from 5 to 36 per cent and more in cv. Nendran in Kerala (Selvarajan *et al.*, 2006). BBrMV cause considerable damage with yield reduction in cv. Robusta (AAA) (70%), followed by cv. Nendran (AAB) (52%) (Cherian *et al.*, 2002).

Plant viruses are most important among the plant pathogens resulting in significant damage and rising threat to crop production. Control measures have been taken to reduce losses caused by viral infections which are inadequate and exclusive. Due to lack of natural resistance source and triploid nature of the popular banana varieties conventional breeding methods have inadequate success in banana improvement programmes. In order to reduce the losses caused by plant pathogens, biotechnological methods have been adopted to engineer resistant plants. Development of resistant varieties is considered the most commercial and ecologically acceptable way of controlling viral diseases (Blackburn and Barker,

2001). Reports on genetic engineering approaches tried for crop improvement in banana are scarce.

During the last decade many researchers have been focusing on genetic modification of plants to deliver increased and robust virus resistance using transgenic machineries. Sanford and Johnston (1985) proposed the concept of pathogen-derived resistance by transferring the viral sequences into the plant genome to produce resistant transgenic plants. The first attempt to develop virus-derived, (coat) protein-mediated resistance against *Tobacco mosaic virus* to develop transgenic tobacco plants was established by Abel *et al.* (1986). Studies indicated that mechanisms responsible for pathogen mediated resistance in plants is due to post-transcriptional gene silencing (PTGS). RNA silencing or PTGS, functions as a powerful defense mechanism against foreign nucleic acid invasions (Voinnet, 2002).

RNAi mediated resistance has emerged as a powerful tool to engineer resistant crops in which small interfering RNAs (siRNAs) silences the expression of genes with homologous sequence specific nucleotide. Degrading viral gene has been successfully achieved used RNAi by expressing cognate dsRNAs for viral transcripts in order to recruit the silencing process. Development of plants resistant to virus infection can be successfully achieved by the application of RNAi in several horticultural and agricultural crops.

In the Department of Plant Biotechnology, RNAi technology has been attempted to develop resistance against BBrMV in banana (*Musa* spp. Var. Nendran) by Lekshmi *et al.* (2016). An ihpRNA cassette targeting the replicase gene of BBrMV was developed in pSTARLIG. The cassette consisted of ubiquitin promoter, ubiquitin intron, sense replicase strand, *cre* intron, antisense replicase strand and termination sequence in the order within the *NotI* restriction site. The ihpRNA cassette was introduced into somatic embryos developed from immature male flower bud and plant regeneration was done through somatic embryogenesis.

The present study envisaged evaluation of the transgenics developed in that study for siRNA mediated resistance by artificial infection and to confirm the expression of siRNA products in infected plants.

It was observed that the glassy elongated monocot transformed embryos developed in half strength semisolid MS with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} showed germination in 3 weeks of inoculation in the dark as reported by Lekshmi *et al.* (2016). When transferred to 14 h photoperiod with light intensity of 31.4 $\mu mol^{-2}S^{-1}$ proper morphogenesis was observed. Germinated embryos transferred to MS medium with BA 2 mg L^{-1} and NAA 1 mg L^{-1} and incubated in light developed green shoots and showed proper root development as reported by Lekshmi *et al.*, (2016).

A total of 8 plants were obtained from the transformed somatic embryos on antibiotic selection medium. Confirmation of transformation was done by amplifying the genomic DNA using primers specific for *nptII* gene (selectable marker), *cre* intron and for the ihpRNA construct covering the sense and antisense fragments of replicase connected through *cre* intron. The PCR result showed a positive result with bands of size 475, 450 and 1300bp showing the expected sizes confirming the integration of ihpRNA construct against BBrMV in the six transformed banana plantlets. PCR analysis was used by many researchers to confirm the presence of the insert. In the experiments conducted by Elayabalan *et al.* (2013) for developing RNAi-mediated resistance to BBTV in banana, the transformation was confirmed by amplification of the transformed template DNA with primers specific for *nptII* and the target gene (replicase gene and coat protein gene) which were used for the hpRNA vector construction and they obtained bands of expected size confirming the transformation. In a study by Ntui *et al.* (2014) to induced resistance to *Cucumber mosaic virus* in tomato plants through RNAi, the transformed plantlets were selected and screened by PCR using replicase gene specific primers used for the ihpRNA construction and NPTII-5P and NPTII-3P for the Kanamycin selectable marker gene. The result showed bands of expected size confirming the transformation. In the present study six

transformed plants yielded amplicons representing the entire ihpRNA construct developed targeting BBrMV replicase.

The transformed plants showed 100% survival rate during hardening. BBrMV infection of the hardened plants was done using viruliferous aphids (*Pentalonia nigronervosa)* through acquisition feeding in contained conditions for 30min, keeping untransformed healthy plants as control. After infection period of 24hr aphids were removed from the plants and new aphid were released in two days interval for 2 weeks.

In a study of the virus-vector relationship of BBrMV using *P. negronervosa* conducted by Herradura *et al.* (2003), it was observed that the optimum acquisition feeding time of 30 min and infection feeding time of 24 hr was sufficient to produce symptom expression on the plants. They found that five aphids can transmit the virus at 15% efficiency.

Similarly, Krishna *et al.* (2013) transmitted BBTV to banana plants transformed with components of the BBTV viral genome. Banana aphids (*Pentalonia nigronervosa*) were collected from infected plants from a banana plantation and reared in the laboratory. Transgenic plants and healthy plants were caged individually and virus transmission was carried out by transferring ten infected viruliferous aphids for three days.

CMV transmission studies were carried out by Dheepa and Paranjothi (2010) using two aphid species *Aphis craccivora* and *A. gossypii*. Aphid were allowed to feed on infected plant for 20 min (acquisition period) and aphids were allowed to feed on healthy *Commelina sp, Nicotiana glutinosa, Vigna radiata, Vigna mungo and Chenopodium spp* for 10-15 min. After infection insects were killed by spraying insecticide. In insect proof cages the plants were sustained for 30 days at 25-30°C and infection was confirmed using the technique, Direct antigen coating enzyme linked immuno sorbent assay (DAC-ELISA) and concluded non-persistent manner of virus transmission efficiently.

RT-PCR was carried out in order to check virus resistance after BBrMV infection by using BBrMV replicase gene specific primers. Amplicon of size 733bp and 110bp sequence were capably detected in infected untransformed control plants. On the other hand, none of the transformed plants produce the amplicon specific to the replicase gene. Absence of BBrMV RNA after aphid inoculation ensured that the transformed plants were resistant to BBrMV infection. These findings were also supported with the findings of Ntui *et al.* (2014) who generated an RNAi construct containing replicase gene from *Cucumber mosaic virus* to produce transgenic tomato plants. Analysis of transgenic plants was done by RT-PCR. No virus was detected in uninfected new leaves of the transformed resistant lines. The resistance was associated with PTGS because of the production of siRNA specific to transgene. Shekhawat *et al.* (2012) revealed the idea of RNAi by using ihpRNA of viral protein essential for initiation of replication to develop transgenic banana plants resistant to BBTV. The construct was fruitfully transformed into banana. To confirm the resistance RT-PCR was carried out to detect cDNAs which codes for viral protein. Virus infected transgenic plants were unsuccessful to detect viral cDNAs.

In order to check the mechanism responsible for the obtained resistance to BBrMV detection of small interfering RNAs (siRNAs) derived from the ihpRNA sequence was done using northern hybridization in the transformed plants which were resistant to BBrMV. In the transformed plants, presence of 21nt long siRNAs specific to BBrMV replicase gene demonstrated that PTGS pathway is responsible for the observed resistance. dsRNA resulted in the synthesis of siRNAs specific to replicase gene was fully functioning in the transformed plants and this eventually led to the BBrMV-resistant. In the experiment conducted by Bonfim *et al.,* (2007) for silencing *AC1* viral gene to generate transgenic common bean plants resistant against *Bean golden mosaic virus* (BGMV), the concept RNA interference was used. They obtained transgene-specific siRNAs in both infected and non-infected transgenic plants.

A rapid and efficient protocol was developed in this study for Evaluation of siRNA mediated *Banana bract mosaic virus* (BBrMV) resistance in banana plants with ihpRNA construct for replicase gene. The transgenic banana plants were confirmed to harbor the ihpRNA construct. The study was also successful in conforming disease resistance in transgenic plants by artificial infecting using aphids. All the transgenic plants containing ihpRNA cassette resistant to BBrMV infection were evaluated further for the production of siRNA. The siRNA detected in the transgenics clearly shows that multiplication of the virus in the host was suppressed due to the silencing of replicase gene.

The RNAi technology was successful in conferring resistance against BBrMV in banana (*Musa* spp. Var. Nendran). The siRNA detected in the transgenics clearly shows that multiplication of the virus in the host was suppressed due to the silencing of replicase gene. This is the first report on siRNA mediated resistance against BBMV in banana. The study suggests the possible use of RNAi technology for developing resistance against other viruses also in banana.

Summary

SUMMARY

The study entitled "Evaluation of siRNA mediated *Banana bract mosaic virus* (BBrMV) resistance in banana plants with ihpRNA construct for replicase gene" was carried out during 2015-2018 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objectives of the study were to evaluate siRNA mediated resistance against *Banana bract mosaic virus* (BBrMV) in banana (*Musa* spp. Var. Nendran) plants harboring ihpRNA construct for replicase gene by artificial infection and to confirm the expression of siRNA products in infected plants.

In the Department of Plant Biotechnology, RNAi technology has been attempted to develop resistance against BBrMV in banana (*Musa* spp. Var. Nendran) by Lekshmi*et al.*(2016). The ihpRNA construct (1300bp) consisted of sense and antisense fragment of BBrMV replicase gene, connected through a *cre* intron under the control of ubi promoter and tmL terminator. The ihpRNA cassette was introduced into somatic embryos developed form the immature male flower buds and shoots were developed through somatic embryogenesis. The present study envisaged evaluation of the transgenics developed for siRNA mediated resistance by artificial infection and to confirm the expression of siRNA products in infected plants.

The transformed embryos were selected on semisolid MS medium supplemented with BA 2 mg L^{-1}, IAA 0.5 mg $L^{-1}$and kanamycin 100 mgL^{-1}. The embryos showed germination in 3 weeks of inoculation in half strength semisolid MS supplemented with the same combination of growth regulators in dark. Germinated embryos when transferred to MS medium with BA 2 mgL^{-1}, NAA 1 mgL^{-1} and kanamycin 100 mgL^{-1} incubated in light developed green shoots with proper root development.

Confirmation of transformation was done by amplifying the genomic DNA using primers specific for *nptII* gene (selectable marker), *cre* intron and for the ihpRNA construct covering the sense and antisense fragments of replicase connected

through *cre* intron. The PCR result showed a positive result with bands of size 475, 450 and 1300bp showing the expected sizes of the respective fragments, confirming the integration of ihpRNA construct against BBrMV in the six transformed banana plantlets. The results indicated successful insertion of the ihpRNA construct in the six transformed banana plantlets developed.

The transformed plants were hardened under protected condition in the glass house. After the acquisition-access of aphids to BBrMV infected banana plant for 30min one month old hardened plants were challenged with 50 infectious viruliferous aphids for 24hrs. After infection period of 24hr aphids were removed from the plants and new aphid were released in two days interval for 2 weeks. Untransformed healthy control plants of the same developmental age were also correspondingly infected with viruliferous aphids. After 2 weeks of infection, amplicons of size 733bp and 110 corresponding to replicase gene were noticed in infected untransformed control plants using RT-PCR, but none of the ihpRNA transformed plants showed infection.

Northern hybridization was carried out to confirm the production of siRNA transcripts in the transgenic plants. The siRNAs isolated from the experimental plants blotted on to nylon membrane were hybridized with probes specific to siRNA targeting replicase gene. Signals equivalent to 21nt long siRNAs were attained in all the transformed plants, clearly indicating the successful siRNA mediated silencing of replicase gene of BBrMV as the cause of virus resistance in transformed plants.

In the present study RNAi technology was found successful in conferring resistance against BBrMV in banana (*Musa* spp. Var. Nendran). The absence of replicase specific amplicon and siRNA detected in the transgenics clearly shows that multiplication of the virus in the host was suppressed due to the silencing of replicase gene. This is the first report on siRNA mediated resistance against BBrMV in banana. In future, clean vector technology can be applied to remove the antibiotic resistance marker also. The study suggests the possible use of RNAi technology for developing resistance against other viruses also in banana.

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Appendices

APPENDIX I

Chemical composition of the media employed: Murashige and Skoog (MS) medium

Macro-nutrients (mg L^{-1})

$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KNO_3	1900
NH_4NO	1650
KH_2PO_4	170

Micro-nutrients (mg L^{-1})

$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$AlCl_3$	0.025
KI	0.83
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25

Iron source (mg L^{-1})

$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25

Vitamins (mg L^{-1})

Nicotinic acid	0.5
Pyridoxin HCl	0.5
Thiamine HCl	0.1

Amino acid sources (mg L^{-1})

Glycine	20

Inositol (mg L^{-1})	100
Sucrose (g L^{-1})	30
Gelrite (g L^{-1})	4.5

APPENDIX II

Chemicals for isolation of DNA from banana leaves

CTAB Buffer

CTAB	2 %
Tris-HCl (pH 8.0)	100 mM
NaCl	1.4 M
EDTA	20 mM
2. β-mercaptoethanol	0.1 %

TE buffer (pH 8.0)

Tris buffer	10 mM
EDTA	1 mM

50X TAE buffer (pH 8.0)- 1000ml

Tris buffer	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA (pH 8.0)	100 ml

APPENDIX III

CTAB RNA extraction buffer (pH 8.5):

CTAB	2%
EDTA (pH 8.0)	25 mM
Tris base	0.1 M
Polyvinylpyrrolidine (PVP)	2 %
NaCl	2 M
To be added just before use:	
β-mercaptoethanol	2%
Chloroform:isoamyl alcohol	24:1
Lithium chloride	8 M

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EVALUATION OF siRNA MEDIATED *Banana bract mosaic virus* (BBrMV) RESISTANCE IN BANANA PLANTS WITH ihpRNA CONSTRUCT FOR REPLICASE GENE

by

HARSHITHA C. K.

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Abstract of the thesis

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ABSTRACT

The study entitled "Evaluation of siRNA mediated *Banana bract mosaic virus* (BBrMV) resistance in banana plants with ihpRNA construct for replicase gene," was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-2018. The objectives of the present study were to evaluate siRNA mediated resistance against *Banana bract mosaic virus* (BBrMV) in banana (*Musa* spp. Var. Nendran) plants harboring ihpRNA construct for replicase gene by artificial infection and to confirm the expression of siRNA products in infected plants.

Somatic embryos transformed with *Agrobacterium tumifaciens* strain LBA 4404 carrying the ihpRNA construct (developed as part of Ph.D. thesis work at the Department of Plant Biotechnology) against the replicase gene of BBrMV was used for the study. The ihpRNA construct (1300bp) consisted of sense and antisense fragment of BBrMV replicase gene, connected through a *cre* intron under the control of ubi promoter and tmL terminator.

The transformed embryos were selected on semisolid MS medium supplemented with BA 2 mg L^{-1}, IAA 0.5 mg $L^{-1}$and kanamycin 100 mgL^{-1}. The embryos showed germination in 3 weeks of inoculation in half strength semisolid MS supplemented with the same combination of growth regulators in dark. Germinated embryos when transferred to MS medium with BA 2 mgL^{-1}, NAA 1 mgL^{-1} and kanamycin 100 $mgL^{-1}$and incubated in light developed green shoots with proper root development.

The transformation of the regenerated plants were confirmed by PCR analysis for the presence of ihpRNA construct, nptII gene (selectable marker) and *cre* intron using the corresponding primers already designed for this purpose. The DNA isolated from the leaves of transformed Nendran shoots showed amplicons of size 475, 450 bp and 1300bp corresponding to nptII gene, *cre* intron and ihpRNA construct respectively. The results indicated successful insertion of the ihpRNA construct in the transformed banana plantlets developed.

The transformed plants were hardened under protected condition in the glass house. Three months old hardened plants were challenged with 50 infectious viruliferous aphids for 24hrs immediately after they were allowed acquisition-access to BBrMV-infected banana plant for 30min. Untransformed control plants of the same developmental stage were also similarly inoculated with viruliferous aphids. After infection period aphids were removed from the plants and new aphid were released in two days interval for 2 weeks. After 2 weeks of infection, the plants were tested for the presence of viral RNA in young leaf by RT-PCR using BBrMV replicase gene specific primers. An amplicon of size 733bp corresponding to replicase gene detected in inoculated untransformed control plants, but none of the ihpRNA transformed plants showed infection.

Northern hybridization was carried out to confirm the production of siRNA transcripts in the transgenic plants. The siRNAs isolated from untransformed control and transformed plants were separated on 1.2% formaldehyde agarose gel, electroblotted on a positively charged nylon membrane and hybridized with probes specific to siRNA targeting replicase gene. Strong signals corresponding to 21nt long siRNAs were obtained in samples from transformed plants, clearly indicating the successful siRNA mediated silencing of replicase gene of BBrMV as the cause of virus resistance in transformed plants.

The RNAi technology was successful in conferring resistance against BBrMV in banana (*Musa* spp. Var. Nendran). The siRNA detected in the transgenics clearly shows that multiplication of the virus in the host was suppressed due to the silencing of replicase gene. This is the first report on siRNA mediated resistance against BBMV in banana. The study suggests the possible use of RNAi technology for developing resistance against other viruses also in banana.