DEVELOPMENT OF SMALL INTERFERING RNA (siRNA) MEDIATED RESISTANCE IN BANANA AGAINST *BANANA BRACT MOSAIC VIRUS*

by

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THESIS
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DECLARATION

I hereby declare that this thesis entitled **"Development of small interfering RNA (siRNA) mediated resistance in banana against *Banana bract mosaic virus*"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

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LIST OF ABBREVIATIONS

BBrMV	- *Banana bract mosaic virus*
RNAi	- RNA interference
miRNA	- microRNA
siRNA	- small interfering RNA
bp	- base pairs
nt	- nucleotide
IMFs	- Immature male flowers
IAA	- Indole-3-acetic acid
NAA	- Naphthalene acetic acid
BA	- Benzyl adenine
2,4-D	- 2,4- Dichlorophenoxy acetic acid
LB medium	- Luria- Bertani medium
E. coli	- *Escherichia coli*
Min	- minutes
h	- hour

Introduction

1. INTRODUCTION

Bananas and plantains (*Musa* spp.) are among the most important fruit crops of the world and the staple food for millions across the globe. Banana holds first place by production volume and is amongst the five most consumed fruits in the world (FAO, 2012). It is a healthy source of carbohydrate, fiber, potassium, vitamin B6, vitamin C and antioxidants and various phytonutrients. In India banana is the second most important fruit crop next to mango. Its year round availability, affordability, varietal range, taste, nutritive and medicinal value and export potential makes it the favorite fruit among all classes of people. Banana is of great importance to small-scale farmers in the developing countries of the tropics and sub-tropics where it is grown both as a staple fruit as well as a cash crop mainly for the local market. The crop can be grown in a range of environments and production systems, and provides source of revenue all year round.

Banana cultivation in India is hampered by various environmental challenges. Viruses are important biotic threats to banana production because of their effect on yield and quality. Four major viruses *viz. Banana bunchy top virus* (BBTV), *Banana streak viruses* (BSVs), *Banana bract mosaic virus* (BBrMV), and *Cucumber mosaic virus* (CMV) are known to affect the production in banana and plantain. BBrMV is a recently described virus disease of banana. In India, incidence of BBrMV has been reported in Kerala, Tamil Nadu, Karnataka 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). In Kerala the disease was first reported in Thrissur district in the variety Nendran, which is found to be highly susceptible. Later the disease was found to affect other varieties like Palayankodan, Kodappanillakunnan, Monthan, Kanchikela, Poovan (Rasthali), Karpooravally and Chenkadali. Incidence of the disease ranges from 5 to 36 per cent and more in cv. Nendran in Kerala (Selvarajan *et al.*, 2006).

BBrMV has been classified as a potyvirus which is transmitted in a non-persistent manner by the aphid species *Pentalonia nigronervous*, *Aphis gossypi* and *Rhopalosiphum maidis*. The virus can also be transmitted by infected planting material. The characteristic symptom of this disease includes discontinuous streaks on the bract of the banana inflorescence. The infected plants flower, but produce smaller bunches bearing brittle fruits. In severe cases 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).

Sources of resistance have not been reported for BBrMV. Phytosanitary measures and use of virus free planting material are the only major methods of virus control (Kumar *et al.*, 2015) but, these preventive measures do not completely control the viruses. The most effective method of controlling plant viruses is through enhancing population resistance. Conventional methods have been used to battle pathogen infections in banana, including cross-protection and utilization of natural resistance in plants. But the lack of natural sources of resistance, low female fertility and poor seed setting make the conventional breeding methods difficult in banana. Most of the popular varieties are triploid in nature. This demands alternate strategies for banana improvement. Advancement of genetic transformation methods in monocots has made it possible to introduce selected genes into plants for controlling plant diseases and pests. The majority of virus-resistant transgenic plants 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.

Recently, RNA silencing has emerged as a successful strategy to control viruses. RNAi has been widely characterized as a conserved regulatory mechanism of gene expression in eukaryotic organisms. This mechanism plays an important regulatory role in plant development. It also functions as a natural antiviral defense mechanism. It suppresses gene expression at the RNA level using double-stranded RNA (dsRNA) expressed as a hairpin RNA construct

which interferes with the expression of both specific endogenous genes and genes encoded by invading pathogens. Small RNAs are key mediators of RNA silencing-related pathways in plants and other eukaryotic organisms. These regulatory molecules are of approximately 21–24 nucleotides originated from transgenes, viruses or endogenous sequences. The double stranded precursors are processed by Dicer enzyme to generate these small RNAs, which act through transcriptional or post-transcriptional gene silencing pathways. It was Waterhouse *et al.* (1998) who first demonstrated RNAi technology for developing virus resistant plants in *Potato virus Y* (PVY). The sense and antisense transcripts of the viral helper component proteinase (HC-Pro) gene was made to express simultaneously in potato plants to develop complete immunity to the virus. Thereafter this technology has been used to develop resistance against several other viruses (Hily *et al.*, 2007; Fahim *et al.*, 2010; Praveen *et al.*, 2010).

The present study envisaged development of siRNA mediated resistance in banana (AAB group, cv. Nendran) against BBrMV in Nendran which is a popular banana cultivar in the southern parts of India and is highly susceptible to BBrMV. Replicase gene of the virus was targeted to silence in this study by developing an ihpRNA construct. The specific objectives of the present study were

- Standardization of a protocol for somatic embryogenesis in banana var. Nendran from immature male inflorescence
- Isolation of partial replicase gene of BBrMV using RT-PCR
- Preparation of ihpRNA construct in siRNA vector with sense and antisense strands of replicase gene linked through *cre* intron
- Mobilization of ihpRNA construct to binary vector and its delivery to banana embryogenic cells via *Agrobacterium* mediated transformation.

Review of literature

2. REVIEW OF LITERATURE

Bananas and plantains are monocotyledonous plants belonging to the genus *Musa* (Musaceae, Zingiberales). The center of origin of banana is in the rainforests of South-East Asia (Simmonds, 1962). The vast majority of banana cultivars are the hybrids developed from inter and intraspecific crosses between wild species i.e. *Musa acuminata* and *Musa balbisiana* (Simmonds *et al.*, 1955). They are having the genome constitution AA (*M. acuminata*) or BB (*M. balbisiana*). Most of the modern domesticated edible parthanocarpic banana cultivars are triploid (2n= 3x = 33). Bananas with AAA genome constitution are mainly the sweet dessert bananas and those with AAB or ABB are considered to be cooking type. Seedless diploids (AA & AB genomic group) and tetraploids (AAAA, AAAB, AABB, ABBB genome group) are also under cultivation (Heslop-Harrison *et al.*, 2007).

Banana is a major crop in the tropical and sub-tropical regions of the world, with an approximate production of 70 million tonnes per annum. India is rich in genetically diverse varieties of banana, cultivated over an area of 802.6 (000Ha), with a production of 297.24 lakh tones contributing to 33.4 per cent of the global production share (Indian Horticulture Database, 2015, National Horticulture Board, Ministry of Agriculture, Govt. of India). The area under banana cultivation in Kerala during 2013-2014 was 62,261 Ha. It occupied 19 per cent of the category of fresh fruits and it has third position in this category. Two per cent area is increased during 2013-14 in banana cultivation than 2012-13. Palakkad, Wayanad and Malappuram districts stands first three positions with areas 28, 19 and 12 per cent respectively during 2013-14 (Agricultural Statistics 2013-14, Department of Economics and Statistics Kerala January, 2015). Area under banana cultivation in Kerala is 61,010 Ha with a production of 515610 million tonnes (Fruit crop-wise data 2012-13, Ministry of Food Processing Industries, Government of India).

The crop can be grown in a range of environments and production systems, and provides source of revenue all year round. Banana is of great importance to small-scale farmers in the developing countries of the tropics and sub-tropics where it is grown both as a staple fruit as well as a cash crop mainly for the local market. Banana cultivation in India is hampered by various environmental challenges. Viruses are important biotic threats to banana production because of their effect on yield and quality

2.1 VIRAL DISEASES OF BANANA

Viral pathogen causes significant losses in the yield and quality of banana crop on an annual basis. Major threatening viruses infecting banana are *Banana bunchy top virus* (BBMV), *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV), *Banana streak virus* (BSV), *Banana mild mosaic virus* and *Banana virus X.*

2.2 BANANA BRACT MOSAIC DISEASE (KOKKAN DISEASE)

Banana bract mosaic virus (BBrMV) is a member of the genus potyvirus of family Potyviridae. It possesses a single stranded positive sense RNA of about 9.7 kb in length (Balasubramanian and Selvarajan, 2012). BBrMV encodes a single polypeptide that is processed by three viral proteinases to release all viral proteins needed for the infection. It was first observed in the philipines in 1988 (Magnaye and Espino, 1990). In India, BBrMV is present in Kerala, Tamil Nadu, Karnataka 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). In Kerala, the first report of Kokkan disease came from Thrissur district in the banana variety Nendran. Eventhough it affects all other varieties, cv. Nendran is a highly susceptible variety. The disease is characterized by spindle shaped streaks and stripes on the pseudostem and mosaic pattern on the bracts. The leaves shows distinct discontinuous streaks along the primary veins, which appear to be irregularly thickened or raised in severe stage of infection. White to yellowish necrotic streaks seen scattered across from the midrib to the margin of

the leaf. The key symptom of the disease is unusual reddish brown or necrotic discontinuous streaks towards the base of the pseudostem. The BBrMV affected plants exhibit necrotic streaks from third month onwards. In certain cases it disappears with the senescence of the affected portion. The infected plants flowers, but produce very small bunches with curved brittle fruits. Severely affected plants may fail to flower and may die by stunted growth and necrosis of pseudostem (Selvarajan *et al.*, 2006). The male buds are dark purple in colour with mosaic patches. There are varietal differences in the symptomatology of the disease. The incidence of disease ranges from 5 to 36 per cent and more in cv. Nendran in Kerala. BBrMV has been reported to cause considerable damage with yield reduction in cv. Robusta (AAA) (70 per cent), followed by cv. Nendran (AAB) (52 per cent) (Cherian *et al.*, 2002). The accessions with ABB genome have maximum incidence of the disease. Besides yield reduction, the fingers become malformed and curved, which reduce the market acceptability of fruits (Cherian *et al.*, 2002). Aphids, *Rhopalosipum maidis* and *Aphis gossypii* transmit BBrMV in a non-persistent manner (Magnaye and Espino, 1990). Banana aphid *Pentalonia nigronervosa* and cowpea aphid *Aphis craccivora* also transmit the virus (Selvarajan *et al.*, 2006).

To find the yield loss due to BBrMV in cv, Nendran (AAB), Selvarajan *et al.* (2006) conducted fixed field plot experiments in Tiruchirapalli District of Tamil Nadu during 1997-1999 and observed the average yield reduction due to BBrMV was 30 per cent. The reduction in bunch weight over healthy plants was highly significant. They also observed malformed bunches of less weight and underdeveloped fingers. Selvarajan *et al.*, 2009 conducted an experiment to find out the effect of increased doses of fertilizer on healthy and BBrMV affected plants of cv. Ney Poovan. The results showed that increased doses, i.e. 125 and 150 per cent of recommended dose of fertilizer have compensated the yield loss in BBrMV infected plants. The disease was mitigated with higher doses of fertilizer application, provided the vector population is managed through insecticides. But

in ratoon crop, due to increased severity of virus infection, plants did not respond to higher doses of fertilizers.

2.3 APPROACHES TO INDUCE RESISTANCE AGAINST VIRUS DISEASES

Viruses are one of the major constraints in banana cultivation resulting in considerable yield loss. Conventional methods have been tried to overcome pathogen infections, including cross-protection and utilization of natural resistance in plants. The specificity of the viruses varies with their ability to infect hosts. Some are able to infect different hosts, whereas others can infect only one defined species. New virus strains emerge due to mutations in their genome (Mangrauthia *et al.*, 2008; Jones, 2009). Knowledge of pathogenic strains is important for the development of disease management strategies. Knowledge of infection process and the effect of infection on host plants will help implement correct control measures. Different approaches have been used to diminish the virus spread throughout the plant, and/or the plantation. Results from epidemiological studies might indicate the main route by which the virus would reach its host and the mechanism(s) of inoculation (Gilligan *et al.*, 2008, Rodrigues *et. al.*, 2009). Virus may be transmitted by contaminated seed, by vectors or during culture by normal agricultural practices (Fereres and Moreno, 2009; Dieryck *et al.*, 2009). The use of certified seeds may significantly reduce the occurrence of certain viruses (Novy *et al.*, 2007). Furthermore, vector population control and the implementation of "clean" agricultural practices can considerably limit the spread of virus diseases (Fereres and Moreno, 2009; Castle *et al.*, 2009).

Virus enters into a viable plant cell by a process known as inoculation by interupting the plasma membrane, (Rodrigues *et al.*, 2009). The virus particles replicate and spread within the host via plasmodesmata and vascular bundles and this requires a compatible interaction between the virus and the plant cell (Taliansky *et al.*, 2008). The relationship between the virus and the host plant determines the intensity of these processes. The set of plant resistance responses

aims to reduce virus replication (Ascencio-Ibáñez *et al.*, 2008). A viable strategy to reduce the virus-induced crop loss is by breeding cultivars with elevated levels of resistance (Ma *et al.*, 2004). Another option to increase the resistance responses is by using the attenuated virus strains (Ichiki *et al.*, 2005). Understanding the biochemistry of virus infection, like RNA silencing, have resulted in advanced methods to effectively control the viral diseases (Tenllado *et al.*, 2004).

2.4 CROP PROTECTION BASED ON ENGINEERED RESISTANCE

Sanford and Johnston in 1985 developed the concept of pathogen derived resistance (PDR). Since then attempts to develop transgenic plants using virus derived genes or genome fragments were made (Lomonossoff, 1995). Beachy lab in 1986 introduced the concept of pathogen-derived resistance (PDR) to *Tobacco mosaic virus* (TMV) and this was the pioneering work on coat protein (CP)-mediated resistance (Powell *et al.*, 1986). A prerequisite for the use of PDR is that, the interference with essential host functions should not be there. PDR can be through protein-mediated resistance or nucleic acid-mediated resistance. Among the viral proteins used for PDR are replicases, movement proteins, proteases and, most often coat protein(s) (CP) (Tepfer, 2002). The observation that transgenic RNA, rather than the expressed viral proteins, was responsible for the observed resistance, created new opportunities based on RNA-mediated resistance (Tenllado *et al.*, 2004).

Multiple strategies to engineer resistant plants were developed rapidly, based on protein or RNA mediated resistance. The initial report on PMR used *Tobacco mosaic virus* (TMV) CP gene expression to produce the resistance in tobacco plants (Powell *et al.*, 1986). Since then, a number of studies have used PMR to confer plant resistance to a variety of viruses. Viral coat protein-mediated resistance can provide either broad or narrow protection (Miller and Hemenway, 1998; Tepfer, 2002; Gharsallah *et al.*, 2008).

The first and the most widely used genes to confer PDR against plant viruses was the viral coat protein (CP) gene (Prins *et al.*, 1995, 2008). Resistance

to virus has been achieved by transforming the plants with viral CP gene and this ultimately showed resistance against infection by the homologous virus. Limited success has been observed in tobacco showing resistance to *Tobacco mosaic virus* (TMV) (Powel *et al.*, 1986) and papaya resistant to *Papaya ring spot virus*. It was later proved that resistance was mediated by the RNAs of the CP transgene, rather than the protein, as an inverse correlation between resistance and the accumulation levels of mRNAs of *CP* transgene were observed. This indicated that PTGS mechanism has played its role involving the *CP* RNA-mediated protection (Jan *et al.*, 1999). In recent report by Amudha *et al.*, 2011, transgenic cotton transformed with antisense coat protein showed considerable resistance against *Cotton leaf curl virus* (CLCuV)

The movement protein (MP) is required by viruses for movement and spread into the host. Some researchers tried to engineer pathogen-derived resistance with dominant negative mutant of viral gene. This strategy was successfully demonstrated in the development of transgenics using dysfunctional MP (Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993). In another experiment, tobacco plants transformed to express a defective TMV movement protein (TMV-MP) showed resistance to *Tobacco rattle tobo virus, Tobacco ring spot nepo virus, Alfalfa mosaic alfamo virus* and *Cucumber mosaic virus* (Cooper *et al.*, 1995). The transgenically expressed functional MP has no effect on virus infection or increased susceptibility (Ziegler-Graff *et al.*, 1991). Transgenic expression of a dysfunctional TMV MP conferred resistance by competing for plasmodesmatal binding sites between the mutant MP and the wild-type MP of the inoculated virus (Lapidot *et al.*, 1993).

The replication of *African cassava mosaic virus* (ACMV) was inhibited in *Nicotiana tabacum* protoplasts by expressing full-length or a truncated N-terminal portion of *Rep* gene. A modest level of ACMV resistance was achieved by the expression of full-length *Rep* gene in experimental plant tobacco. None of these transgenic tobacco plants was resistant to distantly related viruses *Tomato golden mosaic virus* (TGMV) and *Beet curly top virus* (BCTV) (sharing 60 per cent Rep

amino acid sequence identity with ACMV) (Shepherd *et al.*, 2009). The experiment thus suggested that resistance was probably ACMV specific or to its closely related viruses (Hong *et al.*, 1996). Manipulation of *Rep* gene to engineer resistance against *Tomato yellow leaf curl sardinia virus* (TYLCSV) in *N. benthamiana* (Noris *et al.*, 1996) and tomato (Brunetti *et al.*, 1997) were proved successful. Manipulation of *Rep* gene against cotton leaf curl disease in experimental tobacco plants was very successful. Even after 120 days of continuous exposure to viruliferous whiteflies, no observable symptoms were recorded (Asad *et al.*, 2003).

The RNA mediated mechanism (RNA interference pathway), has become a powerful tool for engineering resistance in plants. Besides the regulatory roles in plant development, the siRNA-mediated RNA silencing also functions as a natural antiviral defense mechanism, a process named virus-induced gene silencing (VIGS). Pathogen-derived resistance has also been achieved through the expression of virus sequences, the acquisition of resistance being dependent on the transcribed RNA called Nucleic acid mediated resistance (NMR). This RNA-mediated virus resistance can be considered to be an example of post-transcriptional gene silencing (PTGS) in plants (Prins *et al.*, 2008). Napoli *et al.* (1990) firstly reported PTGS in *Petunia hybrida* transgenically expressing the chalcone synthase gene. They observed a coordinated and reciprocal inactivation of the host gene and the transgene encoding the same RNA. This process has been called RNA silencing or RNA interference (RNAi) and occurs in a variety of eukaryotic organisms (Mlotshwa *et al.*, 2008). The silencing process involves the cleavage of a dsRNA precursor into short nucleotides (21-26nt) RNAs by an enzyme, Dicer that has RNase III domains. These RNAs are known as short interfering RNAs (siRNA) and microRNAs (miRNAs). Both siRNA and miRNA are able to guide an RNA-induced silencing complex (RISC) to destroy single-strand cognate RNA (Naqvi *et al.*, 2009). In addition, longer siRNAs (24-26 nt) have been shown to result in methylation of homologous DNA causing chromatin remodeling and transcriptional gene silencing (TGS).

RNA silencing was first recognized as an antiviral mechanism that protected organisms against RNA viruses (Waterhouse *et al.*, 2001; Prins *et al.*, 2008) or the random integration of transposable elements. However a general role for RNA silencing in the regulation of gene expression only became evident after it had been demonstrated that specific short miRNAs precursor molecules (fold back dsRNA) were actively involved in RNA silencing in plants and animals (Bartel, 2004; Naqvi *et al.*, 2009). Several miRNA genes are evolutionarily conserved. Their function in plants is mainly to cleave the sequence-complementary mRNA.

2.5 RNA INTERFERENCE (RNAi)

RNA silencing is a versatile, complex gene regulation and defense mechanism targeting parasitic or endogenous RNA in a highly sequence-specific manner. RNAi has become one of the most exciting discoveries of molecular biology due to its high specificity, accuracy, and hereditability. The term "RNAi" was initially coined by Fire *et al.* in 1998 while unraveling the mechanism of gene silencing by double stranded RNA in nematode worm, *Caenorhabditis elegans*. Since it was shown for the first time that dsRNA which share complete homology with mRNA can breakdown mRNA (Waterhouse *et al.*, 1998, Fire *et al.*, 1998), this phenomenon has been extensively used to suppress gene function in plants. The silencing effect was first observed in plants in 1990, when the Jorgensen laboratory introduced exogenous transgenes into petunias in an attempt to up-regulate the activity of a gene for chalcone synthase, an enzyme involved in the production of specific pigments (Napoli *et al.*, 1990; Agrawal *et al.*, 2003). Unexpectedly, flower pigmentation did not deepen, but rather showed variegation with complete loss of color in some cases. This indicated that not only were the introduced trangenes themselves inactive, but that the added DNA sequences also affected expression of the endogenous loci (Hannon, 2002). This phenomenon was referred to as "co-suppression" (Napoli *et al.*, 1990; Campbell and Chox, 2005). Two types of RNA play major roles in RNA silencing: dsRNA, which acts as a trigger of RNA break down, and short interfering (siRNA), which is involved

in actual degradation of target mRNA in the final step of the RNAi pathway (Hannon, 2002).

Although various components of the RNAi pathway have been genetically and biochemically identified in several organisms, a complete picture of the RNAi pathway has not yet been revealed. More recently, microRNA has been discovered in various organisms including plants, and it has been shown to play important roles in development (Bartel, 2004). By using RNAi it is possible to target a specific, essential gene(s) in a parasite for 'silencing', and hence incapacitate it. Gene silencing is a ubiquitous form of genetic regulation used by plants and other eukaryotes to tightly control the expression of certain proteins (Baulcombe, 2004; Voinnet *et al.*, 1999). RNAi allows silencing of viral gene without protein expression. RNA-mediated interference occurs in many eukaryotes and functions to regulate gene expression. The gene silencing phenomena follows either at transcriptional level i.e. transcriptional gene silencing (TGS) or post transcriptional gene silencing (PTGS). The transcriptional gene silencing is usually induced by DNA methylation (Rountree *et al.*, 1997 and Mette *et al.*, 2000). The dsRNA mediated gene silencing proceeds via three distinct mechanisms, all involving the cleavage of a dsRNA into short 21–25 nucleotide RNAs by an enzyme called DICER that has Rnase III domains. These RNAs, known as short interfering RNAs (siRNAs) and microRNAs (miRNAs), are the hallmarks of RNAi (Baulcombe, 2004 and Meister *et al.*, 2004).

The first pathway is cytoplasmic siRNA silencing (Hamilton *et al.*, 1999). This pathway is believed to have evolved as a defense mechanism against plant viruses, where the dsRNA could result from a replication intermediate or a secondary-structure feature of single-stranded viral RNA. In plant DNA viruses, dsRNA may be formed by the annealing of overlapping complementary transcripts. The second pathway involves silencing of endogenous messenger RNAs by miRNAs (Bartel, 2004). These miRNAs are involved in negative regulation of gene expression, and they do so by base pairing to specific mRNAs, resulting in either RNA cleavage or termination of protein translation. Like

siRNAs, the miRNAs are short 21–25 nucleotide RNAs derived by DICER cleavage of a precursor. In the third pathway, DNA methylation acts to suppress transcription.

Most groups of eukaryotic organisms contain in their genomes microRNA (miRNA) genes, which are involved in the regulation of gene expression and epigenetic modifications (Ambros, 2001). Genes coding for miRNAs are transcribed by the RNA polymerase II in long non-protein-coding primary-microRNA transcripts (primiRNAs), which are first processed to fold-back precursors (premiRNAs) and then matured to small double-stranded RNAs (Bartel, 2004).The guide and passenger strands forming the RNA duplex are properly unfolded and only the mature miRNA (guide strand) is recruited by an Argonaute protein to form the active RNA-induced silencing complex (RISC). In plants, such as *Arabidopsis thaliana* L., Dicer-like1, an enzyme of the Dicer family with two RNase III domains, mediates cleavage of the pri- and pre-miRNAs. The miRNA loaded in RISC acts as a template to guide the silencing of complementary target mRNAs. In plants, miRNAs are usually highly complementary to their target, which typically triggers the cleavage of the targeted mRNA and subsequent degradation (Bartel, 2004).

RNAi has now been utilized in various fields like elucidation of gene function, inactivation of undesirable genes, has also been reported in the breeding of plants with increased values for nutrition and food processibility. For example, RNAi has been applied to the production of cotton seeds with high stearic and high oleic acids (Liu *et al.*, 2001; Tang *et al.*, 2005). Flores *et al.* (2008) reported soybean producing seed oil with increased stability at high temperatures. Tomato fruits with increased carotenoid and flavonoid content were demonstrated by Davuluri *et al.* (2005) and high lysine corn for animal food was experimentally proved by Houmard *et al.* (2007). Rapeseeds with increased flowers were produced by Byzova *et al.* (2004) and roses with blue-coloured petals were produced by Katsumoto *et al.* (2007) by using RNAi technology.

2.6 RNAi FOR VIRUS RESISTANCE

The mechanism of RNAi targets parasitic or endogenous RNA in a highly sequence-specific manner. RNAi especially in plants works with high accuracy and in a sequence specific manner. RNA silencing (including RNAi) share a common mechanism with plant defense against invasive viruses. Research during the last decade has firmly established that land plants use a RNA-targeting defense mechanism termed RNA silencing (or RNA interference, RNAi) to combat virus infections (Ding, 2010). RNAi has emerged as a powerful tool for battling against viruses and provides a natural defense against viral pathogens. In plants, RNAi has been successfully used to express cognate dsRNAs for viral transcripts in order to initiate the process of viral gene silencing. Despite the wide applicability of RNAi for achieving viral resistance, there are some challenges and constraints that must be addressed to develop RNAi as a more effective tool for virus resistance. There are many different approaches of using RNAi for virus resistance development in various crop plants.

RNAi has been employed for achieving virus resistance in various agricultural and horticultural crops. Zrachya *et al.* (2007) analysed the effect of siRNAs derived from an ihpRNA construct targeted to the coat protein gene of *Tomato yellow leaf curl virus* (TYLCV). Some of the transgenic tomato plants expressing the siRNA did not show disease symptoms seven weeks post-inoculation with the virus, while non-transgenic control plants were infected within two weeks post inoculation. Qu *et al.* (2007) showed that the expression of an artificial miRNA, targeting sequences encoding the silencing suppressor 2b of *Cucumber mosaic virus* (CMV), can efficiently inhibit 2b gene expression and protein suppressor function in transient expression assays and confer effective resistance to CMV infection on transgenic tobacco plants.

Wu *et al.* (2009) engineered melon lines resistant to *Zucchini yellow mosaic virus* (ZYMY), a construct containing the translatable coat protein (CP) sequence coupled with the 3' non-translatable region of the virus was generated

and used to transform a cultivar of oriental melon (Silver light) via *Agrobacterium*. Northern hybridization revealed great variations in the levels of accumulation of the transgene transcripts among transgenic lines, and evidenced an inverse correlation of the levels of accumulation of the transgene transcript to the degrees of virus resistance, indicating PTGS- mediated transgenic resistance.

Yu *et al.* (2011) developed transgenic watermelon resistant to *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ring spot virus* type W (PRSV W) using an untranslatable chimeric construct containing truncated ZYMV CP and PRSV W CP genes via *Agrobacterium*. Green house evaluation of the selected transgenic lines conferred complete resistance to ZYMV and PRSV W, from which virus accumulation were not detected by western blotting. The transgenic transcript was not detected, but small interfering RNA (siRNA) was readily detected, indicating that PTGS is the underlying mechanism for the double-virus resistance. The segregation ratio of progeny of the immune line indicated that the single inserted transgene is nuclearly inherited and associated with the phenotype of double-virus resistance as a dominant trait.

Agrobacterium-mediated transformation of RNAi construct on hill banana cultivar Virupakshi (AAB) for resistance to *Banana bunchy top virus* (BBTV) disease was carried out by Elayabalan *et al.* (2013). They developed RNAi construct targeting the BBTV replicase gene, by cloning *rep* gene fragment in sense and anti-sense orientation in the RNAi intermediate vector, pSTARLING-A. The cloned RNAi gene cassette was released by *Not*I site. Then transformed embryogenic cells of banana via *Agrobacterium*.

Ntui *et al.*, 2014 generated an RNAi construct to produce transgenic tomato plants expressing CMV-specific dsRNA of the replicase gene. Inoculation of transgenic plants with CMV strain O gave plants that showed complete resistance, which were free of symptoms; highly resistant plants, which had mild symptoms, but later recovered because new leaves that emerged were free of symptoms; and susceptible plants, which showed severe symptoms similar to

wild-type plants. The completely resistant lines were selected and challenged with a closely related strain, CMV-Y and found transgenic plant lines either remained immune or showed high levels of resistance to the strain. No virus could be detected in uninoculated new leaves of the resistant lines after RT-PCR and Dot Immuno Binding Assay (DIBA) analyses. They could show that the resistance is correlated with post-transcriptional gene silencing because of the production of transgene specific siRNA.

The successful development of virus resistance using siRNA mediated gene silencing depends on several factors like the selection of target sequences, the inverted repeats, size of the repeats, vectors, spacers or introns used in the RNAi cassette, promoters etc.

2.7 ISOLATION OF TARGET GENE OF THE VIRUS

Selection of target gene is very important in designing ihpRNA construct. Most of the studies used replicase gene for developing resistance. For the preparation of ihpRNA cassette the sequence information of the viral gene targeted is essential. An important characteristic feature of plant miRNA pathway is that, the plant miRNAs are perfectly paired to target RNA and use RNA cleavage rather than translational suppression as the primary silencing mechanism [Rhodes *et al.* (2002) and Llave *et al.* (2002); Jones-Rhodes and Bartel, (2004)]. The RNAi pathway in plants will work only if a perfect pairing between the siRNAs and target gene occurs. The ihpRNA constructs carries inverted repeats of target gene linked through intron. Hence target gene isolation and sequence information is important. Reverse transcriptase-PCR method is commonly used for gene isolation for RNA viruses. The plant miRNAs have targets in the coding sequence or even in the 5' UTR (Lee *et al.*, 1993). BBrMV replicase gene was targeted in the current study.

2.8 RNA ISOLATION IN BANANA

RNA isolation is difficult in banana due to high phenolic content and other interfering compounds. Several protocols have been standardized for RNA isolation from banana. Bateson *et al.* (1995) isolated and purified BBrMV virus particles as described by Moghal and Francki, (1976). RNA was extracted from sucrose gradient purified BBrMV as described by Robertson *et al.* (1991). RNA was precipitated with ethanol. RNA extraction protocols have been developed using insoluble polyvinylpyrrolidone (Woodhead *et al.*, 1997), it was found to be only moderately effective and is bulky, often requiring multiple extractions, with accompanying volumetric losses of RNA. Methods using PVP often require lengthy ultracentrifugation (Woodhead et al., 1997). Rodoni *et al.* (1997) extracted RNA from BBrMV infected banana plants following the protocol developed by Chang *et al.* (1993). This protocol was designed for isolating RNA from pine trees and used CTAB 2 per cent for eliminating polyphenols and Lithium chloride for RNA precipitation.

In the method developed by Salzman *et al.* (1999) phenolic compounds were bound to soluble PVP, and then eliminated by ethanol precipitation of the RNA. Proteins and carbohydrates were subsequently removed by phenol extraction and LiCl precipitation, respectively.

Asif *et al.* (2000) standardized a procedure to isolate high quality RNA from ripening banana. They modified the CTAB/NaCl method of Chang, (1993) by removing PVP from the extraction buffer and including a simple polysaccharide precipitation step that does not affect the RNA yield but removes contaminating polysaccharides.

In the current as the common RNA isolation protocols like CTAB method and Trizol method failed to extract good quality RNA, the protocol standardized by Salzman *et al.* (1999) was followed.

2.9 ISOLATION OF BBrMV REPLICASE GENE USING POLYMERASE CHAIN REACTION

PCR based methods are very commonly used for the isolation of genes. RT-PCR is a method of choice for RNA viruses by designing gene specific primers. Bateson *et al.* (1995) isolated and purified BBrMV virus particles as described by Moghal and Francki, (1976). RNA was extracted from sucrose gradient purified BBrMV as described by Robertson *et al.* (1991). RNA was precipitated with ethanol, resuspended in TE buffer and used immediately for cDNA synthesis and then gene isolation. Rodoni *et al.* (1997) also used RT-PCR to amplify BBrMV gene. The PCR product were cloned to pGEM-T vector and sequenced. Thomas *et al.* (1997) extracted total nucleic acids from plant tissue using a method modified from Pearson *et al.* (1994). First strand cDNA was synthesized by Reverse transcription PCR using *Moloney murine leukemia virus* reverse transcriptase. Degenerate primers from the core of the coat protein were used in PCR. The PCR product was cloned in pCRScriptSK(+).

2.10 INVERSELY REPEATED TARGET GENE SEQUENCES FOR ihpRNA VECTOR CONSTRUCTION

The ihpRNA constructs are composed of inversely repeated (IR) sequences of the target gene. Many studies have demonstrated that IR sequences of partial cDNA from a plant virus can silence the corresponding virus gene. Many studies have demonstrated that IR sequences of partial cDNA from a plant virus can silence the corresponding virus gene. Waterhouse *et al.* (2001) have developed a series of vectors in which the target RNA sequence derived from the target virus is inserted as an inverted repeat separated by an intron. When expressed in a plant it transcribes a dsRNA which is recognised by the plant as an aberrant RNA and is silenced. The plant also becomes immunized for invasion by the homologous virus. The cloned sequence must be different from the host organism, but sufficiently similar to the pathogen to initiate dsRNA mediated

silencing. One of the significant advantages of this approach is that the transgenic plant does not express any recombinant proteins (Goldbach *et al.*, 2003).

Kalantidis *et al.* (2002) developed transgenic tobacco lines containing IR of Cucumber mosaic virus (CMV) cDNA capable of producing intramolecular dsRNA homologous to the 3′ portion of the RNA3 genome. When the transgenic plants were challenge inoculated with CMV, three different types of plants were obtained, including susceptible, recovered plants, and asymptomatic resistant plants. In an another study, transgenic tobacco plants with IR constructs for the cp gene sequences expressed in *Nicotiana benthamiana* plants showed resistance to CMV Chen *et al.*, 2004). Using a similar approach, transgenic sugarbeet plants were generated expressing IR of 400 base pairs (bp) from the replicase gene of the *Beet necrotic yellow vein virus* (BNYVV). Upon challenge inoculation with virus spreading vector species Polymyxabetae, the transgenic lines exhibited resistance, even under high inoculation pressure (Lennefors *et al.*, 2006). IR construct of cp gene used transgenic lines resistant to *Papaya ring spot virus*-W (Krubphachaya *et al.*, 2007), showed 100 per cent efficiency for inducing RNAi.

2.10.1 Identification of suitable target sequences of virus

Target selection for silencing is a key factor determining the success of RNAi-based virus resistance approach. MicroRNAs (miRNAs) are a family of small RNA molecules known in animals and plants, whose conservation among species suggests that they bear conserved biological functions. miRNAs are important regulators of eukaryotic gene expression in most biological processes. They act by guiding the RNAi-induced silencing complex (RISC) to partially complementary sequences in target mRNAs to suppress gene expression by a combination of translation inhibition and mRNA decay. Deep-sequencing has enabled the identification of large numbers of miRNAs and siRNAs, making the high-throughput target identification a main limiting factor in defining their function. Many prediction tools have been developed for identifying miRNA target sites.

Qiu *et al.*, 2007 identified microRNAs and their targets in *Gossypium hirsutum* expressed sequence tags (EST). They used an EST and Genomic survey sequences (GSS) based combined approach for the detection of novel miRNAs in *Gossypium* by using previously known miRNA sequences from *Arabidopsis*, rice and other plant species and an algorithm called miRNA assist to blast the databases of *G. hirsutum* EST and GSS. They found 96 potential miRNA targets in *G. hirsutum*. Singh and Nagaraju (2008) demonstrated a genome-wide computational approach to predict miRNAs and their target(s) in the red flour beetle, *Tribolium castaneum*. They predicted and characterized 45 miRNAs by genome-wide homology search against all the reported miRNAs. Those miRNAs were further validated by statistical and phylogenetic analyses. They also attempted to predict the putative targets of these miRNAs, by making use of 3' untranslated regions of mRNAs from *T. castaneum*.

It is often difficult to perform large-scale validation of miRNA expression that is predicted from genomic regions. Expressed transcripts provide an alternative resource to facilitate identification of miRNAs and their targets. Wen *et al.* (2008) developed a computational pipeline to scan for miRNA genes from polyadenylated transcripts that were associated with limited protein coding potentials, corresponding to the intergenic regions of *Medicago truncatula* genomic sequences. Each predicted miRNA was required to have a near perfect match with target genes. They also searched for miRNA conservation in other plant species, clustered highly similar miRNAs, and provided a functional classification of target genes. The data was represented in the *Medicago*-MIRATdb (**MiR**NA **A**nd **T**arget gene **D**ata **B**ase). The database provided detailed information on the sequences of the predicted miRNAs, their precursors, and potential target genes. It also detailed sequence source information such as the EST library, tissue category, and the number of EST clones. Information regarding miRNA conservation in other species, functional classification of target genes, and clusters of similar miRNAs was also provided. The web interface to the database provides researchers with the ability to narrow their search for

miRNAs and target genes of interest by using a variety of filters. This work represented the first systematic effort to identify candidate miRNAs and targets in the model legume *M. truncatula*.

Using previously known miRNAs from *Arabidopsis*, rice and other plant species against expressed sequence tags (ESTs), genomic survey sequence (GSS) and nucleotide databases, Zhang *et al.* (2009) identified 48 potential miRNAs in *S. tuberosum*, which are involved in floral, leaf, root, and stem development, signal transduction, metabolism pathways, and stress responses.

Sun *et al.*, 2011 developed a protocol which described a computational procedure for plant miRNA target prediction. It involves two key steps: (1) Search of transcript sequence databases for target sequences that have a near-perfect sequence complementarity to the miRNA sequence using the "scan_for_matches" program and (2) evaluation of the miRNA: target sequence pair for pairing complementarity using specific rules, such as positional dependent penalty score and minimum free energy ratio filter, to identify the most likely candidate targets.

High conservation of miRNAs in plant provides the foundation for identification of new miRNAs in other plant species through homology alignment. Muvva *et al.* (2012) by doing BLAST with previous known plant miRNAs against the expressed sequence tag (EST) database of *Raphanus sativus*, and by using a series of filtering criteria, a total of 48 miRNAs belonging to 9 miRNA families were identified, of which 16 potential target genes were subsequently predicted. Most of which encoded transcription factors or enzymes participating in regulation of development, growth and other physiological processes.

Kamarajan *et al.*, 2013 used a computational approach to predict miRNA targets in plant mitochondria. The mitochondrial gene targets identified for miRNAs are located both in mitochondrial and nuclear compartments. Those observations pointed to a fairly early origin of miRNAs. Most of the targets identified could have copies in two compartments and suggested the possibility of miRNA mediated regulation. The study unfurled the possibility of regulating the

plant mitochondrial genes by amending the miRNA genes in the nuclear compartment. In plants, several tools have been developed to predict targets, majority of them being trained on *Arabidopsis* datasets (Srivastava *et al.*, 2014).

2.11 CONSTRUCTION OF ihpRNA VECTOR

Pathogen-derived resistance against viral infection in plants was first demonstrated by Abel *et al.* (1986) by expressing coat protein gene of tobacco mosaic virus in transgenic tobacco plants, which conferred resistance to *Tobacco mosaic virus* infection. Researches indicated that pathogen-derived resistance is due to the phenomenon called RNA silencing. (named post-transcriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in animal systems) [Hannon, (2002); Kooter *et al.* (1999); Waterhouse *et al.* (2001)].Techniques based on RNAi to engineer resistance in crops against parasites have been developed. PTGS in transgenic plants may result from the insertion event leading to formation of an inverted repeat (Scorza *et al.*, 2001) or by careful design and expression of self-complementary hairpin-RNA. These involve cloning the target sequence of the parasite in the sense and antisense directions. Gene fragments ranging from 50 bp to 1000 bp are used to successfully silence genes. Earlier studies suggested that the shorter the fragment, the less effective silencing achieved, while very long fragments increase the chance of recombination. Hence, a fragment length ranging from 98 to 853 nt have been used by Wesley et al. (2001) for the RNA silencing studies and achieved 90-100 per cent silencing of the transformed plants. Helliwell *et al.* (2002) considered 400 and 800 nt long fragments to be a suitable size to maximize silencing efficiency. Functions of rice genes were studied with the help of RNAi technology by Miki *et al.* (2004) by constructing hpRNA vector using sequences of size 300-500 bp. RNAi-induced gene silencing using sequences around 300-700nt was shown to be stable and efficient to suppress the targeted genes in wheat (Travella *et al.*, 2006), rice (Mei *et al.*, 2007) and potato (Bhaskar *et al.*, 2009).

Normally, the sense and the antisense strands are separated with an intron to allow formation of a double-stranded hairpin structure. The intron in the ihpRNA makes the interaction of the two arms of the hairpin more probable. When an intron is cloned between the two complementary regions in a hairpin RNA (ihpRNA), silencing efficiency is significantly enhanced (Wesley *et al.*, 2001). Introduction of an ihpRNA construct in a virus susceptible plant has been a fairly successful strategy in controlling RNA virus infections in plants. Several vectors have been developed for efficient expression and processing of hairpin dsRNA in plants Wesley *et al.* (2001) made a generic vector pHANNIBAL which allowed the construction of efficient silencing vectors with a single PCR product from the gene of interest. They have also created a high-throughput vector pHELLSGATE. Gateway vectors pENTR/D-TOPO and pANDA which carried attL1 and attL2 recombination sites were developed by Miki *et al.* (2004) for RNA interference of rice genes for its functional identification.

RNAi vectors, many sharing overall design but differ in cloning strategies; selectable markers and other elements have been developed during recent years. Commonly used methods for RNAi-mediated gene silencing in plants are transforming plants with hpRNA producing vectors by exogenous dsRNA spray as demonstrated by Tenllado *et al.* (2004)., *Agrobacterium*-mediated plant transformation or particle bombardment to produce stable transgenic plants, and infiltration of *Agrobacterium* cultures harboring hpRNA producing construct for transient gene silencing (Silhavy, (2005) and Senthil-Kumar *et al.* (2010), direct siRNA delivery (Tang *et al.*, 2006) and artificial micro RNA (amiRNA) (Warthmann *et al.*, 2008) based vectors and are also shown to be effective for gene silencing. For the past one decade several novel methods to achieve stable-, transient-, inducible-, specific-, differential-, comprehensive- RNAi have been developed, thus diversifying the application of RNAi.

RNAi vectors were constructed by different researchers in different vectors and in different ways. Miki *et al.* (2004) used RNAi to elucidate gene functions in rice. To generate RNAi constructs, PCR fragments of size 300-500 bp

are cloned into the primary Gateway cloning vector pENTR/D-TOPO by recombinase reaction under maize ubiquitin promoter, and an intron is placed 5' upstream of inverted repeats to enhance RNA expression. This was transferred to *Agrobacterium* via Gateway vector pANDA for further silencing studies.

Fuentes *et al.* (2006) attempted to develop transgenic tomato plants resistant to *Tomato yellow leaf curl virus* (TYLCV). An ihpRNA vector was constructed with 726 nucleotides of the 3' end of the TYLCV, early replication associated protein gene (CI). The CI gene fragment with restriction sites (*Xho*I-*Bam*HI), digested was cloned into the pBPΩ8 plasmid. The TYLCV CI gene with *Bam*HI and *Kpn*I restriction sites were cloned to the plasmid to generate pBP$CI_{\text{antisense}}$- CI_{sense}. Castor bean catalase intron was cloned into pBP$CI_{\text{antisense}}$-CI_{sense} by digesting with *Cla*I, to form the ihpRNA cassette in the primary vector with CaMV 35S promoter.The ihpRNA construct was cloned into a pZ200 – derived binary plasmid at *Hind*III site. They could develop transgenic tomato plants (line 126), that harbored a single transgene copy, which showed immunity to TYLCV, even in extreme conditions of infection.

Construction of ihpRNA vector to confer resistance to *Plum pox virus* (PPV) in herbaceous woody plants by silencing coat protein gene was carried out by Hily *et al.* (2007). Full length coat protein gene was isolated and cloned in sense and antisense orientations in pHELLSGATE vector. The fragment selected for ihpRNA cassette preparation was the highly conserved sequence among the seven PPV sequences analysed. The *Xho*I and *Hind*III sites were added to the 5' ends of the sense and antisense sequences respectively. *Asp*I and *Xba*I sites were added to the 3' end for the sense and antisense sequences respectively. Later the cassette along with the 35S promoter was subcloned to pSTBlue-1 and *Agrobacterium* GV3101 carried the construct to *Nicotiana benthamiana*.

RNAi mediated resistance in Cantaloupe against *Papaya ring spot virus* type W was developed by Krubphachaya *et al.* (2007) developed RNAi mediated resistance in Cantaloupe against *Papaya ring spot virus* type W vector by cloning

the full length coat-protein coding region in the sense and reverse orientation in the primary vector pPRT103. Later this CP expression cassette was inserted at the *Hind* III site between the *nptII* and the *gus* genes of T-DNA to generate plasmid pSA1175. Then intron (*hpr-A*) was inserted between sense and antisense fragments of partial cp gene. This was transferred to the binary vector pSA1304. The binary vectors were introduced into *A. tumefaciens* strain LBA 4404

Qu *et al.* (2007) used an artificial miRNA to target sequences encoding the silencing suppressor 2b of *Cucumber mosaic virus* (CMV). Two plant expression vectors, one harbouring a *Cauliflower mosaic virus* (CaMV) 35S promoter driven miRNA precursor sequence and the other harboring a plant Pol III promoter-driven shRNA construct, to generate an artificial miRNA and an siRNA tentatively named shRNA-derived sRNA (sh-sRNA), respectively, both targeting the same region of the gene coding for the viral suppressor 2b of CMV were designed. The resulting sequences in sense and antisense orientations were synthesized and annealed with overhangs of the *Bam*HI and *Hind*III sites was first cloned into pUC-U6 and then the cassette (U6-sh2b) was transferred into the plant binary vector pCAMBIA 1300 to generate pU6-sh2b to express a 21 nucleotide 2b-specific sh-sRNA in tobacco plant. The DNA vectors were separately moved into *Agrobacterium tumefaciens* strain EHA 105.

An improved strategy was utilized by Zhang *et al.* (2011) to design a vector to express shorter inverted repeats, each containing conserved sequences of one virus, forming dsRNA. Three such short inverted repeats with sequences of unrelated soybean-infecting viruses [*Alfalfa mosaic virus* (AMV), *Bean pod mottle virus* (BPMV), and *Soybean mosaic virus* (SMV)] were assembled into a single transgene. The transgene construct, dsABS, was designed to express three short RNA hairpins in transgenic plants, each with a double-stranded region of 100 to 150 bp and an end loop of about 100 nt, under control of 35S promoter and terminator of cauliflower mosaic virus in the plasmid pRTL2 with modified multicloning sites. The entire P35S-dsABS-T35S cassette was then mobilized into the binary vector pPTN200. They obtained three independent transgenic lines and

all of them exhibited strong systemic resistance to the simultaneous infection of the three viruses. This strategy of construct assembly makes it easy to incorporate additional short inverted repeats in the transgene, thus expanding the spectrum of virus resistance.

Efforts were taken by Shekhawat *et al.* (2012) to silence *Banana bunchy top virus* (BBTV) in banana by designing two ihpRNA constructs ihpRNA-Rep and ihpRNA-ProRep. The primary vector construction was done in various steps. The ihpRNA construct, ihpRNA-Rep was designed by using the complete ORF (861 bp) of the replicase protein. A 222 bp sequence present immediately upstream of the Replicase ORF in DNA-R was taken togeather with 429 bp partial 5' region of Rep ORF (ProRep) was selected to construct a second set of ihpRNA construct, ihpRNA-ProRep. The castor bean intron amplified from pCAMBIA-1301 binary vector was inserted into pTZ57R/T cloning vector to form pTZ57R/T - Int. The sequences of Rep and ProRep were amplified and cloned in *Pst*I and *Bgl*I restriction sites of pTZ57R/T – Int. *Zea mays* polyubiquitin promoters amplified from *Zea mays* genomic DNA was also cloned to pTZ57R/T. Later modified the pCAMBIA-1301 binary vector digested with *Hind*III and *Kpn*I restriction enzymes and maize polyubiquitin promoter and ihpRNA-Rep and ihpRNA-ProRep cassette sequences (released using *Pst*I and *Kpn*I) were ligated directionally in a three way ligation reaction to form two new ihpRNA binary vectors.

Elayabalan *et al.* (2013) developed hair pin RNAi cassette targeting the *Banana bunchy top virus* (BBTV) rep gene to develop resistance in hill banana cultivar Virupakshi (AAB) via *Agrobacterium* mediated transformation. The 440 bp of 5' and 440 bp of 3' end of the BBTV rep gene were utilized. The partial gene fragment was subcloned in sense and antisense orientation in an intermediate RNAi primary vector pSTARLING (CSIRO Plant Industry Australia). This vector contains Ubiquitin promoter, Ubi intron, cre intron, restriction site for sense and antisense orientation. The cloned hair pin RNAi gene cassette with *cre* intron was released by *Not*I enzyme and cloned into the *Not*I site of binary vector pART27

(CSIRO plant industry, Australia). The pART27 contain *nptII* (kanamycin) gene for plant section. Transformed the banana cells via *Agrobacterium* which harbored pART27 vector with ihpRNA cassette.

Transgenic tomato plants were produced by Ntui *et al.* (2014) to develop resistance against *Cucumber mosaic virus* (CMV). They constructed an intron hairpin RNA vector using a 1138 bp fragment of the replicase component of CMV by cloning through RT-PCR technique. The PCR products were then purified and cloned into Gateway entry vector pCR8/GW/TOPO, which contains attL1 and attL2 recombination sites. The gene in sense and antisense orientation were cloned on either end of 278bp fragment of cat1-intron derived from caster bean, to ensure the stability of the construct (Tanaka *et al.*, 1990). The RNAi cassette was subcloned to the binary vector pEKH2IIN2, by eLRclonase™ (Invitrogen, New Zealand and selected on kanamycin-containing LB plates. Clones were verified by digestion with *Eco*RI. The plasmid, which contains marker genes for neomycin phosphotransferase (nptII) and hygromycin phosphotransferease (hpt), the CMV gene being driven by *Cauliflower mosaic virus* (CaMV) 35S promoter, was introduced into *Agrobacterium tumefaciens* strain EHA105 by triparental mating.

It has been now found out that there are many silencing suppressor genes which resist the attacks from its host plants. This inherent ability of virus to evolve, works against induced silencing as well. Inactivating these genes is therefore obviously a potential defensive option. In the study conducted by Chun-zhen *et al.* (2015) they have tried to silence 3 silencing suppressor genes, *p20*, *p23*, *p25*, in *Citrus aurantium* by constructing hairpin RNAs (hpRNAs). A 348 bp long sense and antisense fragments with spacer sequence to act as intron was prepared by PCR and was cloned to the primary vector pTZ57R/T sequentially and transformed to E. coli DH5α. Restriction digestion was used to confirm the insertion and PCR with T7 to check the orientation of the inserts. The pTZ57R-hp20 plasmid extracts were digested with *Sac*I and *Bam*HI to release hp20 was then subcloned into *Sac* I and *Bam*HI digested pCAMBIA 2301 G and the

resulted construct, pCAMBIA 2301G-hp20, was transformed into *E.coli* DH5α. Restriction analysis with *Sac*I and *Bam*HI was performed to identify positive clones. The hp20-carrying binary vector was extracted and transformed into *Agrobacterium* strain EHA105 to infect *C. aurantium.*

In the present study, for the construction of intron hairpin RNA vector and its delivery into *Agrobacterium* for plant transformation, a primary cloning vector and a binary vector system respectively were necessary. The primary cloning vector used was pSTARLING obtained from CSIRO through proper Material Transfer Agreement (MTA). pSTARLING is pUC based primary vector with a novel set of restriction sites. The expression catridge of the primary cloning vector pSTARLING, comprised of maize ubiquitin promoter for high level constitutive hairpin RNAi production in monocot plants. The entire catridge (i.e. ihpRNA cassette) was cloned into the binary vector. The can be removed from pSTARLING as a *Not*I fragment and introduced directly into the binary vector, pART27 at *Not*I site in the lacZ gene. Recombinants can be selected by blue white colony screening.

2.12 TRANSFER OF BINARY VECTOR TO *A. TUMEFACIENS*

The developed RNAi cassette can be introduced into the target plant cell by various methods like Biolistic method, electroporation *Agrobacterium* mediated method. *Agrobacterium* mediated method need a carrier vector for coveying the construct. Usually a binary vector is used for this. Scientists have reported various binary vectors for their experiments. These binary vectors need to be transferred to *A. tumefaciens* for the transformation of the target plant. Various methods are there for the delivery of the binary vectors, like triparental mating, Freeze thaw method, electroporation etc.

Tripathi *et al.* (2005) followed the modified method of Gynheung, (1988) for the transformation of *Agrobacterium*. The *Agrobacterium* strain was grown in YEB (0.1 per cent yeast extract, 0.5 per cent beef extract, 0.5 per cent peptone, 0.5 per cent sucrose, 0.04 per cent MgSO4) in appropriate antibiotics till $OD_{600\ nm}$ =

0.5. The cells were sedimented and resuspended in 10 ml of 0.15 M $CaCl_2$. The cells were again sedimented and resuspended in 0.5 ml of ice-cold 20 mM $CaCl_2$. To this 1µg of plasmid DNA was added, mixed and incubated on ice for 30 mins. The mixture was freezed in liquid N_2 for one min, and then thawed at 37°C water bath. One ml medium was added to the mixture and incubated at 28°C for 2 to 4h with gentle shaking. The cells were collected by centrifuging at 5000 g for 5 mins and resuspended on YEB plates with appropriate antibiotics and incubated at 28°C for 2 to 3 days. The resultant antibiotic resistant colonies were checked for the presence of vector. The engineered strains of *Agrobacterium* were maintained and used for plant transformations.

Krubphachaya *et al.* (2007) developed RNAi mediated resistance in Cantaloupe against *Papaya ring spot virus* type W vector by cloning the full length coat-protein coding region in the sense and reverse orientation in the primary vector pPRT103. Later this CP expression cassete was inserted at the *Hind*III site between the *nptII* and the *gus* genes of T-DNA to generate plasmid pSA1175. Then intron (*hpr-A*) was inserted between sense and antisense fragments of partial cp gene. This was transferred to the binary vector pSA1304. The binary vectors were introduced into *A. tumefaciens* strain LBA 4404 by a freeze-thaw method by Hofgen and Willmitzer, (1988).

Agrobacterium-mediated transformation of banana cultivar 'Rasthali'(AAB) with chitinase gene was reported by Sreeramanan *et al.* (2010). The cDNA of the rice chitinase gene was constructed in the binary vector pBI333-EN4-RCC2. This vector was introduced into the *Agrobacterium tumefaciens* EHA 101 using heat shock method.

Zhang *et al.* (2011) created a transgene construct which is composed of three short inverted repeats (IRs), which contained the specific, highly conserved regions of the replicase genes of AMV, BPMV and SMV respectively. The cDNAs of the three hairpins were linked together in the same order as mentioned above, and cloned between the *Cauliflower mosaic virus* (CaMV) 35S promoter

(P35S) and terminator (T35S) in the plasmid pRTL2 with modified multicloning sites. The entire P35S-dsABS-T35S cassette was then mobilized into the binary vector pPTN200. The resulting construct, pPTN-dsABS, was transformed via triparental mating into *A. tumefaciens* (strain EHA 101).

Shekhawat *et al.* (2012) constructed binary vectors ihpRNA-Rep and ihpRNA-ProRep in pCAMBIA-1301 targeted against replicase gene of BBTV in banana. This binary vector was transformed into *A. tumefaciens* strain EHA105 by electroporation before transforming banana embryogenic cells.

In the current study triparental mating (Cangelosi *et al.*, 1991) was followed initially to carry the binary vector to *A. Tumefaciens* strain LBA4404. But the transformation process was not successful. Later Freeze-thaw method (Jyothishwaran *et al.*, 2007) of *Agrobacterium* transformation was carried out and the transformants were selected on LB agar plates with kanamycin 100 mg L^{-1} and Rifampicin 20 mg L^{-1}.

2.13 SOMATIC EMBRYOGENESIS IN BANANA

Genetic manipulation is a promising technique for introducing desired traits in banana. A rapid and effective *in vitro* regeneration protocol is a prerequisite for any such manipulation techniques. The choice of candidate tissues for genetic engineering in banana is restricted to meristematic tissue and embryogenic cell suspensions. Single-cell origin of the somatic embryos their potential to produce non-chimeric plants makes it a potential target system for genetic manipulation. Sagi *et al.* (2000) reported that transformation of embryogenic cell suspensions (ECS) of bananas and plantains is very efficient. There are many reports on standardization of protocols for somatic embryogenesis in banana. Several explants have been tried for callus induction *viz.*, immature male flower bud, scalp, leaf sheath, rhizome etc. Among them immature male flower bud is known to respond well and produce higher proportion of embryogenic calli with high regeneration potential (Cronauer *et al.*, 1983; Escalant *et al.*, 1994; Cote *et al.*, 1996; Navarro *et al.*, 1997; Ganapathi *et al.*,

1999; Becker *et al.*, 2000; Grapin *et al.*, 2000; Assani *et al.*, 2001; Khalil *et al.*, 2002; Suprasanna *et al.*, 2002; Strosse *et al.*, 2003; Namanya *et al.*, 2004; Houllou-Kido *et al.*, 2005; Kulkarni *et al.*, 2006; Meenakshi *et al.*, 2011). Compared to soil grown suckers, IMFs show less contamination during micropropagation and selection of male buds with desirable characteristics based on visual evaluation of bunch quality, number of hands, pest and disease resistance etc. is possible (Resmi and Nair 2007). As the explants were collected after complete bunch formation, superior traits of the mother plants could be assured for selecting the explants for the study.

Establishing a stable and homogenous embryogenic cell suspension (ECS) is the prerequisite for biotechnological breeding of banana. *In vitro* culturing of banana explants faced a serious hindrance by phenolic exudation from the cut ends. Explants were often browning and necrotic decreasing the possibility for getting ECS. Different protocols were followed by the researchers to overcome the difficulties faced due to phenolic interference. Ganapathi *et al.* (1999) used 0.1 per cent activated charcoal in the MS medium. Use of antioxidants like ascorbic acid was followed by Namanya *et al.* (2004). Browning of explants was reduced by addition of cystein and methionine by Khatri *et al.* (2005). Chang *et al.* (2013) cultured flowers on 1/2 MS or 1/3 MS with ascorbic acid 30 mg L^{-1}, which showed less browning than that cultured on media containing activated charcoal, DTT or $Na_2S_2O_3$, cysteine HCl and methionine alone or in combination. Ascorbic acid is a reducing agent which can prevent the oxidation of phenolics in the medium and is also able to scavenge the oxygen radicals produced during the wounding of tissues. Similar findings have been reported by Khatri *et al.* (2005) in callus induction studies using different banana cultivars. He reported positive correlation of light with browning which may be due to higher physiological activities under light than in dark.

Ganapathi *et al.*, 1999 established embryogenic cultures using male flowers in five cultivars of banana. Rapid proliferation of embryogenic callus and embryo development was obtained on MS medium supplemented with 0.22 mM

BA and 1.14 mM IAA. Embryo germination was observed in six to eight weeks upon transfer to half strength MS medium supplemented with 0.5 g L^{-1} malt extract and subsequent development into complete plantlets were observed. Embryogenic callus were obtained at a percentage of 10.00±0.3 using immature male flower on M1 medium supplemented with 18 µM 2,4-D for 3 months and subsequently transferred to the same media with 9 µM 2,4-D for the next 2-3 months. Plantlets were obtained on MS medium supplemented with 0.8 µM BA, at an average regeneration rate of 13.00±0.58 (Jalil *et al.*, 2003).

Khatri *et al.*, 2005 established a method to induce callus in banana. They could produce proembryo calli from basal sheath and rhizome tissue excised from multiplying shoot clusters. SH medium with 30 µM 3,6 dichloro-2-methoxybenzoic acid and 5 µM TDZ showed best results. Browning of explants was reduced by addition of cystein and methionine. Light and dark treatments were given to reduce the browning in the explants. Strosse *et al.*, 2006 established meristem cultures of 18 varieties of banana by culturing long shoot tips on MS medium supplemented with 100 µM BAP. The meristematic tissue, i.e. scalps, were excised out and embryogenesis was induced on media containing 1-50 µM 2,4-D. Embryogenic responses were obtained for each of the tested concentrations, with an optimum at 5 µM 2,4-D. From 24,375 scalps tested, only 3.3 per cent resulted in an embryogenic response. For cooking bananas (ABB) the average embryogenic frequency was 6.0 per cent, for Cavendish bananas (AAA) 3.8 per cent and for plantains (AAB) 1.8 per cent. Embryogenic calli were transferred to suspension culture for maintenance, and obtained embryogenic cell suspensions with high regeneration capacity of 75 per cent.

Ghosh *et al.*, 2009 separated individual floral whorls from immature male inflorescence and cultured on MS medium supplemented with 2,4-D 4 mg L^{-1}, IAA 1 mg L^{-1}, NAA 1 mg L^{-1}, Biotin 1 mg L^{-1}, 3 per cent sucrose and 2 per cent phytagel with pH 5.7 and were maintained in dark at a temperature of 25 ± 2°C for 3-4 months. The embryogenic callus developed was transferred to semisolid medium supplemented with 2,4-D 1 mg L^{-1}, biotin 1 mg L^{-1}, malt extract 100 mg

L^{-1}, glutamine 100 mg L^{-1} and 4.5 per cent sucrose with pH 5.3 named M2 medium. Three months later friable embryogenic callus were transferred to the liquid M2 medium. Fine cell suspensions obtained after 3 months were again transferred to semisolid M2 medium and 6 days later were transferred to SH medium supplemented with picloram 1 mg L^{-1}, malt extract 100 mg L^{-1}, glutamine 100mg L^{-1} and 4.5 per cent sucrose. Mature somatic embryos were transferred to semisolid MS medium supplemented with BAP 1 mg L^{-1} and 3 per cent sucrose after 3 months. For complete development of plantlets, germinating somatic embryos were transferred to semisolid MS medium supplemented with NAA 1 mg L^{-1} and 3 per cent sucrose with pH 5.7. Around 10.3 per cent of the explants formed white friable embryogenic callus. 100 per cent of germinating embryos formed complete plantlets.

Meenakshi *et al.*, 2011 developed embryogenic callus with high regeneration efficiency from immature male inflorescences of the banana cultivar Lalkela (red banana) on MS medium supplemented with 2,4-D 18.10 µM, IAA 5.71 µM, NAA 5.37 µM, d-biotin 1mg . Somatic embryos developed into plantlets on ½ strength MS basal medium with 100 mg L^{-1} malt extract, 4.52 µM 2,4-D, 1 mg L^{-1} biotin, 100 mg L^{-1} malt extract, 100 mg L^{-1} glutamine, 0.2 per cent gelrite. Torres *et al.*, 2012 evaluated the response of various explants in callus induction media. They experimented on different explants such as scalps from cauliflower-like meristems and meristematic domes of axillary sprouted buds. The best embryogenic response (8 per cent) was noticed with meristematic domes of axillary sprouted buds in culture medium Murashige and Skoog salts at 50 per cent, MS vitamins, 30 g L^{-1} sucrose, 10 mg L^{-1} ascorbic acid, 1 mg L^{-1} 2,4-D, 0.22 mg L^{-1} Zeatin, 100 mg L^{-1} malt extract, 100 mg L^{-1} glutamine, 1 mg L^{-1} biotin, 200 mg L^{-1} casein hydrolysate, 4 mg L^{-1} proline with 2 g L^{-1} gelrite.

Remakanthan *et al.*, 2013 developed an improved, reproducible simple protocol for somatic embryogenesis in banana cv. 'Grand Naine' from 4 week old shoot tips raised *in vitro*. They induced somatic embryos in 15 days in MS medium supplemented with 4.14 µM picloram and 0.22 µM BA. Somatic

embryos formed were converted to plantlets (2-3 per cent) in MS medium containing α- Naphthalene acetic acid (NAA; 0.53-2.68μM) with BA (2.22-44.39μM) and glutamine 200 mg L^{-1}. Secondary embryos were converted to plantlets (3 per cent) in MS liquid medium supplemented with 4.44 μM BA.

2.14 *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF EMBRYOGENIC CALLUS OF BANANA

Genetic transformation has become an important tool for crop improvement. Recent advances in genetic engineering of bananas and plantains enable transfer of foreign genes into plant cells. Eventhough biolistic method of genetic transformation is now routine (Becker *et al.*, 2000; Cote *et al.*, 1996; Sagi *et al.*, 1995) *Agrobacterium*-mediated transformation is advantageous as it reduces copy number of the transgene, leading to fewer problems with transgene co-suppression and instability (Gheysen *et al.*, 1998; Hansen and Wright, 1999; Shibata and Liu, 2000). Banana (*Musa* spp.) were considered outside the host range of *Agrobacterium*, but research reports indicates that banana can be transferred by *Agrobacterium tumefaciens* (May *et al.*, 1995; Ganapathi *et al.*, 2001; Sreeramanan *et al.*, 2002; Khanna *et al.*, 2004). These reports include studies on transient expression of transferred genes, stable transformation, regeneration of transformed plants, and heritability of transgenes in banana. Even then it is still difficult due to long incubation time in the callus induction and difficulty in developing regenerable embryogenic cell suspension. Prolonged incubation in the selection medium lead to very low potential to regenerate in the presence of a selection agent.

Becker *et al.* (2000) developed an effective method for the transformation and regeneration of Cavendish banana (*Musa* spp. AAA) cv. 'Grand Nain' via microprojectile bombardment. The neomycin phosphotransferase (nptII) selectable marker gene under the control of a *Banana bunchy top virus* (BBTV) promoter or the CaMV 35S promoter, and either the β-glucuronidase (*uidA*) reporter gene or BBTV genes under the control of the maize polyubiquitin

promote and transformants were co-bombarded to the embryogenic cells showed stable transgene integration. Matsumatto *et al.* (2002) developed a simple and routine particle bombardment system to generate transgenic bananas which used a herbicide resistance gene as selectable marker. Embryogenic cells were bombarded with a plasmid vector compiling the AHAS gene, under the control of the AHAS promoter from *Arabidopsis thaliana*. The bombarded cells were cultured with the corresponding herbicide containing medium for 30 days, and putative transformed plants were then regenerated.

Sagi, (1997) suggested a method that combined both *Agrobacterium* and micro projectile bombardment methods. Apical meristems and underlying corm tissues were bombarded with naked gold particles and then infected with *Agrobacterium*. Few reports are also available on the biolistic gene gun-mediated transformation of banana. May *et al.* (1995) reported production of transgenic banana Cavendish cv 'Grand Naine' by co-cultivating wounded meristems with *Agrobacterium tumefaciens*.

Ganapathi *et al.* (2001) achieved *Agrobacterium* mediated transformation of embryogenic cell suspensions of banana cv. Rasthali (AAB) using strain EHA 105 containing the binary vector pVGSUN with acetolactase synthase (ALS) gene as a selectable marker and gusA reporter gene. They used 100 µM acetosyringone during co-cultivation. Chakrabarti *et al.* (2003) reported the *Agrobacterium* mediated transformation of Rasthali (AAB) using plant expression binary vectors pMS1164 and pMS1168, imparting fungal disease resistance. Transgenic banana plants showed resistance to *Fusarium oxysporum* f. sp. *cubense* and *Mycosphaerella musicola*.

Tripathi *et al.* (2005) co-cultivated the explants (apical shoot tips of *in vitro* regenerated plantlets) with culture of *Agrobacterium* strains having pCAMBIA 1201 with acetosyringone (100 µM). The explants were then transferred to regeneration medium containing cefotaxime (500 mg L^{-1}) for 7 days. After eliminating bacteria the infected explants were transferred to the

selection medium (regeneration medium containing 25 mg L^{-1} hygromycin and 300 mg L^{-1} cefotaxime. The cultures were transferred to fresh selection medium every two weeks. In the transformed apical shoot tips transient expression of the β-glucoronidase (*uid A*) was achieved. Krubphachaya *et al.* (2007) developed RNAi mediated resistance in Cantaloupe against *Papaya ring spot virus* type W. The construct in the binary vector was introduced to the target plant via *A. tumefaciens* strain LBA 4404 and transformed cantaloupe. Transformants were selected in MS medium with kanamycin 100 mg L^{-1} and claforan 500 mg L^{-1}.

Ghosh *et al.* (2009) transformed Cavendish banana cv. Robusta ECSs with *Agrobacterium tumefaciens* strain EHA 105 harboring vector pCAMBIA 1301 with 35S promoter driven *hptII* gene conferring resistance to hygromycin. After co- cultivation and elimination of infection the transformed ECSs were selected by culturing the cells on MS medium supplemented with carbenicillin 200 mg L^{-1}, cefotaxime 200 mg L^{-1} and hygromycin 5 mg L^{-1}. Later regeneration was carried out in selective regeneration medium ie., MS medium with suitable hormones and hygromycin 5 mg L^{-1}.

Sreeramanan *et al.* (2010) transformed single buds (3mm) of banana cultivar 'Rasthali' (AAB) with chitinase gene via *Agrobacterium.* Precultured mildly injured buds were infected using *Agrobacterium* suspension containing acetosyringone 100 µM for 30 mins. This was subjected for co-cultivation for three days on hormone-free MS medium at 22°C temperature in the dark. Chong-Perez *et al.*, 2012 incubated embryogenic cell suspensions (ECSs) with *A. tumefaciens* strain EHA 101 and studied the effect of spermidine and infection time on transformation efficiency. The highest efficiency was obtained when ECSs were infected for 6 h, in medium supplemented with 200 µM acetosyringone and 1.0 mM spermidine.

Shekhawat *et al.* (2012) constructed the ihpRNA cassette in the cloning vector pTZ57R/T and subcloned it to the binary vector pCAMBIA 1301 containing *hptII* as selectable marker. Hence after transformation via

Agrobacterium the selection agent hygromycin phosphotransferase was used to select and maintain the transformed banana embryos and plantlets. Elayabalan *et al.*, 2013 transformed banana cv. Virupakshi (AAB) via *A. tumefaciens* stin LBA4404. The duration of co-cultivation when reduced to 2 days and the cultures when maintained at lower temperature of 18°C resulted in reduced infection injury to microcalli or ECS. Reduced accumulation of phenolics was also observed. They used the binary vector pART27 for carrying the ihpRNA cassette. It contained *nptII* (kanamycin) gene for plant selection. The selection agent, kanamycin 100 mg L^{-1} was used throughout the selection and regeneration of putative transgenic plants.

2.15 CONFIRMATION OF TRANSFORMANTS WITH ihpRNA CASSETTE

Integration ihpRNA construct in the plant genomic DNA as a whole is required to express the double stranded RNA mediated silencing of the virus gene. Studies conducted in this aspect have reported the confirmation of the presence of the silencing cassette in different ways. Tripathi *et al.* (2005) used PCR with GUS gene specific primers to confirm presence or absence of transgene in the plant genome. PCR analysis showed an amplified product of expected size of 500 bp corresponding to *hpt* gene they used in the binary vector showing stable integration of the transgene. Krubphachaya *et al.* (2007) developed RNAi mediated resistance in Cantaloupe against *Papaya ring spot virus* type W vector by cloning the full length coat-protein coding region in the sense and reverse orientation in the primary vector pPRT103. This was transferred to the binary vector pSA1304 and introduced into *Agrobacterium tumefaciens* strain LBA 4404 for transforming cantaloupe. They used PCR analysis to confirm the transformation using cp gene specific primers and *nptII* gene was amplified using the NPT-F/NPT-R primers.

Ghosh *et al.* (2009) transformed embryogenic callus of banana with *A. tumefaciens* strain EHA 105 harboring plasmid pCAMBIA having ihpRNA cassette. PCR was carried out to confirm the transformation event with specific

primers for β- glucoronidase gene which lies within the T-DNA borders and obtained a positive band of expected size 1585 bp. Zhang *et al.* (2011) created a transgene construct to control *Alfalfa mosaic virus* (AMV), *Bean pod mottle virus* (BPMV), and *Soybean mosaic virus* (SMV) simultaneously. The entire P35S-dsABS-T35S cassette was then mobilized into the binary vector pPTN200 and transformed soybean via *Agrobacterium*. The transformation was confirmed by subjecting to PCR based genotyping to verify the presence of the herbicide resistance (BAR) gene and was found positive.

Shekhawat *et al.* (2012) transformed banana embryogenic cells using *A. tumefaciens* (EHA 105) carrying the ihpRNA cassette in the binary vector pCAMBIA 1301. The genomic DNA was isolated from young leaves of selected transformed banana plants using primers designed to amplify hygromycin phosphotransferase gene sequence present within the T-DNA borders of the binary vector pCAMBIA 1301. The PCR analysis showed the amplification of a single 788 bp fragment of the *hpt* gene.

The genomic DNA isolated from transformed plantlets was used as a template for the amplification of *nptII*, and *replicase* gene for confirming the transformation procedure carried out by Elayabalan *et al.*, 2013. They constructed an intron hairpin RNAi vector using pSTARLING and pART27 as primary and binary vectors respectively and transformed ECS and ECS derived microcalli via *Agrobacterium tumefaciens*. In a study by Ntui *et al.* (2014) to induce RNAi-mediated resistance to *Cucumber mosaic virus* in tomato, 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 selectable marker gene. The result showed bands of expected size confirming the transformation.

2.16 CONSTRAINTS IN RNAi MEDIATED SUPPRESSION OF VIRUSES

RNAi mediated technology is highly sequence specific. Most of the plant viruses exist as a continuum of strains which differ from one another to a greater

or lesser extent in their genome sequence. So RNAi targeted against one strain of a virus may not be effective against a distant strain. Identification of the strain associated with the crop in a region is important to design the transgene to cover the sequence variation likely to be encountered. Another problem encountered is the suppression of transgene. Virtually all plant viruses encode silencing suppressors, proteins that suppress the plant's silencing mechanism. These problems can be overcome by understanding the virus population structures in the target regions and by careful design of the virus derived transgene.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Development of small interfering RNA (siRNA) mediated resistance in banana against *Banana bract mosaic virus*" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during February 2012 to February 2016. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter

3.1 SOMATIC EMBRYOGENESIS

3.1.1 Collection and sterilization of explant

Immature male inflorescence (IMFs) of banana variety Nendran was used as explant to induce somatic embryogenesis. The explant material required for the experiments was collected from the Instructional Farm, College of Agriculture, Vellayani and also from farmers' field. Flower buds were collected from field grown *Musa* sp. cv. Nendran plants after the bunch formation was complete (4 weeks after flowering). The size of the male bud was reduced to 7-8 cm by removing off the bracts. They were surface sterilized by wiping it with sterilized cotton dipped in 90% ethanol inside the laminar hood. The tiny bracts covering the male flowers (hands) were removed with utmost care to avoid damages to the apical dome. Immature male flower clusters from 0-15 position were separated using sterile blade and were used as the explants for somatic embryogenesis.

3.1.2 Culture medium

3.1.2.1 *Chemicals*

All the chemicals used for the preparation of the culture medium 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.

3.1.2.2 ***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 25 mm diameter and 0.2 µm pore size were used for 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 various plant growth substances, *viz.*, 2,4- dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), benzyl adenine (BA), indole acetic acid (IAA), picloram, biotin, proline, malt extract and glutamine 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. Agar and/ gelrite were used at the rate of 5.5 g L^{-1} and 4.5 g L^{-1} respectively 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.

3.1.3 Somatic embryogenesis

3.1.3.1 ***Callus induction***

From the surface sterilized male flower bud, male flower clusters (hands) were excised from position 0- 15 and were cultured onto the MS medium supplemented with hormones like 2,4-D, BA, IAA, NAA, picloram, proline, malt extract, biotin and glutamine alone, or in combination along with 30 g L^{-1} sucrose

and 4.5 g L^{-1}gelrite to induce embryogenic callus (Table 1 & 2). The medium was also supplemented with ascorbic acid 20 mg L^{-1}. The cultures were incubated in dark at 25°C, 50-70 per cent relative humidity. The cultures were maintained by subculturing in the same medium composition at one month interval.

3.1.3.2 *Embryogenesis*

The embryogenic calli developed were transferred to semisolid (3g L^{-1}gelrite) MS medium supplemented with various growth hormones like BA, IAA, malt extract, picloram, glutamine (Table 3) and incubated in dark.

3.1.3.3 *Germination of embryos*

The glassy elongated monocot embryo structures developed were subcultured on to half strength semisolid (3 g L^{-1}gelrite) MS medium supplemented with BA and IAA (Table4). The cultures were incubated in dark and were later transferred to 14 h photoperiod 31.4 $\mu mol^{-2}S^{1}$. For the development of complete plantlets the germinating somatic embryos were transferred to half strength semisolid MS medium with BA and NAA under 14 h photoperiod.

3.1.3.4 *Hardening of the in vitro developed plantlets*

The plantlets developed were transferred to pot trays filled with coir pith compost and shifted to the mist chamber for hardening. After 30 days the plantlets were transferred to polybags with soil and cow dung (1:1) mixture in the mist chamber.

3.2 ISOLATION OF REPLICASE GENE OF BBrMV

It was proposed to construct siRNA vector to develop resistance against *Banana bract mosaic virus* (BBrMV) in banana by silencing the replicase gene of BBrMV. BBrMV is (+) sense RNA virus belonging to the family potyviridae. Total RNA was isolated from BBrMV infected banana plants. cDNA was prepared by RT-PCR for further manipulations.

3.2.1 RNA isolation

The method developed by Salzman *et al.* (1999) was followed to isolate RNA. This method was originally developed for plant tissues containing high levels of phenolic compounds or carbohydrates. The protocol included the following steps:

Spindle leaf of BBrMV infected banana cv. Nendran was used for RNA isolation. The leaf tissue (0.1 g) was ground with liquid nitrogen using motar and pestle. Finely ground tissue was quickly transferred to 1 ml extraction buffer. The extraction buffer was prepared in 0.1 per cent diethyl pyrocarbonate (DEPC) treated, autoclaved water with 4 M guanidine thiocyanate, 100 mM Tris HCl (pH 8.0), 25 mM sodium citrate (pH 8.0) and 0.5 per cent N- lauryl sarcosine (Appendix II). To this mixture Polyvinylpyrrolidone (PVP) 1 per cent and 2, β-mercapto ethanol 20 µL were freshly added. The mixture was shaken vigorously for 1 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added and vigorously shaken for 10-20 min (these steps were carried out in ice). The phases were separated by centrifuging at 16,000 g for 10 min at 4°C. The clear supernatant was transferred to a fresh tube and two volumes of absolute ethanol along with 0.1volume of 5M NaCl were added and kept overnight at -20°C for precipitation.

On the second day it was pelleted by centrifugation at 16000 g for 10 min at 4°C. The pellet was resuspended in 1 ml DEPC treated water. Equal volume of phenol (TE saturated): chloroform: isoamyl alcohol (25: 24: 1) was added to the suspension. This was mixed by inverting the tubes 5-10 min at room temperature. The phases were separated by centrifuging at 13000 g for 10 min at room temperature. The upper phase was transferred to a clean tube. The above steps were repeated until there was no visible interphase. Two volumes of absolute ethanol along with 0.1 volumes of 5M NaCl was added to the supernatant and kept at -20°C overnight for precipitation. On the third day it was pelleted by centrifuging at 16000 g for 15 min at 4°C. The pellet was resuspended in 500 µL

DEPC treated water and the sample volume was made upto 1 ml with DEPC treated water. To the suspension 333 µL, of 8 M LiCl was added and kept for precipitation at -20°C overnight. Fourth day the precipitate was pelleted by centrifuging at 12000 g for 20 min. The pellet was washed with 400 µL of 80 per cent ethanol. The pellet was dried inside the laminar hood and was resuspended in 100 µL DEPC treated water.

3.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit. 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 loading dye reached three fourth length of the gel. The gel was documented using a gel documenting system (BIO-RAD).

3.2.3 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.

3.2.4 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.

3.2.5 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), Ban (Act) Forward and Ban (Act) Reverse 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**	***Actin***	
		Temperature (°C)	**Duration**
1.	Initial denaturation	94	1 min
2.	Denaturation	92	30 sec
3.	Annealing	55	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. One of the wells was loaded with 5 µl of the 100 bp molecular weight marker (Merck Genei) with

required 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 Amplification of replicase gene sequence of BBrMV

Complete genome sequence of BBrMV (Trichy isolate) was retrieved from GenBank [National Centre for Biotechnology Information (NCBI)]. Based on the sequence information, 2 sets of BBrMV replicase gene specific primers were designed manually. Alignment and melting temperature of the manually designed primers were checked using the software BioEdit Sequence Alignment Editor Version 7.2.5 (Hall, T. A. 1999). The sequences of the primers designed for the amplification of the replicase partial gene are given below.

BBrMV- A Forward – 5' TGTAGTCCGCGGGAAATGTC 3'
Reverse – 5' GTGTCTAACGGAGCTGCTGT 3'
BBrMV- B Forward – 5' TTAGCTGAACATAAGGGTGC 3'
Reverse – 5' CTGCTGTGAACACGCGTGTT 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*replicases*pecific primers**	
			Temperature (°C)	**Duration**
1.	Initial denaturation		94	1 min
2.	Denaturation		92	30 sec
3.	Annealing	BBrMV-A	57	30 sec
		BBrMV- B	55	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). One of the wells was loaded with 5 µl of the 100 bp molecular weight marker (Merck Genei) with required 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.

3.2.7 Sequencing of the viral replicase gene partial coding sequence

The PCR products obtained by amplification with the primers BBrMV- A and BBrMV- B were sequenced (Appendix III) at Regional Facility for DNA Fingerprinting (RFDF), Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v 3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (purified)	-	10-20 ng
Primer	-	3.2 pM (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

The sequencing PCR temperature profile consisted of an initial denaturing at 96°C for 2 min followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 min for all the primers.

PCR Clean up

Twelve µl of master mix I (10 µl milli Q and 2 µl 125mM EDTA) was added to each reaction containing 10µl of reaction contents and mixed properly. To this 52 µl of master mix II (2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol) was added. Contents were mixed by inverting and incubated at room temperature for 30 min. The mixture was centrifuged at 14,000 rpm for 30 min. The supernatant was decanted and 100 µl of 70 per cent ethanol was added and centrifuged at 14,000 rpm for 20 min. The supernatant was decanted and this step was repeated. The supernatant was decanted and the pellet was air dried. The cleaned up product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v. (Applied Biosystems).

3.3 ihpRNA VECTOR CONSTRUCTION

3.3.1 miRNA target prediction in the replicase gene of virus

The replicase gene (partial cds) sequence obtained was subjected to miRNA target prediction using RNAi design tool of Integrated DNA Technologies (IDT). The DICER substrates (miRNA target sites) in the target gene were found using the 'Custom Dicer- Substrate siRNA (DsiRNA) inRNAi design tools available at the website of Integrated DNA Technologies (IDT) (Appendix V). The duplex RNA GC per cent of 30-70 per cent and the asymmetrical end stability base pair length of 5 was set along with other target parameters detailed in (Appendix VI). The targets found were selected and analysed for siRNA attributes for higher efficiency.

3.3.2 Restriction mapping

The partial replicase gene sequence isolated was subjected to restriction mapping using the tool 'Restriction Map' in 'Sequence Manipulation Suite' available at the website www.bioinformatics.org. The sequence of interest was converted to FASTA format and submitted for restriction mapping. A textual map showing the positions of restriction endonuclease cut sites was obtained (Appendix VII).

3.3.3 Preparation of sense and antisense fragments

Based on the partial replicase gene sequence of BBrMV Vellayani isolate, another set of primers were designed with specific restriction enzyme recognition sequence anchored to its 5' end. Primers were designed to include miRNA target regions and exclude selected restriction enzyme recognition sites. The primary vector pSTARLING had 3 different sets of restriction enzymes sites on either ends of the *cre* intron. *Bam*HI and *Pac*I were selected for sense strand integration and *Spe*I and *Kpn*I were selected for antisense strand integration. PCR reaction was carried out with the two sets of primers.

Sense	Forward	- 5'AAGGATCCTGGTCCAAGCCGGTT 3'
	Reverse	- 5'TTAATTAAGGAGCTGCTGTGAACAC 3'
Antisense	Forward	- 5'ACTAGTGGAGCTGCTGTGAACAC 3'
	Reverse	- 5'TTGGTACCTGGTCCAAGCCGGTT 3'

A standard PCR mix was prepared with the template DNA (2 µl of the initial PCR product was diluted to 40 µl and 5µl was taken from that as the template DNA), 200 µM dNTP, 10 pM of each primer,1X Taq polymerase buffer and 1 unit of Taq polymerase. The reaction mix was made upto a 1 volume of 20 µl. The DNA was amplified in the Thermal cycler using the program shown in the table below

Step	Stage	*Sense (S) & antisense (AS) primers*	
		Temperature (°C)	Duration
1.	Initial denaturation	94	1 min
2.	Denaturation	92	30 sec
3.	Annealing	59	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 (1.2 per cent) in a horizontal gel electrophoresis unit (as explained above). One of the wells was loaded with 5 µl of the 100 bp molecular weight marker (Merck Genei) with required 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.

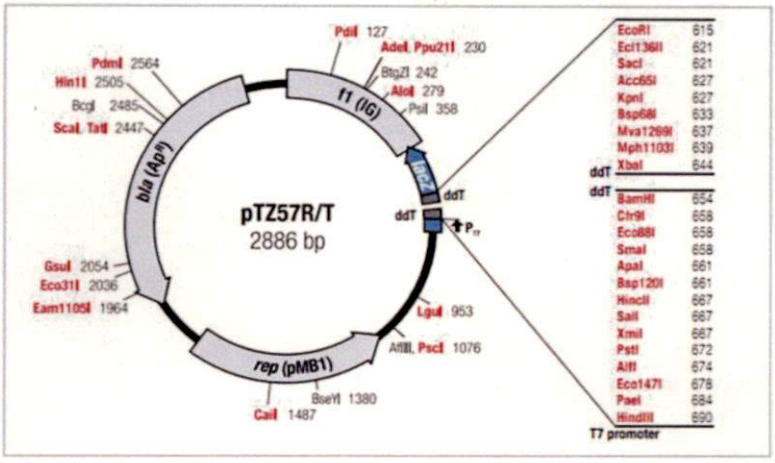
3.3.4 Elution of the sense and antisense fragments from agarose gel and quantification

The sense and antisense fragments separated in low melting agarose (1.2 per cent) gel were sliced out with a clean scalpel and stored in a pre-weighed fresh 1.5 ml vial. Gel extractions of the excised bands were carried out using Nucleospin® Gel and PCR clean-up Kit following the manufacturers' protocol. Eluted PCR product was quantified.

3.3.5 Cloning of sense and antisense fragments in pTZ57R/T

The sense and antisense fragments extracted were ligated to the TA cloning vector pTZ57R/T (MBI, Fermentas). The ligation reaction was carried out by preparing the reaction mix consisting of 0.5 µl of linear pTZ57R/T, 1X T4 DNA ligase buffer, 1 U T4 DNA ligase, 300 ng of insert (the eluted sense and

antisense fragments) and the reaction volume was made upto 15 µl with sterile nuclease free water. The reaction mix was spun briefly and incubated at 22°C for 1h and then stored in 4°C. The cloning site of the vector is within the *lacZ* gene to facilitate blue-white screening of positive clones.



3.3.6 Transforming DH5α with ligated pTZ57R/T

3.3.6.1 *Preparation of stock solutions of Antibiotics*

The antibiotic stock solutions (10^4 mg L^{-1}) were prepared by dissolving them in suitable solvents as shown below. It was then filtered using bacterial membrane filters (Axiva) of 25 mm diameter and 0.2 µm pore size and stored at -20°C as one ml aliquots.

Antibiotics	**Solvents**
Kanamycin	Water
Ampicillin	Water
Spectinomycin	Water
Cefotaxime	Water
Rifampicin	Dimethylsulfoxide (DMSO)

3.3.6.2 *Competent cell preparation*

DH5α competent cells were prepared by calcium chloride ($CaCl_2$) method (Maniatis et al., 1989). The *Escherichia coli* (*E. coli*) cells DH5α were grown in 10 ml of the Luria Bertani (LB) broth (Maniatis *et al.*, 1982) (Appendix VIII) overnight at 37°C on rotary incubator. 1 ml of the overnight grown culture was transferred to 30 ml of fresh LB broth and incubated at 37°C until the O.D. value reached 0.2 at 650 nm. Then the culture was transferred to fresh 2ml tubes and stored in ice. The cells were pelleted at 5000g for 10 min at 5-10°C. The supernatant was decanted off. The pellet was suspended in 300 µl of 0.1M $CaCl_2$ solution. Further 600 µl of 0.1M $CaCl_2$ solution was added. The cells were pelleted at 5000g for 10 min at 5-10°C. The supernatant was discarded. The pellet was suspended in 100 µl of 0.1 M $CaCl_2$ and stored in ice prior to use.

3.3.6.3 *Transformation*

To 100 µl of the competent cells, 5 µl of the ligation mix was added and mixed gently. The tubes were kept on ice for 30 min. Then the tubes were transferred to a water bath and incubated at 42°C for 90 seconds. After incubation the tubes were quickly plunged in ice bath and chilled for 5 min. 1 ml of LB broth at room temperature was added to the tube and incubated at 37°C for 1 hr with gentle shaking. The cells were plated on LB agar with Ampicillin 100 mg L^{-1}, 40µl (20 mg/ml) X-GAL and 40µl 100mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).

3.3.7 Plasmid isolation from transformed DH5α colonies

The white transformed single colonies were picked and inoculated in 5 ml LB broth with ampicillin 100 mgL^{-1} and was kept at 37°C in incubator shaker at 150 rpm overnight. Bacterial plasmid was isolated following the Alkaline lysis method (Birnboim *et al.*, 1979). 1.5 ml of the bacterial culture was taken in a 2 ml microcentrifuge tubes and the cells were pelleted at 5000g for 5 min at 4°C. The cells were pelleted and supernatant discarded. The bacterial pellet was

resuspended completely in 200µl of solution I (Appendix IX) by vortexing and incubated in ice bath for 5 min. To that 300µl of solution II was added, mixed gently by inverting and kept on ice for 2-3 min. To this 300µl of solution III was added and mixed by inverting and kept in ice for 5 min. The white precipitate formed was centrifuged at 12,000g for 10 min at 4°C. The supernatant was transferred to a fresh tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by inverting. The mixture was centrifuged at 12,000g for 5 min at 4°C. The supernatant was transferred to a fresh tube and an equal volume of chilled isopropanol was added, mixed by inverting and incubated at -20°C for 20 min. Pellet was obtained by centrifuging at 10,000g for 10 min at 4°C. The supernatant was discarded and the pellet was washed in 1ml of 70 per cent ethanol. After centrifugation at 8000g for 5 min at room temperature, the supernatant was discarded and the pellet was thoroughly dried and dissolved in 50µl TE Tris EDTA (Ethylene diamine tetra acetic acid) buffer (pH 8.0) (Appendix IV) containing RNase (2mg/ml). Incubated at 37°C for 1h in water bath. The plasmid was quantified and stored in -20°C.

3.3.8 Confirmation of the integration and orientation of the sense and antisense strands by PCR

After plasmid isolation, screening was done to detect the insert by PCR. The plasmids isolated from the white colonies were subjected to PCR with M13 forward primer and the reverse primers of sense and antisense strands simultaneously. The reaction mix was prepared with 1µl of plasmid DNA, 200µM dNTPs, 10 pM of each primers, 1U of Taq polymerase, 1X Taq polymerase buffer and the final reaction volume was made upto 25 µl. The DNA was amplified in the Thermal cycler using the program shown in the table below.

Step	Stage	*M13 forward and S & AS reverse primers*	
		Temperature (°C)	Duration
1.	Initial denaturation	94	1 min
2.	Denaturation	92	30 sec
3.	Annealing	54	30 sec
4.	Extension	72	30 sec
5.	Final extension	72	5 min

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

Gel electrophoresis was carried out in a horizontal gel electrophoresis unit with 1.2 per cent agarose in 1X TAE buffer. The PCR product and gel-loading dye (6 x loading dye i.e., 40 per cent sucrose and 0.25 per cent bromophenol blue) were mixed and loaded in to the wells. One of the wells was loaded with 5 µl of the 100 bp molecular weight marker with required volume of gel loading dye. Electrophoresis was carried out at 50 volts and the gel was documented using a gel documenting system.

3.3.9 Release of sense and antisense fragments from pTZ57R/T

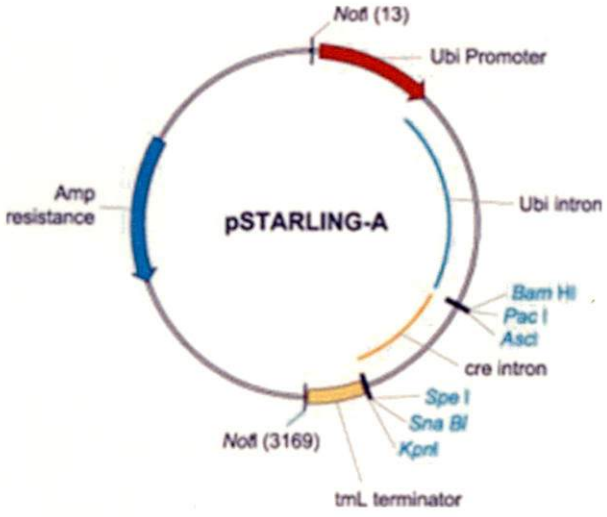
The plasmid DNA isolated was subjected to restriction digestion. The reaction mix was prepared as shown below. The Fast digest enzymes from M/S Thermo scientific were used for this. The final volume of the reaction mix was made upto 60µl. The reaction was carried out at 37°C for 45 min and the reaction was terminated by incubating the reaction at 75°C for 5 min. Gel electrophoresis with 1.2 per cent agarose was carried out to separate out the digested product.

Components for restriction reaction	pTZ57R/T with sense fragment	pTZ57R/T with antisense fragment
Plasmid	4µg	4µg
Fast digest green buffer	1x	1x
Fast digest *Bam*HI	1.5µl	-
Fast digest *Pac*I	1.5µl	-
Fast digest *Spe*I	-	1.5µl
Fast digest *Kpn*I	-	1.5µl

The electrophoretic analysis was documented and the bands of molecular weight 400 bp corresponding to the expected size of the release i.e. the sense and antisense fragments from the pTZ57R/T vector backbone were eluted out separately. The sense and antisense fragments were extracted out using Nucleospin® Gel and PCR clean-up Kit. Eluted products were quantified using spectrophotometer and stored in -20°C.

3.3.10 Insertion of sense and antisense fragments to the primary vector

The primary vector used in the study was pSTARLING (Appendix X) obtained from Commonwealth Scientific and Industrial Research Organisation (CSIRO) through Material Transfer Agreement (MTA). The vector was obtained as stab culture. It was grown on LB medium with antibiotic ampicillin 100 mg L^{-1}. The physical map of the vector is shown below.



The *Not*I fragment contained the components such as the ubiquitin promoter, ubiquitin intron, cyclic AMP response element (*cre*) intron with multiple cloning sites (MCS) comprising of *Bam*HI, *Pac*I and *Asc*I at the 5' end and *Spe*I, *Sna*BI and *Kpn*I at its 3' end and tmL (tumor morphology locus) terminator. For the insertion of the sense and antisense fragments in pSTARLING vector on either side of the cre intron, the primary vector was restricted with the corresponding restriction enzymes. In the first step restriction digestion of pSTARLING was carried out for integration of the sense strand by preparing the reaction mix as shown below.The reaction volume was made up to 30µl and incubated at 37°C for 45 min and the reaction was terminated by incubating the reaction at 75°C for 5 min.

Components for restriction reaction	**Concentrations**
Plasmid (pSTARLING)	1µg
10x Fast digest green buffer	1x
Fast digest BamHI	1U
Fast digest PacI	1U

After the restriction digestion, the linearized pSTARLING was separated on 1 per cent agarose gel and was extracted using Nucleospin® Gel and PCR clean-up Kit. The eluted product was quantified using spectrophotometer. A ligation reaction was carried out to ligate the sense strand on the 5' end of the *cre* intron. Ligation reaction was set up as shown below. The final reaction volume was made upto 20µl, mixed well and incubated at 22°C for 1h and stored at 4°C.

Components for ligation reaction	**Concentration**
Linearized pSTARLING vector	1µg
Insert (sense strand)	1µg
10x T4 DNA ligase buffer	1x
T4 DNA ligase	5U

The ligated product was transformed to *E.coli* DH5α, plated on LB agar with ampicillin 100 mg L^{-1} and incubated overnight at 37°C. After overnight incubation the colonies obtained were grown in LB broth with ampicillin 100 mg L^{-1} and incubated at 37°C in rotary incubator shaker. Plasmid was isolated from the overnight grown culture and subjected to PCR to detect the presence of sense strand insert.

The primary vector with sense fragment insert was subjected to linearization with the second set of restriction enzymes *Kpn*I and *Spe*I to ligate the antisense fragment. The linearization reaction was set as follows. The reaction volume was made up to 30µl and incubated at 37°C for 60 min and the reaction was terminated by incubating the reaction at 75°C for 5 min.

Components for restriction reaction	Concentrations
Plasmid (pSTARLING)	1µg
10x Fast digest green buffer	1x
Fast digest *KpnI*	1U
Fast digest *SpeI*	1U

After the restriction digestion the linearized pSTARLING was separated on 1 per cent agarose gel and was extracted using Nucleospin® Gel and PCR clean-up Kit. The eluted product was quantified using spectrophotometer. A ligation reaction was carried out to ligate the antisense strand on the 3' end of the *cre* intron. Ligation reaction was set up as shown in the table. The final reaction volume was made upto 20µl, mixed well and incubated at 22°C for 1h and stored at 4°C.

Components for ligation reaction	Concentration
Linearized pSTARLING vector	1µg
Insert (antisense strand)	1µg
10x T4 DNA ligase buffer	1x
T4 DNA ligase	5U

The ligated product was transformed to competent *E. Coli* DH5α and plated on LB agar with ampicillin 100 mg L^{-1} and incubated at 37°C overnight. After incubation all the colonies obtained were picked and inoculated in LB broth with ampicillin 100 mg $L^{-1}$and grown overnight at 37°C in incubator shaker. Plasmid was isolated by alkaline lysis method and stored in -20°C.

3.3.10.1 *Confirmation of the integration of sense and antisense fragments in pSTARLING*

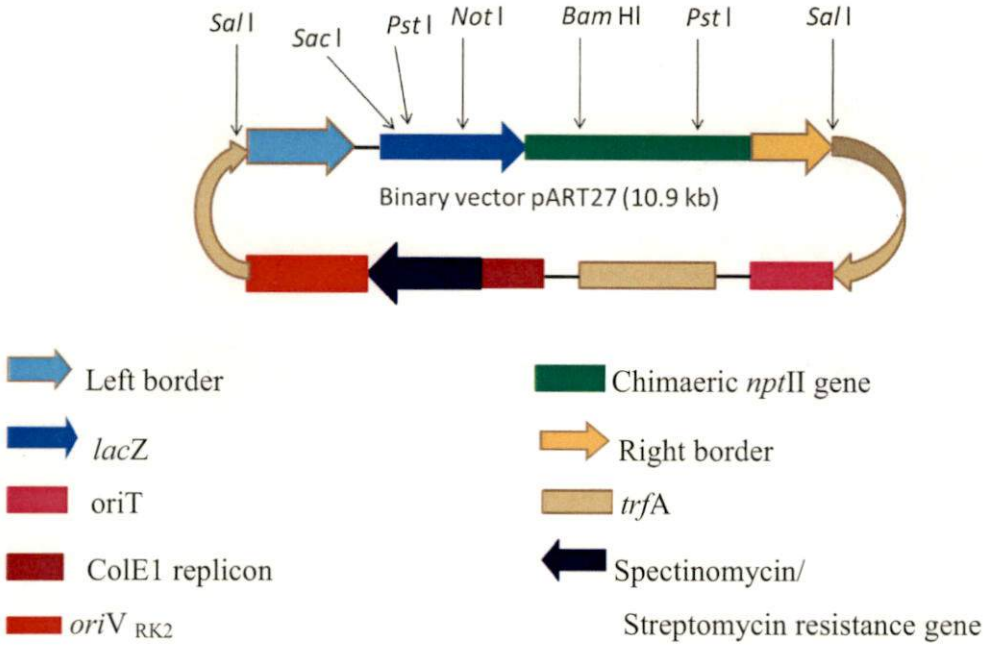
pSTARLING vector isolated from the transformed colony was subjected to restriction digestion reaction to release the sense strand and the antisense strands. Restriction reactions were carried out separately for releasing the sense and antisense fragment inserts. 2µg of plasmid DNA and 1x fast digest buffer in two separate tubes were taken. In the first tube 1U each of *Bam*HI and *Pac*I for the release of sense strand and in the second tube *Spe*I and *Kpn*I for the release of antisense strand. The reaction volume was made upto 30µl and was incubated at 37°C for 45 min. Gel electrophoresis with 1.2 per cent agarose was carried out to separate out the digested products and was documented using a gel documentation unit.

Another restriction digestion was also carried out to release the whole insert along with the *cre* intron. The reaction mix was prepared with 1µg of plasmid DNA, 1X fast digest buffer and 1U each of *Bam*HI and *Kpn*I. The reaction volume was made upto 30µl and was incubated in 37°C for 45 min. Gel electrophoresis with 1.2 per cent agarose was carried out to separate out the digested products. One of the wells was loaded with 5 µl of the 100bp-5 kb molecular weight marker (Merck Genei) with required volume of gel loading dye. After gel separation it was documented using a gel documentation unit.

3.4 TRANSFER OF THE ihpRNA CONSTRUCT TO THE BINARY VECTOR

The *Not*I fragment in the pSTARLING vector comprising of the ubiquitin promoter, ubiquitin intron, sense strand insert, cyclic AMP response element (*cre*) intron, the antisense strand insert and tmL (tumor morphology locus) terminator. This *Not*I fragment from pSTARLING vector was transferred to the *Not*I site of the binary vector pART27 (Appendix XI), which was obtained from The New Zealand Institute for Plant and Food Research through MTA. The binary vector, pART27 was obtained as plasmid mini preparation. It was transformed and cloned

using DH5α and grown in LB plates with the antibiotic spectinomycin 100 mgL^{-1}. The physical map of which is shown below.



3.4.1 Release of *Not*I fragment from pSTARLING

pSTARLING vector containing sense and antisense inserts was subjected to restriction digestion reaction to release out the *Not*I fragment having the ihpRNA construct. Restriction reaction was carried out with 4µg of plasmid DNA, 1X fast digest buffer and 2U of *Not*I fast digest enzyme. The reaction volume was made upto 60µl and was incubated at 37°C for 30 min. Gel electrophoresis with 1 per cent agarose was carried out to separate out the digested products and was documented. The *Not*I fragment was eluted and extracted from the agarose gel using Nucleospin® Gel and PCR clean-up Kit. It was quantified and stored at -20°C.

3.4.2 Insertion of *Not*I fragment to pART27

pART27 was linearized using NotI fast digest enzyme. Restriction reaction was carried out with 4µg of plasmid DNA, 1x fast digest buffer and 2U of *Not*I

fast digest enzyme. The reaction volume was made upto 60µl and was incubated at 37°C for 30 min. Gel electrophoresis with 1 per cent agarose was carried out to separate out the linearized vector and was documented. The linearized vector was extracted from the agarose gel using Nucleospin® Gel and PCR clean-up Kit. It was quantified and stored at -20°C.

For integration of the *Not*I fragment to the *Not*I site of pART27, a ligation reaction was carried out in a 30µl reaction mix containing 1µg each of the insert and the linearized vector pART27 (1:1), 2U T4 DNA ligase enzyme, 1X T4 DNA ligase buffer. The reaction was carried out at 22°C for 1h and stored at 4°C. The ligated plasmid was transformed and cloned using DH5α. The cells were plated on LB agar with Spectinomycin 100 mgL^{-1}, 40µl (20 mg/ml) X-GAL and 40µl 100mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for blue white screening. The white colonies obtained were cultured in LB broth with corresponding antibiotic. The plasmid was isolated by alkaline lysis method and stored in -20°C.

3.4.3 Confirmation of the inserts in pART27

The pART27 plasmid vector was isolated and subjected to restriction digestion to confirm the integration of the *Not*I fragment. The reaction mix (60µl) contained 2µg of plasmid DNA, 1x fast digest buffer and 2U of *Bam*HI, *Kpn*I and *Pac*I fast digest enzymes. The reaction was incubated at 37°C for 30 min. The products were separated out in one per cent agarose gel along with 5µl of 100-5 kb molecular weight marker (Merck Genei) and were documented using a gel documentation unit.

3.5 TRANSFER OF THE BINARY VECTOR TO *AGROBACTERIUM TUMEFACIENS*

3.5.1 Preparation of Competent *A. tumefaciens*

The binary vector was transferred to *A. tumefaciens* strain LBA4404. For that *A. tumefaciens* was made competent to take up the vector DNA. The cells were made competent according to the protocol reported by Jyothishwaran *et al.*

(2007). *A. tumefaciens* strain LBA4404 was streaked out on a LB plate containing 20 mg L^{-1} rifampicin and grown at 28°C overnight. A single colony was inoculated in 3 ml of LB medium containing 20 mg L^{-1} rifampicin and grown overnight at 28°C, shaking at 160 rpm. 0.5 ml (1/100th volume) of the overnight culture was transferred to 50 ml of LB medium with 1 mg L^{-1} rifampicin and grown in an incubator-shaker at 28°C at 160 rpm to obtain cell density of 0.3 at OD600. The cultures were chilled on ice for 15 min and the cells were then harvested by centrifugation at 3000 rpm for 5 min at 4°C, resuspended in 10 ml of sterile ice-cold 100 mM $MgCl_2$ solutions and incubated on ice for 1 h. After another centrifugation step as above, the resulting pellet was resuspended in sterile ice-cold 2 ml of 20 mM $CaCl_2$, and incubated on ice for 6 h to yield the competent cell suspension and aliquots of 100 ml were made.

3.5.2 Transformation of *A. tumefaciens*

The binary vector was to be transferred to competent *A. tumefaciens* strain LBA4404 by the modified freeze-thaw method (Jyothishwaran *et al.*, 2007).One µg of pART27 vector containing the ihpRNA construct was added to 100 µl of freshly prepared competent *A. tumefaciens* kept in ice bath. Mixed gently by tapping and frozen in liquid N_2 for 10 min. Then the vial was thawed at 37°C for 5 min. 10 µl of this mixture was inoculated into 1 ml of pre-warmed LB medium without antibiotics and incubated for 1h at 28°C in a rotary shaker at 160 rpm. This 50 µl of the suspension was spread onto LB plates containing 20 mg L^{-1} rifampicin and 100 mgL^{-1} kanamycin. After incubation at 28°C for 24 h, the colonies were counted for estimating the transformation efficiency.

Transformation efficiency is defined as the number of transformant colony forming units (CFU) obtained per µg of plasmid DNA and is calculated as follows.

$$\text{Transformant CFU} = \frac{[\text{Number of bacterial colonies X dilution ratio X original transformation volume}]}{\text{Plated volume}}$$

Transformation efficiency = Transformant CFU / Plasmid DNA (μg)

3.5.3 Confirmation of transformation

Transformants were tested for the presence of the binary vector, pART27 and ihpRNA cassette in *A. tumefaciens* by PCR analysis with gene-specific primers for *nptII* and *cre* intron respectively. The plasmid DNA from *A. tumefaciens* was isolated by alkaline lysis method. The primers designed for *nptII* gene (universal) and *cre* intron were

Primers for *nptII* gene

(*nptII* –F) 5' GGTGCCCTGAATGAACTG 3'
(*nptII* -R) 5' TAGCCAACGCTATGTCCT 3'.

Primers for *cre* intron

(*cre* –F) 5' ACAGTCCAGCTTTTGTTGTT 3'
(*cre*- R) 5' TGAGCTAGGAATCAGCTAGG 3'

Both the PCR reactions were carried out in 25 μl volume containing 100 ng plasmid DNA, 200 μM dNTPs, 10pM of each primer, 1U of Taq polymerase and 1x Taq polymerase buffer.

Step	Stage		Gene specific primers for *npt*II and *cre* intron	
			Temperature (°C)	Duration
1.	Initial denaturation		94	1 min
2.	Denaturation		92	30 sec
3.	Annealing	*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.

The PCR products were separated on 1.2 per cent agarose gel in a horizontal gel electrophoresis unit. One of the wells was loaded with 5 µl of the 100 bp molecular weight marker with required volume of gel loading dye.

3.6 EVALUATION OF EMBRYOGENIC CALLUS OF BANANA FOR SENSITIVITY TO ANTIBIOTICS

Sensitivity of banana embryogenic callus cultures to antibiotics was evaluated to fix the antibiotic concentration needed for the selection process after transformation. The embryogenic callus produced on MS medium supplemented with BA 0.2 mg L^{-1} and picloram 1 mg L^{-1} were transferred to petriplates containing medium of same composition with different concentrations of kanamycin (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275 and 300 mg L^{-1}) and cefotaxime (0, 5, 25, 50, 75 and 100 mg L^{-1}). The petriplates were sealed with parafilm and kept at 25 ± 2^0 C. The response of the callus to varying concentrations of kanamycin and cefotaxime was evaluated for a period of three weeks.

The following scoring method was used for evaluation.

Score	Culture response
'++++'	Fully green
'+++'	Partially discoloured
'++'	Bleached tissues
'+'	Tissues turning brown and dead

3.7 SCREENING OF *AGROBACTERIUM* STRAINS FOR SENSITIVITY TO ANTIBIOTICS

Agrobacterium maintained in LB medium was transferred to LB medium with different concentrations of kanamycin (0, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg L^{-1} (Table 7) and cefotaxime (0, 5, 10, 15, 20, 25, 50, 75, 100 mg L^{-1}) (Table 8). Petri plates were then sealed with parafilm and incubated at 28°C and bacterial growth was observed for two days.

3.8 TRANSFORMATION OF BANANA EMBRYOGENIC CALLUS

3.8.1 Preparation of *Agrobacterium* Suspension for Co-cultivation

The *A. tumefaciens* LBA4404 with the binary vector pART27 was grown on petriplates containing LB medium with kanamycin 100 mg L^{-1} at 28°C overnight. *A. tumefaciens* suspension for co-cultivation was prepared by picking a single colony from the plate and inoculating into LB broth supplemented with kanamycin 100 mg L^{-1}. It was kept in an incubator shaker at 110 rpm overnight at 28°C and the optical density when reached one, the culture was spun in a centrifuge at 5000 rpm at 4°C for 5 min. The pellet obtained was resuspended in 1 ml of half strength MS broth (with 100 µM acetosyringone) for co-cultivation.

3.8.2 Preparation of Plant Material

The embryogenic calli of banana were pre-cultured on MS medium containing BA 0.2 mg L^{-1} and picloram1 mg L^{-1}, 15 days before co-cultivation to maintain cells in active cell division stage.

3.8.3 Infection of the embryogenic callus

The pre-cultured calli were used for co-cultivation. The pieces of callus were placed in a sterile petriplate and wetted with liquid half MS medium to avoid drying of explants. The calli was then mixed thoroughly with the *Agrobacterium* suspension by gentle swirling for the infection process. Infection was done for different intervals of time i.e. 5, 10, 15, 20 min notated as T1, T2, T3, T4 respectively. Different experiments were included under co-cultivation to study the effect of (a) wounding of callus before infection, (b) addition of inducer acetosyringone100 µM along with *Agrobacterium* suspension and (c) wounding of the callus followed by addition of acetosyringone100 µM along with *Agrobacterium* suspension, on transformation efficiency.

3.8.4 Co-cultivation

After infection the explants were blot dried with sterile filter paper and transferred to a petriplate containing solidified half strength MS medium. Petri plates were sealed with parafilm and kept for co-cultivation in dark for 48 hours at 25 ± 2^0C.

3.8.5 Removal of *Agrobacterium*

After co-cultivation, calli were washed in half strength liquid MS medium containing 100 mg L^{-1}cefotaxime to kill the bacteria. The tissues were blot dried with sterile filter paper and transferred to sterile petriplates containing half MS medium supplemented with 25 mg L^{-1} cefotaxime for eradication of the remaining *Agrobacterium.* The calli were subcultured to new petriplates with semisolid MS medium supplemented with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} and 25 mg L^{-1} fresh

cefotaxime for one week in 2 days interval. Then the cultures were subcultured in the same medium with kanamycin 100 mg L^{-1} and incubated in dark for 40 ± 5 days till embryo formation.

3.8.6 Selection of Transformed Tissues

The transformed tissues were selected on MS medium containing kanamycin 100 mg L^{-1} and cefotaxime 25 mg L^{-1}. The tissues were maintained by subculturing once in seven days in the same medium. After four rounds of subculture, the transformed and non-transformed tissues were scored based on their response in the medium. The embryogenic callus survived in the selection medium were transferred to embryo induction medium and then germinated in the regeneration medium.

3.9 CONFIRMATION OF SOMATIC EMBRYOS 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 carried out to detect 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.

3.9.1 Isolation of genomic DNA

Leaf tissues from the regenerated shoots were used for genomic DNA isolation. Total genomic DNA was isolated using modified protocol of 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 XII). The mixture was homogenized by vortexing for a few seconds. Then the samples were incubated at 55° C for 30 min with occasional mixing. The mixture was cooled to room

temperature 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. Then the extracts were mixed with an equal volume of chloroform: isoamyl alcohol (24:1) and was centrifuged at 5000 rpm for 5 min. The aqueous phase was transferred to a new tube and an equal volume of ice-cold 2-propanol was added. It was kept at –20° C for 1h 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.

3.9.2 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 and *cre* intron**	
			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.

3.9.3 Preparation of positive control

The plasmid pART 27 with the ihpRNA construct was isolated by alkaline-lysis method to act as positive control. The plasmid DNA was subjected to PCR, with *nptII* and *cre* intron specific primer. A standard PCR mix was prepared for 25µl final volumes. The reactions were set up with 100 ng of template DNA, 200 µM dNTPs, 10 pM of each primers, 1 unit of Taq polymerase, 1x Taq polymerase buffer. The final volume was made up with sterile water. The following conditions were provided for the amplification of the specific regions in the plasmid DNA in a Thermal Cycler.

Step	**Stage**		**Gene specific primers for *npt*II and *cre* intron**	
			Temperature (°C)	**Duration**
1.	Initial denaturation		94	1 min
2.	Denaturation		92	30 sec
3.	Annealing	*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.

The PCR products were separated on 1.2 per cent agarose gel in a horizontal gel electrophoresis unit. One of the wells was loaded with 5 µl of the 100 – 5 kb molecular weight marker with required volume of gel loading dye. The gel was documented using a gel documentation unit.

Results

4. RESULTS

The results of the present study on "Development of small interfering RNA (siRNA) mediated resistance in banana against *Banana bract mosaic virus*" carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during February 2012 to February 2016 are presented below. The study included development of ihpRNA vector against the replicase gene of BBrMV and introduction of this construct into embryogenic cells via *Agrobacterium* mediated transformation. For this a protocol was also developed for somatic embryogenesis mediated regeneration suitable for genetic transformation in banana var. Nendran.

4.1 ESTABLISHMENT OF SOMATIC EMBRYOS

4.1.1 Callus initiation

Immature male inflorescences (IMFs) of the commercially important banana cultivar 'Nendran' were used as explants for producing callus (Plate 1). Male flower buds used in the study were collected from the field grown plants soon after the bunch formation was complete. This helped in visually evaluating the bunch quality, number of hands, fruit quality and health of the mother plant before collecting the explants. Contamination free cultures could be established with a simple surface sterilization procedure. Dipping the flower bud in 90 per cent ethanol for one min or wiping it using ethanol soaked cotton swab was found to be effective. The immature flower buds are perfectly and tightly packed within the bracts of the inflorescence. This may be the reason for contamination free cultures with this simple procedure. Endogenous bacterial contamination is very less in inflorescence meristem compared to shoot meristem, which favours selection of this explants for *in vitro* culture.

The presence of polyphenolics was the serious problem encountered in the establishment of IMF culture of Nendran cultivar. All the explants turned brown in 2 to 3 days of inoculation and decayed within a few days. Ascorbic acid was added to the MS medium at a concentration of 20 mg L^{-1} reduced the

blackening of the explants due to phenolic oxidation and subsequent decaying of the explants. Spreading of exudates and phenolics was considerably reduced when agar was replaced with gelrite (Plate 2).

Callus induction was observed on IMFs in 45 days after inoculation in different media compositions tried (Table1). The percentage of callus induction and the morphology of the calli varied in different treatments tried. The explants inoculated on MS medium supplemented with 2, 4-D (0.5 - 4 mg L^{-1}) and IAA (0.5 – 1 mg L^{-1}) turned brown within a week of inoculation (A1 to A16). However, after 4 weeks of inoculation explants exhibited a swelling and after another two weeks, callus initiation was observed (Plate 3). Among the treatments with combination of 2,4-D and IAA, treatment A9 yielded the highest percentage (45.80 per cent) of callus induction while A1 & A13 produced the least (8.30 per cent). Pale white, hard callus was formed in A1 to A10 and watery callus was formed in treatments A11 to A14. Higher concentration of 2,4-D, above 3.5 mg L^{-1}, did not produce any callus (Table 2).

In picloram (0.5-10 mg L^{-1}) supplemented medium (B1 to B11) IMFs became brown at the base within 3 days of inoculation, but showed a response of 15 per cent to 60 percent in 45 days by inducing yellow colored callus in treatments B4 to B11, with maximum induction in B8 (Plate 4).

In MS medium with BA (0.05- 0.5 mg L^{-1}) and picloram (0.5 - 2 mg L^{-1}), IMFs produced small to medium sized yellow, globular, friable callus with dense yellow cytoplasm in treatments C1, C2 and C3 [Plate 5(a)], whereas treatments C4- C10 produced pale white, globular callus with dense cytoplasm [Plate 5(b)] and treatments C11 and C12 [Plate 5(c)] developed yellow, watery callus. Among treatments with BA and picloram combinations in MS medium, C5 showed maximum response of 30 per cent. In the present study, among all the treatments tried for callus initiation, phenolic interference was less in BA and picloram combinations. The callus initiation occurred in 45-55 days of inoculation in this combination.

Plate 1. Immature male flowers inoculated in callus induction medium

Plate 2. Explants in medium solidified with agar (A) and gelrite (B)

Plate 2(A)

Plate 2(B)

Table 1. Response of banana immature male flower (IMFs) in various callus induction medium

Medium: MS (Full strength) + sucrose 30 g L^{-1}+ Ascorbic acid 20 mg L^{-1} + Gelrite 4.5 g L^{-1}

Sl no:	Composition of growth regulators (mg L^{-1})	Response
1	2,4-D -4, NAA-1, IAA- 0.9, Biotin-1 + Agar 5.6 gL^{-1}	-
2	2,4-D -4, NAA-1, IAA- 0.9, Biotin-1 + Gelrite 4.5gL^{-1}	-
3	2,4-D- 4, IAA- 1, NAA- 1, Biotin- 1, Glutamine- 100, ME- 100	-
4	2,4-D- 2, NAA- 0.5, IAA- 0.5	-
5	2,4-D- 2, NAA- 1, IAA- 0.5	-
6	2,4-D- 2, NAA- 0.5, IAA- 1	-
7	2,4-D- 2, NAA- 1, IAA- 1	-
8	BA- 5, NAA- 0.5	-
9	BA-6, NAA- 0.5	-
10	BA-7, NAA- 0.5	-
11	BA-8, NAA- 0.5	-
12	BA-9, NAA- 0.5	-
13	BA-10, NAA- 0.5	-
14	BA-5, NAA- 1	-
15	BA-6, NAA- 1	-
16	BA-7, NAA- 1	-
17	BA-8, NAA- 1	-
18	BA-9, NAA- 1	-
19	BA-10, NAA- 1	-
20	2,4-D- 1, NAA- 1, Biotin- 1, Glutamine- 100, ME- 100,	-
21	2,4-D- 0.5, NAA- 1, Biotin- 1, Glutamine- 100, ME- 100	-
22	2,4-D- 0.5, NAA- 0.5, Biotin- 1, Glutamine- 100, ME- 100	-
23	2,4-D- 1, NAA- 0.5, Biotin- 1, Glutamine- 100, ME- 100	-
24	BA- 1, IAA- 0.5, Casein hydrolysate- 500	-
25	BA- 2, IAA- 0.5, Casein hydrolysate- 500	-
26	BA- 0.5, IAA- 0.5, Casein hydrolysate- 500	-
27	BA- 1.5, IAA- 0.5, Casein hydrolysate- 500	-
28	BA- 2, IAA- 0.5, Casein hydrolysate- 500	-
29	BA- 2.5, IAA- 0.5, Casein hydrolysate- 500	-
30	BA 3.0 + Kn 2.0	-
31	BA 3.0 + Kn 1.0	-
32	BA 3.0 + Kn 0.5 + IAA 1.0	-
33	BA 4.0 + Kn 2.0 + NAA 0.25	-
34	2,4-D- 0.5, IAA- 0.5	+
35	2,4-D- 0.5, IAA- 1	+
36	2,4-D- 1, IAA- 0.5	+

37	2,4-D- 1, IAA- 1	+
38	2,4-D- 1.5, IAA- 0.5	+
39	2,4-D- 1.5, IAA- 1	+
40	2,4-D- 2, IAA- 0.5	+
41	2,4-D- 2, IAA- 1	+
42	2,4-D- 2.5, IAA- 0.5	+
43	2,4-D- 2.5, IAA- 1	+
44	2,4-D- 3, IAA- 0.5	+
45	2,4-D- 3, IAA- 1	+
46	2,4-D- 3.5, IAA- 0.5	+
47	2,4-D- 3.5, IAA- 1	+
48	2,4-D- 4, IAA- 0.5	-
49	2,4-D- 4, IAA- 1	-
50	Picloram- 0.5	-
51	Picloram- 1	-
52	Picloram- 2	-
53	Picloram- 3	+
54	Picloram- 4	+
55	Picloram- 5	+
56	Picloram- 6	+
57	Picloram- 7	+
58	Picloram- 8	+
59	Picloram- 9	+
60	Picloram- 10	+
61	BA- 0.05, Picloram- 0.5	+
62	BA- 0.05, Picloram- 1	+
63	BA- 0.05, Picloram- 2	+
64	BA- 0.1, Picloram- 0.5	+
65	BA- 0.1, Picloram- 1	+
66	BA- 0.1, Picloram- 2	+
67	BA- 0.2, Picloram- 0.5	+
68	BA- 0.2, Picloram- 1	+
69	BA- 0.2, Picloram- 2	+
70	BA- 0.5, Picloram- 0.5	+
71	BA- 0.5, Picloram- 1	+
72	BA- 0.5, Picloram- 2	+

- \- No response or no callus induction
- \+ Callus induction

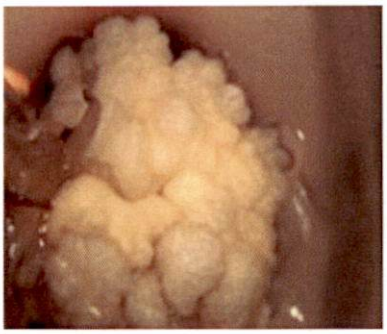
Table 2. Effect of growth hormones on embryogenic callus induction in immature male flower (IMFs) explant of *Musa* spp. cv. Nendran.

Treatments	2,4-D (mg L^{-1})	BA (mg L^{-1})	IAA (mg L^{-1})	Picloram (mg L^{-1})	Morphology of callus	Time taken for callus initiation	Explant Response %
A_1	0.5	-	0.5	-	White		8.3
A_2	0.5	-	1	-	Pale white hard		16.6
A_3	1	-	0.5	-	Pale white hard		25.0
A_4	1	-	1	-	Pale white hard		41.6
A_5	1.5	-	0.5	-	Pale white hard		33.3
A_6	1.5	-	1	-	Pale white hard		29.1
A_7	2	-	0.5	-	Pale white hard	45 days	33.3
A_8	2	-	1	-	Pale white hard		33.3
A_9	2.5	-	0.5	-	Pale white hard		45.8
A_{10}	2.5	-	1	-	Pale white hard		25.0
A_{11}	3	-	0.5	-	Pale white watery		20.8
A_{12}	3	-	1	-	Pale white watery		
A_{13}	3.5	-	0.5	-	Pale white watery		8.3
A_{14}	3.5	-	1	-	Pale white watery		25.0
A_{15}	4	-	0.5	-	-		
A_{16}	4	-	1	-	-		
B_1	-	-	-	0.5	-		
B_2	-	-	-	1	-		
B_3	-	-	-	2	-		
B_4	-	-	-	3	Yellow watery		15.0
B_5	-	-	-	4	Yellow watery	45 days	35.0
B_6	-	-	-	5	Yellow watery		35.0
B_7	-	-	-	6	Yellow watery		50.0
B_8	-	-	-	7	Yellow watery		60.0
B_9	-	-	-	8	Yellow watery		40.0
B_{10}	-	-	-	9	Yellow watery		30.0
B_{11}	-	-	-	10	Yellow watery		40.0
C_1	-	0.05	-	0.5	Yellow globular, friable		8.3
C_2	-	0.05	-	1	Yellow globular, friable		20.8
C_3	-	0.05	-	2	Yellow globular, friable		16.6
C_4	-	0.1	-	0.5	Pale white, globular, friable	45-55 days	16.6
C_5	-	0.1	-	1	Pale white globular, friable		30.0
C_6	-	0.1	-	2	Pale white globular, friable		25.0
C_7	-	0.2	-	0.5	Pale white globular, friable		8.3
C_8	-	0.2	-	1	Pale white globular, friable		12.5
C_9	-	0.2	-	2	Pale white globular, friable		8.3

C_{10}	-	0.5	-	0.5	Pale white globular, friable		16.6
C_{11}	-	0.5	-	1	Tiny yellow globular, friable		12.5
C_{12}	-	0.5	-	2	Tiny yellow globular, friable		8.3

Plate 3. Morphology of calli developed in MS medium supplemented with 2,4-D and IAA

White hard callus (A1 - A10)



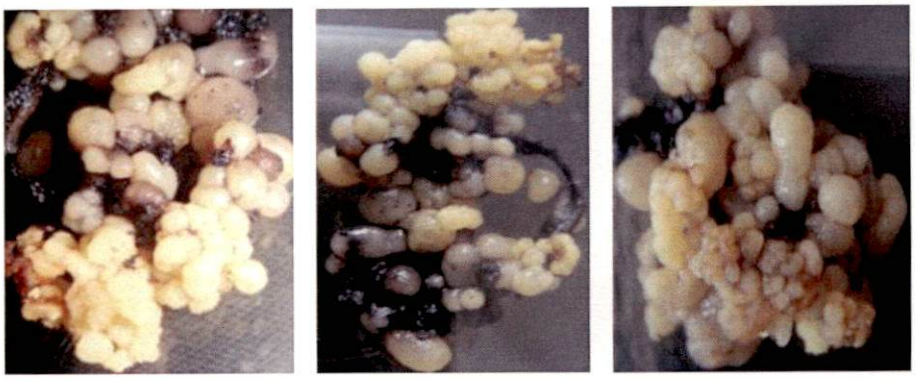
Pale white watery callus (A11 – A14)

Plate 4. Morphology of calli developed in MS medium supplemented with Piclorm

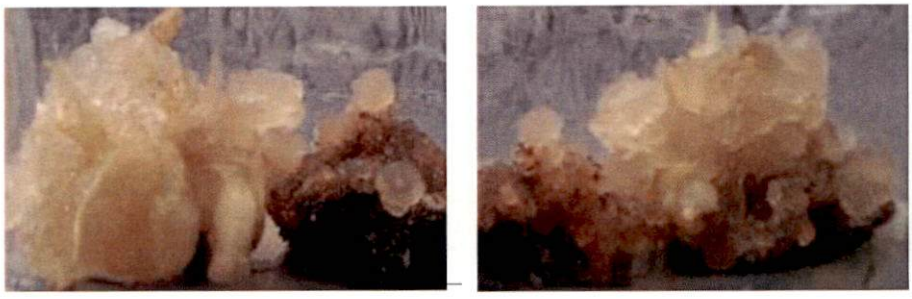
Yellowish watery callus (B4 – B11)

Plate 5. Morphology of calli formed in MS medium supplemented with BA and Picloram

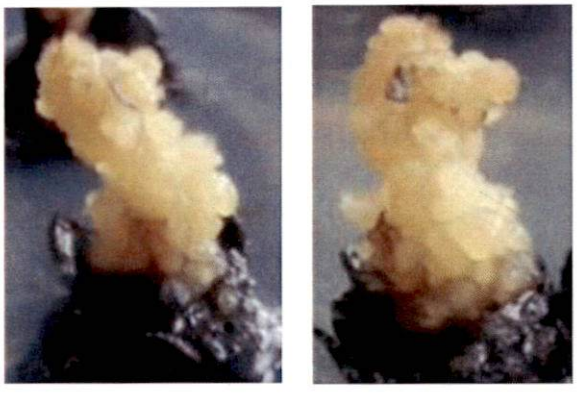
5 (a). Yellow globular friable callus (C1-C3)



5 (b). Pale white globular friable callus (C4 – C10)



5 (c). Yellow callus C11 & C12)



4.1.2 Induction of somatic embryos

Different media were tried for induction of somatic embryos (Table 3). Embryo formation was observed only in semisolid MS medium supplemented with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} and 0.3 per cent gelrite. Calli induced in treatments C8, C9 and C10 produced somatic embryos in this medium in 30 days of inoculation. They showed glassy elongated monocot embryo structures (Plate 6). Callus from C9 gave 60 per cent embryo induction, which was the highest response in this study. These monocot embryos seemed elongated with a glassy appearance. Even though callus induction was high in treatments A and B, they failed to produce embryos. In almost all the reported protocols for callus mediated somatic embryogenesis, an intermediary liquid medium is involved. The maintenance of suspension cultures is a little sophisticated process, even though the rate of multiplication is high. In this study, we could replace the same with a simple semisolid media.

4.1.3 Germination of embryos

The glassy elongated monocot embryos developed in semisolid MS with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} failed to germinate in the same medium. But they showed germination of 60-80 per cent (Table 4) in 3 weeks of inoculation in half strength semisolid MS supplemented with the same combination of growth regulators kept in the dark (Plate 7). During the incubation in the dark for the first 3 weeks, the shoots developed were pale white to pale green in colour. 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 [Plate 8(a) & (b)]. The plantlets after primary hardening in coir pith compost for a month and secondary hardening for one month in soil and cowdung (1:1) mixture in a mist chamber showed 100 per cent survival rate (Plate 9).

Table 3. Embryogenic response of the calli initiated in different treatments in semisolid MS medium with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1}

Treatment	No: of days	Percentage embryogenesis
A_1	-	-
A_2	-	-
A_3	-	-
A_4	-	-
A_5	-	-
A_6	-	-
A_7	-	-
A_8	-	-
A_9	-	-
A_{10}	-	-
A_{11}	-	-
A_{12}	-	-
A_{13}	-	-
A_{14}	-	-
A_{15}	-	-
A_{16}	-	-
B_1	-	-
B_2	-	-
B_3	-	-
B_4	-	-
B_5	-	-
B_6	-	-
B_7	-	-
B_8	-	-
B_9	-	-
B_{10}	-	-
B_{11}	-	-
C_1	-	-
C_2	-	-
C_3	-	-
C_4	-	-
C_5	-	-
C_6	-	-
C_7	-	-
C_8	30-35	33.33
C_9	30-35	60
C_{10}	30-35	50
C_{11}	-	-
C_{12}	-	-

Plate 6. Glassy elongated embryos developed from calli in MS medium (semisolid) + BA 2 mg L^{-1}, IAA 0.5 mg L^{-1}

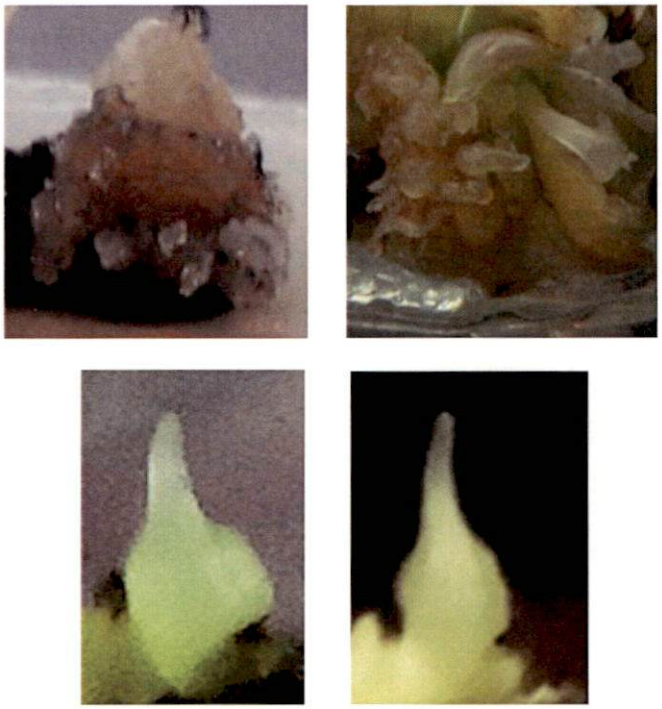


Plate 7. Maturation and germination of embryos in Half MS (semisolid) + BA 2 mg L^{-1}, IAA 0.5 mg L^{-1}

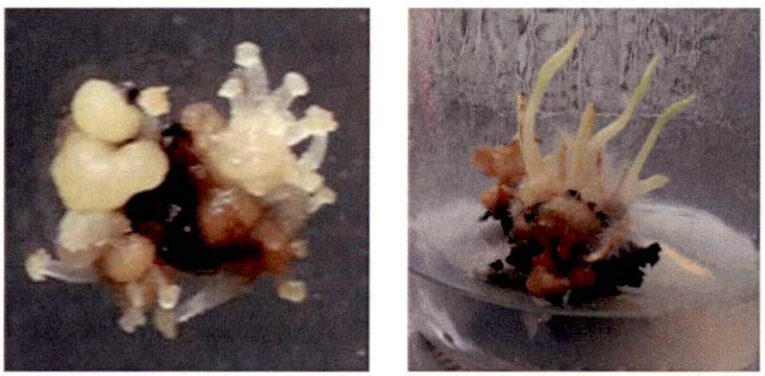


Table 4. Germination of the plated embryos

Treatment	No: of embryo clusters plated	No: of embryo clusters germinated	Percentage germination
C_8	10	6	60
C_9	18	13	72.22
C_{10}	15	11	80

Plate 8 (a). Development of germinated plantletsin MS + BA 2 mg L^{-1}, NAA 1 mg L^{-1}

Plate 8 (b). Development of the plantlet after subculturing in MS + BA 2 mgL^{-1}, NAA 1 mg L^{-1} for 30 days

4.2 ISOLATION OF *REPLICASE* GENE OF BBrMV

4.2.1 RNA isolation

Total RNA was isolated from BBrMV infected banana according to the protocol reported by Salzman *et al.* (1999). 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.

RNA isolated was quantified with UV- visible spectrophotometer. Absorbance at 260 nm (A_{260}) was recorded as 1.262 and absorbance at 280 nm (A_{280}) was recorded as 0.653. The concentration of RNA was calculated as 30.288 µg/µl and A_{260}/A_{280} value was 1.93, indicating good quality of RNA.

4.2.2 cDNA synthesis

cDNA synthesis using AMV RT-PCR kit was confirmed by doing PCR with *actin* (house-keeping gene) 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 size of the *actin* gene (Plate 11). The result confirmed the successful conversion of mRNA to cDNA.

4.2.3 Isolation of partial replicase gene of BBrMV

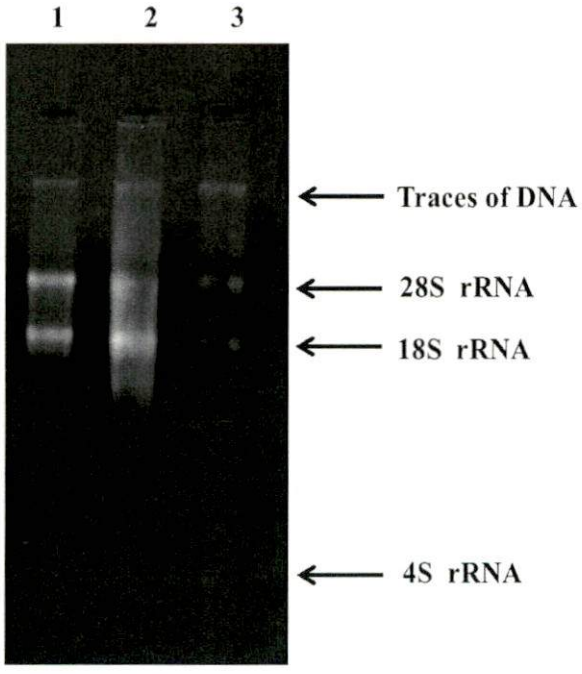
PCR with the 2 sets of BBrMV replicase gene specific primers (BBrMV-A and BBrMv- B) resulted in the amplification of two fragments of size 421bp and 733bp respectively (Plate 12).

4.2.4 Sequence of BBrMV partial coding sequence of replicase gene

The PCR products of size 421 bp and 733 bp obtained by amplification with the primers BBrMV- A and BBrMV – B respectively were sequenced and the sequence information (Appendix III) of partial coding regions of replicase gene of BBrMV, Vellayani isolate were deposited in GenBank, National Centre for Biotechnology Information (NCBI). The accession numbers are GenBank:

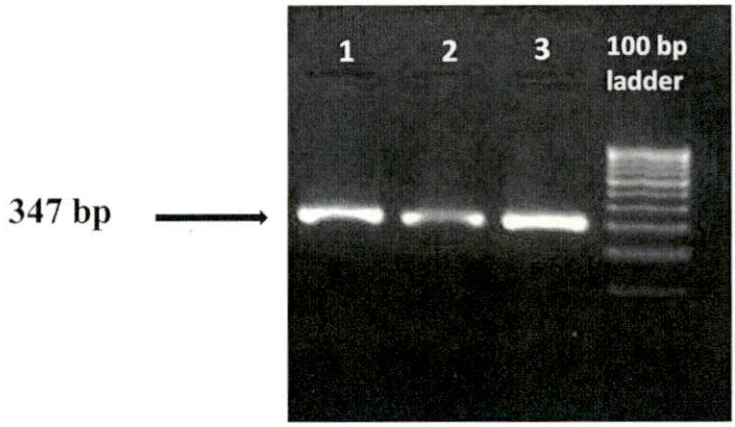
Plate 9. Plantlets kept for hardening

Plate 10. RNA isolated from BBrMV infected Nendran plant



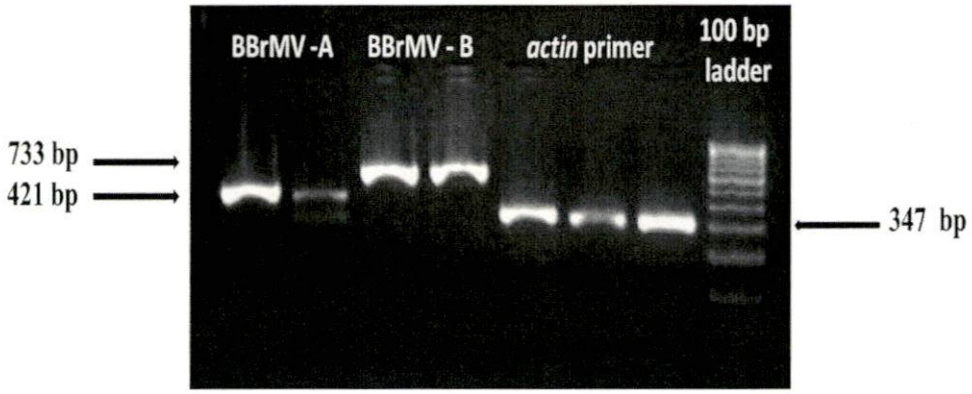
Lane 1, 2 and 3 – Three replications of RNA isolation

Plate 11. cDNAamplified with actin specific primer



Lane 1, 2, and 3 – cDNA prepared from three number of RNA isolates

Plate 12. Partial replicase gene fragments obtained by amplifying cDNA with gene specific primers designed



KM357545.1 and KM357543.1. Analysis of the sequences obtained was done using Basic Local Alignment Search Tool (BLAST) offered by NCBI. The amplified partial coding regions were towards the 5' region of the BBrMV replicase gene. The BLAST result (Appendix IV) showed 96 per cent similarity with BBrMV TRY (Trichy) isolate (Acc No: HM131454.1), complete genome, 94 per cent similarity with BBrMVPhilipine isolate (Acc No: DQ851496.1) and 95 per cent similarity with BBrMV isolate P1 polyprotein mRNA, partial cds (Acc No: AF071591.1).

4.3 ihp RNA VECTOR CONSTRUCTION

4.3.1 miRNA target prediction

Since the sequence towards 5' end of the targeted gene is mostly preferred for ihpRNA construct, the fragment of size 421 bp isolated was used for further studies. RNAi design tool of IDT predicted miRNA targets in the 421 bp coding region of the replicase gene amplified with the BBrMV – A set of primers. Two target regions were identified in the portion isolated and sequenced (Appendix V). The parameters deciding the target regions is detailed in (Appendix VI). Primers for the amplification of the sense and antisense fragments were designed to include maximum miRNA target regions in the amplicons.

4.3.2 Restriction mapping

Restriction map was obtained for the 421 bp long sequence amplified with BBrMV –A set of primers using the tool 'Restriction map' in Sequence Manipulation Suite' available at www.bioinformatics.org. A textual map showing the positions of restriction endonuclease recognition sites was obtained (Appendix VII). According to this result it was confirmed that the 421 bp fragment, selected for the preparation of sense and antisense fragments, did not possess recognition sites for *Bam*HI, *Pac*I, *Kpn*I, *Spe*I or *Not*I restriction enzymes which were used for preparing ihpRNA cassette. It was made clear that these restriction enzymes would make only a single cut during the ihpRNA cassette preparation in the

primary vector pSTARLING and nowhere within the sense or antisense fragments.

Primers for the preparation of sense and antisense fragments were designed based on this information by including maximum number of miRNA targets and excluding selected restriction enzyme (*Bam*HI, *Pac*I, *Kpn*I, *Spe*I and *Not*I) recognition sites.

4.3.3 Viral silencing suppressor prediction

The 421 bp fragment amplied with BBrMV-A set of primers was subjected to viral silencing suppressor prediction using the tool VSupPred. The prediction score obtained was 0.725. The score mentioned that the fragment is a NonViral Silencing supressor Region (NVSR) with a confidence score of 0.725. (The maximum confidence score of the algorithm is +1 and the minimum score is -1).

4.3.4 Preparation of sense and antisense fragments for ihpRNA vector construction

Primers for the preparation of sense fragment was designed so as to get a product size of 400 bp with *Bam*HI and *Pac*I recognition sequences anchoring at the 5'ends of the forward and reverse primers respectively. At the same time primers for the preparation of antisense fragment were designed with *Spe*I and *Kpn*I recognition sequences anchoring at the 5' end of the forward and reverse primers respectively. Colony PCR was performed and the electrophoresis of the PCR products showed an amplicon of size 400 bp which was of expected size (Plate 13).

4.3.5 Elution of the sense and antisense fragments and quantification

The sense and antisense fragments were extracted using Nucleospin® Gel and PCR clean-up Kit following the manufacturers' protocol. The eluted PCR product was quantified with UV- visible spectrophotometer.

Spectrophotometric observations of the eluted sense and antisense fragments.

PCR product	A_{260}	A_{280}	A_{260} / A_{280}	Concentration (µg/µl)
Sense strand	0.188	0.105	1.791	4.7
Antisense strand	0.169	0.098	1.724	4.22

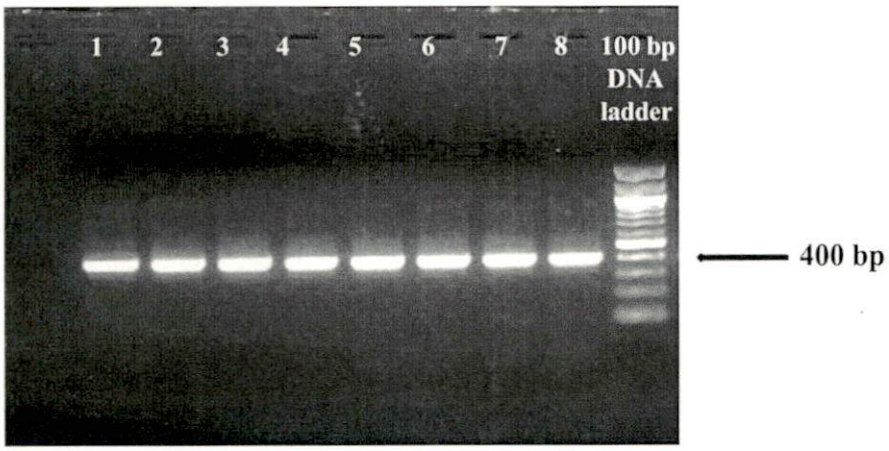
4.3.6 Cloning of the sense and antisense amplicons in pTZ57R/T

The sense and antisense strands were ligated to the linear TA cloning vector pTZ57R/T as separate reactions. After ligation reaction and transformation to competent DH5α, the positive clones were selected on LB agar plates with Ampicillin 100 mg L^{-1}, 40µl (20 mg/ml) X-GAL and 40µl 100mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) by blue white screening (Plate14). Transformed (positive) white colonies were selected and the master plate was made for further use and stored at 4°C.

4.3.7 Confirmation of integration and orientation of the inserted fragments

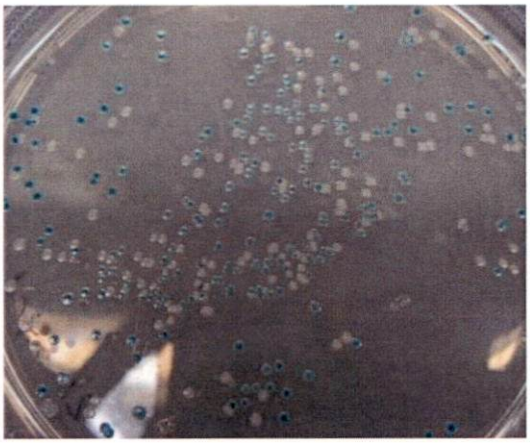
Plasmid was isolated from positive (white) colonies. PCR was carried out to confirm the integration of sense and antisense fragments. As pTZ57R/T, also possesses *Bam*HI site, the orientation of integration was also checked. Hence PCR with M13 forward primer and reverse primers of sense and antisense strands were used respectively. The PCR product when separated electrophoretically showed expected band size of 450 bp (Plate 15). The band was of expected size, confirming the integration as well as proper orientation. Each colony from sense and antisense clones were selected and quantified. Concentration of plasmid with sense strand was quantified to be 3.5 µg/µl and that with antisense strand to be 3.36 µg μl^{-1}.

Plate 13. Amplified products of sense and antisense strands



Lane 1-4 - Sense strand
Lane 5-8 - Antisense strand
Lane 9 - 100 bp DNA ladder

Plate 14. Blue-white screeningof TA cloning vector pTZ57R/T containing sense and antisense strands



White colonies are the transformed DH5α cells carrying the recombinant pTZ57R/Tvector

4.3.8 Release of sense and antisense fragments from pTZ57R/T

Restriction digestion was carried out to release the sense and antisense fragments from pTZ57R/T. Expected strand of 400 bp size was released (Plate 16) from the vector backbone. The sense and antisense strands were eluted out separately and quantified. The concentration of the sense and antisense extract was found to be 2.94 $\mu g\ \mu l^{-1}$ and 2.89 $\mu g\ \mu l^{-1}$ respectively.

4.3.9 Insertion of sense and antisense fragments to the primary vector

pSTARLING was subjected to double digestion using BamHI and PacI. The linearized product was purified from the components of restriction reaction by electrophoretic separation and gel extraction. The linearized pSTARLING extracted from agarose (1 per cent) gel was quantified and the concentration was calculated to be 3.18 µg/µl. Linear pSTARLING with *Bam*HI and *Pac*I sticky ends was subjected to ligation reaction with the sense strand having *Bam*HI and *Pac*I sticky ends (released from pTZ57R/T). The ligated vector was transformed to competent DH5α. Colonies were selected randomly and grown in LB broth to isolate plasmid. The plasmids were screened for the presence of sense strand by PCR with corresponding primers of sense strand and obtained a band of size 400 bp, which was the expected size of the sense strand (Plate 17). A positive colony was selected and the plasmid was quantified to have a concentration of 2.89 $\mu g\ \mu l^{-1}$.

pSTARLING vector carrying the sense insert was subjected to double digestion with SpeI and *Kpn*I and was purified by gel extraction procedure (3.16 µg/µl). The antisense strands both having *Spe*I and *Kpn*I sticky ends were ligated to the linearized pSTARLING vector. The ligated product was transformed to competent DH5α and grown in LB broth with ampicillin 100 mg L^{-1} (Plate 18). Transformed colonies were randomly selected. The plasmids isolated were separated electrophoretically (agarose 1 per cent) and the plasmid corresponding to the band which showed increase in size was selected.

Plate 15. Products of colony PCR with M13 Forward primer and reverse primers of sense and antisense strands

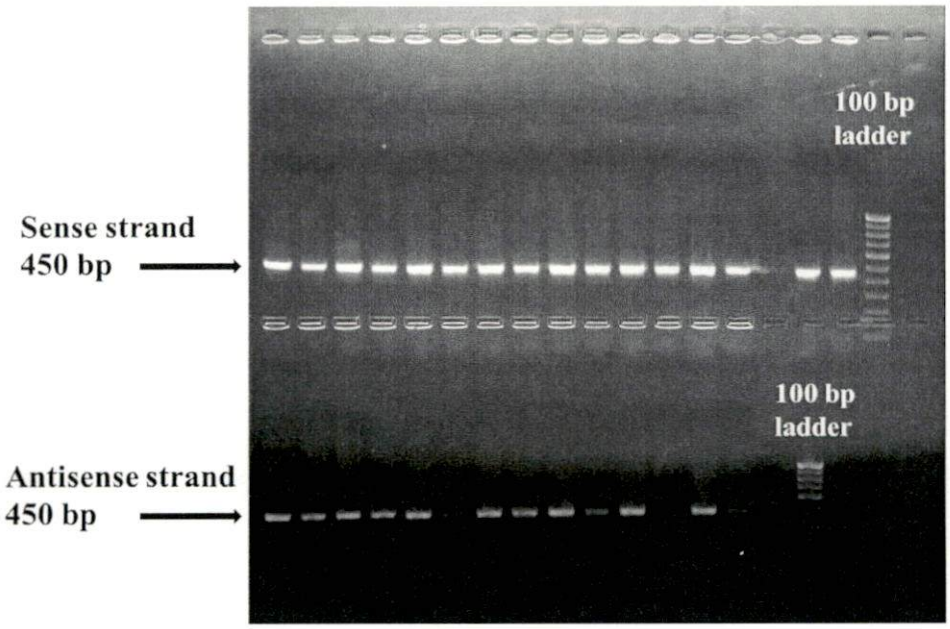
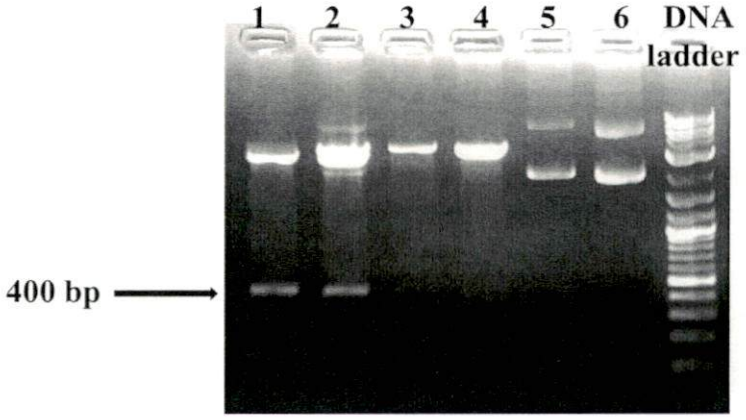
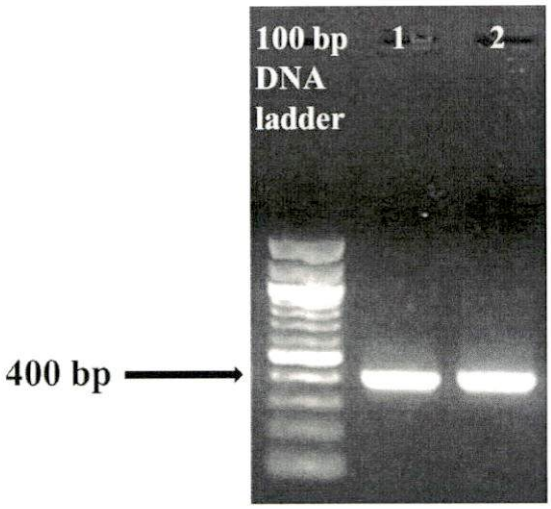


Plate 16. Sense and antisense fragments extracted from pTZ57R/T by Restriction digestion



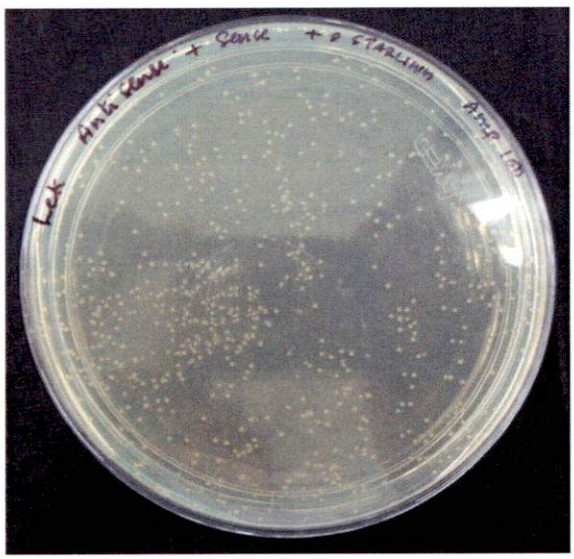
Lane 1.	-	pTZ257R/T with sense strand
Lane 2.	-	pTZ257R/T with antisense strand
Lane 3 & 4.	-	pTZ257R/T without insert, linearized
Lane 5 & 6.	-	pTZ257R/T with insert uncut
Lane 7.	-	100-5 kb DNA ladder

Plate 17. PCR analysis to confirm the ligation of the sense strand in the pSTARLING vector



Lane 1 - colony 1
Lane 2 - colony 2

Plate 18. DH5αcells carrying pSTARLING vector with the ihpRNA cassetteon LB plate containing ampicillin 100 mg L^{-1}



4.3.10 Confirmation of the integration by restriction digestion reaction

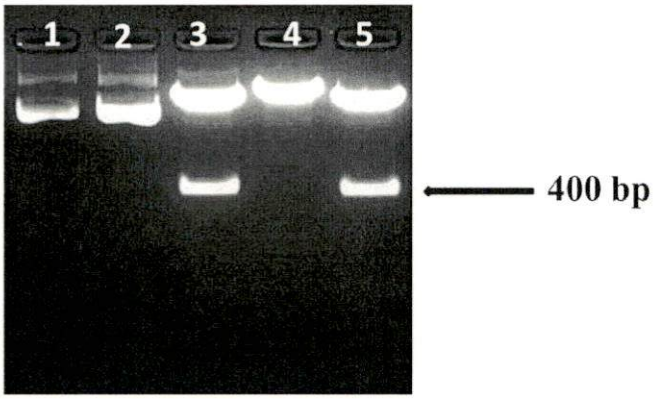
pSTARLING (3.39 μg μl^{-1}) vector harboring the sense and antisense fragments, isolated from transformed clone selected from the previous step, was subjected to restriction digestion for confirmation. The restriction digestion released sense strand fragment (*BamHI* and *Pac*I) and antisense (*Spe*I and *Kpn*I) fragments from the pSTARLING backbone (Plate 19). The products of the restriction reaction could be compared with the control reactions. Second set of restriction reaction released out sense-intron-antisense fragment as a whole from the pSTARLING backbone by digesting with *Bam*HI and *Kpn*I. The release was of size 1300 bp (Plate 20), the expected size of the cassette which included 400 bp sense fragment + 500bp *cre* intron + 400 bp antisense fragment. This result confirmed the successful construction of ihpRNA cassette in the primary vector, pSTARLING.

4.4 TRANSFER OF ihpRNA CONSTRUCT TO THE BINARY VECTOR

pSTARLING was subjected to restriction reaction with *Not*I restriction enzyme and this resulted in digestion of the plasmid into two fragments of size 3.9 kb and 2.8 kb (Plate 21). Considering the size of the inserts and other components within the NotI site, it was confirmed that the fragment of size 3.9 kb harbored the ihpRNA construct. This fragment eluted out from the agarose gel showed a concentration of 4.2 μg μl^{-1}.

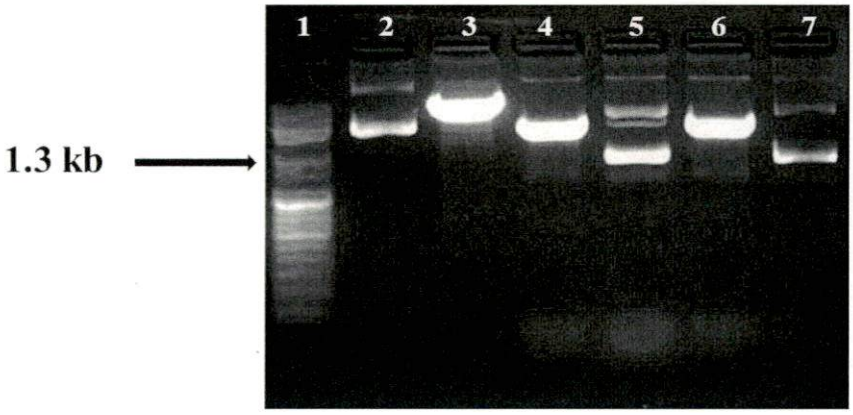
The 3.9 kb fragment was ligated to the linearized (*Not*I) pART27. The recombinant vector was transformed to competent DH5α and plated on to LB plate with Spectinomycin 100 mg L^{-1}, X-GAL 40 μl (20 mg ml^{-1}) and 40 μl 100 mM (IPTG) for blue white screening (Plate 22). A restriction reaction was carried out to confirm the integration of the *Not*I fragment. The agarose gel electrophoresis showed (Plate 23 & 24) bands of sizes 400 bp which was the expected size of sense and antisense fragments. Band of size 900 bp represented sense strand fragment with intron and antisense strand with intron. Amplicon of

Plate 19. pSTARLINGdigested with restriction enzymes showing the integration of sense and antisense fragments



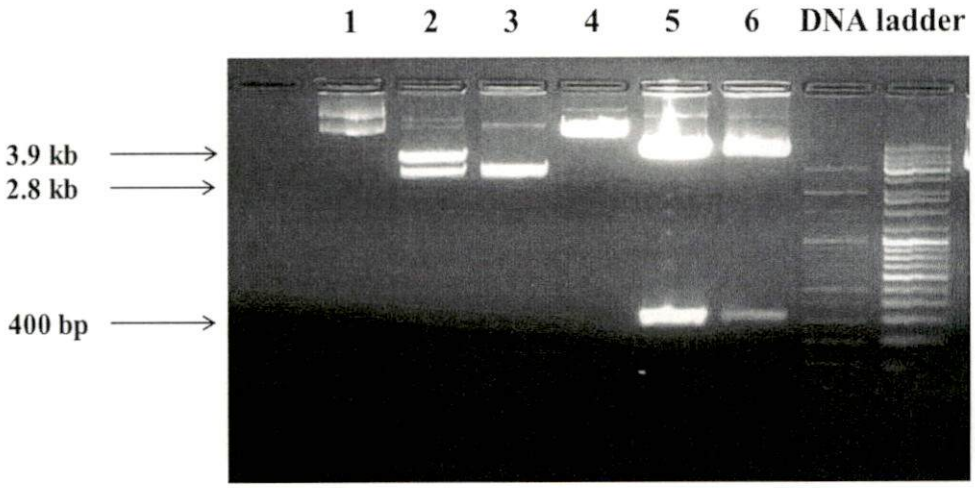
Lane 1 & 2 - pSTARLING without inserts
Lane 3 - pSTARLING – sense strand
Lane 4 - pSTARLING + sense and antisense strands
Lane 5 - pSTARLING – antisense strand

Plate 20. pSTARLING vector digested with *Bam*HI and *Kpn*I showing the release of ihpRNA cassette



Lane 1 - DNA ladder (100 – 5kb)
Lane 2, 4 & 6 - pSTARLING
Lane 3 - pSTARLING + Sense strand + antisense strand
Lane 5 & 7 - pSTARLING+Sense + *cre*intron + antisense strand)

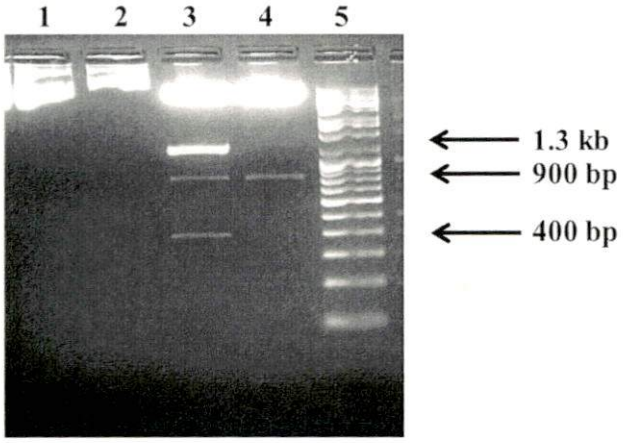
Plate 21. Digestion reaction to separate out *Not*I fragment (ihpRNA cassette) from pSTARLING



Lane 1	- pART27 uncut
Lane 2 & 3	- pSTARLING cut with *Not*I
Lane 4	- pART27 linearized
Lane 5	- pSTARLING with sense strand released
Lane 6	- pSTARLING with antisense strand released
Lane 7	- DNA ladder 100 – 3 kb
Lane 8	- DNA ladder 100 bp– 5 kb

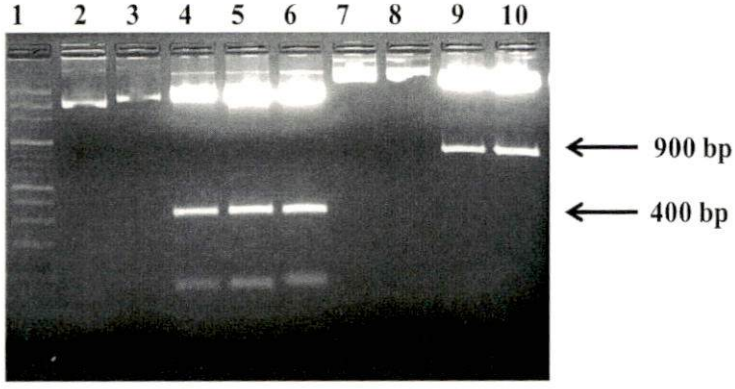
Plate 22. Blue- white screening of DH5αcells transformed with pART27 containing ihpRNA cassette on LB medium with Spectinomycin100mgL^{-1}

Plate 23. Restriction digestion showing the presence of inserts in the binary vector pART27



Lane 1 & 2	- pART27 uncut
Lane 3	- pART27cut with *Kpn I,Bam HI&Pac I*
Lane 4	- pART27 cut with *SpeI* and *KpnI*
Lane 5	- 100 – 5 kb DNA ladder

Plate 24. Restriction digestion showing the presence of inserts in the binary vector pART27



Lane 1	- 100bp DNA ladder
Lane 2 & 3	- pART27
Lane 4	- pART27 – sense strand
Lane 5	- pART27– antisense strand
Lane 6	- pART27– intron
Lane 7 & 8	- pART27+ Inserts
Lane 9	- pART27– (sense + intron)
Lane 10	- pART27 – (antisense + intron)

size 1.3 kb band corresponds to the fragment containing sense-intron-antisense sequences together.

4.5 TRANSFER OF THE BINARY VECTOR TO *A. TUMEFACIENS*

The binary vector containing the ihpRNA cassette was transferred to *A. tumefaciens* by freeze-thaw method. The transformed cells were plated on to LB plates containing 20 mg L^{-1} rifampicin and 100 mg L^{-1} kanamycin. After incubation at 28°C for 24 h, 38 colonies appeared on the plate (Plate 25). Transformation efficiency was found to be 7.6 X 10^3 transformant CFU μg^{-1}.

4.5.1 Confirmation of transformation

Transformation of the binary vector to *A. tumefaciens* was confirmed by PCR analysis. PCR analysis of the plasmid DNA from *A. tumefaciens* with gene-specific primers for nptII and *cre* intron was carried out. PCR with *nptII* primers yielded product of size 475 bp (Plate 26) and PCR with *cre* intron specific primer resulted in a product of 450 bp, which were of the expected size. PCR was conducted to amplify sense-intron segment and antisense-intron segment. This resulted in a band showing an expected product size of approximately 830 bp (Plate 27).

4.6 EVALUATION OF EMBRYOGENIC CALLUS OF BANANA FOR SENSITIVITY TO ANTIBIOTICS

The sensitivity of banana embryogenic callus to different doses of antibiotics in MS medium was tested.

4.6.1 Kanamycin

The sensitivity of the callus to different doses of kanamycin (0-300 mg L^{-1}) is shown in Plate 28 and Table 5. In the presence on kanamycin 25 mg L^{-1}, the tissues remained pale white up to four weeks and became yellow after six weeks. The percentage survival of callus in kanamycin 25 mg L^{-1}upto six weeks was 68.75.

Plate 25. *Agrobacterium tumefaciens* (LBA 4404) colonies transformed with binary vector containing ihpRNA construct

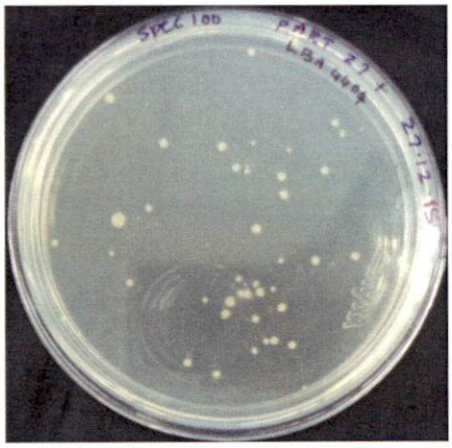
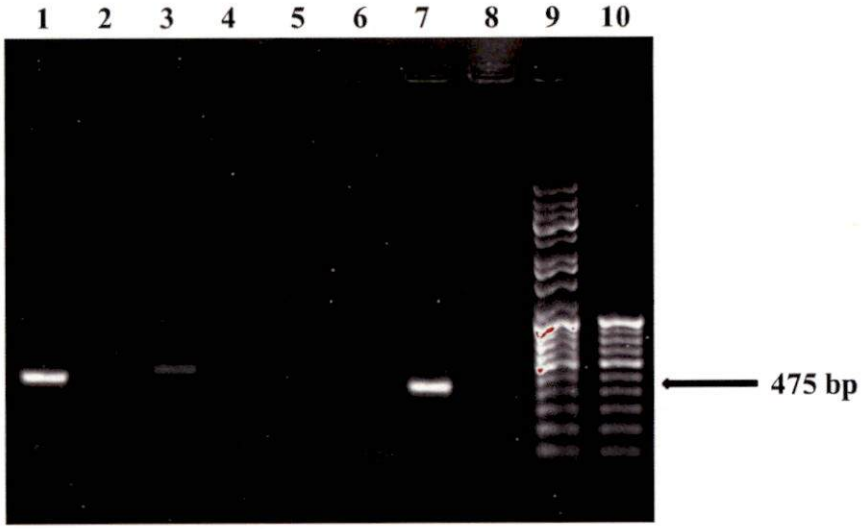
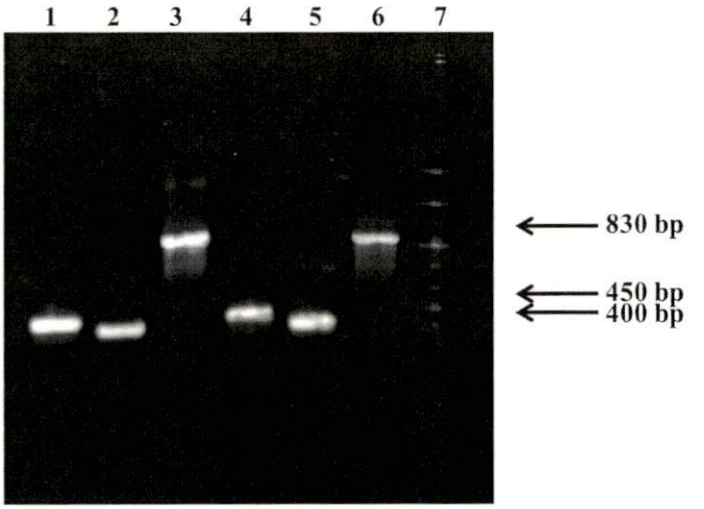


Plate 26. PCR analysis of plasmid isolated from *Agrobacterium* using *nptII* gene specific primers



Lane 1-8	- randomly selected transformed *Agrobacterium* colonies
Lane 9	- 100 – 5 kb DNA ladder
Lane 10	- 100 bp DNA ladder

Plate 27. PCR analysis of the binary vector from transformed *Agrobacterium* cells with ihpRNA specific primers



Lane 1 - cre intron amplified
Lane 2 - sense strand amplified
Lane 3 - cre intron + sense strand amplified
Lane 4 - cre intron amplified
Lane 5 - antisense strand amplified
Lane 6 - cre intron + antisense strand amplified
Lane 7 - 100 bp ladder

after six weeks. Only 37.5 per cent of the callus survived by the sixth week in this concentration.

The tissues were white up to two weeks, became yellow after three weeks and started browning after four weeks in cefotaxime 50 and 75 mg L^{-1}. The tissues turned brown in six weeks. The percentage survival of callus at the end of the sixth week was 25 and 12.5 respectively.

The tissues were white up to seven ±2 days, became yellow after two weeks. The callus turned brown and dry after four weeks in medium supplemented with cefotaxime 100 mg L^{-1}. The percentage survival of callus on sixth week was zero.

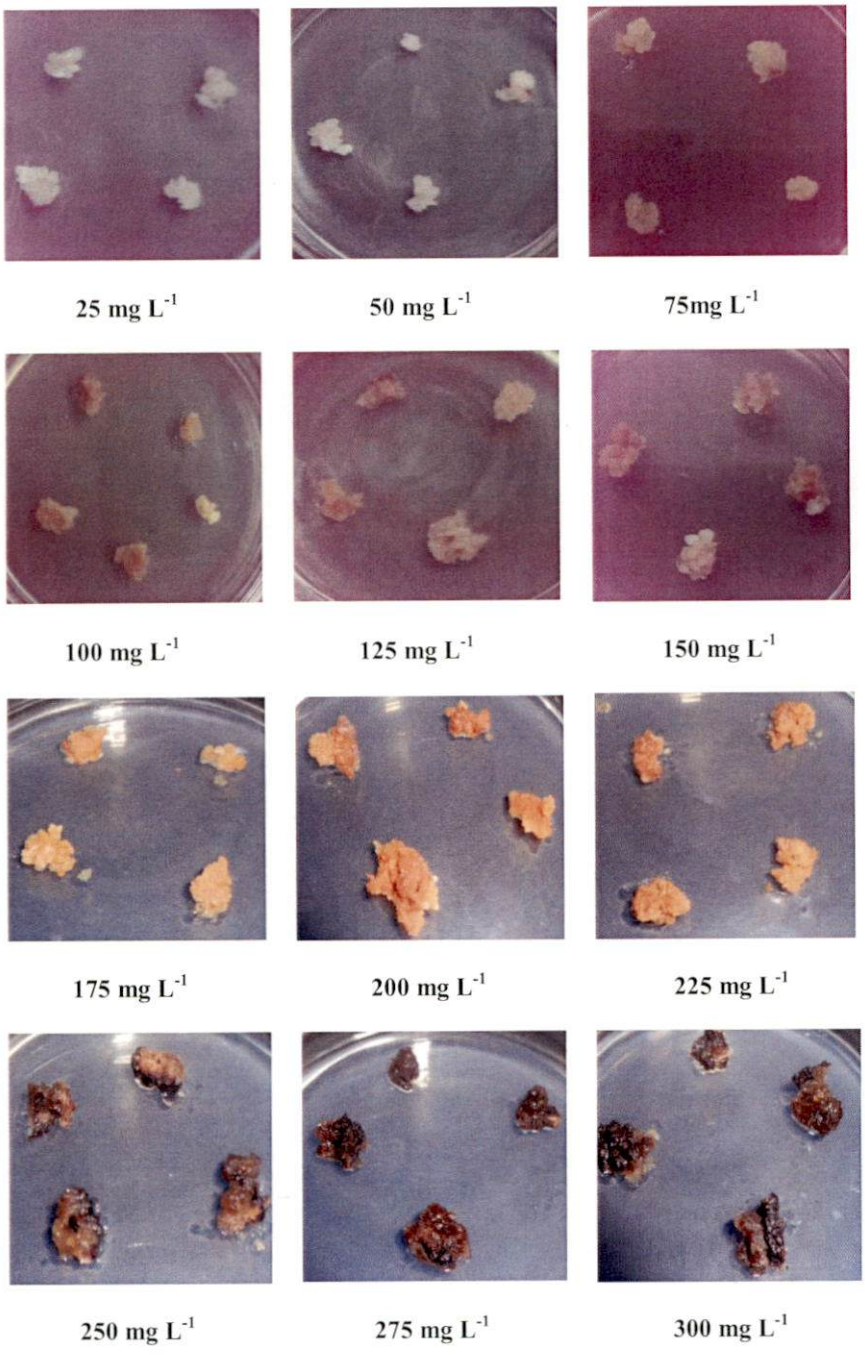
Cefotaxime was found to be toxic to tissues at 50 mg L^{-1} (Figure 2). Hence, for the elimination of *Agrobacterium* after co-cultivation, cefotaxime was used at a concentration of 25 mg L^{-1}.

Table 5. Sensitivity of banana embryogenic callus to different doses of Kanamycin

Sl No.	Kanamycin (mg L^{-1})	Sensitivity						Survival (%) after 6 weeks
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
1	0	++++	++++	++++	++++	++++	++++	100
2	25	++++	++++	++++	++++	++++	+++	68.75
3	50	++++	++++	+++	+++	+++	++	43.75
4	75	++++	++++	+++	+++	+++	++	31.25
.5	100	++++	++++	+++	+++	++	+	12.5
6	125	++++	+++	++	++	++	+	0
7	150	++++	+++	++	++	++	+	0
8	175	++++	+++	++	++	+	+	0
9	200	++++	+++	++	+	+	+	0
10	225	++++	+++	++	+	+	+	0
11	250	++++	+++	++	+	+	+	0
12	275	++++	++	+	+	+	+	0
13	300	++++	++	+	+	+	+	0

++++ Pale whitecoloured callus
+++ Yellowing of the callus
++ Tissue started browning
+ Tissue turning brown and dead

Plate 28. Response of banana embryogeniccalli to different doses of Kanamycin



25 mg L^{-1} 50 mg L^{-1} 75mg L^{-1}

100 mg L^{-1} 125 mg L^{-1} 150 mg L^{-1}

175 mg L^{-1} 200 mg L^{-1} 225 mg L^{-1}

250 mg L^{-1} 275 mg L^{-1} 300 mg L^{-1}

Fig. 1 Sensitivity of banana embryogenic callus to different concentrations of kanamycin

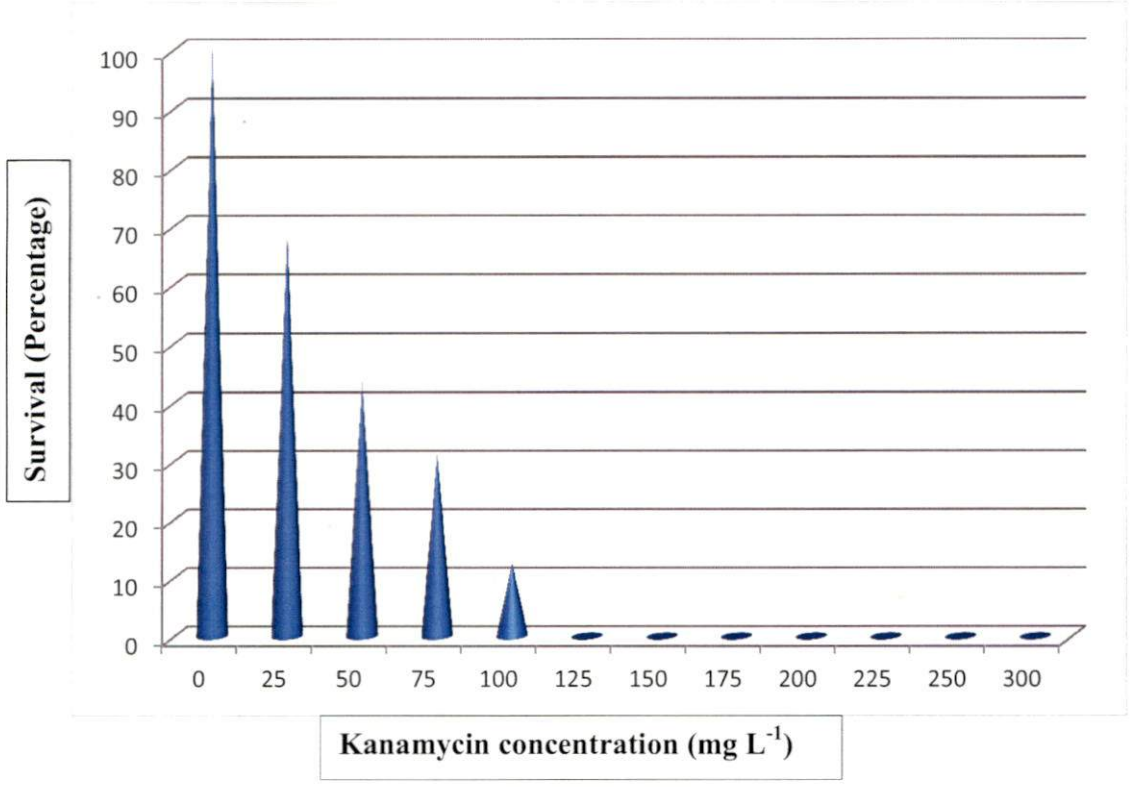
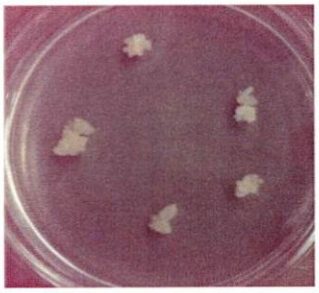


Table 6. Sensitivity of banana embryogenic callus to different doses of cefotaxime

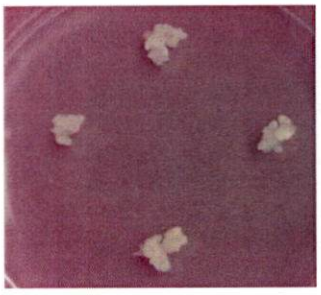
Sl No.	Cefotaxime (mg L^{-1})	Sensitivity						Survival (%) after 6 weeks
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	
1	0	++++	++++	++++	++++	++++	++++	100
2	5	++++	++++	++++	++++	+++	+++	75
3	25	++++	++++	+++	+++	+++	++	37.5
4	50	++++	++++	+++	+++	++	++	25
.5	75	++++	+++	+++	++	++	+	12.5
6	100	++++	+++	++	+	+	+	0

++++ Pale white coloured callus
+++ Yellowing of the callus
++ Tissue started browning
+ Tissue turning brown and dead

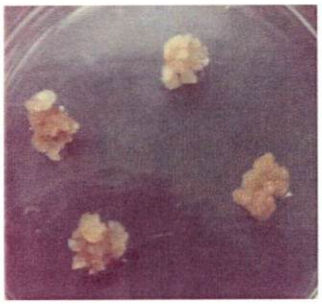
Plate 29. Response of banana embryogenic calli to different doses of Cefotaxime



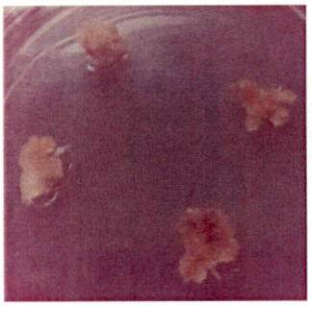
0 mg L^{-1}



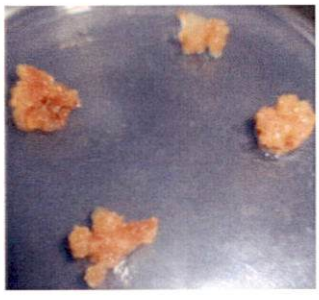
5 mg L^{-1}



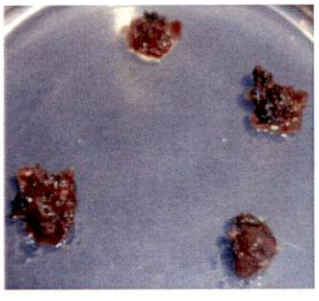
25 mg L^{-1}



50 mg L^{-1}

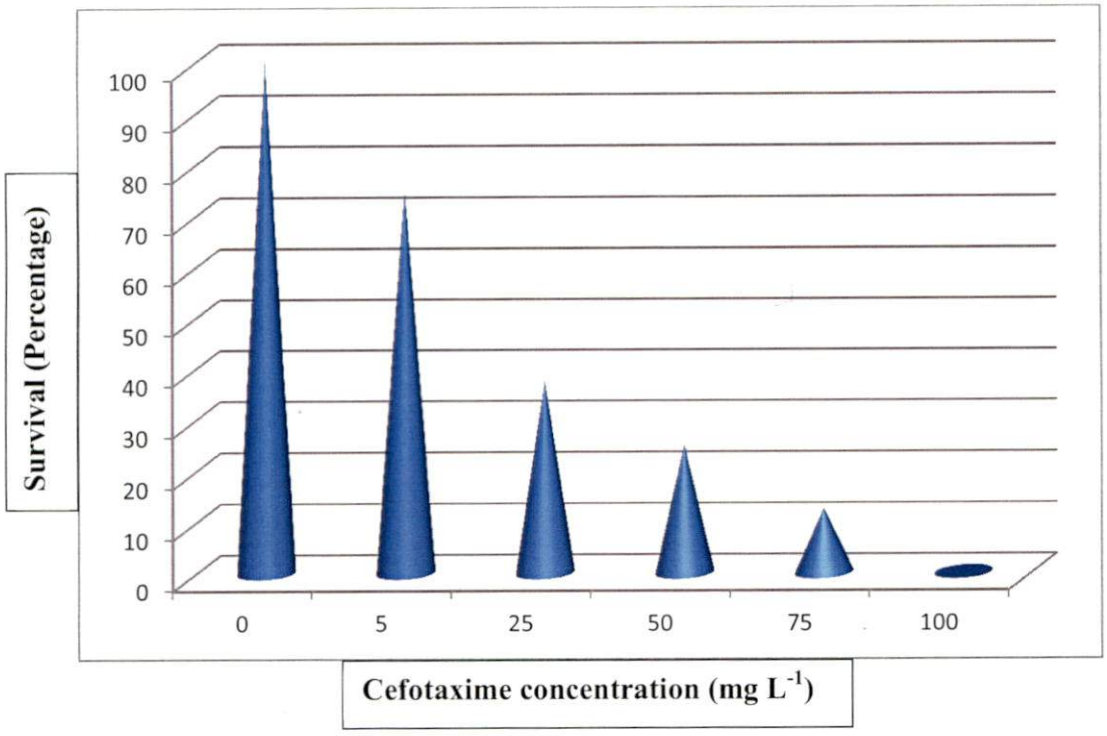


75 mg L^{-1}



100 mg L^{-1}

Fig. 2 Sensitivity of banana embryogenic callus to different concentrations of cefotaxime



4.7 SENSITIVITY OF *AGROBACTERIUM* STRAINS TO ANTIBIOTICS

The sensitivity of the bacterial strain LBA4404 harboring pART27 to different doses of antibiotics is presented below.

4.7.1 Kanamycin

The growth of *Agrobacterium* strain LBA4404 containing the plasmid vector pART27 in LB medium containing different concentrations of kanamycin (0 - 500 mg L^{-1}) after two days of culture is shown in Table 7. The bacterial cultures survived in the presence of kanamycin up to a concentration of 300 mg L^{-1}. No bacterial growth was observed in LB medium containing kanamycin 350, 400, 450 and 500 mg L^{-1}.

4.7.2 Cefotaxime

The growth of *Agrobacterium* strain LBA4404 containing the plasmid vector pART27 in LB medium containing different concentrations of cefotaxime (5 - 100 mg L^{-1}) after two days of culture is shown in Table 8. Bacterial growth was observed in LB medium containing cefotaxime 5 and 10 mg L^{-1}. No bacterial growth was observed in LB medium containing cefotaxime at concentrations 15 mg L^{-1} and above.

Table 7. Sensitivity of *A. tumefaciens* strain LBA4404 (pART27) to kanamycin

Sl. No.	Kanamycin (mg L-1)	Bacterial growth
1	0	+
2	5	+
3	25	+
4	50	+
5	75	+
6	100	+
7	125	+
8	150	+
9	175	+
10	200	+
11	225	+
12	250	+
13	275	+
14	300	-
15	350	-
16	400	-
17	450	-
18	500	-

\+ Bacterial growth

\- No bacterial growth

Table 8. Bactericidal activity of cefotaxime on *Agrobacterium* strain LBA 4404

Sl. No.	Cefotaxime (mg L-1)	Bacterial growth
1	0	+
2	5	+
3	10	+
4	15	-
5	20	-
6	25	-
7	50	-
8	75	-
9	100	-

\+ Bacterial growth

\- No bacterial growth

4.8 GENETIC TRANSFORMATION OF BANANA EMBRYOGENIC CALLUS

4.8.1 Identification of the transformed embryogenic callus by selection

The tissues after transformation were placed on selection medium (Plate 30) and survival percentage was recorded three weeks after elimination of the *Agrobacterium* infection (Table 9). A survival percentage of 12.5 were obtained in selection medium containing kanamycin 100 mg L^{-1} for the treatment T1(c). The survival percentage of 16.66 was observed in selection medium for the treatment T2(a), 25 for the treatment T2(b) and 12.5 for the treatment T2(c). Survived calli were used for embryogenesis (Plate 31) in embryo induction medium with kanamycin 100 mg L^{-1}. The embryos induced in 45 ± 5 days were transferred to germination medium with kanamycin 100 mg L^{-1} (Plate 32). The germinated plantlets were maintained by subculturing in MS medium with BA 2 mg L^{-1} and kanamycin 100 mg L^{-1} (Plate 33).

4.9 CONFIRMATION OF TRANSFORMATION

The transgenes were confirmed by PCR using *nptII* gene and *cre* intron specific primers. The DNA isolated from the leaves of transformed Nendran shoots (tissues selected on kanamycin 100 mg L^{-1}) showed amplicons of size 475 and 450 bp respectively. The sizes of the products were comparable to the positive control (Plate 34 & 35). 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 of sense strand reverse primer of antisense strand produced a band of size approximately 1300bp (Plate 36). This band matched with the size of the ihpRNA cassette (covering sense and antisense strands of replicase gene connected through *cre* intron) transferred. So the result obtained confirmed the presence of ihpRNA cassette in the transformed plants.

Plate 30. Transformed embryogenic calli in MS medium containing Kanamycin100 mgL^{-1} and cefotaxime 25 mg L^{-1}

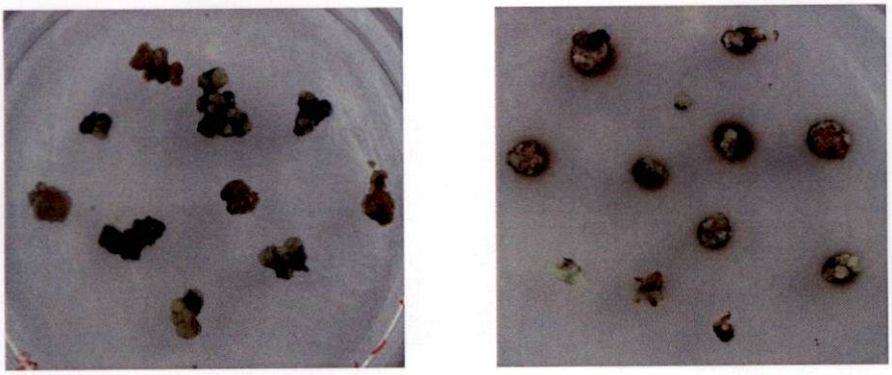


Plate 31. Embryogenesis from the transformed calli in semisolid MS containing BA 2 mg L^{-1}, IAA 0.5 mg L^{-1}Kanamycin 100 mg L^{-1}

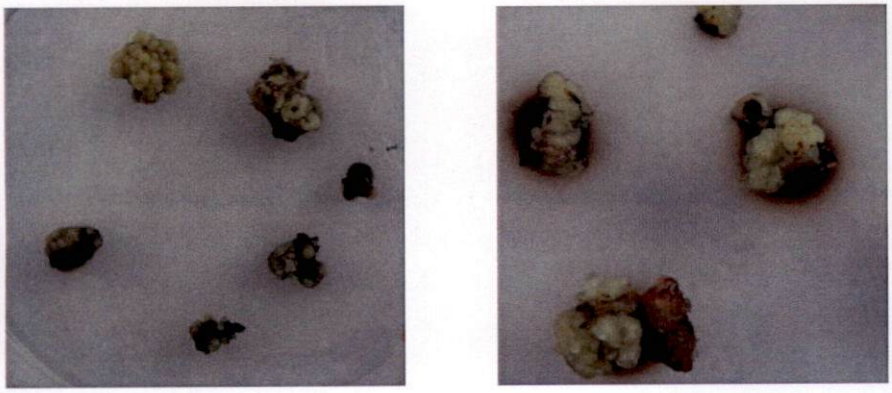


Table 9. Survival of tissues transformed with *Agrobacterium* LBA4404 (pART27) in selection medium (Kanamycin 100 mg L^{-1})

Treatments	No: of tissues survived in selection medium	No: of tissues turned brown and dead in selection medium	Survival of tissues in selection medium (%)
T1(a)	0	24	0
T1(b)	3	21	12.5
T1(c)	0	24	0
T2(a)	2	22	8.33
T2(b)	6	18	25
T2(c)	3	21	12.5
T3(a)	0	24	0
T3(b)	0	24	0
T3(c)	0	24	0
T4(a)	0	24	0
T4(b)	0	24	0
T4(c)	0	24	0

Plate 32. Maturation and germination of transformed embryos in Half MS (semisolid) + BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} , Kanamycin100 mg L^{-1}

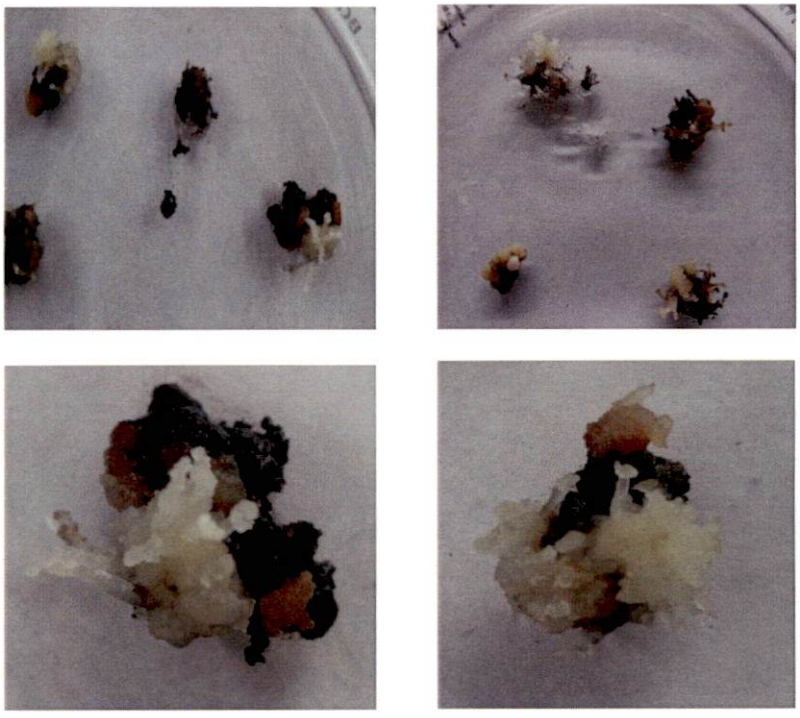
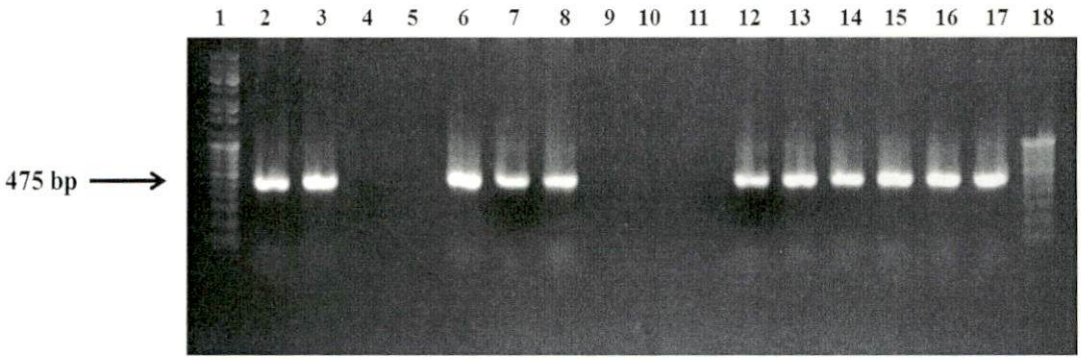


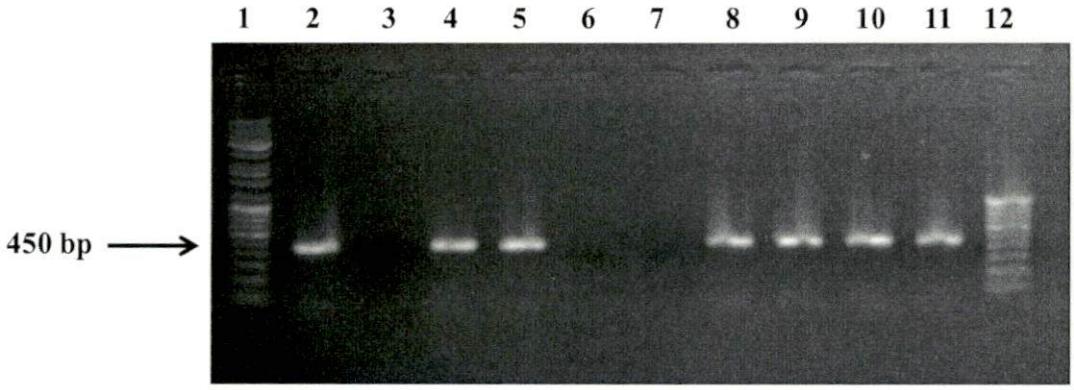
Plate 33. Transformed plantlets maintained by subculturing in MS medium containing BA 2 mg L^{-1}, NAA 0.5 mg L^{-1} and kanamycin 100 mg L^{-1}

Plate 34. Confirmation of transformed plants by PCR using nptII gene Specific primers



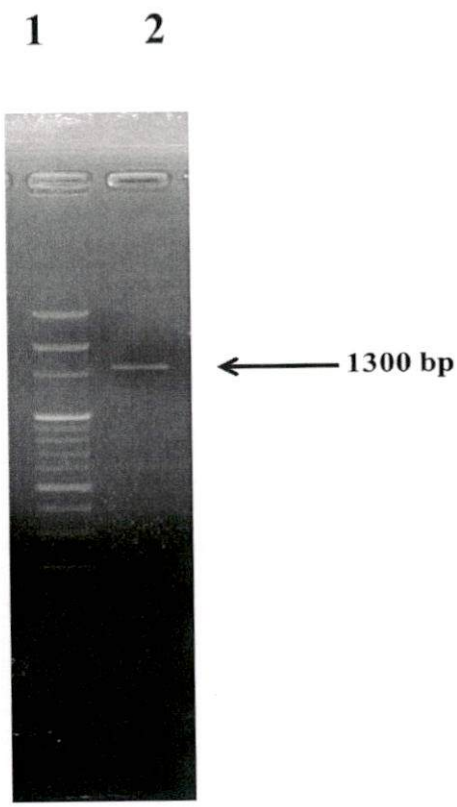
Lane 1	- 100-5 kb ladder
Lane 2 & 3	- positive control (Plasmid)
Lane 4 & 5	- negative control (nontransformed plant)
Lane 6, 7, 8	- T1(b)
Lane 9,10,11	- T2(a)
Lane 12,13,14	- T2(b)
Lane 15, 16, 17	- T2(c)
Lane 18	- 100 bp ladder

Plate 35. Confirmation of presence of the insert by PCR with *cre* intron Specific primer



Lane 1	- 100 – 5 kb ladder
Lane 2	- positive control
Lane 3	- negative control
Lane 4,5	- T1(b)
Lane 6,7	- T2(a)
Lane 8,9	- T2(b)
Lane 10,11	- T2(c)
Lane 12	- 100 bp ladder

Plate 36. PCRconfirmation of presence of the ihpRNA cassette by PCR



Lane 1 - 100 - 2 kb DNA ladder
Lane 2 - Transformed plantlet

Discussion

5. DISCUSSION

Bananas and plantains belonging to the *Musa* genus of the Musaceae family are perennial herbaceous monocots. It is a major crop in the tropical and sub-tropical regions of the world. India has a rich genetic diversity of banana with more than 90 distinct clones. Banana cultivation faces numerous environmental challenges, particularly with fungal, bacterial and viral pathogens. Viral diseases cause significant loss in the yield and quality of banana crop on an annual basis. Major threatening viruses infecting banana are *Banana bunchy top virus* (BBMV), *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV), *Banana streak virus* (BSV), *Banana mild mosaic virus*, *Banana virus X*. Banana bract mosaic disease (BBrMD) is a recently described virus disease of banana. In India, BBrMD is reported in Kerala, Tamil Nadu, Karnataka and Andhra Pradesh (Rodoni *et al.*, 1997; Thomas and Magnaye, 1996; Selvarajan *et al.*, 1997; Thangavelu *et al.*, 2000; Cherian *et al.*, 2002; Kiranmani *et al.*, 2005). In Kerala the disease was first reported from Thrissur district in the variety Nendran, which found to be a highly susceptible variety. The incidence of disease ranges from 5 to 36 per cent and more in cv. Nendran in Kerala. Later on, the disease was found to affect other varieties like Palayankodan, Kodappanillakunnan, Monthan, Kanchikela, Poovan (Rasthali), Karpooravally and Chenkadali. There are varietal differences in the symptomatology of the disease. BBrMV has been reported to cause considerable damage with yield reduction in cv. Robusta (AAA) (70per cent), followed by cv. Nendran (AAB) (52per cent) (Cherian *et al.*, 2002).

Plant viruses are among the most important plant pathogens resulting in substantial damage to crop production. Currently there is no strategy available to protect bananas against the viruses. Conventional breeding methods have limited success in banana improvement programmes due to lack of natural resistance source and triploid nature of the popular banana varieties. Reports on genetic engineering approaches tried for crop improvement in banana are scarce.

During the last decade many researchers have tried to introduce resistance to plant viruses by genetic engineering, mainly by the expression of functional or dysfunctional coat protein, movement protein or polymerase gene. The first attempt to develop virus-derived, (coat) protein-mediated resistance to develop transgenic tobacco plants resistant to *Tobacco mosaic virus* was demonstrated by Abel *et al.* (1986). Studies indicated that pathogen-derived resistance in plants is due to post-transcriptional gene silencing (PTGS). Voinnet (2001) reported that RNA silencing or PTGS, functions as a defense mechanism against foreign nucleic acid invasions.

Small interfering RNA (siRNA) mediated gene silencing has been emerged as a powerful defense mechanism targeting the pathogens in a highly sequence specific manner. Endogenous or exogenous small RNAs can either guide post-transcriptional gene silencing by mRNA cleavage or degradation or guide transcriptional gene silencing by DNA methylation and chromatin modifications. The most important application of RNAi in plant biology is development of virus resistance in agricultural and horticultural crops.

In the current study an attempt has been made to develop an intron hairpin RNA construct against the replicase gene of BBrMV to suppress the replication of the virus inside the host. The siRNA vector, pSTARLING was used for preparing the ihpRNA construct by integrating a short fragment (400 bp) of BBrMV replicase gene as inverted repeats linked through an intron. This ihpRNA construct was mobilized to the binary vector pART27. The binary vector was transferred to *Agrobacterium tumefaciens* (LBA4404) by freeze-thaw method and then introduced into the host plant banana (cv. Nendran was focused in the study). The expression of dsRNA inside the host is expected to initiate RNA silencing against the BBrMV replicase gene and the transformed plants will be resistant to virus infection.

The genetic transformation can be successful only with an efficient and reliable *in vitro* regeneration system. Somatic embryos are mostly preferred for

genetic transformation studies in plants because of their single cell origin and the potential to produce non-chimeric plants. In banana var. Nendran, a commercially important banana cultivar of Kerala, a reliable protocol for somatic embryogenesis has not been reported. The protocols reported in other varieties require 18-24 months for regeneration via somatic embryogenesis, involving sophisticated procedures (Khatri *et al.*, 1997; Ganapathi *et al.*, 1999; Meenakshi *et al.*, 2011). Hence in this study attempts were made to standardize a protocol for somatic embryogenesis mediated regeneration in var. Nendran. Immature male flowers (IMFs) were used as explants. Compared to soil grown suckers, IMFs show less contamination during micropropagation. The explants were collected soon after bunch formation is complete and as reported by Resmi and Nair, (2007), this helped in visually evaluating the bunch quality, number of hands, fruit quality and health of the mother plant before collecting the explants. Several explants have been tried for callus induction in banana *viz.*, immature male flower bud, scalp, leaf sheath, rhizome etc. Among them immature male flower bud is known to respond well and produce higher proportion of embryogenic calli with high regeneration potential (Escalant *et al.*, 1994; Cote *et al.*, 1996; Navarro *et al.*, 1997; Sagi *et al.*, 1998; Ganapathi *et al.*, 1999; Becker *et al.*, 2000; Khalil *et al.*, 2002; Strosse *et al.*, 2003; Namanya *et al.*, 2004; Houllou-Kido *et al.*, 2005; Meenakshi *et al.*, 2011).

Contamination free cultures could be established with a simple surface sterilization procedure. Dipping the flower bud in 90 per cent ethanol for one min or wiping it using ethanol soaked cotton swab was found to be effective. The immature flower buds are perfectly and tightly packed within the bracts of the inflorescence. This may be the reason for contamination free cultures with this simple procedure. Endogenous bacterial contamination is very less in inflorescence meristem compared to shoot meristem, which favours selection of this explants for *in vitro* culture.

A serious problem encountered in the establishment of IMF cultures of cv. Nendran was the presence of polyphenolics. The explant turned brown in 2 to 3

days after inoculation and decayed later. In this study, 20 mg L^{-1} ascorbic acid was added invariably to all the treatments which reduced the blackening of the explants due to phenolic oxidation and prevented the decaying of the explants. Use of antioxidants like ascorbic acid (Namanya *et al.*, 2004), cysteine HCl and methionine (Khatri *et al.*, 1997), alone or in combination has been shown to avoid browning and improve callus formation in banana. Ascorbic acid is a reducing agent which can prevent the oxidation of phenolics in the medium and is also able to scavenge the oxygen radicals produced during the wounding of tissues. By replacing agar with gelrite, the spreading of phenolic exudates could be considerably reduced and thereby preventing the decaying of the explants. Similar findings have been reported by Khatri *et al.* (2005) in callus induction studies using different banana cultivars. He had also reported positive correlation of light with browning which may be due to higher physiological activities under light than in dark. In the present study as well, the cultures were incubated in the dark to overcome the effect of phenolics.

Different media compositions were tried for callus induction from IMF. MS medium supplemented with a combination of 2,4-D and IAA produced callus in 45 days after inoculation, even though the explants turned brown initially. The highest callus induction was 45.80 per cent. The morphology of the calli varied with concentrations of growth regulators. It was observed that as the concentration of 2,4-D increased, the nature of the callus changed from hard to watery. When the concentration exceeded 3.5 mg L^{-1} no callus formation occurred. Callus initiation from IMF using different growth regulators have been reported by Meenakshi *et al.* (2011) and they could induce 77.70 per cent callus formation from IMF within a few weeks of inoculation in 2,4-D supplemented MS medium. In a study to find out the effect of 2,4-D from IMF in seven banana cultivars, Karintanyakit *et al.* (2014) observed that callus induction was genome specific.

The IMFs innoculated in picloram supplemented medium also turned brown initially, but later showed callus induction in 45 days, with a maximum of 60 per cent. A similar observation was made by Houllou-kido *et al.* (2005) when

IMFs were inoculated in MS medium supplemented with picloram. The explants developed necrosis in first two weeks, however, after two months of inoculation 80 per cent callus initiation was observed beneath the bracts. Smitha *et al.* (2011) reported the use of picloram for callus induction from leaf sheath explants. They obtained brown spongy callus with yellow globular structures with lower concentrations of picloram in MS medium and at higher concentrations of picloram, brown compact callus with white globular structures were developed. In the present study picloram produced watery callus.

In treatments with BA and picloram IMFs produced small to medium sized yellow globular friable callus with dense yellow cytoplasm a maximum response of 30 per cent. In the present study, among all the treatments tried for callus initiation, phenolic interference was less in BA and picloram combinations. The callus initiation occurred in 45-55 days of inoculation in this combination.

Among the calli formed in various combinations of growth regulators, only the calli induced in treatments C8, C9 and C10 (combination of BA and picloram) showed embryogenic potential. The monocot embryos seemed elongated with a glassy appearance. According to Arnold *et al.* (2002), the competence for embryogenic induction might be the result of the varying auxin sensitivity of the cells. It was observed in the study that though the callus initiation was comparatively higher in treatments with 2,4-D and IAA and that with picloram alone, they failed to induce somatic embryos. Hence, it can be inferred that somatic embryogenesis might have been influenced by the effect of picloram and BA used for callus initiation. Remakanthan *et al.* (2014) obtained direct embryogenesis from split shoot tips of Grand Naine in MS medium supplemented with picloram and BA, used alone or in combination, with the best embryogenic response when they were used in combination. When they used BA alone, the explants produced only shoots.

In almost all the reported protocols for somatic embryogenesis from callus, an intermediary liquid medium is involved. The maintenance of suspension

cultures is a little sophisticated process, even though the rate of multiplication is high. In this study, we could replace the same with a simple semisolid media. Shorter protocols are useful for genetic manipulation studies where early evaluations of transformants are possible.

Somatic embryos induced in semisolid MS with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} failed to germinate in the same medium. But when the strength of MS medium was reduced to half 60-80 per cent germination was obtained. This is in line with the studies made by Ganapathi *et al.* (1999), where they observed that development of embryo to plantlet was good in IAA and BA combination. They obtained germination of somatic embryos in half strength MS basal medium without any growth regulators. Meenakshi *et al.* (2011) used BA and IAA supplemented in MS medium with 0.2 per cent gelrite for the conversion of somatic embryo to plantlet.

In the present study it was observed that the germination of somatic embryos was better when incubated in dark. But the shoots were pale white to pale green in colour. When the cultures were shifted to 14h photoperiod they turned green. Better development of plantlets was observed when IAA was substituted with NAA 1 mg L^{-1}. A similar observation was made by Meenakshi *et al.* (2011), by supplementing MS medium with NAA and 0.2 per cent gelrite for root development. The plantlets after primary hardening in coir pith compost and secondary hardening for one month in soil and cowdung (1:1) mixture in mist chamber showed 100 per cent survival rate.

The current study demonstrated the potential of male flower buds as explants for raising highly proliferative, embryogenic cultures with simple hormone combination. The studies revealed that the growth hormones have an important role in inducing calli with embryogenic potential. The combination of growth hormones is critical in deciding the germination of embryos. In most of the protocols liquid medium is used for the proliferation of the embryos. Maintenance of the embryogenic cell suspensions is a laborious and time-

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consuming. The frequent subculturing needed in this procedure may lead to somaclonal variation, microbial contamination, and eventually the loss of morphogenetic potential (Kulkarni and Ganapathi, 2009). The protocol described in the present study does not require any intermediate cell suspension system, making it more convenient and very effective method for small labs lacking facilities for continuous agitation. According to Georget *et al.* (2000), a common feature of many banana cell culture protocols is the slow development of cell clusters, making the plant regeneration time-consuming (18-24 months). The protocols standardized in the present study required only 6 months to produce plantlets from the explant.

RNAi has emerged as a powerful tool for defending against viruses and it share a common mechanism with plant defense mechanism against invasive viruses (Ding, 2010 and Qu, 2010). The RNAi silencing pathway in plants starts with the formation of long dsRNA precursors which are cleaved into 21-, 22- and 24-nt short interference RNAs (siRNAs) by distinct DICER-like enzymes (Parent *et al.*, 2012) . The 21-nt siRNAs mediate post-transcriptional gene silencing (PTGS), whereas the 24-nt siRNAs guide RNA-directed DNA methylation (RdDM) of homologous DNA (Hamilton *et al.*, 2002). In plants, RNAi has been successfully used to express cognate dsRNAs to initiate the process of viral gene silencing.

The design of the ihpRNA vectors is a critical factor in producing gene silencing. An important feature of plant miRNA pathway is that, the plant miRNAs are perfectly paired to target RNA and use RNA cleavage rather than translational suppression as the primary silencing mechanism (Rhodes *et al.*, 2002; Llave *et al.*, 2002; Jones-Rhodes and Bartel, 2004). Hence the accuracy of silencing depends on the selection of target gene sequence for the construction of ihpRNA vector. In the current study the replicase gene of BBrMV was targeted. BBrMv is an RNA virus infecting banana leading to yield loss upto 40 per cent in Nendran. Replicase gene was isolated from the BBrMV infected plants located in the Instructional Farm, College of Agriculture, Vellayni. Total RNA was isolated

from the spindle leaf of the BBrMV infected banana plant using the protocol reported by Salzman *et al.* (1999). Tissues of banana contain large amount of polyphenols. In var. Nendran the problems associated with high phenolics were found severe during embryogenesis. In the protocol described by Salzman *et al.* soluble poly vinyl-pyrrolidone (PVP) polymers are used which will immediately bind the phenolic compounds upon homogenization of the tissues, which are eliminated during the ethanol precipitation of the RNA. Proteins and carbohydrates are subsequently removed by phenol extraction and lithium chloride precipitation respectively. This protocol yielded good quality RNA without any phenolic or protein contamination.

The primers for amplification of BBrMV replicase were designed based on the sequence information of BBrMV Trichy isolate retrieved from GenBank. Two partial replicase gene fragments of size 421bp and other 733 bp were isolated and these sequences were published in the NCBI database. Gene fragments ranging from 50 bp to 1000 bp are used to successfully silence genes. Earlier studies suggested that the shorter the fragment, the less effective silencing achieved, while very long fragments increase the chance of recombination. Hence, a fragment length of between 400 and 800 bp was considered to be a suitable size to maximize silencing efficiency (Helliwell *et al.*, 2002). RNAi-induced gene silencing using sequences around 300 -700nt was shown to be stable and efficient to suppress the targeted genes in wheat (Travella *et al.*, 2006), rice (Mei *et al.*, 2007) and potato (Bhaskar *et al.*, 2009). But there is no report on the accurate size of the sequence required for virus resistance. In the present study, the 421bp region was fond to code for the C terminus of the replicase protein. So this sequence was selected for the preparation of ihpRNA construct. Before proceeding further, the sequence was subjected to restriction mapping and miRNA target prediction. MicroRNAs (miRNAs) are important regulators ofeukaryotic gene expression in most biological processes. They act by guiding the RNAi-induced silencing complex (RISC) to the complementary sites (DICER substrates) in target mRNAs to suppress gene expressionby mRNA decay.

Inclusion of miRNA target may enhance the silencing process. Many tools have been reported for predicting miRNA target regions. Krek *et al.* (2005) reported 'Pic Tar', a computational method for identifying the miRNA targets and have experimentally validated it. An algorithm called miRNA assist was used by Qiu *et al.* (2007) to blast the miRNA databases of *Gossypium hirsutum*. In the current study RNAi design tools provided by Integrated DNA Technologies (IDT) was used to find out the location of the Dicer substrate.

For preparing ihpRNA construct pSTARLING was used as the primary vector. In plants, ihpRNA constructs were shown to give higher gene silencing efficiency than intron-free hpRNA as the intron spacer makes the interaction of the two arms of the hairpin more probable (Wesley *et al.*, 2001 ; Yan *et al.*, 2012). Many studies revealedthat splicing of the intron promotes formation of a dsRNA structure of nascent transcripts with inverted repeat (IR) sequences (Reichhart *et al.*, 2002; Wesley *et al.*, 2001). The RNA silencing cassette in pSTARLING is designed to contain a cyclic AMP Response Element (*cre* intron) intron. The sense and antisense fragments of the targeted gene are linked through this intron to facilitate the hairpin structure formation. Many studies have demonstrated that inverted repeat (IR) sequences of partial cDNA from a plant virus can silence the corresponding virus gene. Transgenic tobacco lines containing IR of *Cucumber mosaic virus* (CMV) cDNA when challenge inoculated with CMV, three different types of plants were obtained, including susceptible, recovered plants, and asymptomatic resistant plants (Kalantidis *et al.*, 2002).

Zea mays polyubiquitin promoter is used in pSTARLING to drive the expression of ihpRNA sequence. This promoter has been shown to be more efficient than CaMV 35S based promoters in banana tissues (Hermann *et al.*, 2001). The information obtained from the restriction map and the insilico predicted miRNA targets of the 421 bp replicase gene fragment were considered while designing primers for extracting the sense and antisense fragments required for the silencing cassette. Restriction sites for *Bam*HI, *Pac*I, *Kpn*I, *Spe*I and *Not*I were excluded and two miRNA targets were included to get a product of size 400

bp. Homology between small RNA and target gene determines the siRNA mediated degradation of target sequence. In a study conducted by Gaba *et al.* (2010) homozygous tobacco plants containing a 597 nucleotide hairpin RNA construct of the PVY *rep* sequence when challenged with a variety of PVY strains showed immunity to five potato PVY strains with 88.3–99.5 per cent sequence similarity to the transgene. In turn, infection with more distant tomato and pepper PVY field strains (86 – 87 per cent sequence similarity) caused delayed symptom appearance in the transgenic tobacco.

Orientation of the sense and antisense strands in the silencing cassette is very important to for the formation of small double stranded RNA species. The primers designed in this study were able to amplify viral replicase gene fragment which can be integrated in the primary vector in inverted orientations to form the sense and antisense strands. Sense and antisense fragments amplified were initially cloned into T/A cloning vector pTZ57R/T providing the anchorage site for the restriction enzymes. These fragments were later released from pTZ57R/T vector using corresponding restriction enzymes for ligating to the primary vector pSTARLING on the 5' and 3' ends of the *cre* intron.

Replicase gene has been targeted for developing virus resistance in banana by other workers also. Shekhawat *et al.* (2012) developed ihpRNA vector to silence BBTV in banana by targeting replicase gene. They used castor bean catalase intron and *Zea mays* polyubiquitin. Elayabalan *et al.* (2013) also reported a similar work to transform banana to make it resistant to *banana bunchy top virus* (BBTV) by developing RNAi mediated resistance against replicase gene. Ntui *et al.* (2014) constructed RNAi vector based on the replicase gene of *Cucumber mosaic virus.*

pART 27 was used as the binary vector for transformation in this study. The *Not*I fragment containing the ihpRNA construct was released from pSTARLING and mobilized to the binary vector. *Not*I site in pART27 within the lacZ gene and so the insertional inactivation of lacZ gene facilitated blue white

screening of the positive colonies. Similar kind of work was reported by Elayabalan *et al.* (2013) for developing resistance against BBTV. The ihpRNA construct prepared in the notI fragment in the primary vector pSTARLING was transferred to the *Not*I site of the binary vector pART27. The ihpRNA cassette constructed by Ntui *et al.* (2014) in the Gateway entry vector, pCR®8/GW/TOPO® was subcloned to the binary vector pEKH2IN2 by eLRclonase™ recombination reaction.

Binary vector pART27 containing ihpRNA construct was transferred to *Agrobacterium* strain LBA 4404 by Freeze-thaw method (Jyothishwaran *et al.*, 2007). Different methods are reported for the *Agrobacterium* transformation *viz.* triparental mating (Ditta *et al.*, 1980), direct introduction of the genetically engineered binary vector construct into *Agrobacterium* by electroporation (Ryu and Hartin, 1999) or freeze and thaw method (Holsters and Willmitzer, 1978). Triparental mating requires at least five to seven days in order to determine the successful mobilization into *Agrobacterium* and is confined to strains harbouring plasmids that carry the mobilizing gene. Electroporation is faster and more efficient than triparental mating, but requires special equipment. Transformation frequency by freeze-thaw method in *Agrobacterium* has remained low as per the reports of Holsters *et al.* (1978); Mersereau *et al.* (1990) and Hofgen *et al.* (1988). Shekhawat *et al.* (2012) used electroporation for mobilizing the binary vector pCAMBIA 1301 with ihpRNA construct into *A. tumefaciens* (EHA 105) for transforming banana embryogenic cells. Ntui *et al.* (2014) reported triparental mating for transferring binary vector pEKH2IN2 to *A. tumefaciens* strain EHA105. In the present study, a modified freeze-thaw method reported by Jyothishwaran *et al.* (2007) has been followed for competent cell preparation and transformation of *A. tumefaciens* (LBA 4404), which was found to be effective. As the binary vector carried *nptII* gene as selectable marker the transformation of *Agrobacterium tumefaciens* strain LBA4404 was confirmed using *nptII*. The presence of ihpRNA cassette was confirmed by amplifying *cre* intron.

The embryogenic calli of banana were precultured to MS medium supplemented with BA 0.2 mg L^{-1} and picloram 1 mg L^{-1}, 15 days before co-cultivation. This step is necessary to maintain cells in active cell division stage. Ganapathi *et al.* (2001) and Ghosh *et al.* (2009) have also used similar procedure in *Agrobacterium*-mediated transformation of embryogenic cells of banana. In both these studies the cells were pre-cultured seven days before to maintain it in actively dividing stage. Elayabalan *et al.* (2013) precultured the embryogenic cells of banana cv. Virupakshi 7-10 days prior to co-cultivation while they were transforming banana for developing resistance to BBTV disease through RNAi. In the current study, as phenolic interference was severe in variety Nendran and hence cells neededmore time to establish in a fresh medium, the embryogenic cells were precultured 15 days prior to co-cultivation.

For the transformation of embryogenic calli different treatments were tried for finding out the optimum time for infection and the effect of inducer for improving the transformation efficiency. The transformed embryos were regenerated in half strength semisolid MS medium supplemented with BA 2 mg L^{1}, IAA 0.5 mg L^{-1} and kanamycin 100 mg L^{1}. Results of survival of calli in selection medium showed that an infection time of 10 min and co cultivation for 48h was the optimum for transformation. Incubation time of 5 min was not sufficient for proper infection as reflected the PCR result. Addition of 100 µM acetosyringone and wounding of calli was found to enhance the transformation efficiency.

Cefotaxime 25 mg L^{-1} was effective in eliminating the *Agrobacterium* after co-cultivation in this study. Use of carbenicillin for elimination of bacteria has also been reported in other studies (Ganapathi *et al.*, 2001; Ghosh *et al.*, 2009). The transformed calli were transferred to semisolid MS medium supplemented with BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} and kanamycin 100 mg L^{-1} and germinated in semisolid half strength MS medium BA 2 mg L^{-1}, IAA 0.5 mg L^{-1} and kanamycin 100 mg L^{-1}. A maximum of 25 per cent survival was obtained in the treatment T2(b) in which wounded embryogenic calli was subjected to

infection for 10 min with the *Agrobacterium* suspension containing 100 µM acetosyringone.

Transformed plantlets were confirmed by amplifying the genomic DNA using sense-forward and antisense-reverse primers (for detecting the ihpRNA cassette), *nptII* (for the selectable marker) and *cre* intron specific primers. The PCR result showed a positive result with bands of size 1300, 475 and 450 bp showing the expected sizes confirming the integration of ihpRNA construct against BBrMV in banana. 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 induce RNAi-mediated resistance to *Cucumber mosaic virus* in tomato, 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 selectable marker gene. The result showed bands of expected size confirming the transformation. In the present study confirmation of transformation was made by amplifying the regions corresponding to (i) selectable marker gene (ii) *cre* intron (iii) full ihpRNA insert (sense and antisense fragments linked through *cre* intron).

A rapid and efficient protocol was developed in this study for somatic embryogenesis mediated regeneration in banana var. Nendran. The study was also successful in developing an ihpRNA construct against replicase gene of BBrMV and delivering it into embryogenic calli to develop into a complete plant. The transgenic banana plants were confirmed to harbor the ihpRNA construct. The plants need to be confirmed for disease resistance by infecting with BBrMV and evaluated further for the production of siRNA.

Summary

SUMMARY

The study entitled "Development of small interfering RNA (siRNA) mediated resistance in banana against *Banana bract mosaic virus* (*BBrMV*)" was carried out during 2012-2016 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to develop siRNA mediated technology for the development of banana plants resistant to *BBrMV*. Banana (*Musa* spp. AAB) var. Nendran was used for the study.

The study envisaged development of transgenic banana plants harbouring ihpRNA construct to silence the replicase gene of BBMrV, to suppress the replication of the virus inside the plant. Embryogenic cells were used as the targets for *Agrobacterium*-mediated transformation, because of their single-cell origin and ability to produce non-chimeric plants. In the present study a protocol for somatic embryogenesis mediated regeneration in banana var. Nendran was developed. Immature male flowers (IMF) were used as explants. Wiping the flower bud with 90 per cent ethanol was sufficient to establish contamination free cultures. A serious problem encountered in the establishment of IMF cultures of cv. Nendran was the presence of polyphenolics. The explant turned brown in 2 to 3 days after inoculation and decayed later. In this study, 20 mg L^{-1}ascorbic acid was added invariably to all the treatments which reduced the blackening of the explants and prevented the decaying of the explants. Incubation of cultures in dark helped to overcome the problems associated with phenolic oxidation. Spreading of exudates was found reduced when agar was replaced with gelrite.

For callus induction from IMF, MS medium with thirty nine combinations of growth regulators *viz*. 2,4-D, IAA, BA and picloram were tried. Callus induction was observed on IMFs in 45 days after inoculation in the treatments tried. Callus induction percentage and the morphology of the calli obtained in the different treatments varied. In treatments with 2, 4-D (0.5 - 4 mg L^{-1}) and IAA (0.5 – 1 mgL^{-1}), IMFs turned brown within a week of inoculation (A1 to A16), but later developed pale white hard callus. The highest callus induction (45.80 per

cent) was observed in A9 and higher concentration of 2,4-D, above 3.5 mg L^{-1} did not favour callus induction. IMFs inoculated in picloram (0.5-10 mg L^{-1}) supplemented medium (B1 to B11) produced mostly watery callus in 45 days with the maximum response (60 percent) in treatment B8.In treatments with BA and picloram (C1 to C12), IMFs produced globular callus with dense cytoplasm with maximum response (30 per cent) in treatment C5.

The calli developed in different treatments were transferred to semisolid MS medium supplemented with BA 2 mg L^{-1} and IAA 0.5 mg L^{-1} for embryogenesis. Calli developed in the treatment C9 showed maximum (60 per cent) response in this medium. The glassy elongated monocot embryos when subcultured in half strength semisolid MS medium supplemented with BA 2 mg L^{-1} and IAA 0.5 mg L^{-1} and incubated in dark showed a maximum germination rate of 80 per cent. Better development of plantlets was observed when IAA was substituted with NAA 1mg L^{-1}. The plantlets were transferred to coirpith compost in pot trays in mist chamber for one month for primary hardening and then transferred to polybags with soil and cowdung (1:1) mixture.

The embryogenic potential of immature male flower was demonstrated in this study. The protocol developed was able to produce 60 per cent embryogenesis without the requirement of intermediate suspension culture with 80 per cent germination in a period of six months. 100 per cent survival of germinated plantlets was obtained.

The intron hairpin RNA (ihpRNA) construct required for silencing replicase gene of BBrMV needed sense and antisense fragments of BBrMV replicase gene with an intron in between to form a hairpin structure. For this replicase gene of BBrMV was isolated by RT-PCR. Total RNA was isolated (30.288 $\mu g\ \mu l^{-1}$) from the leaves of infected Nendran plants, cDNA and amplified using BBrMV replicase gene specific primers designed based on the whole genome sequence information retrieved from NCBI. Two partial sequences towards the 5' region of the BBrMV replicase gene (421bp and 733bp) were

isolated was sequenced. These sequences on BLAST analysis showed 96 per cent similarity with BBrMV Trichy isolate and 94 per cent similarity with Philipine isolate. These two replicase gene sequences were deposited in GenBank (NCBI) with accession numbers; GenBank: KM357545.1 and KM357543.1.

The 421bp sequence corresponds to the C-terminus of the BBrMV replicase protein. Hence this fragment was selected for the further vector construction procedures. The sequence was subjected to miRNA target prediction and restriction mapping. Based on this information another set of primers were designed with selected restriction enzyme recognition sequences anchored to the 5' end of the primers. Selected regions were amplified to form the sense and antisense fragments and were cloned to pTZ57R/T cloning vector. Further pTZ57R/T was digested with restriction enzymes *Bam*HI and *Pac*I to release the sense strand and *Kpn*I and *Spe*I to release the antisense strand. The ihpRNA vector, pSTARLING was also restricted with the corresponding enzymes and the sense and antisense fragments were ligated at the *Not*I restriction site of the vector. The complete ihpRNA construct in pSTARLING contained ubiquitin promoter, ubiquitin intron, sense replicase strand, *cre* intron, antisense replicase strand and termination sequence in the order within the *Not*I restriction sites. The integration of the insert was confirmed by restriction digestion.

The ihpRNA construct was mobilized to the binary vector pART 27, for the genetic transformation. For this the *Not*I fragment was released from pSTARLING and ligated to the *Not*I site of the binary vector pART 27 and maintained in DH5α cells. By blue white screening the colonies with ihpRNA cassette were confirmed. From these colonies the binary vector with the insert was transferred to *Agrobacterium tumefaciens* strain LBA 4404 via freeze-thaw method with a transformation efficiency of 7.6 X 10^3 CFUμg^{-1}. Transformed colonies were picked up and the presence of the vector and the ihpRNA insert was confirmed by PCR.

A protocol for *Agrobacterium*-mediated transformation of embryogenic calli of banana cv. Nendran was standardized. Embryogenic calli were transformed with LBA 4404 carrying the ihpRNA construct and the transformed calli were selected with antibiotic pressure of kanamycin 100 mg L^{-1}. Infection of wounded embryogenic calli for 10 mins in the presence of acetosyringone 100 µM and co-cultivation for 48 h was found effective for transformation. Transformed calli showed 25 per cent embryogenesis in semisolid MS medium supplemented with BA 2 mg L^{-1} and IAA 0.5 mg L^{-1} and developed plantlets. DNA was isolated from these plantlets and the presence of ihpRNA was confirmed by PCR with *nptII* specific primer (reporter gene) and *cre* intron specific primers.

In banana, where traditional breeding for virus resistance is very difficult, this technology is a promising alternative. Since only a small fragment of the target gene is used in the ihpRNA construct used for genetic transfer, the possibility of producing any functional protein by the transgene is also very less. In future, clean vector technology can be applied to remove the antibiotic resistance marker also.

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Appendices

APPENDIX I

Chemical composition of the media employed for the in vitro culture of Banana (Musa spp.) cv. Nendran: Murashige and Skoog medium (MS)

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

$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KNO_3	1900
NH_4NO_3	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 sources (mg L^{-1})

$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25

Vitamins (mg L^{-1})

Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

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

Glycine	2.0
Inositol (mg L^{-1})	100
Sucrose (g L^{-1})	30
Gelrite (g L^{-1})	4.5

APPENDIX II

RNA EXTRACTION BUFFER (Salzman et al., 1999)

Guanidine thiocyanate	4 M
Tris HCl (pH 8.0)	100 mM
Sodium citrate (pH 8.0)	25 mM
N- lauryl sarcosine	0.5 %
Polyvinylpyrrolidone (PVP)	1 %
2,β- mercapto ethanol	20 µL
RNase free water (DEPC) 0.1 %	Treated with Diethyl pyrocarbonate

APPENDIX III

Sequence 1 (421 bp): BBrMV replicase gene partial cds amplified with BBrMV- A set of primers

ATGAGGCCGCGGTATGTCTCATTATGGTCCAGCCGGTTAAATCGCAAGGC
ATTCTTGAAAGATTTACTAAAATATTCTGGCGAGCTGATTGTTGGTGTAG
TTGATTGTGATACATTCGAAAGTGCATACAACTTCACTGCATCATTGTTA
CGTAGCCATGGGTTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGAT
ATTTCAGAGTTTGAATATGAAGGCCGCTGTTGGAGCAATGTACGCTGGGA
AGAAGAAGGAATATTTTGAGGGCTTCACTAATCAACAAAAGGATGAGATT
ATATTCCAGAGTTGTCTTCGCCTATATAAAGGACACTTAGGCATATGGAA
CGGAGTCTTGAAAGCTGAATTACGGCCAAATGAAAAGAATGAGTTAAACA
AAACACGCGTGTTCACAGCAG

Sequence 2 (733 bp): BBrMV replicase gene partial cds amplified with BBrMV- B set of primers

CGATTTAGCTGACATAAGGGTGCTGAGATTTCTTTAGGCCGCTCATGTCT
CATTATGGTCCAAGCCGGTTAAATCGCAAGGCATTCTTGAAAGATTTACT
AAAATATTCTGGCGAGCTGATTGTTGGTGTAGTTGATTGTGATACATTCG
AAAGTGCATACAACTTCACTGCATCATTGTTACGTAGCCATGGGTTTGAA
GGAAGGAAGTTCATCACTGATACTGACGAGATATTTCAGAGTTTGAATAT
GAAGGCCGCTGTTGGAGCAATGTACGCTGGGAAGAAGAAGGAATATTTTG
AGGGCTTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTT
CGCCTATATAAAGGACACTTAGGCATATGGAACGGATCCTTGAAAGCTGA
ATTACGGCCAAATGAAAAGAATGAGTTAAACAAAACACGCGTGTTTACAG
CAGCTCCTTTAGAAAAAACAAAAAGTTTTGTTGAGAGGGGGTCTATTCAA
TTGGCCTAATTCACTTCAGGGATCGTTGCGTATCCCATTTCCTATTGGCA
AAGACACTCTGGAAATAACTCACCCTTTTGGATAGTGAAGCCTCATAATA
TTCCATCTTCTTCCGGCCGAGATGGGTCACGGCGCCGCTCAAATCCCCAC
CCTTAAAAATTGGGCGGTGTGAATAAAAGAAATTCCCCCCCCCCCACCCC
CGGGCCCCTTAACATGAAGCCCGTGTGAATTTT

APPENDIX IV

Analysis of partial cDNA sequences obtained using Basic Local Alignment Search Tool (BLAST) NCBI

BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - GDX5FR4W01R**
Formatting options
Download
Blast report description

Nucleotide Sequence (421 letters)

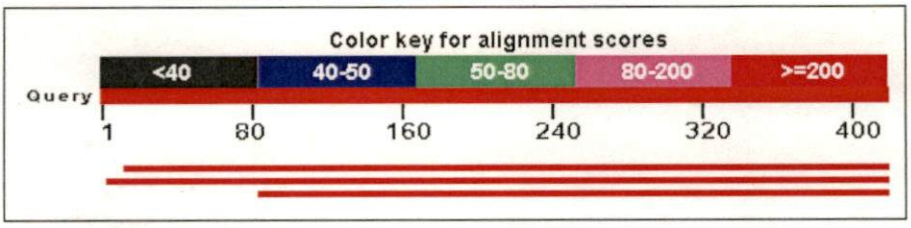
RID GDX5FR4W01R (Expires on 02-22 13:39 pm)

Query ID lcl|113101
Description None
Molecule type nucleic acid
Query Length 421

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+

Graphic Summary

Distribution of 3 Blast Hits on the Query Sequence



178

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Banana bract mosaic virus isolate TRY, complete genome	660	660	96%	0.0	96%	HM131454.1
Banana bract mosaic virus, complete genome	623	623	99%	4e-175	94%	DQ851496.1
Banana bract mosaic virus isolate P1 polyprotein mRNA, partial cds	529	529	80%	1e-146	95%	AF071591.1

Alignments

Banana bract mosaic virus isolate TRY, complete genome

Sequence ID: **gb|HM131454.1|** Length: 9711 Number of Matches: 1

Range 1: 7209 to 7617

Score	Expect	Identities	Gaps	Strand	Frame
660 bits(357)	0.0()	392/409(96%)	1/409(0%)	Plus/Plus	

Features:

```
Query  14    ATGTCTCATTATGGTCC-AGCCGGTTAAATCGCAAGGCATTCTTGAAAGATTTACTAAAA  72
Sbjct  7209  ATGTCTCATTATGGTCCAAGCCGGTTAAATCGCAAAGCATTCTTGAAAGATTTATTAAAA  7268
Query  73    TATTCTGGCGAGCTGATTGTTGGTGTAGTTGATTGTGATACATTCGAAAGTGCATACAAC  132
Sbjct  7269  TATTCTGGCGAGCTGATTGTTGGTGTAGTTGACTGTGATACATTCGAAAGCGCATACAAT  7328
Query  133   TTCACTGCATCATTGTTACGTAGCCATGGGTTTGAAGGAAGGAAGTTCATCACTGATACT  192
Sbjct  7329  TTCACTGCATCATTGTTACGTAACCATGGGTTTGAAGGAAGGAAGTTCATCACTGATACT  7388
Query  193   GACGAGATATTTCAGAGTTTGAATATGAAGGCCGCTGTTGGAGCAATGTACGCTGGGAAG  252
Sbjct  7389  GACGAGATATTTCAGAGTTTGAATATGAAGGCCGTTGTTGGAGCACTGTACGCTGGGAAA  7448
Query  253   AAGAAGGAATATTTTGAGGGCTTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGT  312
Sbjct  7449  AAGAGGGATTATTTTGAGGGCTTCACTACTCACCAAAAGGATGAGATTATATTCCAGAGT  7508
Query  313   TGTCTTCGCCTATATAAAGGACACTTAGGCATATGGAACGGATCCTTGAAAGCTGAATTA  372
Sbjct  7509  TGTCTTCGCCTATACAAAGGACATTTAGGCATATGGAACGGATCCTTGAAAGCTGAATTA  7568
Query  373   CGGCCAAATGAAAAGAATGAGTTAAACAAAACACGCGTGTTCACAGCAG  421
Sbjct  7569  CGGCCAAATGAAAAGAATGAGTTAAACAAAACACGCGTGTTTACAGCAG  7617
```

Banana bract mosaic virus, complete genome

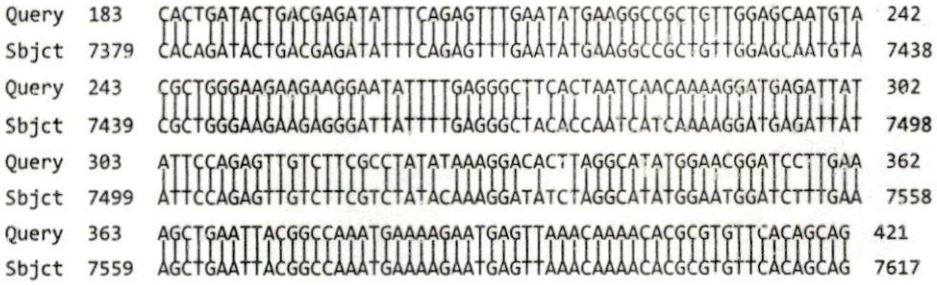
Sequence ID: **gb|DQ851496.1|** Length: 9711 Number of Matches: 1

Range 1: 7200 to 7617

Score	Expect	Identities	Gaps	Strand	Frame
623 bits(337)	4e-175()	392/419(94%)	2/419(0%)	Plus/Plus	

Features:

```
Query  4     AGGCCGCGGTATGTCTCATTATGGTCC-AGCCGGTTAAATCGCAAGGCATTCTTGAAAGA  62
Sbjct  7200  AGGCCGC-TCATGTCTCATTATGGTCCAAGCCGGTTAAATCGCAAGGCATTCTTGAAAGA  7258
Query  63    TTTACTAAAATATTCTGGCGAGCTGATTGTTGGTGTAGTTGATTGTGATACATTCGAAAG  122
Sbjct  7259  TTTGTTAAAATACTCTGGCGAGCTAATTGTTGGCGTGGTTGACTGTGATACATTCGAAAA  7318
Query  123   TGCATACAACTTCACTGCATCATTGTTACGTAGCCATGGGTTTGAAGGAAGGAAGTTCAT  182
Sbjct  7319  TGCATACAATTTCACTGCTTCATTGTTACGTAGCCATGGGTTTGAAGGAAGGAAGTTCAT  7378
```

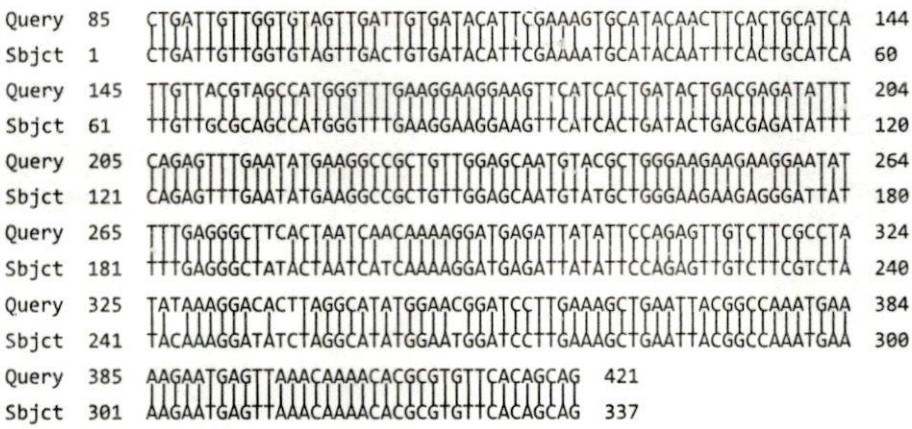


Banana bract mosaic virus isolate P1 polyprotein mRNA, partial cds

Sequence ID: **gb|AF071591.1|AF071591** Length: 1326 Number of Matches: 1
Range 1: 1 to 337

Score	Expect	Identities	Gaps	Strand	Frame
529 bits(286)	1e-146()	320/337(95%)	0/337(0%)	Plus/Plus	

Features:



BLAST ®

Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - GDWZ2C3101R**
Formatting options
Download
Blast report description

Nucleotide Sequence (733 letters)

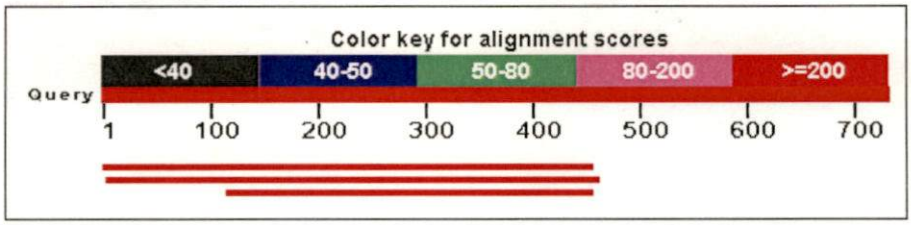
RID GDWZ2C3101R (Expires on 02-22 13:36 pm)

Query ID lcl|16159
Description None
Molecule type nucleic acid
Query Length 733

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.29+

Graphic Summary

Distribution of 3 Blast Hits on the Query Sequence



181

Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident	Accession
Banana bract mosaic virus isolate TRY, complete genome	737	737	62%	0.0	96%	HM131454.1
Banana bract mosaic virus, complete genome	701	701	62%	0.0	94%	DQ851496.1
Banana bract mosaic virus isolate P1 polyprotein mRNA, partial cds	531	531	46%	5e-147	95%	AF071591.1

Alignments

Banana bract mosaic virus isolate TRY, complete genome

Sequence ID: **gb|HM131454.1|** Length: 9711 Number of Matches: 1

Range 1: 7165 to 7621

Score	Expect	Identities	Gaps	Strand	Frame
737 bits(399)	0.0()	438/457(96%)	2/457(0%)	Plus/Plus	

Features:

```
Query  3     ATTTAGCTG-ACATAAGGGTGCTG-AGATTTCTTTAGGCCGCTCATGTCTCATTATGGTC  60
Sbjct  7165  ATTTAGCTGAACATAAGGGCGCTGAAGATTTCTTTAGACCGCTCATGTCTCATTATGGTC  7224
Query  61    CAAGCCGGTTAAATCGCAAGGCATTCTTGAAAGATTTACTAAAATATTCTGGCGAGCTGA  120
Sbjct  7225  CAAGCCGGTTAAATCGCAAAGCATTCTTGAAAGATTTATTAAAATATTCTGGCGAGCTGA  7284
Query  121   TTGTTGGTGTAGTTGATTGTGATACATTCGAAAGTGCATACAACTTCACTGCATCATTGT  180
Sbjct  7285  TTGTTGGTGTAGTTGACTGTGATACATTCGAAAGCGCATACAATTTCACTGCATCATTGT  7344
Query  181   TACGTAGCCATGGGTTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGATATTTCAGA  240
Sbjct  7345  TACGTAACCATGGGTTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGATATTTCAGA  7404
Query  241   GTTTGAATATGAAGGCCGCTGTTGGAGCAATGTACGCTGGGAAGAAGAAGGAATATTTTG  300
Sbjct  7405  GTTTGAATATGAAGGCCGTTGTTGGAGCACTGTACGCTGGGAAAAAGAGGGATTATTTTG  7464
Query  301   AGGGCTTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCCTATATA  360
Sbjct  7465  AGGGCTTCACTACTCACCAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCCTATACA  7524
Query  361   AAGGACACTTAGGCATATGGAACGGATCCTTGAAAGCTGAATTACGGCCAAATGAAAAGA  420
Sbjct  7525  AAGGACATTTAGGCATATGGAACGGATCCTTGAAAGCTGAATTACGGCCAAATGAAAAGA  7584
Query  421   ATGAGTTAAACAAAACACGCGTGTTTACAGCAGCTCC  457
Sbjct  7585  ATGAGTTAAACAAAACACGCGTGTTTACAGCAGCTCC  7621
```

Banana bract mosaic virus, complete genome

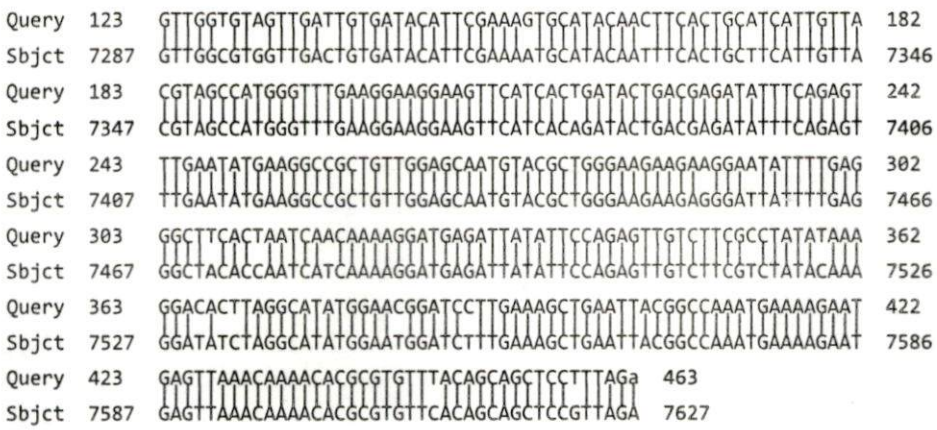
Sequence ID: **gb|DQ851496.1|** Length: 9711 Number of Matches: 1

Range 1: 7167 to 7627

Score	Expect	Identities	Gaps	Strand	Frame
701 bits(379)	0.0()	434/461(94%)	2/461(0%)	Plus/Plus	

Features:

```
Query  5     TTAGCTG-ACATAAGGGTGCTG-AGATTTCTTTAGGCCGCTCATGTCTCATTATGGTCCA  62
Sbjct  7167  TTAGCTGAACATAAGGGTGCTGAAGATTTCTTTAGGCCGCTCATGTCTCATTATGGTCCA  7226
Query  63    AGCCGGTTAAATCGCAAGGCATTCTTGAAAGATTTACTAAAATATTCTGGCGAGCTGATT  122
Sbjct  7227  AGCCGGTTAAATCGCAAGGCATTCTTGAAAGATTTGTTAAAATACTCTGGCGAGCTAATT  7286
```



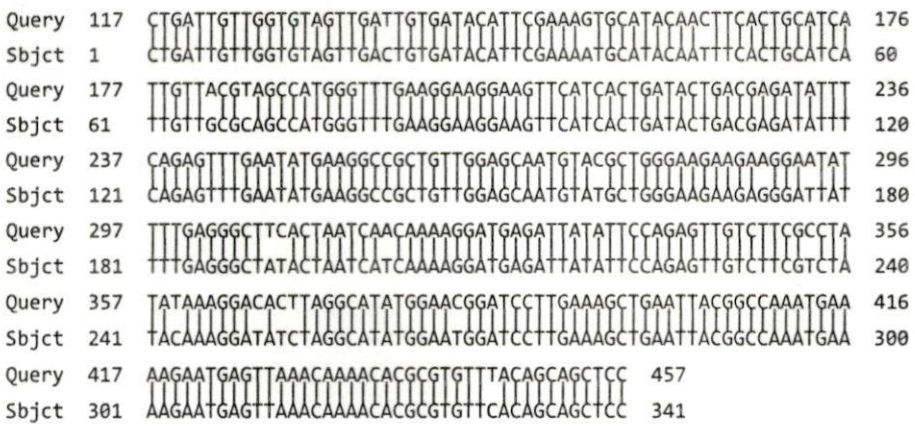
Banana bract mosaic virus isolate P1 polyprotein mRNA, partial cds

Sequence ID: **gb|AF071591.1|AF071591** Length: 1326 Number of Matches: 1

Range 1: 1 to 341

Score	Expect	Identities	Gaps	Strand	Frame
531 bits(287)	5e-147()	323/341(95%)	0/341(0%)	Plus/Plus	

Features:



APPENDIX V

miRNA targets (Dicer substrate) prediction result

INTEGRATED DNA TECHNOLOGIES
(/)

Sign In (/site/account)

Get Help | 0 Items € 0,00 (/site/order/cart)

Custom Dicer-Substrate siRNA (DsiRNA)

Generate DsiRNAs for any sequence from any species.

For technical assistance or to reorder using a Design ID generated before February 2016, contact applicationsupport@idtdna.com (mailto:applicationsupport@idtdna.com).

Search for Predesigned DsiRNAs Generate Custom DsiRNA

Input Format:

Sequence

Paste/Type Input

Enter an Accession Number:

ex: NM_001234

Retrieve Sequence

Or Paste FASTA Sequence (≤10 kb):

TATTTTGAGGGCTTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCCTATATA
AAGGACACTTAGGCATATGGAACGGATCCTTGAAAGCTGAATTACGGCCAAATGAAAAGAATGAG
TTAAACAAAACACGCGTGTTCACAGCAG

BLAST Species

- ○ Human
- ○ Mouse
- ○ Rat
- ◉ Other (Manual BLAST)

Search

Clear and Reset

185

Showing **5** results for . Clear results »

Select All Results (5 selected)

Actions: To perform a BLAST search against your species of interest: choose **BLAST** from the dropdown menu

DsiRNA - Custom

Search Input	Cross-Reacting Species	Design ID	2 nmol
Custom Input	Other (Manual BLAST)	CD.Ri.21060.13.1	**€ 75,00**
			Add to Order

Hide product details -

1	210	421

Cross-Reacting Transcripts
none
Sequence Positions
46-71
Sequence Details
Sense and antisense sequences

Sequence	Strand
5' rArArGrGrCrArUrUrCrUrUrGrArArArGrArUrUrUrArCrUAA 3'	+
5' rUrUrArGrUrArArArUrCrUrUrUrCrArArGrArArUrGrCrCrUrUrGrC 3'	-

DsiRNA - Custom

Search Input	Cross-Reacting Species	Design ID	2 nmol
Custom Input	Other (Manual BLAST)	CD.Ri.21060.13.2	**€ 75,00**
			Add to Order

Hide product details -

1	210	421

Cross-Reacting Transcripts
none
Sequence Positions
47-72
Sequence Details
Sense and antisense sequences

Sequence	Strand
5' rArGrGrCrArUrUrCrUrUrGrArArArGrArUrUrUrArCrUrAAA 3'	+
5' rUrUrUrArGrUrArArArArUrCrUrUrUrCrArArGrArArUrGrCrCrUrUrG 3'	-

☑ **DsiRNA - Custom**

Search Input	Cross-Reacting Species	Design ID	2 nmol ▾
Custom Input	Other (Manual BLAST)	CD.Ri.21060.13.3	**€ 75,00**

Add to Order

Hide product details -

1	210	421

Cross-Reacting Transcripts
none
Sequence Positions
193-218
Sequence Details
Sense and antisense sequences

Sequence	Strand
5' rGrArCrGrArGrArUrArUrUrUrCrArGrArGrUrUrUrGrArATA 3'	+
5' rUrArUrUrCrArArArCrUrCrUrGrArArArUrArUrCrUrCrGrUrCrArG 3'	-

☑ **DsiRNA - Custom**

Search Input	Cross-Reacting Species	Design ID	2 nmol ▾
Custom Input	Other (Manual BLAST)	CD.Ri.21060.13.4	**€ 75,00**

Add to Order

Hide product details -

1	210	421

Cross-Reacting Transcripts
none
Sequence Positions
50-75
Sequence Details
Sense and antisense sequences

Sequence	Strand
5' rCrArUrUrCrUrUrGrArArArGrArUrUrUrArCrUrArArArATA 3'	+
5' rUrArUrUrUrUrArGrUrArArArUrCrUrUrUrCrArArGrArArUrGrCrC 3'	-

☑ **DsiRNA - Custom**

Search Input	Cross-Reacting Species	Design ID	2 nmol ▾
Custom Input	Other (Manual BLAST)	CD.Ri.21060.13.5	**€ 75,00**

Add to Order

Hide product details -

1	210	421

Cross-Reacting Transcripts
none
Sequence Positions
45-70
Sequence Details
Sense and antisense sequences

Sequence	Strand
5' rCrArArGrGrCrArUrUrCrUrUrGrArArArGrArUrUrUrArCTA 3'	+
5' rUrArGrUrArArArUrCrUrUrUrCrArArGrArArUrGrCrCrUrUrGrCrG 3'	-

More

APPENDIX VI

miRNA target (DICER substrates) prediction parameters : RNAi design tool, Integrated DNA Technologies (IDT)

siRNA target prediction in virus genome

The siRNA targets in the whole genome of virus or full gene sequence of target gene were found using the RNAi design in Sci Tools available at the website of Integrated DNA Technologies (IDT). The duplex RNA GC% of 30-70% and the asymmetrical end stability base pair length of 5 was set along with other target parameters detailed in Table. The targets found in the CP region were selected and analysed for siRNA attributes described by Reynolds et al. (2004) for higher efficiency.

Parameters used for siRNA target prediction in DsMV genome

Method	Pattern	Position	Weight
Must Exclude	GGGG	0	N/A
Must Exclude	CCCC	0	N/A
Must Exclude	AAAA	0	N/A
Must Exclude	TTTT	0	N/A
Must Exclude	SSSS	0	N/A
Suggest Include	AA	1	0.5
Suggest Include	TT	-2	0.5
Suggest Include	S	3	0.5
Suggest Include	A	5	0.5
Suggest Include	A	8	0.5
Suggest Include	S	13	0.5

Suggest Include	W	15	0.5
Suggest Include	G	18	0.5
Suggest Include	H	21	0.5
Suggest Exclude	T	3	0.5
Suggest Exclude	C	8	0.5
Suggest Exclude	W	13	0.5
Suggest Exclude	G	15	0.5
Suggest Exclude	S	21	0.5

Attributes in selected siRNA target region

No.	Reynolds' Criteria	Attributes in selected siRNA target
I	30%-52% G/C content	✓
II	At least 3 'A/U' bases at positions 15-19 (sense strand)	✓
III	Absence of internal repeats	✓
IV	An 'A' base at position 19 (sense strand)	×
V	An 'A' base at position 3 (sense strand)	✓
VI	A 'U' base at position 10 (sense strand)	✓
VII	A base other than 'G' or 'C' at 19 (sense strand)	✓
VIII	A base other than 'G' at position 13 (sense strand)	✓

APPENDIX VII

Textual map showing positions of restriction endonuclease recognition sites

Restriction Map results

cuts once
cuts twice

Results for linear 421 residue sequence "Untitled" starting "ATGAGGCCGC"

```
                                                MseI t|taa 38
                                             MspI c|cgg 34
                                             HpaII c|cgg 34
                      SacII ccgc|gg 11
                      SstII ccgc|gg 11
                     BstUI cg|cg 10
                     AccII cg|cg 10
                  PhoI gg|cc 7
                  HaeIII gg|cc 7
  1   E  A  A  V  C  L  I  M  V  Q  P  V  K  S  Q  G  I  L  E  R
  1   *  G  R  G  M  S  H  Y  G  P  A  G  *  I  A  R  H  S  *  K
  1 M  R  P  R  Y  V  S  L  W  S  S  R  L  N  R  K  A  F  L  K
  1 ATGAGGCCGCGGTATGTCTCATTATGGTCCAGCCGGTTAAATCGCAAGGCATTCTTGAAA
  1          10        20        30        40        50
  1 TACTCCGGCGCCATACAGAGTAATACCAGGTCGGCCAATTTAGCGTTCCGTAAGAACTTT
                                                              AsuII tt|cgaa 117
                                                              TaqI t|cga 117
                              AluI ag|ct 85
                    SspI aat|att 74
 21   F  T  K  I  F  W  R  A  D  C  W  C  S  *  L  *  Y  I  R  K
 21   I  Y  *  N  I  L  A  S  *  L  L  V  *  L  I  V  I  H  S  K
 21 D  L  L  K  Y  S  G  E  L  I  V  G  V  V  D  C  D  T  F  E
 61 GATTTACTAAAATATTCTGGCGAGCTGATTGTTGGTGTAGTTGATTGTGATACATTCGAA
 61          70        80        90        100       110
 61 CTAAATGATTTTATAAGACCGCTCGACTAACAACCACATCAACTAACACTATGTAAGCTT
                                          NlaIII catg| 161
                                       Bsp19I c|catgg 157
                                       NcoI c|catgg 157
                                  SnaBI tac|gta 152
 41   C  I  Q  L  H  C  I  I  V  T  *  P  W  V  *  R  K  E  V  H
 41   V  H  T  T  S  L  H  H  C  Y  V  A  M  G  L  K  E  G  S  S
 41 S  A  Y  N  F  T  A  S  L  L  R  S  H  G  F  E  G  R  K  F
121 AGTGCATACAACTTCACTGCATCATTGTTACGTAGCCATGGGTTTGAAGGAAGGAAGTTC
121          130       140       150       160       170
121 TCACGTATGTTGAAGTGACGTAGTAACAATGCATCGGTACCCAAACTTCCTTCCTTCAAG
                                             PhoI gg|cc 224
                                             HaeIII gg|cc 224
 61   H  *  Y  *  R  D  I  S  E  F  E  Y  E  G  R  C  W  S  N  V
 61   S  L  I  L  T  R  Y  F  R  V  *  I  *  R  P  L  L  E  Q  C
 61 I  T  D  T  D  E  I  F  Q  S  L  N  M  K  A  A  V  G  A  M
181 ATCACTGATACTGACGAGATATTTCAGAGTTTGAATATGAAGGCCGCTGTTGGAGCAATG
181          190       200       210       220       230
181 TAGTGACTATGACTGCTCTATAAAGTCTCAAACTTATACTTCCGGCGACAACCTCGTTAC
                         SspI aat|att 263
    RsaI gt|ac 242
    AfaI gt|ac 242
 81   R  W  E  E  E  G  I  F  *  G  L  H  *  S  T  K  G  *  D  Y
 81   T  L  G  R  R  R  N  I  L  R  A  S  L  I  N  K  R  M  R  L
 81 Y  A  G  K  K  K  E  Y  F  E  G  F  T  N  Q  Q  K  D  E  I
241 TACGCTGGGAAGAAGAAGGAATATTTTGAGGGCTTCACTAATCAACAAAAGGATGAGATT
241          250       260       270       280       290
```


192

```
241 ATGCGACCCTTCTTCTTCCTTATAAAACTCCCGAAGTGATTAGTTGTTTTCCTACTCTAA
                                                    HinfI g|antc 354
                                                NdeI ca|tatg 344
101   I   P   E   L   S   S   P   I   *   R   T   L   R   H   M   E   R   S   L   E
101  Y   S   R   V   V   F   A   Y   I   K   D   T   *   A   Y   G   T   E   S   *
101 I   F   Q   S   C   L   R   L   Y   K   G   H   L   G   I   W   N   G   V   L
301 ATATTCCAGAGTTGTCTTCGCCTATATAAAGGACACTTAGGCATATGGAACGGAGTCTTG
301          310       320       330       340       350
301 TATAAGGTCTCAACAGAAGCGGATATATTTCCTGTGAATCCGTATACCTTGCCTCAGAAC
                                                      BstUI cg|cg 408
                                                      AccII cg|cg 408
                                                    MluI a|cgcgt 406
                                          MseI t|taa 395
                    PhoI gg|cc 376
                    HaeIII gg|cc 376
        AluI ag|ct 365
121   S   *   I   T   A   K   *   K   E   *   V   K   Q   N   T   R   V   H   S
121  K   L   N   Y   G   Q   M   K   R   M   S   *   T   K   H   A   C   S   Q   Q
121 K   A   E   L   R   P   N   E   K   N   E   L   N   K   T   R   V   F   T   A
361 AAAGCTGAATTACGGCCAAATGAAAAGAATGAGTTAAACAAAACACGCGTGTTCACAGCA
361          370       380       390       400       410
361 TTTCGACTTAATGCCGGTTTACTTTTCTTACTCAATTTGTTTTGTGCGCACAAGTGTCGT
141
141
141
421 G
421
421 C
```

Site:	Positions:
AatI agg|cct	none
AatII gacgt|c	none
Acc16I tgc|gca	none
AccII cg|cg	10, 408
AccIII t|ccgga	none
AclI aa|cgtt	none
AcvI cac|gtg	none
AfaI gt|ac	242
AfeI agc|gct	none
AflII c|ttaag	none
AgeI a|ccggt	none
AhlI a|ctagt	none
Alw441 g|tgcac	none
AluI ag|ct	85, 365
Aor51HI agc|gct	none
ApaI gggcc|c	none
ApaLI g|tgcac	none
AscI gg|cgcgcc	none
AseI at|taat	none
Asp718I g|gtacc	none

AsuII tt|cgaa	117
AvaI c|ycgrg	none
AviII tgc|gca	none
AvrII c|ctagg	none
BalI tgg|cca	none
BamHI g|gatcc	none
BanIII at|cgat	none
BbeI ggcgc|c	none
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BfrBI atg|cat	none
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BlnI c|ctagg	none
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Bsp19I c|catgg	157
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BspEI t|ccgga	none
BsrGI t|gtaca	none
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BstUI cg|cg	10, 408
ClaI at|cgat	none
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DraI ttt|aaa	none
EagI c|ggccg	none
EcoRI g|aattc	none
EcoRV gat|atc	none
EgeI ggc|gcc	none
FseI ggccgg|cc	none
FspI tgc|gca	none
HaeIII gg|cc	7, 224, 376
HincII gty|rac	none
HindIII a|agctt	none
HinfI g|antc	354
HpaI gtt|aac	none
HpaII c|cgg	34
KasI g|gcgcc	none

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MscI tgg|cca	none
MseI t|taa	38, 395
MspI c|cgg	34
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NarI gg|cgcc	none
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NdeI ca|tatg	344
NdeII |gatc	none
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NheI g|ctagc	none
NlaIII catg|	161
NotI gc|ggccgc	none
NruI tcg|cga	none
NsiI atgca|t	none
PacI ttaat|taa	none
PciI a|catgt	none
PhoI gg|cc	7, 224, 376
PmeI gttt|aaac	none
PmlI cac|gtg	none
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PvuI cgat|cg	none
PvuII cag|ctg	none
RsaI gt|ac	242
SacI gagct|c	none
SacII ccgc|gg	11
SalI g|tcgac	none
SbfI cctgca|gg	none
ScaI agt|act	none
SfoI ggc|gcc	none
SmaI ccc|ggg	none
SnaBI tac|gta	152
SpeI a|ctagt	none
SphI gcatg|c	none
SspI aat|att	74, 263
SstI gagct|c	none
SstII ccgc|gg	11
StuI agg|cct	none
SwaI attt|aaat	none

TaqI t|cga	117
TliI c|tcgag	none
VspI at|taat	none
XbaI t|ctaga	none
XhoI c|tcgag	none
XmaI c|ccggg	none

APPENDIX VIII

Luria-Bertani (LB) medium (g L^{-1})

Yeast extract	5
Tryptone	10
Sodium chloride	10
Agar	15
pH	7

APPENDIX IX

Chemicals for isolation of plasmid DNA from bacteria

(i) Solution I

20 % glucose	2.25 ml
0.5 M EDTA (pH 8.0)	1 ml
1M Tris (pH 8.0)	1.25 ml
Sterile distilled water	45.50 ml

(ii) Solution II

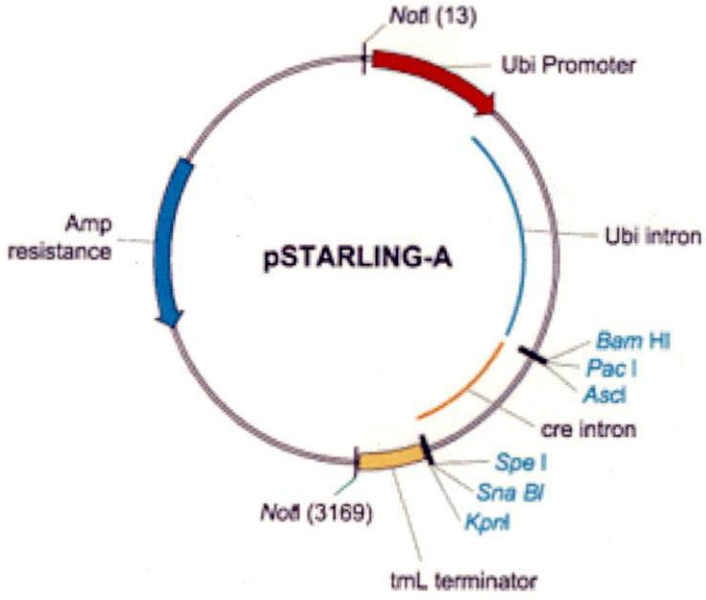
10 N NaOH	0.4 ml
20 % SDS	1 ml
Sterile distilled water	18.6 ml

(iii) Solution III

5 M sodium acetate	60 ml
Glacial acetic acid	11.5 ml
Sterile distilled water	28.5 ml

APPENDIX X

Nucleotide sequence and structure of the ihpRNA vector pSTARLING, (CSIRO)



Vector description:

This is a pUC based vector with a novel set of restriction sites. The cassete pepared can be mobilised to the binary vector as NotI fragment. The *Not*I fragment contained the components such as the *Zea mays* Ubiquitin promoter, ubiquitin intron, cyclic AMP response element (*cre*) intron with multiple cloning sites (MCS) comprising of *Bam*HI, *Pac*I and *Asc*I at the 5' end and *Spe*I, *Sna*BI and *Kpn*I at its 3' end and tmL (tumor morphology locus) terminator.

```
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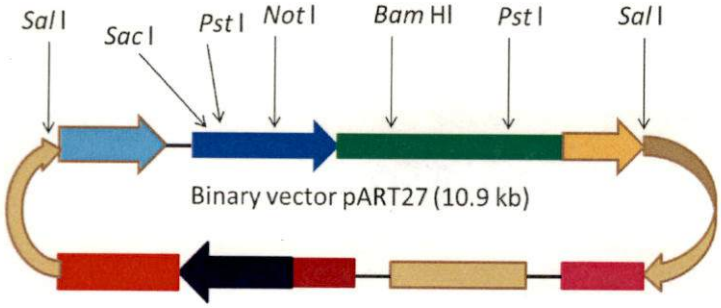
APPENDIX XI

Physical map and nucleotide sequence of the binary vector pART27.



Left border

lacZ

oriT

ColE1 replicon

oriV RK2

Chimaeric *npt*II gene

Right border

*trf*A

Spectinomycin/ Streptomycin resistance gene

>pART27

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APPENDIX XII

Chemicals for isolation of DNA from banana leaves

CTAB Buffer

CTAB	2 %
Tris-HCl (pH8.0)	100 mM
NaCl	1.4 M
EDTA	20 mM
2, β- mercapto ethanol	0.1 %

TE buffer (pH 8.0)

Tris buffer	10 mM
EDTA	1 mM

50X TAE buffer (pH 8.0)- 1000 ml

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

DEVELOPMENT OF SMALL INTERFERING RNA (siRNA) MEDIATED RESISTANCE IN BANANA AGAINST *BANANA BRACT MOSAIC VIRUS*

by

LEKSHMI R. S.
(2011-21-103)

Abstract of the
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ABSTRACT

The present study entitled "Development of small interfering RNA (siRNA) mediated resistance in banana against *Banana bract mosaic virus* (BBrMV)" was carried out during 2012-2016 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The study was carried out with an objective to develop siRNA mediated technology for the development of banana plants resistant to *Banana Bract Mosaic Virus* (BBrMV). The study was conducted in banana cv. Nendran.

A protocol for somatic embryogenesis in banana cv. Nendran was standardized by using immature male flowers as explants. Pale white friable callus with rich cytoplasm was initiated in Murashige and Skooge (MS) medium supplemented with BA (0.1 – 0.5 mgL^{-1}) and picloram (0.5 – 2 mgL^{-1}) incubated in dark with a maximum explant response of 30 per cent. For embryogenesis, the developed embryogenic calli were transferred to semisolid MS medium supplemented with BA 2 mgL^{-1} and IAA 0.5 mgL^{-1} which resulted in a maximum of 60 per cent embryogenesis. The glassy elongated monocot embryos were germinated in half strength semisolid MS medium (0.3 per cent Gelrite) supplemented with BA 2 mg L^{-1} and IAA 0.5 mg L^{-1} and incubated in dark. A maximum germination rate of 80 per cent was obtained in this medium. The germinated embryos were transferred to MS medium with BA 2 mg L^{-1} and NAA 1 mg L^{-1} resulted in 100 per cent Plant regeneration. The plantlets were transferred to coirpith compost in pot trays in mist chamber for one month for hardening and then transferred to polybags with soil and cowdung (1:1) mixture.

To develop siRNA technology to silence the replicase gene of BBrMV, an intron hairpin RNA (ihpRNA) construct was developed. For this a partial mRNA sequence of replicase gene was isolated from BBrMV banana plants. Gene specific primers designed based on the whole genome sequence information retrieved from the GenBank, NCBI. Total RNA from infected banana leaves was isolated and cDNA was prepared using RT-PCR. The partial gene fragment

isolated was sequenced and analysed using the bioinformatics tool BLAST. The sequence was subjected to miRNA target prediction and restriction mapping to select suitable region for the construct and further processing. Based on this information a fragment of 400 bp towards the 5' end was amplified by designing a set of primers with anchored restriction sites. The primers anchored with *Bam*HI and *Pac*I sites were used for the amplification of sense strand and primers anchored with *Kpn*I and *Spe*I sites were used for antisense strand amplification. The sense and antisense fragments amplified were cloned to pTZ57R/T cloning vector.

In the next step the inserts were released from pTZ57R/T using the corresponding restriction enzymes and were integrated in pSTARLING (primary vector), on either side of the *cre* intron which facilitated the formation of the hairpin (ihpRNA) construct. Presence of the inserts was confirmed by restriction digestion and electrophoresis. The ihpRNA construct in pSTARLING now contained ubiquitin promoter, ubiquitin intron, sense strand of replicase gene, *cre* intron, antisense strand of replicase and termination sequence in the order with the *Not*I restriction sites. This construct was released from pSTARLING and ligated to the digested *Not*I site in the lacZ gene of the binary vector pART27 containing antibiotic resistance marker *nptII* and *spec*. The binary vector was confirmed for the insert by transferring to DH5α and colony selection by blue-white screening. The binary vector with the insert isolated from the white colony, was transferred to *Agrobacterium tumefaciens* strain LBA 4404 via freeze-thaw method. Transformed colonies were picked up and confirmed the presence of the vector and the ihpRNA insert by PCR.

Somatic embryos were transformed with LBA 4404 carrying the ihpRNA construct the ihpRNA construct and the transformed embryos were selected with antibiotic pressure (Kanamycin 100 mg L^{-1}). Transformed embryos were subjected to regeneration. A maximum regeneration of 25 per cent was obtained after transformation. The regenerants were confirmed for the presence of ihpRNA construct using PCR with specific primers for sense-intron-antisense fragment,

npt II and *cre* intron. The study was successful in developing a siRNA construct for resistance against BBrMV and obtaining transformed Nendran banana plantlets.

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