# ihpRNA MEDIATED RESISTANCE FOR *BANANA BRACT MOSAIC VIRUS* IN *MUSA* SPP. BY TARGETING REPLICASE AND MOVEMENT PROTEIN GENES

**EKATPURE SACHIN CHANDRAKANT**
**(2016-21-022)**

**DEPARTMENT OF PLANT BIOTECHNOLOGY**
**COLLEGE OF AGRICULTURE, VELLAYANI,**
**THIRUVANANTHAPURAM-695 522**
**KERALA, INDIA**

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*By*

**EKATPURE SACHIN CHANDRAKANT**
**(2016-21-022)**

**THESIS**

**Submitted in partial fulfillment of the requirement**

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**DEPARTMENT OF PLANT BIOTECHNOLOGY**
**COLLEGE OF AGRICULTURE, VELLAYANI,**
**THIRUVANANTHAPURAM-695 522**
**KERALA, INDIA**
**2020**

## DECLARATION

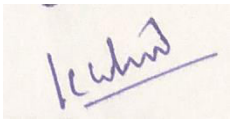
I hereby declare that the thesis entitled **"ihpRNA mediated resistance for *Banana bract mosaic virus* in *Musa* spp. by targeting replicase and movement protein genes."** is a bonafide record of research work done by me during the course of research and that the thesis has not been previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

**Place: Vellayani**
**Date: 18/08/2020**

**EKATPURE SACHIN CHANDRAKANT**
(2016-21-022)

## CERTIFICATE

Certified that the thesis entitled **"ihpRNA mediated resistance for *Banana bract mosaic virus* in *Musa* spp. by targeting replicase and movement protein genes."** is a record of research work done independently by **Mr. Ekatpure Sachin Chandrakant** (2016-21-022) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.



**Dr. K. B. Soni**
(Chairman, Advisory Committee)
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram 695 522

Place: Vellayani
Date: 18/08/2020

## CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Ekatpure Sachin Chandrakant** a candidate for the degree of **Doctor of Philosophy in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"ihpRNA mediated resistance for *Banana bract mosaic virus* in *Musa* spp. by targeting replicase and movement protein genes."** may be submitted by **Mr. Ekatpure Sachin Chandrakant** in partial fulfilment of the requirement for the degree.

**Dr. K. B. Soni**
(Major advisor, Advisory Committee
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani

**Dr. Swapna Alex**
Member (Advisory committee)
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani,
Thiruvananthapuram, Kerala

**Dr. Joy M.**
Member (Advisory committee)
Associate Professor and Head
Department of Plant Pathology
College of Agriculture, Vellayani,
Thiruvananthapuram, Kerala

**Dr. R. V. Manju**
Member (Advisory committee)
Professor,
Department of Plant Physiology
College of Agriculture, Vellayani,
Thiruvananthapuram, Kerala

**Dr. T. Makeshkumar**
Member (Advisory committee)
Principal Scientist,
Division of Crop Protection
ICAR- Central Tuber Crop Research
Institute, Thiruvananthapuram, Kerala

**Dr. J. Ramalingam**
(External Examiner)
Professor and Head
Department of Biotechnology
Agricultural College and Research Institute
TNAU, Madurai 625 104

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF PLATES

## LIST OF APPENDICES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| @ | At the rate |
| = | Equal to |
| 2,4-D | 2,4-Dichlorophenoxy acetic acid |
| BA | Benzyl adenine |
| BBrMV | *Banana bract mosaic virus* |
| bp | Base pair |
| CP | Coat protein |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| cv. | Cultivar |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dsRNA | Double stranded RNA |
| E. coli | *Escherichia coli* |
| EST | Expressed sequence Tags |
| h | Hour |
| IAA | Indol-3-Acetic Acid |
| IMFs | Immature Male Flowers |
| L | Litre |
| LB medium | Luria – Bertani medium |
| M | Molar |
| mg | Milligram |
| Min | Minute |
| miRNA | Micro RNA |
| mM | Milli Molar |
| MP | Movement protein |
| MS | Murashige and Skoog medium |
| NAA | Naphthalene acetic acid |

| | |
|---|---|
| nt | Nucleotide |
| ºC | Degree Celsius |
| PCR | Polymerase chain reaction |
| PDR | Pathogen derived resistance |
| pH | Hydrogen ion concentration |
| pM | Pico Mole |
| PTGS | Post transcriptional gene silencing |
| PVP | Poly vinyl pyrrolidone |
| Rep | Replicase |
| RISC | RNA induced silencing complex |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| s | Second |
| siRNA | Small interfering RNA |
| TAE | Tris Acetate EDTA |
| TBE | Tris Borate EDTA |
| TDZ | Thidiazuron |
| TE | Tris-EDTA buffer |
| TGS | Transcriptional gene silencing |
| UV | Ultra violet |
| V | Volt |
| µg | Micro gram |
| µl | Micro litre |

# *Introduction*

# 1. INTRODUCTION

Banana and plantains (*Musa* spp.) are the world's most important fruit crops and the staple meal for millions of people. There are more than 1000 varieties of banana produced around the world. Banana holds the first place by production volume and is among the five most consumed fruits in the world (FAO, 2018). It is a nutritious and healthy source of carbohydrate, potassium, vitamin B6 and C, fibers, antioxidants and various useful plant nutrients. In India, banana ranks second among the most important fruit crops next to mango. Banana crops can be grown in a diverse range of environments and production systems. Its availability round the year, less price with diverse varietal range, flavor, nutritive and therapeutic value and high export prospective make it preferred fruit among all classes of people. Small scale farmers from developing countries (tropical and subtropical regions) grow bananas as staple fruit as well as cash crop generally for the local market.

Banana cultivation in India is hindered by a variety of environmental challenges. Among them, viruses are the important biotic threats due to their serious effects on yield and quality of banana. Four major viruses *viz., Banana bunchy top virus* (BBTV), *Banana streak virus* (BSVs), *Banana bract mosaic virus* (BBrMV) and *Cucumber mosaic virus* (CMV) are identified as serious threats that affect the production of banana and plantain. Banana bract mosaic disease (Kokkan disease) caused by BBrMV is a lately described viral 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*., 1997, Selvarajan and Singh, 1997, Thangavelu *et al*., 2000, Cherian *et al*., 2002, Kiranmani *et al*., 2005). In Kerala the disease was first reported in Thrissur region in the variety Nendran, which is found to be highly vulnerable (Samraj *et al*., 1966). The prevalence of this disease was between 5 - 36 per cent and more in cv. Nendran in Kerala (Selvarajan *et al*., 2006). Later the disease was found to have an effect on other varieties like Palayankodan, Karpooravally, Monthan, Chenkadali, Poovan (Rasthali), Kodappanillakunnan and Kanchikela.

BBrMV has been identified as a potyvirus which is transmitted by the aphid species *Pentalonia nigronervosa*, *Aphis gossypii* and *Rhopalosiphum maidis* in non-persistent manner. The virus can also be spread through 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 very smaller bunches bearing brittle fruits. In severe cases, infected 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).

As of now no resistance sources have been reported for BBrMV disease (Rodoni *et al*., 1997). Use of virus free planting material and phytosanitary precautions are the only preventive measures (Kumar *et al*., 2015). However these preventive measures are inadequate to completely control the viruses. Enhancing plant population resistance is the most effective method for control of viral diseases. Cross-protection and utilization of natural resistance sources in plants are the conventional methods to combat infectious pathogens in bananas. But the lack of natural sources of resistance, low female fertility and poor seed setting make the conventional breeding methods difficult in banana (Kleidon *et al*., 2020). Most of the popular varieties are triploid in nature and this demands alternate strategies for banana improvement. Advancement in genetic engineering in monocot plants has made it possible to transfer the identified genes into plants against diseases and pests (Lal, 2020). The majority of the virus resistant transgenic plants are developed through the pathogen derived resistance (PDR), which is mediated either by proteins determined by transgenes or by the transcripts produced from the inserted transgene.

RNA silencing has turned out to be an advanced molecular approach for controlling viral diseases. The RNAi technique was commonly characterized in eukaryotic organisms as a conserved regulatory mechanism for gene expression. This

mechanism plays an important regulatory role in plant development and also functions as a natural antiviral defense mechanism. It suppresses gene expression at RNA level using double-stranded RNA (dsRNA) expressed as a hairpin RNA (ihpRNA) construct which interferes with the expression of specific endogenous genes or genes encoded by invading pathogens (Uslu and Wassenegger, 2020). In plants, ihpRNA constructs were preferred as they give higher gene silencing efficiency than intron-free hpRNA ones since, the intron spacer makes the interaction of the two arms of the hairpin more probable (Dang *et al*., 2014).

Small RNAs are the most important mediators of RNA silencing- associated 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 produced by transcriptional or post-transcriptional pathways which are later processed by dicer enzyme to generate small RNAs which leads to gene silencing. Waterhouse *et al*. (1998) first showed the RNAi technology for developing virus resistance in plants against *Potato virus Y* (PVY). This technique was later used to develop resistance against a number of other viruses (Hilly *et al*., 2007; Fahim *et al*., 2010; Praveen *et al*., 2010; Rupp *et al.*, 2019; Worrall *et al*., 2019; Luan *et al*., 2020; Tatineni *et al*., 2020).

The concept of RNAi was investigated in the present study with the objectives of developing the ihpRNA construct targeting replicase and movement protein genes of the *Banana bract mosaic virus*, generating transformants of the banana cultivar Grand Naine carrying the ihpRNA cassette and confirming the transformants.

# Review of Literature

## 2. REVIEW OF LITERATURE

Bananas and plantains are monocotyledonous herbaceous plants belonging to the genus *Musa* (Musaceae, Zingiberales). Center of origin of bananas is the Southeast Asian rainforest (Simmonds, 1962). The vast majority of banana cultivars are the hybrids of two wild species i.e. *Musa acuminata* and *Musa balbisiana* developed from inter and intraspecific crosses (Simmonds and Shepherd, 1955), with the genome constitution AA (*M. acuminata*) or BB (*M. balbisiana*). Most of the modern domesticated edible parthenocarpic 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 types. Seedless diploids (AA & AB genomic group) and tetraploids (AAAA, AAAB, AABB, ABBB genome group) are also under cultivation (Heslop-Harrison and Schwarzacher, 2007).

Banana is a major crop in the tropical and subtropical regions of the world, with an approximate production of 148 million tonnes per annum. India is rich in genetically diverse varieties of banana, cultivated over an area of 898 thousand hectares, with a production of 31747 thousand Metric tonnes (MT) (2018-19). India leads the production of bananas by supplying more than a quarter (25.58 per cent) of total production globally. The area under banana cultivation in Kerala during 2017-2018 was 109.26 thousand hectares with 1119.16 thousand MT production and 10.24 MT/Hector productivity (Agricultural Statistics, 2018-19).

The banana crop can be cultivated in a variety of environments and production systems, and provides revenue sources throughout the year. Banana is of great importance to small scale farmers in the tropical and sub-tropical developing countries, where it is cultivated as a staple fruit and as a cash crop primarily for the local market. In India, banana cultivation is hampered by diverse environmental challenges. Viruses pose major biotic threats to banana cultivation due to their negative impact on yield and quality.

## 2.1 VIRAL DISEASES OF BANANA

Viruses cause substantial losses in the annual yield and quality of banana crops. *Banana bunchy top virus* (BBTV) of the genus *Babuvirus*, *Banana streak virus* (BSV) of the genus *Badnavirus, Banana mild mosaic virus* (BanMMV) of the genus *Banana virus X* (BVX), *Cucumber mosaic virus* (CMV) of the genus *Cucumovirus* and *Banana bract mosaic virus* (BBrMV) of the genus *Potyvirus* are relatively well characterized and the major threatening viruses of banana (Diekmann and Putter, 1996).

## 2.2 BANANA BRACT MOSAIC DISEASE (KOKKAN DISEASE)

Banana bract mosaic disease, also known as Kokkan disease, is caused by *Banana bract mosaic virus* (BBrMV), which is a member of the genus *potyvirus* and family *Potyviridae*. BBrMV is a 750 nm long flexuous filamentous virus (Singh *et al*., 2000) with a single stranded positive-sense RNA of about 9.7 kb in length. It serves as messenger RNA and can be translated into protein in the host cell (Balasubramanian and Shelvarajan, 2012). The RNA dependant RNA polymerase (RdRP), a viral protein encoded by the virus synthesizes RNA from a RNA template while host cell proteins (RNA binding protein, chaperon protein, membrane remodeling and lipid synthesis proteins) are employed by virus for its replication. BBrMV codes for a single polypeptide processed with three viral proteinases (P1, HC-Pro and NIa-Pro) produce 11 mature protein products necessary for infection (Chung *et al*., 2008; Wei *et al*., 2010).

Such viral proteins are polyproteins from N to C terminus: P1 (first protein), HC-Pro (helper component/proteases), P3 (third protein), P3N-PIPO (from the frame-shift in the P3 cistron), 6K1 (first 6 kDa peptide), CI (cylindrical inclusion protein), 6K2 (second 6 kDa peptide), NIa-VPg (nuclear inclusion ‘a’ - viral genome-linked protein; also VPg), NIa-Pro nuclear inclusion ‘a’ protein – the protease), NIb (nuclear

inclusion ‘b’ protein), and CP (coat protein) (Riechmann *et al*., 1992; Urcuqui-Inchima *et al*., 2001; Wei *et al*., 2010).

NIa-VPg (N-terminal component of NIa) is also called as the VPg domain of 11 mature proteins, which is active in the transcription and transport of viral genomes (Urcuqui- Inchima *et al*., 2001). The protein PIPO (pretty interesting potyviridae ORF) is formed in the P3 cistron by +2 frame shifting. These proteins’ crucial role includes cell to cell movement and replication or combo of these functions (Chung *et al*., 2008). Nevertheless, other potyviral proteins, including HC-Pro, Cl, VPg, and P3N-PIPO, have been shown to be active in the viral cell to cell movement (Dunoyer *et al*., 2004; Hofius *et al*., 2007; Wei *et al*., 2010; Vijayapalani *et al*., 2012; Ivanov *et al*., 2014).

BBrMV disease was first observed in the Philippines in 1988 (Magnaye and Espino, 1990). In India, BBrMV is observed in Kerala, Andhra Pradesh, Karnataka, Tamil Nadu and Andhra Pradesh (Rodoni *et al*., 1997, Thomas *et al*., 1996, Selvarajan and Singh, 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 (Samraj *et al*., 1966). Even though it affects all the varieties, Nendran cultivar is an extremely susceptible variety. The disease has also been reported in Sri Lanka, Vietnam, Thailand and Western Samoa (Rodoni *et al*., 1999) and included in the list of quarantine importance (Ferison and Putter, 1989). Whole genome sequence of the Indian BBrMV-TRY (Trichy) isolate and BBrMV-PHI (The Philippines) isolates showed 94 percent sequence similarity and 88-98 per cent amino acid identities (Balasubramanian and Selvarajan, 2012)

### 2.2.1 Symptoms

In most cultivars, disease is marked by lesions, lines, strips or sometimes spindle shaped discoloration on pseudostems that are seen after the removal of dead

sheath of the leaves. The most obvious sign during flowering is mosaic patterns in bracts. On petiole, particularly at the position where they emerge from the pseudostem, chlorotic or reddish streaks or spindle formed patterns might also be seen. Those patterns may later turn dark brown. Peduncles can also be seen with chlorotic streaks and stripes, and even sometimes in leaves (Thomas *et al*., 1999). Leaf veins can also become conspicuous, although this occurrence has been linked with other plant diseases.

From the third month onwards, the BBrMV infected plant exhibits necrotic streaks. With the senescence of the affected portion, it disappears most of the time. The infected plants flowers, but produce very small bunches with curved weak fruits. Severely affected plants may fail to produce the flowers and may die by stunted growth and necrosis of pseudostem (Selvarajan *et al.*, 2006). The male buds will appear dark purple in colour with mosaic patterns. There are varietal differences in the symptomatology of the disease.

### 2.2.2 Transmission

BBrMV can affect the banana plant at various growth stages. The BBrMV virus is transmitted by three species of aphids, *Pentalonia nigronervosa*, *Aphis gossypii* and *Rhopalosiphum maidis*, non-persistently (i.e. virus particles are maintained by the vector for a short period of time) (Magnaye and Espino, 1990). Cowpea aphid, *Aphis craccivora* can also transmit the virus (Selvarajan *et al.*, 2006). Infected planting materials can also spread the virus.

Selvarajan *et al*. (2020) showed the distribution of virus in the various parts of the seeds, flower and leaf tissues of seedlings from banana plants of a synthetic diploid (H-201) by Plate-trapped antigen coating enzyme –linked immunosorbent assay (PTA-ELISA), reverse transcriptase PCR (RT-PCR), quantitative RT-PCR (qRT-PCR) and sequencing. RT-PCR and qRT-PCR showed the presence of BBrMV

in embryo, endosperm and seed coat of seeds from the infected plants. BBrMV infection was confirmed by sequencing the RT_PCR amplicons. qRT-PCR results showed that virus load was maximum in the endosperm followed by seed coat and embryo. According to the seed growth test, thirteen seedlings raised *in vitro* from infected embryos expressed prominent symptoms of the BBrMV and also confirmed positive for virus through RT-PCR. Natural host of BBrMV includes *Musa* spp. and small cardamom in India (Siljo *et al*., 2011) and flowering ginger (*Alpinia purpurata*) in Hawaii (Wang *et al*., 2010).

### 2.2.3 Losses

BBrMV has been reported to cause considerable damage with yield reduction in cv. Robusta (AAA) (70 percent), followed by cv. Nendran (AAB) (52 per cent). 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).

In order to find out the yield loss due to BBrMV during 1997-1999 Shelvarajan *et al.* (2006) performed fixed field plot trials in Tiruchirapalli District of Tamil Nadu and recorded an average yield loss of 30 per cent in cv. Nendran (AAB). The reduction in bunch weight over healthy plants was highly significant. There were also symptoms of this disease in many other commercial cultivars in the subgroups AA, AB, AAA, AAB and ABB. They also observed malformed bunches of less weight and underdeveloped fingers. Roperos and Magnaye (1991) reported high yield losses of up to 40% due to this disease.

### 2.2.4 Management of *Banana Bract Mosaic Virus*

The BBrMV disease management has been categorized in to following ways:

#### 2.2.4.1 Cultural practices

Selvarajan *et al.* (2009) conducted an experiment to find out the effect of higher fertilizer doses on healthy and BBrMV infected plants of cv. Ney Poovan. The results indicated that increased doses of fertilizer, i.e. 125 and 150 per cent, have reduced the yield loss in plants infected with BBrMV. Higher dose of fertilizer application and managing the vector population through insecticide could effectively mitigate the disease. Due to virus severity in ratoon crops, plants were unable to respond for higher doses of the fertilizer.

#### 2.2.4.2 Biological Control

The use of fungal bio-control agent *Verticillium lecanii* is suggested to reduce aphid's populations. Soap solution sprays were also used to control the aphid population (Alavo, 2015).

#### 2.2.4.3 Chemical control

For aphids vector control integrated approach of preventive measures alongside the biological treatments can also be utilized as there is no direct chemical treatment available for viral disease control. Nevertheless, pesticide use can regulate aphid populations to some extent (for example cypermethrin, acetamid and chlorpyrifos). Herbicides may be used to destroy infected plants or growing young shoots from the infected plants (Abdel-Moniem, 2003).

## 2.3 APPROACHES TO INDUCE RESISTANCE AGAINST VIRUS DISEASES

Viral diseases in agricultural crops constitute a major problem to food safety in the developing world. Plant breeding for virus resistance is often considered the most competent and simplest way to avoid the losses due to viral diseases. Resistance mechanisms are very diverse and interrelate with various stages of the virus cycle in the host plant. Resistances may also differ in their specificity, constancy and

durability (Lecoq *et al*., 2004). There are two major strategies followed against viruses: one which includes developing resistance against viruses by introducing the resistance gene in the host plant. Another strategy is preventing the virus transmission by targeting vectors (Groen *et al*., 2017). The specificity of the viruses varies with their ability to infect hosts. Some can infect different hosts while others can infect one specified species only.

New virus strains emerge due to mutations in their genome (Mangrauthia *et al.*, 2008; Jones, 2009). Knowledge of pathogenic strains and their mode of infection in the host plants are essential for developing the strategies for controlling the disease. Different approaches were used to limit the spread of the virus throughout the plant or plant population. Results from epidemiological studies could indicate the principal route by which the virus reaches to its host and its mechanism of inoculation (Gilligan and Van den Bosch, 2008, Rodrigues *et al.*, 2009). Viruses may be transmitted by infected seed, by vectors or by normal agricultural practices (Fereres and Moreno, 2009; Dieryck *et al.*, 2009). The use of certified planting or seed material may significantly reduce the occurrence of certain viruses of seed borne nature (Novy *et al.*, 2007). However, vector control and the introduction of clean agricultural practices will significantly limit the spread of virus diseases (Castle *et al.*, 2009; Fereres and Moreno, 2009).

Virus reaches a healthy plant cell by interrupting the plasma membrane by a process known as inoculation (Rodrigues *et al.*, 2009). Particles of virus replicate and spread within the host cell via plasmodesmata (PD) and vascular bundles and this requires a compatible interaction between the virus and the host plant cell (Taliansky *et al.*, 2008). The virus-host plant partnership dictates the strength of those processes. The set of plant resistance responses intended to reduce the virus replication (Ascencio-Ibanez *et al.*, 2008). Movement of a virus particle within the host cell requires support from virus and host encoded proteins. Many viruses attain their

movement through virus encode movement proteins (MP) by attaching to the nucleic acid and target which dilates the PD (Lucas, 2006).

A feasible strategy to reduce the virus-induced crop damage is to develop high resistant cultivars (Ma *et al.*, 2004). Another way to increase the resistance responses is by using the attenuated strains of the virus (Ichiki *et al.*, 2005). Understanding the biochemistry of virus infection, such as RNA silencing, has contributed to advanced methods for successfully controlling viral diseases (Tenllado *et al*., 2003).

## 2.4 CROP PROTECTION BASED ON ENGINEERED RESISTANCE

Natural sources of resistance to many viral diseases of tropical crops are known and already employed in the plant breeding programme. In most scenarios, however, resistance sources are deficient, or the genetic complication and complicatedness in introgressing resistance genes to cultivars by crossing obstruct the efforts of cop improvement. Hence, development and transfer of resistance to crop plants by biotechnological means offers a striking alternative solution.

Sanford and Johnston (1985) developed the concept of pathogen derived resistance (PDR). Since then attempts have been made to develop transgenic plants using virus derived genes or genome fragments (Lomonossoff, 1995). Beachy's 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 precondition for using PDR is that there shouldn't be interaction with important host functions. PDR can be induced resistance by protein or by nucleic acid. Replicase, movement protein, proteases and most notably CP are the viral proteins used for PDR (Tepfer, 2002). The observation that transgenic RNA, rather than the expressed viral proteins, was responsible for the observed resistance, created new opportunities on RNA-mediated resistance (Tenllado *et al.*, 2004).

Multiple strategies to engineer resistant plants were developed rapidly, based on RNA or protein mediated resistance (PMR). The preliminary PMR study used CP gene expression of the *Tobacco mosaic virus* (TMV) to develop 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 viral CP gene was the first and most commonly used gene to confer PDR against plant viruses (Prins *et al*., 1995, 2008). Virus resistance has been achieved by transforming the plants with viral CP genes which ultimately showed resistance against viral infection; similarly transformation of plants to express non-structural viral proteins (Golemboski *et al*., 1990), truncated and defective viral genes also provided the protection against the homologous viruses (Anderson *et al*., 1992). The studies of Abel *et al*. (1986) showed that transformation of tobacco plants to express the coat protein of TMV made the plants resistant to this viral disease. Resistance was later proven to be mediated by the CP transgene RNAs rather than the protein as there was an inverse correlation between resistance and aggregation rates of CP transgene mRNAs. These results created much enthusiasm and hope for a rapid answer to viral disease problems in crop plants. This mechanism of resistance indicated that post-transcriptional gene silencing (PTGS) (i.e., RNA silencing or RNAi) (Brodersen and Voinnet, 2006) played its role involving the *CP* RNA-mediated protection (Jan *et al.*, 1999). Amudha *et al.* (2011), developed transgenic cotton with antisense coat protein gene which showed significant resistance against *Cotton leaf curl virus* (CLCuV).

Systemic infection of plant cells by viruses requires several critical steps. An important step in this process is the capacity of the virus to encode one or more movement proteins (MPs) that interact with PD, increase the PD size exclusion limit and mediate the cell-to cell movement of viral nucleic acids i.e. viral ribonucleoprotein (vRNP) such as DNA or RNA and virions. Viruses are then

transported to other plant organs by long distance movement (systemic movement) within the host plant (Zwart *et al*., 2012). Researchers attempted to develop pathogen-derived resistance with dominant negative mutants of viral genes. This approach was successfully demonstrated in producing transgenics using dysfunctional MP (Lapidot *et al*., 1993; Malyshenko *et al*., 1993). In another study, tobacco plants transformed to express a defective TMV movement protein (TMV-MP) showed resistance to *Tobacco rattle tobo virus, Tobacco ringspot nepo virus, Alfalfa mosaic alfamo virus* and *Cucumber mosaic virus* (Cooper *et al.*, 1995). The transgenically expressed functional MP had 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 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 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 nucleotide (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 degrade the 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 heritability. 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, 1999; Fire *et al.*, 1998, 1999), this phenomenon has been extensively used to suppress gene function in plants.

Napoli *et al*. (1990) shown the silencing effect in plants for the first time, when exogenous transgenes were introduced 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. 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 transgenes themselves inactive, but that the added DNA sequences also affected expression of the endogenous loci (Hannon, 2003). This phenomenon was referred to as "co-suppression" (Napoli *et al*., 1990). 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, 2003).

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 technique, particular, important genes

can be targeted in a parasite for silencing and thus it is disabled. 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 genes without protein expression. RNA interference exists in many eukaryotes and regulates the gene expression functions. 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). dsRNA induced silencing of the gene occurs by three distinct pathways. The first mechanism is silencing of cytoplasmic siRNA (Hamilton *et al.*, 1999). This pathway is thought to have developed as a defense mechanism against plant viruses, where the dsRNA may originate from a single stranded viral intermediate replication or secondary structure characteristics. dsRNA may be formed in plants by the DNA viruses through the annealing of complementary transcripts that overlap. 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. Short term interfering RNAs (siRNAs) and microRNAs (miRNAs), are RNAi hallmarks (Baulcombe, 2004 and Meister *et al.*, 2004).

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 processability. 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) suggested 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 colored petals were produced by Katsumoto *et al.* (2007) by using RNAi technology.

Shekhawat *et al*. (2012) successfully transformed the susceptible cultivar of banana (cv. Rasthali), with replication protein gene 'Rep' full coding sequence and 'Pro-Rep' which is partially coding replicase gene with 5' partial upstream regulatory region of the BBTV. Generated plants were resistant to BBTV up to six months post inoculation with viruliferous aphids.

## 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) shares 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 fighting 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, which includes hairpin RNA expressing vectors, virus induced gene silencing (VIGS), *Agrobacterium*-mediated plant transformation and gene gun method (Burch-Smith *et al*., 2004).

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 ringspot 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 showed complete resistance to ZYMV and PRSV W, from which virus accumulation was 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.

Rodriguez-hernandez *et al*. (2012) developed the transgenic melon (*Cucumis melo* L.) plants through RNAi technique by targeting the eukaryotic translation initiation factor (elF) (175 nt), as the 4E family factor is necessary for the multiplication of the diverse viruses. They generated the transformants through *Agrobacterium* mediated transformation (strain EHA105) by knocking down *Cm-elF4E*, and characterized the T2 generation genetically and phenotypically. In

transformed plants they found that the *Cm-elF4E* gene was silenced and decreased accumulation of *Cm-elF4E* mRNA. They challenged the transformants with eight agronomically valuable melon infecting viruses, and identified that plants were resistant to *Cucumber vein yellowing virus* (CVYV), *Melon necrotic spot virus* (MNSV), *Moroccan watermelon mosaic virus (*MWMV*)* and *Zucchini yellow mosaic virus* (ZYMV), which indicated that *Cm-elF4E* controls the melon crop susceptibility to these four viruses. Also they suggested that *Cm-elF4E* was an efficient target for the identification of new resistance alleles for broad spectrum virus resistance.

Chen *et al*. (2016) developed the transgenic tomato plants through RNAi technique by targeting six segments of the monopartite genome of *Tomato leaf curl Taiwan virus* (ToLCTWV). Transformants were tested for their capacity to lower symptoms and inhibit viral DNA accumulation. A RNAi construct containing fusions of C1, C3 (encodes proteins responsible for virus replication) and C2 (regulation of gene expression) gene sections of ToLCTWV and the corresponding segments of the bi-partite *Tomato yellow leaf curl Thailand virus* (TYLCTHV) DNA genome were introgressed into tomato line CLN1621L. Tomato R1 and R2 lines were challenged with viruliferous whiteflies in separate screen houses for ToLCTWV and TYLCTHV. ToLCTWV (16 plants) and TYLCTHV (12 plants) R2 plants from one primary transformant remained symptomless up to three weeks after the exposure to the respective viruses. Real time PCR showed very low titres of viral DNA and their results suggested that expression of bi-viral RNAi constructs in tomato plants can lead to resistance against two different tomatoes affecting begomovirus spp.

Kumar *et al*. (2017) employed RNAi technique to control the Cowpea infecting *Mungbean yellow mosaic India virus* (MYMIV). They developed transgenic cowpea plants containing three different ihpRNA constructs harbouring *AC2, AC4* and combination of *AC2* and *AC4* (*AC2+AC4*) of seven cowpea infecting begomoviruses. All the generated T0 and T1 transgenic cowpea lines of the three

RNAi constructs accumulate virus transgene specific siRNAs. They assessed transgenic plants up to T1 generation to check resistance to MYMIV using agro infectious clones. They found 100 per cent resistance to MYMIV infection in transgenic lines expressing *AC2-hp* and *AC2+AC-hpRNA* when compared to the non transformed control plants.

Ramesh *et al*. (2019) showed the effectiveness of *Agrobacterium*-mediated transient expression of short hairpin RNA (shRNA) by targeting the conserved region of *AC2* ORF of MYMIV which confer virus resistance in soybean. Transient expression of shRNA showed reduction in virus titre. Also they found the newly emerged leaves showing symptom recovery. They proved that the *AC2* gene of MYMIV is a strong target gene for imparting ihpRNA mediated resistance in the soybean crop.

Rupp *et al*. (2019) developed transgenic wheat lines by targeting TaeIF(iso)4E and TaeIF4G elongation factors involved in the potyviral [*Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV)] genome translation initiation. RNAi construct targeting TaeIF(iso)4E generating three transgenic lines and construct targeting TaeIF4G produced four transgenic lines. They found early generation lines are resistant to the WSMV and TriMV and co-inoculation of both. RT-qPCR analysis using T5 generation lines showed 18 fold reduction in WSMV and TriMV viral RNA as compared to the control plants.

Tatineni *et al*. (2020) developed dual resistant wheat lines against two important wheat viruses (WSMV and TriMV). An ihpRNA construct was developed against the replicase gene of both viruses with 202 bp fragment size (404 bp in total). RNAi fragments were cloned into a binary vector and used to transform the spring wheat CB037. Phenotyping of T1 lines from eight independent transgenic events for resistance showed that two events were resistant to both the viruses, four events provided resistance to either WSMV or TriMV and in two lines there was no

resistance found. Northern blot analysis of RNA, isolated from transgenic wheat lines showed virus specific small RNAs (vsRNAs) buildup with increase in the temperature, with no traceable levels of vsRNA accumulation at 20ºC.

### 2.6.1 RNAi for virus resistance targeting replicase gene

Fuentes *et al*. (2006) developed transgenic tomato plants, transformed with an intron hairpin genetic construction to induce post- transcriptional gene silencing for early *Tomato Yellow Leaf Curl Virus* (TYLCV) replication associated protein gene (C1). They used 726 nt of the 3' end of TYLCV C1 gene to develop the sense and antisense fragments with caster bean catalase intron. Transgenic line 126 showed the single copy transgene and tolerance to TYLCV.

Hu *et al*. (2011) developed transgenic tobacco by intermolecular intron-hairpin RNA construct against *Cucumber mosaic virus*, targeting partial replicase gene. Agrobacterium mediated transformation was carried out and found three transgenic tobacco lines (Rep15-1-1, Rep15-1-7 and Rep 15-1-32) immune to the CMV infection. Also they reported that there was no significant correlation between resistance and copy number of the transgene.

*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 the RNAi construct targeting the BBTV replicase gene, by cloning *rep* gene fragments in sense and antisense orientation in the RNAi intermediate vector, pSTARLING-A. The cloned RNAi gene cassette was released by *Not*I site. Then embryogenic cells of banana were transformed using *Agrobacterium tumefaciens*. The presence of transgene was confirmed through PCR and RT-PCR analysis. Transgenic plants developed in this study showed BBTV resistance upon challenging BBTV infection in the greenhouse condition.

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 was correlated with post-transcriptional gene silencing because of the production of transgene specific siRNA.

Suppaiah *et al*. (2015) developed hpRNA construct against *Tobacco streak virus* (TSV) by targeting replicase gene. Nucleotide sequence of replicase gene of virus showed 99 percent sequence similarity with the Tamil Nadu okra isolate. hpRNA construct was prepared in pHANNIBAL vector and then Rep hairpin construct was cloned to binary vector pART27, vector then mobilized to the *Agrobacterium* strain LBA4404 for plant transformation. Regenerated T0 plantlets were screened with PCR and southern blot analysis using isolated genomic DNA from transformed shoots. PCR results showed 299 and 340 bp fragments corresponding to *npt*II and *Rep* gene respectively. Southern blot analysis demonstrated multiple and single copy integration of the transgene. Transgenics (T0 plants) generated in this study showed resistance against TSV when challenged mechanically without producing any observable symptoms.

Lekshmi (2016) developed small interfering RNA (siRNA) mediated resistance against *banana bract mosaic virus* (BBrMV) in *Musa* (AAB) cv. Nendran. The ihpRNA construct targeting BBrMV replicase gene was prepared in

pSTARLING by linking the 420 bp inverted repeats of BBrMV replicase through *cre* intron. The complete ihpRNA cassette was cloned to binary vector pART27 and mobilized to *A. tumefaciens* strain LBA4404 for transforming somatic embryos. The plants developed from the transformed somatic embryos confirmed the presence of ihpRNA cassette and showed resistance against BBrMV.

Yang *et al*. (2017) developed transgenic soybean lines containing inverted repeats of replicase (nuclear inclusion b, *NIb*) gene of *Soybean mosaic virus* (SMV) strain SC3. 248 bp inverted repeats were selected for the development of RNAi construct. Transgenic lines showed lower average disease indices than the non-transformed control plants.

Yu *et al*. (2018) designed specific siRNAs and miRNAs to target the replicase gene (*NIb*) of *Potato virus Y* common strain ($PVY^O$) and *Potato Virus Y* necrotic strain ($PVY^N$). Eight plant expression vectors constructed with one or two sRNAs. Designed sRNAs activity was checked with luciferase assay for cleavage of the *NIb* gene of $PVY^O$ and $PVY^N$. Vector harbouring combined siRNA and miRNA based shRNA showed stronger inhibitory effect.

### 2.6.2 RNAi for virus resistance targeting movement protein gene

Tobacco plants transformed with inverted repeat sequences of the partial TMV movement protein (MP) gene and the partial sequence of CMV replicase protein (RP) gene showed PTGS of the respective genes (Hu *et al*., 2011). The transgenic plants exhibited complete resistance to TMV or CMV infection. Additionally, the authors confirmed that the silencing was stably inherited through self-pollination in T4 progeny and that viral resistance was unaffected by low temperature.

Shimizu *et al*. (2011) analysed the effects of possible target sequences in each of the coding genes in the *Rice Stripe Virus* (RSV) genome, using a set of inverted-

repeat (IR) constructs. Transgenic plants having IR constructs specific for the gene for pC3, which encodes nucleocapsid protein, and for pC4, which code for a viral movement protein, showed immunity to infection by RSV and were more resistant to infection than the natural resistant cultivars that have been used to control the disease in the field.

Winterhagen *et al*. (2009) established RNAi in *Nicotiana benthamiana* plants by introducing silencing construct with a fragment of the defective interfering (DDI) sequence from *Tomato bushy stunt virus* (TBSV) and the *Grapevine fanleaf virus* (GFLV) derived fragment from the core region of the movement protein (MPc) under the control of CaMV 35S promoter. They confirmed the silencing in plants by *Agrobacterium* – mediated infiltration of GFP sensors containing the GFLV- derived target sequence.

Shimizu *et al*. (2013) transformed rice plants by introducing an RNAi construct of the *Rice grassy stunt virus* (RGSV) genes for the nucleocapsid protein pC5 or movement protein pC6. They showed that all the progenies from self-fertilized transgenic plants had strong resistance against RGSV infection and did not allow the proliferation of RGSV.

### 2.6.3 RNAi for virus resistance by targeting multiple genes of virus

Nicola-Negri *et al*. (2005) developed the ihpRNA construct against the *Plum Pox Virus* (PPV) infecting *N. benthamiana*. P1 and the HC-Pro genes were selected as potential RNA silencing targets, as they are involved in the suppression of the RNA silencing and amplification and movement of the virus. Four constructs were developed containing UTR/P1, P1/Hc-Pro, Hc-Pro and Hc-Pro/P3 genes. Each PPV gene was placed as two inverted repeats separated by Dof Affecting Germination (DAG1) intron. DAG1 intron amplified with *Bam*HI/*Sma*I site and cloned into linearized pBluescript II KS generating pBluescript-DAG. Each PPV sequence was

amplified as *Sac*I- *Xba*I for sense and *Pst*I- *Kpn*I for antisense fragments. Each sense-DAG-antisense fragment was excised as *Sac*I-*Kpn*I fragment and cloned into the *Sma*I site of linearized pBIN19-35S. *N. benthamiana* leaf discs were co-cultivated with Agrobacterium tumefaciens LBA4404. They found that 38 out of 40 T0 transgenic plants were resistant to PPV infection. Two hundred forty eight out of 253 T1 transgenic plants were resistant to local and systemic PPV infection.

Bucher *et al*. (2006) developed a multiple virus resistance at a high frequency using a transgene construct, by arranging viral transgenes as inverted repeats. To obtain broad spectrum virus resistance at high frequency they used N gene sequence of major tomato infecting tospoviruses (*Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV) and *Watermelon silver mottle virus* (WSMoV), in a single small chimaeric hairpin (hp) RNA construct. Two hairpin RNA constructs IR-IN and IR-OUT are used to obtain transgenic resistance against four tospoviruses. The inward-facing construct is named IR-IN and the outward construct IROUT. Primers were designed to amplify sequential 150 bp N gene segments of TSWV, GRSV, TCSV and WSMoV and to overlap where the segments of the cassette should be fused. After a first round of PCR, the products were used in another round of PCR, resulting in the fusion of the two segments. Using this technique, the four segments were fused, resulting in a chimaeric N gene cassette of 600 bp. Developed constructs were cloned into pBIN19 binary vector which having *Asc*I and *Pac*I sites between left and right borders. Binary vector was transformed into *A. tumefaciens* LBA4404 and used to transform the small leaf explants of *N benthamiana*. They found that 82% of the transformed plant lines heritably resistant against all four viruses.

Nahid *et al*. (2011) developed hairpin RNA construct against *Chickpea chlorotic dwarf Pakistan virus* (CpCDPKV) which is a legume-infecting *mastrevirus* that affects chickpea and other leguminous crops in Pakistan. They constructed

CpCDPKV one hpRNA construct (Chp6pFGC5941), targeting replicase gene and part of the movement protein (MP) gene (both 730 bp) which was separated by large intergenic region (LIR) as an intron. Sense and antisense fragments were cloned into pTZ57R/T. pFGC5941 was used as the RNAi vector to clone the sense and antisense fragments. *N. benthamiana* was transformed with the RNAi construct (ChP6pFGC5941) by leaf disc method using *Agrobacterium tumefaciens* strain LBA4404. They also reported that the transgenic tobacco showed normal phenotype and viable seed set without adverse effect of transgene. Plants transformed with hpRNA construct when artificially inoculated with CpCDPKV did not show symptoms; whereas uninoculated plants were heavily infected.

Yan *et al*. (2012) developed a novel restriction ligation technique that provides a simple but proficient construction of intron containing hpRNA (ihpRNA) vectors. This system is based on the type II restriction enzyme *Bsa*I and new plant RNAi vector pRNAi-GG through Golden Gate (GG) cloning. By using this strategy an ihpRNA namely pRNAi-GFP/PDS, targeting both GFP and PDS were successfully constructed. Both the inserts were flanked with *Bsa*I sites whose cleavage site sequences were designed in such a way that the two inserts can be ligated after the digestion. *Agrobacterium* cultures with pRNAi-GFP/PDS were co-infiltrated into the *N. benthamiana* plants and simultaneous silencing of GFP and PDS was observed in 3 days post inoculation.

Arif *et al*. (2012) developed a broad-spectrum resistance against RNA viruses in potatoes. In order to exploit the effectiveness and flexibility of the vector-based siRNA approach, they constructed a chimeric expression vector containing three partial gene sequences derived from the ORF2 gene of *Potato virus X* (300 bp), Helper Component Protease gene of *Potato virus Y* (365 bp) and Coat protein gene of *Potato leaf roll virus* (300 bp). *Solanum tuberosum* cv. Desiree and Kuroda were transformed with this chimeric gene cassette via *Agrobacterium tumefaciens*-

mediated transformation. A total of 965 bp long chimeric gene fragment was arranged in sense and antisense orientation in a pN6 vector separated by an intron, downstream of a single CaMV 35S promoter. The whole 4.96 kb expression cassette was transferred to binary vector pGreen at *Not*I site, later moved into *A. tumefaciens* LBA4404 for plant transformation. Expression of partial triple gene sequence cassette showed 20% of the transgenic plants were immune against all three viruses. Thus, they suggested that the expression of a single transgene construct can be effectively conferring resistance to multiple viruses in transgenic plants.

Sharma *et al*. (2015) utilized RNAi technique to control the chilli infecting begomoviruses (CIBs) infection. They developed transgenic *N. benthamiana* plants containing two different intron hairpin RNAi constructs named as TR1 (*AC1/AC2*) and TR2 (*AC1/AC2/βC1*), where TR is target region and *AC1* was the replication initiation protein, *AC2* acts as a transcriptional activator for virion sense gene of begomovirus and *βC1* for beta satellites encoded protein. Based on the alignment results they selected 417 bp conserved region for AC1 and AC2 genes of CIBs and 193 bp for beta satellite encoded ORF for *βC1*. After plant transformation they found that two lines carrying TR1 construct (13-1 and 2-4) and one line harbouring TR2 construct (5-1) showed resistance to most of the predominant Indian CIBs. Resistant lines showed transgene specific siRNAs and confirmed the RNAi mediated resistance against these viruses.

Patil *et al*. (2016) developed the RNAi constructs for all the genes of genomic components (DNA-A and DNA-B) of *African cassava mosaic virus* (ACMV-CM) which is the most destructive geminiviruses causing cassava mosaic disease (CMD) in Africa. Using transient agro-infiltration studies they evaluated RNAi constructs for their ability to trigger gene silencing against the invading viruses and protection against it. Also they found that the DNA target sequence is an important determinant for the amount of siRNA produced and the range of resistance. The ACMV genes

AC1, AC2, AC4 from DNA-A and BC1 from DNA-B were the most effective target sites for RNAi mediated resistance and their siRNA expression was higher compared to other RNAi constructs. Also they found that the AC2 component of RNAi construct gave the highest level of resistance to ACMV.

Hameed *et al*. (2017) developed transgenic potato lines (*Solanum tuberosum* cv. Desiree) which expresses the fused viral coat protein genes form *Potato virus Y* (PVY), *Potato virus X* (PVX) and *Potato virus S* (PVS) as a 600 bp inverted repeats with a constitutive 35S promoter. The expression cassette designed was named as *Ec1/p5941* to develop the dsRNA having the hairpin loops. The transgene incorporation in plants was confirmed by the glufosinate resistance, gene specific PCR and southern blotting. Also the regenerated potato lines were analysed for the resistance to virus inoculation for two continuous crop seasons. They found 100 percent resistance against PVX, PVY and PVS infection as compared to untransformed control potato plants. These experimental outcomes set up the viability of RNAi technique utilizing the coat protein gene as a potential target site for the successful induction of stable antiviral immunity in potatoes.

Beyene *et al*. (2017) utilized RNAi technology to integrate Cassava brown streak disease (CBSD) resistance into the Ugandan farmer preferred cassava cultivar TME 204. They developed transgenic lines using *Agrobacterium* mediated transformation with LBA4404 strain in friable embryogenic callus which expressed an inverted repeats construct (p5001) derived from coat-protein genes of CBSV and *Ugandan brown streak virus* (UCBSV) which fused in tandem. 169 independent transgenic lines were screened with northern blot analysis for the presence of CP-derived siRNA. They found that low siRNA expressing lines were susceptible to CBSV and UCBSV, while medium to high siRNA accumulating plant lines were resistant to both the viruses.

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. Studies on these aspects were conducted for the improvement of hpRNA construct to increase the silencing efficiency.

## 2.7 IMPROVEMENT IN hpRNA CONSTRUCTS

### 2.7.1 Selection of target gene sequence

Overall efficiency of RNAi is dependent on RNA-protein interactions during siRNA-RISC assembly and activation (Khvorova *et al.,* 2003; Schwarz *et al.,* 2003). Hence, a systematic analysis of 180 siRNAs targeting the mRNA of two genes was done to identify siRNA-specific features that are likely to contribute to efficient processing at each step of RNAi (Reynolds *et al.,* 2004). Based on the analysis, characters like low G/C content, a bias towards low internal stability at the sense strand 3′ terminus, lack of inverted repeats, and sense strand base preferences (positions 3, 10, 13 and 19), etc. were found to be disturbing siRNA functionality. An algorithm was designed to incorporate all the eight criteria for the selection of potent siRNA for facilitating the functional gene knockdown studies.

Similar study was conducted to decipher the relationship between short interfering RNA (siRNA) sequence and RNA interference (RNAi) in three mammalian and *Drosophila* cells by analyzing 62 targets of four exogenous and two endogenous genes (Kumiko *et al.,* 2004). Based on analysis certain rules were formulated for designing effective siRNAs capable of inducing highly effective gene silencing in mammalian cells. Rules included the sequence conditions *viz*; presence of A/U at the 5′ end of the antisense strand; and G/C at the 5′ end of the sense strand; presence of at least five A/U residues in one-third of the antisense strand towards 5’ end; and the absence of any GC stretch of more than 9 nt in length. These rules

indicated that siRNAs which satisfy all these conditions will increase the gene silencing efficiency in mammalian cells.

The investigation was done on the use of RNA interference (RNAi) for obtaining resistance against *Cotton leaf curl Multan virus* (CLCuMV) (Mubin *et al.,* 2011). Three hairpin RNAi constructs were produced containing either complementary-sense genes essential for replication/pathogenicity or non-coding regulatory sequences of CLCuMV. All three RNAi constructs significantly reduced the replication of the virus in inoculated tissues. However, the systemic movement of the virus was controlled by only one of the constructs (CLCRNAiRepTrAPREn/ pFGC), possibly because it spanned three virus-encoded genes; the replication-associated protein (Rep), the replication enhancer protein (Ren) and the transcriptional activator protein (TrAP). Also, the ability of viruses to infect plants was compromised as the expression of TrAP was down regulated. TrAP is a transcription factor possibly involved in suppression of silencing machinery. Hence, both the target sequence, as well as the levels of identity between the construct and target sequence, determined the outcome of RNAi-based resistance against virus.

On studying the effects of the structure, position and sequence of a target RNA on RNAi using 47 constructs for inhibition of firefly *luciferase* activities by siRNAs targeted to TAR motif, Yoshinari *et al.* (2004) observed that the efficacy of siRNAs is mainly dependent on the target sequence. Statistical analysis of the data collected on the sequence preferences indicated that some nucleotides at specific positions are positively or negatively correlated with the efficiencies of siRNAs, for example the siRNAs with an A residue at the 19$^{th}$ nucleotide position from the 5′ end of the sense strand showed relatively high suppressive activities, while siRNAs with a G residue at the 19$^{th}$ nucleotide in the sense strand tend to be less effective. Similar preferences were noted for miRNA sequences by Reinhart and Bartel (2002). This preference suggests the importance of the low internal stability and a possible functional

contribution of a U at the 5′ end of inverted repeats for the activities of both siRNA and miRNA (Schwarz *et al.,* 2003; Khvorova *et al.,* 2003). The GC contents of the 3′ half of siRNAs (the $12^{th}$ to the $19^{th}$ nucleotide) and the activities of siRNAs were inversely correlated. Similar observations were also seen in a report by Vickers *et al.* (2003).

The first commercialized transgenic papaya carrying the PRSV CP gene was introduced to Hawaii in 1998 to save the remains of the papaya industry (Ferreira *et al.,* 2002). However, transgenic papaya cultivars showed varying levels of resistance against PRSV isolates from distinct topographical regions. For example, isolates from the Bahamas, Florida and Mexico have delayed mild symptoms. Isolates from Brazil and Thailand also have delayed symptoms, but the virus eventually overcomes their resistance. The CP hemizygous line, 'Rainbow,' is also susceptible to PRSV isolates from Taiwan (Tennant *et al.,* 1994). Therefore, the resistance levels were found to be dependent on the variability among CP genes of the isolates (Gonsalves, 1998; Bau *et al.,* 2003; Davis and Ying, 2004).

The high levels of genetic divergence in PRSV isolates from Hainan caused the failure of transgenic papaya lines that target specific viral CP gene (Zhao *et al.,* 2016). CP transgenic resistance of papaya is dependent on the homology to the viral nucleotide sequence (Kung *et al.,* 2015; Jia *et al.*, 2017). The proper selection of a target sequence for a given gene of interest remains one of the most critical components of successful gene knockdown regardless of the RNAi methodology.

### 2.7.2 Size of the inverted repeats

Jacobs *et al.* (1999) described the silencing efficiency to be proportional to the size of target sequence and the terminal regions (5' and 3') were found to be unaffected by the silencing mechanism. This description was based on the experiment

conducted to identify the target regions and relative efficiencies of various target regions for silencing of gn1 (β-*1,3 glucanase*) gene in transgenic tobacco line T17.

Similarly, the effect of length of inverted repeats in tobacco BY-2 cells was tested by co-transformation of a *luciferase* gene construct and a *luciferase* dsRNA expression plasmid (Akashi *et al.,* 2001). The dsRNA expression plasmids targeted to the firefly *luciferase* gene were constructed with 500 and 300 nt inverted repeats. However, no significant difference in silencing efficiency was observed and the presence of 300 bp dsRNA was found to be sufficient to suppress the *luciferase* activity in cultured plant cells. A longer dsRNA did not show any enhancement in RNAi effect.

Helliwell and Waterhouse (2003) reported the less frequency in silencing when shorter fragments were used. It was based on constructs with fragments of range 50 bp to 1 kb targeted to silence two Arabidopsis genes, *flowering locus C* and *phytoene desaturase* successfully. The use of fragments between 300 to 600 bp was recommended to achieve effective silencing.

A difference in silencing efficiency was also observed in *Neurospora crassa* upon introduction of varied size of inverted repeat constructs targeting the *albino-1* gene (Goldoni *et al.,* 2004). Higher silencing frequencies were obtained when the length of the repeat for the targ*et al* bino-1 gene in *Neurospora crassa* was kept above 200 and below 900 nucleotides. A substantial decrease in the silencing efficiency was observed when the repeat size was reduced below 200 nucleotides.

Effect of size of granule-bound starch synthase (GBSSI) sequence in inverted repeat constructs was evaluated and it was found that, for GBSSI, the small inverted repeat constructs (500 to 600 bp repeat size and 150 bp spacer size) were more efficient silencing inducers than the large inverted repeat constructs (1.1 or 1.3 kb repeat size and 1.3 or 1.1 kb as spacer size) (Heilersig *et al.,* 2006). Together, these

reports suggest that 300 to 500 bp is the optimal size for inverted repeat for an effective silencing mechanism.

### 2.7.3 Vectors for RNAi

Construction of RNAi vectors takes considerable time and is a tedious task since it involves tedious conventional cloning technology based on restriction digestion and ligation of two fragments corresponding to the antisense and sense region of the stem and sub cloning into a binary vector. Hence, there is demand for high-throughput plant RNAi vectors for rapid and easy construction.

For instance, a technique involving a single step for construction of an RNAi vector has been developed that facilitates fast and reliable DNA cloning. It is called gateway cloning technique which is also available with compatibility for *Agrobacterium* sp. binary vector system. The backbone of all Gateway-compatible destination vectors is derived from the pCAMBIA series of binary vectors for *Agrobacterium* sp. mediated plant transformation. The Gateway recombination site for introduction of a DNA fragment of interest is placed towards the right border of the T-DNA in the pCAMBIA vectors. Most of the T-DNA destination vectors described contain the *hygromycin phosphotransferase* (*hpt*) gene as a plant-selectable marker. This selectable marker was chosen so that these vectors would be compatible with a large number of insertion lines that are Kanamycin resistant.

For the construction of RNAi vectors using Gateway recombination technology, the PCR products of the target gene are generated with primers flanking attB1 and attB2 sites for recombination with two cloning sites with attP1 and attP2 sequences using BP clonase. pHELLSGATE (Helliwell and Waterhouse, 2003) and pANDA (Miki and Shimamoto, 2004) are the vectors that allow the assembly of an inverted repeat structure by Gateway recombination technology (Karimi *et al*., 2007). Several pHELLSGATE related RNAi vectors have been developed for RNAi in

monocotyledonous plants and for inducible RNAi (Earley *et al.,* 2006; Karimi *et al*., 2007).

For conventional cloning, pHANNIBAL (Wesley *et al*., 2001), pKANNIBAL (Helliwell and Waterhouse, 2005), pSAT (Yelin *et al*., 2007) and pSH (Hirai *et al*., 2007) are available. In these RNAi vectors, PCR fragments of the target gene are produced by using primers with restriction sites, and cloned successively into both upstream and downstream regions of the spacer to become the two arms of the hairpin construct. Simultaneously work was done to enhance the efficiency in cloning inverted repeats for RNAi. An all-purpose vector, pGEM-WIZ, for assembling the repeat for any Drosophila gene was developed (Sujin and Ross, 2006). The inverted repeats in pGEM-WIZ are stable in common *E. coli* strains and have a method for fast selection of clones with the repeat.

For a simple and efficient construction of intron-containing hpRNA (ihpRNA) vectors a novel restriction-ligation approach was developed. The system was designed based on the type IIs restriction enzyme *Bsa*I and plant RNAi vector pRNAi-GG based on the Golden Gate (GG) cloning (Yan, 2012). It required only a single PCR product of the gene of interest flanked with *Bsa*I recognition sequence which can then be cloned into pRNAi-GG at both sense and antisense orientations simultaneously to form ihpRNA construct. The ihpRNA construction process could be completed in one tube with one restriction-ligation step.

### 2.7.4 Use of spacers or introns

The silencing efficiency of RNAi vectors was reportedly enhanced by the addition of an intron interposed between the inverted flanking target sequences in the vector. The spacer fragment was observed to give the stability to the inverted-repeat sequences. It didn't play any role in PTGS and was spliced out during pre-mRNA processing. The process of intron excision from the construct by the spliceosome

might help to align the complementary arms of the hairpin in an environment favoring RNA hybridization and promoting the formation of a duplex. Also, splicing may contribute in increasing the amount of hairpin RNA by preventing the hairpin's passage from the nucleus, or by creating a smaller, less nuclease-sensitive loop. (Smith *et al.,* 2000).

Smith *et al.* (2000) showed that transgene constructs encoding a splicable intron within a hairpin RNA structure can induce PTGS with almost 100% efficiency. For the sense gene 7% PVY-resistant tobacco plants were obtained by targeting the nuclear inclusion a (NIa) protease gene, for the antisense gene 4% resistance was observed. For the hpRNA with a non spliceable loop separating the sense and antisense arms 58% resistance was obtained. Higher per cent resistance (96%) was for the same hpRNA with a spliceable intron. The high efficiency of intron hpRNA constructs was confirmed against *Cucumber mosaic virus* (CMV) for inducing PTGS and developing resistant transgenic plants against CMV (Kalantidis *et al.,* 2002; Chen *et al.,* 2004).

Sayaka *et al.* (2007) demonstrated the effect of spacer sequences on silencing potential of RNAi constructs. It was tested by an *in vivo* assay of the *α*-linolenic acid content in hairy roots of tobacco with RNAi vectors against *ω-3 fatty acid desaturase* (NtFAD3) gene responsible for production of *α*-linolenic acid of root membrane lipids. The frequency of RNA silencing was observed to be more affected by spacer sequences than by spacer length (100 to 1800 bp). They concluded that it is possible to change the degree of silencing by replacing spacer sequences. They predicted the interaction of spacer sequences with stem sequences of the hpRNA which affect the formation of a hairpin structure.

Furthermore, it was shown that RNA silencing can be induced by applying plant viral dsRNA externally as a spray to protect plants against potyviruses (Tenllado *et al.,* 2003). However, the reliability of RNA silencing-mediated

resistance is to be checked in the field under the pressure of heterologous virus infections and changing environmental conditions.

### 2.7.5 Promoters

The degree of silencing is deceptively proportional to the level of siRNAs, hence, the strong promoters have been used in the construction of RNAi vectors as follows, the *Cauliflower mosaic virus* 35S promoter (p35S) (Gavilano *et al.,* 2006; Rutherford *et al*., 2004; Sin *et al*., 2006), soybean lectin promoter (Liu *et al*., 2002), Arabidopsis rbcS promoter (Rohr *et al*., 2004; Li *et al*., 2008), rice ubiquitin promoter (Miki *et al*., 2005; Travella *et al*., 2006) and Chrysanthemum rbcS1 (Outchkourov *et al*., 2003).

RNAi technique cannot be applied to genes whose silencing interferes with plant regeneration or causes embryo lethality or severe pleiotropic phenotypes. In such cases the inducible RNAi vectors are used which can confer transient and local silencing. Ethanol or estrogen inducible vectors were developed for transient RNAi expression. In the case of an ethanol-inducible vector, a transcriptional regulator, AlcR, is constitutively expressed and the RNAi cassette is inserted behind the alcA promoter. On treatment with ethanol, AlcR binds to the alcA promoter and transcription of the downstream RNAi sequences is activated (Lo *et al*., 2005). Also, a Cre/loxP-mediated recombination and a chemical inducing RNAi vector were developed for the stringent control of expression of an RNAi cassette. A chimeric transcriptional factor, XVE, was constitutively expressed which when bound to estrogen induces the transcription of a *Cre recombinase* gene. The resulting Cre recombinase removes a fragment that blocks transcription of an RNAi cassette (Guo *et al*., 2003). Similarly, for controlled expression, the pOp6 promoter was used which could allow the rapid induction of RNAi across the whole plant or in limited tissues under investigation. The induction was controlled by the expression of a synthetic transcription factor, LhGR which can bind to pOp6 in the presence of

dexamethasone and initiate the transcription of downstream RNAi cassette (Craft *et al*., 2005). The pHELLSGATE vector-based inducible RNAi vectors are also available (Wielopolska *et al*., 2005).

## 2.8 ISOLATION OF TARGET GENE OF THE VIRUS

Selection of the target gene is very important in designing ihpRNA construct. Most of the studies used replicase genes 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; 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 construct carries inverted repeats of the 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).

## 2.9 RNA ISOLATION IN BANANA

RNA isolation is difficult in bananas due to high phenolic content and other interfering compounds. Several protocols have been standardized for RNA isolation from bananas. 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 bananas. 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. Commercial kits are available to isolate the RNA, usually clogging in column and column leakage makes it unsuitable for mucilaginous crop RNA isolation (Barman *et al*., 2017). However, a suitable method specifically for recalcitrant plant tissues is not yet available (MacRae, 2007).

In the current study, common RNA isolation protocols like CTAB and Trizol method failed to extract good quality viral RNA, the protocol standardized by Rodriguez-Garcia *et al*. (2010) was followed with the modifications.

## 2.10 ISOLATION OF BBrMV REPLICASE AND MOVEMENT PROTEIN GENES 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 products were cloned to the 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(+).

## 2.11 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.

Waterhouse *et al.* (2001) had 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 similar enough 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

challenged ith CMV, three different types of plants were obtained, including susceptible, recovered plants, and asymptomatic resistant plants.

In 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 sugar beet 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 fungal 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 ringspot virus*-W (Krubphachaya *et al.*, 2007), showed 100 per cent efficiency for inducing RNAi.

Hu *et al*. (2011) developed transgenic tobacco plants expressing partial TMV movement protein gene (MP) and partial CMV replicase gene (Rep) in the form of an intermolecular intron-hairpin RNA and showed complete resistance to TMV or CMV infection.

### 2.11.1 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 EST, GSS and nucleotide databases, Zhang *et al.* (2009) identified 48 potential miRNAs in *Solanum 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 plants 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 them encoded transcription factors or enzymes participating in regulation of development, growth and other physiological processes.

Kamarajan *et al.* (2012) used a computational approach to predict miRNA targets in plant mitochondria. The mitochondrial gene targets identified for miRNAs were 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)

## 2.12 CONSTRUCTION OF ihpRNA VECTOR

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 and Shimamoto (2004) for RNA interference in rice genes for its functional identification.

Pathogen-derived resistance against viral infection in plants was first demonstrated by Abel *et al.* (1986) by expressing coat protein gene (478 bp) of *Tobacco mosaic virus* (TMV) in transgenic tobacco plants, which conferred resistance to TMV infection. cDNA of the CP cistron of TMV-RNA was generated and cloned to the plasmid vector pUC9 and subsequently used for the *Agrobacterium* mediated transformation. 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, 2003; Kooter *et al.* 1999; Waterhouse *et al.* 2001).

RNAi techniques have been developed to avoid parasites in crops. 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. This includes the cloning of the parasites target sequence 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 has been used by Wesley *et al*. (2001) for the RNA silencing studies and achieved 90 to 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 and Shimamoto (2004) by constructing hpRNA vector using sequences of size 300-500 bp. RNAi-induced gene silencing using sequences around 300-700 nt 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).

The sense and antisense strands are usually divided by an intron to facilitate the creation 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.

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 microRNA (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 and Shimamoto (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$CI_{\text{antisense}}$- $CI_{\text{ sense.}}$ Castor bean catalase intron was cloned into pBP$CI_{\text{antisense}}$- $CI_{\text{ sense}}$ 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 Hilly *et al.* (2007). Full-length coat protein gene has been extracted and cloned in the pHELLSGATE vector in sense and antisense manner. 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 sub cloned to pSTBlue-1 and *Agrobacterium* GV3101 carried the construct to *N. benthamiana*.

RNAi mediated resistance in Cantaloupe against *Papaya ringspot virus* type W was developed by Krubphachaya *et al.* (2007) developed RNAi mediated resistance in Cantaloupe against *Papaya ringspot 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 *HindIII* 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 LBA4404

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 *A. tumefaciens* strain EHA105.

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.

Hu *et al*. (2011) developed transgenic tobacco plants using inverted repeats of the partial *Tobacco mosaic virus* (TMV) movement protein (MP) gene and the partial *Cucumber mosaic virus* (CMV) replication protein (Rep) gene. Transgenic tobacco plants expressing hairpin RNA derived from TMV ΔMP or CMV ΔRep gene were generated by tri parental mating *in A. tumefaciens*-mediated transformation using EHA101 strain. They analyzed the resistance of T0 to T4 transgenic plants, and found that T4 transgenic lines with single copy were completely resistant to the corresponding virus, and viral resistance of transgenic plants did not be affected by the low temperature (15°C). Also they did not find the significant correlation between the resistance and the copy number of the transgene.

Efforts were taken by Shekhawat *et al.* (2012) to silence *Banana bunchy top virus* (BBTV) in bananas 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 together with a 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 hairpin 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 contains *npt*II (kanamycin) gene for the plant section. Transformed 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 CMV. They constructed an intron hairpin RNA vector using an 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 a 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$^{TM}$ (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 (*npt*II) and hygromycin phosphotransferase (*hpt*), the CMV gene being driven by CaMV 35S promoter, was introduced into *A. 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 the 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 sub cloned into *Sac*I and *Bam*HI digested pCAMBIA 2301 G and the resulted construct, pCAMBIA2301-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 Material Transfer Agreement (MTA). pSTARLING is a pUC based primary vector with a novel set of restriction sites. The expression cartridge of the primary cloning vector pSTARLING, composed of maize ubiquitin promoter for high level constitutive hairpin RNAi production in monocot plants. The entire cartridge (i.e. ihpRNA cassette) was cloned into the binary vector. This can be removed from pSTARLING as a *Not*I fragment and introduced directly into the binary vector, pART27 at the NotI site in the lacZ gene. Recombinants can be selected by blue white colony screening.

## 2.13 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 methods need a carrier vector for conveying 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 min. The mixture was

freezed in liquid $N_2$ for one min, and then thawed at 37ºC water bath. One ml medium was added to the mixture and incubated at 28ºC for 2 to 4 hr with gentle shaking. The cells were collected by centrifugation at 5000 g for 5 min and resuspended on YEB plates with appropriate antibiotics and incubated at 28ºC 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 ringspot 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 LBA4404 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* EHA101 using a heat shock method.

Zhang *et al.* (2011) created a transgene construct that is composed of three short inverted repeats (IRs), each containing 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 order of dsABS, 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 EHA101).

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

In the current study freeze-thaw method (Jyothishwaran *et al.*, 2007) mediated *Agrobacterium* transformation was carried out and the transformants were selected on LB agar plates with spectinomycin 100 mg $L^{-1}$ and rifampicin 20 mg $L^{-1}$.

## 2.14 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 and 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. Among the several explants tried in banana, immature male flower bud is known to respond well and produce higher proportion of embryogenic calli with high regeneration potential (Cronauer and Krikorian, 1983; Escalant *et al.*, 1994; Cote *et al.*, 1996; Navarro *et al.*, 1997; Ganapathi *et al.*, 1999; Becker *et al.*, 2000; Grapin *et al.*, 1996, 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 are 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 face a serious hindrance by phenolic exudation from the cut ends. Explants often brown and become 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 MS medium. Use of antioxidants like ascorbic acid was followed by Namanya *et al.* (2004). Khatri *et al.* (2005) minimized browning of explant by incorporating cysteine and methionine. Chang and Shu (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) produced embryogenic cultures using male flowers in five cultivars of banana. Rapid proliferation of embryogenic callus and embryo development was obtained on MS medium incorporated with BA 0.22 mM and IAA 1.14 mM. IN 6 to 8 weeks, embryo germination was observed after the transfer to half strength of the MS medium supplemented with malt extract 0.5 mg $L^{-1}$ and

subsequent development into complete plantlet. Embryogenic callus was collected at a percentage of 10.00±0.3 using immature male flowers on M1 medium supplemented with 18 µM 2,4-D for 3 months and then moved to the same media with 9 µM 2,4-D for the next 2-3 months. On MS media, enriched 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 may develop proembryo calli excised from basal sheath and rhizome tissue from growing clusters of shoots, Good results were found in SH medium of 30 µM 3,6 dichloro-2-methoxybenzoic acid and 5 µM TDZ. Addition of cysteine and methionine reduced the browning of explants. Light and dark treatments effectively reduced the browning in the explants. Strosse *et al.*(2006) developed meristem cultures of 18 banana varieties by culturing elongated shoots on MS medium enriched with 100 µM BAP. The top layers comprising the meristematic tissue, i.e. scalps, were excised and induced for embryogenesis on media containing 1-50 µM 2,4-D. Embryogenic responses were observed with an optimum at 5 µM 2,4-D for each of the concentrations examined. From 24, 375 scalps tested, only 3.3 per cent resulted in an embryogenic response. The average embryogenic frequency was 6.0 per cent for cooking bananas (ABB), 3.8 per cent for Cavendish bananas (AAA) and 1.8 per cent for plantains (AAB). Embryogenic complexes were transferred to liquid maintenance medium, embryogenic cell suspensions with high regeneration capacity of 75 per cent were obtained.

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 enriched with 1 mg $L^{-1}$ picloram, 100 mg $L^{-1}$ malt extract, 100 mg $L^{-1}$ glutamine and 4.5 per cent sucrose. After 3 months of culture in this medium, mature somatic embryos were transferred to semisolid MS medium supplemented with BAP 1 mg $L^{-1}$ and 3 per cent sucrose. 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 converted into plants on half 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 with various explants such as scalps from cauliflower-like meristems and meristematic domes of axillary sprouted buds. The best embryogenic response (8 %) was observed with meristematic domes of sprouted axillary buds in 50% MS medium salt, MS vitamins, sucrose 30 g $L^{-1}$, ascorbic acid 10 mg $L^{-1}$, 2,4-D 1 mg $L^{-1}$, Zeatin 0.22 mg $L^{-1}$, malt extract 100 mg $L^{-1}$, glutamine 100 mg $L^{-1}$, biotin 1 mg $L^{-1}$, casein hydrolysate 200 mg $L^{-1}$, proline 4 mg $L^{-1}$ with gelrite 2 g $L^{-1}$.

Remakanthan *et al.* (2014) 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; 0.53-2.68 µM) with BA (2.22-44.39 µM) and glutamine 200 mg $L^{-1}$. Secondary embryos were converted to plantlets (3 percent) in MS liquid medium supplemented with 4.44 µM BA.

## 2.15 *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. Even though biolistic method of genetic transformation is now routine (Sagi *et al.*, 1995; Cote *et al.*, 1996; Becker *et al.*, 2000; Wu *et al*., 2015; Ibandalin *et al*., 2016; Hamada *et al*., 2017; Ismagul *et al*., 2018; Sobańska *et al*., 2019; Hanin *et al*., 2020; Loc *et al*., 2020) *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; Khanna *et al.*, 2004; Sreeramanan *et al.*, 2010). 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 leads to very low potential to regenerate in the presence of a selection agent.

Becker *et al.* (2000) developed an effective method for the stable transformation and regeneration of Cavendish banana (*Musa* spp. AAA) cv. 'Grand Nain' by microprojectile bombardment. Embryogenic cells were co-bombarded with

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 showed stable transgene integration. Matsumatto *et al.* (2002) developed a simple and routine particle bombardment system to generate transgenic bananas using 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 herbicide containing medium for 30 days, and putative transformed plants were regenerated.

Sagi (1997) suggested a method that combined both *Agrobacterium* and microprojectile 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 a 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 a fresh selection medium every two weeks. In the transformed apical shoot tips transient expression of the β-glucuronidase (*uid A*) was achieved. Krubphachaya *et al.* (2007) developed RNAi mediated resistance in Cantaloupe against *Papaya ringspot 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 EHA105 harboring vector pCAMBIA1301 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 a selective regeneration medium i.e., 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 min. This was subjected to co-cultivation for three days on hormone-free MS medium at 22ºC temperature in the dark. Chong-Perez *et al.*, 2012 incubated embryogenic cell suspensions (ECSs) with *A. tumefaciens* strain EHA101 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* strain LBA4404. The duration of co-cultivation when reduced to 2 days and the cultures when maintained at lower temperature of 18°C, infection injury to microcalli or ECS was reduced. 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.

## 2.16 CONFIRMATION OF TRANSFORMANTS WITH ihpRNA CASSETTE

Integration of the 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 the 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 ringspot 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-glucuronidase 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 into 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 a 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.

## 2.17 CONFIRMATION OF TRANSFORMANTS FOR SYNTHESIS OF siRNAs SPECIFIC FOR ihpRNA CASSETTES

In engineered plants, hpRNA that are transcribed from IR constructs are efficiently processed into siRNA by dicer like proteins. The siRNA are loaded into the RISC complex containing *Argonaute* 1 (AGO1). The siRNA guides RISC to the viral genome by recognizing the complementary RNA sequence. The AGO1 slicer activity cleaves the viral RNA, thereby plants getting resistance to the virus.

Gaba *et al*. (2010) studied the specificity of gene silencing based resistance using homozygous tobacco (*Nicotiana tabacum* L.) plants. They used 597-nt hairpin RNA construct of the Potato Virus Y (PVY) targeting replicase gene sequence. The transgene carrying tobacco line was immune to five potato PVY strains with high sequence similarity (88.3–99.5%) to the transgene. Transgene production of small interfering (si) RNA was detected by northern blot and measured using a custom-designed microarray for the detection of small RNAs. siRNA accumulation peaks were observed in microarray throughout the inverted repeat transgene. In the resistance-breaking tomato and pepper strains there were nucleotide differences in the sequences correlated to siRNA transgene accumulation, indicating the role of siRNA specificity in resistance breaking. The log of transgene siRNA signal intensity increased with probe GC content, indicating that the accumulating siRNA molecules were GC-rich. Sequence similarity of highly accumulating siRNAs with the target virus strain appears to be important for both resistance and resistance-breaking characteristics.

Li *et al*. (2016) studied the RNAi impact in *Rice stripe virus* (RSV) resistant transgenic rice, by introducing an inverted repeat construct that targets RSV nucleocapsid protein (NCP) gene. They developed three independent RSV-resistant transgenic rice lines and their stable integration of the T-DNA fragment and the expression of siRNAs were confirmed by Southern blotting and Northern blotting analyses. Also they found that majority of siRNAs were in lengths of 21, 22, and 24 nucleotides (nt), which have validated a connection between the presence of the RSV NCP homologous siRNAs and the RSV resistance in those transgenic rice lines. Bioinformatics analysis of small RNA-Seq data on the T4-B1 line also confirmed the large population of NCP-derived siRNAs in transgenic plants, and the RSV-infected library (T4-B1-V) possessed more siRNAs than its mock inoculated libraries (T4-B1-VF), these results indicating the inverted repeat construct and RSV could introduce abundance of siRNAs in transgenic rice.

Oiao *et al*. (2018) developed the transgenic *Nicotiana benthamiana* plants through hairpin RNA construct by targeting the RNA-dependent RNA polymerase (RdRp) (801 bp) of the *Lettuce infectious yellows virus* (LIYV), RdRp sequence showed immunity to systemic LIYV infection. Deep sequencing analysis was performed to characterize virus-derived small interfering RNAs (vsiRNAs) generated on systemic LIYV infection in non-transgenic *N. benthamiana* plants as well as transgene derived siRNAs (t-siRNAs) derived from the immune-transgenic plants before and after LIYV inoculation. Interestingly, a similar sequence distribution pattern was obtained with t-siRNAs and vsiRNAs mapped to the transgene region in both immune and susceptible plants, except for a significant increase in t-siRNAs of 24 nucleotides in length, which was consistent with small RNA northern blot results that showed the abundance of t-siRNAs of 21, 22 and 24 nucleotides in length. The accumulated 24- nucleotide sequences have not yet been reported in transgenic plants partially resistant to criniviruses, and thus may indicate their correlation with crinivirus immunity.

Harshitha (2018) evaluated the transgenic banana (cv. Nendran) plants for siRNA mediated resistance by artificial infection and confirmed the expression of siRNA in the *Banana bract mosaic virus* (BBrMV) infected plants using Northern hybridization. The siRNA isolated from the experimental plants blotted on the nylon membrane and hybridized with the biotin labeled probes specific to siRNA targeting viral replicase gene. 21 nt long siRNA was obtained in all the transformed plants and it clearly showed the successful siRNA mediated silencing of the replicase gene of BBrMV.

Jadhav (2019) developed an ihpRNA construct against the coat protein gene of BBrMV and delivered it into embryogenic calli of *Musa* spp. var. Grand Naine. The developed transgenic banana plants were confirmed to harbor the ihpRNA construct and evaluated for the presence of siRNA. The siRNAs were detected by northern blotting using target specific biotin labelled probes which confirmed the expression of target siRNA in the transformed plants.

## 2.18 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 “ihpRNA mediated resistance for *Banana bract mosaic virus* in *Musa* spp. by targeting replicase and movement protein genes” was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during September 2016 to February 2020. 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 cv. Grand Naine was used as explant for somatic embryogenesis, which was collected from the Instructional Farm, College of Agriculture, Vellayani. Flower buds were collected after the bunch formation was complete (Four weeks after flowering). The size of the male bud was trimmed to 7-8 cm by removing off the bracts. They were surface sterilized by wiping it with sterilized cotton dipped in 90 percent 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 a 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 glassware and DNase, RNase and protease free micro centrifuge tubes, PCR tubes and tips (Tarsons Products Pvt. Ltd.) were used for the study. Schott Duran screw capped bottles were used for storing stock solutions of antibiotics, plant hormones and buffers. Bacterial membrane filters (Axiva Sichem Biotech) 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 Thidiazuron (TDZ) and Benzyl Adenine (BA) was used for the development of somatic embryos (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 melt 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 100 kPa (15 psi) and a temperature of 121ºC for 15 min and the autoclaved medium was 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 BA (8 mg $L^{-1}$) and TDZ (0.6 mg $L^{-1}$) with 30 g $L^{-1}$ sucrose and 4.5 g $L^{-1}$ gelrite to induce embryogenic callus (Lekshmi *et al*., 2017). The

cultures were incubated in dark at 25°C and 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 Germination of embryos

The glassy elongated monocot embryo structures developed were subcultured on to MS medium supplemented with 2 mg $L^{-1}$ BA for plantlet regeneration (Lekshmi *et al*., 2017). The cultures were incubated in light for 16 h photoperiod.

## 3.2 ISOLATION OF MOVEMENT PROTEIN (Mvp) AND REPLICASE (Rep) GENES OF BBrMV

It was envisaged to develop BBrMV resistance in Grand Naine by inserting ihpRNA constructs to silence the replicase and movement protein genes of the virus. Coding sequences of replicase movement protein genes were isolated for the preparation of ihpRNA constructs. BBrMV is a (+) sense RNA virus belonging to the family potyviridae. Total RNA was isolated from BBrMV infected banana plants and cDNA was prepared by RT-PCR for further manipulations.

### 3.2.1 RNA isolation

The method developed by Rodriguez-Garcia *et al*. (2010) was followed with a slight modification to isolate RNA. This method was originally developed for fungal RNA isolation from plant tissues containing high levels of phenolic compounds or carbohydrates with the following steps:

BBrMV infected banana bract sample (0.5 g) was taken in a previously chilled mortar and ground to a fine powder using liquid nitrogen. 3 ml extraction buffer (Appendix II) was added to the homogenate and kept in a hot water bath at 65 ºC for 30 min. Homogenate (approx. 750 µl) was transferred to a sterile 2 ml

Eppendorf tube using the cut tip to avoid the RNA damage and was precipitated using 5 mM potassium acetate (66 µl) and absolute ethanol (150 µl).

The tube was vortexed briefly for 1 min. An equal volume of chloroform-isoamyl alcohol (49:1, v/v) was added and vortexed briefly and centrifuged at 16,000 g for 20 min at room temperature. The supernatant was gently moved to a fresh tube and repeated this process to eliminate any cell debris that was suspended. The supernatant was taken in a fresh tube, equal volume (850 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) added and vortexed for 10 s and centrifuged at 16000 g for 15 min at room temperature (RT). The supernatant was collected in a sterile fresh tube and 850 µl of chloroform-isoamyl alcohol (49:1, v/v) was added. Tube was briefly vortexed for 10 s and centrifuged at 16000 g for 15 min at 4ºC. The supernatant was then collected and 3 M LiCl was added to it, mixed by inversion and kept at 4ºC overnight. The content was then spun at 16000 g for 20 min at 4ºC. Pellet was recovered at this stage and washed twice with 70 per cent ethanol. Pellet was air dried at room temperature under laminar hood and resuspended in 30 µl of DEPC treated sterile distilled water. RNA was quantified in a UV-light spectrophotometer and stored at -80ºC until use.

### 3.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was done in a horizontal gel electrophoresis unit. Agarose (1 per cent) was weighed out and melted in the 1X TAE buffer (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0) prepared in (0.1 percent) DEPC treated water. Ethidium bromide ($10^{-3}$ mg $ml^{-1}$) was added to the agarose after cooling it at about 50°C. The mixture was then poured with the appropriate comb onto a pre set base. The comb and sealing tape were removed after the gel was set and the gel was placed in an electrophoresis tank. 1X TAE buffer was filled to around 1mm above the gel surface. RNA sample (5 µl) and gel-loading dye (2 µl) (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 documentation 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 wavelengths. 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 RNA isolated using Verso cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturers' protocol. The kit contained reverse transcriptase enzyme and Oligo (dT) primers. The kit also contained an RNase inhibitor which prevents the degradation of the isolated RNA. For removal of DNA contamination, RT enhancer was used. It effectively destroys the double stranded DNA during the process hence, DNase I treatment was not needed. It was disabled at 95 ºC.

| Components | Volume | Final concentration |
|---|---|---|
| 5X cDNA synthesis buffer | 4 µl | 1X |
| dNTP Mix | 2 µl | 500 µM each |
| RNA Primer | 1 µl | 2 µM |
| RT Enhancer | 1 µl | 20 U/ µl |
| Verso Enzyme Mix | 1 µl | 20 U/ µl |
| Template (RNA) | 1-5 µl | 1 ng |
| Nuclease-free water | To 20 µl | |
| **Total volume** | 20 µl | |

Reverse transcription cycling program:

| Steps | Temperature | Time | Number of cycles |
|---|---|---|---|
| cDNA synthesis | 42 ℃ | 30 min | 1 |
| Inactivation | 95 ℃ | 2 min | 1 |

According to the length of template strand and degree of secondary structure, the efficiency of the first strand synthesis may be improved by optimizing temperature and time (42-57 °C for 5-60 minutes).

**3.2.5 Confirmation of cDNA synthesis**

PCR analysis with actin gene (house-keeping gene), specific primers was done 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 primer (Forward primer 5'- ACATTGTTCTCA GCGGTGGAT and Reverse primer 5' CCACCTTAATCTTCATGCTGCTT), 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 (T100™ Thermal Cycler, Bio-Rad).

| Step | Stage | *Actin* | |
|---|---|---|---|
| | | **Temperature (°C)** | **Duration** |
| 1. | Initial denaturation | 95 | 1 min |
| 2. | Denaturation | 95 | 30 sec |
| 3. | Annealing | 55 | 30 sec |
| 4. | Extension | 72 | 30 sec |
| 5. | Final extension | 72 | 5 min |
| 6. | Storage | 12 | Infinite |

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

The PCR product was electrophoretically analyzed in agarose gel (one per cent) in a horizontal gel electrophoresis unit as explained above (section 3.2.2). One of the wells was loaded with 5 µl of the 100 bp molecular weight marker (Himedia Laboratories, Mumbai, India) 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 Rep and Mvp gene sequence of BBrMV

Complete genome sequence of BBrMV (Trichy isolate, Accession no. HM131454.1) was retrieved from GenBank [National Centre for Biotechnology Information (NCBI)]. Based on the sequence information, BBrMV Mvp gene specific primer was designed manually. For the Rep gene, primers already designed in the previous study in the department were used (Lekshmi, 2016). Alignment and melting temperature of the manually designed primer 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 Mvp and Rep partial genes are given below.

| Primer Name | Length | GC content (%) | Sequence |
|---|---|---|---|
| Mvp F | 20 | 55 | GAGGAAGTACGCGCATCAAC |
| Mvp R | 20 | 50 | ATCTCCATACCACGCCGTAA |
| Rep F | 20 | 55 | TGTAGTCCGCGGGAAATGTC |
| Rep R | 20 | 55 | GTGTCTAACGGAGCTGCTGT |

A standard PCR mix was prepared for a total volume of 20 µl containing 100 ng of template cDNA, 200 µM dNTPs, 10 pM of each primer, 1 unit of Taq polymerase, and 1x Taq polymerase buffer. The cDNA was amplified using sets of primers in a Thermal cycler (T100™ Thermal Cycler, Bio-Rad) using the program shown in the table below.

| Step | Stage | | BBrMV Mvp and Rep gene specific primers | |
|---|---|---|---|---|
| | | | Temperature (°C) | Duration |
| 1. | Initial denaturation | | 95 | 3 min |
| 2. | Denaturation | | 95 | 15 sec |
| 3. | Annealing | Mvp | 60 | 15 sec |
| | | Rep | 60 | 15 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 analyzed in agarose gel (one per cent) in a horizontal gel electrophoresis unit (section 3.2.2). One of the wells was loaded with 5 µl of the 100 bp molecular weight marker (Himedia Laboratories, Mumbai, India) 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 partial coding sequences of viral Rep and Mvp genes

The PCR products obtained by amplification with the BBrMV Mvp and Rep primers were sequenced (Appendix III) at SciGenom Labs, Cochin, Kerala.

## 3.3 ihpRNA VECTOR CONSTRUCTION

### 3.3.1 miRNA target prediction in the Mvp and Rep gene sequences of the virus

Partial sequences of Mvp and Rep encoding genes (partial cds) were 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 identified using the 'Custom Dicer- Substrate siRNA (DsiRNA) in RNAi design tools available at the website of Integrated DNA Technologies (IDT) (Appendix IV). The duplex RNA GC content (30-70 percent) and the asymmetrical end stability base pair length of 5 were set along with other target parameters detailed in (Appendix V). The targets found were selected and analyzed for siRNA attributes for higher efficiency.

### 3.3.2 Restriction mapping

The partial Mvp and Rep gene sequences isolated were 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 VI).

### 3.3.3 Viral silencing suppressor prediction

The partial sequences obtained for the Mvp and Rep gene were subjected to viral silencing suppressor prediction using the tool VSupPred. The nucleotide sequences were converted to protein sequence in FASTA format and uploaded for prediction of Non Viral Silencing suppressor Regions (NVSR).

### 3.3.4 Preparation of sense and antisense fragments

#### 3.3.4.1 Amplification of Rep and Mvp gene fragments

Based on the partial Mvp and Rep gene sequence of BBrMV Vellayani isolate, a separate set of primers were designed with restriction enzyme recognition sequence anchored to its 5' end. Primers were designed to include miRNA target regions and excluded selected restriction enzyme recognition sites. The primary vector pSTARLING has 3 different sets of restriction enzyme sites on either end of the *cre* intron. *Kpn*I and *Spe*I were selected for sense strand integration and *Asc*I and *Pac*I were selected for antisense strand integration.

| Gene | Direction | Sequence |
|---|---|---|
| Mvp Sense | Forward | 5' GGGGTACCCCTAAACGCAGAATGGAGAGCA 3' |
| | Reverse | 5'GACTAGTCTACTGTTCCGGTGGCGTACT 3' |
| Mvp Antisense | Forward | 5'AGGCGCGCCTTACTGTTCCGGTGGCGTACT 3' |
| | Reverse | 5'CCTTAATTAAGGTAAACGCAGAATGGAGAGCA 3' |
| Rep Sense | Forward | 5'GGGGTACCCCATGGTCCAAGCCGGTTAAAT3' |
| | Reverse | 5'GACTAGTCTTGGCCGTAATTCAGCTTTC3' |
| Rep Antisense | Forward | 5'AGGCGCGCCTTTGGCCGTAATTCAGCTTTC3' |
| | Reverse | 5'CCTTAATTAAGGATGGTCCAAGCCGGTTAAAT3' |

#### 3.3.4.2 Amplification of fragments containing both Rep and Mvp gene sequences

Partial Mvp and Rep gene sequences of BBrMV Vellayani isolate were subjected to NEBuilder Assembly Tool to generate the overlapping primers. The primers generated were anchored with respective restriction enzyme sites of the cloning sites of the vector. PCR reaction was carried out with the two sets of primers to assemble Mvp and Rep genes.

| Gene | Direction | Sequence |
|---|---|---|
| ihp Mvp | Forward | 5'GGGGTACCCCAAAATATCAAGACCTATTAAACGC3' |
| | Reverse | 5'ACCCTTATGTCTAGATGCTGTTCCGGTG3' |
| ihp Rep | Forward | 5'CAGCATCTAGACATAAGGGTGCTGAGAG3' |
| | Reverse | 5'GACTAGTC TTTGGCCGTAATTCAGCTTTC3' |
| ihp Mvp-Rep AS | Forward | 5'AGGCGCGCCT TTTGGCCGTAATTCAGCTTTC3' |
| | Reverse | 5'CCTTAATTAAGGAAAATATCAAGACCTATTAAACGC3' |

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 up to a 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 | 3 min |
| 2. | Denaturation | 94 | 30 sec |
| 3. | Annealing | 60 | 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 analyzed in agarose gel (1 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 (Himedia Laboratories, Mumbai, India) with 2 µl of 6x 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.

### 3.3.5 Elution of the sense and antisense fragments from agarose gel and quantification

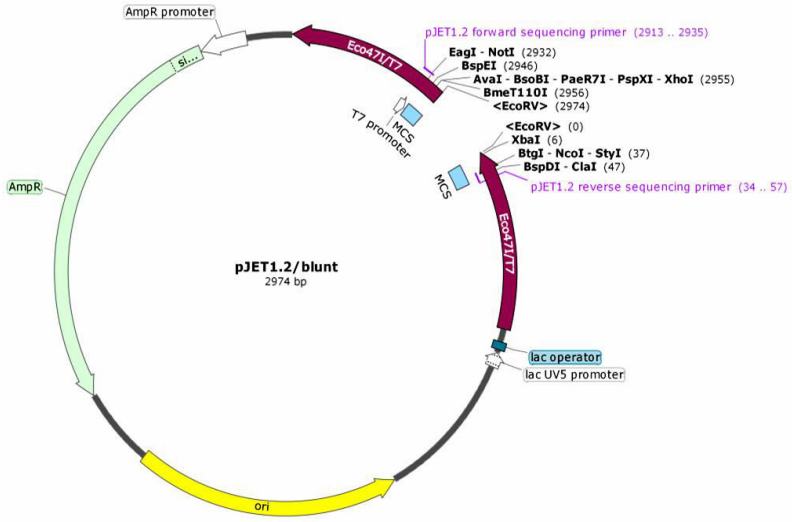
The sense and antisense fragments separated in low melting agarose (1.2 per cent) gel were sliced out with a clean scalpel and stored in a pre-weighed fresh 1.5 ml vial. Gel extractions of the excised bands were carried out using Thermo Scientific GeneJET Gel Extraction Kit following the manufacturers'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

### 3.3.6 Cloning of sense and antisense fragments in pJET1.2/Blunt vector

The sense and antisense fragments extracted were ligated to the linear cloning vector pJET1.2/Blunt (Thermo Fisher Scientific, USA). The ligation reaction was carried out by preparing the reaction mix consisting of

| **Components** | **Volume** |
|---|---|
| 2X Reaction Buffer | 10 µL |
| Eluted and purified PCR product | 0.15 pmol ends |
| pJET1.2/blunt Cloning Vector (50 ng/µL) | 1 µL (0.05 pmol ends) |
| Nuclease free water | Up to 19 µL |
| T4 DNA Ligase | 1 µL |
| **Total volume** | **20 µL** |

The reaction mix was spun briefly and incubated at 22°C for 1 h and then stored in 4ºC. The cloning site of the insert is within the *eco47IR* gene. It is the lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies. Recircularized pJET1.2/blunt vector molecules lacking an insert express a lethal restriction enzyme, which kills the host *E. coli* cell after transformation. Due to the positive selection process, colony screening was done on ampicillin amended medium and thus not required blue/white selection.




### 3.3.7 Transforming DH5α with ligated pJET1.2/Blunt vector

#### 3.3.7.1 Competent cell preparation

DH5α competent cells were prepared by calcium chloride ($CaCl_2$) method (Sambrook *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 VII) overnight at 37ºC on a rotary incubator. 1 ml of the overnight grown culture was transferred to 30 ml of fresh LB broth and incubated at 37ºC until the O.D. value reached 0.2 at 650 nm. Then the culture was transferred to fresh 2 ml tubes and stored in ice. The cells were pelleted at 5000g for 10 min at 5-10ºC. The supernatant was decanted and 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 5-10ºC. The supernatant was discarded. The pellet was resuspended in 100 µl of 0.1 M $CaCl_2$ and stored on ice prior to use.

#### 3.3.7.2 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 42ºC for 90 s. After incubation the tubes were quickly plunged in the ice bath and chilled for 5 min. 1 ml of LB broth at room temperature was added to the tube and incubated at 37ºC for 1 h with gentle shaking. The cells were plated on LB agar with Ampicillin 100 mg $L^{-1}$.

### 3.3.8 Plasmid isolation from transformed DH5α colonies

The transformed single colonies were picked and inoculated in 5 ml LB broth with ampicillin 100 mg $L^{-1}$ and were kept at 37ºC in the incubator shaker at 150 rpm overnight. Bacterial plasmid was isolated following the alkaline lysis method (Birnboim and Doly, 1979). 1.5 ml of the bacterial culture was taken in 2 ml microcentrifuge tubes and the cells were pelleted at 5000g for 5 min at 4ºC. The bacterial pellet was resuspended completely in 200 µl of solution I (Appendix VIII) by vortexing and incubated in an ice bath for 5 min. To that 300 µl 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 4ºC. 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 4ºC. The supernatant was transferred to a fresh tube and an equal volume of chilled isopropanol was added, mixed by inverting and incubated at -20ºC for 20 min. Pellet was obtained by centrifuging at 10,000g for 10 min at 4ºC. 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 dried and dissolved in 50µl TE Tris EDTA (Ethylene diamine tetra acetic acid) buffer (pH 8.0) (Appendix IX) containing RNase (2mg/ml) and incubated at 37ºC for 1h in water bath. The plasmid was quantified and stored in -20ºC.

### 3.3.9 Confirmation of the integration of sense and antisense strands by PCR

After plasmid isolation, screening was done to detect the insert using PCR. The plasmids isolated from the colonies grown on ampicillin medium were subjected to PCR with pJET forward primer and reverse primer. The standard 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 up to 20 µl. The DNA was amplified in the Thermal cycler using the program shown in the table below.

| **Step** | **Stage** | **pJET forward and reverse primers** | |
|---|---|---|---|
| | | **Temperature (°C)** | **Duration** |
| 1. | Initial denaturation | 94 | 1 min |
| 2. | Denaturation | 92 | 30 sec |
| 3. | Annealing | 60 | 30 sec |
| 4. | Extension | 72 | 45 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 into 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 documentation system.

### 3.3.10 Release of sense and antisense fragments from pJET1.2

The isolated plasmid DNA was subjected to restriction digestion to release the sense and antisense strands from pJET1.2 vector with sticky ends. The reaction mix

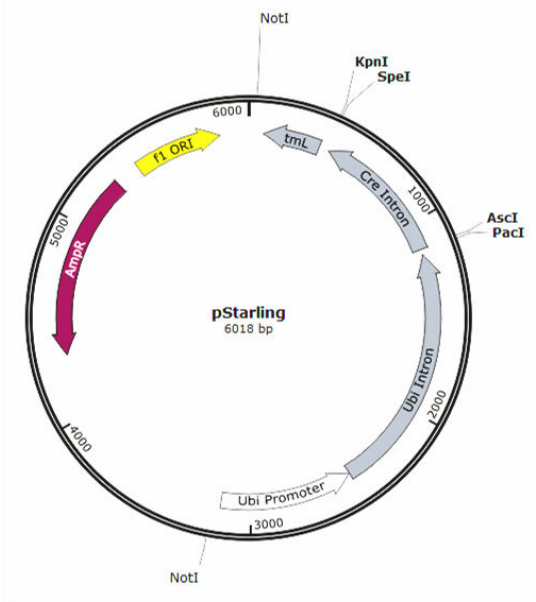
was prepared as shown below. The High Fidelity (HF) restriction enzymes from M/S New England BioLabs were used. The final volume of the reaction mix was made up to 50 µl. The reaction was carried out at 37ºC for 60 min and the reaction was terminated by adding 8 µl 6x purple gel loading dye. Gel electrophoresis with 1.2 per cent agarose was carried out to separate out the digested product.

| **Components for restriction reaction** | **pJET1.2 with sense fragments of Mvp-Rep, Rep, Mvp** | **pJET1.2 with antisense fragments of Mvp-Rep, Rep, Mvp** |
|---|---|---|
| Plasmid | 1 µg | 1µg |
| CutSmart Buffer | 1µl (1x) | 1µl (1x) |
| *Spe*I - HF® | 1.0 µl (or 10 units) | - |
| *Kpn*I - HF® | 1.0 µl (or 10 units) | - |
| *Pac*I - HF® | - | 1.0 µl (or 10 units) |
| *Asc*I - HF® | - | 1.0 µl (or 10 units) |

The electrophoretic analysis was documented and the bands of expected molecular weight i.e., 657 bp representing move-rep fragment, 357 bp for replicase and 224 bp for movement protein gene fragment (both sense and antisense fragments) from the pJET1.2 vector backbone were eluted out separately. The sense and antisense fragments were extracted using Thermo Scientific™ GeneJET Gel Extraction Kit. Eluted products were quantified using spectrophotometer and stored at -20ºC.

**3.3.11 Insertion of sense and antisense fragments to the primary vector**

The primary vector used in the study was pSTARLING (Appendix X), obtained from the Commonwealth Scientific and Industrial Research Organization (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 *Pac*I and *Asc*I at the 5' end and *Spe*I 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 digested with the corresponding restriction enzymes. In the first step restriction digestion of pSTARLING was carried out for integration of the antisense strand by preparing the reaction mix as shown below. The reaction volume was made up to 50µl and incubated at 37ºC for 60 min and the reaction was terminated by adding 8 µl 6x purple gel loading dye.

| **Components for restriction reaction** | **Concentrations** |
|---|---|
| Plasmid (pSTARLING) | 1µg |
| CutSmart Buffer | 5 µl (1x) |
| *Pac*I - HF® | 1.0 µl (or 10 units) |
| *Asc*I - HF® | 1.0 µl (or 10 units) |

After the restriction digestion, the linearized pSTARLING was separated on 1 percent agarose gel and was extracted using Thermo Scientific™ GeneJET Gel Extraction Kit. The eluted product was quantified using a spectrophotometer. A ligation reaction was carried out to ligate the antisense strand on the 5' end of the *cre* intron. The molar ratio of 1:3 vectors to insert was used to set the reaction. The ligation reaction was set up as shown below. The final reaction mixture was gently mixed by pipetting up and down and microfuged briefly and incubated at 16ºC for overnight and later heat inactivated at 65°C for 10 min and chilled on ice.

| **Components for the ligation reaction** | **Concentration** |
|---|---|
| T4 DNA ligase buffer (10x) | 2 µl |
| Linearized pSTARLING vector | 50 ng (0.020 pmol) |
| Insert (antisense strands) | 37.5 ng (0.060 pmol) |
| Nuclease free water | To 20 µl |
| T4 DNA ligase | 1 µl |

5 µl ligated product was transformed to 100 µl competent *E.coli* DH5α, plated on LB agar with ampicillin 100 mg $L^{-1}$ and incubated overnight at 37ºC. After overnight incubation, the colonies obtained were grown in LB broth with ampicillin 100 mg $L^{-1}$ and incubated at 37ºC in a rotary incubator shaker at 120 rpm. Plasmid was isolated from the overnight grown culture and subjected to PCR to detect the presence of antisense strand insert.

The primary vector with antisense fragment insert was subjected to linearization with the second set of restriction enzymes *Kpn*I and *Spe*I to ligate the sense fragment. The linearization reaction was set as follows. The reaction volume was made up to 50 µl and incubated at 37ºC for 60 min and the reaction was terminated by adding 8 µl 6x purple gel loading dye.

| Components for restriction reaction | Concentrations |
|---|---|
| Plasmid (pSTARLING + antisense) | 1µg |
| CutSmart Buffer | 5 µl (1x) |
| *Kpn*I - HF® | 1.0 µl (or 10 units) |
| *Spe*I - HF® | 1.0 µl (or 10 units) |

After restriction digestion, linearized pSTARLING was separated on 1 per cent agarose gel and was extracted using Thermo Scientific™ GeneJET Gel Extraction Kit. The eluted product was quantified using a spectrophotometer. A ligation reaction was carried out to ligate the sense strand on the 3' end of the *cre* intron. Ligation reaction was set up as shown in the table. Ligation reaction was set up as shown below. The components were mixed gently by pipetting up and down and spun briefly and incubated at 16ºC for overnight and later heat inactivated at 65°C for 10 min and chilled on ice.

| Components for the ligation reaction | Concentration |
|---|---|
| T4 DNA ligase buffer (10x) | 2 µl |
| Linearized pSTARLING vector with antisense | 50 ng (0.020 pmol) |
| Insert (sense strand) | 37.5 ng (0.060 pmol) |
| Nuclease free water | To 20 µl |
| T4 DNA ligase | 1 µl |

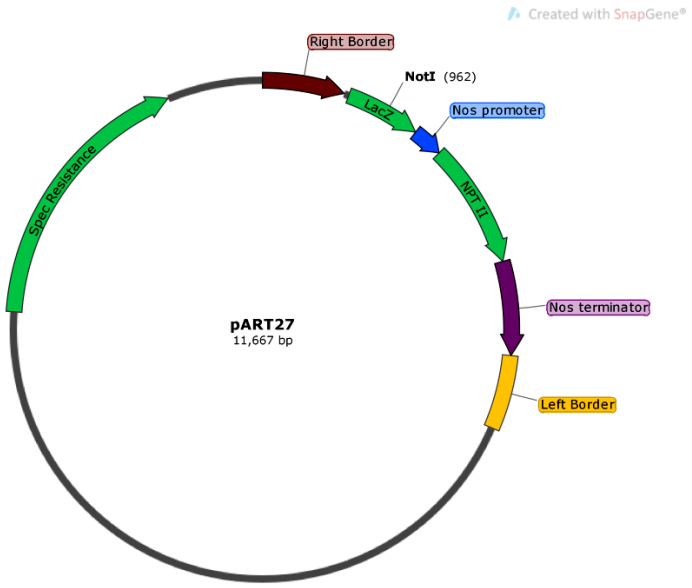
5 µl ligated product was transformed to competent *E.coli* DH5α (100 µl) and plated on LB agar with ampicillin 100 mg $L^{-1}$ and incubated at 37ºC overnight. After incubation, colonies obtained were picked and inoculated in LB broth with ampicillin 100 mg $L^{-1}$and grown overnight at 37ºC in the incubator shaker. The plasmid was isolated by alkaline lysis method and stored at -20ºC.

### 3.3.11.1 Confirmation of the integration of sense and antisense fragments in pSTARLING

From the transformed colonies pSTARLING vector carrying, Rep, Mvp and Mvp-Rep gene fragments were isolated and subjected to restriction digestion to release the sense and antisense strands. Restriction reactions were carried out separately for releasing the sense and antisense fragment inserts. Two µg of plasmid DNA and 1x CutSmart Buffer in two separate tubes were taken. In the first tube 1U each of *Asc*I and *Pac*I enzymes were used to release the antisense strand and in the second tube *Spe*I and *Kpn*I used to release the sense strand. The reaction volume was made up to 30 µl and was incubated at 37ºC for 60 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.

## 3.4 TRANSFER OF THE ihpRNA CONSTRUCT TO THE BINARY VECTOR

The *Not*I fragment in the pSTARLING vector comprised 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 Commonwealth Scientific and Industrial Research Organisation (CSIRO), Canberra, Australia through MTA. pART27 was obtained as *E.coli* stab culture. It was grown in LB plates with the antibiotic spectinomycin 100 mg $L^{-1}$. The physical map of which is shown below.




### 3.4.1 Release of *Not*I fragment from pSTARLING

pSTARLING vectors containing the three sense and antisense inserts were subjected to restriction digestion reaction to release out the *Not*I fragment having the ihpRNA construct. Restriction reaction was carried out with 2 µg of plasmid DNA, 5 µl (1X) CutSmart buffer and 2U of *Not*I- HF® enzyme. The reaction volume was made up to 50 µl and was incubated at 37ºC for 60 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 GeneJET Gel Extraction Kit. It was quantified and stored at -20ºC.

### 3.4.2 Insertion of *Not*I fragment to pART27

pART27 was linearized using *Not*I enzyme. Restriction reaction was carried out with 2 µg of plasmid DNA, 5 µl (1x) CutSmart buffer and 2U of *Not*I- HF® enzyme. The reaction volume was made up to 50 µl and was incubated at 37ºC for 60 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 GeneJET Gel Extraction Kit. It was quantified and stored at -20ºC.

For the integration of *Not*I fragment to the *Not*I site of pART27, a ligation reaction was carried out in a 20 µl 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 final reaction volume gently mixed by pipetting up and down and microfuge briefly and incubated at 16ºC for overnight and later heat inactivated at 65°C for 10 min and chilled on ice. The ligated plasmid was transformed and cloned using DH5α. The cells were plated on LB agar with spectinomycin 100 $mgL^{-1}$, 40 µl (20 mg/ml) X-gal and 40 µl 100mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for blue-white screening. The white colonies obtained were cultured in LB broth with corresponding antibiotics. The plasmid was isolated by alkaline lysis method and stored at -20ºC.

### 3.4.3 Confirmation of the inserts in pART27

The pART27 plasmid vectors carrying the three different constructs were isolated and subjected to restriction digestion to confirm the integration of the *Not*I fragment. Three separate reactions were kept to release the sense fragment, antisense fragment and *Not*I fragment. The reaction mixture contained 2µg of plasmid DNA, 5 µl (1x) CutSmart buffer and 2U of *Not*I- HF® enzyme to release the *Not*I fragment, *Kpn*I and *Spe*I to release the sense fragment and *Asc*I and *Pac*I to release the antisense fragment. The 50 µl reaction volume was incubated at 37ºC for 60 min. The products were separated out in one per cent agarose gel along with 5µl of 1-10 kb and 100 bp DNA molecular weight marker (Himedia Laboratories, Mumbai, India) and were documented using a gel documentation unit.

The pART27 plasmid vectors carrying the three different constructs were subjected to PCR analysis to confirm the presence of sense, antisense, *cre* and *npt*II gene. Also, the constructs were confirmed through the plasmid sequencing, using

sense forward and antisense reverse primers. Resulting sequences were analyzed using CLUSTALW sequence alignment online tool for the orientation of the fragments (Appendix XII).

## 3.5 TRANSFER OF THE BINARY VECTOR TO *AGROBACTERIUM TUMEFACIENS*

### 3.5.1 Preparation of competent *Agrobacterium tumefaciens*

The binary vector was transferred to *A. tumefaciens* strain GV3103. For that *A. tumefaciens* was made competent to take up the vector DNA according to the protocol reported by Jyothishwaran *et al*. (2007).

*A. tumefaciens* strain GV3103 was streaked out on a LB plate containing 25 mg $L^{-1}$ rifampicin and grown at 28°C overnight. A single colony was inoculated in 3 ml of LB medium containing 25 mg $L^{-1}$ rifampicin and grown overnight at 28°C, shaking at 160 rpm. 0.5 ml ($1/100^{th}$ volume) of the overnight culture was transferred to 50 ml of LB medium with 25 mg $L^{-1}$ rifampicin and grown in an incubator-shaker at 28°C at 160 rpm to obtain cell density of 0.3 at $OD_{600}$. The cultures were chilled on ice for 15 min and the cells were then harvested by centrifugation at 3000 rpm for 10 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 30 minutes to yield the competent cell suspension and aliquots of 100 µl were made.

### 3.5.2 Transformation of *A. tumefaciens*

The binary vector was transferred to the competent *A. tumefaciens* strain GV3103 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 an ice bath. Mixed gently by tapping and

frozen in liquid $N_2$ for 10 min. Then the vial was thawed at 37ºC for 5 min. 10 µl of this mixture was inoculated into 1 ml of pre-warmed LB medium without antibiotics and incubated for 1 h at 28ºC in a rotary shaker at 160 rpm. This 50 µl of the suspension was spread onto LB plates containing 25 mg $L^{-1}$ rifampicin and 100 mg $L^{-1}$ kanamycin. After incubation at 28ºC 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.

**Transformation efficiency = Colonies / µg / Dilution**

Colonies : The number of colonies grown on the plate

µg : The amount of DNA transformed

Dilution : The total dilution of the DNA before plating

### 3.5.3 Confirmation of transformation

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

| **Primers** | **Length** | **GC content (%)** | **Sequence 5'-3'** |
|---|---|---|---|
| *nptII* –F | 18 | 55 | GGTGCCCTGAATGAACTG |
| *nptII* -R | 18 | 50 | TAGCCAACGCTATGTCCT |
| *cre* –F | 20 | 40 | ACAGTCCAGCTTTTGTTGTT |
| *cre*- R | 20 | 50 | TGAGCTAGGAATCAGCTAGG |

Both the PCR reactions were carried out in 20 µl volume containing 100 ng plasmid DNA, 200 µM dNTPs, 10 pM 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 50 bp molecular weight marker with required volume of gel loading dye.

## 3.6 TRANSFORMATION OF BANANA EMBRYOGENIC CALLUS

### 3.6.1 Preparation of *Agrobacterium* Suspension for Co-cultivation

The *A. tumefaciens* GV3103 with the binary vector pART27 was grown on petri plates containing LB medium with spectinomycin 100 mg $L^{-1}$ at 28ºC overnight. *A. tumefaciens* suspension for co-cultivation was prepared by picking a single colony from the plate and inoculating into LB broth supplemented with spectinomycin 100 mg $L^{-1}$. 0.5 ml of the overnight culture was transferred to 30 ml of LB medium with 25 mg $L^{-1}$ rifampicin and 100 mg $L^{-1}$ spectinomycin and grown in an incubator shaker

at 28 °C at 160 rpm to obtain a cell density of 0.2 at $OD_{600}$. Then the culture was spun in a centrifuge at 5000 rpm at 4ºC for 5 min. The pellet obtained was resuspended in 1 ml of half strength MS broth (with 100 µM Acetosyringone) for co-cultivation.

### 3.6.2 Preparation of plant material

The embryogenic calli of banana were pre-cultured on fresh MS medium containing 8 mg $L^{-1}$ BA and 0.6 mg $L^{-1}$ TDZ for 15 days before co-cultivation to maintain cells in the active cell division stage.

### 3.6.3 Infection of the embryogenic callus by *A. tumefaciens*

The pieces of callus were placed in a sterile petri plate and wetted with liquid half MS medium to avoid drying of explants. The calli were then mixed thoroughly with the *Agrobacterium* suspension ($OD_{600}$, 0.2) with inducer acetosyringone (100 µM) by gentle swirling for 10 min.

### 3.6.4 Co-cultivation

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

### 3.6.5 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 petri plates containing half MS medium supplemented with 25 mg $L^{-1}$ cefotaxime for the eradication of remaining *Agrobacterium.* The calli were subcultured in semisolid MS medium supplemented with BA 8 mg $L^{-1}$, TDZ 0.6 mg $L^{-1}$ and 25 mg $L^{-1}$ cefotaxime in 2 days interval up to 2 weeks. Then the cultures were transferred to a fresh medium of the same composition (No cefotaxime) with kanamycin 200 mg $L^{-1}$.

### 3.6.6 Selection of Transformed calli

The transformed calli were selected on MS medium supplemented with BA 8 mg $L^{-1}$, TDZ 0.6 mg $L^{-1}$ and kanamycin 200 mg $L^{-1}$. The tissues were maintained by subculturing once in seven days in a fresh medium of the same composition. After four rounds of a subculture, the transformed and non-transformed tissues were scored based on their response in the medium. The embryogenic calli survived in the selection medium were transferred to MS medium supplemented with BA 2 mg $L^{-1}$ and 200 mg $L^{-1}$ kanamycin and incubated in light (14 h photoperiod 31.4 $\mu mol^{-2}$ $S^{1}$) for embryo formation and plantlet development.

## 3.7 CONFIRMATION OF TRANSFORMED SHOOTS

The binary vector was having *nptII* gene which confer kanamycin resistance as a 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 that survived in the selection medium (kanamycin 200 mg $L^{-1}$). The presence of ihpRNA cassette in the transformed shoots was also confirmed by PCR analysis.

### 3.7.1 Isolation of genomic DNA

Leaf tissues from the regenerated shoots were used for genomic DNA isolation. Total genomic DNA was isolated using a modified protocol of Chong-Perez *et al*. (2012). Leaf tissue (0.2 g) was washed in distilled water and blot dried. It was ground into fine powder using liquid nitrogen with mortar and pestle. It was then transferred quickly to a 2 ml centrifuge tube and 2 ml of pre-warmed extraction buffer was added (Appendix XIII). The mixture was homogenized by vortexing for a few seconds. 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 (200 µg $ml^{-1}$) for 15 min at 37ºC for removing the RNA contamination. The extracts were mixed with an equal volume of chloroform: isoamyl alcohol (24:1) and were 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 1 h and centrifuged for 15 min at 11,000 rpm, at 4ºC. The pellet was washed twice using 70 per cent ethanol; air dried and was dissolved in 100 µl water.

### 3.7.2 Polymerase Chain Reaction (PCR)

PCR mix (20 µl) was prepared which contained 100 ng of template DNA, 200 µM dNTPs, 10 pM of each primer, 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 primers specific to ihpRNA insert and *nptII* gene. The following conditions were provided for the amplification of the specific region in the genomic DNA in a Thermal cycler

| **Step** | **Stage** | | **Gene specific primer for *npt*II, Sense fragment and *cre* intron** | |
|---|---|---|---|---|
| | | | **Temperature (°C)** | **Duration** |
| 1. | Initial denaturation | | 94 | 2 min |
| 2. | Denaturation | | 92 | 30 sec |
| 3. | Annealing | *npt*II | 55 | 30 sec |
| | | Sense strand | 60 | |
| | | *cre* intron | 53 | |
| 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. For reference 100 bp ladder was kept as a marker.

### 3.8 Detection of siRNA synthesis by the transgenic plants towards the target genes

#### 3.8.1 Small RNA isolation

Small RNAs were isolated and purified using RNASure fusion miRNA mini kit (Genetic Nucleopore). This kit utilizes the lysis method (phenol and guanidium salt) and purification method based on the glass fiber membrane technology.

#### 3.8.2 Primer designing for Mvp and Rep specific siRNA

miRBase online database (www. mirbase.org) was used to predict the miRNA specific to the *Banana bract mosaic virus*. BBrMV Rep and Mvp gene sequences were converted to RNA sequence and searched for stem-loop sequences within the given viral sequence using BLASTn tool which is provided by miRBase database. With the BLASTn result stem-loop sequences were retrieved and used to find out the mature miRNAs in it. Mature miRNA sequences were used to design the forward primers and stem-loop primers. Reported conserved stem-loop sequence and reverse primers were selected as suggested by Kramer (2011). List of the designed primers given below.

| Primer name | Sequence |
|---|---|
| Rep_Stem-loop | 5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CAACTA 3' |
| Rep_siRNA F | 5' CGG GTG G GA GCT GAT TGT TGG 3‘ |
| Mvp_Stem-loop | 5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC TCTGGC 3' |
| Mvp_siRNA F | 5' GGC CGG GG A AAA GAA AGT CGA TC 3' |
| Universal R | 5’ CCA GTG CAG GGT CCG AGG TA 3’ |

### 3.8.3 Synthesis of first strand of cDNA

Complementary DNA (cDNA) was synthesized using 1 µg of isolated small RNA with stem loop primers specific for mature miRNA sequences in Rep and Mvp sequences in two separate 20 µl reaction volume with dNTP mix (10 mM) and Verso enzyme mix (Thermo Scientific). The reaction mixture was incubated for 30 min at 42ºC followed by 95ºC for 2 min to stop the reaction. The cDNA was stored at -20 ºC until use.

### 3.8.4 PCR with siRNA specific primers

A standard PCR mix was prepared in 20 µl total volume containing 100 ng of template cDNA, 200 µM dNTPs, 10 pM of stem-loop forward and universal reverse primer, 1 unit of Taq DNA polymerase and 1 X Taq polymerase buffer. The cDNA was amplified in a Thermal cycler (T100$^{TM}$ Thermal Cycler, Bio-Rad) using the programme shown in the table below.

| **Step** | **Stage** | | **BBrMV siRNA specific primers** | |
|---|---|---|---|---|
| | | | **Temperature (ºC)** | **Duration** |
| 1 | Initial denaturation | | 95 | 3 min |
| 2 | Denaturation | | 95 | 30 sec |
| 3 | Annealing | Rep_siRNA | 58 | 30 sec |
| | | Mvp_siRNA | 58 | 30 sec |
| 4 | Extension | | 72 | 45 sec |
| 5 | Final extension | | 72 | 5 min |

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

The PCR product was electrophoretically analysed in agarose gel (1.5 per cent) in a horizontal gel electrophoresis along with 50 bp DNA marker (Himedia Laboratories, Mumbai, India).

# Results

# 4. RESULTS

The results of the present study on "ihpRNA mediated resistance for *Banana bract mosaic virus* in *Musa* spp. by targeting replicase and movement protein genes" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during September 2016 to February 2020 are presented below. The study aimed to develop ihpRNA constructs targeting replicase and movement protein genes of *Banana bract mosaic virus*, to generate transformants of banana cultivar Grand Naine carrying the ihpRNA cassette and to confirm the transformants.

## 4.1 ESTABLISHMENT OF SOMATIC EMBRYOS

### 4.1.1 Embryogenic callus initiation and regeneration

Immature male inflorescences (IMFs) of the commercially important banana cultivar 'Grand Naine' were used as explant for producing callus (Plate 1A). 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. The explants were sterilized by wiping it using an ethanol soaked cotton swab.

IMFs produced callus under dark conditions in 45 days after inoculation in the MS medium with 8 mg $L^{-1}$ Benzyl adenine (BA) and 0.6 mg $L^{-1}$ Thidiazuron (TDZ) with 5 gm $L^{-1}$ gelrite (Plate 1B). This medium composition yielded 12 per cent callus induction. Embryogenic callus developed was small to medium sized, yellow, globular and friable with dense cytoplasm. The glassy friable globular embryos were developed on the same medium after three subcultures (80-90 days after inoculation) (Plate 2). These embryos were germinated in 15 days of inoculation on MS medium supplemented with 2 mg $L^{-1}$ BA and 5 gm $L^{-1}$ gelrite and 16 h

**Plate 1A.** Immature male flowers (IMFs) bud inoculated on MS medium supplemented with BA (8 mg $L^{-1}$) and TDZ (0.6 mg $L^{-1}$).

**Plate 1B.** Embryogenic calli development in 45 days on MS medium supplemented with BA (8 mg $L^{-1}$) and TDZ (0.6 mg $L^{-1}$).

photoperiod with light intensity of 31.4 µmol $m^{-2}$ $s^{-1}$ showing elongated monocot embryo structures and proper morphogenesis (Plate 3A and B).

## 4.2 ISOLATION OF REPLICASE (Rep) AND MOVEMENT PROTEIN (Mvp) GENES OF BBrMV

### 4.2.1 RNA isolation

The gel picture of the total RNA isolated showed a typical eukaryotic RNA profile with bands representing 23S, 18S and 5S rRNA (Plate 4). A near ratio of 2:1 for 28S and 18S rRNA when resolved by electrophoresis and ethidium bromide staining indicated that no significant degradation of RNA has occurred. The crisp bands indicated the intact nature of the nucleic acid isolated.

RNA isolated was quantified with UV visible spectrophotometer. Absorbance ratio $A_{260}/A_{230}$ was recorded as 2.31 and $A_{260}/A_{280}$ was recorded as 2.06, indicating good quality of RNA. The concentration of RNA was calculated as 202.293 µg $g^{-1}$ fresh weight.

### 4.2.2 cDNA synthesis

cDNA synthesis using Thermo Scientific 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 650 bp which was the expected size of the actin gene (Plate 5). The result confirmed the successful conversion of mRNA to cDNA.

### 4.2.3 Isolation of partial coding fragments of Rep and Mvp genes of BBrMV

PCR using the Rep and Mvp gene specific primers resulted in the amplification of fragments of expected size 421 and 456 bp respectively (Plate 6).

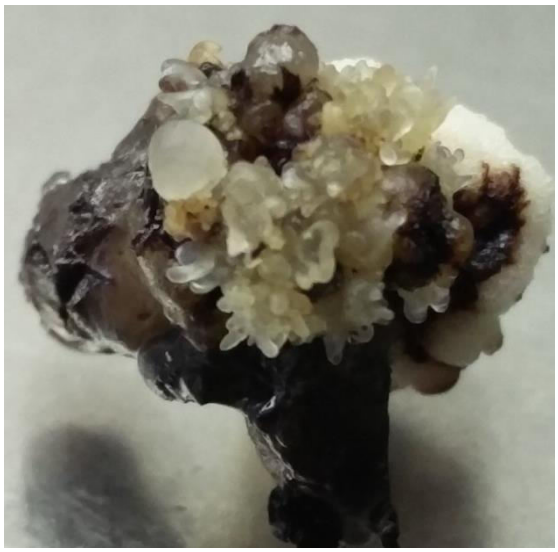
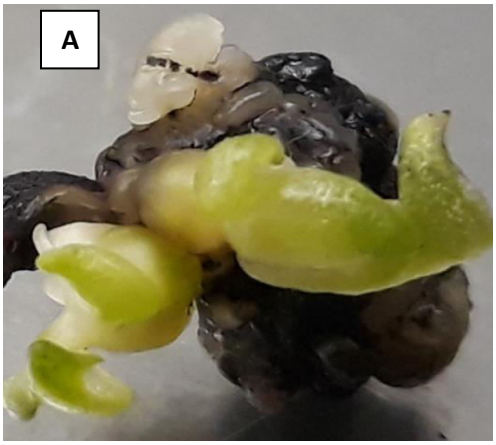
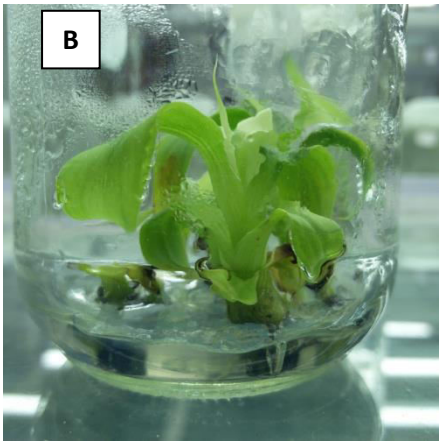


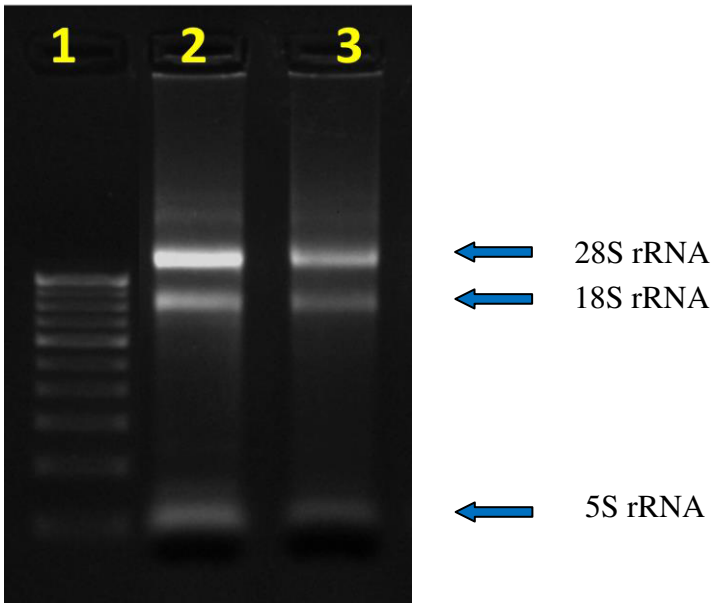
**Plate 2**. Development of Somatic embryos on MS medium supplemented with BA (8 mg $L^{-1}$) and TDZ (0.6 mg $L^{-1}$).



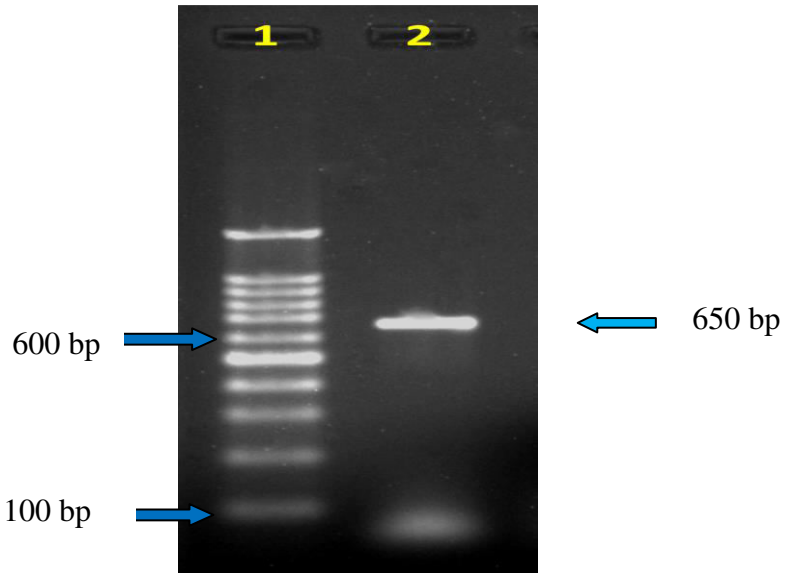

A) Germinating somatic embryos B) Regenerated plantlet

**Plate 3A and B**. Germination (A) and regeneration (B) of somatic embryos in MS medium supplemented with BA (2 mg $L^{-1}$)



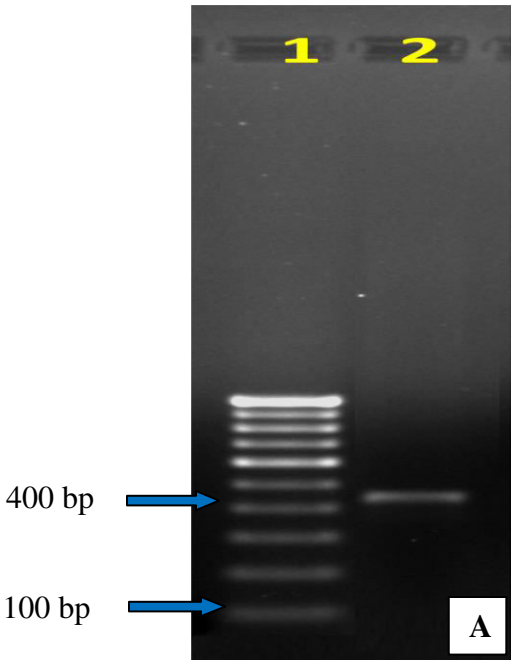

Lane 1: 100 bp DNA ladder
Lane 2-3: Replications of total RNA isolated

**Plate 4**. Total RNA isolated from BBrMV infected banana flower bract tissue cv. Grand Naine

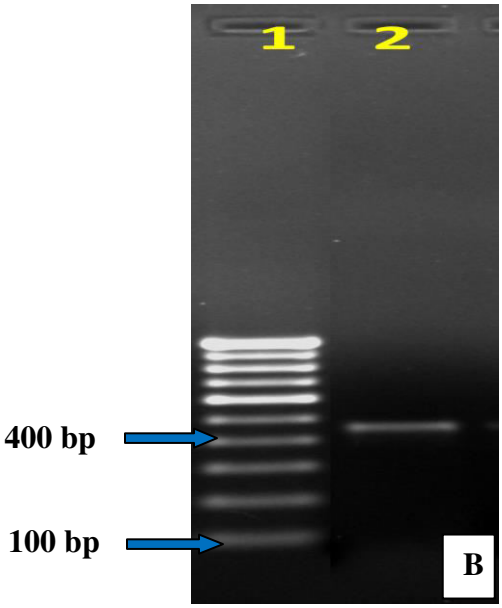



Lane 1: 100 bp DNA marker
Lane 2: Amplified *Actin* gene fragment at 650 bp

**Plate 5**. Confirmation of cDNA formation with actin specific primer




Lane 1: 100 bp DNA ladder
Lane 2: Amplified Rep gene fragment (421 bp)




Lane 1: 100 bp DNA ladder
Lane 2: Amplified Mvp gene fragment (456 bp)

**Plate 6.** Gel profiles showing PCR amplified fragments of A) Rep and B) Mvp genes of BBrMV

### 4.2.4 Sequencing and analysis of partial coding fragments of BBrMV Rep and Mvp genes

PCR products representing Rep and Mvp genes were sequenced and generated 401 bp for Rep and 339 bp for Mvp gene nucleotide sequence. The sequence information (Appendix III) of Rep and Mvp gene of BBrMV, Vellayani isolate was deposited in GenBank, National Centre for Biotechnology Information (NCBI) with accession number MH253671 and MH253670. Both the sequences were analysed using Basic Local Alignment Search Tool (BLAST) offered by NCBI.

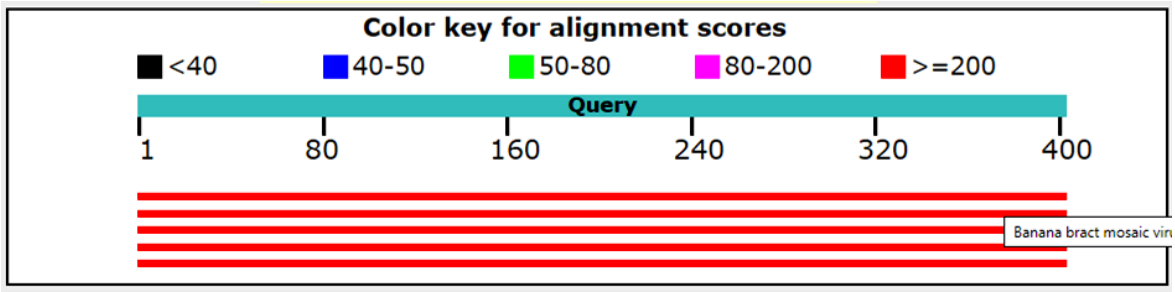
BLASTn results for Rep gene fragment showed a maximum of 96.37 per cent sequence identity with zero E-value for Trichy isolate (Tamil Nadu) (Acc. No.: HM131454.1) in Nendran variety and 95.34 per cent similarity with Vellayani isolate (Acc. No. KM357543.1) (Kerala) in Nendran variety (Plate 7A).

Mvp gene fragment showed 100 per cent similarity in BLAST result with complete genome sequence of BBrMV Philippine isolate (Acc. No.: DQ851496.1). Nucleotide sequence of isolates from various regions of India and worldwide shared 95-98 per cent sequence identity with the movement protein gene of BBrMV isolate from Vellayani (Plate 7B).

## 4.3 ihpRNA VECTOR CONSTRUCTION

### 4.3.1 miRNA target prediction

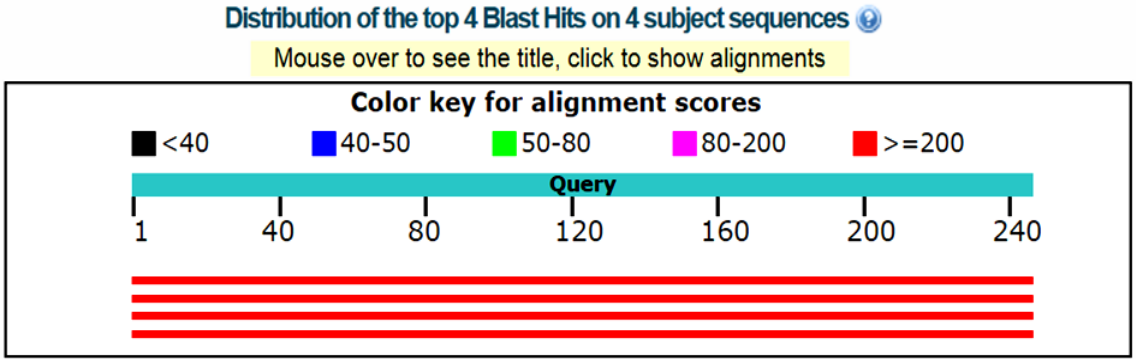
Partial Rep and Mvp gene sequences were subjected to further analysis. RNAi design tool from the IDT site predicted three miRNA target sites in the coding region of the Rep gene (401 bp) and Mvp gene (246 bp). The sequence at 5’ terminus of the target gene is mostly preferred for ihpRNA construct and hence the target region identified in this portion was selected (Appendix IV). The parameters deciding the target regions are detailed in Appendix V. Primers for the amplification of the sense




Alignments | Download | GenBank | Graphics | Distance tree of results

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Banana bract mosaic virus isolate TRY, complete genome | 652 | 652 | 100% | 0.0 | 96.37% | HM131454.1 |
| Banana bract mosaic virus isolate Wayanad, complete genome | 647 | 647 | 100% | 0.0 | 95.77% | MG758140.1 |
| Banana bract mosaic virus isolate A nonfunctional replicase mRNA, partial sequence | 641 | 641 | 100% | 0.0 | 95.51% | KM357543.1 |
| Banana bract mosaic virus, complete genome | 586 | 586 | 100% | 4e-170 | 93.03% | DQ851496.1 |
| Banana bract mosaic virus isolate BBrMV-Ginger, complete genome | 580 | 580 | 100% | 2e-168 | 92.79% | KT456531.1 |

**Plate 7A**. BLAST analysis of Rep gene sequence of BBrMV




Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| | Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|---|
| ☐ | Banana bract mosaic virus, complete genome | 455 | 455 | 100% | 7e-131 | 100.00% | DQ851496.1 |
| ☐ | Banana bract mosaic virus isolate BBrMV-Ginger, complete genome | 433 | 433 | 100% | 3e-124 | 98.37% | KT456531.1 |
| ☐ | Banana bract mosaic virus isolate Wayanad, complete genome | 416 | 416 | 100% | 3e-119 | 97.15% | MG758140.1 |
| ☐ | Banana bract mosaic virus isolate TRY, complete genome | 411 | 411 | 100% | 2e-117 | 96.75% | HM131454.1 |

**Plate 7B.** BLAST analysis of Mvp gene sequence of BBrMV

and antisense fragments were designed to include maximum miRNA target regions in the amplicon.

### 4.3.2 Restriction mapping

Restriction map for Rep (401 bp) and Mvp gene (246 bp) was obtained 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 VI). According to the result it was confirmed that the sequence selected for the preparation of sense and antisense fragments, did not possess recognition sites for *Asc*I, *Pac*I, *Kpn*I, *Spe*I or *Not*I restriction enzymes which are present at the cloning site of the vector. It was thus made clear that these restriction enzymes would make a cut only in the primary vector pSTARLING and nowhere within the sense or antisense fragments.

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

### 4.3.3 Viral silencing suppressor prediction

The partial Rep and Mvp gene sequences were subjected to viral silencing suppressor prediction using the tool VSupPred. The maximum confidence score of the algorithm is +1 and the minimum score is -1. The score mentioned that the fragment is a Non Viral Silencing suppressor Region (NVSR) with a confidence score of 0.775 for Rep and 0.725 for Mvp gene.

### 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 357 bp for Rep, 224 bp for Mvp and 647 bp for combination of Mvp-Rep with *Spe*I and *Kpn*I recognition sequences anchoring at the 5' ends of the forward and reverse primers respectively. At the same time primers for the amplification of antisense fragments were designed with *Asc*I and *Pac*I recognition sequences anchored at the 5' end of the forward and reverse primers respectively. Electrophoresis of the PCR products showed amplicons of expected size i.e., 357 bp for Rep, 224 bp for Mvp and 647 bp for Mvp-Rep (Plate 8).

### 4.3.5 Elution of the sense and antisense fragments and quantification

The sense and antisense fragments were extracted using GeneJet gel elution kit. The eluted PCR product was quantified using UV-visible spectrophotometer. The concentration of eluted sense and antisense fragments is given in Table 1.

**Table1. Spectrophotometric observations of the eluted sense and antisense fragments**

| Construct | Fragment | Concentration (µg µl$^{-1}$) |
|---|---|---|
| Rep | Sense | 3.6 |
| | Antisense | 3.36 |
| Mvp | Sense | 3.4 |
| | Antisense | 4.8 |
| Mvp-Rep | Sense | 4.7 |
| | Antisense | 4.22 |

### 4.3.6 Cloning of the sense and antisense amplicons in pJET1.2/ Blunt vector

The sense and antisense fragments of Rep, Mvp and Mvp-Rep were ligated to the linear blunt cloning vector pJET1.2 in separate reactions. After ligation reaction

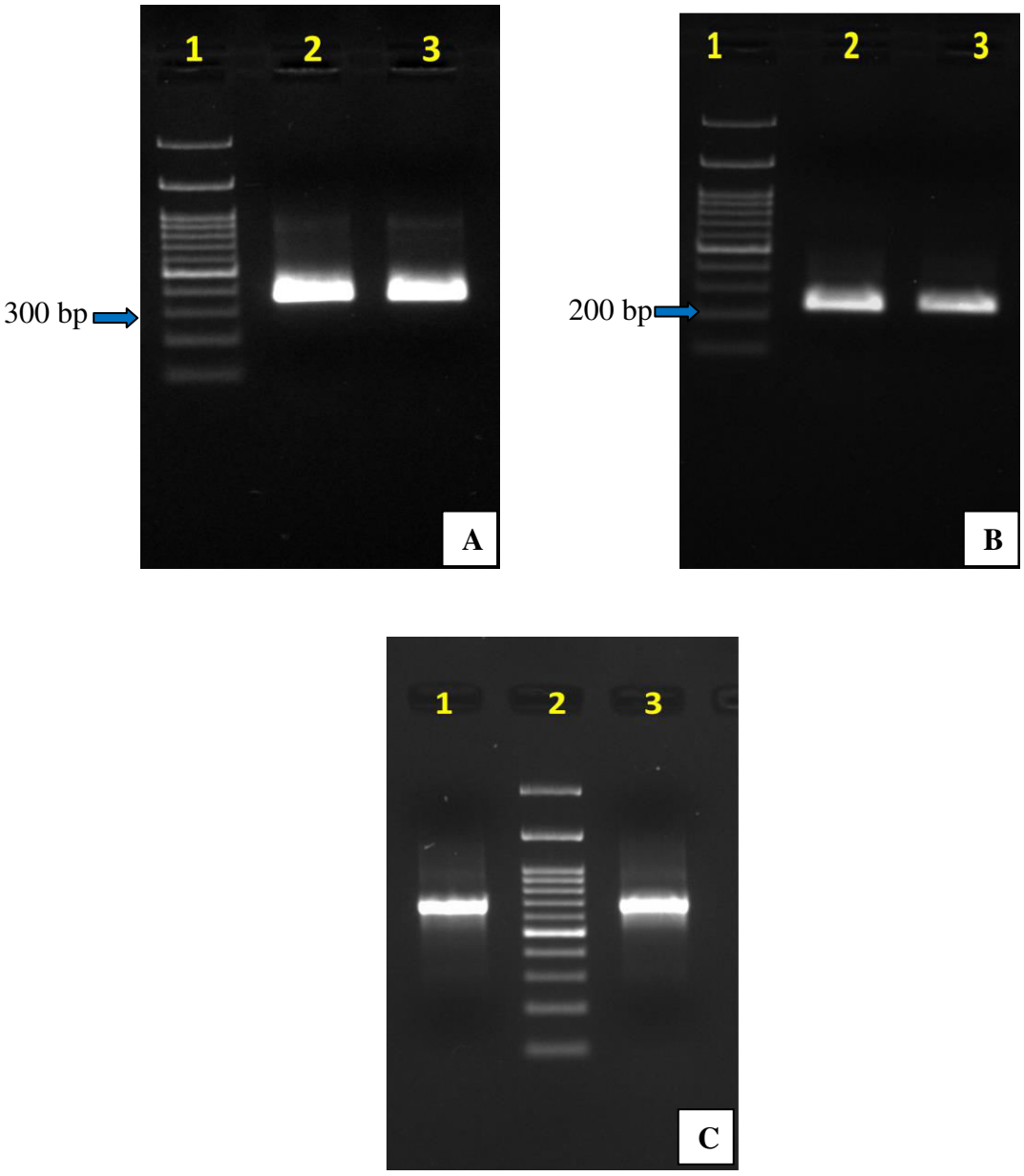



**Plate 8**. Gel profile showing amplicons of sense and antisense fragments of (A) Rep (B) Mvp and (C) Mvp-Rep:
***A) Rep:*** Lane 1: 100 bp DNA ladder, Lane 2: Amplified Rep sense fragment (357 bp), Lane 3: Amplified Rep antisense fragment (357 bp); ***B) Mvp:*** Lane 1: 100 bp DNA ladder, Lane 2: Amplified Mvp sense fragment (224 bp), Lane 3: Amplified Mvp antisense fragment (224 bp); ***C) Mvp-Rep:*** Lane 1: Amplified Mvp-Rep sense fragment (647 bp), Lane 2: 100 bp DNA ladder, Lane 3: Amplified Mvp-Rep antisense fragment (647 bp)

transformation was done in competent DH5α and the positive clones obtained on selection medium (LB plates with ampicillin 100 mg $L^{-1}$) are shown in Plate 9. They were picked and the master plate was made for further use and stored at 4°C.

### 4.3.7 Confirmation of integration of the sense and antisense fragments

Plasmid was isolated from transformed colonies. PCR was carried out using pJET forward and reverse primes to confirm the integration of sense and antisense fragments. pJET primers possess 59 bases extra on both sides, so when amplified the product shows the extra length of 118 bp. The PCR product when separated electrophoretically showed expected band size of 475 bp for Rep, 342 bp for Mvp and 765 bp for Mvp-Rep (Plate 10) confirming the integration.

### 4.3.8 Release of sense and antisense fragments from pJET1.2

The sense and antisense fragments from pJET1.2 were released by digesting the vector DNA using the respective restriction enzymes. Fragments of expected size i.e. 357 bp for Rep, 224 bp for Mvp and 647 bp for Mvp-Rep were released from the vector backbone (Plate 11). These sense and antisense strands were eluted out separately and quantified. The concentration of eluted sense and antisense fragments is given in Table 2.

**Table2. Spectrophotometric observations of the eluted sense and antisense fragments from pJET1.2 vector**

| Constructs | Fragment | Concentration (µg $µl^{-1}$) |
|---|---|---|
| Rep | Sense | 3.50 |
| | Antisense | 3.36 |
| Mvp | Sense | 2.15 |
| | Antisense | 2.36 |
| Mvp-Rep | Sense | 3.18 |
| | Antisense | 4.20 |

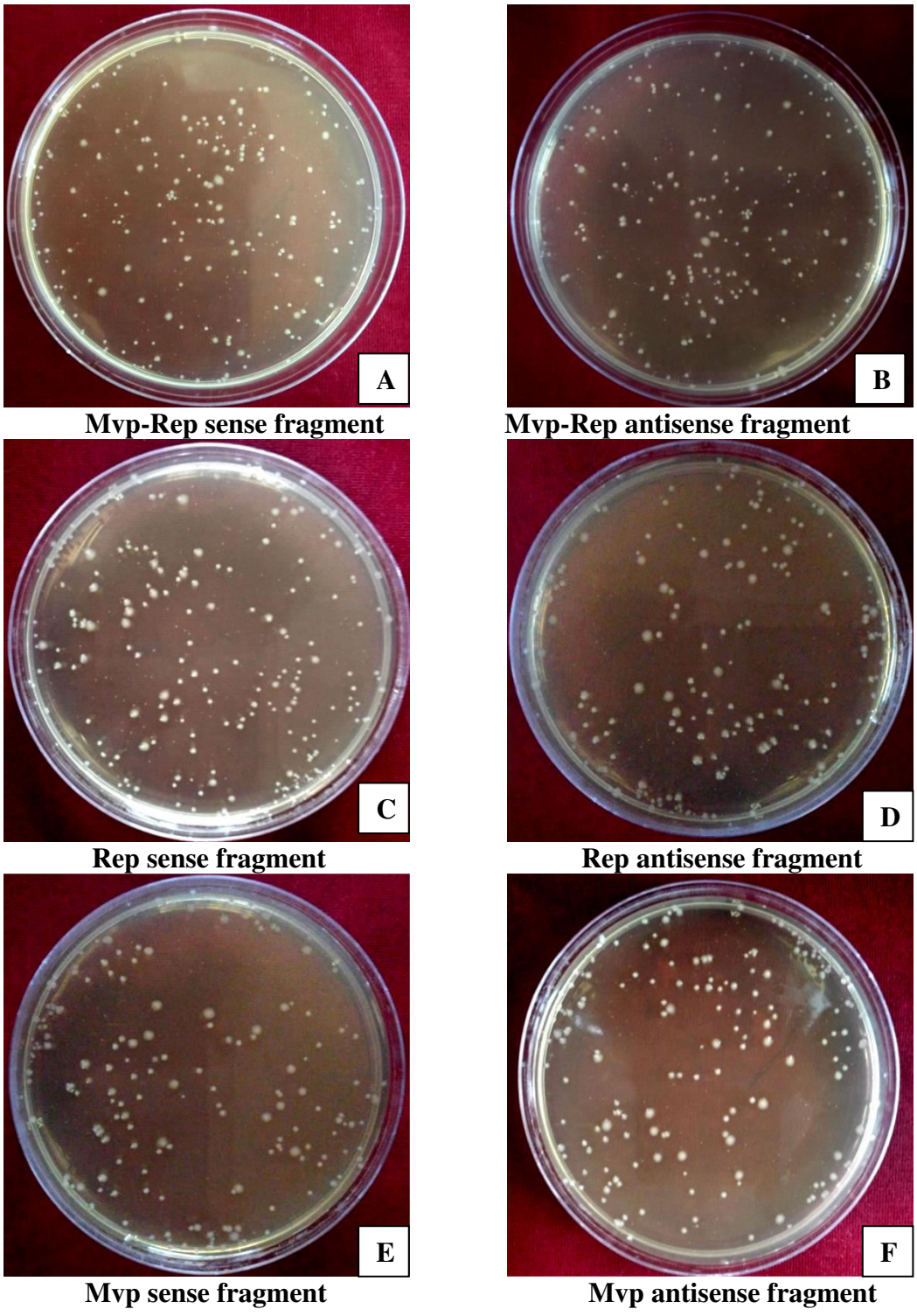



**Plate 9:** Colonies of pJET1.2 vectors (A-F) with sense and antisense fragment

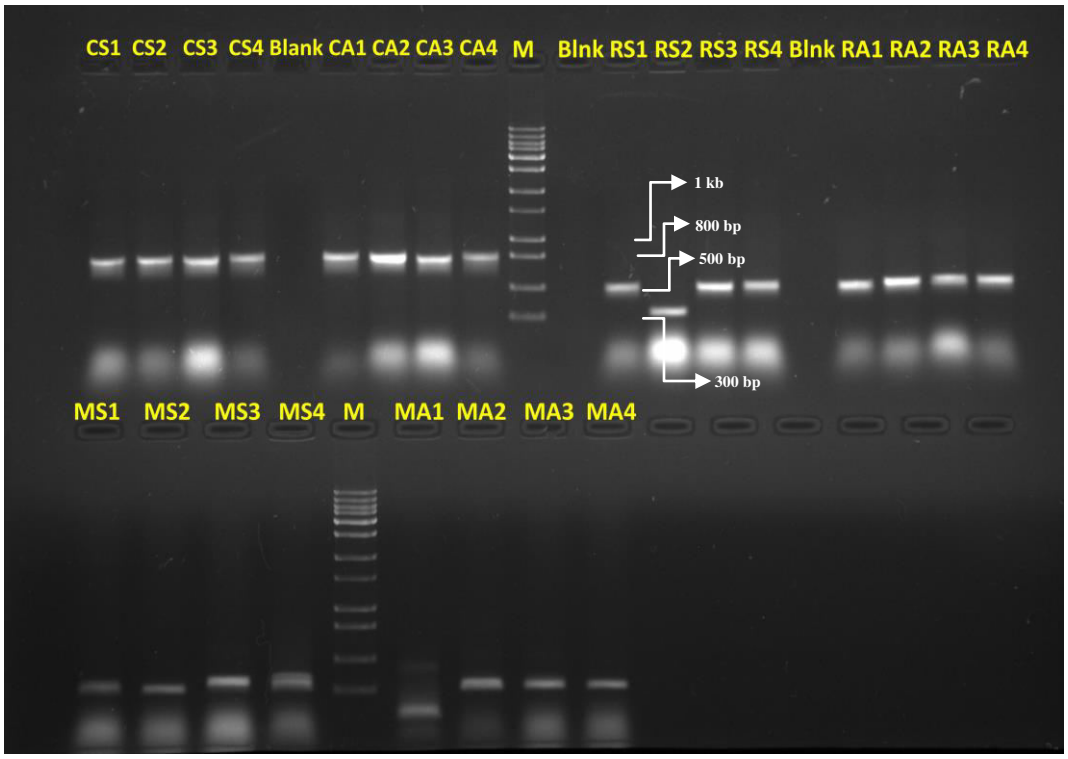



**Plate 10**: Gel profile showing amplicons of colony PCR to confirming integration of sense and antisense strands in pJET1.2 vector using pJET forward and reverse sequencing primers: Lane CS: Amplified Mvp-Rep sense fragment (765 bp), Lane CA: Amplified Mvp-Rep antisense fragment (765 bp), Lane M: 1 Kb plus DNA ladder, Lane RS: Amplified Rep sense fragment (475 bp), Lane RA: Amplified Rep antisense fragment (475 bp), Lane MS: Amplified Mvp sense fragment (342 bp), Lane MA: Amplified Mvp antisense fragment (342 bp). Lane 1-4: Randomly selected colonies.

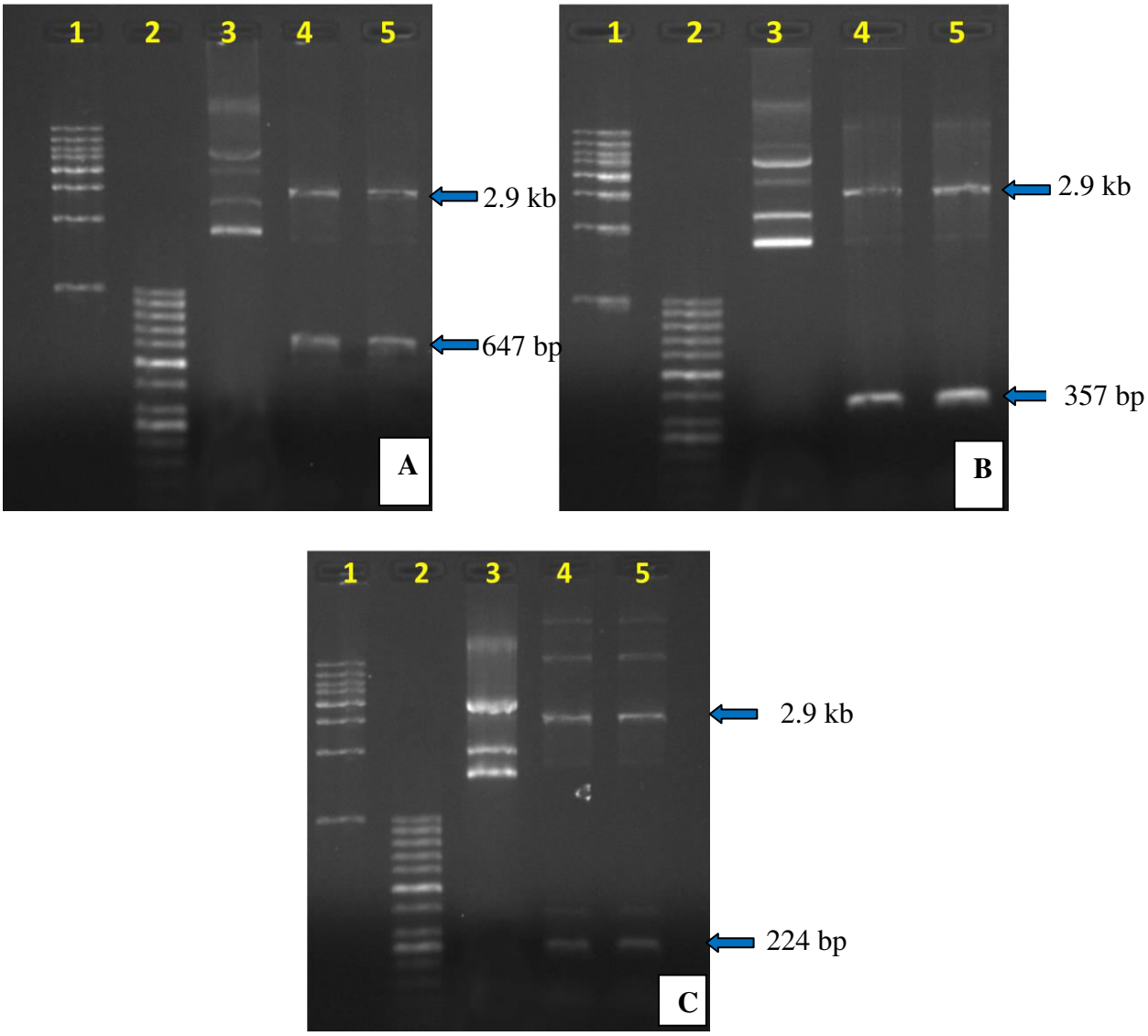



**Plate 11**: Gel profile showing the released sense and antisense fragments from the pJET vector by restriction digestion using *Spe*I and *Kpn*I to release the sense fragment and *Asc*I and *PacI* to release antisense fragment: **(A) Mvp-Rep (B) Rep (C) Mvp.** Lane 1: 1 kb DNA ladder, Lane 2: 50 bp DNA ladder, Lane 3: pJET1.2 with insert uncut, Lane 4: Sense fragment released from pJET 1.2 (2974 bp) with *Spe*I and *Kpn*I Lane 5: Antisense fragment released from Pjet1.2 with *Asc*I and *Pac*I.

### 4.3.9 Insertion of sense and antisense fragments to the primary vector

pSTARLING was subjected to double digestion using the *Asc*I and *Pac*I for linearization (Plate 12). The linearized product was purified from the components of restriction reaction by electrophoretic separation and gel extraction. The concentration of extracted linear pSTARLING obtained was 3.18µg $\mu l^{-1}$. Linear pSTARLING with *Asc*I and *Pac*I sticky ends was subjected to ligation reaction with the released antisense fragment from pJET1.2 having *Asc*I and *Pac*I sticky ends. The ligated vector was transformed to competent DH5α. From the colonies selected on LB broth containing ampicillin 100 mg $L^{-1}$, plasmids were isolated and screened for the presence of antisense strands by PCR. With the corresponding primers of antisense strands bands of expected sizes were obtained i.e. 357 bp for Rep, 224 bp for Mvp and 647 bp for Mvp-Rep (Plate 13). The plasmids were found to have the concentration of 2.89 µg $\mu l^{-1}$ for Rep, 3.16 µg $\mu l^{-1}$ for Mvp and 3.39 µg $\mu l^{-1}$ for Mvp-Rep.

pSTARLING vectors carrying the antisense inserts were subjected to double digestion with *Spe*I and *Kpn*I (Plate 14) and were purified by gel elution procedure. The sense fragments having strand *Spe*I and *Kpn*I sticky ends were ligated to the linearized pSTARLING vector and transformed to competent DH5α. Plate 15 shows the transformed colonies grown on LB plates containing ampicillin 100 mg $L^{-1}$. The plasmids were isolated from transformed colonies and separated electrophoretically on 1 per cent agarose gel (Plate 16).

### 4.3.10 Confirmation of integration of the sense and antisense fragments of target genes by restriction digestion reaction

pSTARLING vector carrying the sense and antisense fragments of Rep, Mvp and Mvp-Rep, on restriction digestion released sense (*Spe*I and *Kpn*I) and antisense (*Asc*I and *Pac*I) fragments of size 357, 224 and 647 bp respectively from the

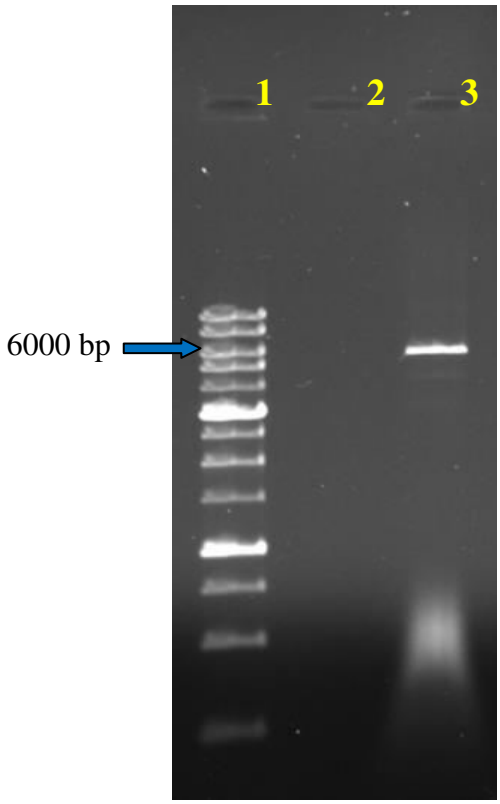



**Plate 12:** Gel profile showing linearized pSTARLING vector with restriction enzymes *Asc*I *and Pac*I. Lane 1: 10 kb DNA ladder, Lane 2: Blank, Lane 3: linearized pSTARLING (6018 bp) vector with *Asc*I and *Pac*I restriction enzymes.

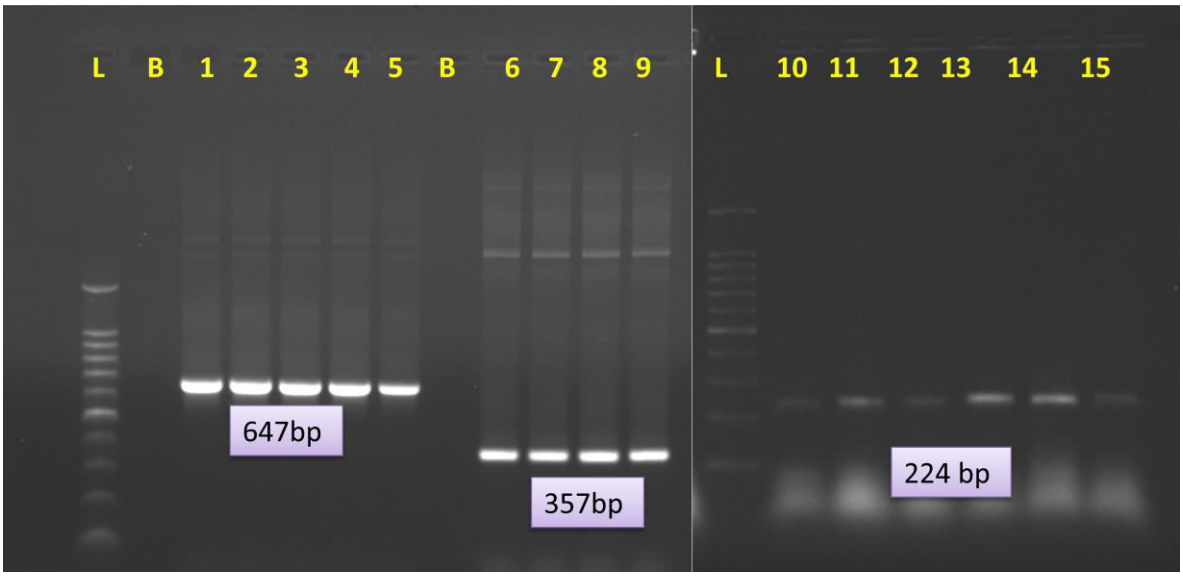



**Plate 13**: Gel profile showing amplicons of colony PCR confirming the integration of antisense fragment in the pSTARLING vector. Lane L:100 bp ladder; Lane B: Blank, Lane 1-5: Amplified Mvp-Rep Antisense fragment (647 bp), Lane 6-9: Rep antisense fragment (357 bp), Lane 10-15: Mvp antisense fragment (224 bp).

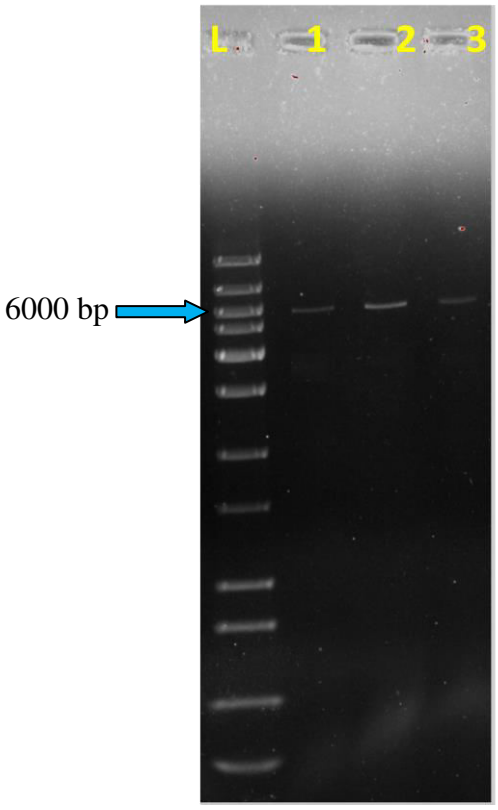
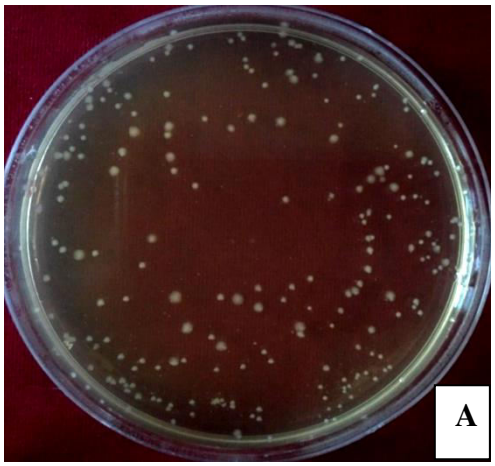
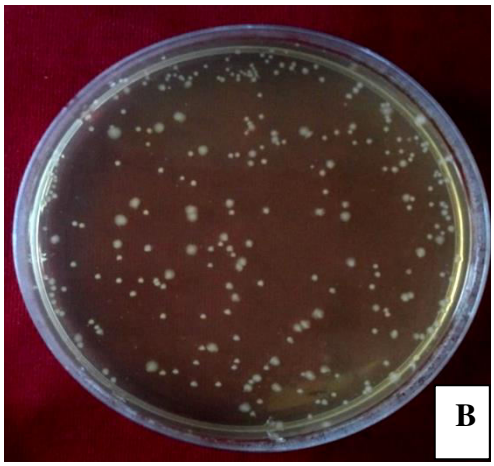


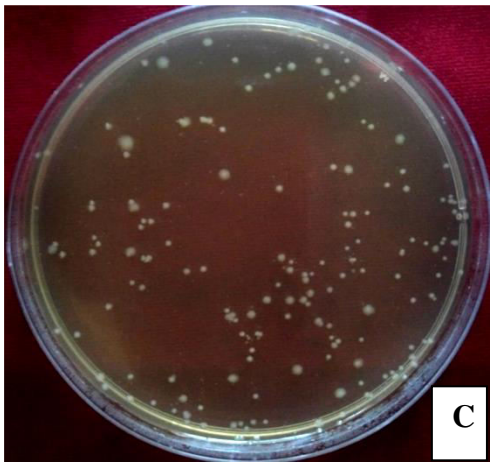

**Plate 14**: Gel profile showing transformed pSTARLING with antisense fragment digested with *Kpn*I and *Spe*I. Lane L: 1 kb plus DNA ladder, Lane 1: pSTARLING with Mvp antisense, Lane 2: pSTARLING with Rep antisense, Lane 3: pSTARLING with Mvp-Rep antisense.




Colonies of *E. coli* DH5α carrying pSTARLING vector with ihpRNA Mvp-Rep cassette




Colonies of *E. coli* DH5α carrying pSTARLING vector with ihpRNA Rep cassette




Colonies of *E. coli* DH5α carrying pSTARLING vector with ihpRNA Mvp cassette

**Plate 15:** pSTARLING vector with the ihpRNA cassette cloned to DH5α in LB plates (A-C) containing ampicillin 100 mg $L^{-1}$

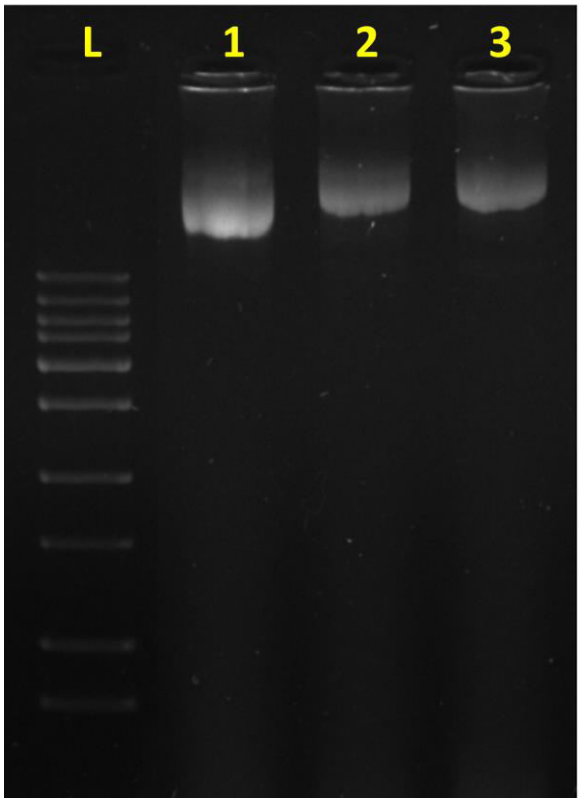



**Plate 16:** Gel profile showing the isolated plasmid from sense and antisense integration confirmed *E. coli* cells. Lane L: 1kb plus DNA ladder, Lane 1: Plasmid isolated from *E. coli* carrying pSTARLING vector with Mvp ihpRNA cassette, Lane 2: Plasmid isolated from *E. coli* carrying pSTARLING vector with Rep ihpRNA cassette, Lane 3: Plasmid isolated from *E. coli* carrying pSTARLING vector with Mvp-Rep ihpRNA cassette.

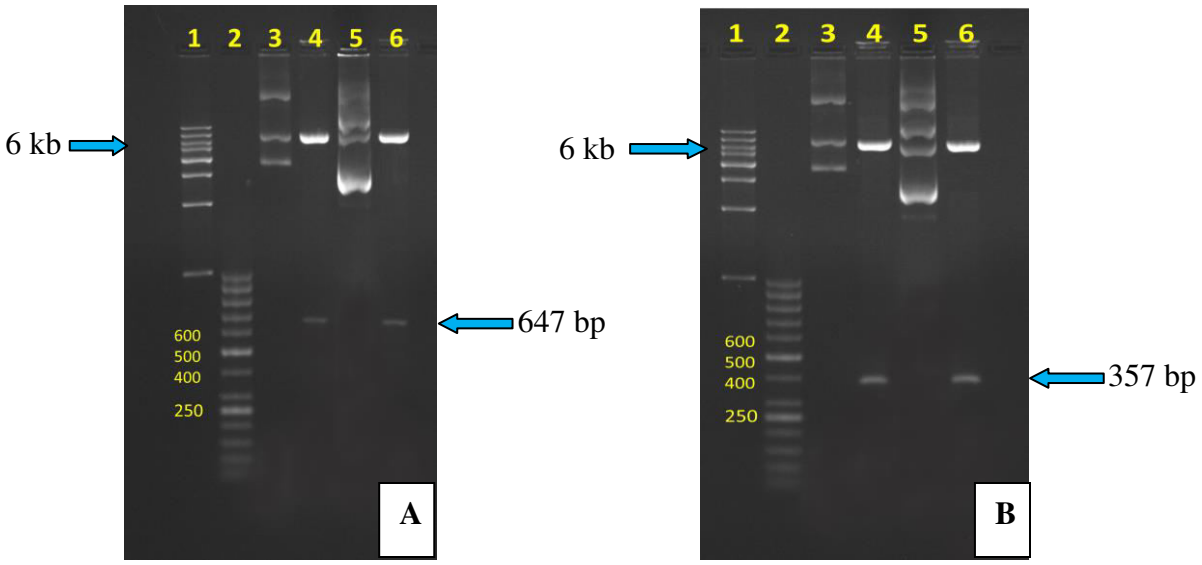
pSTARLING backbone (Plate 17). These results confirmed the successful integration of the gene fragments to form ihpRNA constructs in the primary vector pSTARLING.

**4.4 Transfer of ihpRNA cassette to the binary vector**

pSTARLING vector subjected to restriction reaction with *Not*I restriction enzyme resulted in the digestion of the plasmid into two fragments of size 3862 bp (fragment with the ihpRNA cassette for Rep gene), 3596 bp (fragment with the ihpRNA cassette for Mvp gene), 4450 bp (fragment with the ihpRNA cassette for Mvp-Rep) and 2864 bp (Empty vector backbone which is common) (Plate 18). From the size of the original vector and the inserts within the *Not*I site, it was confirmed that the fragments of size 3862 bp, 3596 bp and 4450 bp harbor the ihpRNA construct for Rep gene, Mvp gene and Mvp-Rep gene respectively. These fragments eluted out from the agarose gel showed a concentration of 4.2 µg/µl for Rep, 3.9 µg $\mu l^{-1}$ for Mvp and 3.5 µg $\mu l^{-1}$ for Mvp-Rep respectively. pART27 linearized with *Not*I restriction enzyme (Plate 19) eluted from the gel showed a concentration of 4.82 µg $\mu l^{-1}$.

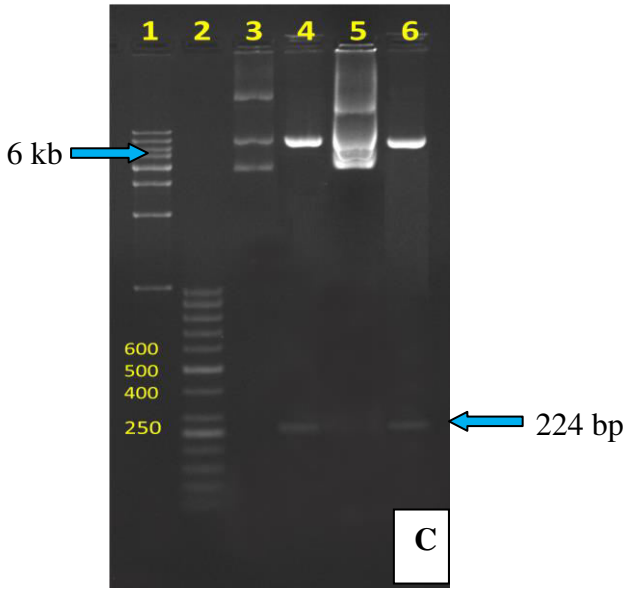
The *Not*I fragments of size 3862 bp (ihpRNA-Rep), 3596 bp (ihpRNA-Mvp) and 4450 bp (ihpRNA-Mvp-Rep) were ligated separately to the linearized (*Not*I) pART27. Plate 20 shows the recombinant vector in DH5α. The recombinant plasmids isolated from DH5α, digested with *Not*I, yielded fragments of expected size (Plate 21), i.e. 3862 bp (for Rep ihpRNA cassette and 11667 bp for pART vector backbone). Similarly 3596 bp fragment size represented Mvp ihpRNA cassette and 4450 bp for Mvp-Rep ihpRNA cassette.

Restriction digestion of recombinant pART27 using combination of enzymes i.e. *Spe*I + *Kpn*I and *Asc*I + *Pac*I, released the sense and antisense fragments (Plate 21). Fragments were of expected size i.e. 357, 224 and 647 bp represented the sense




**pSTARLING with ihpRNA Mvp-Rep cassette**

**pSTARLING with ihpRNA Rep cassette**




**pSTARLING with ihpRNA Mvp cassette**

**Plate 17**: Gel profile A, B and C showing released sense and antisense fragments with linear pSTARLING vector. Lane 1: 1 kb DNA ladder, Lane 2: 50 bp DNA ladder, Lane 3: pSTARLING control, Lane 4: pSTARLING – Sense strand released, Lane 5: pSTARLING with insert uncut, Lane 6: pSTARLING - Antisense strand released

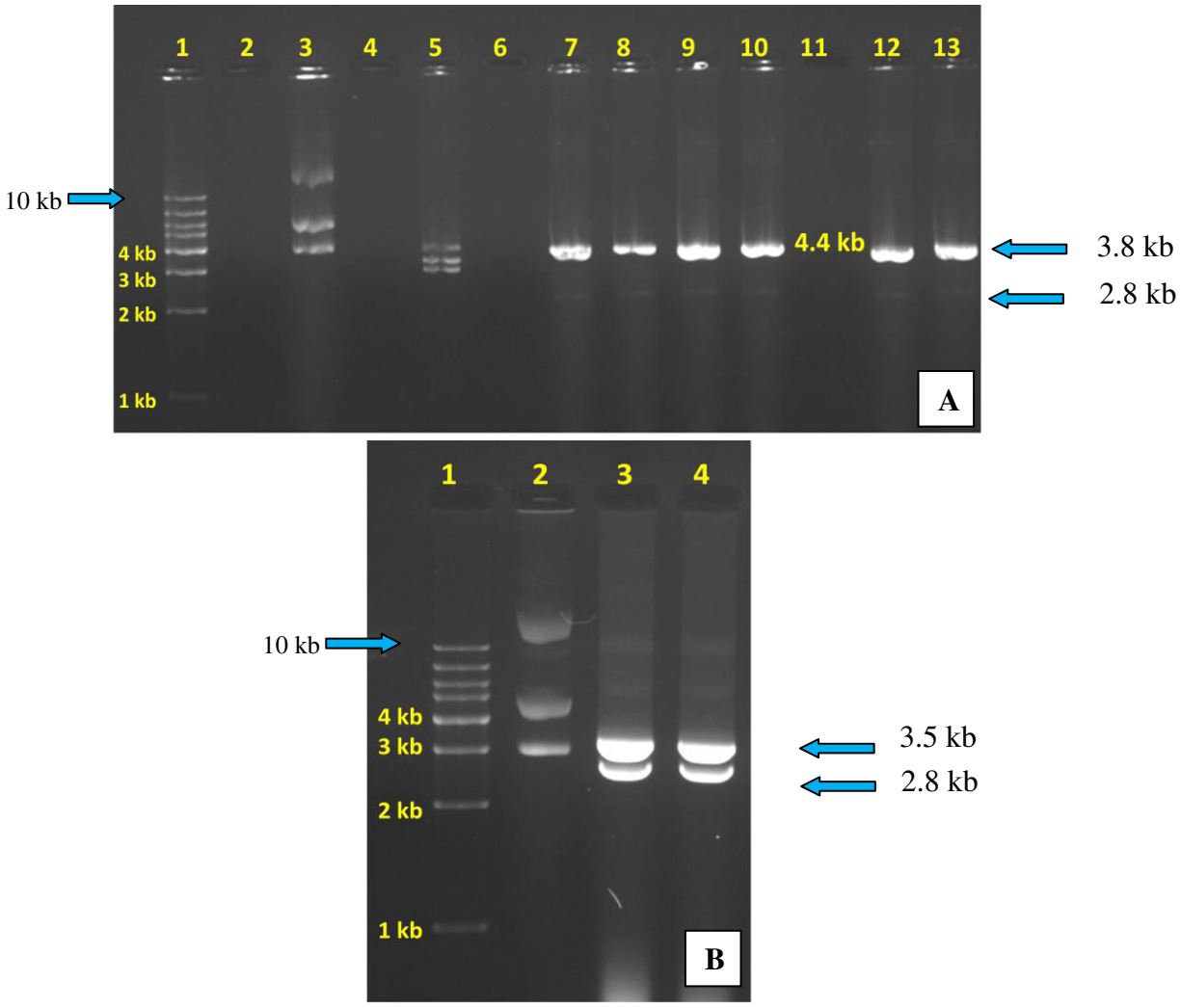



**Plate 18**: Gel profiles showing the restriction reaction to release the ihpRNA cassette from pSTARLING vector by *Not*I enzyme: **(A) Gel profile for Mvp-Rep and Rep ihpRNA cassette:** Lane 1: DNA ladder 1 kb to 10 kb, Lane 3: pSTARLING uncut, Lane 5: pSTARLING cut with *NotI,* Lane 7-10: pSTARLING with ihpRNA Mvp-rep Cassette cut with *Not I* enzyme (4450 bp), Lane 12-13: pSTARLING with ihpRNA Rep cassette cut with *Not*I enzyme(3862 bp), Lane 2,4,6,11: Blank, **(B) Gel profile for Mvp ihpRNA cassette**: Lane 1: DNA ladder 1 kb to 10 kb, Lane 2: pSTARLING uncut, Lane 3 and 4: pSTARLING with ihpRNA Mvp cassette cut with *Not*I (3596 bp)

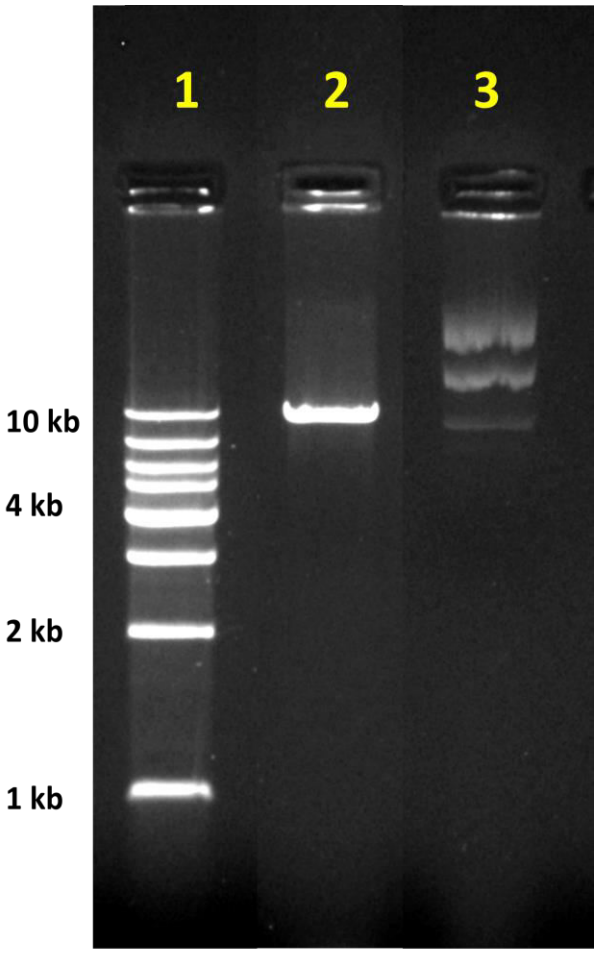
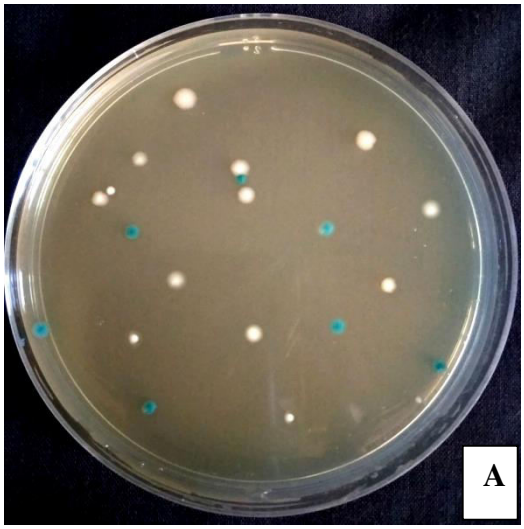



**Plate 19**: Gel profile showing pART27 linearized with *Not*I enzyme

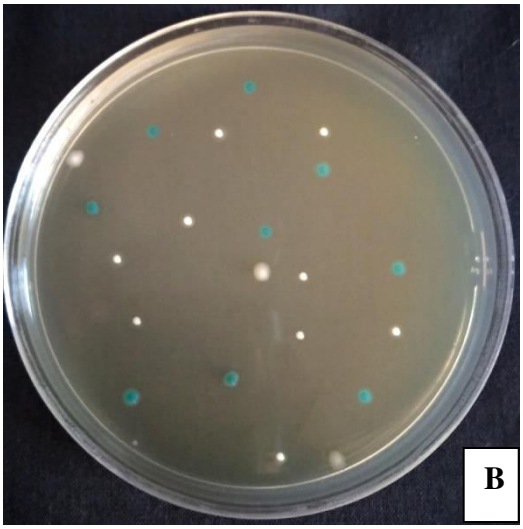
Lane 1: DNA ladder 1 kb to 10 kb,

Lane 2: pART27 cut with *Not*I enzyme (11667 bp)

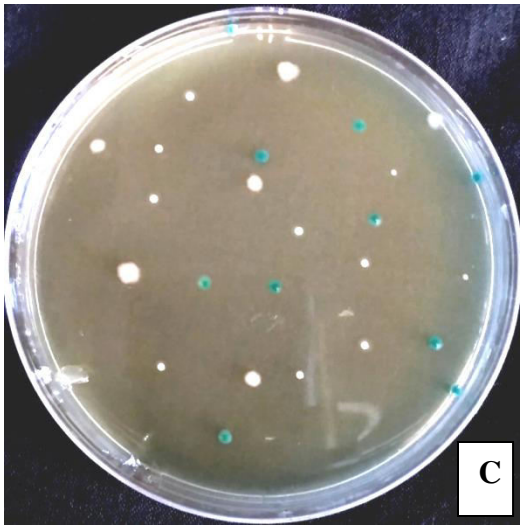
Lane 3: pART27 uncut




Colonies of *E. coli* DH5α carrying pART27 vector with ihpRNA-Mvp-Rep construct




Colonies of *E. coli* DH5α carrying pART27 vector with ihpRNA-Rep construct




Colonies of *E. coli* DH5α carrying pART27 vector with ihpRNA-Mvp construct

**Plate 20:** Blue- white screening of pART27 with ihpRNA cassette (A-C).

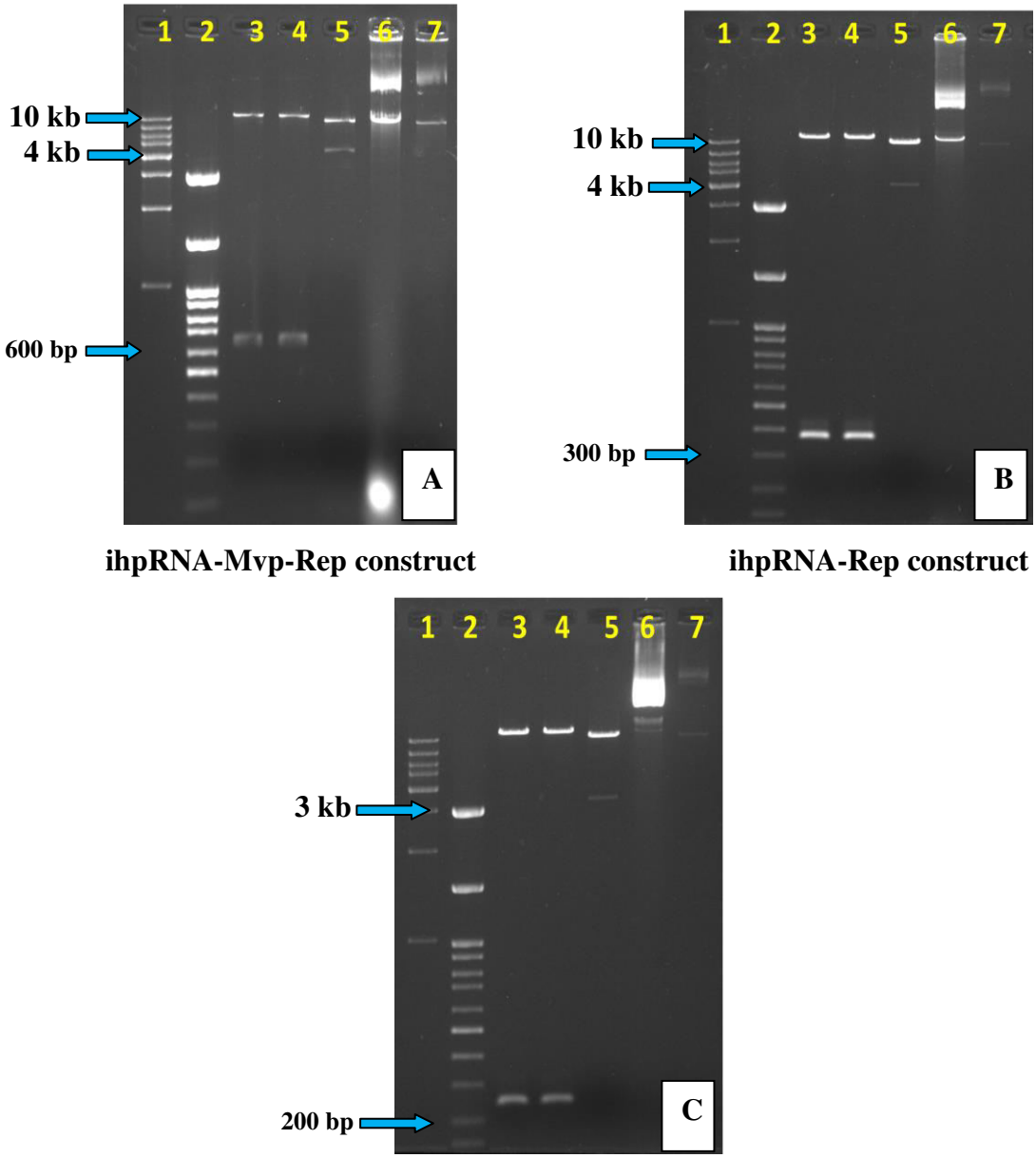



**Plate 21**: Gel profile A-C showing the presence of ihpRNA cassette in pART27 by restriction digestion. Lane 1: 1 Kb DNA ladder, Lane 2: 100 bp DNA ladder, Lane 3: Restriction digestion with *Spe*I and *Kpn*I to release sense fragment, Lane 4: Restriction digestion with *Asc*I and *Pac*I to release antisense fragment, Lane 5: Restriction digestion with *Not*I enzyme to release whole ihpRNA cassette, Lane 6: pART27 with insert uncut, Lane 7: PART27 control uncut.

and antisense fragments of Rep, Mvp and Mvp-Rep gene respectively in the three ihpRNA constructs.

### 4.5 Transfer of the binary vector to *Agrobacterium tumefaciens* and confirmation of transformation

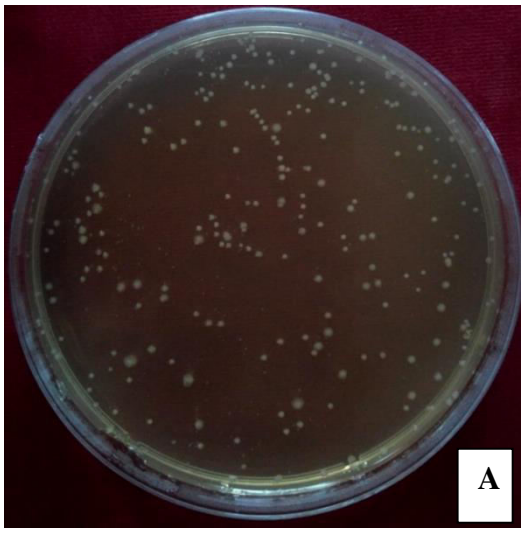
The binary vector containing the ihpRNA cassettes transferred to *A. tumefaciens* when plated on to LB medium supplemented with antibiotics showed 232 colonies for ihpRNA-Rep (with transformation efficiency 2.3 X $10^{-3}$ CFU/µg), 195 colonies for ihpRNA-Mvp (1.9 X $10^{-3}$ CFU/µg) and 254 colonies for ihpRNA-Mvp-Rep (2.5 X $10^{-3}$ CFU/µg) (Plate 22).

PCR amplicons of the plasmid DNA isolated from the transformed *A. tumefaciens* with gene specific primers for sense and antisense fragments, *npt*II and *cre* intron are shown in Plate 23. PCR with *npt*II specific primers yielded a product of size 475 bp and with *cre* intron specific primers yielded a product of 450 bp. The three amplicons of size 357, 224 and 647 bp were as expected representing sense and antisense fragment of Rep, Mvp and Mvp-Rep in the ihpRNA cassettes.

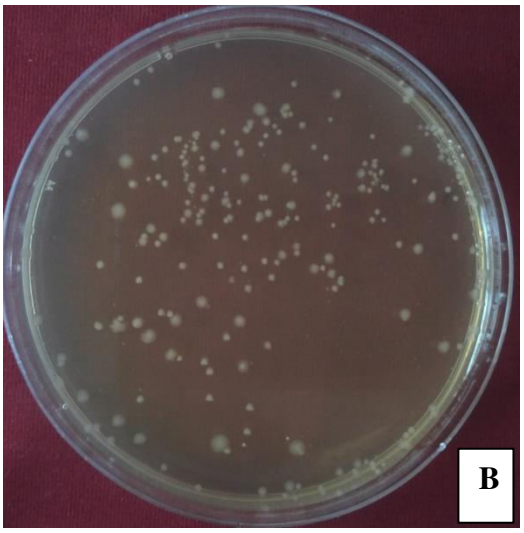
### 4.6 Genetic transformation of banana embryogenic callus

#### 4.6.1 Identification of the transformed embryogenic callus on selection medium

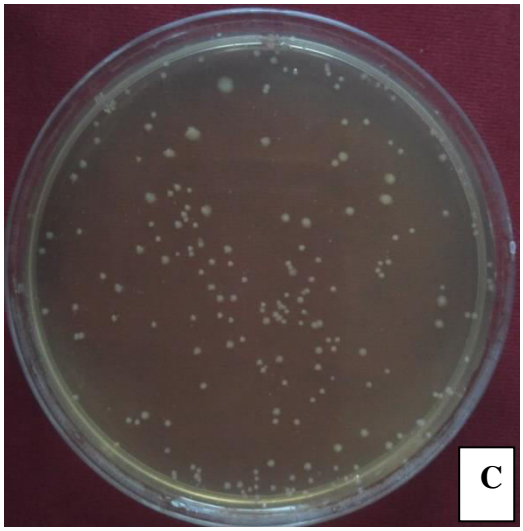
Plate 24 shows the transformed embryogenic calli on selection medium. Survival percentage of calli transformed with ihpRNA-Rep, ihpRNA-Mvp and ihpRNA-Mvp-Rep in a selection medium containing kanamycin 200 mg $L^{-1}$was 11, 8 and 12 percent respectively (Table 3). The embryos were induced in 45 ±5 days and were cultured on germination medium with kanamycin 200 mg $L^{-1}$. The germinated plantlets were maintained by sub-culturing in MS medium with BA 2 mg $L^{-1}$ and kanamycin 200 mg $L^{-1}$ (Plate 25).




**Transformed colonies of ihpRNA-Mvp-Rep construct**




**Transformed colonies of ihpRNA-Rep construct**




**Transformed colonies of ihpRNA-Mvp construct**

**Plate 22:** Transformed *Agrobacterium tumefaciens* GV3103 colonies (A-C) containing pART27 with ihpRNA constructs selected on 25 mg $L^{-1}$ rifampicin and 100 mg $L^{-1}$ spectinomycin

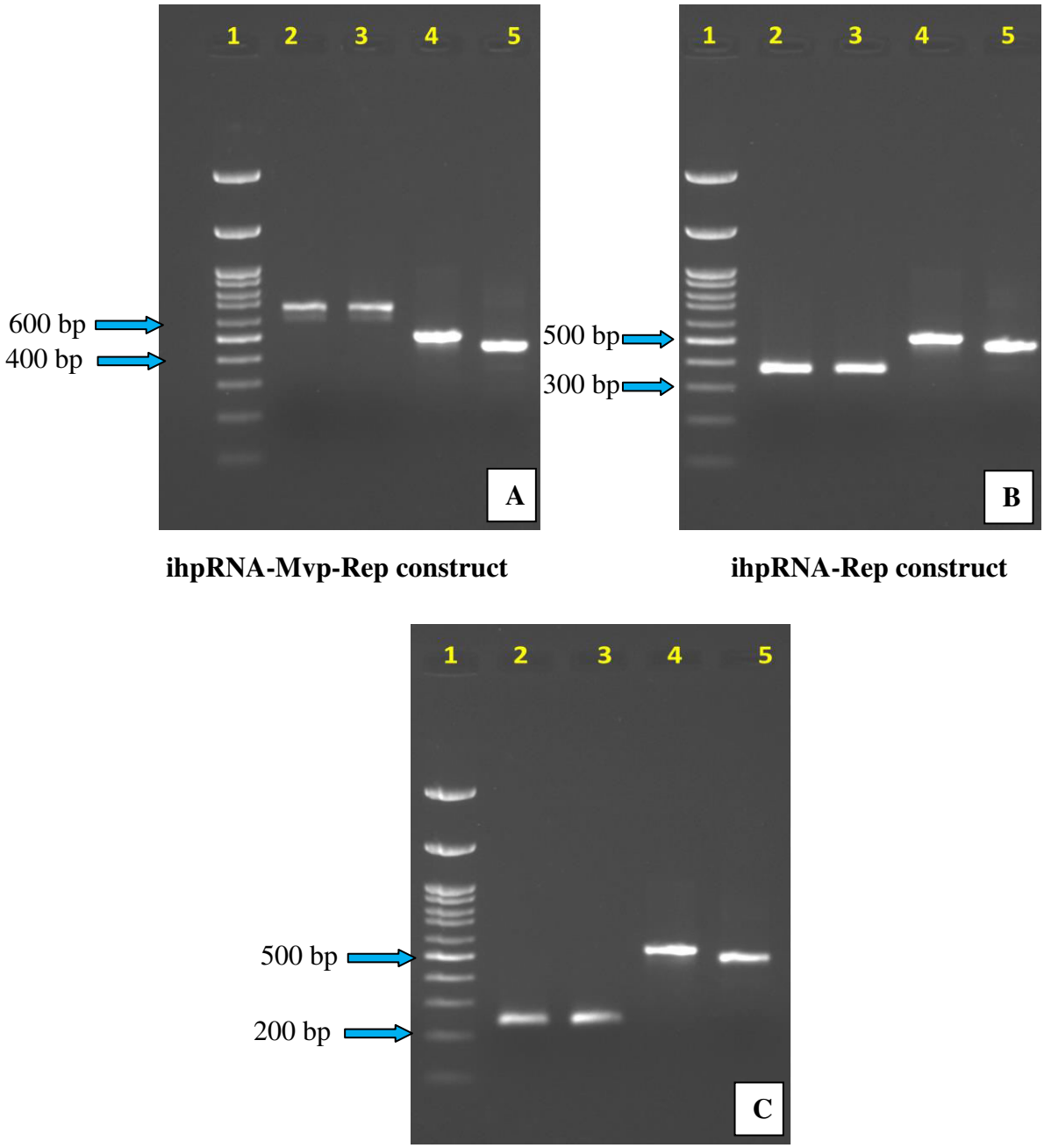
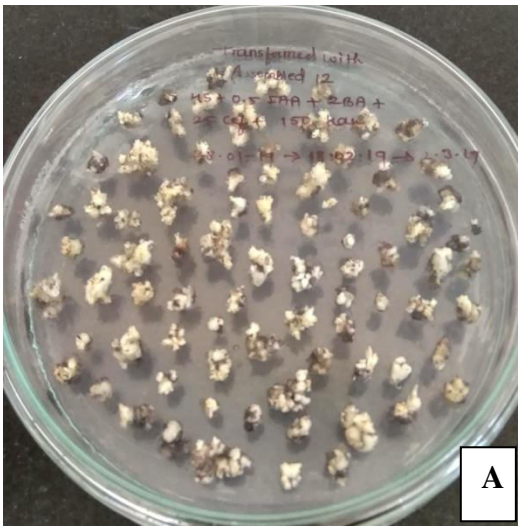
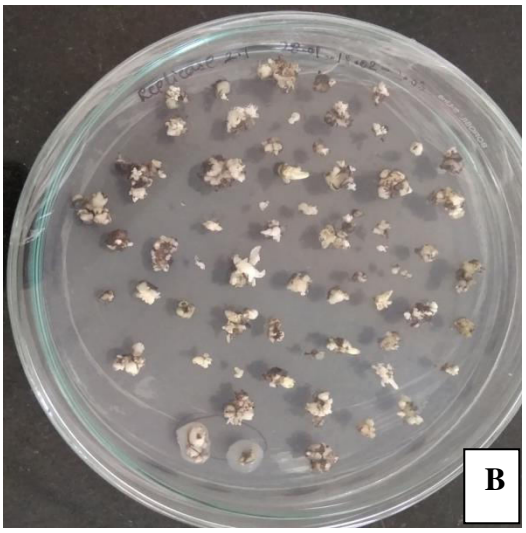


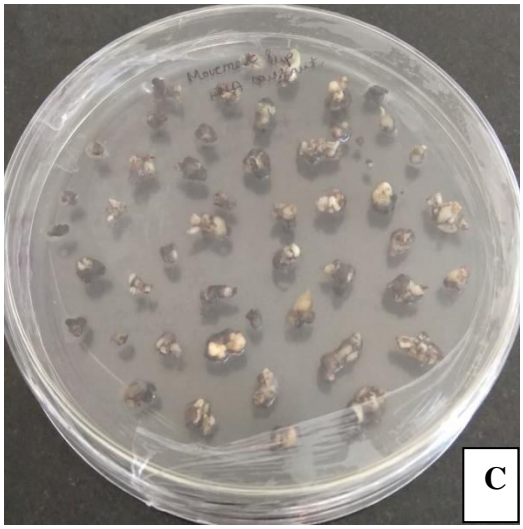

**Plate 23:** Gel profiles (A, B, C) showing amplified sense and antisense fragments, *npt*II and *cre* intron from *Agrobacterium* GV3103 confirming the presence of pART27 having ihpRNA construct. Lane 1: 100 bp DNA ladder, Lane 2: Sense strand, Lane 3: Antisense strand, Lane 4: *npt*II, Lane 5: *Cre* Intron.




**Embryogenic calli transformed with ihpRNA-Mvp-Rep construct**

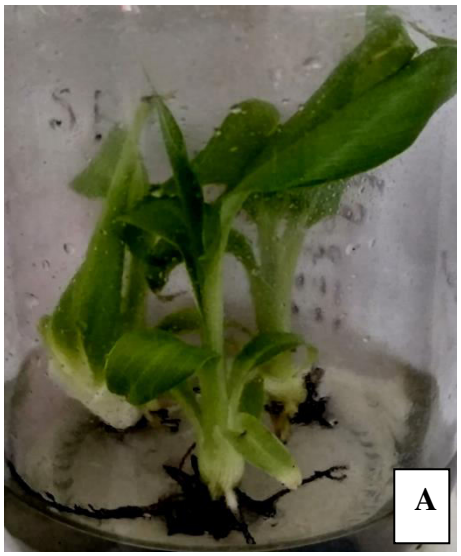



**Embryogenic calli transformed with ihpRNA-Rep construct**




**Embryogenic calli transformed with ihpRNA-Mvp construct**

**Plate 24:** Embryogenic calli (A, B and C) in MS medium with kanamycin 200 mg $L^{-1}$ and cefotaxime 100 mg $L^{-1}$ for the selection of transformants




**Plants transformed with ihpRNA-Mvp-Rep construct**


**Plants transformed with ihpRNA-Rep construct**


**Plants transformed with ihpRNA-Mvp construct**

**Plate 25:** Regenerated plants (A, B and C) grown under selection pressure of 200 mg $L^{-1}$ kanamycin in MS medium containing 2 mg $L^{-1}$ BA

**Table 3. Survival and percentage germination of transformed embryos**

| Construct | No. of embryogenic calli transformed | No. of embryogenic calli survived after transformation | Transformed somatic embryos (Percentage) |
|---|---|---|---|
| ihpRNA-Rep | 200 | 18 | 11 |
| ihpRNA-Mvp | 180 | 15 | 8 |
| ihpRNA-Mvp-Rep | 250 | 30 | 12 |

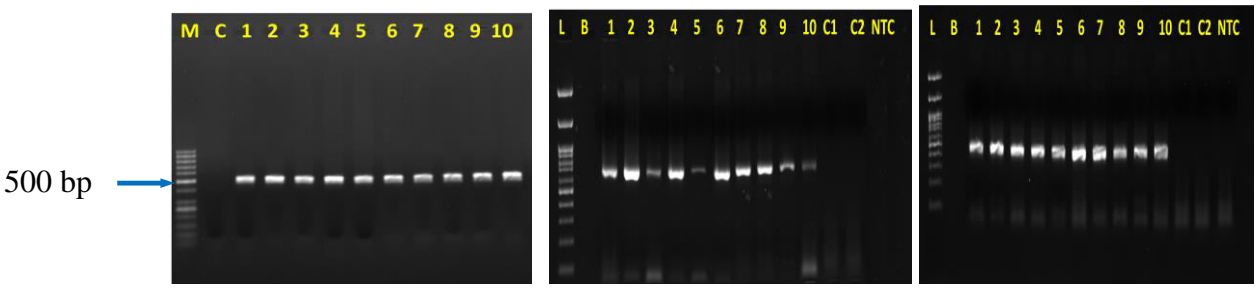
### 4.6.2 Confirmation of transformation in regenerated plants

Regenerated plantlets were screened by PCR for the presence of transgenes using primers specific for the different components in ihpRNA cassette viz., *npt*II gene, sense fragments of the three genes and *cre* intron. In Plate 26, amplicon of size 475 bp indicated *npt*II gene. Amplicons of size 357 and 450 bp represented Rep sense fragment and *cre* intron respectively.

Similarly plants transformed with ihpRNA-Mvp and ihpRNA-Mvp-Rep showed the amplicons of size 224 and 657 bp representing the sense fragment of each. The amplicons of size 475 bp and 450 bp showed the presence of *npt*II gene and *cre* intron (Plate 26). The results indicated successful integration of the ihpRNA cassettes in the transgenic banana plantlets developed.

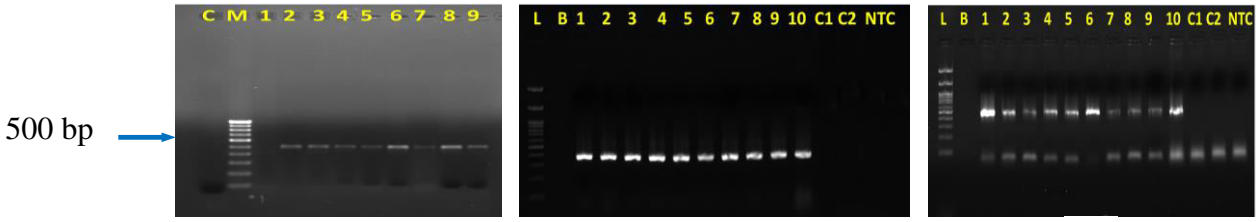
## 4.7 RT-PCR analysis of synthesis of siRNA products by the ihpRNA cassettes using stem-loop primers

Synthesis of siRNA by the ihpRNA cassettes in the putative transformants towards the targeted genes was confirmed by the stem-loop primers designed for DICER substrate present in the gene fragment of each ihpRNA cassette. The primers designed were found to be efficient in amplifying the specific siRNA against Rep and Mvp genes of BBrMV. Amplicons of size 64 bp and 66 bp represented siRNAs synthesized against Rep and Mvp genes respectively (Plate 27).



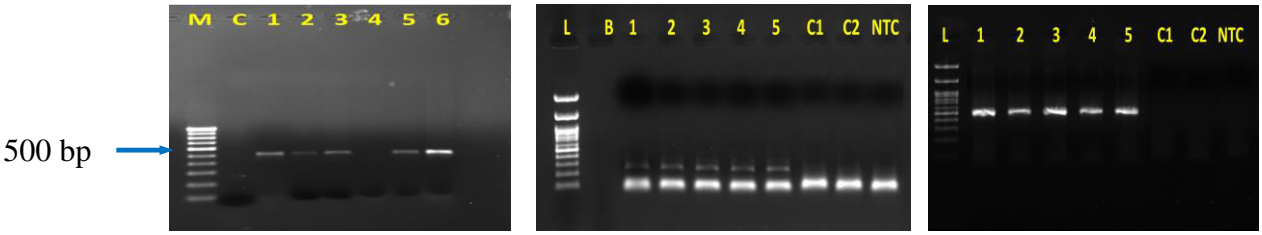

**A** **B** **C**

**Plants transformed with ihpRNA-Mvp-Rep construct** (A) *npt*II gene amplified (475 bp), (B) Mvp-Rep sense fragment amplified (647 bp), (C) *cre* intron amplified (450 bp)




**A** **B** **C**

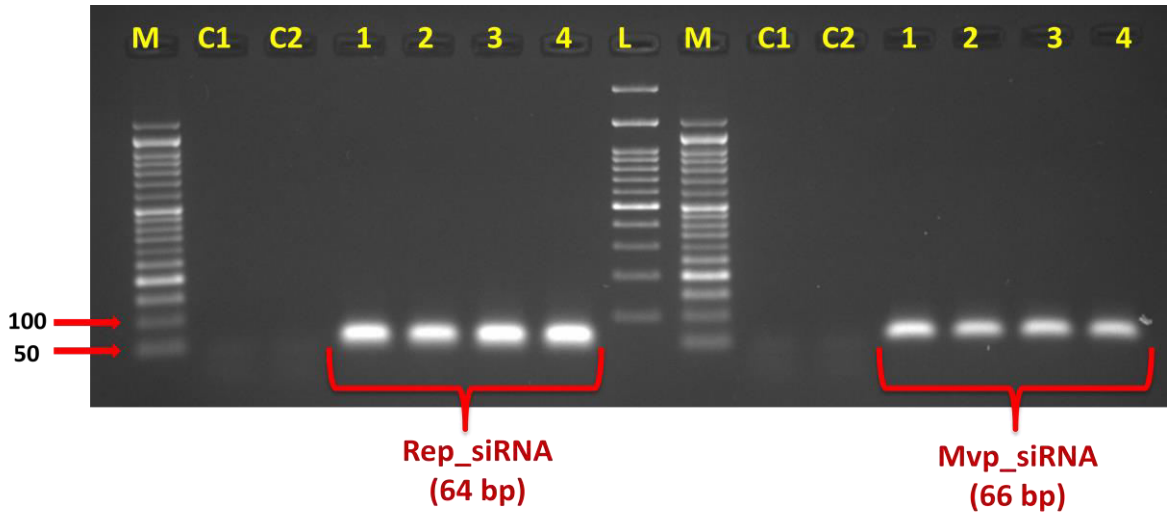
**Plants transformed with ihpRNA-Rep construct** (A) *npt*II gene amplified (475 bp), (B) Rep sense fragment amplified (357 bp), (C) *cre* intron amplified (450 bp)



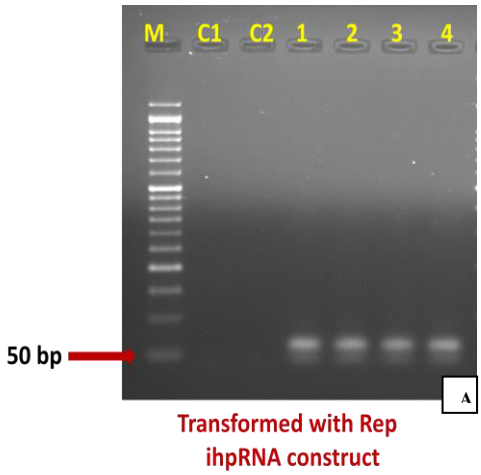

**A** **B** **C**

**Plants transformed with ihpRNA-Mvp construct** (A) *npt*II gene amplified (475 bp), (B) Mvp sense fragment amplified (224 bp), (C) *cre* intron amplified (450 bp)

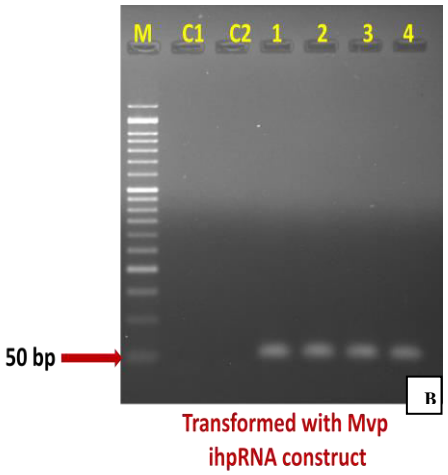
**Plate 26**: PCR products of ihpRNA construct in transformed plant




**Plants transformed with ihpRNA-Mvp-Rep construct:**
Lane M: 50 bp DNA ladder,
Lane L: 100 bp DNA ladder
Lane C1, C2: Control- untransformed
Lane 1, 2, 3, 4: Transformed with Mvp-Rep construct




Lane M: 50 bp DNA ladder,
Lane C1&C2: Control untransformed
Lane 1-4: Transformed plants amplified (64 bp)




Lane M: 50 bp DNA ladder,
Lane C1&C2: Control untransformed
Lane 1-4: Transformed plants amplified (66 bp)

**Plate 27:** Gel profiles showing PCR amplified product of siRNA in transformed plants

# Discussion

# 5. DISCUSSION

Banana is a monocot, perennial herbaceous crop belonging to the genus *Musa* of *Musaceae* family. It is the most important fruit crop in India, grown in tropical and subtropical regions with great genetic diversity of more than 90 distinct clones. Banana cultivation occupies about 20 percent of the total area under cultivation in India (NHB, 2019). However, banana cultivation encounters a large number of environmental challenges, mostly bacterial, fungal and viral pathogens. Viral diseases cause huge losses in the yield and quality of banana crops. Major viruses that infect bananas are *Banana bunchy top virus* (BBMV), *Banana bract mosaic virus* (BBrMV), *Cucumber mosaic virus* (CMV) and *Banana streak virus* (BSV).

*Banana bract mosaic virus* (BBrMV) is reported to cause considerable damage by reducing yield in cv. Robusta (70 %) followed by Nendran (52 %) (Cherian *et al*., 2002). In India, BBrMV is reported in Kerala, Tamil Nadu, Karnataka and Andhra Pradesh (Rodoni *et al*., 1997; Thomas and Magnaye, 1996; Selvarajan and Singh, 1997; Thangavelu *et al*., 2000; Cherian *et al*., 2002; Kiranmani *et al*., 2005; Darshan *et al*., 2019; Bhat *et al*., 2018, 2020; Sankaranarayanan *et al*., 2020). In Kerala, the disease was first reported in Thrissur district in the variety Nendran, which was later found prevalent in almost all the varieties with varietal difference in the symptomatology. During 2012, mosaic pattern in flower bract and bunch deformation of Cavendish bananas was observed in the commercial field in Ecuador. Twenty one symptomatic plants out of 35 were found positive for BBrMV in Cavendish bananas using double antibody sandwich ELISA (Quito-Avila *et al*., 2013).

Currently there is no strategy available to completely protect banana crops against viral diseases. Phytosanitary measures include use of virus free planting material from certified labs and strict regulation on movement of infected planting material through international exchange of germplasm. Conventional breeding

methods have restrictions in banana improvement programmes due to lack of natural resistance and triploid nature of banana varieties. Reports on genetic engineering approaches tried for crop improvement in bananas are limited.

Throughout the last decade many research groups have attempted to develop virus mediated resistance against viruses using genetic engineering techniques. Thus pathogen derived resistance (PDR) was used to engineer virus resistance in plants by transforming plants with various viral genes, and led to successful development of virus resistant crops for commercial applications (Baulcombe, 1996). The first PDR in plants was shown by transformation of Tobacco plants with *Tobacco mosaic virus* (TMV) coat protein gene (Abel *et al*., 1986). PDR in plants is due to the post-transcriptional gene silencing (PTGS) (Baulcombe, 1996). According to Voinnet (2001) RNA silencing or PTGS, functions as a defense mechanism against foreign nucleic acid invasions. Most of the techniques include expression of inverted repeat (IR) or hairpin RNA (hpRNA) that involves mutual homology with the viruses that infect the plants. hpRNA transcribed from IR constructs are effectively translated by Dicer like proteins into small interfering RNA (siRNA).

siRNA mediated gene silencing has emerged as a powerful tool for defense mechanism by targeting the pathogen in a highly sequence specific manner. Endogenous or exogenous small RNAs can either guide PTGS by cleaving the target mRNA and rendering its degradation or recruiting cofactors and inhibiting mRNA translation, or recruiting DNA and histone modifiers and inhibiting the transcription of target gene. Thus siRNA mediated sequence specific gene silencing mechanism plays important role in antiviral defense and maintenance of genome integrity (Baulcombe, 2004; Dugas and Bartel, 2004; Wassenegger and Krczal, 2006; Van der Krol *et al*., 1990; Lindbo and Dougherty, 1992; Voinnet, 2002, Baulcombe, 2004).

A free RNA complementarity with the target RNA, initiates the silencing machinery. This understanding encouraged researchers to develop the hpRNA

construct against plant viruses which has become an important approach to develop the resistance against viruses in agricultural and horticultural crops. Similar attempt has been made in the current study to develop intron hairpin RNA (ihpRNA) constructs against replicase (Rep), movement (Mvp) genes of BBrMV to suppress the virus replication in the host either by silencing a single gene at a time or both simultaneously. The primary silencing mechanism in plant miRNA pathway involves the perfect pairing of plant miRNAs with the target RNA to activate the silencing machinery (Rhodes *et al*., 2002; Llave *et al*., 2002; Jones-Rhodes and Bartel, 2004). The silencing efficiency thus depends on the complementary pairing of siRNA produced from ihpRNA introduced in the plant with the target gene sequence. Hence, in the current study to get the sequence accuracy with the target gene, the Rep and Mvp genes of BBrMV were amplified from the infected Grand Naine plants and sequenced.

In the present study for the isolation of single stranded RNA of BBrMV the protocol of Rodriguez-Garcia *et al.* (2010) was used with some modifications. This protocol was originally developed for the isolation of fungal RNA from *Mycosphaerella fijiensis* inoculated banana leaves. For this study, the incubation time was increased from 10 min to 30 min at 65ºC. Chloroform: Isoamyl treatment was given twice to completely remove the cell debris, protein and phenolic compounds. Samples were kept for precipitation in 3M LiCl overnight at 4ºC instead of -20ºC. The modified protocol was effective to give an appreciable RNA yield of 127-200 µg $g^{-1}$ of fresh tissue.

Three ihpRNA constructs were prepared in the study to silence either replicase or movement protein gene individually or both the genes simultaneously. For constructing ihpRNA, inverted repeat sequences (sense and antisense) of Rep and Mvp genes were amplified (357 bp for Rep, 224 bp for Mvp gene and 647 bp for Mvp-Rep). Earlier reports suggested the potential size range of 98-853 bp for

effective silencing mechanism (Wesley *et al*., 2001; Helliwell *et al*., 2002; Travella *et al*., 2006; Mei *et al*., 2007; Bhaskar *et al*., 2009). The regions selected belonged to the 5' terminus of the gene sequences. The preference was given to the 5' terminus of the target sequence to maintain the internal stability of the antisense strand and to get a possible functional contribution of uracil (U) at the 5' end for the activities of siRNA (Schwarz *et al*., 2003; Khvorova *et al*., 2003). These regions were also selected based on the results of restriction mapping, virus suppressor prediction and miRNA target prediction.

The miRNA/siRNA guides the RNAi induced silencing complex (RISC) to the complementary DICER substrate site. So it is preferred to have miRNA target sites in the sequence. Many tools and algorithms have been reported for predicting miRNA target regions (Reynolds *et al*., 2004; Krek *et al*., 2005; Qiu *et al*., 2007). In the current study the tool provided by Integrated DNA technologies (IDT) was used to find out the location of the DICER substrate. The restriction site sequence for *Asc*I, *Pac*I, *Kpn*I, *Spe*I and *Not*I were not detected in the miRNA targets, making it safer to use these cloning sites in the vector.

Nucleotide sequences of the selected target genes were analysed with the computational algorithm of VsupPred tool. This tool is used to predict viral Suppressors of RNA silencing (VSRs). VSRs are the viral proteins evolved with the capability to block RNA silencing response of the host as a counter defensive strategy. They have been identified in almost all plants, insects or mammalian viruses and showed diversity within and across the kingdoms. The prediction score value +1 is the maximum and -1 is the lowest i.e. the lower values indicate that the sequence is prone to silencing suppressor. In the gene sequences used, the prediction score gave a statistical value 0.775 for Rep and 0.725 for Mvp, denoting the least probability of presence of viral suppressor of RNA silencing. The primers designed for amplifying the inverted repeats were anchored with the restriction sites at both ends, which were

used for cloning in the primary vector, pSTARLING. The restriction sites added to the primer corresponding to the primary vector ensures the correct orientation of the resulting sense and antisense arms (Weasley *et al*., 2001).

pSTARLING vector was selected as the primary vector as it contained a cyclic AMP response element (*cre* intron) intron. Intron sequence present between the inverted repeats are reported to give the stability and aid in aligning the complementary arms of the hairpin in an environment, favoring RNA hybridization and thus promoting the formation of a stable duplex (Smith *et al*., 2000; Wesley *et al*., 2001; Reichhart *et al*., 2002; Kalantidis *et al*., 2002; Yan *et al*., 2012). In plants, ihpRNA constructs were shown to give higher gene silencing efficiency than intron free hpRNA. Moreover, pSTARLING has *Zea mays* polyubiquitin promoter, more efficient than CaMV 35S based promoter in banana tissues (Hermann *et al*., 2001).

Sense and antisense fragments were amplified using the primers designed for directional cloning in the primary vector and cloned into blunt end cloning vector pJET1.2/Blunt to anchor the restriction sites of primary vector pSTARLING. Direct cloning in the primary vector was avoided so as to favour long term storage of individual sense and antisense fragments in *E. coli* as glycerol stock for easy recovery of the fragments. It is also useful in checking the orientation of the inserted fragments and vector provides the insert specific forward and reverse primers for easy sequencing. The pJET1.2/Blunt vector contains a lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies. Recircularized pJET1.2/blunt vector molecules lacking an insert express a lethal restriction enzyme, which kills the host *E. coli* cell after transformation. Due to the positive selection process, colony screening was done on ampicillin.

Later these fragments were released from pJET1.2 vectors using the respective restriction enzymes. This process helped in ligating the inverted repeats to

pSTARLING on the 5' end and 3' ends of the *cre* intron in correct orientation. The ihpRNA constructs prepared in the *Not*I fragment in the pSTARLING was transferred to the *Not*I site within the lacZ gene of the binary vector pART27 for *Agrobacterium* mediated transformation and transformed colonies were selected through the blue-white screening. Binary vector pART27 containing ihpRNA constructs were transferred to *Agrobacterium*, strain GV3103 by a modified freeze thaw method reported by Jothishwaran *et al*. (2007) and then introduced in to the embryogenic calli of host plant banana. The use of pART27 has been reported by Elayabalan *et al*. (2013) for delivering ihpRNA construct into banana for developing the resistance against BBTV.

Somatic embryos are preferred for genetic transformation studies in plants due to their ability to express introduced DNA at a high level as well as ability to develop plants from single cells. In this study, the protocol reported by Jadhav (2019) was used for somatic embryogenesis mediated regeneration in cv. Grand Naine. Embryogenic calli developed from immature male flowers (IMFs) were used for transformation. Compared to soil grown suckers, IMFs shows less contamination during micropropagation and the response of IMFs for somatic embryo generation was reported to be higher (Esclant *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; Lekshmi *et al*., 2019). The flower buds were sterilized by wiping the flower bud with ethanol soaked cotton swab was reported to be effective (Lekshmi *et al*., 2019). Tightly packed immature flower buds within the bracts of the inflorescence may be a reason for preventing the entry of the microbes in the bract. Also endogenous bacterial contamination was not observed in inflorescence meristem compared to shoot meristem. Explants for inoculation were selected soon after the bunch formation was complete. It helped in visually evaluating the bunch quality, number of hands, fruit quality and health of the mother plant before collecting the

explants. The protocol used in this study (Jadhav, 2019) was efficient for raising highly proliferative, embryogenic calli with simple hormone combination of BA (8 mg $L^{-1}$) and TDZ (0.6 mg $L^{-1}$). The growth hormones BA with TDZ have the potential to induce somatic embryogenesis as TDZ is supposed to modulate the endogenous plant growth regulators (Visser *et al*., 1992; Hunchinson and Saxena, 1996; Sadik *et al*., 2014). Also, a high concentration of BA and TDZ is reported to induce both shoot organogenesis and somatic embryogenesis (Yao *et al*., 2016). In the present study the semisolid medium was used for the germination of somatic embryos, to make the process simple. The somatic embryos were germinated on BA 2 mg $L^{-1}$ media at 16 hours photoperiod.

For *Agrobacterium* mediated transformation, the embryogenic calli of banana were pre cultured 15 days before co-cultivation on fresh half strength MS medium supplemented with BA 8 mg $L^{-1}$ and TDZ 0.6 mg $L^{-1}$. Pre Culturing maintains the cells in active cell division stage (Ganpathi *et al*., 2001; Ghosh *et al*., 2012; Elayabalan *et al*., 2013). Acetosyringone is known to activate the virulence genes of the Ti plasmid and to initiate the transfer of T- DNA. It enhances chemotactic movement and attachment of *Agrobacterium tumefaciens* to the tissue of banana. So in this study, for transformation, embryogenic calli were treated with half strength MS medium supplemented with 100 µM Acetosyringone. Co-cultivation was done for 48 h for transformation in full strength MS medium. After three subcultures, *Agrobacterium* was completely eliminated using Cefotaxim 100 mg $L^{-1}$ (Ganapathi *et al*., 2001; Ghosh *et al*., 2009). The transformed embryos could be successfully regenerated in MS medium supplemented with BA 2 mg $L^{-1}$ and kanamycin 200 mg $L^{-1}$. The results on the survival of calli in the selection medium showed that an infection time of 10 min and co-cultivation for 48 h were most suitable for transformation.

Transformed plantlets were confirmed for the insertion of the ihpRNA cassette. The complete ihpRNA cassettes were of size 3.8 kb, 3.5 kb and 4.4 kb for those targeting replicase, movement protein alone and both simultaneously. Hence for checking integration of the complete ihpRNA cassette, PCR was carried out for the amplification of different components of ihpRNA, like sense fragment of the two genes, *npt*II gene (Selectable marker) and *cre* intron. PCR amplification using primers designed specifically for these components showed the positive results with expected band size and confirmed the integration of ihpRNA construct against the two targeted genes of BBrMV in bananas. In the experiment conducted by Elayabalan *et al*. (2013) and Ntui *et al*. (2014) for developing RNAi-mediated resistance against virus in banana and tomato respectively, the transformation was confirmed by amplification of the transformed template DNA with the primer specific for *npt*II and the target gene used for the hpRNA vector construction.

In engineered plants, hpRNA transcribed from the inverted repeat region of the constructs are efficiently processed into siRNA by dicer like proteins. The siRNA is loaded into the RISC complex containing *Argonaute* 1 (AGO1). The siRNA guides RISC to the viral genome by recognizing the complementary RNA sequence. The AGO1 slicer activity cleaves the viral RNA, thereby plants getting resistance to the virus (Dunoyer *et al*., 2005; Fusaro *et al*., 2006; Wesley *et al*., 2001). So it is necessary to check the synthesis of siRNA by the ihpRNA construct for invoking virus resistance. A number of techniques have been developed to detect the siRNA which includes Northern blot (Várallyay, *et al*. 2008; Cissell and Deo, 2009), primer extension (Zeng and Cullen, 2003), microarrays (Li and Ruan, 2009) and real-time PCR (Cirera and Busk, 2014). In *G. lamblia*, fluorescence *in situ* hybridization (FISH) was used to find the cellular location of the snoRNA GlsR17 and its product, miR2 (Saraiya and Wang, 2008). Shekhawat *et al*. (2012) found the presence of 21-24 nt long siRNA in banana phenotype transformed with BBTV ihpRNA-Rep construct using northern blot analysis showing BBTV resistance confirming the

operation of Post-transcriptional gene silencing (PTGS) pathway from the synthesized specific siRNAs in the transformed plants. However, these techniques require time, and they are difficult, costly, and have low sensitivity (Cissell and Deo, 2009). Stem-loop quantitative reverse transcription PCR (RT-qPCR) is an efficient strategy that has been used to detect and amplify mature miRNAs and siRNAs (Czimmerer *et al*., 2013; Yang *et al*., 2014; Gautam *et al*., 2016). The stem-loop is designed such that it is shaped as a hairpin and possesses a 30 bp overhang complementary to the miRNA. Also, a miRNA-specific forward primer with a 50 bp adapter and a universal reverse primer are designed for PCR amplification; these features allow high specificity and increased flexibility in the design of primers for identification of miRNAs (Busk, 2014).

Similarly, in the present study siRNA specific to dicer substrates of BBrMV Rep and Mvp genes in plants transformed with ihpRNA cassette was detected with RT-PCR using stem-loop primers. An amplicon of 64 bp was obtained using Rep-siRNA forward and universal reverse primers and 66 bp amplicon with Mvp-siRNA. It indicates the synthesis of the corresponding siRNA from the ihpRNA construct in the transformed banana plants.

The present study was successful in developing three ihpRNA constructs against Rep and Mvp genes of BBrMV and delivering it into embryogenic calli of *Musa* spp. var. Grand Naine to develop into a complete plant. The putative transformants were confirmed to harbor the ihpRNA construct. They were also confirmed for the synthesis of siRNA corresponding to Rep and Mvp genes. These transgenic plants carrying ihpRNA constructs need to be evaluated by challenging with viruliferous aphids to confirm the development of virus resistance in Grand Naine. Further the comparison of silencing potential of the three different ihpRNA constructs developed in this study also need to be carried out for selecting the most efficient one for imparting BBrMV resistance in Grand Naine.

# *Summary*

## SUMMARY

The study entitled "ihpRNA mediated resistance for *Banana bract mosaic virus* in *Musa* spp. by targeting replicase and movement protein genes" was carried out during 2016-2020 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to develop ihpRNA constructs targeting replicase and movement protein genes of *Banana bract mosaic virus*, to generate transformants of banana cultivar Grand Naine carrying the ihpRNA cassettes and to confirm the transformants.

In the study, replicase and movement protein genes of BBrMV were targeted to suppress the replication of the virus inside the host plant and to arrest the cell to cell movement of the virus particles. Embryogenic cells were used as the targets for *Agrobacterium*-mediated transformation, because of their single-cell origin and ability to produce non-chimeric plants.

Embryogenic calli of banana cv. Grand Naine required for the transformation process. Callus developed using the immature male flower buds in 45 days when inoculated on MS medium supplemented with BA and TDZ under dark conditions. Twelve per cent of them were embryogenic calli. The embryogenic calli were transferred to light (16 h) in MS medium containing BA (2 mg $L^{-1}$) for germination and regeneration.

Total RNA was extracted for developing ihpRNA constructs from the BBrMV infected flower bracts of the Grand Naine banana plants. The cDNA was prepared and Rep and Mvp of BBrMV were amplified using gene specific primers designed based on the gene sequence of BBrMV of Trichy isolate available in NCBI. The amplified fragments were sequenced and analysed using BLASTn bioinformatics tool for similarity. The BLAST result for Rep (421 bp) showed 96.37 percent similarity

with BBrMV infecting Nendran banana (Trichy). Mvp gene (456 bp) showed 100 percent similarity with BBrMV of banana from Philippine isolates. The partial Rep and Mvp gene sequences were submitted in the GenBank (NCBI) with accession number MH253671.1 and MH253670.1 respectively.

In the Rep (401 bp) and Mvp (246 bp) gene sequences, presence of miRNA targets were confirmed (five each) using prediction tools. Restriction mapping was done to avoid recognition sequences of the cloning enzymes within the gene sequence. Viral silencing suppressors prediction (prediction score 0.775 for Rep and 0.725 for Mvp) indicated that the selected sequences are non viral silencing suppressors. Based on these analyses, primers were designed for amplification of sense and antisense fragments of the Rep and Mvp genes of the BBrMV and were anchored with selected restriction enzymes to the 5' end. Sense and antisense fragments of the selected regions of the genes were amplified for use as inverted repeats and were cloned to pJET1.2/Blunt cloning vector. These fragments were later released from pJET1.2 with restriction enzymes *Kpn*I and *Spe*I (for sense strand) and *Asc*I and *Pac*I (for antisense strand). These fragments were gel eluted and ligated to the pSTARLING vector linking through *cre* intron. The complete ihpRNA cassette in pSTARLING contained ubiquitin promoter, ubiquitin intron, sense fragment, *cre* intron, antisense fragment and terminator sequence in the order within the *Not*I restriction site. The integration of inserts in pSTARLING was confirmed by restriction digestion. Three ihpRNA constructs were prepared, one for targeting Rep, second for Mvp and the third one for targeting both the genes.

The ihpRNA cassette (*Not*I fragment) in the pSTARLING vector was released and mobilized to the *Not*I site of the binary vector pART27. By blue white screening the positive colonies with ihpRNA cassette were selected and confirmed by PCR. The binary vectors carrying the three different ihpRNA constructs were transferred to *Agrobacterium tumefaciens* strain GV3103 via freeze thaw method with

transformation efficiency of 2.3 x $10^3$, 1.9 x $10^3$ and 2.5 x $10^3$ CFU/µg, for those carrying Rep, Mvp and Rep-Mov construct respectively. Transformed colonies were selected on spectinomycin (100 mg $L^{-1}$) and rifampicin (25 mg $L^{-1}$) and confirmed by PCR for the presence of ihpRNA insert.

Embryogenic calli of Grand Naine were transformed with *Agrobacterium* strain GV3103 carrying the ihpRNA cassette. The transformed calli were selected on medium containing kanamycin (200 mg $L^{-1}$) and germinated and regenerated into plantlets on MS medium supplemented with BA (2 mg $L^{-1}$). These plantlets were confirmed for the presence of ihpRNA cassettes by PCR using primers specific for *npt*II, *cre* intron and sense fragment in the ihpRNA cassette. The plantlets were also confirmed for the synthesis of small RNAs targeting Rep and Mvp genes by real time PCR using stem-loop primers.

The study was successful in developing ihpRNA constructs targeting Rep and Mvp genes of BBrMV in *Musa* spp. cv. Grand Naine. The putative transformants were confirmed to harbor the ihpRNA construct and they were also evaluated for the synthesis of siRNA corresponding to Rep and Mvp genes. Comparison of silencing potential of three different ihpRNA constructs developed in this study need to be evaluated for BBrMV resistance by challenging the transgenic plants with viruliferous aphids and study also need to be carried out for selecting the most efficient construct for imparting BBrMV resistance in Grand Naine. As compared to other recombinant DNA technologies RNAi has target specificity and efficacy in gene silencing.

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# *Appendices*

## APPENDIX I

### Chemical composition of the media employed for the in vitro culture of banana (Musa spp.) cv. Grand Naine: 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 |
| $AlCl_3$ | 0.83 |
| $H_3BO_3$ | 6.2 |
| $Na_4MoO_4.2H_2O$ | 0.25 |
| **Iron sources (mg $L^{-1}$)** | |
| $FeSO_4$.7H2O | 27.85 |
| $Na_2EDTA$ | 37.25 |
| **Vitamins (mg $L^{-1}$)** | |
| Nicotinic acid | 0.5 |
| Pyridoxine HCL | 0.5 |
| Thiamine HCL | 0.1 |
| **Amino acid source (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 (Rodriguez- Garcia *et al*., 2010)

| | |
|---|---|
| Tris HCl (pH 8.0) | 150 mM |
| SDS | 4 % ( w/v) |
| EDTA (pH 7.5) | 100 mM |
| β- mercaptoethanol | 2 % (v/v) |
| Polyvinyl pyrrolidone (PVP) | 4 % (w/v) |
| RNase free water | Treated with Diethyl pyrocarbonate (DEPC) 0.1 % |

## APPENDIX III

**A) Replicase gene sequence (401 bp): BBrMV replicase partial cds amplified with BBrMV-Rep primers**

>ACATAAGGGTGCTGAGAGTTCTTTAGACCGCTCATGTCTCATTATGGTCC
AAGCCGGTTAAATCGCAAGGCATTCTTGAAAGATTTATTAAAATATTCTG
GCGAGCTGATTGTTGGTGTAGTTGACTGTGATACATTTGAAAGCGCGTAC
AATTTCACTGCATCATTGTTACGTAGCCATGGATTTGAAGGAAGGAAGTT
CATCACTGATACTGACGAGATATTTCAGAGTTTGAATATGAAGGCTGCTG
TTGGGGCACTGTATGCTGGGAAGAAGAGGGATTATTTTGAGGGCTTCACT
AATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCCTATACAA
AGGACATTTAGGCGTATGGAACGGATCCTTGAAAGCTGAATTACGGCCAA
A

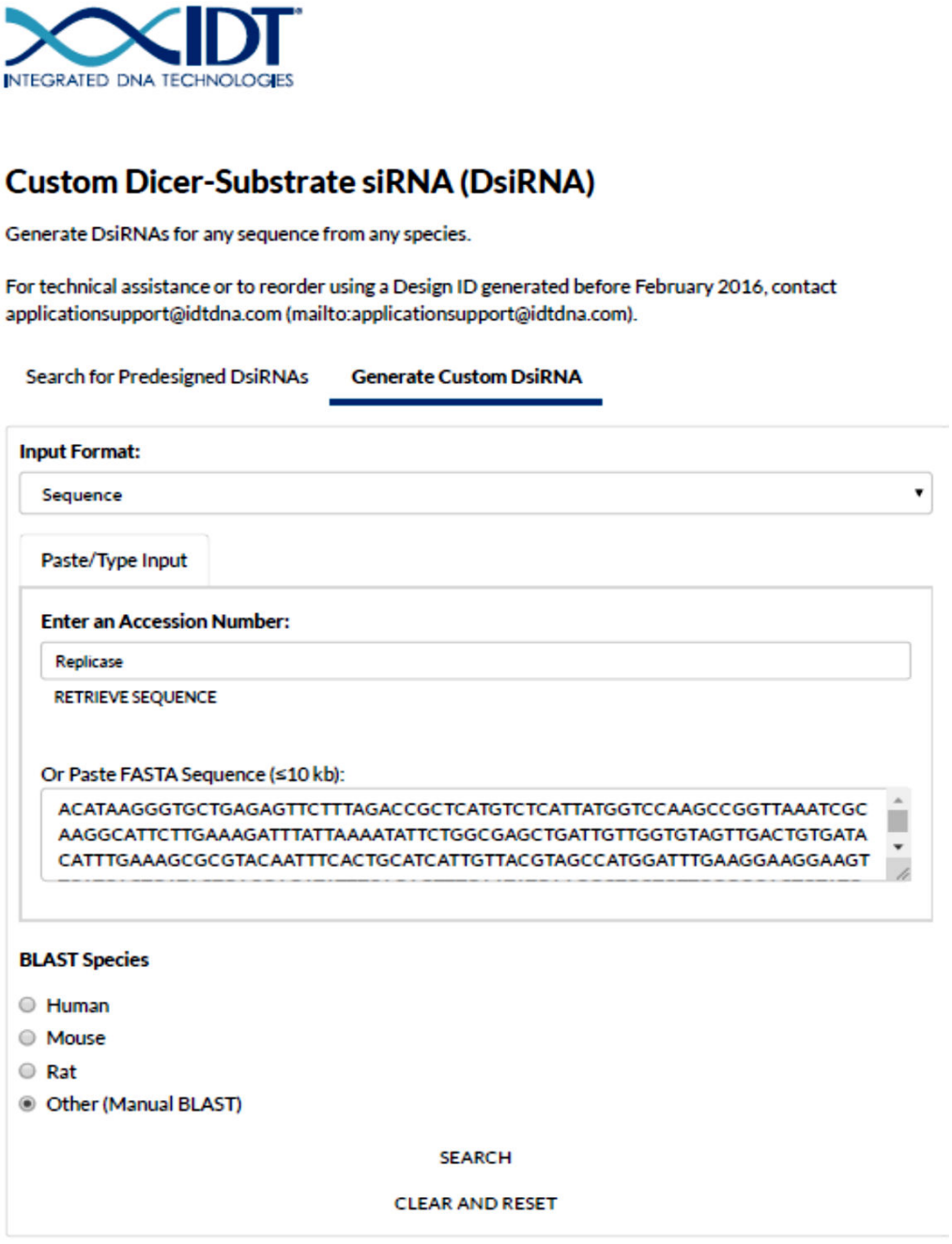
**B) Movement protein gene sequence (339 bp): BBrMV movement protein gene partial cds amplified with BBrMV-Move primers**

>GCTTAGGATGGCTCAAGATGTAGAAATGAATGAATCACTTTTAGATGCC
GGTTTCGCAACGATGAATTTGGTGTATTATGCATTTTCAGAAAAAAAATA
TCAAGACCTATTAAACGCAGAATGGAGAGCATTAAAATTGTCGGAAAAA
TTCTCACGAATCATGCATACATCATACTACAGAATCTCATCAACACTCTCA
TTCTCCGAAAAGAAAGTCGATCTGCCAGATTTGAAAGAACGAAGCACCA
CATCGCTCAAGCGCTCTTTTGGTGCAGTGAAGGGGGTTTGGAATGGCGCC
CTAACCGGTGGGTGGAAGTACGCCACCGGAACAGTATCTAG

## APPENDIX IV

## miRNA target (DICER substrate) prediction results

### Custom dicer substrate sites for Rep



Select All Results

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.218116.13.1 | **($1.00) USD** |

ADD TO CART

Hide product details -

1 200 401

**Cross-Reacting Transcripts**

none

**Sequence Positions**

214-239

**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.218116.13.2 | **($1.00) USD** |

ADD TO CART

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1 200 401

**Cross-Reacting Transcripts**

**Sequence Positions** none

**Sequence Details** 203-228

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rUrCrArCrUrGrArUrArCrUrGrArCrGrArGrArUrArUrUrUCA 3' | + |
| 5' rUrGrArArArUrArUrCrUrCrGrUrCrArGrUrArUrCrArGrUrGrArUrG 3' | - |

DsiRNA Custom

| Search Input | Cross-Reacting Species | Design ID | 2 nmol |
|---|---|---|---|
| Custom Input | Other (Manual BLAST) | CD.Ri.218116.13.3 | ($1.00) USD |

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1 200 401

**Cross-Reacting Transcripts** none

**Sequence Positions** 204-229

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rCrArCrUrGrArUrArCrUrGrArCrGrArGrArUrArUrUrUrCAG 3' | + |
| 5' rCrUrGrArArArUrArUrCrUrCrGrUrCrArGrUrArUrCrArGrUrGrArU 3' | - |

DsiRNA Custom

| Search Input | Cross-Reacting Species | Design ID | 2 nmol |
|---|---|---|---|
| Custom Input | Other (Manual BLAST) | CD.Ri.218116.13.4 | ($1.00) USD |

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**Cross-Reacting Transcripts**

none

**Sequence Positions**

196-221

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rArArGrUrUrCrArUrCrArCrUrGrArUrArCrUrGrArCrGrAGA 3' | + |
| 5' rUrCrUrCrGrUrCrArGrUrArUrCrArGrUrGrArUrGrArArCrUrUrCrC 3' | - |

DsiRNA Custom

| Search Input | Cross-Reacting Species | Design ID | 2 nmol |
|---|---|---|---|
| Custom Input | Other (Manual BLAST) | CD.Ri.218116.13.5 | ($1.00) USD |

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**Cross-Reacting Transcripts**

none

**Sequence Positions**

194-219

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rGrGrArArGrUrUrCrArUrCrArCrUrGrArUrArCrUrGrArCGA 3' | + |
| 5' rUrCrGrUrCrArGrUrArUrCrArGrUrGrArUrGrArArCrUrUrCrCrUrU 3' | - |

**Custom dicer substrate sites for Mvp**


## 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:**

Movement

RETRIEVE SEQUENCE

Or Paste FASTA Sequence (≤10 kb):

AAAATATCAAGACCTATTAAACGCAGAATGGAGAGCATTAAAATTGTCGGAAAAATTTTCACGAAT
CATGCATACATCATATTACAGAATTTCATCAACACTCTCATTCTCCGAAAGGAAAGTCGATCTGCCA
GATTTGAAAGAACGAAGCACCACATCGCTCAAGCACTCTTTTGGTGCAATGAAGAGAGTTTGGAA

**BLAST Species**

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

SEARCH

CLEAR AND RESET

Showing 5 results for . Clear results »

Select All Results

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.218117.13.1 | **($1.00) USD** |

ADD TO CART

Hide product details -

1 123 246

**Cross-Reacting Transcripts**

none

**Sequence Positions**

81-106

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rArUrUrArCrArGrArArUrUrUrCrArUrCrArArCrArCrUrCTC 3' | + |
| 5' rGrArGrArGrUrGrUrUrGrArUrGrArArArUrUrCrUrGrUrArArUrArU 3' | - |

**DsiRNA** Custom

| Search Input | Cross-Reacting Species | Design ID | 2 nmol |
|---|---|---|---|
| Custom Input | Other (Manual BLAST) | CD.Ri.218117.13.2 | **($1.00) USD** |

ADD TO CART

Hide product details -

1 123 246

**Cross-Reacting Transcripts**

**Cross-Reacting Transcripts**

none

**Sequence Positions**

23-48

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rGrCrArGrArArUrGrGrArGrArGrCrArUrUrArArArArUrUGT 3' | + |
| 5' rArCrArArUrUrUrUrArArUrGrCrUrCrUrCrCrArUrUrCrUrGrCrGrU 3' | - |

**DsiRNA** Custom

| Search Input | Cross-Reacting Species | Design ID | 2 nmol ▾ |
|---|---|---|---|
| Custom Input | Other (Manual BLAST) | CD.Ri.218117.13.3 | **($1.00) USD** |

ADD TO CART

Hide product details -

1 123 246

**Cross-Reacting Transcripts**

none

**Sequence Positions**

86-111

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rArGrArArUrUrUrCrArUrCrArArCrArCrUrCrUrCrArUrUCT 3' | + |
| 5' rArGrArArUrGrArGrArGrUrGrUrUrGrArUrGrArArArUrUrCrUrGrU 3' | - |

**DsiRNA** Custom

| Search Input | Cross-Reacting Species | Design ID | 2 nmol ▾ |
|---|---|---|---|
| Custom Input | Other (Manual BLAST) | CD.Ri.218117.13.4 | **($1.00) USD** |

ADD TO CART

Hide product details -

1 123 246

**Cross-Reacting Transcripts**

none

**Sequence Positions**

76-101

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rArUrCrArUrArUrUrArCrArGrArArUrUrUrCrArUrCrArACA 3' | + |
| 5' rUrGrUrUrGrArUrGrArArArArUrUrCrUrGrUrArArUrArUrGrArArUrGrU 3' | - |

**DsiRNA** Custom

| Search Input | Cross-Reacting Species | Design ID | 2 nmol |
|---|---|---|---|
| Custom Input | Other (Manual BLAST) | CD.Ri.218117.13.5 | **($1.00) USD** |

ADD TO CART

Hide product details -

1 123 246

**Cross-Reacting Transcripts**

none

**Sequence Positions**

78-103

**Sequence Details**

Sense and antisense sequences

| Sequence | Strand |
|---|---|
| 5' rCrArUrArUrUrArCrArGrArArUrUrUrCrArUrCrArArCrACT 3' | + |
| 5' rArGrUrGrUrUrGrArUrGrArArArArUrUrCrUrGrUrArArUrArUrGrArU 3' | - |

## APPENDIX V

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

### siRNA target prediction in virus genome

The siRNA target in the whole genome of virus or full gene sequence of target gene were found using the RNAi design in Sci Tool 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 the 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**

| Sr. No. | Reynolds' Criteria | Attributes in selected siRNA target |
|---|---|---|
| I | 30-50% GC content | ✓ |
| II | At least 3 A/U bases at position 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' of 'C' at 19 (sense strand) | ✓ |
| VIII | A base other than 'G' at position 13 ( sense strand) | ✓ |

# APPENDIX VI

## Textual map showing positions of restriction endonuclease recognition sites

## A) Replicase gene partial cds:

Restriction Map results

**Results for linear 401 residue sequence "Replicase" starting "ACATAAGGGT"**

```
MseI t|taa 59
                                                                    HpaII
c|cgg 55
                                                                    MspI
c|cgg 55
                                                NlaIII catg| 37
       1   I  R  V  L  R  V  L  *  T  A  H  V  S  L  W  S  K  P  V
K
       1  H  K  G  A  E  S  S  L  D  R  S  C  L  I  M  V  Q  A  G  *
       1 T  *  G  C  *  E  F  F  R  P  L  M  S  H  Y  G  P  S  R  L
       1
ACATAAGGGTGCTGAGAGTTCTTTAGACCGCTCATGTCTCATTATGGTCCAAGCCGGTTA
       1          10        20        30        40        50
       1
TGTATTCCCACGACTCTCAAGAAATCTGGCGAGTACAGAGTAATACCAGGTTCGGCCAAT
                                                      AluI ag|ct 106
                                           SspI aat|att 95
                                     MseI t|taa 89
      21   S  Q  G  I  L  E  R  F  I  K  I  F  W  R  A  D  C  W  C
S
      21  I  A  R  H  S  *  K  I  Y  *  N  I  L  A  S  *  L  L  V  *
      21 N  R  K  A  F  L  K  D  L  L  K  Y  S  G  E  L  I  V  G  V
      61
AATCGCAAGGCATTCTTGAAAGATTTATTAAAATATTCTGGCGAGCTGATTGTTGGTGTA
      61          70        80        90        100       110
      61
TTAGCGTTCCGTAAGAACTTTCTAAATAATTTTATAAGACCGCTCGACTAACAACCACAT

Bsp19I c|catgg 178

NcoI c|catgg 178
                                                                  SnaBI
tac|gta 173
                                     AfaI gt|ac 149
                                     RsaI gt|ac 149
                                  AccII cg|cg 146
                                  BstUI cg|cg 146
           HincII gty|rac 124
      41   *  L  *  Y  I  *  K  R  V  Q  F  H  C  I  I  V  T  *  P
W
      41  L  T  V  I  H  L  K  A  R  T  I  S  L  H  H  C  Y  V  A  M
      41 V  D  C  D  T  F  E  S  A  Y  N  F  T  A  S  L  L  R  S  H
     121
GTTGACTGTGATACATTTGAAAGCGCGTACAATTTCACTGCATCATTGTTACGTAGCCAT
     121          130       140       150       160       170
```

```
     121
CAACTGACACTATGTAAACTTTCGCGCATGTTAAAGTGACGTAGTAACAATGCATCGGTA
          NlaIII catg| 182
      61   I  *  R  K  E  V  H  H  *  Y  *  R  D  I  S  E  F  E  Y
E
      61  D  L  K  E  G  S  S  S  L  I  L  T  R  Y  F  R  V  *  I  *
      61 G  F  E  G  R  K  F  I  T  D  T  D  E  I  F  Q  S  L  N  M
     181
GGATTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGATATTTCAGAGTTTGAATATG
     181          190       200       210       220       230
     181
CCTAAACTTCCTTCCTTCAAGTAGTGACTATGACTGCTCTATAAAGTCTCAAACTTATAC
      81   G  C  C  W  G  T  V  C  W  E  E  E  G  L  F  *  G  L  H
*
      81  R  L  L  L  G  H  C  M  L  G  R  R  G  I  I  L  R  A  S  L
      81 K  A  A  V  G  A  L  Y  A  G  K  K  R  D  Y  F  E  G  F  T
     241
AAGGCTGCTGTTGGGGCACTGTATGCTGGGAAGAAGAGGGATTATTTTGAGGGCTTCACT
     241          250       260       270       280       290
     241
TTCCGACGACAACCCCGTGACATACGACCCTTCTTCTCCCTAATAAAACTCCCGAAGTGA
     101   S  T  K  G  *  D  Y  I  P  E  L  S  S  P  I  Q  R  T  F
R
     101  I  N  K  R  M  R  L  Y  S  R  V  V  F  A  Y  T  K  D  I  *
     101 N  Q  Q  K  D  E  I  I  F  Q  S  C  L  R  L  Y  K  G  H  L
     301
AATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCCTATACAAAGGACATTTA
     301          310       320       330       340       350
     301
TTAGTTGTTTTCCTACTCTAATATAAGGTCTCAACAGAAGCGGATATGTTTCCTGTAAAT
                                           HaeIII gg|cc 397
                                           PhoI gg|cc 397
                                  AluI ag|ct 386
                      BamHI g|gatcc 374
                      DpnII |gatc 374
                      MboI |gatc 374
                      NdeII |gatc 374
     121   R  M  E  R  I  L  E  S  *  I  T  A  K
     121  A  Y  G  T  D  P  *  K  L  N  Y  G  Q
     121 G  V  W  N  G  S  L  K  A  E  L  R  P
     361 GGCGTATGGAACGGATCCTTGAAAGCTGAATTACGGCCAAA
     361          370       380       390       400
     361 CCGCATACCTTGCCTAGGAACTTTCGACTTAATGCCGGTTT
```

| Site: | Positions: |
|---|---|
| AatI agg\|cct | none |
| AatII gacgt\|c | none |
| Acc16I tgc\|gca | none |
| AccII cg\|cg | 146 |
| AccIII t\|ccgga | none |
| AclI aa\|cgtt | none |
| AcvI cac\|gtg | none |

| | |
|---|---|
| AfaI gt\|ac | 149 |
| AfeI agc\|gct | none |
| AflII c\|ttaag | none |
| AgeI a\|ccggt | none |
| AhlI a\|ctagt | none |
| Alw441 g\|tgcac | none |
| AluI ag\|ct | 106, 386 |
| 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 | none |
| AvaI c\|ycgrg | none |
| AviII tgc\|gca | none |
| AvrII c\|ctagg | none |
| BalI tgg\|cca | none |
| BamHI g\|gatcc | 374 |
| BanIII at\|cgat | none |
| BbeI ggcgc\|c | none |
| BbrPI cac\|gtg | none |
| BbuI gcatg\|c | none |
| BcuI a\|ctagt | none |
| BclI t\|gatca | none |
| BfaI c\|tag | none |
| BfrI c\|ttaag | none |
| BfrBI atg\|cat | none |
| BglII a\|gatct | none |
| BlnI c\|ctagg | none |
| BseCI at\|cgat | none |
| BsePI g\|cgcgc | none |
| BseX3I c\|ggccg | none |
| BshTI a\|ccggt | none |
| Bsp1407I t\|gtaca | none |
| Bsp19I c\|catgg | 178 |
| BspDI at\|cgat | none |
| BspEI t\|ccgga | none |
| BsrGI t\|gtaca | none |

| | |
|---|---|
| BssHII g\|cgcgc | none |
| BstUI cg\|cg | 146 |
| ClaI at\|cgat | none |
| DpnII \|gatc | 374 |
| 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 | 397 |
| HincII gty\|rac | 124 |
| HindIII a\|agctt | none |
| HinfI g\|antc | none |
| HpaI gtt\|aac | none |
| HpaII c\|cgg | 55 |
| KasI g\|gcgcc | none |
| KpnI ggtac\|c | none |
| MboI \|gatc | 374 |
| MfeI c\|aattg | none |
| MluI a\|cgcgt | none |
| MscI tgg\|cca | none |
| MseI t\|taa | 59, 89 |
| MspI c\|cgg | 55 |
| NaeI gcc\|ggc | none |
| NarI gg\|cgcc | none |
| NcoI c\|catgg | 178 |
| NdeI ca\|tatg | none |
| NdeII \|gatc | 374 |
| NgoMIV g\|ccggc | none |
| NheI g\|ctagc | none |
| NlaIII catg\| | 37, 182 |
| NotI gc\|ggccgc | none |
| NruI tcg\|cga | none |
| NsiI atgca\|t | none |
| PacI ttaat\|taa | none |
| PciI a\|catgt | none |
| PhoI gg\|cc | 397 |

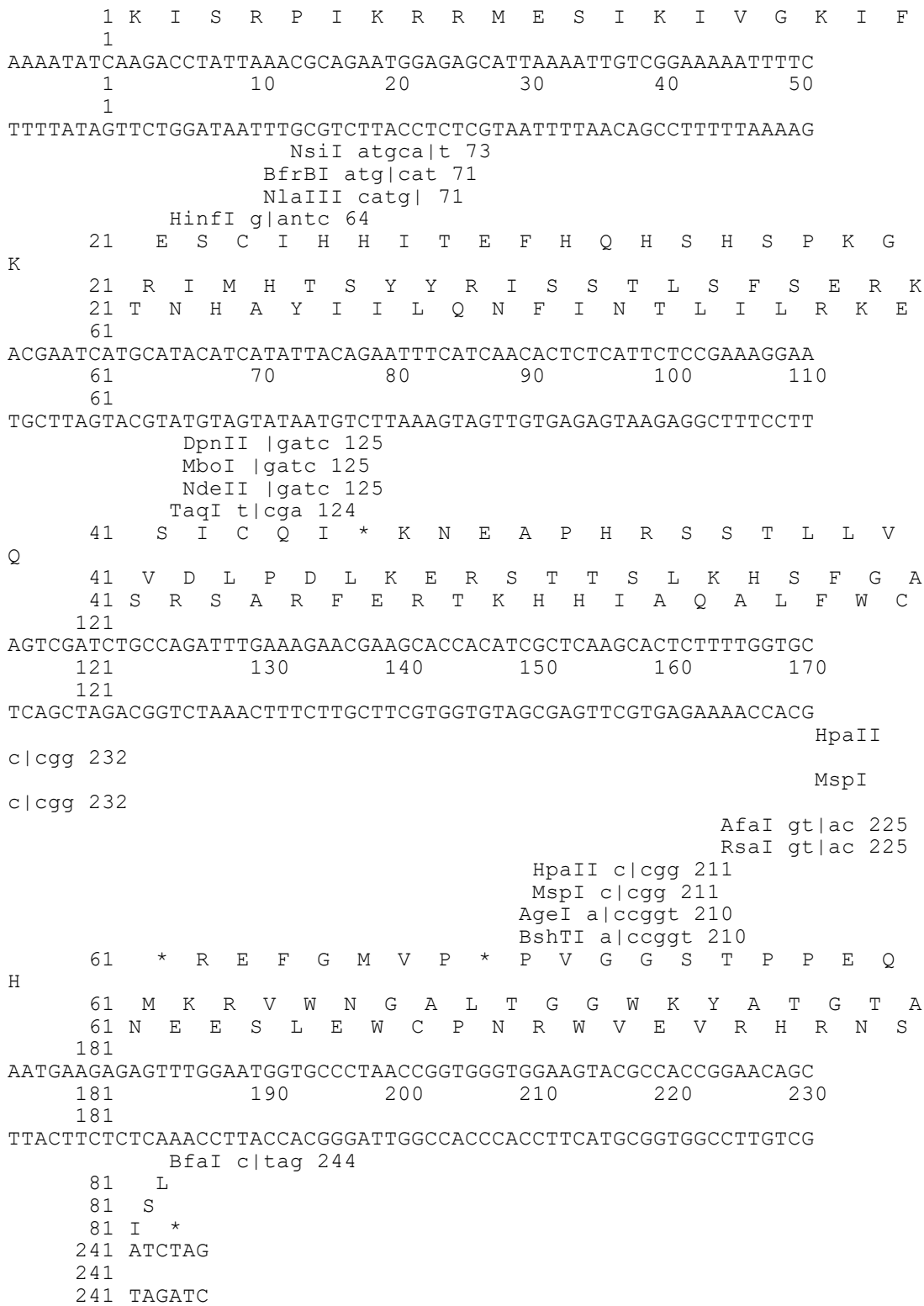
| | |
|---|---|
| PmeI gttt\|aaac | none |
| PmlI cac\|gtg | none |
| PsiI tta\|taa | none |
| PstI ctgca\|g | none |
| PvuI cgat\|cg | none |
| PvuII cag\|ctg | none |
| RsaI gt\|ac | 149 |
| SacI gagct\|c | none |
| SacII ccgc\|gg | none |
| SalI g\|tcgac | none |
| SbfI cctgca\|gg | none |
| ScaI agt\|act | none |
| SfoI ggc\|gcc | none |
| SmaI ccc\|ggg | none |
| SnaBI tac\|gta | 173 |
| SpeI a\|ctagt | none |
| SphI gcatg\|c | none |
| SspI aat\|att | 95 |
| SstI gagct\|c | none |
| SstII ccgc\|gg | none |
| StuI agg\|cct | none |
| SwaI attt\|aaat | none |
| TaqI t\|cga | none |
| TliI c\|tcgag | none |
| VspI at\|taat | none |
| XbaI t\|ctaga | none |
| XhoI c\|tcgag | none |
| XmaI c\|ccggg | none |

## B) Movement protein gene partial cds:

Restriction Map results

**Results for linear 246 residue sequence "Movement" starting "AAAATATCAA"**

```
                                                    MseI t|taa 39
                          MseI t|taa 18
       1   N   I   K   T   Y   *   T   Q   N   G   E   H   *   N   C   R   K   N   F
H
       1   K   Y   Q   D   L   L   N   A   E   W   R   A   L   K   L   S   E   K   F   S
```


1 K I S R P I K R R M E S I K I V G K I F
1
AAAATATCAAGACCTATTAAACGCAGAATGGAGAGCATTAAAATTGTCGGAAAAATTTTC
1 10 20 30 40 50
1
TTTTATAGTTCTGGATAATTTGCGTCTTACCTCTCGTAATTTTAACAGCCTTTTTAAAAG
NsiI atgca|t 73
BfrBI atg|cat 71
NlaIII catg| 71
HinfI g|antc 64
21 E S C I H H I T E F H Q H S H S P K G
K
21 R I M H T S Y Y R I S S T L S F S E R K
21 T N H A Y I I L Q N F I N T L I L R K E
61
ACGAATCATGCATACATCATATTACAGAATTTCATCAACACTCTCATTCTCCGAAAGGAA
61 70 80 90 100 110
61
TGCTTAGTACGTATGTAGTATAATGTCTTAAAGTAGTTGTGAGAGTAAGAGGCTTTCCTT
DpnII |gatc 125
MboI |gatc 125
NdeII |gatc 125
TaqI t|cga 124
41 S I C Q I * K N E A P H R S S T L L V
Q
41 V D L P D L K E R S T T S L K H S F G A
41 S R S A R F E R T K H H I A Q A L F W C
121
AGTCGATCTGCCAGATTTGAAAGAACGAAGCACCACATCGCTCAAGCACTCTTTTGGTGC
121 130 140 150 160 170
121
TCAGCTAGACGGTCTAAACTTTCTTGCTTCGTGGTGTAGCGAGTTCGTGAGAAAACCACG
HpaII
c|cgg 232
MspI
c|cgg 232
AfaI gt|ac 225
RsaI gt|ac 225
HpaII c|cgg 211
MspI c|cgg 211
AgeI a|ccggt 210
BshTI a|ccggt 210
61 * R E F G M V P * P V G G S T P P E Q
H
61 M K R V W N G A L T G G W K Y A T G T A
61 N E E S L E W C P N R W V E V R H R N S
181
AATGAAGAGAGTTTGGAATGGTGCCCTAACCGGTGGGTGGAAGTACGCCACCGGAACAGC
181 190 200 210 220 230
181
TTACTTCTCTCAAACCTTACCACGGGATTGGCCACCCACCTTCATGCGGTGGCCTTGTCG
BfaI c|tag 244
81 L
81 S
81 I *
241 ATCTAG
241
241 TAGATC

| Site: | Positions: |
|---|---|
| AatI agg\|cct | none |
| AatII gacgt\|c | none |
| Acc16I tgc\|gca | none |
| AccII cg\|cg | none |
| AccIII t\|ccgga | none |
| AclI aa\|cgtt | none |
| AcvI cac\|gtg | none |
| AfaI gt\|ac | 225 |
| AfeI agc\|gct | none |
| AflII c\|ttaag | none |
| AgeI a\|ccggt | 210 |
| AhlI a\|ctagt | none |
| Alw441 g\|tgcac | none |
| AluI ag\|ct | none |
| 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 | none |
| 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 |
| BbrPI cac\|gtg | none |
| BbuI gcatg\|c | none |
| BcuI a\|ctagt | none |
| BclI t\|gatca | none |
| BfaI c\|tag | 244 |
| BfrI c\|ttaag | none |
| BfrBI atg\|cat | 71 |

| | |
|---|---|
| BglII a\|gatct | none |
| BlnI c\|ctagg | none |
| BseCI at\|cgat | none |
| BsePI g\|cgcgc | none |
| BseX3I c\|ggccg | none |
| BshTI a\|ccggt | 210 |
| Bsp1407I t\|gtaca | none |
| Bsp19I c\|catgg | none |
| BspDI at\|cgat | none |
| BspEI t\|ccgga | none |
| BsrGI t\|gtaca | none |
| BssHII g\|cgcgc | none |
| BstUI cg\|cg | none |
| ClaI at\|cgat | none |
| DpnII \|gatc | 125 |
| 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 | none |
| HincII gty\|rac | none |
| HindIII a\|agctt | none |
| HinfI g\|antc | 64 |
| HpaI gtt\|aac | none |
| HpaII c\|cgg | 211, 232 |
| KasI g\|gcgcc | none |
| KpnI ggtac\|c | none |
| MboI \|gatc | 125 |
| MfeI c\|aattg | none |
| MluI a\|cgcgt | none |
| MscI tgg\|cca | none |
| MseI t\|taa | 18, 39 |
| MspI c\|cgg | 211, 232 |

| | |
|---|---|
| NaeI gcc\|ggc | none |
| NarI gg\|cgcc | none |
| NcoI c\|catgg | none |
| NdeI ca\|tatg | none |
| NdeII \|gatc | 125 |
| NgoMIV g\|ccggc | none |
| NheI g\|ctagc | none |
| NlaIII catg\| | 71 |
| NotI gc\|ggccgc | none |
| NruI tcg\|cga | none |
| NsiI atgca\|t | 73 |
| PacI ttaat\|taa | none |
| PciI a\|catgt | none |
| PhoI gg\|cc | none |
| PmeI gttt\|aaac | none |
| PmlI cac\|gtg | none |
| PsiI tta\|taa | none |
| PstI ctgca\|g | none |
| PvuI cgat\|cg | none |
| PvuII cag\|ctg | none |
| RsaI gt\|ac | 225 |
| SacI gagct\|c | none |
| SacII ccgc\|gg | none |
| SalI g\|tcgac | none |
| SbfI cctgca\|gg | none |
| ScaI agt\|act | none |
| SfoI ggc\|gcc | none |
| SmaI ccc\|ggg | none |
| SnaBI tac\|gta | none |
| SpeI a\|ctagt | none |
| SphI gcatg\|c | none |
| SspI aat\|att | none |
| SstI gagct\|c | none |
| SstII ccgc\|gg | none |
| StuI agg\|cct | none |
| SwaI attt\|aaat | none |

| | |
|---|---|
| TaqI t\|cga | 124 |
| TliI c\|tcgag | none |
| VspI at\|taat | none |
| XbaI t\|ctaga | none |
| XhoI c\|tcgag | none |
| XmaI c\|ccggg | none |

## APPENDIX VII

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

| | |
|---|---|
| Yeast extract | 5 |
| Tryptone | 10 |
| Sodium chloride | 10 |

| | |
|---|---|
| Agar | 15 |
| pH | 7 |

## Appendix VIII

## Chemicals for plasmid DNA isolation from bacteria

### (i) Solution I

| | |
|---|---|
| 20% Glucose | 2.25 ml |
| 0.5 M EDTA (pH 8.0) | 1 ml |
| 1 M 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 |

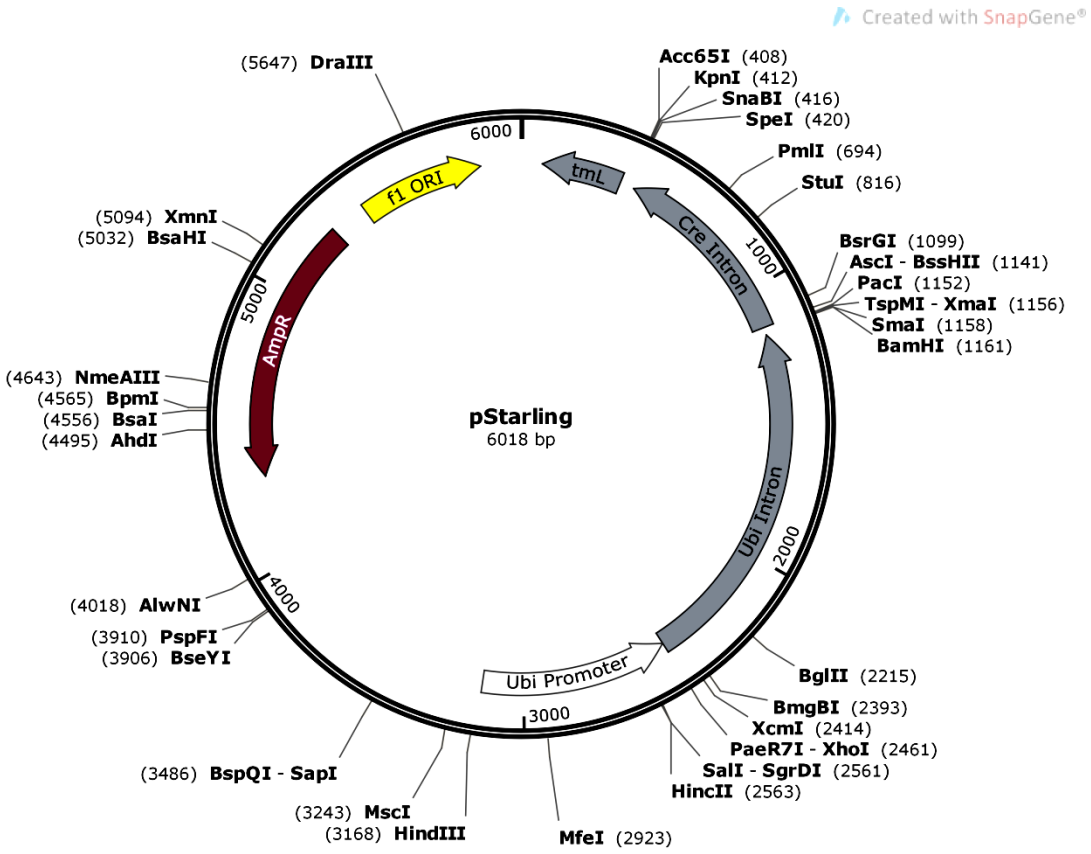
## Appendix IX

**TE buffer recipe**

| | |
|---|---|
| 1 M Tris -Cl | 1 ml |
| 0.5 M EDTA (pH 8.0) | 200 l |
| Sterile distilled water | 98.8 ml |

## APPENDIX X

**Nucleotide sequence and structure of the ihpRNA vector pSTARLING**




**Vector description:**

This is a pUC based vector with a novel set of restriction sites. The cassette prepared can be mobilized to the binary vector as *Not*I fragment. The *Not*I fragment contained the components such as *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 SpeI, *Sna*BI and *Kpn*I at its 3' end and tmL (tumor morphology locus) terminator.

>pSTARLING

gttgtaaaacgacggccagtgccaagctgacttggtcagcggccgcatctagagggcccggatctgatgggcgagctcg
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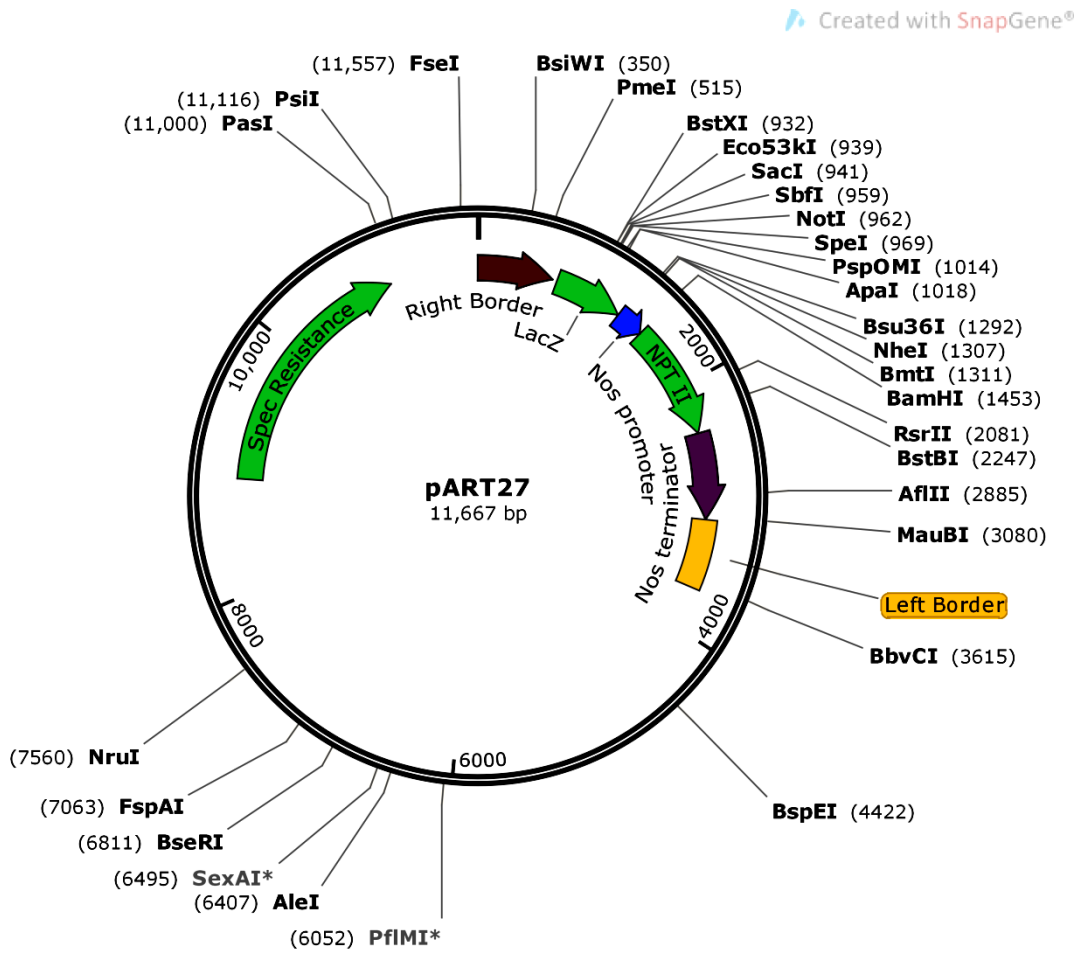
## APPENDIX XI

**Physical map and nucleotide sequence of the binary vector pART27**

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**APPENDIX XII**

CLUSTAL W alignment to check orientation of the sense and antisense fragments

**Replicase sense**

```
CLUSTAL O(1.2.4) multiple sequence alignment


OriginalRep-S      ATGGTCCAAGCCGGTTAAATCGCAAGGCATTCTTGAAAGATTTATTAAAATATTCTGGCG 60
TestRep-S          ----------------------------------------TTAAAATATTCTGGCG 16
                                                           ***************

OriginalRep-S      AGCTGATTGTTGGTGTAGTTGACTGTGATACATTTGAAAGCGCGTACAATTTCACTGCAT 120
TestRep-S          AGCTGATTGTTGGTGTAGTTGACTGTGATACATTTGAAAGCGCGTACAATTTCACTGCAT 76
                   ************************************************************

OriginalRep-S      CATTGTTACGTAGCCATGGATTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGATAT 180
TestRep-S          CATTGTTACGTAGCCATGGATTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGATAT 136
                   ************************************************************

OriginalRep-S      TTCAGAGTTTGAATATGAAGGCTGCTGTTGGGGCACTGTATGCTGGGAAGAAGAGGGATT 240
TestRep-S          TTCAGAGTTTGAATATGAAGGCTGCTGTTGGGGCACTGTATGCTGGGAAGAAGAGGGATT 196
                   ************************************************************

OriginalRep-S      ATTTTGAGGGCTTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCC 300
TestRep-S          ATTTTGAGGGCTTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCC 256
                   ************************************************************

OriginalRep-S      TATACAAAGGACATTTAGGCGTATGGAACGGATCCTTGAAAGCTGAATTACGGCCAA--- 357
TestRep-S          TATACAAAGGACATTTAGGCGTATGGAACGGATCCTTGAAAGCTGAATTACGGCCAAAGG 316
                   ***********************************************************

OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          CGCGCCTATCTTTCTAGAAGATCTCCTACAATATTCTCAGCTGCCATGGAAAATCGATGT 376

OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          TCTTCTTTTATTCTCTCAAGATTTTCAGGCTGTATATTAAAACTTATATTAAGAACTATG 436

OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          CTAACCACCTCATCAGGAACCGTTGTAGGTGGCGTGGGTTTTCTTGGCAATCGACTCTCA 496

OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          TGAAAACTACGAGCTAAATATTCAATATGTTCCTCTTGACCAACTTTATTCTGCATTTTT 556
OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          TTTGAACGAGGTTTAGAGCAAGCTTCAGGAAACTGAGACAGGAATTTTATTAAAAATTTA 616

OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          AATTTTGAAGAAAGTTCAGGGTTAATAGCATCCATTTTTTGCTTTGCAAGTTCCTCAGCA 676

OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          TTCTTAACAAAAGACGTCTCTTTTGACATGTTTAAAGTTTAAACCTCCTGTGTGAAATTA 736

OriginalRep-S      ------------------------------------------------------------ 357
TestRep-S          TTATCCGCTCATAATTCCACACATTATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGG 796

OriginalRep-S      ----------   357
TestRep-S          TGCCTAATGA   806
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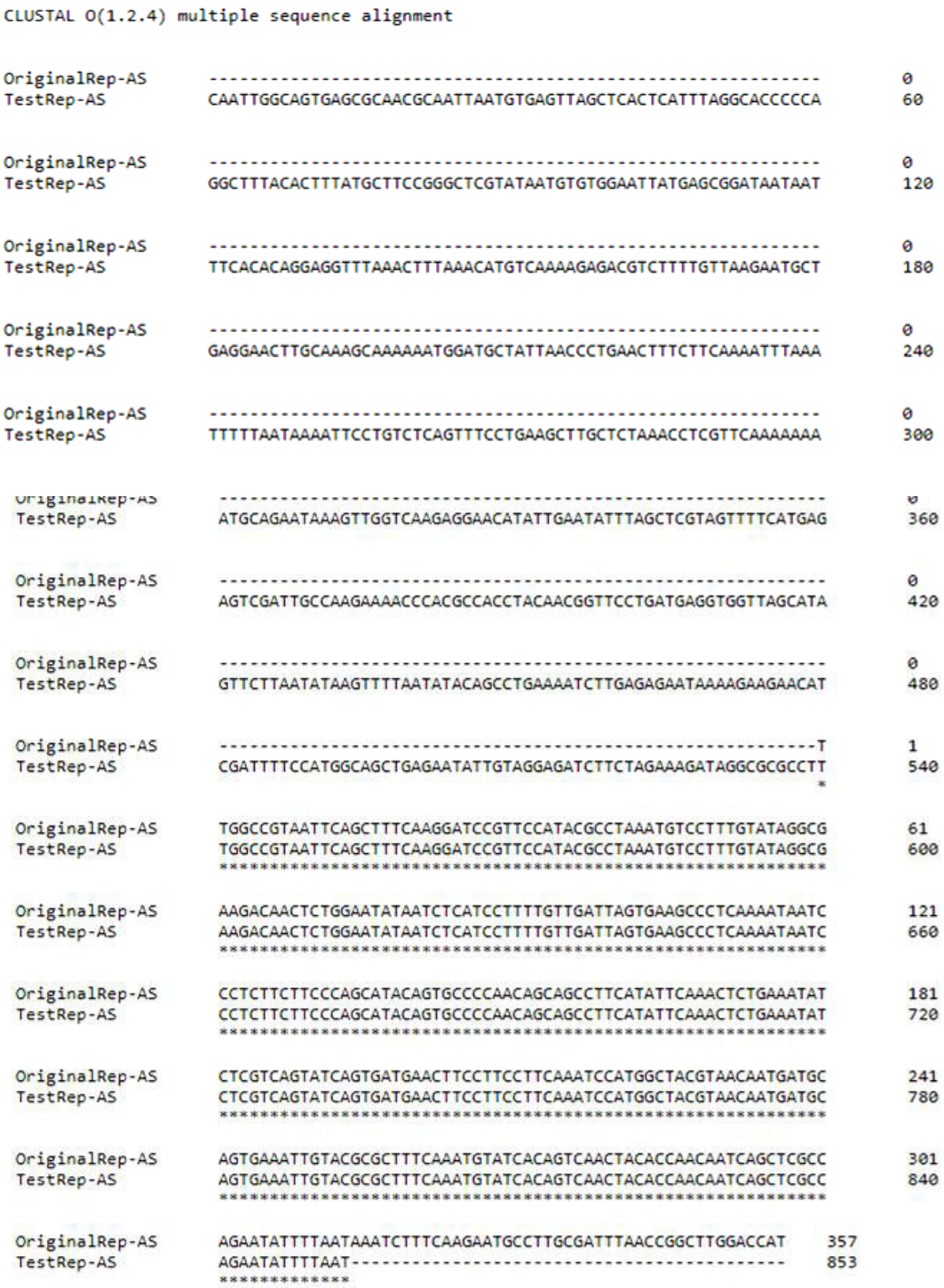
**Replicase antisense**




**Movement protein sense**


**Movement protein antisense**

```
CLUSTAL O(1.2.4) multiple sequence alignment


OriginalMvp-AS      ------------------------------------------------------------        0
TestMvp-AS          CATGTTCTTTCGTGCGTTATCCCATGTTTCTGTGGATAACCGTATTACCGCTTTTGAGTG        60

OriginalMvp-AS      ------------------------------------------------------------        0
TestMvp-AS          AGCAGATACCGCTTGCCGCAGCCGAACGAACGAGCGCAGCGAGTCAGTGAGCGTGGAAGC        120

OriginalMvp-AS      ------------------------------------------------------------        0
TestMvp-AS          CGAAGAGCTTACAATTCCAAAGCCCTCGATACGTGGATTGAATGATTCATTTATTCAGAT        180

OriginalMvp-AS      ------------------------------------------------------------        0
TestMvp-AS          GGCTCGCCAGGTTTATACAATGGAAAGTGGACGGCGATTCCCTTGCTATTTGGTGGAGCT        240

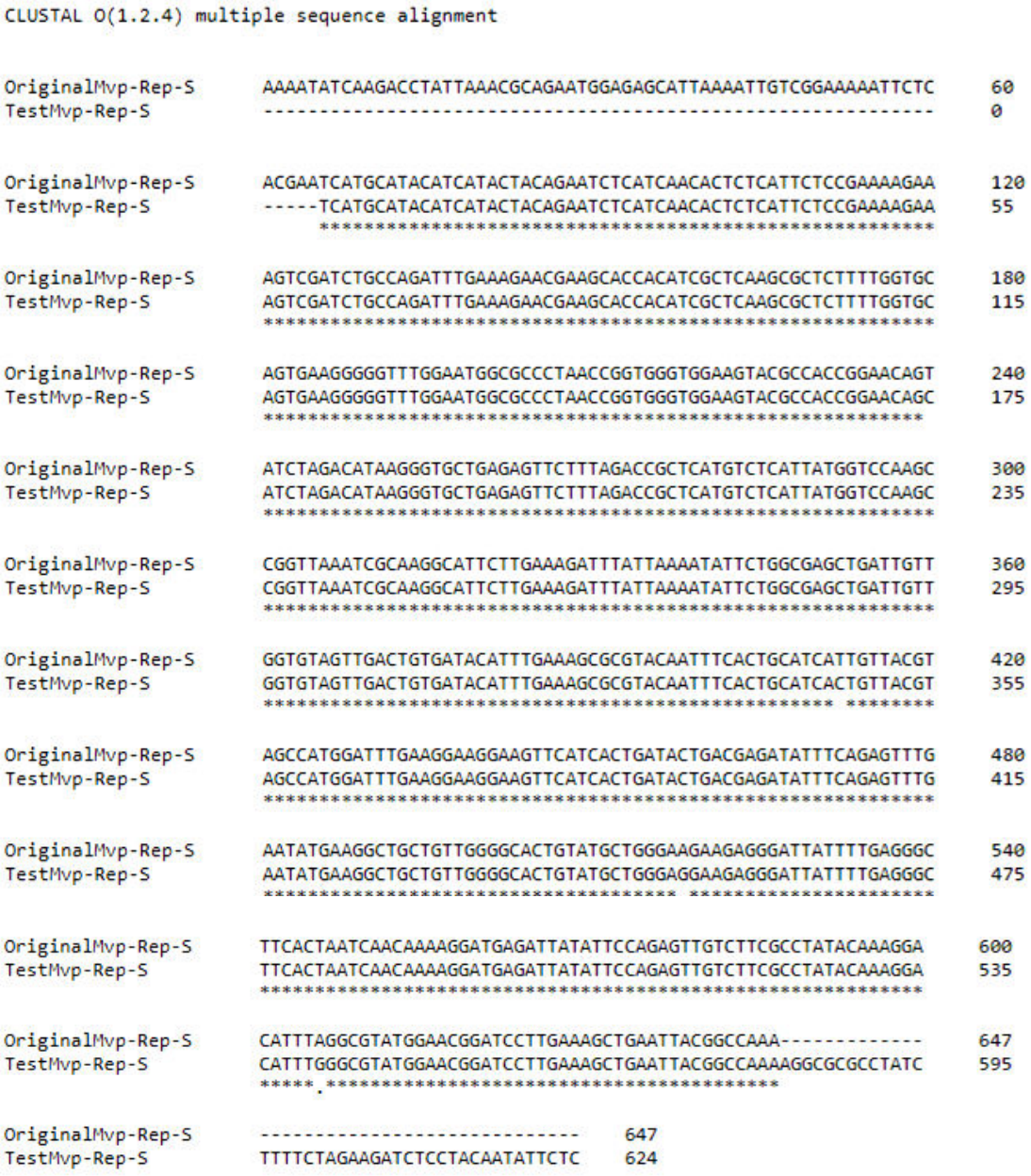
OriginalMvp-AS      ------------ACTGTTCCGGTGGCGTACTTCCACCCACCGGTTAGGGCGCCATTCCAA        48
TestMvp-AS          GGAGCATTCTTTAGGCTTCCCGGGGCGTACTTCCACCCACCGGTTAGGGCGCCATTCCAA        300
                               *   **** * ************************************

OriginalMvp-AS      ACCCCCTTCACTGCACCAAAAGAGCGCTTGAGCGATGTGGTGCTTCGTTCTTTCAAATCT        108
TestMvp-AS          ACCCCCTTCACTGCACCAAAAGAGCGCTTGAGCGATGTGGTGCTTCGTTCTTTCAAATCT        360
                    ************************************************************

OriginalMvp-AS      GGCAGATCGACTTTCTTTTCGGAGAATGAGAGTGTTGATGAGATTCTGTAGTATGATGTA        168
TestMvp-AS          GGCAGATCGACTTTCTTTTCGGAGAATGAGAGTGTTGATGAGATTCTGTAGTATGATGTA        420
                    ************************************************************

OriginalMvp-AS      TGCATGATTCGTGAGAATTTTTCCGACAATTTTAATGCTCTCCATTCTGCGTTTA     223
TestMvp-AS          TGCATGATTCG-------------------------------------------     431
                    ***********
```

**Mvp-Rep-Sense**


CLUSTAL O(1.2.4) multiple sequence alignment
OriginalMvp-Rep-S AAAATATCAAGACCTATTAAACGCAGAATGGAGAGCATTAAAATTGTCGGAAAAATTCTC 60
TestMvp-Rep-S ---------- 0
OriginalMvp-Rep-S ACGAATCATGCATACATCATACTACAGAATCTCATCAACACTCTCATTCTCCGAAAAGAA 120
TestMvp-Rep-S -----TCATGCATACATCATACTACAGAATCTCATCAACACTCTCATTCTCCGAAAAGAA 55
OriginalMvp-Rep-S AGTCGATCTGCCAGATTTGAAAGAACGAAGCACCACATCGCTCAAGCGCTCTTTTGGTGC 180
TestMvp-Rep-S AGTCGATCTGCCAGATTTGAAAGAACGAAGCACCACATCGCTCAAGCGCTCTTTTGGTGC 115
OriginalMvp-Rep-S AGTGAAGGGGGTTTGGAATGGCGCCCTAACCGGTGGGTGGAAGTACGCCACCGGAACAGT 240
TestMvp-Rep-S AGTGAAGGGGGTTTGGAATGGCGCCCTAACCGGTGGGTGGAAGTACGCCACCGGAACAGC 175
OriginalMvp-Rep-S ATCTAGACATAAGGGTGCTGAGAGTTCTTTAGACCGCTCATGTCTCATTATGGTCCAAGC 300
TestMvp-Rep-S ATCTAGACATAAGGGTGCTGAGAGTTCTTTAGACCGCTCATGTCTCATTATGGTCCAAGC 235
OriginalMvp-Rep-S CGGTTAAATCGCAAGGCATTCTTGAAAGATTTATTAAAATATTCTGGCGAGCTGATTGTT 360
TestMvp-Rep-S CGGTTAAATCGCAAGGCATTCTTGAAAGATTTATTAAAATATTCTGGCGAGCTGATTGTT 295
OriginalMvp-Rep-S GGTGTAGTTGACTGTGATACATTTGAAAGCGCGTACAATTTCACTGCATCATTGTTACGT 420
TestMvp-Rep-S GGTGTAGTTGACTGTGATACATTTGAAAGCGCGTACAATTTCACTGCATCACTGTTACGT 355
OriginalMvp-Rep-S AGCCATGGATTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGATATTTCAGAGTTTG 480
TestMvp-Rep-S AGCCATGGATTTGAAGGAAGGAAGTTCATCACTGATACTGACGAGATATTTCAGAGTTTG 415
OriginalMvp-Rep-S AATATGAAGGCTGCTGTTGGGGCACTGTATGCTGGGAAGAAGAGGGATTATTTTGAGGGC 540
TestMvp-Rep-S AATATGAAGGCTGCTGTTGGGGCACTGTATGCTGGGAGGAAGAGGGATTATTTTGAGGGC 475
OriginalMvp-Rep-S TTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCCTATACAAAGGA 600
TestMvp-Rep-S TTCACTAATCAACAAAAGGATGAGATTATATTCCAGAGTTGTCTTCGCCTATACAAAGGA 535
OriginalMvp-Rep-S CATTTAGGCGTATGGAACGGATCCTTGAAAGCTGAATTACGGCCAAA---------- 647
TestMvp-Rep-S CATTTGGGCGTATGGAACGGATCCTTGAAAGCTGAATTACGGCCAAAAGGCGCGCCTATC 595
OriginalMvp-Rep-S ---------- 647
TestMvp-Rep-S TTTTCTAGAAGATCTCCTACAATATTCTC 624

**Mvp-Rep-AS**

```
CLUSTAL O(1.2.4) multiple sequence alignment

OriginalMvp-Rep-AS      ------------------------------------------------------------    0
TestMvp-Rep-AS          TCAAAAAAAATGCAGAATAAAGTTGGTCAAGAGGAACATATTGAATAATTTAGCTCGTAG    60

OriginalMvp-Rep-AS      ------------------------------------------------------------    0
TestMvp-Rep-AS          TTTTCATGAGAGTCGATTGCCAAGAAAAACCACGCCACCTACAACGGTTCCTGATGAGGT    120

OriginalMvp-Rep-AS      ------------------------------------------------------------    0
TestMvp-Rep-AS          GGTTAGCATAGTTCTTAATATAAGTTTTAATATACAGCCTGAAAATCTTGAGAGAATAAA    180

OriginalMvp-Rep-AS      ------------------------------------------------------------    0
TestMvp-Rep-AS          AGAAGAACATCGATTTTTCCATGGCAGCTGAGAATATTGTAGGAGATCTTCTAGAAAGAT    240

OriginalMvp-Rep-AS      ----------TTTGGCCGTAATTCAGCTTTCAAGGATCCGTTCCATACGCCTAAATGTCC    50
TestMvp-Rep-AS          AGGCGCGCCTTTTGGCCGTAATTCAGCTTTCAAGGATCCGTTCCATACGCCCAAATGTCC    300
                                  **************************************** ********

OriginalMvp-Rep-AS      TTTGTATAGGCGAAGACAACTCTGGAATATAATCTCATCCTTTTGTTGATTAGTGAAGCC    110
TestMvp-Rep-AS          TTTGTATAGGCGAAGACAACTCTGGAATATAATCTCATCCTTTTGTTGATTAGTGAAGCC    360
                        ************************************************************

OriginalMvp-Rep-AS      CTCAAAATAATCCCTCTTCTTCCCAGCATACAGTGCCCCAACAGCAGCCTTCATATTCAA    170
TestMvp-Rep-AS          CTCAAAATAATCCCTCTTCCTCCCAGCATACAGTGCCCCAACAGCAGCCTTCATATTCAA    420
                        ****************** ****************************************

OriginalMvp-Rep-AS      ACTCTGAAATATCTCGTCAGTATCAGTGATGAACTTCCTTCCTTCAAATCCATGGCTACG    230
TestMvp-Rep-AS          ACTCTGAAATATCTCGTCAGTATCAGTGATGAACTTCCTTCCTTCAAATCCATGGCTACG    480
                        ************************************************************

OriginalMvp-Rep-AS      TAACAATGATGCAGTGAAATTGTACGCGCTTTCAAATGTATCACAGTCAACTACACCAAC    290
TestMvp-Rep-AS          TAACAGTGATGCAGTGAAATTGTACGCGCTTTCAAATGTATCACAGTCAACTACACCAAC    540
                        ***** ********************************************************

OriginalMvp-Rep-AS      AATCAGCTCGCCAGAATATTTTAATAAATCTTTCAAGAATGCCTTGCGATTTAACCGGCT    350
TestMvp-Rep-AS          AATCAGCTCGCCAGAATATTTTAATAAATCTTTCAAGAATGCCTTGCGATTTAACCGGCT    600
                        ************************************************************

OriginalMvp-Rep-AS      TGGACCATAATGAGACATGAGCGGTCTAAAGAACTCTCAGCACCCTTATGTCTAGATACT    410
TestMvp-Rep-AS          TGGACCATAATGAGACATGAGCGGTCTAAAGAACTCTCAGCACCCTTATGTCTAGATGCT    660
                        ************************************************************ **

OriginalMvp-Rep-AS      GTTCCGGTGGCGTACTTCCACCCACCGGTTAGGGCGCCATTCCAAACCCCCTTCACTGCA    470
TestMvp-Rep-AS          GTTCCGGTGGCGTACTTCCACCCACCGGTTAGGGCGCCATTCCAAACCCCCTTCACTGCA    720
                        ************************************************************

OriginalMvp-Rep-AS      CCAAAAGAGCGCTTGAGCGATGTGGTGCTTCGTTCTTTCAAATCTGGCAGATCGACTTTC    530
TestMvp-Rep-AS          CCAAAAGAGCGCTTGAGCGATGTGGTGCTTCGTTCTTTCAAATCTGGCAGATCGACTTTC    780
                        ************************************************************

OriginalMvp-Rep-AS      TTTTCGGAGAATGAGAGTGTTGATGAGATTCTGTAGTATGATGTATGCATGATTCGTGAG    590
TestMvp-Rep-AS          TTTTCGGAGAATGAGAGTGTTGATGAGATTCTGTAGTATGATGTATGCATGATTCGTGAG    840
                        ************************************************************

OriginalMvp-Rep-AS      AATTTTTCCGACAATTTTAATGCTCTCCATTCTGCGTTTAATAGGTCTTGATATTTT      647
TestMvp-Rep-AS          AATTTTCCG-----------------------------------------------      849
                        ****** *
```

## APPENDIX XIII

## Chemicals for DNA isolation from banana leave (CTAB method)

### CTAB buffer

| | |
|---|---|
| CTAB | 2 % |
| Tris- HCL (pH 8.0) | 100 mM |
| NaCl | 1.4 M |
| EDTA (pH 8.0) | 20 mM |
| 2, β- mercaptoethanol | 0.1 % |

### TE buffer (pH 8.0)

| | |
|---|---|
| Tris buffer | 10 mM |
| EDTA | 1 mM |

### 50X TAE buffer (pH 8.0)

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

# ihpRNA MEDIATED RESISTANCE FOR *BANANA BRACT MOSAIC VIRUS* IN *MUSA* SPP. BY TARGETING REPLICASE AND MOVEMENT PROTEIN GENES

*By*

**EKATPURE SACHIN CHANDRAKANT**
**(2016-21-022)**

**ABSTRACT OF THE THESIS**

**Submitted in partial fulfillment of the requirement**

**for the degree of**

## *DOCTOR OF PHILOSOPHY IN AGRICULTURE*

**(PLANT BIOTECHNOLOGY)**

**Faculty of Agriculture**

**Kerala Agricultural University**


**DEPARTMENT OF PLANT BIOTECHNOLOGY**

**COLLEGE OF AGRICULTURE, VELLAYANI,**

**THIRUVANANTHAPURAM-695 522**

**KERALA, INDIA**

**2020**

## Abstract

The study entitled “ihpRNA mediated resistance for *Banana bract mosaic virus* in *Musa* spp. by targeting replicase and movement protein genes” was carried out during 2016-2020 in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to develop ihpRNA constructs targeting replicase and movement protein genes of *Banana bract mosaic virus*, and to generate transformants of banana cultivar Grand Naine carrying the ihpRNA cassette and confirm the transformation.

Embryogenic calli of banana var. Grand Naine were developed using the protocol standardised for Nendran in the Department of Plant Biotechnology, College of Agriculture, Vellayani.

Intron hairpin RNA (ihpRNA) vectors were constructed in pSTARLING to produce small interfering RNA (siRNA) against the replicase and movement protein genes of *Banana bract mosaic virus* (BBrMV). Movement protein (Mvp) and replicase (Rep) gene fragments were amplified from the RNA isolated from the BBrMV infected plants by reverse transcriptase (RT) PCR. The partially amplified gene fragments were sequenced and analysed using BLASTn tool. Replicase gene sequence showed 96.37 per cent similarity with BBrMV infecting banana (Trichy isolate), while movement protein gene showed 100 per cent similarity to BBrMV infecting banana (Philippine isolate). These sequences were analysed using Dicer substrate siRNA prediction tool for identifying Dicer substrates for finding any virus suppressing sequences. Restriction mapping was carried out to avoid recognition sequences present in the cloning site. Based on the results of these analyses replicase gene fragment of 357 bp and movement protein gene fragment of 224 bp were selected for preparing the construct.

Primers were designed to amplify the fragment of 357 bp of Rep and 224 bp of Mvp towards the 5' end. NEBuilder tool was used to design the primers for amplifying the fragment containing a combination of Mvp and Rep (Mvp-Rep) with a fragment size of 647 bp. Sense fragments were amplified with the primers anchored with *Kpn*I and *Spe*I sites so as to ligate them to the corresponding cloning site of siRNA vector pSTARLING. The antisense amplified fragments of Rep (357 bp), Mvp (224 bp) and Mvp-Rep (647 bp) were amplified using primers anchored with *Asc*I and *Pac*I sites. The amplified sense and antisense fragments were eluted from agarose gel and cloned in pJET1.2 cloning vector. Later, the cloned fragments with sticky ends were released from pJET1.2 using the corresponding restriction enzymes and integrated into the *Not*I cloning site of pSTARLING vector flanking the *cre* intron to favour the formation of the hairpin structure. Presence of the inserts was confirmed by restriction digestion and PCR.

For *Agrobacterium* mediated transformation, all the three ihpRNA cassettes containing ubiquitin promoter, ubiquitin intron, sense strand, *cre* intron, antisense strand and termination sequence in the order within the *Not*I restriction site were released from pSTARLING by digesting with *Not*I enzyme. Then ligated at *Not*I site within the lacZ gene of the binary vector pART27 having antibiotic resistance markers *npt*II and *Spec*. Integration of cassette within lacZ gene facilitated the selection of transformed colonies by blue-white screening. The white positive colonies were confirmed for the integration of ihpRNA cassette using PCR. The binary vector with the insert was transferred to *Agrobacterium tumefaciens* strain GV3103 and ihpRNA insert was confirmed by PCR and restriction digestion.

Embryogenic calli were transformed with *Agrobacterium* strain GV3103 containing three different ihpRNA cassettes and transformed embryos were selected on kanamycin (200 $mgL^{-1}$) media. Transformed embryogenic calli carrying Rep, Mvp and Rep-Mvp ihpRNA cassettes when transferred to fresh MS medium with 2 $mgL^{-1}$

BA yielded a regeneration of 9, 8 and 12 percent respectively. The regenerants were confirmed for the presence of ihpRNA construct using PCR with the primers for *npt*II gene. Integration of each ihpRNA cassette was also confirmed by Real-Time PCR using sense strand and *cre* intron specific primers. Syntheses of the respective siRNAs in the regenerated plants were also confirmed through PCR using siRNA specific stem loop primers.

The study was successful in developing transgenic Grand Naine plants carrying ihpRNA constructs targeting the replicase and movement protein genes of BBrMV. Synthesis of siRNA by the transgenics indicate that the constructs integrated are functional. The silencing potential of the constructs need to be validated by artificially inoculating the plants with BBrMV.