RNA MEDIATED RESISTANCE TO *Yellow vein mosaic virus* IN OKRA

By

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(2017-21-013)

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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KERALA, INDIA
2021

DECLARATION

I hereby declare that the thesis entitled **"RNA mediated resistance to *Yellow vein mosaic virus* in okra"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara
Date: 15/9/2024

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(2017-21-013)

CERTIFICATE

Certified that the thesis entitled **"RNA mediated resistance to *Yellow vein mosaic virus* in okra"** is a record of research work done independently by **Mr. Kelkar Vipul Ganesh (2017-21-013)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

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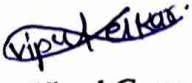

Kelkar Vipul Ganesh

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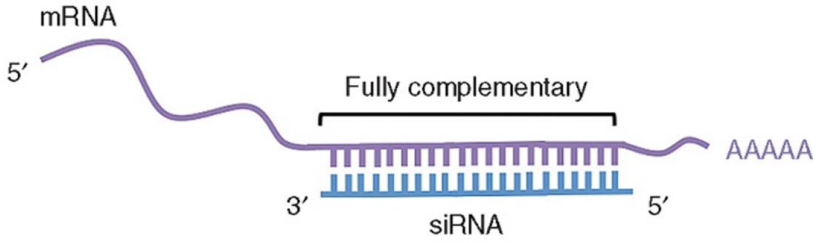
%	Per cent
@	At the rate
=	Equal to
2,4-D	2,4-Dichlorophenoxy acetic acid
BAP	Benzyl adenine purine
bp	Base pair
cDNA	Complementary DNA
CTAB	Cetyl Trimethyl Ammonium Bromide
cv	Cultivar
dATP	Deoxyadenosine triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxynucleic acid
DNase	Deoxyribonuclease
dsRNA	Double stranded RNA
E. coli	*Escherichia coli*
EDTA	Ethylenediamine Tetraacetic Acid
h	Hour
hpRNA	Hair pin Ribonucleic Acid
IAA	Indole -3-Acetic Acid
IBA	Indole Butryic Acid
Kb	Kilo base
L	Litre
LB medium	Luria-Bertani medium
LIC	Ligation Independent Cloning
M	Molar
mg	milligram
Min	Minute
miRNA	Micro RNA
mL	Milli Litre

mM	Milli Molar
MP	Movement Protein
MS	Murashige and Skoog medium
NAA	Naphthalene Acetic acid
ng	Nanogram
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	Revolution Per Minute
s	Second
siRNA	Small interfering RNA
TAE	Triss Acetate EDTA
TDZ	Thidiazuron
TE	Triss-EDTA Buffer
TGS	Transcriptional Gene Silencing
UV	Ulta Violet
V	Volt
µg	Micro gram
µM	Micro Molar
µL	Micro Litre



INTRODUCTION

1. INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench, Malvaceae) is one of the oldest cultivated crops grown extensively in the tropical to subtropical regions of the world. Though it originated in the Eastern African countries, India is the leader in its production and consumption. Its tender fruits are rich in vitamins, calcium, potassium and other minerals. Seed oil is a rich source of linoleic acid, the crude fibre in the mature fruits and stems is used in paper industry.

Genome sequencing of okra has been quite complicated due to its huge size (16,000 Mb), spanning over 65 linkage groups, and the complex polyploidy (Sanwal *et al*., 2014). *A. esculentus* (2n=130) is an allodiploid of *A. tuberculatus* (2n=58) and *A. ficulneus* (2n=72) (Datta and Naug, 1968).

Globally, okra occupies an area of 2.73 million ha, yielding 9.95 million metric ton (MT) fruits annually, with an average yield of 3.6 MT ha^{-1}. India ranks first in the world with a production of 6.1 MT (61 per cent of the total world production) from 0.51 million hectares, with a productivity of 12.03 tons ha^{-1} (FAO, 2019).

In India and Africa, okra is mostly grown for its vegetable fruits. Middle East and European countries are the largest importers of okra from India and account for around 60 per cent of total import (Singh *et al*., 2014). Hot and humid climate is suitable for okra cultivation, and unfortunately this condition is conducive for many pests and diseases. Okra is susceptible to viruses such as *Yellow vein mosaic virus* (YVMV) and *Enation leaf curl virus* (ELCV) caused by *Begomovirus* (family Geminiviridae), which severely damage the crop. Because of favourable conditions prevailing in the coastal region, the losses in Kerala state are 60-100 per cent, depending upon the stage of plant growth and the severity of virus infection (Venkataravanappa *et al*., 2014; Sanwal *et al*., 2014; Khaskheli *et al*., 2017). In India, Yellow vein mosaic disease (YVMD) was first reported by Kulkarni (1924), which was subsequently named *Yellow vein mosaic of okra* by Uppal *et al*. (1940). YVMV is

characterised by the yellowing of veins, chlorosis, smaller leaves and fruits and stunted growth of the plants (Venkataravanappa *et al.*, 2012a). The severity of the disease depends on location and environment. Incidence is higher in north India during rainy season but in central and south India it is during summer (Deshmukh *et al*., 2011). For resistance breeding, suitable source of resistance is necessary (Dhankhar *et al*., 2005). Since the virus is emerging with new strains (Venkataravanappa *et al*., 2012b), even the varieties released for YVMV resistance have become gradually susceptible. Once the disease symptoms appear in the plants, none of the plant protection measures can save the plant.

RNAi is one of the promising approach in molecular biology against viral diseases. Naturally, RNAi is being harboured by eukaryotic organisms as conserved regulatory mechanism of gene expression. This mechanism plays an important role in plant growth and development as well as antiviral defense mechanism. The mechanism supresses the gene expression at RNA level through dsRNA (double-stranded RNA) produced by gene expression of ihpRNA (hairpin RNA) construct. The dsRNA processed by RISC (RNA-induced silencing complex), interferes with the expression of specific endogenous or viral genes (Castel and Martienssen, 2013).

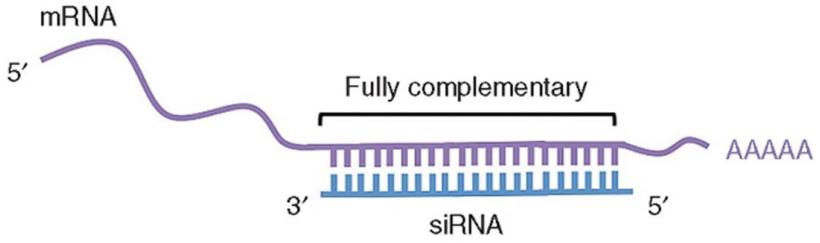
In nature, plants harbour the RNAi mechanism against viruses. Non-Viral Silencing Suppressor Region (NVSR) in the virus genome can manipulate the miRNA pathway and regulate the host plant gene expression, resulting in disease development (Verchot-Lubicz and Carr, 2008).

The viruses coming under *Geminiviridae* family can either have monopartite or bipartite genomic organization. The *Begomovirus* infecting okra, consists of a monopartite genome with small satellite DNA β component, that induces typical symptoms of YVMV (Jose and Usha, 2003). Genome of monopartite *Begomoviruses* resemble that of DNA-A of bipartite *Begomoviruses* but they will have satellite DNA (beta-satellite) associated which is about half the size of *Begomovirus* genome components. DNA-A is responsible for multiple protein production involved in

rolling circle replication, gene transcription, cell-to-cell and long-distance movement, suppression of host gene silencing, and encapsidation of the viral genome (Lazarowitz, 1992). Beta-satellites have been found associated with monopartite *Begomoviruse*s in the Old World. Beta-satellites encode the βC1 protein in the complementary-sense strand, which has important role in symptom induction and suppression of transcriptional and post-transcriptional gene silencing (Akhtar *et al.*, 2017). Success of RNAi is highly dependent on the ihpRNA cassette and method of construct designing. The construct pRNAi-LIC developed by Xu *et al.* (2010) enhanced the efficiency of RNAi mechanism. The ligation of sense and antisense strand in pRNAi-LIC vector, is one step ligation with T4 DNA polymerase. Restriction enzymes are not involved during ligation which increases possibilities of getting ihpRNA cassette with correct insertion.

Transformation in okra has been a challenging task, due to high polyphenolic and mucilage content (Ganesan *et al*., 2007; Kabir *et al.*, 2008; Anisuzzaman *et al*., 2010; Narendran *et al.*, 2013). Different protocols reported for the okra tissue culture (Malella *et al*., 2009; Dhande *et al*., 2012; Irshad *et al*., 2017) were not helpful for the somatic embryogenesis for *Agrobacterium* mediated transformation. In the recent years, *in-planta* mediated *Agrobacterium* transformation is reported, which is more promising in okra (Narendran *et al*., 2013; Manickavasagam *et al*., 2015; Menon *et al*., 2018; Anandan *et al*., 2019).

An attempt to develop okra lines with YVMV resistance through RNAi is made in this study with the objectives of developing the ihpRNA construct targeting *βC1* gene of *Yellow vein mosaic virus*, generating transformants of the okra cv. Salkeerthi harbouring ihpRNA cassette and evaluation of resistance under the controlled conditions. Cultivar Salkeerthi used in the study is a high yielding variety released from Kerala Agricultural University, with characteristics of light green long fruits (27 cm) with average yield 16.2 tonnes per hectare, but is highly susceptible to YVMV.



REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The investigations on 'RNA mediated resistance to *Yellow vein mosaic virus* in okra' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Kerala Agricultural University, Thrissur, with the objective of development of okra lines transformed with RNAi constructs of *βC1* ORF sequence of *Begomovirus* causing yellow vein mosaic disease and to evaluate their silencing potential. A detailed review on the available literature in this line is presented below.

2.1 VIRAL DISEASES OF OKRA

2.1.1 Yellow vein mosaic disease caused by *Yellow vein mosaic virus* (YVMV)

In okra, *Yellow vein mosaic virus* (YVMV) has always been a serious problem, inflicting yield losses to the tune of 20-50 per cent. Loss in yield may increase up to 90 per cent in case of early infection. The virus is classified under *Begomovirus*, which can either have monopartite or bipartite genomic organization. Bipartite *Begomovirus* genomes are made up of two ssDNA molecules of approximately 2.6 kb, includes DNA-A and DNA-B. Both DNA have separate function during the infection (Jose and Usha, 2003). According to Lazarowitz (1992), the genes present on DNA-A are responsible for encapsidation and replication, and the genes present on DNA-B are involved in systemic movement, host range determination, and symptom expression of virus inside host plant.

In monopartite *Begomovirus*, along with DNA-A, some satellite DNA molecules are also reported by Briddon *et al.* (2003) which is known as DNA-b or betasatellites (1,350 bp). These betasatellites depend on DNA-A for encapsidation, replication, and insect borne-transmission (Saunders *et al*., 2000; Rojas *et al*., 2005; Briddon and Stanley, 2006; Brown *et al*., 2012). YVMV is a monopartite *Begomovirus* belonging to *Geminiviridae* family.

2.1.1.1 Symptom of YVMV

Symptoms of this virus are characterized by a homogenous interwoven network of yellow mosaic pattern enclosing islands of green tissue in leaf blades. With higher virus load, infected leaves become yellowish, plants stunted and fruits become small sized with pale yellow colour (Gupta and Paul, 2001; Sanwal, *et al*., 2014; Khaskheli *et al*., 2017). The symptoms are varying according to time of infection, during late rainy season symptoms are lesser compared to the summer season. This is due to the lower vector population in rainy season (Sanwal *et al*., 2016).

2.1.1.2 Transmission of YVMV

Naturally, virus transmission among the plants occurs through the insect vector whitefly (*Bemisia tabaci* Genn.) (Seal *et al*., 2006; Venkataravanappa *et al*., 2014). Sanwal *et al*. (2014) have observed that the female whiteflies transmit the virus more effectively compared to male whiteflies. The study by Chattopadhyay *et al*. (2011) has revealed that the population of whiteflies is increasing from February to August and the highest population is noticed in June and July. Disease occurrence was higher at optimum temperature with bright sunshine and lower relative humidity (Dhankar, 2012; Ali *et al*., 2005).

2.1.1.3 Yield loss due to YVMV

YVMV is a serious disease of okra causing 20 to 80 per cent losses and which may extended upto 100 per cent losses are observed, depending upon the stage of infection. The losses shall be heavier when the infection happens within 35-50 days after sowing (Sanwal *et al*., 2014). Khaskheli *et al*. (2017) observed a significant reduction in the height (48.67 cm) in infected plants compared to healthy plants (62.96 cm). The damage was not restricted to the plant biomass but also affected the fruit quality parameters.

The survey carried out by Venkataravanappa (2008) revealed that the YVMV disease incidence range from 42.45 to 75.64 per cent in Kerala, 23 to 85.64 per cent in Maharashtra, 35.76 to 57 per cent in Uttar Pradesh, 45.89 to 56.78 per cent in Andhra Pradesh, 23.0 to 67.67 per cent in Karnataka, 24.85 to 65.78 per cent in Haryana,

67.78 per cent in Chandigarh, 23 to 75.64 per cent in Tamil Nadu, 45.89 to 66.78 per cent in Rajasthan and 45.45 per cent in Delhi.

2.1.1.4 Management of YVMV

Managing the whiteflies is the way of managing this disease. Whiteflies have a wide range of host plants, due to which it is difficult to control them throughout the crop growth. Few strategies applied for virus control are discussed here.

2.1.1.4.1 Cultural practices

Field cleaning and removal of the infected plants is a common practice for reducing the vector borne spread of the disease.

2.1.1.4.2 Biological control

Plant growth promoting Rhizobacteria (PGPR) can be used to control the population of whiteflies. Genes encoding *chitinase*, *beta-1,3 glucans*, *peroxidase*, *PALase*, and other enzymes are activated by *Rhizobacteria* and hence it reduces the YVMV incidence up to 86.6 per cent by systemic defence mechanism (Patil *et al*., 2011). Fungal bio-control agent *Verticillium lecanii* is also effective to manage the YVMV in okra (Alavo, 2015).

2.1.1.4.3 Management using organic compounds

Patil *et al*. (2011) observed that uses of biological products like Azadirachtin, at the interval of 15 days is effective to control whitefly population up to 79.2 per cent. Application of Crozophera oil at 1.0 ml/L, followed by Palmrosa oil at 1.0 ml/L are effective to control whitefly population and ultimately reduction of disease incidence (Biswas *et al*., 2008). Soap solution (0.5 per cent) and neem oil spray is also used to control YVMV disease in okra (Ansar *et al*., 2014).

Singh *et al*. (2010) tried to reduce YVMV incidence under natural condition, using the root extract of *Boerhaavia diffusa* @ 4 per cent at 7 days interval from seedling stage of plants. Regular spraying is more effective which reduced the YVMV incidence by 80 per cent and reduced the crop losses.

2.1.1.4.4 Chemical control

Imidacloprid, Acetamiprid, and Trizophos are the most suggested chemicals against whiteflies (Gowdar *et al*., 2007). Alam *et al*. (2010) have observed that two sprays of Acetamiprid 20SP @ 40 g ai/ha is effective in reducing the incidence of YVMV. The systemic insecticide Imidachloprid 17.8 per cent, under the category of neonicotinoids, reduces the pest population up to 90.2 per cent (Ansar *et al*., 2014).

2.1.1.4.5 Legal control

The virus infection may occur due to infected planting material, insect vector or common agricultural practices (Dieryck *et al.*, 2009; Fereres and Moreno, 2009). Novy *et al.* (2007) suggested that use of certified planting material is the only way to restrict virus transmission through planting material. Vector control and good agricultural practices are useful to control the virus transmission (Castle *et al.*, 2009; Fereres and Moreno, 2009).

2.1.2 Enation leaf curl disease caused by *Okra enation leaf curl virus* (OELCV)

The incidence of ELCV disease in okra was reported in Karnataka in 1980 (Singh and Dutta, 1986). The same virus got reported from many countries such as Nigeria (Atiri, 1984), Pakistan (Nadeem *et al*., 1997), Saudi Arabia (Ghanem, 2003), China (Lubin *et al*., 2005) and Iran (Bananej *et al*., 2016). This disease is caused by monopartite *Begomovirus* similar to YVMV, but the symptoms are different. Leaf curling, pin-headed enations on leaves and main stem twisting are the main symptoms caused by *Okra enation leaf curl virus* (OELCV) (Singh, 1996; Venkataravanappa *et al*., 2015) and 30-100 per cent losses are recorded (Singh, 1996). Generally, ELCV and YVMV are the major viral diseases of okra in southern part of India (Sohrab *et al*., 2013). Hot weather and no rainfall is favourable environment for increasing population of white fly which is transmitting vector in both viruses (Singh, 1996; Ali *et al*., 2005; Anita and Nandihalli, 2008).

2.2 APPROACHES TO INDUCE RESISTANCE AGAINST VIRAL DISEASE

2.2.1 Breeding in okra for YVMV resistance

Food security is a major goal for developing countries and viral diseases of crop plants is a major constraint in its achievement. The yield losses due to viruses could be reduced by the development of resistant varieties through plant breeding, but okra genetic resources having resistance to YVMV are yet to be reported (Dhankhar *et al*., 2005). Though the wild species *A. manihot*, *A. crinitus*, *A. angulosus*, *A. tetraphyllus* ssp. *tetraphyllus* are reported to harbour the resistance genes and few varieties are bred using them, the genetic distances among the different species are limiting the hybridization success (Singh *et al*., 2007; Lecoq *et al*., 2004). Parbhani Kranti (*A. esculentus* × *A. manihot*) was released as resistant variety (Jambhale and Nerkar, 1986) but lost the resistance after two decades due to mutation in the virus genome. A small change/mutation in the virus genome may result in the emergence of new strains with higher infectivity (Mangrauthia *et al*., 2008; Jones, 2009).

2.2.2 Crop protection based on engineered resistance

The development of resistance in plants may be achieved by introducing the resistance gene against virus or by introducing the resistance gene against the vector (Groen *et al*., 2017). Manipulating the host-pathogen interaction is an effective way to develop resistance in plants (Gilligan and Van den Bosch, 2008; Rodrigues *et al*., 2009).

The entry of virus particles into plant cell by interrupting the plasma membrane is known as inoculation or infection (Rodrigues *et al*., 2009). The effectiveness of a virus inside the host plant is based on its compatibility with the host plant cell, plasmodesmata (PD) and the vascular bundles which have key roles during cell interaction (Taliansky *et al*., 2008). Internal immunity of the host plant is responsible for the trigger of resistance, leading to reduced viral replication (Ascencio-Ibanez *et al*., 2008). The movement of virus inside plant cell is controlled by virus encoded movement protein (MP), through the attachment of MP with the nucleic acid and target (Lucas, 2006).

Ma *et al.* (2004) opined that developing the cultivars with high level resistance is the only way to reduce crop losses due to virus infection. The study of the biochemistry of virus infection by Tenllado *et al*. (2003) has revealed that advanced methods such as RNA silencing have great potential for developing virus resistant plants. The recombinant DNA technologies and standardization of transformation protocols have opened the door for biotechnological approaches for virus resistance in plants (Lapidot and Friedmann, 2002). The transgenic okra lines with shoot and fruit borer resistance have been developed by Narendran *et al*. (2013). Replication protein and Movement protein genes can be promising targets for transgenesis for virus resistance in plants.

2.2.2.1 RNAi

RNA interference is a conserved regulatory mechanism of gene expression observed in eukaryotic organisms (Bartel, 2004). This mechanism involves gene silencing by sequence specific degradation of complementary mRNA which is triggered by dsRNA. On the basis of the stage of gene silencing, RNAi is divided into two categories, post-transcriptional gene silencing (Vaucheret *et al*., 2001; Vaucheret and Fagard, 2001) and transcriptional gene silencing (induced by DNA methylation) (Rountree and Selkar, 1997; Mette *et al*., 2000; Castel and Martienssen, 2013).

Wang and Chekanova (2016) have shown that RNA silencing plays an important role in the cellular mechanisms of eukaryotic organisms. It can control almost all the cellular processes including growth and physiological activities through gene silencing using small noncoding RNAs (sRNAs). RNAi is also associated with the regulation of genome stability, viral infection, epigenetic modification, curbing of transposons and movement and regulation of heterochromatin (Castel and Martienssen, 2013).

Double stranded RNA (dsRNA) act as trigger in gene silencing by RNA-dependent RNA polymerases (RDRPs) of plant-infecting RNA viruses (Ruiz-Ferrer and Voinnet, 2009). The mechanism of RNAi is induced with the incorporation of dsRNA into RNA-induced silencing complex (RISC) containing an Argonaute (AGO)

protein as sRNA-binding domain and an RNaseIII-like enzyme called Dicer having cleavage activity of target sequence (Hamilton and Baulcombe, 1999; Baulcombe, 2004; Vaucheret *et al*., 2004; Krek *et al.*, 2005; Qiu *et al.*, 2007; Ketting, 2011). This mechanism specifically depends on guide RNA (dsRNA) and RISC and the reaction is ATP dependent (Brodersen and Voinnet, 2006; Ghildiyal and Zamore, 2009; Liu and Paroo, 2010). The term "RNAi" was initially coined by Fire *et al.* in 1998, during the studies on gene silencing in nematode worm *Caenorhabditis elegans.* The mechanism was quickly and widely accepted for gene silencing in plants due to its high specificity, accuracy, and heritability (Waterhouse *et al*., 1998; Fire *et al*., 1998; Fire, 1999; Voinnet *et al*., 1999; Waterhouse *et al*., 1999; Baulcombe, 2004).

Napoli *et al*. (1990) have accidentally observed RNAi while attempting to overexpress *Chalcone synthase* (*CHS*) gene in pigmented petunia petals by introducing a chimeric petunia *CHS* gene. Unexpectedly, the introduced gene created a block in anthocyanin biosynthesis, producing totally white flowers and/or patterned flowers with white or pale nonclonal sectors on a wild-type pigmented background. The mechanism responsible for the reversible co-suppression of homologous genes was later identified as RNAi. Both dsRNA which act as a trigger of RNAi and siRNA which is directly involved in cleavage with RISC complex can be employed in RNAi (Hannon and Zamore, 2003).

In earlier studies it was reported that DNA methylation is the inducing factor for gene silencing at transcriptional level (Rountree and Selkar, 1997; Mette *et al*., 2000). RNA induced gene silencing occurs through siRNA and miRNAs (Bartel, 2004; Hamilton *et al.*, 1999). In case of siRNA mechanism, dsRNA originates from single stranded viral intermediate replication and the miRNA is involved in negative regulation of gene expression (Baulcombe, 2004; Bartel, 2004; Meister *et al.*, 2004).

At application level, RNAi is used in various field of agriculture for increasing the nutritional level and protection of crop plants. RNAi has been successfully used in many crops for developing lines with desirable traits such as cotton seeds with high stearic and high oleic acids (Liu *et al.*, 2001; Tang *et al.*, 2005), soybean with increased oil stability at high temperature (Flores *et al.*, 2008), tomato with increased

carotenoid and flavonoid content (Davuluri *et al.,* 2005), corn with high lysine content (Houmard *et al.*, 2007), rapeseed with increasing flowers (Byzova *et al.*, 2004) and rose with blue petals (Katsumoto *et al.,* 2007).

2.2.2.2 RNAi for virus resistance

RNAi is effective against virus gene suppression in plants because it is sequence specific. The effect of siRNA derived from ihpRNA construct targeted to the coat protein gene to generate tomato lines with resistance to *Tomato yellow leaf curl virus* (TYLCV) was shown by Zrachya *et al.* (2007). In few of the transformed tomato plants, disease symptoms were not observed even after seven weeks of inoculation. Transgenic tomato plants were developed by Chen *et al*. (2016) by targeting six segments of monopartite genome of *Tomato leaf curl taiwan virus* (ToLCTWV). The resistance against multiple *Begomovirus* strains in tomato was achieved through RNAi (Chen *et al*., 2016).

Expression of miRNA targeting the *2b* gene of *Cucumber mosaic virus* (CMV) was found to confer effective resistance against CMV in tobacco plants (Qu *et al.* 2007). Resistance to *Zucchini yellow mosaic virus* (ZYMY) in melon was developed through RNAi against *Coat protein* (CP) gene (Wu *et al.,* 2009). Similarly, ZYMV resistant watermelon lines were developed by targeting CP gene. Transgenic melon was developed by Rodriguez-Hernandez *et al*. (2012) by targeting eukaryotic translation initiation factor (TIF) which developed resistance against various viruses such as *Melon necrotic spot virus* (MNSV), *Moroccan watermelon mosaic virus* (MWMV), *Cucumber vein yellowing virus* (CVYV) and *Zucchini yellow mosaic virus* (ZYMV).

Coat protein (CP) gene of *Papaya ringspot virus* type W was targeted for developing PRSV-W resistance in papaya. Virus accumulation was not found in western blotting but siRNA was seen in post-transcriptional gene silencing (Yu *et al.*, 2011). BBTV resistant banana (cv. Rasthali) plants were developed through RNAi strategy (Shekhawat *et al*., 2012). Leksmi *et al*. (2020) develop resistance in banana plants against *Banana bract mosaic virus* by transferring ihpRNA cassette targeting

viral replicase gene.

An RNAi construct was developed by fusions of C1, C3 (encodes proteins responsible for virus replication) and C2 (regulation of gene expression) gene sections. The resistance against *Mungbean yellow mosaic india virus* (MYMIV) in cowpea was developed by targeting *AC2*, *AC4* and combination of *AC2* and *AC4* (*AC2+AC4*) (Kumar *et al*., 2017). Virus resistance was developed in soybean by targeting the conserved region of *AC2* ORF of MYMIV (Ramesh *et al*. 2019).

Translation Initiation Factors TaeIF(iso)4E and TaeIF4G are necessary for the translation process in wheat potyviruses, *Wheat streak mosaic virus* (WSMV) and *Triticum mosaic virus* (TriMV). Transgenic wheat lines with resistance to these viruses were developed through RNA interference targeting these genes (Rupp *et al*., 2019). Through RNAi targeting the *Replicase* gene of wheat viruses WSMV and TriMV, Tatineni *et al*. (2020) have developed the resistant transgenic lines. RNAi was also used to develop *Cotton Leaf Curl* (CLC) virus resistance in tobacco (*Nicotina tabacum*) by targeting the *BC1* region of *Cotton leaf curl multam betasatellites* (CLCuMuB). Resistance was observed in transgenic plants even after 90 days of virus inoculation (Akhtar *et al*., 2017). Hashmi *et al*. (2011) developed CLCuMuB resistance in cotton by targeting truncated *Rep* gene of the virus through RNA interference. RNAi can be used as an efficient tool to develop cotton varieties resistant to CLCuD (Sattar *et al*., 2013).

Few viruses are known to have established their defence mechanism by targeting the RISC complex of the plants (Ding, 2010). Even with all these constraints, RNAi had emerged as a powerful tool against viruses in crop plants and currently the leading strategies employ hairpin RNA expressing vectors and virus induced gene silencing (VIGS) (Burch-Smith *et al*., 2004). Whiteflies are phloem feeders and most of the *Begomovirus* strains are phloem limited. Considering this, RNAi mechanisms aimed at *Begomovirus* resistance in okra should target the genes in the related pathways (Thakur *et al*., 2014; Zaidi *et al*., 2017).

2.3 IMPROVEMENT IN hpRNA CONSTRUCTS

2.3.1 Selection of target gene sequence

The efficiency of RNAi is increased by better accuracy in RNA-protein interaction which is required during siRNA-RISC assembly and activation of RNAi mechanism (Khvorova *et al.,* 2003; Schwarz *et al.,* 2003). Lots of guidelines including G/C content, position of G/C (3, 10, 13 and 19), increased stability with G/C at 3' terminal etc., are made after the analysis of large number of siRNAs (Reynolds *et al.,* 2004). Bioinformatics tools such as GenScript was also developed to find out siRNA for gene silencing which is easier for researcher to find target region. Kumiko *et al.* (2004) have studied 62 targets for two endogenous and four exogenous genes in *Drosophila* cells and based on analysis they formulated some rules for more accuracy and activity of siRNA. The rules include sequence conditions such as presence of G/C at the 5' end of the sense strand and A/U at the 5' end of the antisense 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 have increased the efficiency of siRNA in mammalian cells.

Mubin *et al.* (2011) worked on *Cotton leaf curl multan virus* (CLCuMV) and they noticed that the RNAi mechanism will be more effective if level of identity between target sequence and construct is high. They develop resistance through RNAi by targeting virus-encoded genes such as the *Replication- associated protein* (Rep), *Transcriptional activator protein* (TrAP) and the *Replication enhancer protein* (Ren). Yoshinari *et al.* (2004) have noticed that siRNAs with an A residue at the 19th nucleotide position from the 5′ end of the sense strand showed relatively high gene silencing activities and similar observation were recorded by Schwarz *et al.* (2003), Khvorova *et al.* (2003) and Vickers *et al.* (2003).

During 1998, *Papaya ringspot virus* has heavily damaged the papaya industry in Hawaii. Rainbow variety was susceptible to PRSV isolates from Taiwan and Hainan due to the different localization of viruses. First commercialized transgenic papaya was developed by RNAi targeting PRSV *Coat protein* gene (Tennant *et al.,* 1994; Gonsalves, 1998; Ferreira *et al.,* 2002; Bau *et al.,* 2003; Davis and Ying, 2004; Zhao

et al., 2016). The CP transgenic resistance was dependent on homology to the viral nucleotide sequence (Kung *et al.,* 2015; Jia *et al.,* 2017).

2.3.2 Size of the inverted repeats

Generally preferable size of sense and antisense strands is 200 to 400 bp. Akashi *et al.* (2001) have observed that increasing the size of sense and antisense strands up to 500 bp had no significant effect on *Luciferase* gene expression. Similarly, Helliwell and Waterhouse (2003), while testing 50 to 1000 bp of *Flowering Locus C* and *Phytoene Desaturase* in *Arabidopsis,* have shown that 200 to 400 bp sense and antisense strand is more effective. In *Neurospora crassa albino-1* gene, higher silencing frequency was observed when the length of the target region has been kept at 200-500 bp (Goldoni *et al*., 2004). Heilersig *et al*. (2006) have tried different sizes of target gene from 500-600 bp and 1.1 or 1.3 kb, and observed that 500-600 bp sequence is more efficient than 1.1 or 1.3 kb. From all these reports, it can be concluded that 200-400 bp size of target gene is more effective for gene suppression through RNAi.

2.3.3 Vectors for RNAi

Construction of vector for RNAi mechanism is still difficult and time consuming task because of conventional cloning strategies. Vector construction with restriction digestion cloning includes a number of steps such as sense and antisense strand cloning in vector backbone and after that cloning into binary vector. To overcome this tedious process there is a need to standardize high throughput technology. Xu *et al*. (2010) discovered new strategy for cloning where restriction digestion is not required during sense and antisense cloning. The new strategy was named ‘One-step, zero-background ligation-independent cloning (OZ-LIC)’. They developed the pRNAi-LIC vector is a derivative of binary vector pCAMBIA, specially designed for *Agrobacterium* mediated transformation. Gateway cloning is also widely accepted for RNAi construct designing, which include T-DNA with *Hygromycin phosphotransferase* (*hpt*) and Kanamycin resistance as selectable markers. pHELLSGATE (Helliwell and Waterhouse, 2003) and pANDA (Miki and

Shimamoto, 2004) are plasmid vectors commonly used for the RNAi through Gateway recombination technology (Earley *et al*., 2006; Karimi *et al*., 2007). Plasmids pHANNIBAL, pKANNIBAL, pSAT, pSH, pGEM-WIZ *etc.* are the conventional cloning vectors which are available at Commonwealth Scientific and Industrial Research Organisation (CSIRO) (Wesley *et al*., 2001; Helliwell and Waterhouse, 2005; Bao and Cagan, 2006; Hirai *et al*., 2007; Yelin *et al*., 2007). In all these RNAi vectors, target region will be isolated by PCR amplification with primers which are gene specific as well as having restriction site overhangs. These primers are very useful to increase the efficiency of RNAi construct designing.

OZ-LIC technique developed by Xu *et al*. (2010) solves the limitations of the earlier recombination techniques. This technique needs only one restriction to open the backbone of construct. In this technique, two PCR products ligated with dATP and open vector backbone ligated with dTTP are mixed together to form construct. OZ-LIC method uses gene-specific primers with shorter adaptors (LIC adaptors LIC1 ⁄ LIC2 are 14 nt) whereas GATEWAY uses longer adaptors (attB1 ⁄ attB2 are 29 nt). In OZ-LIC method, one universal primer set can be used to amplify the PCR Product 2 of any target genes.

2.3.4 Use of spacers or introns

Inverted repeats are important to form loop like structure in RNAi vector. Most of the plasmids cannot tolerate the inverted repeats and in such cases spacers or introns play important role in stability (Smith *et al*., 2000). In RNAi mechanism, intron does not have any direct role, because intron will be removed or separated after splicing with the DICER. The silencing efficiency of RNAi was enhanced by keeping spacer or intron in between the sense and antisense inserts of the target gene, making a hairpin loop. Introns also increase the stability of inverted repeats. The process of intron excision from the construct by the spliceosome might help to align the complementary arms of the hairpin under an environment that favours the RNA hybridization and promoting the duplex formation. 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) observed that PTGS efficiency of hairpin RNA is almost 100 per cent when transgene construct contains spliceable intron. In case of PVY-resistant tobacco plants, only 58 per cent resistance was observed when non-spliceable intron has been used but the efficiency increased up to 96 per cent when spliceable intron was used. In transgenic tobacco plants resistance to *Cucumber mosaic virus* was enhanced with the use of splicable intron (Kalantidis *et al.,* 2002; Chen *et al.,* 2004).

While developing transgenic lines against *ω-3 fatty acid desaturase* (*NtFAD3*) gene which is responsible for α-linolenic acid production of root membrane lipids, Sayaka *et al*. (2007) have observed that the spacer sequence is more important as compared to the spacer size. Antibiotic resistance gene inside spacer or intron can dramatically increase the efficiency of getting true transformed plants (Conley *et al*., 1986; Wesley *et al*., 2001).

2.3.5 Promoters

To increase the RNAi efficiency, continuous high level siRNA production can be achieved with the strong promoter such as *Cauliflower mosaic virus* 35S promoter (p35S) (Gavilano *et al*., 2006; Rutherford *et al*., 2004; Sin *et al*., 2006). Choice of promoters mainly depend on the plant (dicots or monocots). Tissue specific promoters such as soybean lectin promoter (Liu *et al*., 2002), Chrysanthemum rbcS1 (Outchkourov *et al*., 2003), *Arabidopsis* rbcS promoter (Rohr *et al*., 2004; Li *et al*., 2008) and rice ubiquitin promoter (Miki *et al*., 2005; Travella *et al*., 2006) are frequently used in RNAi cassette.

Lo *et al*. (2005) studied the effect of ethanol inducible transcriptional regulator using alcA promoter in RNAi construct. On the treatment with ethanol, TF AlcR binds to the alcA promoter and the transcription of the downstream RNAi sequences is activated. The pHELLSGATE vector with tissue specific promoter such as pOp6 is shown to increase the RNAi efficiency at a particular tissue throughout the plant (Craft *et al*., 2005; Wielopolska *et al*., 2005). Guo *et al*. (2003) studied continuous

expression of transcription factor XVE which, after binding with estrogen, activates the transcription of *Cre recombinase* gene. *Cre recombinase* is the enzyme which cleaves the fragment that blocks transcription of RNAi cassette.

2.4 ISOLATION OF TARGET GENE OF VIRUS

The overall success of RNAi is dependent on the target gene selected. In many projects, the *Replicase* gene was targeted for making resistance against viruses. Target gene selection and designing of ihpRNA cassette require sequence information of virus genes (Rhodes *et al.,* 2002; Llave *et al.*, 2002; Jones-Rhodes and Bartel, 2004).

Geminivirus with ssDNA genome replicate through rolling circle mechanism and forms dsRNA structure, by which they induce RNA silencing in plants. Vector with specific viral genes can be used to induce RNA silencing or PTGS in many plants (Covey *et al*., 1997).

Elmer *et al*. (1988) reported that the replication and encapsidation of a bipartite virus is carried out by DNA-A which has five ORFs (*Rep, TrAp, REn, AC4* and *CP*) and movement of virus in the host is carried out by DNA-B which has two ORFs (*MP* and *NSP*). In a monopartite virus, the *βC1* ORF in beta-satellite DNA (β DNA) is responsible for countering host defence response and symptom development in host plant (Saunders *et al*., 2000; Briddon *et al*., 2001; Jose and Usha, 2003; Saeed *et al*., 2005; Gaur and Rathore, 2009).

2.4.1 DNA isolation

The success of DNA isolation depends on the plant cellular structure. Malvaceous plants have high mucilage which negatively influence the DNA quality (Bayer *et al*., 1999). Secondary metabolites in the mucilage, the phenolics and alkaloids, are major factors reducing the DNA quality. These components co-precipitate with DNA and interfere with the downstream processes such as restriction digestion, thermal cycling (Fang *et al*., 1992).

Ghosh *et al*. (2009) have slightly modified the protocol of DNA isolation for reducing mucilage content. Use of extra volume of extraction buffer and dissolving

the crude pellet in 1M NaCl was found to reduce the mucilage content drastically. In the final precipitation with isopropanol, large yield of good quality DNA was found. The role of NaCl in reducing the viscosity of mucilage contents was earlier reported by Chen and Chen (2004). Higher levels of phenol-binding reagent (PVP) and NaCl were shown to reduce the polyphenols and polysaccharides in DNA. Similarly, glucose has reduced the contamination and browning of DNA pellet (Ali *et al*., 2019). Potassium acetate was used by Greco *et al.* (2014) to reduce the concentrations of polysaccharides (which are precipitated as potassium salts) during DNA isolation from *Ectocarpus siliculosus*.

2.4.2 Isolation of gene using PCR

The easiest method for virus target gene isolation is thermal cycling. Jose and Usha (2003) have isolated DNA from YVMV infected okra, 1.35 kbp DNA was amplified using non-overlapping PCR primers (Beta1.F and Beta1.R) and cloned into pGEM-T (pBeta1). The association of DNA-β with symptoms of YVMV was confirmed with southern blotting. To amplify DNA-β, forward primer (BYCRH.F) and reverse primer (BYCRH.R) were designed on the intergenic region of nucleotide sequence of the YVMV.

The abnormal pathogenicity expressions in *βC1* transgenic tobacco lines against *Ageratum yellow vein virus* (Saunders *et al*., 2004), *Tomato yellow leaf curl china virus* (Cui *et al*., 2004) and *Cotton Leaf Curl Multan Virus* (Saeed *et al*., 2005) suggested that this ORF encodes an essential pathogenicity protein.

2.5 ihpRNA VECTOR CONSTRUCTION

The generation of ihpRNA construct can be done through restriction digestion based and ligation based methods. In the earlier RNAi projects, plasmid vectors such as pHANNIBAL and pKANNIBAL were used to develop ihpRNA construct.

Chen *et al*. (2009) used PCR based technology to prepare the ihpRNAi construct and they concluded that this method is faster, accurate and cost effective compared to the GATEWAY method. The ihpRNA cassette was further transferred into the plant transformation vector with TA cloning. In PCR based ihpRNA cassette

preparation, accuracy can be increased by selecting the target region with more GC content.

Golden Gate (GG) cloning is used for making RNAi constructs with multiple DNA fragments in a single ligation reaction (Engler *et al*., 2008; Engler *et al*., 2009). Yan *et al*. (2012) have developed a Golden gate cloning based technique for quick preparation of ihpRNA construct. In this technique, PCR product containing the gene of interest flanked with *Bsa*I recognition sequence and Pdk intron were ligated to the vector backbone (pRNAi-GG). This method is suggested to be advantageous over the traditional ligase methods.

Xu *et al*. (2010) used the ligation independent cloning technique for making of ihpRNA cassette against both exogenous and endogenous genes of *Nicotiana benthamiana*. In this experiment target region was amplified with the primer with adapters and PCR product used as template for next PCR with universal primers. Products from first and second PCRs were ligated with *Sma*I digested vector backbone in the presence of T4 DNA ligase.

2.5.1 ihpRNA vector cloning into *E. coli*

Initially, RecA-independent λ phage- and Rac prophage-based systems (λ Red and RecET, respectively) were used in the cloning experiments. DH5α strain of *E. coli* is more advantageous over *S. cerevisiae* with its faster growth rate, higher plasmid yield and better stability during cloning. Hence, *E. coli* is commonly used in cloning experiments (Zhang *et al*., 1998; Yu *et al*., 2000; Thomason *et al*., 2014).

Three important regions of *E. coli* plasmids are origin of replication, antibiotic resistance gene and the region where DNA can be inserted with no interference in plasmid replication. When plasmid is incubating with the competent cells of *E. coli*, single copy of the plasmid will be taken up by few cells. This can be screened on the media containing specific antibiotic. Transformed colonies may be re-streaked on fresh plates with antibiotics and confirmed with colony PCR (Maniatis *et al.*, 1982).

2.5.2 Transfer of ihpRNA vector to *A. tumefaciens*

Agrobacterium is a well-known and commonly used bacterium for the transfer of foreign gene into plants. The plasmid transformation into agrobacterium is easier compared to the transformation to *E. coli.* Three methods suggested to transfer the plasmid into *A. tumefaciens* are electroporation, freeze and thaw and triparental mating (in case of mobile plasmids) (Wise *et al*., 2006).

The freeze and thaw method (Gynheung *et al*., 1988) was widely used for the transformation of binary vectors into *Agrobacterium* strains. In this method, fresh colony of *Agrobacterium* grown in YEM broth containing Rifampicin (25 mgL^{-1}) as antibiotic and incubated until OD reaches to 0.5 at 600 nm. Centrifuge at 5000 rpm for 5 min., suspend the pellet in 1.0 mL of 100 mM $MgCl_2$ and incubate on ice for 5-10 min. Centrifuge the tubes at 5000 rpm for 5 min, re-suspend the pellet in 1.0 mL of 20 mM $CaCl_2$ and incubate on ice for 5 min. Add 100 ng plasmid to 100 µL $CaCl_2$ and immediately incubate the tubes in liquid nitrogen for 5 min. Add 1.0 mL YEM broth to the tubes, incubate at 37ºC for 1-2 h. and plate on YEM medium containing appropriate antibiotics. Similar protocol was followed by Tripathi *et al*. (2005), Jyothishwaran *et al*., (2007), Krubphachaya *et al*. (2007), Sreeramanan *et al*. (2010), Lekshmi (2016), Jadhav (2019), and Ekatpure (2020).

2.6 *IN VITRO* CULTURE IN OKRA

Tissue culture in okra plants was first reported by Mangat and Roy (1986) and in the past three decades, many studies have been carried out in different areas of *in vitro* culture of this crop. Few studies are on the selection of explants because okra is known for more phenolic compounds and recalcitrant nature which are the constraints in its tissue culture (Kabir *et al*., 2008; Anisuzzaman *et al*., 2010; Narendran *et al*., 2013). Anisuzzaman *et al*. (2010) have established a protocol for the large-scale production of okra tissue culture plantlets. The surface sterilised seeds of okra were sown on MS medium with no hormones. Shoot tips (0.3-0.5 cm) of three-week old plants were surface sterilized and inoculated on paper bridge in MS liquid medium enriched with 1.0 mgL^{-1} BAP. Established meristems were transferred to semisolid

MS medium with 1.0 mgL^{-1} BAP, which gave maximum shooting. Faster multiplication was observed when the node explants were grown on medium with 1.0 mgL^{-1} BAP and 0.5 mgL^{-1} GA_3. The rooting was done on MS solid medium with 1 mgL^{-1} IBA and more than 75 per cent plants were successfully hardened in the hardening medium containing soil, sand and FYM (1:1:1).

Mallela *et al*. (2009) have developed organogenic callus on MS medium supplemented with low concentrations of TDZ, NAA and 2,4-D. Shooting from the calli derived from leaves and cotyledons was obtained on MS medium with BAP (1.0 mgL^{-1}), NAA (0.5 mgL^{-1}) and TDZ (0.04 mgL^{-1}). Better rooting was obtained with NAA (1.0 mgL^{-1}) or IAA (1.0 mgL^{-1}).

Dhande *et al*. (2012) were isolated the shoot tips from nine-day old okra seedlings and cultured on MS medium with IBA (1 mgL^{-1}) and NAA (0.5 mgL^{-1}) had shown maximum regeneration response. Maximum elongation of the shoots was obtained with kinetin (0.5 mgL^{-1}) and best rooting was in medium with IBA (0.5 mgL^{-1}) and 1.0 g activated charcoal.

Irshad *et al*. (2017) have experimented the effect of anti-browning agents on *in vitro* regeneration of okra. For callus induction from hypocotyl and cotyledon explants, MS medium with activated charcoal AC (200 mgL^{-1}), citric acid CA (10 mgL^{-1}) and ascorbic acid AA (10 mgL^{-1}) was best by controlling the phenolic secretion. MS with 2,4-D (0.5 mgL^{-1}) and BA (1.5 mgL^{-1}) yielded best callusing from hypocotyl whereas MS with NAA (1.5 mgL^{-1}) and BAP (0.5 mgL^{-1}) gave best callusing from cotyledon explants. Maximum shooting was on MS with BAP (2 mgL^{-1}) and IBA (0.1 mgL^{-1}) whereas best rooting was on MS supplemented with IBA (2 mgL^{-1}) and AC (200 mgL^{-1}).

MS supplemented with TDZ (0.044 μM) has given 64 per cent shooting within 8.4 days with up to 6.8 shoots per explants (Kabir *et al*., 2016). Up to 83.3 per cent shoots have rooted on half MS medium containing IBA (2.46 μM), after 9.7 days.

Okra somatic embryos have been developed on MS liquid medium supplemented with B5 vitamins, 2,4-D (2.0 mgL^{-1}) and kinetin (1.0 mgL^{-1}) (Ganesan

et al., 2007). Embryos (67.30%) were regenerated into plantlets on MS solid medium with B5 vitamins, BAP (2.0 mgL^{-1}) and GA_3 (1.0 mgL^{-1}).

MS medium with different levels of auxin and cytokinin were used for the plant regeneration through embryogenic calli derived from hypocotyl and cotyledonary explants (Rahman *et al*., 2008). The MS media supplemented with 2,4-D (1.5 mgL^{-1}) + BAP (0.1 mgL^{-1}) showed highest response (82.6%) in the callus induction from cotyledonary explant, whereas MS media supplemented with BAP (2.0 mgL^{-1}) + IAA (0.1 mgL^{-1}) showed better response (72.1%) in the shoot induction from hypocotyl calli. Maximum rooting was observed on half strength MS medium with IBA (0.1 mgL^{-1}). Nearly 70 per cent plants survived after hardening in the greenhouse.

Rizwan *et al*. (2018) standardised the protocol for the regeneration of okra using silver nitrate ($AgNO_3$) in the culture media. During the experiment they found that MS medium with BAP (2.0 mgL^{-1}) showed highest (75%) shoot bud regeneration. The highest (85%) shoot multiplication and proliferation was observed in the MS media with BAP (1.5 mgL^{-1}), KIN (1.5 mgL^{-1}) and $AgNO_3$ (3.0 mgL^{-1}), whereas 53 per cent shoot multiplication observed in medium with no $AgNO_3$. Nearly 83 per cent rooting was observed on MS based rooting medium containing IBA (1 mgL^{-1}) and activated charcoal (200 mgL^{-1}).

2.7 PLANT GENETIC TRANSFORMATION IN OKRA

Transformation of any foreign gene to okra plant is reported to be difficult due to its *in vitro* recalcitrant nature. Even though many reports exist (as discussed under 2.10) on *in vitro* regeneration of okra, its regeneration through callus is still a great challenge (Ganesan *et al*., 2007; Mallela *et al*., 2009). Among the several methods of genetic transformation, *Agrobacterium* mediated transformation is reported successful in okra.

2.7.1 *Agrobacterium* mediated transformation

Agrobacterium mediated plant transformation requires two distinct gene harbouring regions in the plasmid, T-DNA region and virulence region made up of about seven different genes (*virA, virB, virC, virD, virE, virF*, and *virG*). All *vir* genes

are controlled by *virA* and *virB*, while remaining *vir* genes (*virC, virD, virE, virF, and virG*) help the transformation and integration of T-DNA into the host plant (Hwang *et al*., 2017).

2.7.1.1 *Transformation through embryogenic callus of okra*

Okra callus was transformed by co-cultivation with *Agrobacterium* carrying plasmid pBI121 which harboured selectable marker *npt*II (neomycin phosphotransferase) (Mallela *et al*., 2009). Transformed calli were proliferated on MS media containing growth regulators and Kanamycin (50 mgL^{-1}). Cefatoxime (300 mgL^{-1}) was used to supress the excess growth of *Agrobacterium* and proliferated callus was transferred on shooting and rooting media subsequently. The GUS (β-glucuronidase) expression was observed in around 50-60 per cent plants.

2.7.1.2 *In planta transformation*

In planta transformation is an easier strategy for *in vitro* recalcitrant plants. Embryos and the shoot buds which are just emerging from the plumule are shown to be the best for agro-inoculation (Narendran *et al*., 2013). Transformed plants were inoculated on shoot bud regeneration medium to obtain multiple shoots from the plumule. Binary vector pCAMBIA 1301-bar was transferred to okra through *in planta* transformation using *Agrobacterium* strain EHA 105 (Manickavasagam *et al*., 2015). Okra seed were initially sonicated for 30 min. and then vacuum infiltrated for 3 min. with *Agrobacterium* suspension containing 100 µM acetosyringone. The transformed plants were co-cultivated on medium containing acetosyringone (100 µM) and transferred to shooting and rooting media.

Okra seeds were surface sterilized and soaked overnight in orbital shaker (100 rpm). Apical meristematic region of the embryo plumules were pricked with syringe needle and immediately immersed in the *Agrobacterium* suspension for 60 min. at room temperature. Seeds were thoroughly washed with sterile water and grown in potting mixture (3:1 ratio of soil and FYM). Plants were maintained in the greenhouse and transformation confirmed with PCR (Menon *et al*., 2018).

In another study, *Agrobacterium* strain LBA4404 with binary vector pBinAR carrying *cry3a* was co-cultivated with sonicated and needle pricked sprouted seeds of okra in liquid media containing acetosyringone (100 µM). Seeds were incubated in shooting and rooting MS media supplemented with growth regulators. PCR analysis had shown that 12.5 per cent plants are transformed (Anandan *et al*., 2019).

2.8 CONFIRMATION OF TRANSFORMATION WITH ihpRNA CASSETTE

Cui *et al*. (2004) amplified *C1* gene from transgenic plants of *N. benthamiana* and *N. tabacum*. The sequence results revealed that all the plants contain unaltered sequence of *C1* gene. Narendran *et al*. (2013) have screened the transgenic plants with ELISA followed by PCR. PCR results confirmed that all the plants positive for ELISA contain both *npt*II and *cry*1Ac gene at the expected size.

Sahu *et al*. (2014) have PCR analysed the transgenic plants of *N. benthamiana* with *βC1* gene (750 bp). Bands were obtained in all the transgenic lines whereas it was missing in wild types. Similarly, Manickavasagam *et al*. (2015) have evaluated the transformed lines of okra to confirm the integration *bar* gene (462 bp). All the transformed plants have developed marker at 462 bp whereas it was absent in control. In a parallel study, Menon *et al*. (2018) performed PCR amplification of *Cry1Ac* gene to screen the transgenic lines. Of the 1036 plants, one plant each from Pusa Sawani and Punjab Padmini were positive, with the marker generated at 927 bp. Plasmid isolated from *Agrobacterium* was used as positive control and non-transformed plants as negative control.

Anandan *et al*. (2019) have performed PCR to screen the plants transformed with *cry3a* gene. DNA was isolated from 25-day-old transformed plants and PCR was performed using *cry3a* gene specific primers. On an average 10 per cent plants were found transformed.

2.9 CONFIRMATION OF TRANSFORMANTS FOR SYNTHESIS OF siRNAs

Cui *et al*. (2004) have studied the expression of *C1* transgene in the transformed plants of *N. benthamiana* and *N. tabacum*. Total RNA was isolated and hybridized with radiolabelled *C1* gene probe, and the probe hybridized with 380 bp transcript of

N. benthamiana and *N. tabacum*. The results have shown that there is strong relationship between the severity of virus like infection and *C1* gene expression, with almost 10 times greater expression in transformed lines compared to control lines.

Narendran *et al*. (2013) have carried out southern blot analysis of transgenic line with *Cry1Ac* gene. Total DNA was digested with *Hind*III, which has cut the T-DNA at one place. Fragment of *Cry1Ac* gene (1357 bp) was labelled using DIG and used as probe. Result of the hybridization has shown that the transgenic plants contain single or multiple copies of transgene integration and no copies in non-transgenic lines.

Manickavasagam *et al*. (2015) have used southern blot analysis to confirm the integration and copy number of *bar* gene in the genomic DNA of positively transformed okra plants. The T-DNA region was digested with *Eco*RI enzyme and hybridized with gene specific probe. Results have indicated that there are 2-3 *bar* gene copies integrated into the genome of transformed lines.

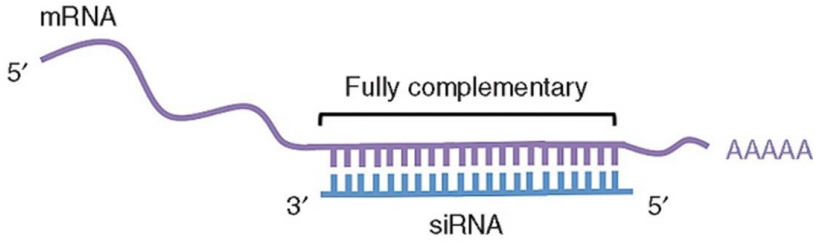
Thu *et al*. (2016) have produced transgenic lines of tobacco by inserting RNAi vector pK7GW-CPi for coat protein gene against *Soybean mosaic virus* (SMV) and *Bean Yellow mosaic virus* (BYMV). qRT-PCR was used to evaluate the silencing potential of transgenic lines. Around 1.84 times higher copy number was observed in wild types as compare with transgenic lines, showing that RNAi is activated in the transgenic lines.

2.10 EVALUATION OF RESISTANCE IN TRANSFORMANTS

Sharma (2020) produced transgenic lines of two hairpin RNAi constructs harbouring overlapping regions of AC1/AC2 and AC1/AC4 genes of DNA-A genome of *Begomovirus*. Transgenic lines were screened in T_1 generation to observe silencing potential. The plants were kept in containment facility along with the YVMV infected plants and viruliferous whiteflies were released into containment facility. Observations were collected after 4 weeks of infestation and revealed that about 90 per cent resistance shown by transgenic lines. Some transgenic lines also shown symptoms of YVMV but there was no effect on plant yield.

2.11 CONSTRAINTS IN RNAi MEDIATED SUPPRESSION OF VIRUS

Virus evolution is the major constraint for RNAi, which may be resolved by designing the construct that is effective against a spectrum of strains. The multiple target sites must be good enough so that transgenic lines can show resistance for more days. When the viral strains differ regionally, there is need to identify common genes for designing of RNAi cassettes. When the virus is capable to produce suppressor proteins against RNAi, cassette should carry the ligand genes against suppressor proteins.



MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "RNA mediated resistance to *Yellow vein mosaic virus* in okra" carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Kerala Agricultural University, Thrissur, during September 2017 to May 2021. 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

Healthy seeds of okra (cv. Salkeerthi) were procured from Department of Vegetable Science, College of Agriculture, KAU, Thrissur. The seeds were then sterilized using combinations of three treatments including Carbendazim (0.5, 1.0 and 1.5% for 10 min.) followed by mercuric chloride (0.1% for 10 min) and ethanol (70%). The seeds were rinsed with autoclaved distilled water after each treatment. Sterilized seeds were soaked overnight in distilled water, and later incubated in test tubes containing moist cotton, for 6-8 days.

3.1.2 Culture medium

3.1.2.1 *Chemicals*

All the chemicals, antibiotics and growth regulators used for somatic embryogenesis were analytical grade, procured from Sisco Research Laboratories (SRL) and HIMEDIA. Plasmid isolation and PCR clean-up gel extraction kit was procured from Macherey and Nagel. Taq DNA polymerase, dNTPs, Taq buffer A, and molecular grade water were procured from GeNei Pvt. Ltd. T4 DNA polymerase, dATP, dTTP, restriction enzymes and competent cell preparation kit were procured from Thermo Scientific Pvt. Ltd. De-salted oligonucliotide primers were procured from Sigma Pvt. Ltd.

3.1.2.2 *Glassware, plastic ware and other materials*

Glassware required for the media preparation and storage of stock solutions were procured from Borosil Pvt. Ltd. Microcentrifuge tube, microtips, screw capped tubes (DNase, RNase Protease free) were procured from Tarson Pvt. Ltd.

3.1.2.3 *Composition of the medium for in vitro cultures*

Basal MS medium (Murashige and Skoog, 1962) supplemented with growth regulators (BAP, IAA, NAA, 2,4-D, Kinetin) and anti-browning agents (ascorbic acid, activated charcoal) was used for the development of somatic embryos (Appendix I).

3.1.2.4 *Preparation of medium*

Standard MS medium was used with different combinations of growth regulators for callus initiation (Table 3.1) and embryogenesis (Table 3.2).

Table 3.1 Composition of media for callus initiation

Treatment	Media composition
Control	MS (Without growth regulators)
CIM 1	MS + BAP (1 mgL^{-1})
CIM 2	MS + BAP (2 mgL^{-1})
CIM 3	MS + BAP (3 mgL^{-1})
CIM 4	MS + BAP (1 mgL^{-1}) + 2,4-D (1 mgL^{-1})
CIM 5	MS + BAP (2 mgL^{-1}) + 2,4-D (1 mgL^{-1})
CIM 6	MS + BAP (3 mgL^{-1}) + 2,4-D (1 mgL^{-1})
CIM 7	MS + BAP (1 mgL^{-1}) + IAA (1 mgL^{-1})
CIM 8	MS + BAP (2 mgL^{-1}) + IAA (1 mgL^{-1})
CIM 9	MS + BAP (3 mgL^{-1}) + IAA (1 mgL^{-1})

Table 3.2 Composition of the media used for induction of callus maturation and somatic embryogenesis

Treatment	Media composition
Control	MS (Without growth regulators)
CEM 1	MS + BAP (1 mgL^{-1})
CEM 2	MS + BAP (2 mgL^{-1})
CEM 3	MS + BAP (3 mgL^{-1})
CEM 4	MS + BAP (1 mgL^{-1}) + NAA (0.1 mgL^{-1})
CEM 5	MS + BAP (2 mgL^{-1}) + NAA (0.1 mgL^{-1})
CEM 6	MS + BAP (3 mgL^{-1}) + NAA (0.1 mgL^{-1})
CEM 7	MS + Kinetin (1 mgL^{-1})
CEM 8	MS + Kinetin (2 mgL^{-1})
CEM 9	MS + Kinetin (3 mgL^{-1})

The pH of the medium was adjusted to 5.8 using 0.1N NaOH/ HCl. Agar was used as solidifying agent at the rate of 8 gL^{-1} and the medium was heated to melt the agar. It was then dispensed into clean culture bottles (50 mL/bottle), autoclaved at a pressure of 15 psi at 121ºC for 15 min and the autoclaved medium was stored at 25±2°C under sterile conditions.

3.1.3 Somatic embryogenesis from callus

3.1.3.1 *Callus initiation*

Hypocotyl explants were excised from 6-8 days old germinated seeds. These were further cut into 0.5-1.0 cm pieces using sterile scalpel and inoculated in MS media containing growth regulators (Table 3.1). Culture was incubated at 25±2ºC in dark for 6-10 days. Light period (16 h) was provided as soon as the callus initiation started and incubated at 25±2ºC in light (16 h) up to 21 days.

3.1.3.2 *Embryogenesis through callus*

The callus was transferred to Callus Embryogenesis Media (CEM 1-9) (Table 3.2), incubated at 25±2ºC in light (16 h) and subcultured on same media after 21 days.

3.2 ISOLATION OF TARGET GENE (*βC1* GENE OF YVMV)

3.2.1 DNA isolation from YVMV infected plant

DNA was isolated from YVMV infected plant using CTAB method (Ghosh *et al*., 2009) with modifications. Procedure for DNA isolation is given below.

Reagents used

Liquid nitrogen, CTAB Buffer (2%), Polyvinylpyrilidone (PVP) (3%), EDTA (20 mM), Tris Buffer (100 mM), β- mercaptoethanol, Chloroform, Isoamyl alcohol, RNaseA (50 µg/mL), Potassium acetate (0.3M), 2-Isopropanol, and Ethanol.

Procedure

- YVMV infected okra leaf (100 mg) was wiped with 100 per cent ethanol and ground in sterilized pre-chilled mortar and pestle with liquid nitrogen.
- Pre-warmed working CTAB buffer (1.5 mL) (Appendix II), PVP (3%) and β-mercaptoethanol (50 µL) were added to the samples, samples transferred to 2 mL centrifuge tubes (microtip was cut for easy sucking of sample) and incubated the tubes at 65ºC for 45 min, with gentle inversions after every 15 min.
- The tubes were centrifuged at 10,000 rpm for 10 min at 4ºC, supernatant was transferred to fresh 2 mL centrifuge tubes, Chloroform : Isoamyl alcohol (24:1) (400 µL) was added and tubes were centrifuged at 10,000 rpm for 10 min at 4ºC.
- Supernatant was transferred to fresh tubes, 2 µL RNaseA (50 µg/mL) was added and incubated at 37ºC for 30 min.
- Sodium chloride (3M) 300 µL and potassium acetate (0.3M) 200 µL was added and incubated in ice for 10 min.
- Chloroform : Isoamyl alcohol (24:1) (400 µL) was added in tubes and tubes were centrifuged at 10,000 rpm for 10 min at 4ºC.
- Supernatant was transferred in fresh 1.5 mL tubes, chilled isopropanol (500 µL) was added to the tubes and tubes were incubated for overnight at -20 ºC.
- Tubes were centrifuged at 10,000 rpm for 10 min at 4ºC, supernatant was

discarded, pellet was washed with 70% ethanol (100 µL), centrifuged tubes at 10,000 rpm for 3 min at 4ºC and pellet was air dried in laminar air flow.

- Dissolved the pellet in 100 µL sterile molecular grade nuclease free water, incubated at 37ºC for 10 min and stored at -20ºC.

(**Modifications:** The sodium chloride concentration was increased up to 3M, 0.3M Potassium acetate was added and twice Chloroform : Isoamyl alcohol treatment was used.)

3.2.2 Agarose gel electrophoresis for DNA

The quality of DNA was checked using horizontal gel electrophoresis. Agarose (0.8 g) was added to 100 mL of 1X TAE buffer (Appendix II) and heated to melt the agarose. Ethidium bromide (0.5 µg/mL) was added to the melted gel when it cools down to 45ºC and after proper mixing, the gel was poured into gel tray containing comb inside caster tray and kept in steady position for 30 min at room temperature. Then, comb was removed gently and gel was placed in gel tank (BioRad, India) containing 1X TAE buffer. DNA (5 µL) mixed with bromophenol blue (2 µL) (20 mg/mL) was loaded into the well. Electrophoresis was carried out at 80 volts until loading dye reached three-fourth of the gel.

3.2.3 Gel Documentation

The electrophoresed gel was documented under UV with (BioRad GelDoc™ XR^+ system using PDQuest™ software).

3.2.4 Quantification of DNA

Quantification was carried out using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) at wavelengths 260 and 280 nm. Initially the reading was set to zero with molecular grade nuclease free water as blank. DNA sample (1.0 µL) was loaded and the quality of the DNA was judged from the ratio of the absorbance values at 260 and 280 nm. A_{260}/A_{280} values between 1.8 and 2.0 indicated good quality DNA. Quantity of DNA (ng/µL) was also recorded.

3.2.5 Amplification of *βC1* ORF sequence of YVMV

Complete sequence of *βC1* gene (Accession no. EF417919.1) was retrieved from GenBank available at National Center for Biotechnology Information (NCBI). Based on the sequence information, larger ORF was found using ORF finder. The *βC1* ORF was amplified using primers designed with Primer3 Input (version 0.4.0).

Name	Sequence (5'-3')	Annealing Temperature
VβC1F	TTCTGCTTTTGAACCCCAGT	58ºC
VβC1R	GCTCATGCAACAACAAAGGA	

Standard PCR components were used to prepare 20.0 µL reaction mixture, containing 100 ng of template DNA (2.0 µL), 200 µM dNTPs (1.0 µL), 10 pM of each primer (1.0 µL), 1 unit of Taq polymerase (0.4 µL), 1x Taq polymerase buffer (2.5 µL) and molecular grade nuclease free water to make volume up to 20.0 µL (All components from GeNei, India). The following conditions were provided for the amplification of the specific region of *βC1* ORF in the Thermal cycler (SureCycler 8800, Agilent Technologies), steps 2 to 4 were repeated 35 times.

Step	Stage	Temperature (ºC)	Duration	
1.	Initial denaturation	94	4 min	
2.	Denaturation	94	30 s	35 Cycles
3.	Annealing	58	30 s	
4.	Extension	72	45 s	
5.	Final extension	72	8 min	
6.	Hold	4	Infinite	

The PCR product was analysed in agarose gel (1.6%) using horizontal gel electrophoresis unit as explained in section 3.2.2 and 3.2.3. First well was loaded with 4 µL of the 100 bp molecular weight marker (Step-up 100 bp ladder, GeNei) and gel was documented as explained in section 3.2.3.

3.2.6 Sequencing of the partial coding sequences of viral *βC1* ORF

The PCR product obtained using the βC1 ORF primers was sequenced through Sanger sequencing.

3.3 ihpRNA VECTOR CONSTRUCTION

3.3.1 Prediction of siRNA in the *βC1* ORF sequence of the virus

Partial sequences of *βC1* encoding genes (partial cds) were subjected to siRNA target prediction using RNAi design tool of Integrated DNA Technologies (IDT). The DICER substrates (siRNA 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). The duplex RNA GC content (30-70%) and the asymmetrical end stability base pair length of 5 were set along with other target parameters detailed in (Appendix III). The targets found were selected and analysed for siRNA attributes for higher efficiency.

3.3.2 Restriction mapping

The partial *βC1* 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.

3.3.3 Viral silencing suppressor prediction

The partial sequences obtained for the *βC1* 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 pRNAi-LIC plasmid isolation (CD3-1285)

Backbone of the construct pRNAi-LIC (Genbank GQ870263, CD3-1285, 12kb) (Fig 3.1) (Appendix IV), a derivative of pCAMBIA2300, was procured (ABRC

Arabidopsis Biological Resource Centre, The Ohio State University) and plasmid was isolated using plasmid DNA purification kit (Macherey-Nagel).

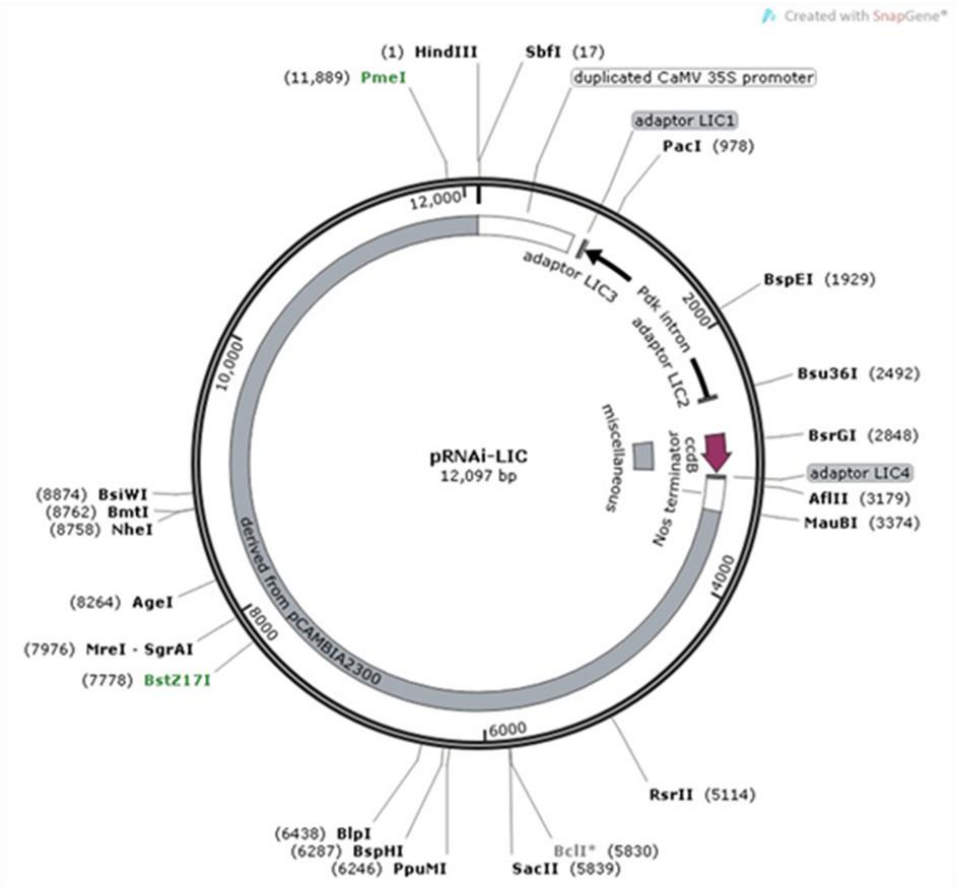


Figure 3.1 Physical map of pRNAi-LIC (CD3-1285)

Requirements

Resuspension buffer A1 (containing RNase), Lysis buffer A2, Neutralization buffer A3, Wash buffer A4 (containing ethanol), and Elution buffer (all provided with Macherey-Nagel DNA purification kit).

Procedure

- The single colony of pRNAi-LIC CD3-1285 (not more than 10 days old) was inoculated in 10 mL of LB broth containing Kanamycin (50 mgL^{-1}), incubated at 37ºC in orbital shaker at 120 rpm for overnight.
- Overnight grown culture (1 mL) was added to 25 mL of LB broth containing

Kanamycin (50 mgL^{-1}) and incubated at 37ºC in orbital shaker at 120 rpm until O.D reaches to 0.3.

- Culture was centrifuged at 11,000 rpm for 1 min, pellet was re-suspended in Buffer A1 (250 µL) and mixed by vortexing.
- Buffer A2 (250 µL) was added, mixed by inverting tubes 8-10 times and incubated at room temperature for 5 min.
- Buffer A3 (300 µL) was added and mixed by inverting tubes until blue colour solution changes to colourless (do not vortex to avoid shearing of plasmid).
- The tubes were centrifuged at 11,000 rpm for 5 min (repeated if clear supernatant was not found), Nucliospin® plasmid column was placed in 2 mL collection tube and supernatant (max. 700 µL) was added into column.
- The tubes centrifuged at 11,000 rpm for 1 min and Buffer A4 (600 µL) was added.
- Silica membrane was dried by spining the tubes at 11,000 rpm for 2 min.
- Silica column was fixed into 1.5 mL microcentrifuge tubes, molecular grade DNase free water (50 µL) was added in the column and incubated for 1 min at room temperature.
- Plasmid DNA was eluted by spinning tubes at 11,000 rpm and stored at -20ºC.

3.3.5 *Sma*I Digestion of pRNAi-LIC (CD3-1285) plasmid vector

Isolated plasmid (pRNAi-LIC CD3-1285) was digested with *Sma*I (Thermo Scientific, India) by incubating at 30 ºC for over 3 hours and to inactivate *Sma*I, incubation was continued at 65ºC for 20 min. The reaction mixture had 30.0 µL (5.0 µg) plasmid, 10 µL 10x Buffer Tango, 5 µL *Sma*I (10U/µL) with final volume made to 100 µL using sterile molecular grade nuclease free water (Fig. 3.2).

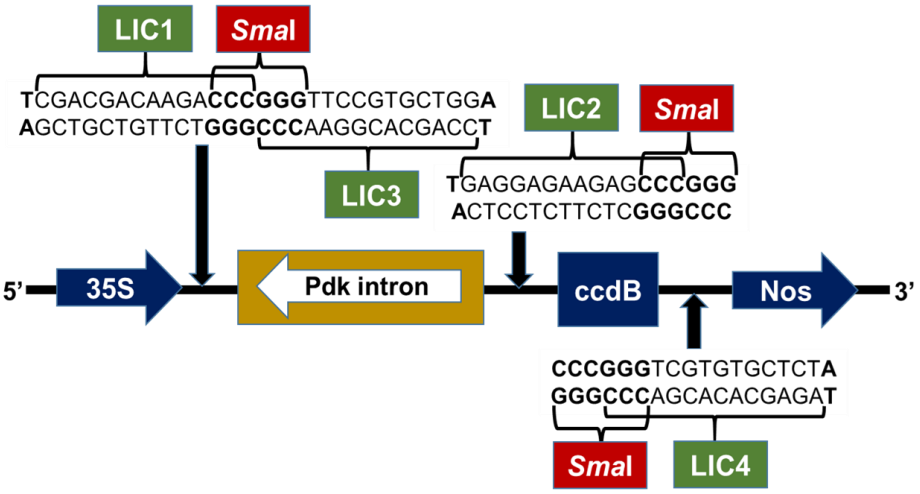


Figure 3.2 Position of *Sma*I and LIC adapters on pRNAi-LIC (CD3-1285)

3.3.6 Preparation of sense strand through PCR

The *βC1* ORF was amplified using the primers specially designed for inserting sense and antisense strands in vector backbone. Sense strand was amplified by using DNA of YVMV infected plant as template for PCR and oligo pair of primer VLIC1 and VLIC2 were used to get PCR product-1 (Sense strand). A standard PCR components and temperature profile was used as mentioned in section 3.2.5.

Name	Sequence (5'-3')	Annealing Temperature
VLIC1	CGACGACAAGACCCTTTCTGCTTTTGAACCCCAGT	58ºC
VLIC2	GAGGAGAAGAGCCCTGCTCATGCAACAACAAAGGA	

(Underlined sequence are adapters to ligate sense strand with pRNAi-LIC backbone, namely LIC1 and LIC2. These adapter sequence in VLIC1 primer was complementary to the 3' region of the plasmid backbone at the first *Sma*I site. Adapter sequence in VLIC2 primer was complementary to the 5' region of the Pdk intron).

The underlined sequence in the primers are the sequences of the primers used for initially amplifying the *βC1* region of YVMV.

3.3.7 Preparation of antisense strand through PCR

Antisense strand was amplified by using PCR product-1 (Sense strand) as template for PCR and oligo pair of primer VLIC3 and VLIC4 to get PCR product-2 (Antisense strand). The LIC3 and LIC4 are adapters to ligate antisense strand with pRNAi-LIC backbone. Standard PCR components and temperature profile were used as mentioned in section 3.2.5.

Name	**Sequence (5'-3')**	**Annealing Temperature**
VLIC3	CCAGCACGGAACCCTTGAGGAGAAGAGCCCT	58ºC
VLIC4	AGAGCACACGACCCTTCGACGACAAGACCCT	

The underlined sequence in VLIC4 primer was similar to the underlined sequence in VLIC1 primer. Similarly, the underlined sequence in VLIC3 was similar to the underlined sequence in VLIC2 primer. Rest of the nucleotides in theVLIC3 primer were complementary to the 3' region of the Pdk intron of the second *Sma*I restriction site in the plasmid backbone and rest of the nucleotides in VLIC4 primers were complementary to the 5' region of the third *Sma*I restriction site in the plasmid backbone.

3.3.8 Elution of the sense and antisense fragments from agarose gel

The sense and antisense fragments were separated in agarose gel (1.4%) and bands were sliced out with a clean scalpel under UV transilluminator. Sliced gel was stored in a pre-weighed fresh 1.5 ml microcentrifuge tube. Gel extractions of the excised bands were carried out using NucleoSpin® PCR Clean-up gel extraction kit (Macherey-Nagel). Eluted PCR product was quantified using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The detailed procedure for the band elution from agarose gel is given below.

Reagents used

Buffer NTI, Buffer NT3, Molecular grade nuclease free water (all supplied with NucleoSpin® PCR Clean-up gel extraction kit).

Procedure

- DNA fragment was excised from agarose gel with scalpel (with minimum amount of agarose), weight (around 150 mg) was taken and transferred gel slice to 1.5 mL tube.
- Buffer NTI (300 µL) was added and the tubes were heated to 40ºC for 5 min to dissolve gel slice completely.
- NucleoSpin® gel and PCR clean-up column (Silica column) was placed in 2 mL collection tube, 700 µL sample was added and tubes were centrifuged at 11,000 rpm for 30 s.
- Flow-through was discarded and silica column was placed back in collection tube, buffer NT3 (700 µL) was added and tubes were centrifuged at 11,000 rpm for 30 s.
- Flow-through was discarded and silica column was placed back in collection tube, column was dried by spinning tubes at 11,000 rpm for 1 min.
- Silica column was placed in fresh autoclaved 1.5 mL microcentrifuge tube, molecular grade nuclease free water (30 µL) was added and tubes were incubated at room temperature for 1 min.
- Tubes were centrifuged tubes at 11,000 rpm for 1 min and eluted DNA was stored at -20ºC.

3.3.9 T4 DNA polymerase treatment of PCR Products

PCR product-1 and PCR product-2 were treated with dATP. The tubes were incubated at 22ºC for 30 min and inactivation was done at 75ºC for 20 min. The details of PCR components are given below.

Sl. No.	Components	Volume (µL)
1.	Purified PCR product (~ 50 ng)	10.0
2.	5X T4 polymerase buffer	4.0
3.	100 mM dATP	1.0
4.	T4 DNA polymerase (5U/µL)	0.4
5.	Molecular grade water	4.6

3.3.10 T4 DNA polymerase treatment of *Sma*I digested pRNAi-LIC

*Sma*I digested pRNAi-LIC vector components were treated with dTTP. The tubes were incubated at 22ºC for 30 min and inactivation done at 75ºC for 20 min. The details of PCR components are given below.

Sl. No.	Components	Volume (µL)
1.	*Sma*I-digested pRNAi-LIC (purified)	14.6
2.	5X T4 polymerase buffer	4.0
3.	100 mM dTTP	1.0
4.	T4 DNA polymerase (5U/µL)	0.4

3.3.11 Cloning of sense and antisense fragments in pRNAi-LIC vector

10 µL (~50 ng) each of dATP treated PCR products 1 and 2 along with 6 µL (~100 ng) of dTTP treated *Sma*I digested vector backbone were incubated at 65ºC for 5 min. in a thermal cycler and then at 22ºC for 15-30 min (Fig. 3.3).

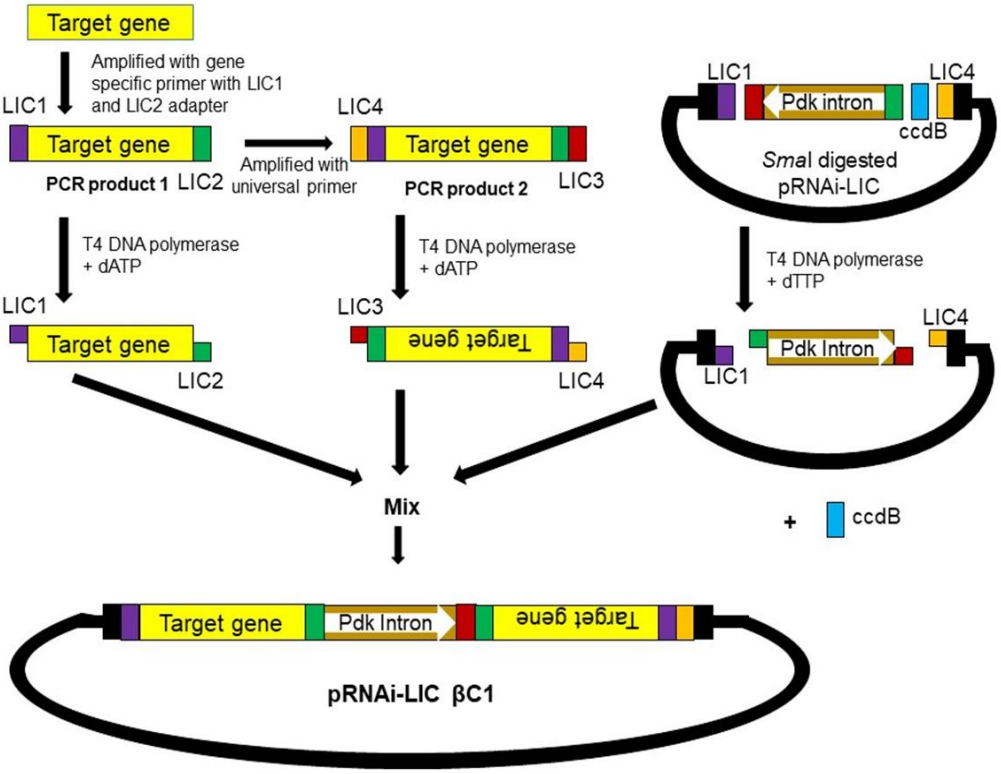


Figure 3.3 Schematic diagram of hairpin RNA (ihpRNA) construction

The target fragment of the gene of interest is PCR amplified using gene-specific primers carrying the adaptors (VLIC1 and VLIC2) to obtain PCR Product 1, which has terminal adapter sequences LIC1 and LIC2. PCR Product 2 is amplified by using PCR Product 1 as template and primers (VLIC3 and VLIC4) and thus has terminal sequences LIC3 and LIC4. PCR Products 1 and 2 are further treated with T4 DNA polymerase in the presence of dATP. pRNAi-LIC vector is digested with *Sma*I and treated with T4 DNA polymerase at the presence of dTTP. The treated vector and two PCR products with sticky ends are mixed and then transformed into *Escherichia coli* cells (DH5α).

3.3.12 pRNAi-LIC vector transformation into DH5α

The competent cells of *E. coli* DH5α were prepared using TransformAid Bacterial Transformation Kit (Thermo Scientific). The protocol used for preparation of competent cells is given below.

Reagents used

C-medium, T-solution (A) and T-solution (B), LB agar, and Kanamycin and Chloramphenicol (all supplied with TransformAid Bacterial Transformation Kit, Thermo Scientific).

Procedure

- The single colony of fresh DH5α (not more than 10 days old) was inoculated in 1 mL of C-medium without any antibiotic, incubated at 37ºC in orbital shaker at 120 rpm for 3h.
- Bacterial cells were pelleted by 1 min centrifugation at 10,000 rpm, supernatant was discarded and pellet was re-suspended in 300 µL of T-solution and incubated on ice for 5 min. [T-solution prepared fresh by mixing equal quantity of T-solution (A) and T-solution (B)]
- Tubes were centrifuged for 1 min at 10,000 rpm, supernatant was discarded and the pellet was re-suspended in 120 µL of T-solution and incubated on ice for 5 min.
- 10 µl of ligation mixture (containing 80-100 ng vector DNA) was added into

new microcentrifuge tubes, incubated on ice for 2 min.

- 50 µL of the prepared competent cells added into tube containing ligation mixture, mixed and incubated on ice for 5 min.
- Immediately 20 µL culture was plated on fresh LB agar plates (prepared 1 h before) containing antibiotics (Kanamycin 50 mgL^{-1} and Chloramphenicol 5 mgL^{-1}) and incubated overnight at 37ºC.

3.3.13 Confirmation of insert by colony PCR and sequencing

The transformed colonies were confirmed by colony PCR using same primer combination (Forward- VLIC1 and reverse- VLIC2) used to perform PCR products 1. PCR components and temperature profile required for amplification are mentioned in section 3.2.4. The positive DH5α cells with the cloned pRNAi-LIC plasmids were grown overnight in LB broth containing Kamamycin (50 mgL^{-1}) and Chloramphenicol (5 mgL^{-1}) for plasmid isolation (as per section 3.3.4). Plasmid sequenced with two reverse primers VLIC5 and VLIC6 [one for sense strand (VLIC5) which is located on intron region and second for antisense strand (VLIC6) which is located on Nos terminator of plasmid backbone (Xu *et al*., 2010)]. Sequences from the sense and antisense strands were aligned to confirm the direction and position of the inserts.

Primer Name	Sequence (5'-3')
VLIC5	TCTTCTTCGTCTTACACATC
VLIC6	AAGACCGGCAACAGGATTC

3.4 TRANSFER OF THE BINARY VECTOR TO *Agrobacterium tumefaciens*

3.4.1 Preparation of competent *A. tumefaciens* cells

Single colony from fresh plate of GV3101 was inoculated in LB broth (50 mL) containing Rifampicin (25 mgL^{-1}) and incubated overnight at 28ºC and 120 rpm. Overnight incubated culture (1 mL) of GV3101 was transferred to autoclaved LB broth (50 mL) containing Rifampicin (25 mgL^{-1}) and maintained at 28ºC and 120 rpm till OD reached 0.3. Then the culture was chilled on ice for 10 min and pelleted by

centrifugation at 3000 rpm for 3 min. The pellet was re-suspended in 10 mL of $MgCl_2$ (100 mM) and kept on ice for 1h. Then the tubes were centrifuged at 3000 rpm for 3 min, pellet was re-suspended in 1 mL of $CaCl_2$ (20 mM) and tubes were incubated on ice for 30 min. Aliquots (100 µL) were made by adding glycerol (20 µL) and stored at -80ºC.

3.4.2 Isolation of plasmid with ihpRNA cassette from *E. coli*

Plasmid (pRNAi-LIC with βC1 gene) was isolated from *E. coli* as mentioned in section 3.3.4.

3.4.3 Transformation of *Agrobacterium tumefaciens*

Stock of aliquots was thawed on ice for 10 min and 10 µL of plasmid (100 ng/ µL) was added. The tubes were frozen in liquid nitrogen for 2-3 min and incubated in water bath at 37ºC for 5 min. LB broth (1 mL) without any antibiotic was added in the tube and incubated at 28ºC for 1 h. at 160 rpm. 10 µL culture was spread on LB agar plate containing Kanamycin (50 mgL^{-1}), Chloramphenicol (5 mgL^{-1}) and Rifampicin (25 mgL^{-1}) and incubated for 20 h. at 28ºC.

3.4.4 Confirmation of transformation

3.4.4.1 *Confirmation through colony PCR*

The transformed colonies were confirmed by colony PCR using same primer combination (VLIC1 and VLIC2) used to perform PCR products 1. PCR components and temperature profile required for amplification are mentioned in section 3.2.4.

3.4.4.2 *Confirmation through Sangers sequencing*

The positive GV3101 cells with the cloned pRNAi-LIC plasmids were grown overnight in LB broth containing Kamamycin (50 mgL^{-1}), Chloramphenicol (5 mgL^{-1}) and Rifampicin (25 mgL^{-1}) for plasmid isolation (as per section 3.3.4).

Sense and antisense strand was sequenced with two reverse primers VLIC5 and VLIC6 (Section 3.3.13). Sequences from the sense and antisense strands were aligned to confirm the direction and position of the inserts.

3.5 *IN PLANTA* TRANSFORMATION OF OKRA

3.5.1 Preparation of *Agrobacterium* suspension for co-cultivation

Single colony of *Agrobacterium* strain GV3101 with binary vector pRNAi-LIC was inoculated in 10 mL LB broth containing Kanamycin (50 mgL^{-1}), Chloramphenicol (5 mgL^{-1}) and Rifampicin (25 mgL^{-1}) and incubated for overnight, at 28ºC and 160 rpm in orbital shaker. Overnight incubated culture (1 mL) of GV3101 was transferred in 50 mL LB broth containing Kanamycin (50 mgL^{-1}), Chloramphenicol (5 mgL^{-1}) and Rifampicin (25 mgL^{-1}) and incubated in orbital shaker until OD 0.3 at 28ºC and 160 rpm. After this, the culture was centrifuged at 5000 rpm for 5 min and the pellet was re-suspended in 100 mL MS liquid medium containing 5 per cent sucrose and acetosyringone (100 µM).

3.5.2 Preparation of plant material and co-cultivation with *Agrobacterium*

Okra cv. Salkeerthi seeds were sterilised with Carbendazim (1% for 10 min), followed by mercuric chloride (0.1% for 10 min) and ethanol (70% for 1 min) and rinsed 2 to 3 times with autoclaved distilled water after every treatment. Sterilized seeds were soaked in autoclaved distilled water for overnight and then incubated on moist tissue paper for 4 h. Well germinated seeds were selected for transformation. Injury was made to the germinated seeds near to plumule with a sterile syringe needle. Prickled seeds were co-cultivated with *Agrobacterium* culture in incubator shaker at 28ºC for 30 min and 100 rpm. Co-cultivated seeds were washed once with Cefotaxime (500 mgL^{-1}) solution and sown in paper cup containing soil and FYM (1:1).

3.6 CONFIRMATION OF TRANSFORMED PLANTS

3.6.1 Isolation of genomic DNA

DNA was isolated from 15-days old plants (protocol for DNA isolation mentioned in section 3.2.1), quantified using NanoDrop spectrophotometer (section 3.3.4) and the quality checked by agarose gel electrophoresis (section 3.3.2 and 3.3.3).

3.6.2 Confirmation by polymerase chain reaction (PCR)

PCR was performed using sense strand specific primers VLIC1 and VLIC5 (VLIC1-5'CGACGACAAGACCCTTTCTGCTTTTGAACCCCAGT-3' and VLIC5-5'-TCTTCTTCGTCTTACACATC-3'; Annealing temperature 58ºC). Housekeeping gene Ubiquitin was also amplified using primer UBQ7 (F-5'-CTCCACCTCGTCCTCCGTCTG-3' and R-5'-CGCCGCACTCCGCATTAAGG-3'; Annealing temperature 58ºC). PCR components and temperature profile required for amplification are mentioned in section 3.2.5.

3.7 CONFIRMATION OF mRNA ENCODING FOR siRNA

3.7.1 RNA isolation

Total RNA was isolated from transgenic plants using Purelink® Plant RNA Purification Reagent (Invitrogen, India) (Sreedevi, 2019). The detailed procedure used for RNA isolation is given below.

Reagents used

Liquid nitrogen, Purelink® Plant RNA Purification Reagent (Invitrogen), DEPC (0.1%), Sodium chloride (1.5M), Chloroform, 2-isopropanol, and Ethanol (70%).

Procedure

- Young leaves of transgenic plants (100 mg) were ground well with liquid nitrogen using DEPC treated mortar and pestle.
- Plant RNA purification reagent (1 mL) was added and the homogenate was transferred to a 1.5 mL DEPC treated tube.
- Sample was homogenised by vortexing for two minutes and tubes were incubated horizontally at room temperature (21ºC) for 5 min.
- The tubes were centrifuged at 12,000 rpm for 2 min at room temperature and supernatant was transferred to a 2 mL centrifuge tube.

- Sodium chloride 1.5M (100 µL) was added and homogenised by inverting the tube gently, thereafter chloroform (300 µL) was added and the content was mixed by inverting eight times.
- Tubes were centrifuged at 12,000 rpm for 10 min at 4ºC, supernatant was transferred to a new tube, 2-isopropanol (500 µL) was added, gently inverted and incubated at room temperature for 10 min.
- Tubes were centrifuged at 12,000 rpm for 10 min at 4ºC, 75 per cent ethanol (1mL) was added to wash pellet, thereafter centrifuged the tubes at 12,000 rpm for 1 min. at room temperature.
- Supernatant was discarded, pellet was air dried for 5 min and pellet was dissolved in 70 µL of autoclaved DEPC water or nuclease-free water.
- The tubes were stored at -80ºC for further use.

3.7.2 Agarose gel electrophoresis for RNA

RNA was checked for quality in horizontal gel electrophoresis unit. Melted 1 per cent agarose in 1X TAE buffer (Tris buffer, Glacial acetic acid, EDTA give concentrations) (Buffer was prepared using DEPC water). Ethidium bromide was added into melted gel after cooling down to 45ºC. After proper mixing, the gel was poured into gel tray containing comb inside caster tray and kept steady position for 30 min. at room temperature. Gel was placed in gel tank (BioRad) containing 1X TAE buffer (Buffer was prepared using DEPC water) and the DNA (5 µL) mixed with bromophenol blue (2 µL) was loaded into the well. Electrophoresis was carried out at 80 volts until loading dye reached three fourth of the gel.

3.7.3 Quantification of RNA

RNA quantification was carried out using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) at wavelength 260 and 280 nm. RNA sample (1 µL) was loaded after setting DEPC treated molecular biology grade nuclease free water as blank. 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 near 2.0 indicated

the best quality of RNA. The amount of RNA was recorded in unit ng/µL for further use.

3.7.4 First strand cDNA synthesis by RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

cDNA was synthesized from the total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) 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. The cDNA preparation was carried out in two steps, in first step DNA was removed and in second step RNA was converted to cDNA using thermal cycler (Agilent SureCycler 8800).

Step-I: Removal of DNA and preparation of RNA for cDNA synthesis.

In the RNase free PCR tube, total RNA (1000 ng),10X Reaction Buffer with $MgCl_2$ (1 µL), DNase I (1 µL or 1 U), Nuclease free water to make up volume 10 µL were added and incubated at 37ºC for 30 min followed by inactivation DNase activity at 65ºC for 5 min.

Step-II: First strand cDNA synthesis.

In RNase free PCR tube, 500 ng total RNA (10 µL) (DNase free), 15 pmol Oligo (dT) primer (1 µL), 5X Reaction Buffer (4 µL), 20 U/µL RiboLock RNase Inhibitor (1 µL), 10 mM dNTP Mix (2 µL), 200 U/µL RevertAid M-MuLV RT (1.0 µL), Nuclease free water to make up volume 10 µL were added and incubated at 42ºC for 60 min and terminated the reaction by heating at 70ºC for 5 min. cDNA sample were stored at -70ºC for further use.

3.7.5 Confirmation of mRNA encoding for siRNA synthesis using PCR

PCR was performed using sense strand specific primers VLIC1 and VLIC5 (VLIC1-5'-CGACGACAAGACCCTTTCTGCTTTTGAACCCCAGT-3' and VLIC5-5'-TCTTCTTCGTCTTACACATC-3'; Annealing temperature 58ºC). Housekeeping gene Ubiquitin was also amplified using primer UBQ7-okra (F-5'-CTCCACCTCGTCCTCCGTCTG-3' and R-5'-CGCCGCACTCCGCATTAAGG-3';

Annealing temperature 58ºC). PCR components and temperature profile required for amplification are mentioned in section 3.2.5.

3.8 CONFIRMATION OF siRNA SYNTHESIS USING RT-PCR

3.8.1 Small RNA isolation

Total RNA was isolated (section 3.7.1) and first strand was synthesized using stem loop primers designed specifically for siRNA found in target sequence.

3.8.2 Primer designing for *βC1* gene specific siRNA

The siRNA found in *βC1* gene revealed by IDT software (section 3.3.1) was used for designing of 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
VStem	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC CACATC-3'
VqPCR-F	5'-GCGGCCGTAATGAAATTT-3'
VqPCR-R	5'-CCAGTGCAGGGTCCGAGGTA-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 siRNA sequences in *βC1* gene sequence In RNase free PCR tube, 500 ng RNA (10 µL) (DNase free), 15 pmol stem loop primer (1 µL), 5X Reaction Buffer (4 µL), 20 U/µL RiboLock RNase Inhibitor (1 µL), 10 mM dNTP Mix (2 µL), 200 U/µL RevertAid M-MuLV RT (1.0 µL), Nuclease free water to make up volume 10 µL were added and 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 siRNA specific forward and reverse primer, 1 unit of Taq DNA polymerase and 1 X Taq polymerase buffer and molecular grade nuclease free water to make volume up to 20.0 µL (All components from GeNei, India). The cDNA was amplified in a Thermal cycler (SureCycler 8800, Agilent Technologies), using the programme shown in the table below.

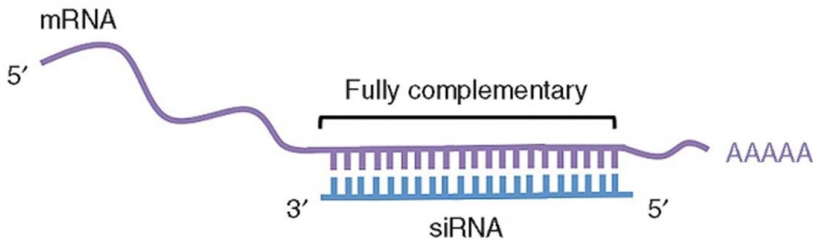
Step	Stage	Temperature (ºC)	Duration	
1.	Initial denaturation	94	2 min	
2.	Denaturation	94	30 s	35 Cycles
3.	Annealing	58	30 s	
4.	Extension	72	30 s	
5.	Final extension	72	2 min	
6.	Hold	4	Infinite	

The steps 2-4 were allowed to repeat 35 times.

The PCR product was electrophoretically analysed in agarose gel (2 %) in a horizontal gel electrophoresis along with 100 bp DNA marker (GeNei).

3.9 EVALUATION OF RESISTANCE IN TRANSFORMANTS

Twenty-days old transgenic plants along with control and YVMV infected plant were kept inside insect cage within the containment, for one week and white flies were released inside the insect cage. After one week plants were kept within the containment under observation for one month.



RESULTS

4. RESULTS

The results of the study on "RNA mediated resistance to *Yellow vein mosaic virus* in okra" carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Kerala Agricultural University, Thrissur, during September 2017 to May 2021, are presented below. The study aimed to develop okra lines transformed with RNAi constructs of βC1 ORF sequence of *Begomovirus* causing yellow vein mosaic disease and to evaluate their silencing potential.

4.1 SOMATIC EMBRYOGENESIS

4.1.1 Sterilization of explant

Surface sterilization with Carbendazim (1.0 % for 10 min.) followed by mercuric chloride (0.1 % for 10 min.) and ethanol (70.0 % for 1 min.) showed maximum sterilization of seeds (90.0 %) with good germination percentage (78.0 %) (Table 4.1) (Plate 4.1). The germination was significantly influenced with the concentration of chemicals and the duration of treatment.

Table 4.1 Effect of chemical treatments on seeds sterilization

Treatment	Combination	Time (Min.)	Sterilization (%)	Germination (%)
T1	Carbendazim (0.5 %)	10	65.0	80.0
	Mercuric chloride (0.1 %)	10		
	Ethanol (70.0 %)	1		
T2	Carbendazim (1.0 %)	10	90.0	78.0
	Mercuric chloride (0.1 %)	10		
	Ethanol (70.0 %)	1		
T3	Carbendazim (1.5 %)	10	92.0	62.0
	Mercuric chloride (0.1 %)	10		
	Ethanol (70.0 %)	1		

4.1.2. Callus initiation

Among the nine callus initiation medium, CIM 6 containing MS + BAP (3 mgL^{-1}) + 2,4-D (1 mgL^{-1}) has shown the minimum days for callus initiation (5.8 days) (Table 4.2). Callus initiation was observed within 9.4 days and green callus was initiated in the media with BAP (3 mgL^{-1}) alone (Table 4.2). Media with 2,4-D or IAA has quickly proliferated the calli but the calli were white and non-regenerative. In some cases, rooting was also observed when the concentration of IAA was increased from 0.1 to 1 mgL^{-1} (Plate 4.2).

Table 4.2 Callus initiation from okra seeds on different media combinations

Treatment	**Media composition**	**Mean number of days taken for callus initiation***	**Nature of the callus**
Control	MS (Without growth regulators)	19.0	White Friable
CIM 1	MS + BAP (1 mgL^{-1})	14.2	Light green compact
CIM 2	MS + BAP (2 mgL^{-1})	10.0	Green compact
CIM 3	MS + BAP (3 mgL^{-1})	9.4	Green compact
CIM 4	MS + BAP (1 mgL^{-1}) + 2,4-D (1 mgL^{-1})	8.3	Yellowish compact
CIM 5	MS + BAP (2 mgL^{-1}) + 2,4-D (1 mgL^{-1})	6.0	White friable
CIM 6	MS + BAP (3 mgL^{-1}) + 2,4-D (1 mgL^{-1})	5.8	White compact
CIM 7	MS + BAP (1 mgL^{-1}) + IAA (1 mgL^{-1})	9.8	Yellowish compact
CIM 8	MS + BAP (2 mgL^{-1}) + IAA (1 mgL^{-1})	8.4	White compact with rooting
CIM 9	MS + BAP (3 mgL^{-1}) + IAA (1 mgL^{-1})	7.8	White compact
C.D.		0.697	
SE(m)		0.243	
SE(d)		0.344	
C.V.		5.503	

* Mean was taken from 5 replications

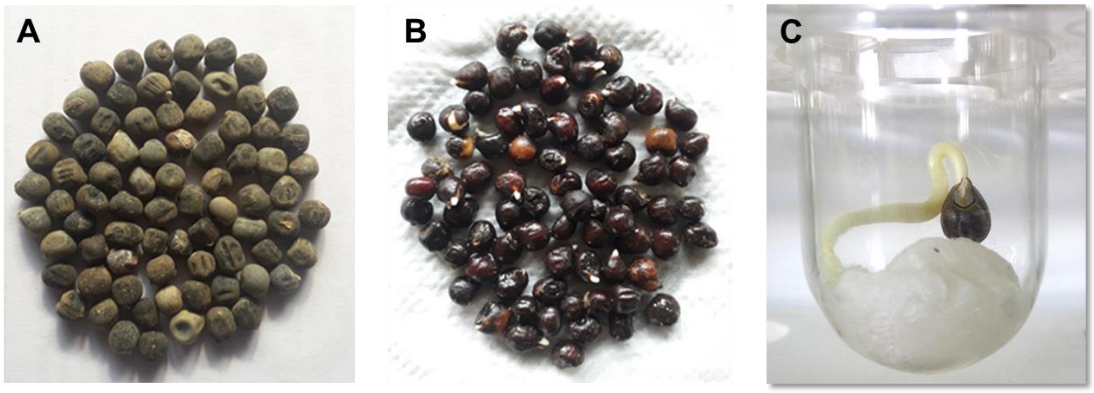


Plate 4.1 Explant preparation for callusing

A. Okra seeds cv. Salkeerthi, B. Sterilised and overnight water soaked okra seeds, C. Hypocotyl formation after 6 days

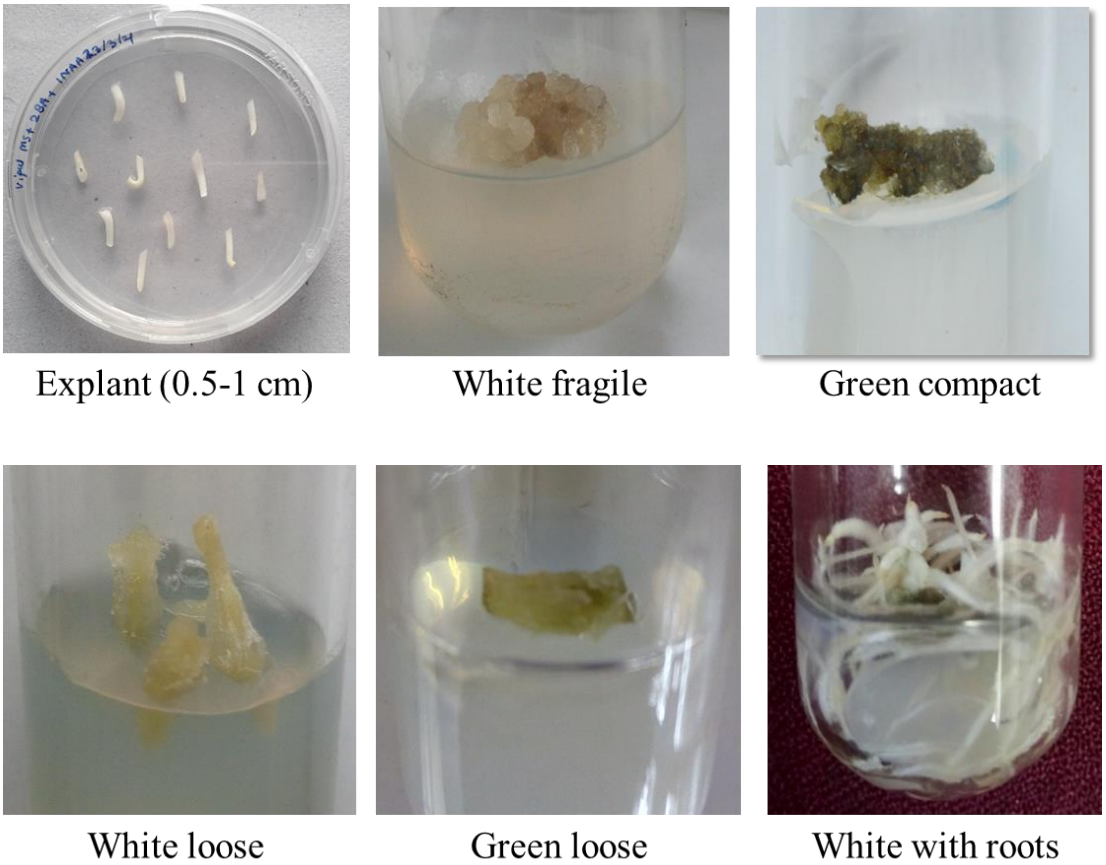


Plate 4.2 Different nature of callus on callus initiation media

4.1.3. Callus maturation and embryogenesis

Media supplemented with different growth regulators have shown positive response in callus growth compared with the control treatment. The best time for callus sub-culturing on Callus Embryogenesis Media (CEM) was 10 to 12 days after callus initiation. After 21 days of sub-culturing and the medium CEM 6 containing MS + BAP (3 mgL^{-1}) + NAA (0.1 mgL^{-1}) produced the highest callus weight (0.66 g) (Table 4.3). Development of embryogenic callus required optimal levels of growth regulators, along with the anti-browning agents ascorbic acid and activated charcoal. On CEM medium with no ascorbic acid or activated charcoal, calli were found turning brown after 6 days of sub-culturing. All the nine medium with ascorbic acid or activated charcoal have enhanced the callus growth. Sub-culturing in every 21 days has also improved the nutrient uptake compared to control. On CEM with 0.1 mM ascorbic acid, survival rate of the calli has increased up to 85.0 per cent and the mean weight increased by 0.55 g. With 0.1 mM activated charcoal, survival rate increased to 90.0 per cent and the mean callus weight increased by 0.60 g (Table 4.3) (Plate 4.3).

Table 4.3 Effect of different growth regulators on callus maturation and embryogenesis with respect to anti-browning agents

Treatment	Media composition	Mean weight of callus (g)	Mean weight of callus with ascorbic acid (g)	Mean weight of callus with activated charcoal (g)
Control	MS (With no growth regulators)	0.200	0.210	0.190
CEM 1	MS + BAP (1 mgL^{-1})	0.328	0.382	0.402
CEM 2	MS + BAP (2 mgL^{-1})	0.524	0.608	0.724
CEM 3	MS + BAP (3 mgL^{-1})	0.616	0.682	0.688
CEM 4	MS + BAP (1 mgL^{-1}) + NAA (0.1 mgL^{-1})	0.578	0.644	0.718

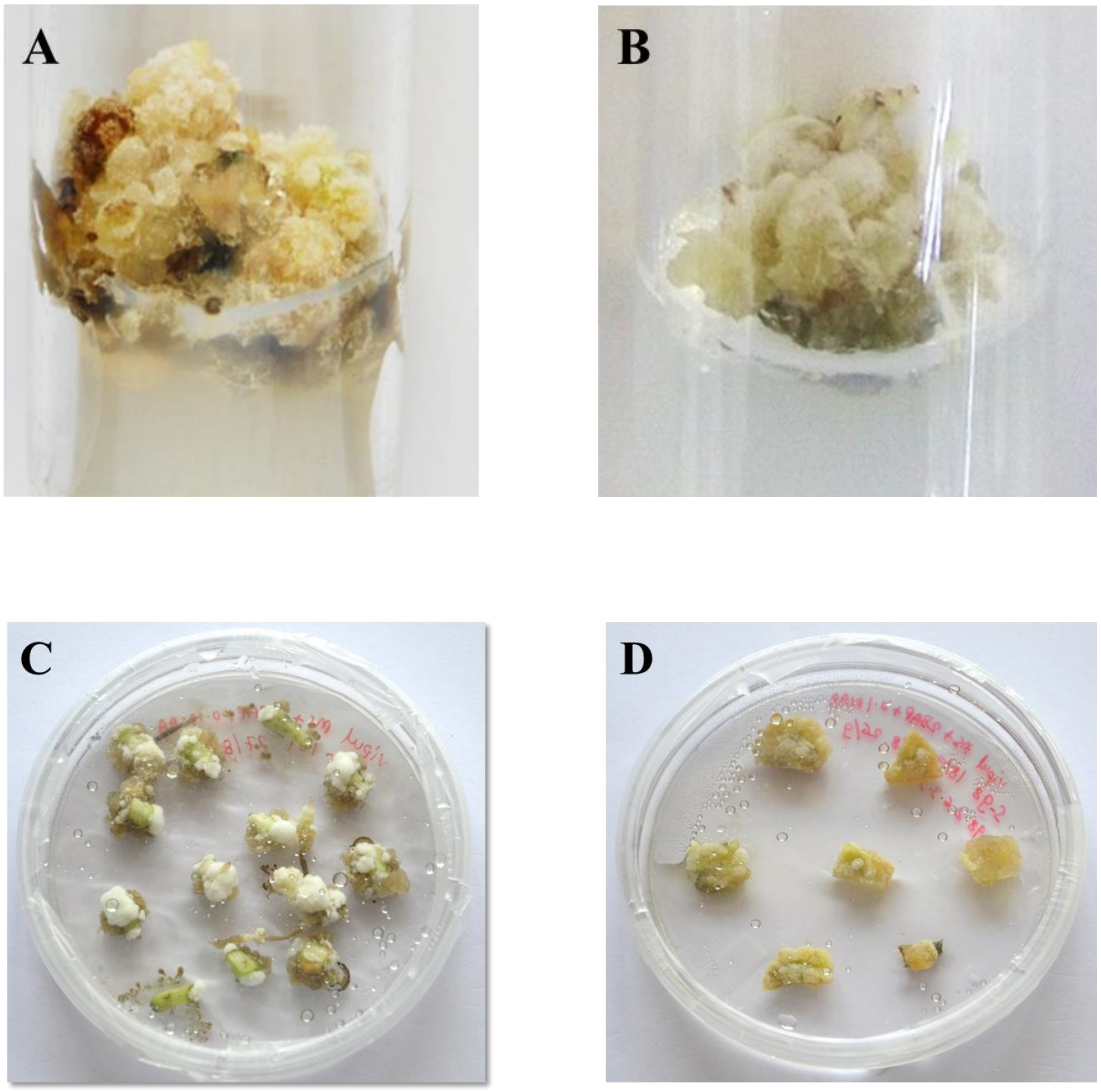


Plate 4.3 Response of callus on callus maturation media

A: Callus on MS + BAP (3 mgL^{-1}) + NAA (0.1 mgL^{-1}) media

B: Callus on MS + BAP (2 mgL^{-1}) media

C: Callus on MS + BAP (2 mgL^{-1}) + NAA (0.1 mgL^{-1}) media

D: Callus on MS + Kinetin (2 mgL^{-1}) media

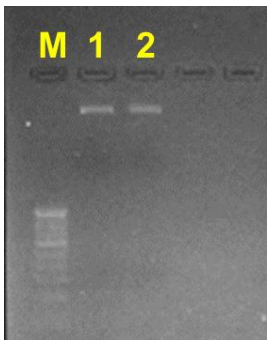


Plate 4.4 Gel photograph of DNA isolated from YVMV infected plant

M- Step-up 100 bp ladder (GeNei), 1-2 Okra DNA

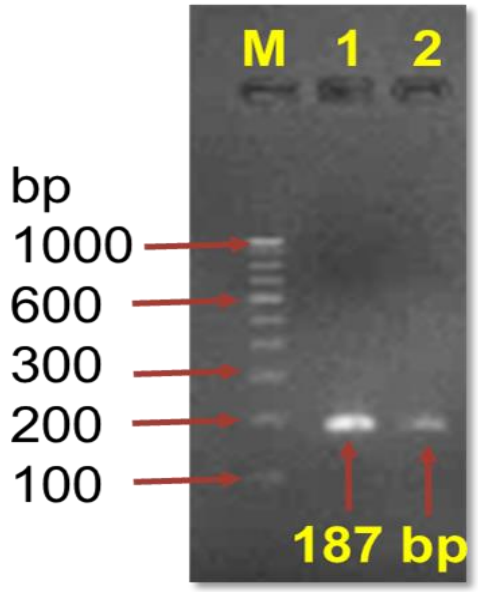


Plate 4.5 Gel photograph of *βC1* gene amplified with gene specific primer (VβC1)

M- Step-up 100 bp ladder (GeNei), 1 -2 *βC1* gene (187 bp)

CEM 5	MS + BAP (2 mgL^{-1}) + NAA (0.1 mgL^{-1})	0.644	0.706	0.744
CEM 6	MS + BAP (3 mgL^{-1}) + NAA (0.1 mgL^{-1})	0.660	0.714	0.740
CEM 7	MS + Kinetin (1 mgL^{-1})	0.408	0.472	0.478
CEM 8	MS + Kinetin (2 mgL^{-1})	0.472	0.534	0.596
CEM 9	MS + Kinetin (3 mgL^{-1})	0.514	0.578	0.704
	C.D.	0.025	0.058	0.050
	SE(m)	0.009	0.020	0.017
	SE(d)	0.012	0.029	0.024
	C.V.	3.917	8.173	6.397

Even the calli cultured on CEM 6 medium containing MS + BAP (3 mgL^{-1}) + NAA (0.1 mgL^{-1}) and supplemented with activated charcoal and sub-cultured every after 21 days, have failed to produce somatic embryos.

4.2 ISOLATION OF TARGET GENE (*βC1* GENE OF YVMV)

4.2.1 Total DNA isolation from YVMV infected plant

DNA isolation was done with CTAB method (Ghosh, *et al.* 2009) but the quality of the DNA obtained was not good enough, and hence few modifications were made such as treatment with sodium chloride and precipitation with potassium acetate. Chloroform : Isoamyl alcohol treatment twice has reduced the pigment contamination, without affecting the DNA quality. Treatment with 3M NaCl has reduced the mucilage and 0.3M potassium acetate has reduced polysaccharide content, which made the DNA available for precipitation.

The agarose gel picture of DNA showed a typical eukaryotic intact DNA. Isolated DNA was free from RNA and protein contamination (Plate 4.4). The Absorbance ratio of A_{260}/A_{280} was 1.85 which is indication of good quality DNA and the concentration of DNA was 320.0 ng/µL.

4.2.2 Amplification of *βC1* ORF sequence of YVMV

The output of Primer 3.0 has yielded the forward primer starting from 297 bp and reverse primer starting at 483 bp in the total 1354 bp *βC1* gene of YVMV (Fig. 4.1). The product size was 187 bp, containing ORF1 (84 bp) and ORF2 (187 bp), as revealed through ORF finder analysis. The ORF2 (187 bp) is coding for 60 amino acids (Fig. 4.2). PCR amplification with designed primers has shown expected size 187 bp in the agarose gel (Plate 4.5).

Primer3 Output

```
PRIMER PICKING RESULTS FOR EF417919.1 Bhendi yellow vein mosaic virus satellite DNA beta isolate Barrackpore, complete sequence

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER        297   20   59.71   45.00  3.00  1.00 TTCTGCTTTTGAACCCCAGT
RIGHT PRIMER       483   20   59.85   45.00  4.00  0.00 GCTCATGCAACAACAAAGGA
SEQUENCE SIZE: 1354
INCLUDED REGION SIZE: 1354

PRODUCT SIZE: 187, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 ACCGTGGGCGAGCGGAGTCTGAGCCGTTGTGGGACCCACAGATGAATGAAGTTCATGGGT

   61 GATTTCAAGTATATGGAGGAAATTGTGGATGAGAAAAATGAATCATGTTTGTTTTTGCAA

  121 ATTATTTTAGATAACAGTCTCCTAATAATAATTAATATGCAAACATATTACTAACAAAAT

  181 TAAATTATTATCTTATTATCAATAGTTACTGGTTCGTTTACATCCATTCCCATAATCTCT

  241 GGGTTTTCAATAATAATAATATCCACCATATGTATGATGTCTTCTTCTAATATTTCTTCT
                                                                >>>>

  301 GCTTTTGAACCCCAGTAATGAAACTTGAAGGTTGATGTGATCGTTCCTTCTAAACCGTTG
      >>>>>>>>>>>>>>>>

  361 AAGTCGAATGGAATATGTAATTGTTGGTATGTATAGTCGATGATGAATGTTTTTGTTGAT

  421 ATTGCTGGAGATCTTGTTGAACTGATCTGCATCTGGACTGATATCCTTTGTTGTTGCATG
                                                  <<<<<<<<<<<<<<<<<

  481 AGCTTGACATCTACTTTGAAGACGATCCCCTCCCTGTTTTTGCTGAACCTTGTCATTTTG
      <<<
```

Figure 4.1 *βC1* gene specific primers designed using primer3

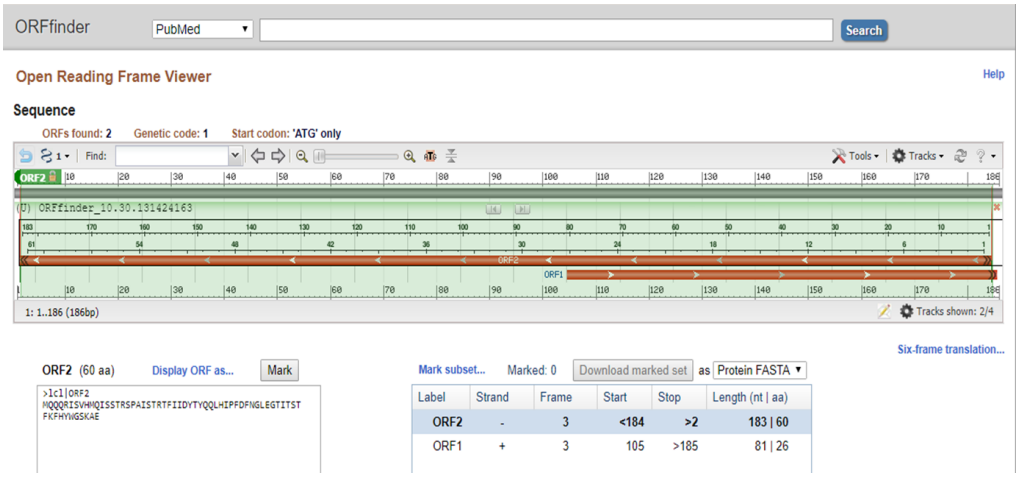
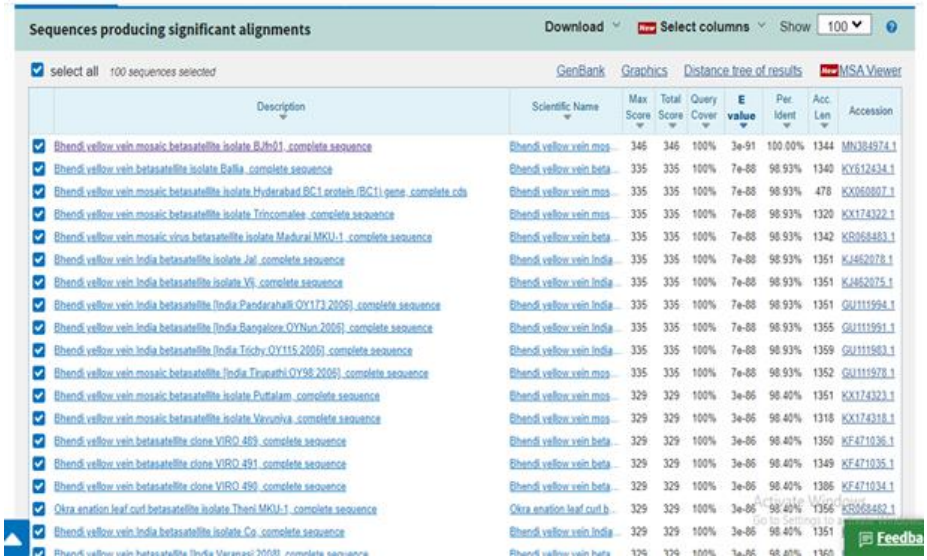


Figure 4.2 Output of ORF finder for *βC1* gene

4.2.3 Sequencing of the PCR amplified region of *βC1* gene

The PCR product was Sanger sequenced and the sequence information (Appendix V) of *βC1* gene was deposited in GenBank, National Centre for Biotechnology Information (NCBI) with accession number MK164881. The sequence was analysed using BLASTn which showed 100 per cent sequence identity (E-value - $3e^{-91}$) with Telangana isolate (Sri Lanka) (Acc. No.:MN384974.1) (Fig. 4.3) and 94.65 to 98.93 similarity with Indian isolates.



Bhendi yellow vein mosaic betasatellite isolate BJfn01, complete sequence

Sequence ID: MN384974.1 Length: 1344 Number of Matches: 1

Range 1: 294 to 480 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
346 bits(187)	3e-91	187/187(100%)	0/187(0%)	Plus/Plus

```
Query  1    TTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTGATGTGATTGTTCCTTCTAGACC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  TTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTGATGTGATTGTTCCTTCTAGACC  353

Query  61   GTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGTAGTCGATGATGAATGTTTTTGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  GTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGTAGTCGATGATGAATGTTTTTGT  413

Query  121  TGATATGGCTGGAGATTTTGTTGAACTGATCTGCATATGCACCGATATCCTTTGTTGTTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  TGATATGGCTGGAGATTTTGTTGAACTGATCTGCATATGCACCGATATCCTTTGTTGTTG  473

Query  181  CATGAGC  187
            |||||||
Sbjct  474  CATGAGC  480
```

Figure 4.3 Output of BLASTn for sequence of *βC1* gene

4.3 ihpRNA VECTOR CONSTRUCTION

4.3.1 Prediction of siRNA in the βC1 ORF sequence of the virus

Partial *βC1* gene sequence was subjected to analysis in RNAi design tool from the IDT site. This has predicted three siRNA target sites in the coding region of the *βC1* (187 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. Custom Dicer-Substrate siRNA analysis on the βC1 region has yielded 10 siRNAs, showing that the target is ideal for RNA interference. Among them, three siRNAs had different sizes (19-44, 34-59, 99-124 bp), increasing the target sites for cleavage (Appendix VI.). The parameters deciding the target regions are detailed in (Appendix III). Primers for the amplification of the sense and antisense fragments were designed to include maximum miRNA target regions in the amplicon.

4.3.2 Restriction mapping

Restriction map for partial sequence of βC1 (187 bp) was obtained using the tool 'Restriction mapper' in Sequence Manipulation Suite' available at https://www.bioinformatics.org>sms2>rest_map. A textual map showing the positions of restriction endonuclease recognition sites was obtained (Appendix VII). Output of Restriction Mapper has confirmed that the sequence selected for the preparation of sense and antisense fragments, did not possess recognition sites for *Sma*I, *Hind*III amd *Mau*BI restriction enzymes which are present in the pRNAiLIC vector.

4.3.3 Viral silencing suppressor prediction

The partial βC1 (187 bp) gene sequences were subjected to viral silencing suppressor prediction using the tool VSupPred available at http://bioinfo.icgeb.res.in>pvsup>submit. The prediction score ranges from 0 to 1 and higher the prediction score, better the confidence level of prediction. The score mentioned that the fragment is a Non Viral Silencing suppressor Region (NVSR) with a prediction score of 0.625 for partial sequence of *βC1* gene (Appendix VIII).

4.3.4 Isolation and *Sma*I digestion of vector plasmid pRNAi-LIC (CD3-1285)

Good quality plasmid was isolated from DH5α using plasmid DNA purification kit (Macherey-Nagel). The Absorption ratio (A_{260}/A_{280}) was 1.87 and quantity was 220 ng μL^{-1}. The *Sma*I digested plasmid was run on agarose gel which produced three fragments such as vector backbone (9842 bp), Pdk intron (1641 bp) and ccdB gene (614 bp) (Plate 4.6).

4.3.5 Preparation of sense and antisense strand through PCR

The sense strand was amplified using primers VLIC1 (Forward) and VLIC2 (Reverse) which produced fragment size of 215 bp. The antisense strand was amplified using primers VLIC3 (Forward) and VLIC4 (Reverse) which produced fragment size of 243 bp (Plate 4.7). Both fragments were eluted from gel by using NucleoSpin® PCR Clean-up gel extraction kit (Macherey-Nagel). The Absorption ratio (A_{260}/A_{280}) was 1.8 and quantity was 16 ng μL^{-1} for sense strand and 1.82 and 40 ng μL^{-1}, respectively for antisense strand.

4.3.6 Ligating the sense and antisense strands of *βC1* gene into pRNAi-LIC vector

The *Sma*I digested plasmid which contain vector backbone (9842 bp), Pdk intron (1641 bp) and ccdB gene (614 bp) were successfully treated with dTTP. Sense strand (215 bp) and antisense strand (243 bp) were successfully treated separately with dATP. The dATP and dTTP treated products were mixed and added in competent cells of DH5α and screened on antibiotic media.

4.3.7 pRNAi-LIC vector transformation into DH5α and confirmation

Efficient competent cell preparation and quick transformation was obtained by the Transform Aid Bacterial Transformation Kit (Thermo Scientific). The competent cells used were *E. coli* laboratory strain DH5α and were transformed using the pRNAi-LIC plasmid with Kanamycin and Chloramphenicol resistance. Control experiment was also set up to see false positive result.

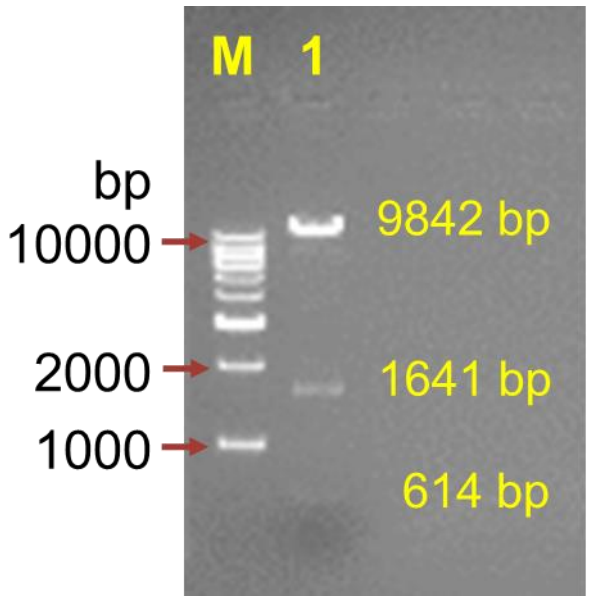


Plate 4.6 Gel photograph of *Sma*I digested pRNAi-LIC (CD3-1285)

M- 1Kb DNA ladder (GeNei), 1) *Sma*I digested plasmid

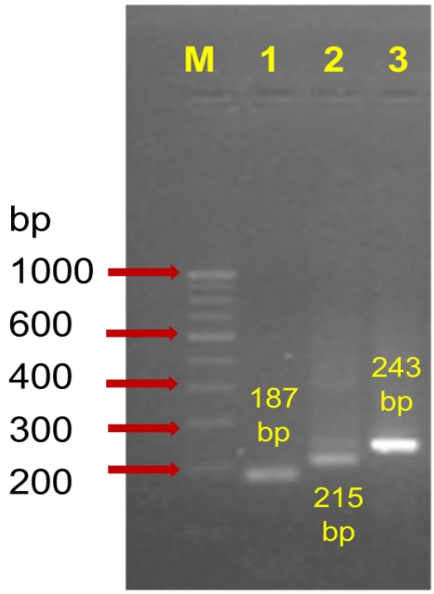


Plate 4.7 Gel photograph gene amplified with gene specific primers

M- Step-up 100 bp ladder (GeNei), 1) *βC1* gene amplified with primer VβC1F and VβC1R, 2) Sense strand amplified with primer VLIC1 and VLIC2, 3) Antisense strand amplified with primer VLIC3 and VLIC4.

About 50-60 positive colonies were found in the overnight culture of DH5α (Plate 4.8) whereas no colonies were found in control plate. All positive colonies were tested using colony PCR with same primers used to produce PCR product1 (VLIC1 and VLIC2), which has shown that around 80 per cent colonies are transformed (Plate 4.9). For the maintenance of transformed colonies, colony grid was prepared from the colonies confirmed by colony PCR.

Using plasmid DNA purification kit (Macherey-Nagel), plasmid was isolated from a positive colony. The Absorption ratio (A_{260}/A_{280}) was 1.85 and quantity was 234 ng μL^{-1}. The plasmids were sequenced using VLIC5 and VLIC6 primers and the sequences aligned in Clustal Omega has confirmed that both the sense and antisense strands are in right position and direction (Appendix IX).

4.3.8 Transfer of plasmid containing ihpRNAi cassette into *Agrobacterium*

Efficient competent cells of *A. tumefaciens* laboratory strain GV3101 were prepared using $CaCl_2$ and stored at -80ºC for further use. Quick transformation was obtained by the freeze and thaw method. The competent cells having Rifampicin resistance and were transformed using the pRNAi-LIC plasmid which was having Kanamycin and Chloramphenicol resistance. Control experiment was also set up to see false positive result.

Thirteen positive colonies were found in the overnight culture of GV3101 (Plate 4.10) whereas no colonies were seen in control plate. Positive colonies were tested for colony PCR with sense specific sequencing primers (VLIC1 and VLIC5) and antisense specific sequencing primers (VLIC3 and VLIC6), which has shown that 100 per cent colonies contain sense and antisense strands (Plate 4.11). For the maintenance of transformed colonies, colony grid was prepared from the colonies confirmed by colony PCR.

Using plasmid DNA purification kit (Macherey-Nagel), plasmid was isolated from the positive colony which was confirmed by colony PCR. The Absorption ratio (A_{260}/A_{280}) was 1.88 and quantity was 164 ng μL^{-1}. Sequencing results were aligned

using Clustal Omega tool which confirmed that both sense and antisense strands are in right position and direction (Appendix X).

4.4 *IN PLANTA* TRANSFORMATION AND CONFIRMATION OF TRANSGENEISS

4.4.1 Co-cultivation with *Agrobacterium tumefaciens* and germination

One hundred seeds of cv. Salkeerthi were sterilised and soaked overnight in autoclaved distilled water. Of the 65 seeds germinated, 35 were used for the co-cultivation with *Agrobacterium*. Out of 35 seeds used for the *in planta* transformation (Plate 4.12), 20 have germinated in a mixture of soil and FYM. All the plants were grown in a containment glass house, under controlled environmental conditions.

4.4.2 Confirmation of transgenic plants through thermal cycling

The DNA was isolated from 20 fifteen-days old plants and amplified with sense strand specific primer (VLIC1 and VLIC5). Out of 20 plants, 4 plants (plant no. 2, 3, 4 and 11) have shown amplification at the expected size of 400 bp (Plate 4.13).

4.5 siRNA SYNTHESIS IN TRANSGENIC PLANTS

4.5.1 Total RNA isolation and cDNA preparation

Total RNA was isolated from three transformed plants and one control plant (Non transformed) using Purelink® Plant RNA purification reagent (Invitrogen, India). Three intact bands were obtained corresponding to 28S, 18S and 5S rRNA (Plate 4.14).

Table 4.4 Quality estimation of isolated RNA using NanoDrop

Sl. No.	Sample (RNA)	$OD_{260/280}$	Concentration (ng/µL)
1	Control	1.90	345.4
2	$T_0$2	1.96	323.7
3	$T_0$3	1.83	288.2
4	$T_0$4	1.87	264.6

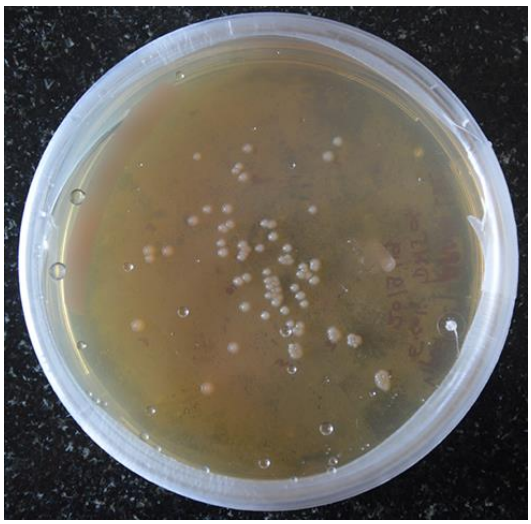


Plate 4.8 ihpRNA transformed DH5α colonies on LB agar medium containing Kanamycin (50 mgL^{-1}) and Chloramphenicol (5 mgL^{-1})

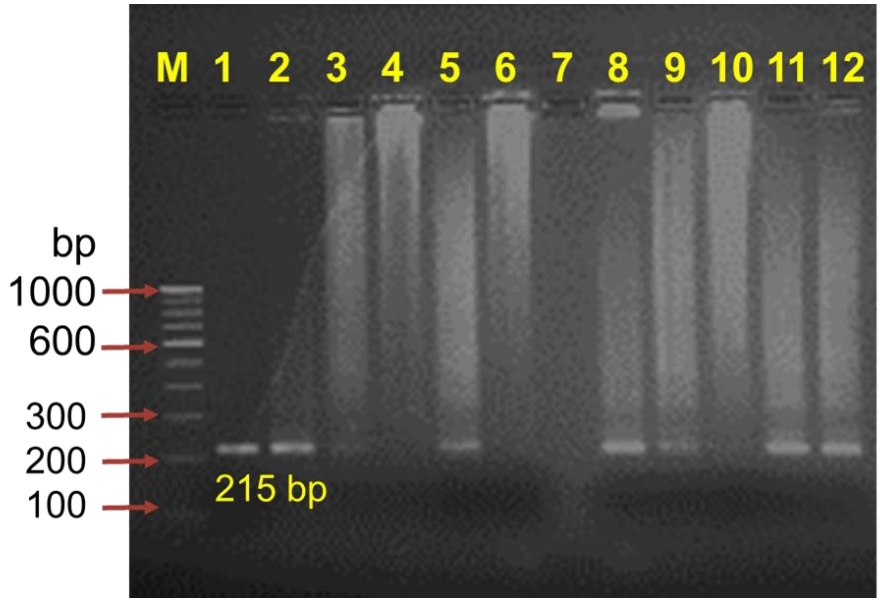


Plate 4.9 Gel photograph of colony PCR of DH5α containing ihpRNA-*βC1* amplified with primer VLIC1 and VLIC2

M- Step-up 100 bp ladder (GeNei), 1) Positive control (Target gene *βC1*),
2) to 12) DH5α colonies

Plate 4.10 ihpRNA transformed GV3101 colonies on LB agar medium containing Kanamycin (50 mgL^{-1}), Chloramphenicol (5 mgL^{-1}) and Rifampicin (25 mgL^{-1})

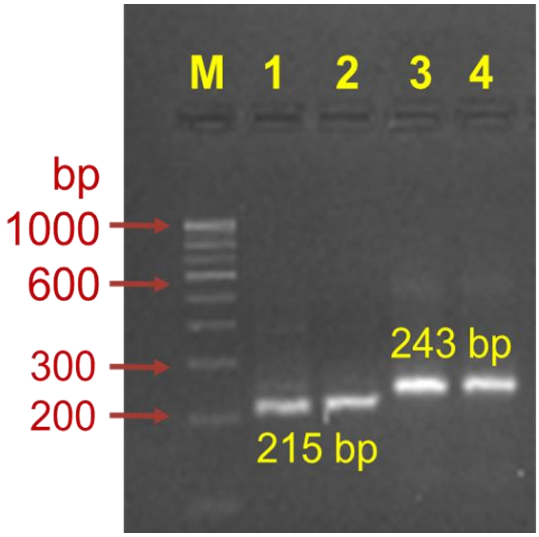


Plate 4.11 Gel photograph of colony PCR of GV3101 containing ihpRNA-*βC1* amplified with primer VLIC1 and VLIC2, VLIC3 and VLIC4

M- Step-up 100 bp ladder (GeNei), 1) and 2) colonies of GV3101 amplified with gene specific primer for sense strand (VLIC1 and VLIC2, 215 bp), 3) to 4) colonies of GV3101 amplified with gene specific primer for antisense strand (VLIC3 and VLIC4, 243 bp)

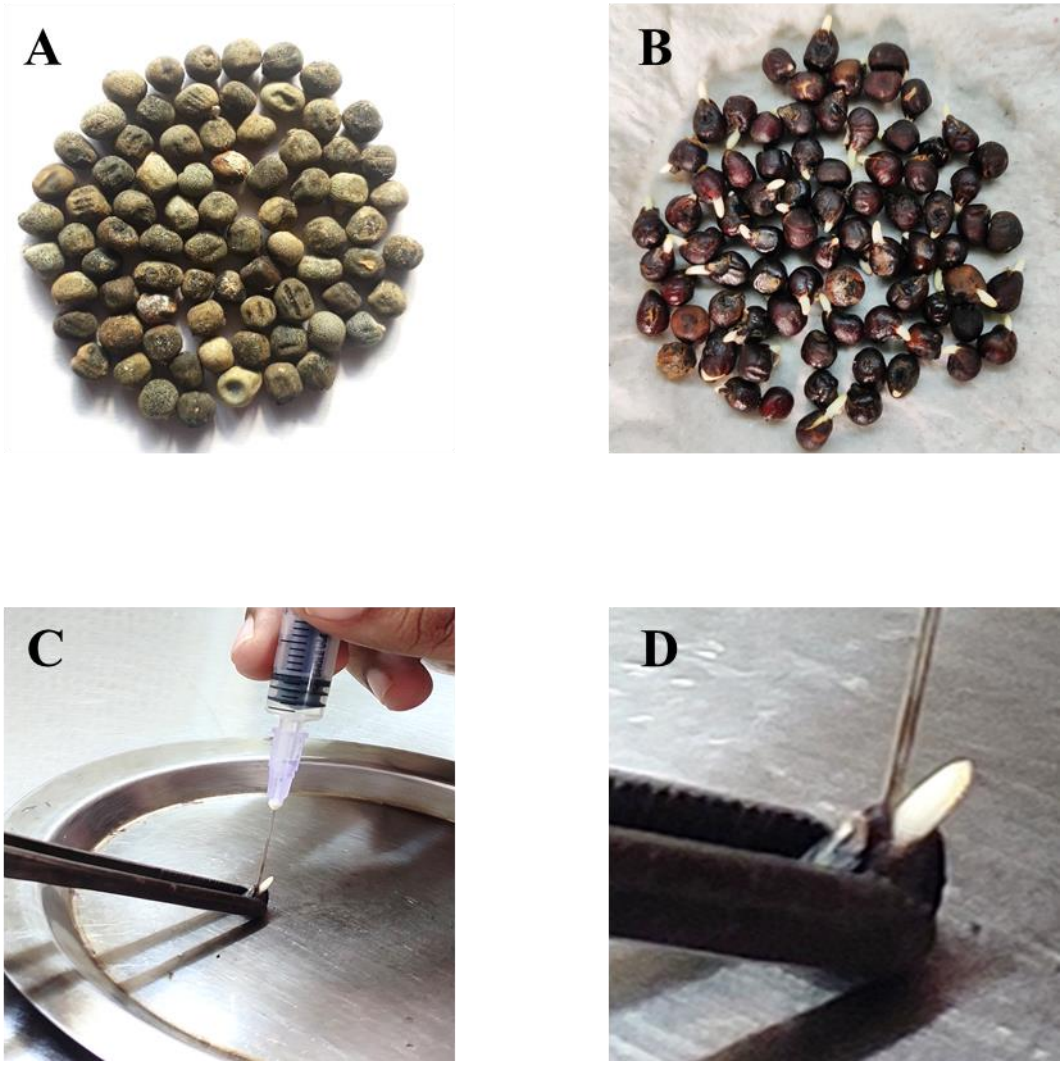


Plate 4.12 *In planta* transformation in okra cv. Salkeerthi

A) Okra seeds cv Salkeerthi,

B) Sterilised, overnight soaked okra seeds germinated on sterilised moist tissue paper for 4 h.

C) Plumule injured with syringe,

D) Magnified image of plumule injury

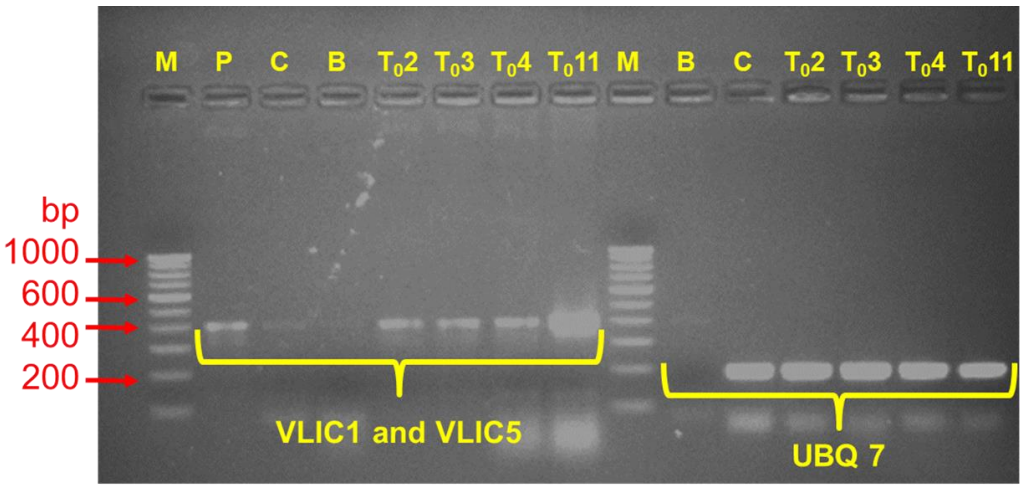


Plate 4.13 Gel photograph of transformed plants amplified with sense strand specific primer (400 bp) and *Ubiquitin* gene specific primer (UBQ7-187 bp)

M- Step-up 100 bp ladder (GeNei), P) Plsmid DNA, C) Control plant DNA (Non transformed), B) Blank, $T_0$2, $T_0$3, $T_0$4 and $T_0$11 transformed plants)

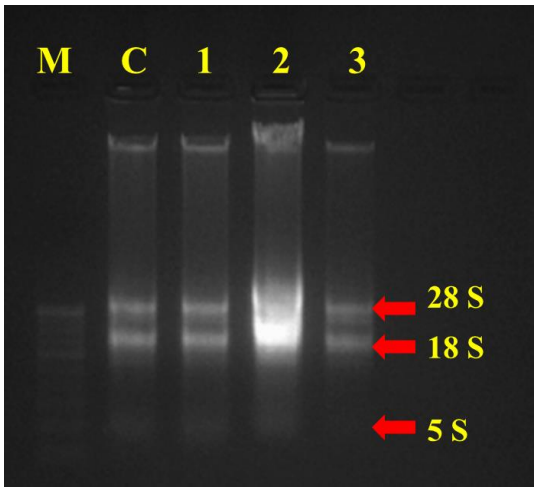


Plate 4.14 Gel photograph of total RNA isolated from transgenic plants

M- Step-up 100 bp ladder (GeNei), C) Control plant, 1) $T_0$2, 2) $T_0$3, and 3) $T_0$4

cDNA was synthesised from isolated RNA (1000 ng) using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific), as per the instruction provided by Thermo scientific.

4.5.2 Confirmation of mRNA encoding for siRNA

Synthesis of mRNA encoding for siRNA in the putative transformants towards the targeted gene was confirmed by PCR using sense strand specific primers (VLIC1-VLIC5) (400 bp). The gene expression was confirmed in the transformed plants and housekeeping gene (UBQ7) was also amplified at expected size (187 bp) (Plate 4.15).

4.6 RT-PCR ANALYSIS OF siRNA SYNTHESIS USING STEM-LOOP PRIMERS

Synthesis of siRNA by the ihpRNA cassettes in the putative transformants towards the *βC1* gene was confirmed by the stem-loop primers. The primers designed were found to be efficient in amplifying the specific siRNA against *βC1* gene of YVMV. Amplicons of size 68 bp represented siRNA synthesized against *βC1* gene (Plate 4.16).

4.7 EVALUATION OF RESISTANCE IN TRANSFORMANTS

Twenty-days old transgenic plants along with control (Non-transformed) and YVMV infected plant were kept inside insect cage within the containment, for one week and white flies were released inside the insect cage (Plates 4.17 and 4.18). All the control plants and one transgenic plant have shown the symptoms of YVMV after 10 days. Other three transgenic plants were healthy and did not show any symptoms of YVMV even after 30 days of infection (Plate 4.19 and 4.20).

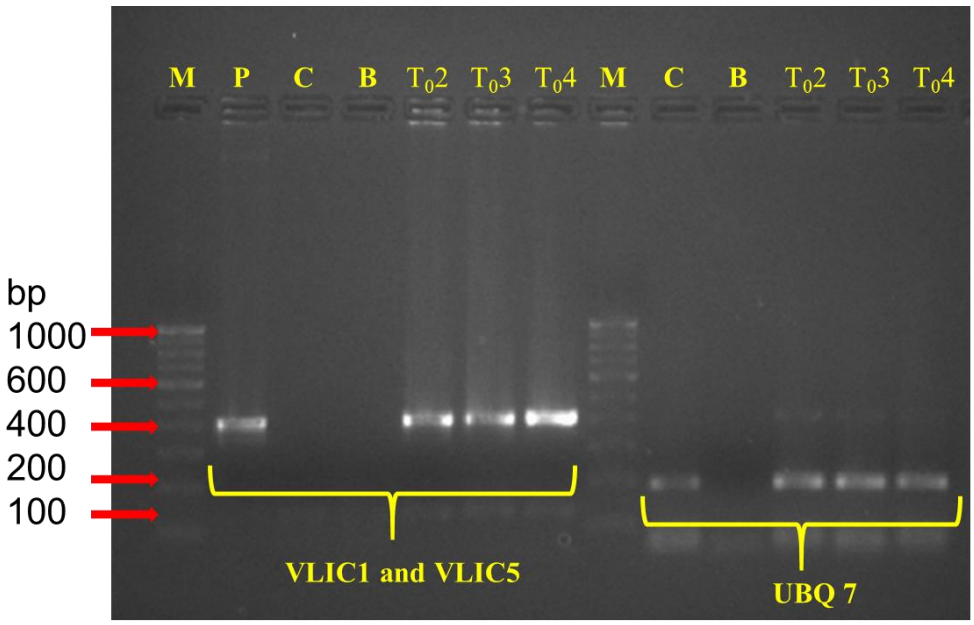


Plate 4.15 Gel photograph of RT-PCR amplified product of siRNA producing gene in transformed plants

M- Step-up 100 bp ladder (GeNei), P) Plsmid DNA, C) Control plant DNA (Non transformed), B) Blank, T02, T03, and T04 – transformed plants

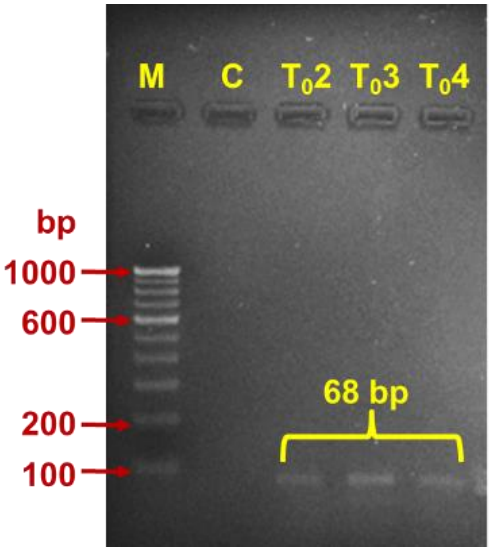


Plate 4.16 Gel photograph of siRNA amplified with stem loop primers

M: Marker (Steup up 100 bp ladder, GeNei), C: Negative control,

T02, T03 and T04: Transformed plants

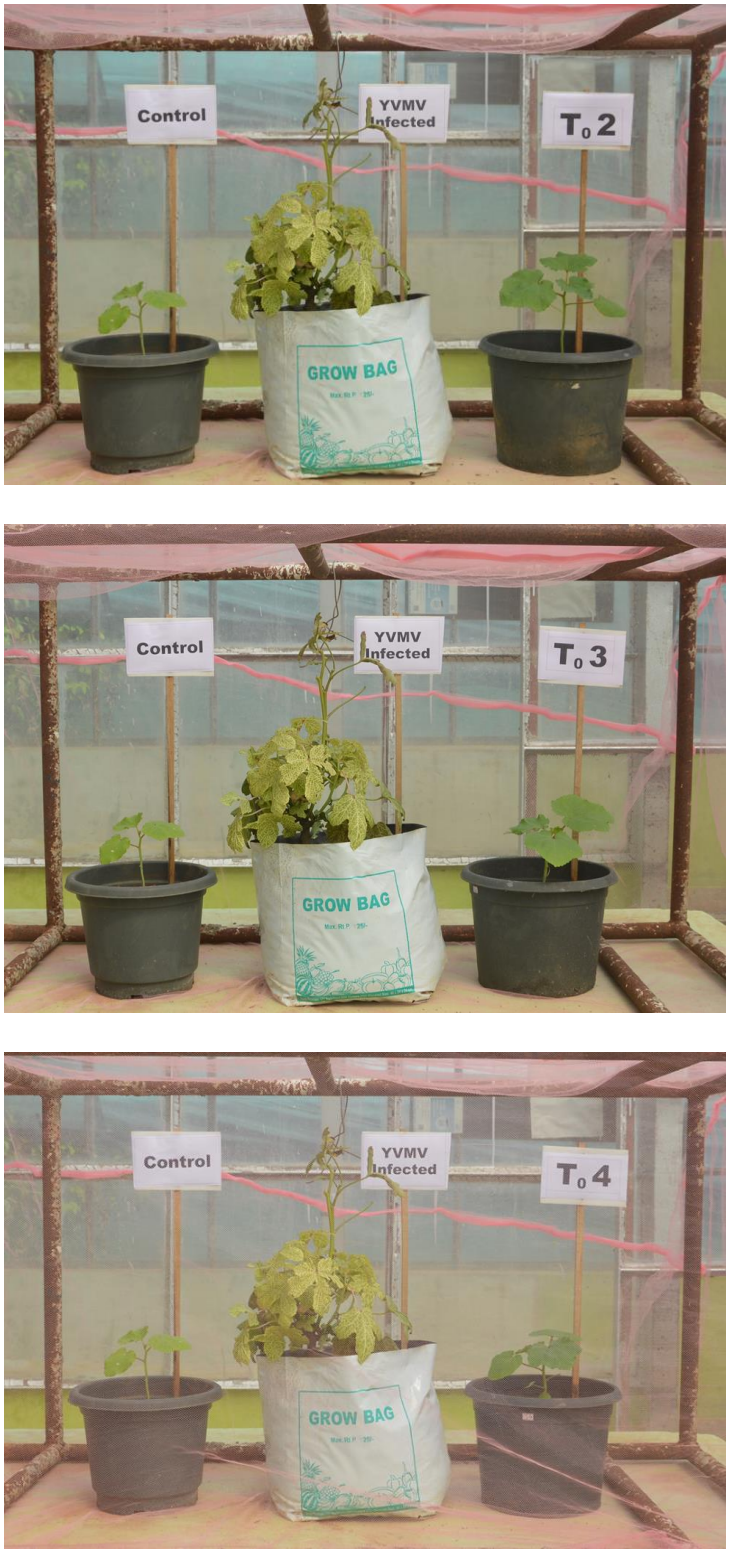


Plate 4.17 Transformed plants (15 days old) kept inside insect cage for whitefly infection

Plate 4.18 Whiteflies released for infection inside cages

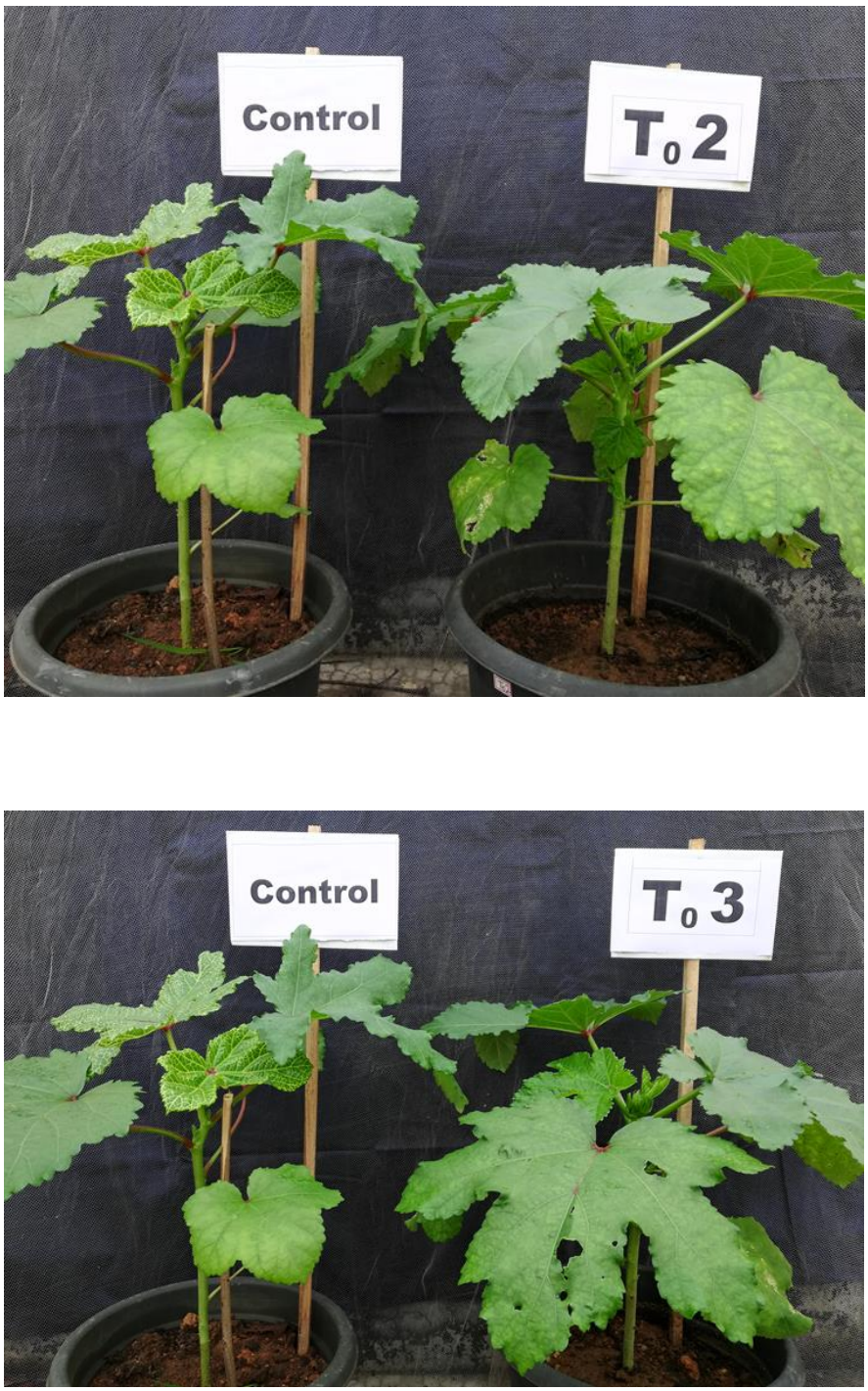
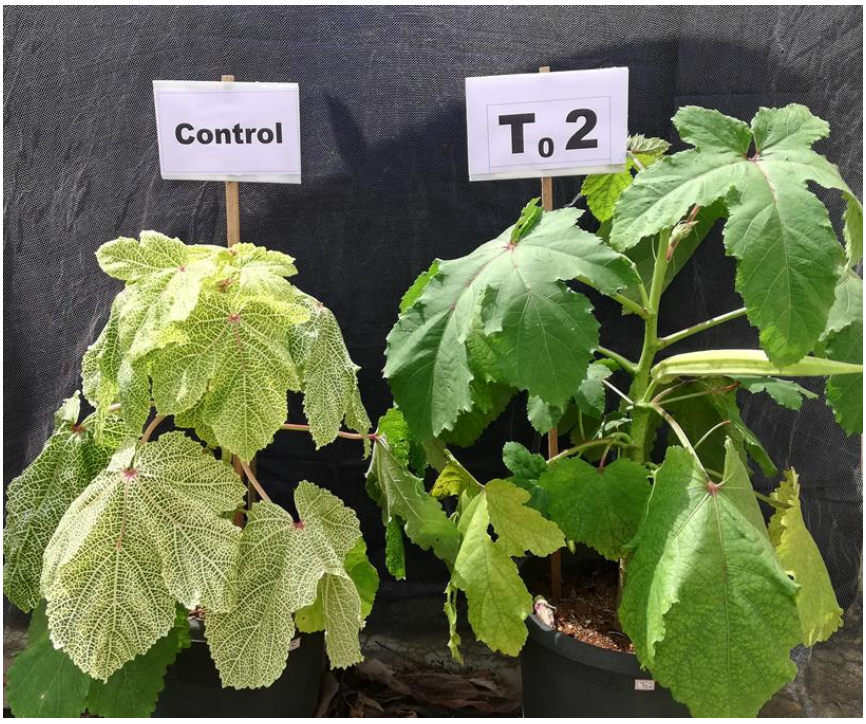


Plate 4.19 Transformed plants after 10 days of infection



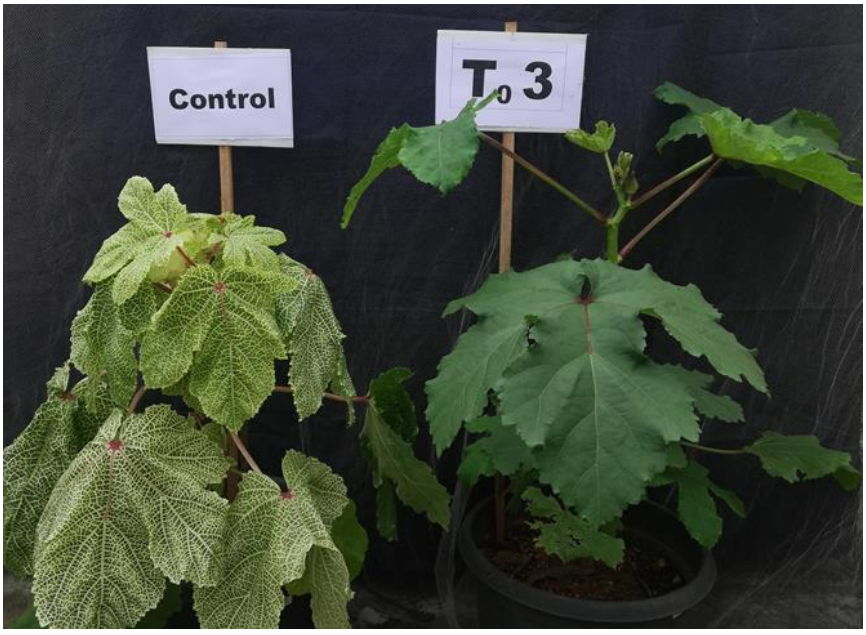
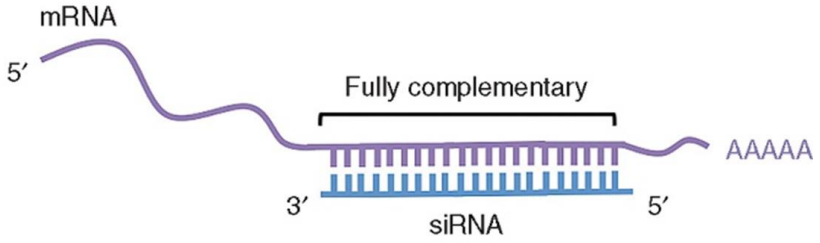


Plate 4.20 Transformed plants after 30 days of infection



DISCUSSION

5. DISCUSSION

Okra (*Abelmoschus esculentus* L., Malvaceae) is one of the popular vegetables in the tropical and subtropical regions of the world. Okra usually has the chromosome number 2n=130, and according to Datta and Naug (1968), 2n=72, 108, 120, 132 and 144 are also observed with x=12. Area under okra cultivation is 2.73 million ha in the world and 0.5 million ha in India (FAO, 2019). India has secured the first rank in the world in the case of okra production and productivity. Humid and hot climate is favourable for the okra cultivation, but it is also favourable for many pests and diseases. Among the pests, whitefly (*Bemisia tabaci*) is the most destructive because it transmits the *Begomoviruses*, *Yellow vein mosaic virus* (YVMV) and *Okra enation leaf curl virus* (OELCV) (Kochlar, 1986; Kumar *et al*., 2010).

Viral diseases of okra are serious, causing 20-80 per cent and sometimes 100 per cent yield losses, depending on the stage of the plant at infection (Sanwal *et al*., 2014; Khaskheli *et al*., 2017; Venkataravanappa, 2008). Virus infection in the initial days (up to 20 days after sowing) results in complete crop loss. Management of whitefly is the key for YVMV management but they have a wide range of host plants. Spraying with Plant Growth Promoting Rhizobacteria (PGPR) at 15 days interval was effective to manage the whitefly population up to 79.2 per cent by activating the translation of defence enzymes (Patil *et al*., 2011). Insecticides such as Imidacloprid, Acetamiprid, and Trizophos are recommended for managing the whiteflies (Gowdar *et al*., 2007; Alam, *et al*., 2010; Ansar *et al*., 2014). The sustainable solution is the development of virus resistant cultivars (Dhankhar *et al*., 2005). A few wild species, *A. manihot, A. crinitus, A. angulosus,* and *A. tetraphyllus* are identified as the sources of resistance for the breeding programmes, but their genetic distance lead to reduced hybridization success (Singh *et al*., 2007). Jambhale and Nerkar (1986) have developed YVMV resistant cultivar Parbhani Kranti by crossing *A. esculentus* × *A. manihot,* but it lost resistance due to mutation in *Begomovirus* genome leading to the development of new strains.

Biotechnological interventions can incorporate resistance in to crop lines which can be used as cultivar or a parent in further breeding programmes (Ma *et al*.,

2004). RNAi is found in eukaryotic organism as conserved regulatory mechanism of gene expression, which acts against viruses (Bartel, 2004). Tenllado *et al*. (2003) suggested that advance methods like RNA silencing have the potential of developing virus resistant plants. RNA interference was used by Hashmi *et al*. (2011) to produce virus resistance in cotton by targeting the truncated *Rep* gene of virus and also recommended RNAi for virus resistance. Similarly, Chen *et al*. (2016) also have produced tomato plants resistant against *Begomoviruses*. The mechanism of RNAi has emerged quickly due to its high specificity, accuracy, and heritability (Waterhouse *et al*., 1998; Fire *et al*., 1998; Fire, 1999; Voinnet *et al*., 1999; Waterhouse *et al*., 1999, Baulcombe, 2004). Studies by Thakur *et al*. (2014) and Zaidi *et al*. (2017) suggested that RNAi can produce *Begomovirus* resistance in okra.

RNAi involves the expression of an inverted repeat (IR) or hairpin RNA (hpRNA) that has homology with the virus. The hpRNA transcribed from IR constructs will be converted 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 or recruiting DNA and histone modifiers and inhibiting the transcription of target gene. Thus, siRNA mediated sequence specific gene silencing mechanism plays an important role in antiviral defense (Fire, 1999; Baulcombe, 2004; Voinnet, 2009, Voinnet *et al.*, 1999).

Complementarity of a free mRNA in the cytoplasm, with the target mRNA, initiates the silencing machinery. A similar attempt is made in the current study to develop intron hairpin RNA (ihpRNA) constructs for the *βC1* gene of the virus and to transform the okra plants, to develop YVMV resistant lines.

5.1 SOMATIC EMBRYOGENESIS

Prerequisite for the genetic transformation is an efficient tissue culture protocol, and unfortunately recalcitrant nature of okra reduces the regeneration through callus (Ganesan *et al*., 2007). Selection of explants is an important factor in okra, because it is known for high level of phenolic compounds and recalcitrant

nature, which are major constraints in tissue culture (Anisuzzaman *et al*., 2010; Kabir *et al*., 2008; Narendran *et al*., 2013). Somatic embryogenesis is more preferred in genetic transformation because of their single cell origin and the ability to produce non-chimeric plants.

Seed treatment with 1.0 per cent Carbendazim, 0.1 per cent mercuric chloride and 70 per cent ethanol was effective to reduce the contamination without affecting the germination. Higher levels of Carbendazim reduces the germination percentage. Similarly, Ganesan *et al*. (2007) found that the germination percentage increased up to 85 per cent when seeds were sterilised with Carbendazim (1.0%) and mercuric chloride (0.1%). Anisuzzaman *et al.* (2010) also observed contamination free explant after mercuric chloride treatment.

Hypocotyl was used as explants for callusing and callus was initiated within 5.8 days on MS medium supplemented with BAP (3 mgL^{-1}) and 2,4-D (1 mgL^{-1}). Combination of BAP and 2,4-D gave early callus initiation compared to other combinations. Cytokinin in combination with auxin is necessary for callus growth (Mallela *et al.* 2009). Callus formed was green and compact when the media was supplemented with only BAP and green and compact calli are usually more efficient to produce somatic embryos. Observations with respect to the narure of callus were more similar with those reported by Anisuzzaman *et al.* (2010). Colour of the calli has gradually changed to green on MS medium suplemented with BAP (1 mgL^{-1}). Mallela *et al.* (2009) also observed that calli were white, light green and friable on MS supplemented with auxine alone (2,4-D, NAA, TDZ), while cytokinins such as BAP prduced green nodular regenerative calli.

In okra, calli have turned brown, due to the polyphenols. Supplemetation of activated charcoal (0.1 mM) and ascorbic acid (0.1 mM) in callus maturaton media increased the callus weight by 0.60 and 0.55 g, respectively. Survival of calli also increased to 85 per cent in CEM conataining ascorbic acid and CEM containing acivated charcoal. Activated charcoal was effective as antibrowning agent with respect to callus weight and germintion percentage. Similar observations were recorded by Irshad *et al*. (2017) when they used ascorbic acid, charcoal and combination of both during the callus initiation stage to obtain 67.7 per cent callus

generation. Dhande *et al*. (2012) have also observed that the activated charcoal increased the *in vitro* growth of okra during rooting.

The best time for callus sub-culturing on Callus Embryogenesis Media (CEM) was 10 to 12 days after callus initiation. After 21 days, highest callus weight was found in CEM 6 which had the highest concentration of cytokinin. All the nine treatments were tried could not induce somatic embryogenesis. However, Mallela *et al.* (2009) obtained somatic embryos from leaf and cotyledonary explants, on MS medium with low concentration of TDZ, NAA and 2,4-D. For shooting, they transferred the calli to MS medium with BAP (1 mg/L), NAA (0.5 mg/L) and TDZ (0.04 mg/L). Better rooting was observed with NAA (1 mg/L) or IAA (1 mg/L). The use of seeds as explant may be the reason for the failure in somatic embryogenesis in the current study.

5.2 ISOLATION OF TARGET GENE (*βC1* GENE OF YVMV)

The primary silencing mechanism in a miRNA pathway involves the pairing of plant miRNAs with the target RNA to activate the silencing machinery (Llave *et al*., 2002; Jones-Rhodes and Bartel, 2004). Hence the accuracy of gene silencing depends on the selection of target sequence for construction of ihpRNA cassette. The *βC1* gene was selected as target gene to develop resistance against YVMV through RNAi, because satellite DNA (β DNA) and *βC1*-ORF are responsible for countering host defence responses and symptoms development in host plants (Saunders *et al*., 2000; Briddon *et al*., 2001; Jose and Usha, 2003; Saeed *et al*., 2005; Gaur and Rathore, 2009).

DNA was isolated following the standard protocol (Ghosh *et al*., 2009) with slight modification. The protocol was originally designed for isolation of *Begomovirus* DNA from jute and other mucilaginous plants. High mucilage content of okra is always a constraint in DNA isolation. During the isolation process, sodium chloride 3M was additionally used to reduce the mucilage content, and potassium acetate 0.3M helped to precipitate polysaccharides. Chloroform : Isoamy alcohol treatment was repeated after the sodium chloride treatment to reduce the pigments and NaCl molecules. Similarly, Chen and Chen (2004) also have observed that NaCl is

reducing the viscosity of mucilage contents. Higher levels of phenol-binding reagent PVP and NaCl were shown to reduce the polyphenols and polysaccharides in DNA (Ali *et al*., 2019). Greco *et al*. (2014) found that potassium acetate is more effective for precipitation of DNA contaminants such as polysaccharides during DNA isolation from *Ectocarpus siliculosus*.

The ORF2 (187 bp) was selected for the preparation sense strand, because the sense strand size of less than 400 bp is reported to increase the efficiency of RNAi (Akashi *et al.,* 2001). Similarly, Helliwell and Waterhouse (2003), while testing 50 to 1000 bp of *Flowering Locus C* and *Phytoene desaturase* in *Arabidopsis*, have shown that 200 to 400 bp sense and antisense strands, is more effective. The ORF2 of *βC1* gene was also selected based on the results of restriction mapping, virus suppressor prediction and siRNA target prediction by IDT.

The siRNA guides the RNAi induced silencing complex (RISC) to the complementary DICER substrate site. Hence it is preferred to have siRNA target sites in the sequence. Many tools and algorithms have been reported for predicting siRNA target regions (Krek *et al.,* 2005; Qiu *et al.,* 2007; Ketting, 2011). In the current study, the tool provided by Integrated DNA technologies (IDT) was used to find out the location of the DICER substrate. Restriction mapper was performed to see the presence of restriction enzyme sites present in the sense and antisense region. In this project, the ligation independent technology was used for the preparation of ihpRNA cassette, hence restriction sites are not making any trouble during ihpRNA construct designing.

Viral Suppression Regions (VSRs) present in the sense strand will affect the RNAi mechanism. VSRs are the viral proteins having capability to block the siRNA produced during RNA interference, which is a counter defence mechanism. This VSRs region was found in almost all plants, mammalian or insect viruses, and showed diversity within and across the kingdoms (Ekatpure, 2020). VsupPred was used to find the presence of viral suppressor regions. Scores for the prediction of VSRs region should lie between 0.0 and 1.0, higher the score, better the confidence of prediction. In the ORF2 of *βC1* gene, the prediction score had a statistical value 0.625, showing very low probability for the presence of VSRs.

Freely available online software, Primer3 (Input version 0.4.0), was used for designing the primers to amplify ORF2, instead of manual designing for more accuracy. The BLASTn result had shown that the sequenced region was present in YVMV isolate and in some OELV isolates also.

5.3 ihpRNA VECTOR CONSTRUCTION

The pRNAi-LIC consisted of three *Sma*I restriction sites, hence digestion with *Sma*I yielded three different blunt ended fragments viz. (i) vector backbone (9842 bp), (ii) Pdk intron (1641 bp) and (iii) *ccdB* gene (614 bp). Four different adapters were essential for the easy and correct insertion of sense and antisense strands with Pdk intron during ligation. LIC-1 and LIC-2 adapters were necessary for the ligation of sense strand with CaMV 35S promoter region of backbone and Pdk intron. LIC-3 and LIC-4 adapters were necessary for the ligation of antisense strand with Pdk intron and Nos terminator region of backbone. According to Xu *et al*. (2010), pRNAi-LIC contain Pdk intron region with chloramphenicol gene placed between LIC3 and LIC2 which allows the growth of transformed colonies. Conley *et al*. (1986) and Wesley *et al*. (2001) reported that Pdk intron without antibiotic gene allows false-positive transformants.

During ligation, the *ccdB* gene positioned between LIC2 and LIC4 will be replaced by antisense strand, allowing the screening of the putative recombinant colonies. pHELLSGATE (Helliwell and Waterhouse, 2003) and pANDA (Miki and Shimamoto, 2004) are the frequently used Gateway recombination based plasmid vectors in RNAi studies (Earley *et al*., 2006; Karimi *et al*., 2007). pHANNIBAL, pKANNIBAL, pSAT, pSH, pGEM-WIZ *etc.* are the conventional cloning vectors based on restriction digestion and cloning technology, available at Commonwealth Scientific and Industrial Research Organisation (CSIRO) (Wesley *et al*., 2001, Helliwell and Waterhouse, 2005; Bao and Cagan, 2006; Hirai *et al*., 2007). These vectors require several rounds of restriction digestion and ligation which is time consuming and costly.

PCR product-1 (Sense strand), PCR product-2 (Antisense strand) and vector backbone were ligated using T4 DNA ligase. Addition of dATP and dTTP to the blunt

ends of PCR products and the vector respectively, has effected the ligation. T4 DNA polymerase is popular in the cloning of blunt ended fragments because of its strong exonuclease and polymerase activity. Single stranded exonuclease activity of T4 DNA polymerase not only repairs the ends of the PCR product but also removes the remaining primers in the reaction, and PCR product purification is not required after the reaction (Maniatis *et al*., 1982). In this study, vector with two antibiotic resistance genes was used. Multiple antibiotic resistance genes help to improve the selection of transformed colonies on selection media (Xu *et al*.,2010).

The ligated mixture was incubated with the competent cells of *E. coli* (DH5α), cells and spread on LB media containing Kanamycin and Chloramphenicol as antibiotics. Preparation of competent calls of DH5α is critical task which made easy by using Transform Aid Bacterial Transformation Kit (Thermo scientific). DH5α strain of *E. coli* is more advantageous over *S. cerevisiae* with its faster growth rate, higher plasmid yield and better stability during cloning (Zhang *et al*., 1998; Yu *et al*., 2000; Thomasan *et al*., 2014). Hence, *E. coli* (DH5α) was used in cloning of ihpRNA cassette. Grown colonies were confirmed using colony PCR and plasmid was reconfirmed through sequencing for the alignment and orientation of sense and antisense strands in the plasmid. Similar method of screening of transformed colonies on antibiotic medium reported by Ekatpure (2020), where the transformed colonies grown on the media containing Rifampicin (25 mg L^{-1}) and Spectinomycin (100 mg L^{-1}) antibiotic. Transformed colonies were re-streaked on fresh plates with antibiotics and confirmed with colony PCR.

Agrobacterium is a well-known and commonly used bacterium for the transfer of foreign gene into plants. The plasmid transformation into agrobacterium is easier compared to the transformation to *E. coli.* According to Gynheung (1988), freeze and thaw method of transforming plasmid into *Agrobacterium* was easy quick and with high transformation efficiency. *Agrobacterium* strain GV3101, most reliable for the gene cloning works in the dicots, was used in the study. The plasmids were incubated with the competent cells of GV3101 cells and spread on LB medium containing Kanamycin, Chloramphenicol and Rifampicin as antibiotics. Colonies were confirmed using colony PCR and through sequencing and alignment, the orientation of sense and

antisense strands in the plasmid was verified. Similar protocol of plasmid transformation into *Agrobacterium* was followed by Tripathi *et al*. (2005), Krubphachaya *et al*. (2007), Sreeramanan *et al*. (2010), Jyothishwaran *et al*. (2007), Lekshmi (2016), Jadhav (2019) and Ekatpure (2020).

5.4 *IN PLANTA* TRANSFORMATION AND CONFIRMATION OF TRANSGENEISS

In planta transformation is an easier strategy for *in vitro* recalcitrant plants such as okra. Surface sterilised overnight water soaked germinated seeds were used for co-cultivation with *Agrobacterium* in MS containing 5 per cent sucrose and 100 µM acetosyringone. Well germinated seeds were carefully prickled with sterile needle syringe. Needle was inserted into seed as much as possible to make injury to plumule region of the germinated seeds. During co-cultivation, prickled seeds were incubated with *Agrobacterium* in a shaker at 100 rpm for 30 min. Co-cultivated seeds were sown in FYM and soil mixture and maintained in containment facility. Similarly, Narendran *et al.* (2013) have transformed the okra plants with *cry1Ac* gene against okra shoot and fruit borer through *in planta* transformation technique and reported that embryos and the shoot buds just emerging from the plumule are best for agro-inoculation. Similar observations were recorded by Manickavasagam *et al*. (2015), when pCAMBIA1301 with *bar* gene was transferred through *Agrobacterium tumefaciens* (EHA 105). Maximum transformation efficiency (18.3%) was observed when pre-cultured okra seeds were sonicated for 30 min. and vacuum infiltrated for 3 min. in *Agrobacterium* suspension containing 100 µM acetosyringone.

DNA was isolated from 20 fifteen-day old transformed plants and amplified with sense strand specific primers to confirm the ihpRNA cassette integration into okra genome. Of them, four were positive (11.42% transformation efficiency). The efficiency is almost similar with that (18.3%) reported by Manickavasagam *et al*. (2015).

5.5 CONFIRMATION OF mRNA CODING FOR siRNA SYNTHESIS IN TRANSGENIC PLANTS

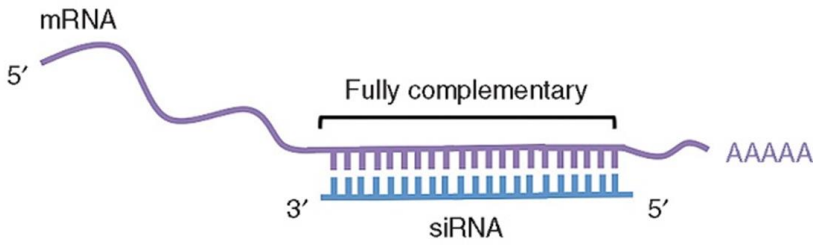
Isolation of good quality and quantity of RNA was essential for the cDNA preparation. Total RNA isolation from okra was quite difficult due to mucilage content present in the sample. To reduce the mucilage content, 1.5 M NaCl treatment was given before the chloroform treatment and incubated at room temperature for 10 min. Putative transformants were evaluated for the siRNA synthesis through RT-PCR and observed that *βC1* gene is expressed in the transformed plants. Similarly, in tobacco, using qRT-PCR, Thu *et al*. (2016) have observed the silencing potential of the pK7GW-CPi for coat protein gene of *Soybean mosaic virus* (SMV) and *Bean Yellow mosaic virus* (BYMV).

5.6 EVALUATION OF RESISTANCE IN TRANSFORMANTS

Silencing of the target gene and resistance development in transformants were evaluated under controlled conditions. All putative transformants with control and YVMV infected plants were placed together under insect cage within double walled glasshouse for one week. Viruliferous whiteflies were released into insect cage and YVMV symptoms were observed in control plants and one transformed plant after 10 days of infection. The infection observed in transformed plant was may be due to the failure in gene expression. Other than that all the three infected transformed plants were healthy with normal growth. Similar screening methodology was used by Sharma (2020) to evaluate the transgenic plants produce by inserting hairpin RNAi construct containing AC1/AC2 and AC1/AC4 gene against YVMV. They have reported nearly 90 per cent success through this method.

5.7 CONCLUSION

The present investigation was successful in the development of ihpRNA- βC1 using pRNAi-LIC (CD3-1285) plasmid vector. Four transgenic plants were successfully screened for ihpRNA- βC1 vector and three transgenic plants identified for YVMV resistance under controlled condition. The further evaluation is needed in next generation for identification of stable transformants.



SUMMARY

6. SUMMARY

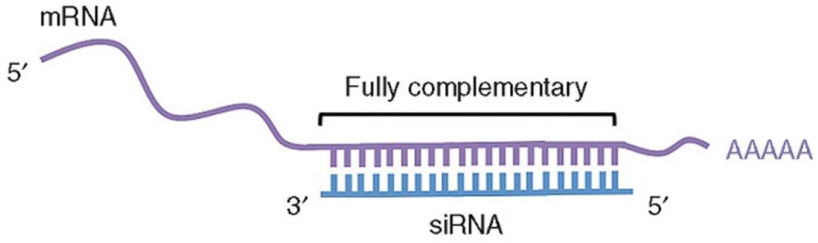
The study entitled “RNA mediated resistance to yellow vein mosaic virus in okra” was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Kerala Agricultural University, Thrissur, during September 2017 to May 2021. The objective of the study was to develop ihpRNA constructs targeting *βC1* gene of *Yellow vein mosaic virus*, to generate transformants of okra cultivar Salkeerthi carrying the ihpRNA cassettes and to confirm the transgenesis.

- The high yielding and YVMV susceptible popular okra cv. Salkeerthi was selected for development of YVMV resistance using RNAi mechanism.
- Total DNA was isolated from the YVMV infected plant and *βC1* gene of the virus was amplified using primers VβC1F and VβC1R. Sequence information of PCR product has revealed that the gene is 90-95 per cent identical with Indian isolates.
- The *βC1* gene sequence was analysed using IDT software and 10 siRNAs were found at three different positions (19-44, 34-59, 99-124 bp).
- Through Restriction Mapper, it was confirmed that the sequence selected for the preparation of sense and antisense strand, do not possess recognition sites for *Sma*I, *Hind*III amd *Mau*BI restriction enzymes which are present in the pRNAiLIC.
- The output of VSupPred revealed that the fragment is a Non-Viral Silencing Suppressor Region (NVSR) with a prediction score of 0.625.
- The efficient and ligation independent plasmid pRNAi-LIC (CD3-1285) procured from ABRC was used for making ihpRNA cassette.

- The *Sma*I digested plasmid produces three fragments, vector backbone (9842 bp), Pdk intron (1641 bp) and ccdB gene (614 bp) and the digested plasmid was treated with dTTP.
- Product-1 was PCR amplified with VLIC1 and VLIC2 primers, using DNA of YVMV infected plant as template.
- Product-2 was PCR amplified with VLIC3 and VLIC4 primers using product-1 as template.
- PCR product-1 and PCR product-2 were eluted from gel using NucleoSpin® PCR Clean-up gel extraction kit and treated with dATP.
- The dATP treated PCR products and dTTP treated *Sma*I digested plasmid were mixed together and incubated at 65ºC for 5 min followed by 22ºC for 15 min. for ligation.
- Ligated product was successfully transformed in competent cells of *E. coli* (DH5α), incubated on LB media containing Kanamycin and Chloramphenicol.
- Plasmid was isolated from positive DH5α colony confirmed by colony PCR and sequenced using the primers VLIC5 and VLIC6. Sequence data had shown that both sense and antisense strands are at right position and direction.
- Plasmid containing ihpRNA cassette was successfully transformed in competent cells of *Agrobacterium* (GV3101), incubated on LB media containing Kanamycin, Chloramphenicol and Rifampicin with 100 per cent transformation efficiency.
- Plasmid was isolated from positive GV3101 colony confirmed by colony PCR and sequenced using the primers VLIC5 and VLIC6. Sequence data revealed that both sense and antisense strands are at right position and direction.
- ihpRNA cassette was successfully transformed into okra cv. Salkeerthi using *in planta* method of *Agrobacterium* mediated transformation with transformation

efficiency of 11.42 per cent. Transformation was confirmed through amplification of sense strand using the primer (VLIC1 and VLIC5).

- cDNA was prepared from the total RNA isolated from transformed and control plants. siRNA and Ubiquitin coding mRNA was confirmed using the primers (VLIC1 and VLIC5).
- Silencing potential of the plants was evaluated by keeping the transformed and control plants along with YVMV infected plants inside containment facility and whiteflies released into insect cage for infection.
- All the control plants and one transgenic plant have shown the YVMV symptoms after 10 days. Three transgenic plants were healthy with no symptoms.



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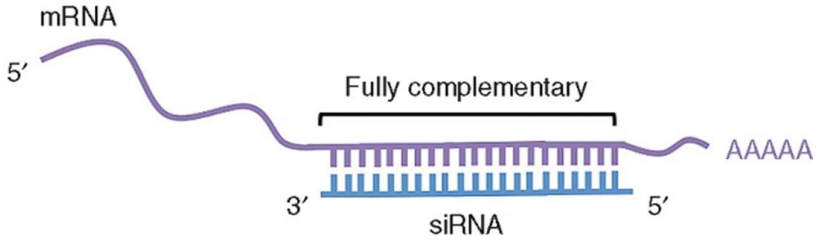

APPENDICES

APPENDIX I

Chemical composition of the media employed for the *in vitro* culture of okra (*Abelmoschus esculentus* L. Moench) cv. Salkeerthi, Murashige and Skoog medium (MS)

Stock I- Macro nutrients (mgL^{-1})	
$MgSO_4.7H_2O$	370.00
$CaCl_2.2H_2O$	440.00
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
Stock II- Micro nutrients (mgL^{-1})	
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$CuSO_4.5H_2O$	0.025
$AlCl_3$	0.83
H_3BO_3	6.20
$Na_4MoO_4.2H_2O$	0.25
Stock III- Iron Sources (mgL^{-1})	
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Stock IV- Vitamins (mgL^{-1})	
Nicotinic acid	0.50
Pyridoxine HCL	0.50
Thiamine HCL	0.10
Glycine	2.00
Myo-Inositol	100.00
Sucrose (gL^{-1})	30.00
Agar (gL^{-1})	8.00

APPENDIX II

Chemicals for DNA isolation from okra leaf (CTAB method) and agarose gel electrophoresis

A)	**CTAB buffer (pH 8.0)**	**Concentration**	**For 100 mL**
	CTAB (MW=364.45)	2 %	2 g
	Tris- Buffer (MW=121.1)	100 mM	1.2114 g
	NaCl (MW=58.44)	1.4 M	8.186 g
	EDTA (MW=372.24)	20 mM	0.7448
B)	**TE buffer (pH 8.0)**		**For 100 mL**
	Tris buffer (MW=121.1)	10 mM	121 mg
	EDTA (MW=372.24)	1 mM	37 mg
C)	**50X TAE buffer (pH 8.0)**		**For 500 mL**
	Tris buffer (MW=121.1)	2 M	121 g
	Glacial acetic acid (MW=60.05)	5.71 %	28.5 mL
	EDTA (MW=372.24)	0.5 M	50 mL

APPENDIX III

siRNA target (DICER substrate) prediction parameters

siRNA target prediction in virus genome

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 βC1 region were selected and analysed for siRNA attributes described by Reynolds et al. (2004) for higher efficiency.

Attributes in selected siRNA target region

Sl. No.	Reynolds' Criteria	Attributes in selected siRNA target
1.	30-50% GC content	✓
2.	At least 3 A/U bases at position 15-19 (sense strand	✓
3.	Absence of internal repeats	✓
4.	An 'A' base at position 19 (sense strand)	X
5.	An 'A' base at position 3 (sense strand)	X
6.	A 'U' base at position 10 (sense strand)	X
7.	A base other than 'G' of 'C' at 19 (sense strand)	✓
8.	A base other than 'G' at position 13 (sense strand)	✓

APPENDIX IV

>GQ870263.1 Plant RNAi vector pRNAi-LIC, complete sequence

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AAGGCACCGCGCCGCCTGGCCTCGGTGGGCGTCACTTCCTCGCTGCGCTC
AAGTGCGCGGTACAGGGTCGAGCGATGCACGCCAAGCAGTGCAGCCGCC
TCTTTCACGGTGCGGCCTTCCTGGTCGATCAGCTCGCGGGCGTGCGCGATC
TGTGCCGGGGTGAGGGTAGGGCGGGGGCCAAACTTCACGCCTCGGGCCTT

GGCGGCCTCGCGCCCGCTCCGGGTGCGGTCGATGATTAGGGAACGCTCGA
ACTCGGCAATGCCGGCGAACACGGTCAACACCATGCGGCCGGCCGGCGTG
GTGGTGTCGGCCCACGGCTCTGCCAGGCTACGCAGGCCCGCGCCGGCCTC
CTGGATGCGCTCGGCAATGTCCAGTAGGTCGCGGGTGCTGCGGGCCAGGC
GGTCTAGCCTGGTCACTGTCACAACGTCGCCAGGGCGTAGGTGGTCAAGC
ATCCTGGCCAGCTCCGGGCGGTCGCGCCTGGTGCCGGTGATCTTCTCGGA
AAACAGCTTGGTGCAGCCGGCCGCGTGCAGTTCGGCCCGTTGGTTGGTCA
AGTCCTGGTCGTCGGTGCTGACGCGGGCATAGCCCAGCAGGCCAGCGGCG
GCGCTCTTGTTCATGGCGTAATGTCTCCGGTTCTAGTCGCAAGTATTCTAC
TTTATGCGACTAAAACACGCGACAAGAAAACGCCAGGAAAAGGGCAGGG
CGGCAGCCTGTCGCGTAACTTAGGACTTGTGCGACATGTCGTTTTCAGAA
GACGGCTGCACTGAACGTCAGAAGCCGACTGCACTATAGCAGCGGAGGG
GTTGGATCAAAGTACTTTGATCCCGAGGGGAACCCTGTGGTTGGCATGCA
CATACAAATGGACGAACGGATAAACCTTTTCACGCCCTTTTAAATATCCGT
TATTCTAATAAACGCTCTTTTCTCTTAGGTTTACCCGCCAATATATCCTGTC
AAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCTGATCCAAGC
TCAAGCTGCTCTAGCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGG
CGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATG
TGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGAC
GTTGTAAAACGACGGCCAGTGCC

APPENDIX V

Sequence information of βC1 gene sequenced through sanger sequencing

>Forward βC1 YVMV

TTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTGATGTGATTGTTCC
TTCTAGACCGTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGTAGTC
GATGATGAATGTTTTTGTTGATATGGCTGGAGATTTTGTTGAACTGATCTG
CATATGCACCGATATCCTTTGTTGTTGCATGAGC

>Reverse βC1 YVMV

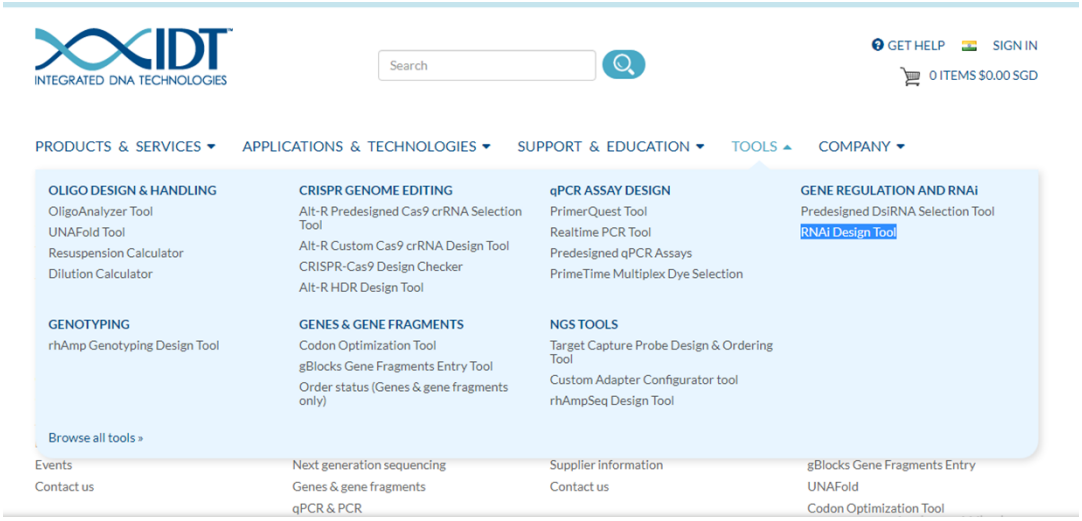
GCTCATGCAACAACAAAGGATATCGGTGCATATGCAGATCAGTTCAACAA
AATCTCCAGCCATATCAACAAAAACATTCATCATCGACTACACATACCAA
CAATTACACATTCCATTCGACTTCAACGGTCTAGAAGGAACAATCACATC
AACCTTCAAATTTCATTACTGGGGTTCAAAAGCAGAA

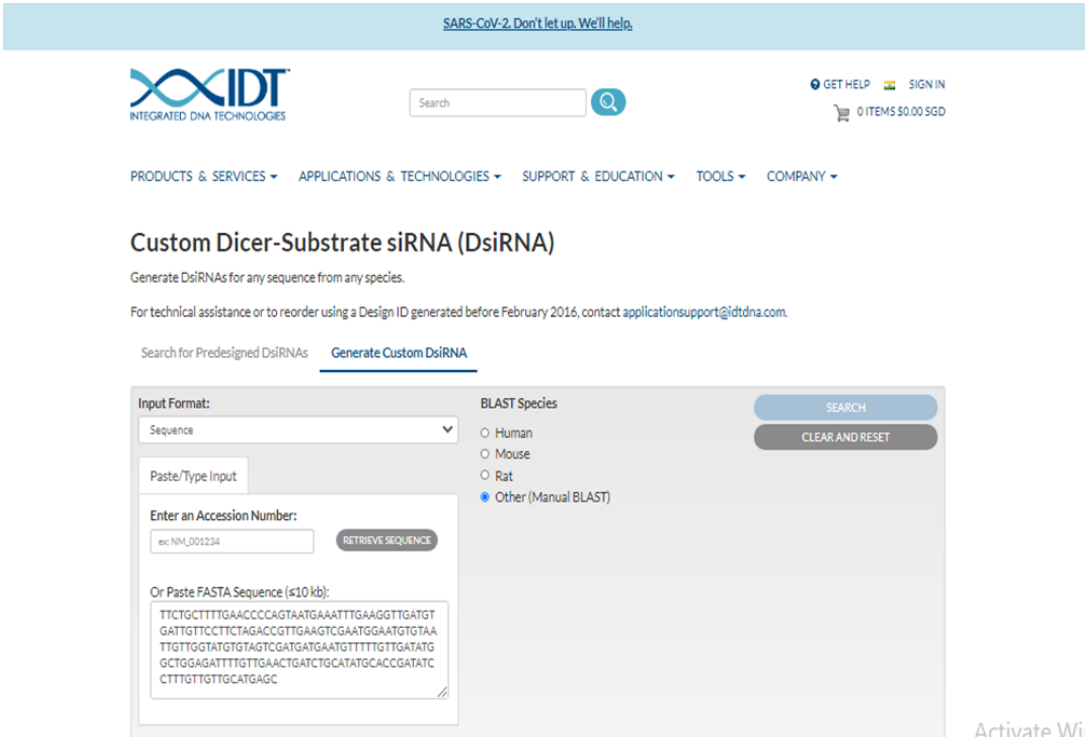
>Contig

TTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTGATGTGATTGTTCC
TTCTAGACCGTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGTAGTC
GATGATGAATGTTTTTGTTGATATGGCTGGAGATTTTGTTGAACTGATCTG
CATATGCACCGATATCCTTTGTTGTTGCATGAGC

APPENDIX VI

siRNA in the *βC1*, analyse with Integrated DNA Technologies (IDT)



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Search Input
Cross-Reacting Species
Design ID
2 nmol
Custom Input
Other (Manual BLAST)
CD.Ri.231488.13.1
$145.00 SGD
ADD TO CART
Hide product details -
1
93
187
Cross-Reacting Transcripts none
Sequence Positions 99-124
Sequence Details Sense and antisense sequences
Sequence
Strand
5' rArGrUrCrGrArUrGrArUrGrArArUrGrUrUrUrUrGrUrUGA 3'
+
5' rUrCrArArCrArArArArArCrArUrUrCrArUrCrArUrCrGrArCrUrArC 3'
-


DsiRNA
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Search Input
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Design ID
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Custom Input
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Sequence Details Sense and antisense sequences
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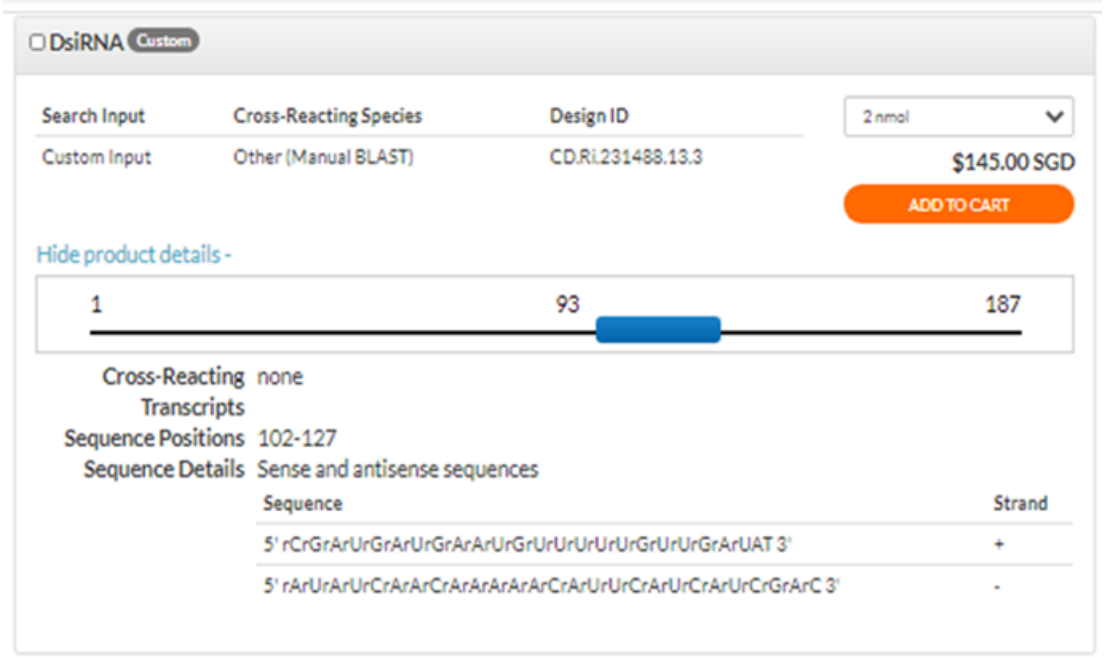
+
5' rCrArCrArUrCrArArCrCrUrUrCrArArArUrUrUrCrArUrUrArCrUrG 3'
-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Search Input
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Design ID
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ADD TO CART
Hide product details -
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93
187
Cross-Reacting Transcripts
none
Sequence Positions
102-127
Sequence Details
Sense and antisense sequences
Sequence
Strand
5' rCrGrArUrGrArUrGrArArUrGrUrUrUrUrGrUrUrGrArUAT 3'
+
5' rArUrArUrCrArArCrArArArArArCrArUrUrCrArUrCrArUrCrGrArC 3'
-


DsiRNA
Custom
Search Input
Cross-Reacting Species
Design ID
2 nmol
Custom Input
Other (Manual BLAST)
CD.Ri.231488.13.4
$145.00 SGD
ADD TO CART
Hide product details -
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Cross-Reacting Transcripts
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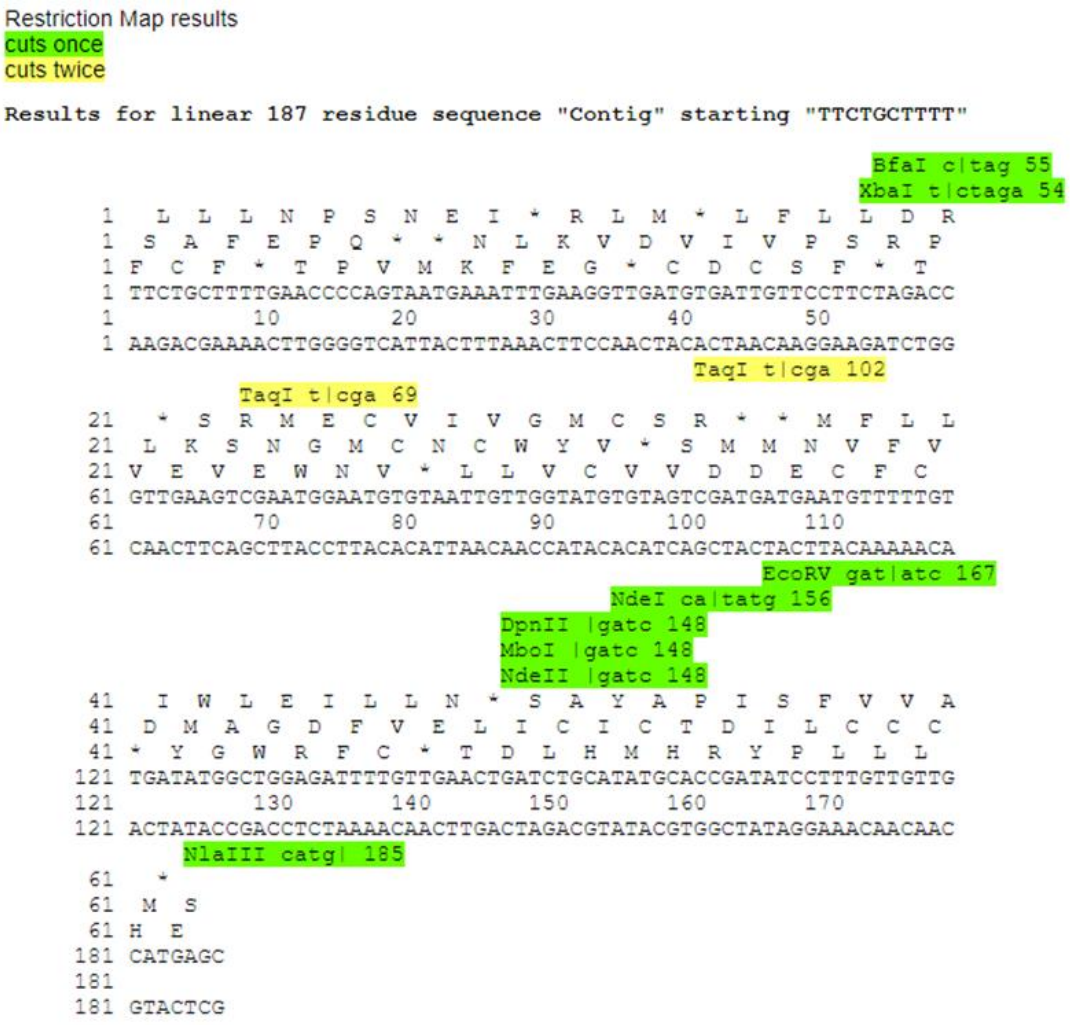
Sequence Positions
34-59
Sequence Details
Sense and antisense sequences
Sequence
Strand
5' rGrGrUrUrGrArUrGrUrGrArUrUrGrUrUrCrCrUrUrCrUrAGA 3'
+
5' rUrCrUrArGrArArGrGrArArCrArArUrCrArCrArUrCrArArCrCrUrU 3'
-

APPENDIX VII

A textual map showing the positions of restriction endonuclease recognition sites



Restriction endonuclease recognition sites and position in βC1 sequence

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	none
AfeI agc|gct	none
AflII c|ttaag	none
AgeI a|ccggt	none
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

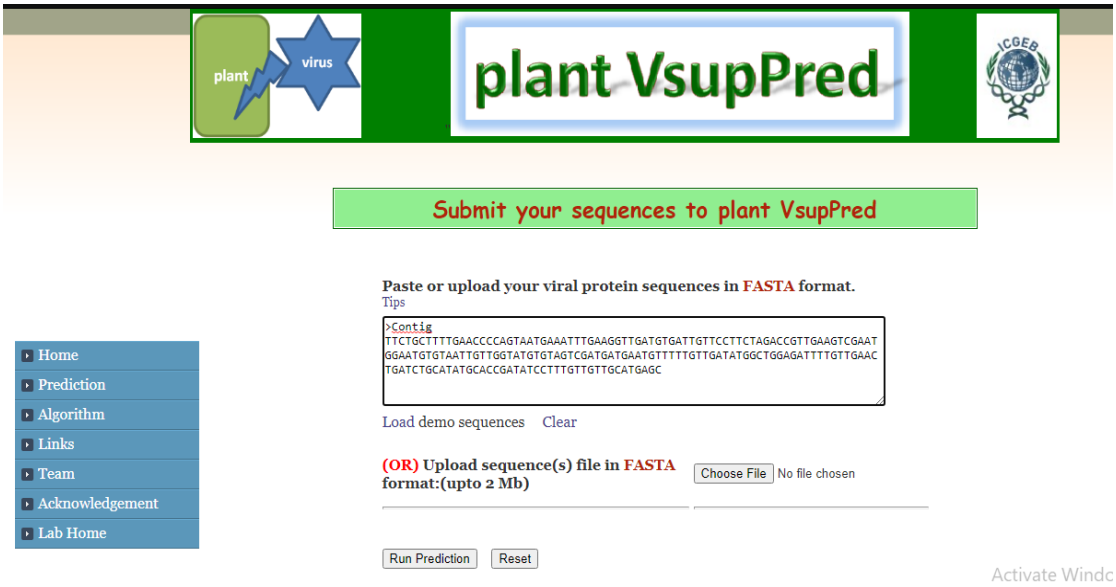
BbuI gcatg|c	none
BcuI a|ctagt	none
BclI t|gatca	none
BfaI c|tag	55
BfrI c|ttaag	none
BfrBI atg|cat	none
BglII a|gatct	none
BlnI c|ctagg	none
BseCI at|cgat	none
BsePI g|cgcgc	none
BseX3I c|ggccg	none
BshTI a|ccggt	none
Bsp1407I t|gtaca	none
Bsp19I c|catgg	none
BspDI at|cgat	none
BspEI t|ccgga	none
BsrGI t|gtaca	none
BssHII g|cgcgc	none
BstUI cg|cg	none
ClaI at|cgat	none
DpnII |gatc	148
DraI ttt|aaa	none
EagI c|ggccg	none
EcoRI g|aattc	none
EcoRV gat|atc	167
EgeI ggc|gcc	none
FseI ggccgg|cc	none
FspI tgc|gca	none
HaeIII gg|cc	none
HincII gty|rac	none

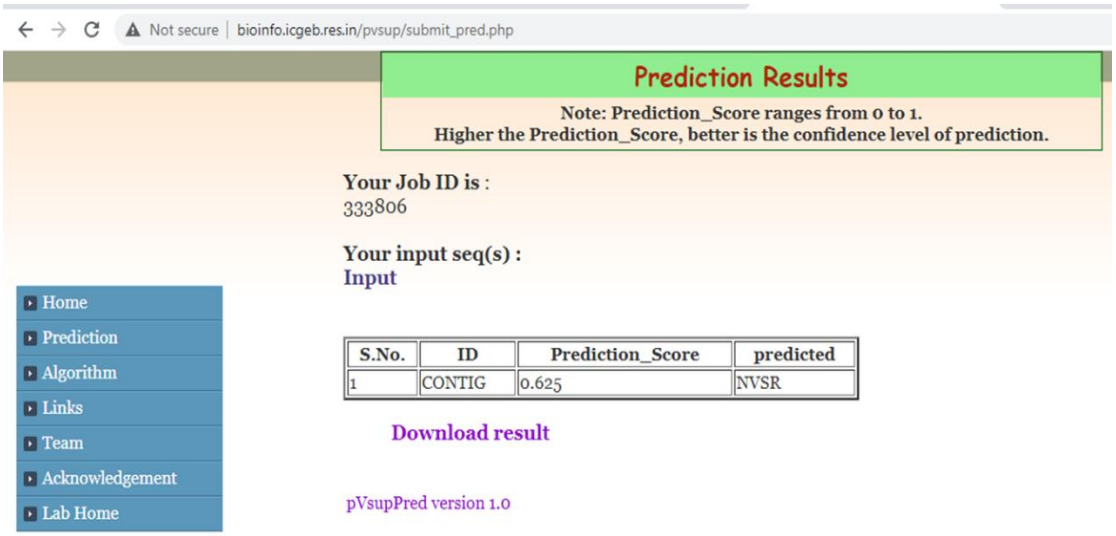
HindIII a|agctt	none
HinfI g|antc	none
HpaI gtt|aac	none
HpaII c|cgg	none
KasI g|gcgcc	none
KpnI ggtac|c	none
MboI |gatc	148
MfeI c|aattg	none
MluI a|cgcgt	none
MscI tgg|cca	none
MseI t|taa	none
MspI c|cgg	none
NaeI gcc|ggc	none
NarI gg|cgcc	none
NcoI c|catgg	none
NdeI ca|tatg	156
NdeII |gatc	148
NgoMIV g|ccggc	none
NheI g|ctagc	none
NlaIII catg|	185
NotI gc|ggccgc	none
NruI tcg|cga	none
NsiI atgca|t	none
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	none
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	69, 102
TliI c|tcgag	none
VspI at|taat	none
XbaI t|ctaga	54
XhoI c|tcgag	none
XmaI c|ccggg	none

APPENDIX VIII

Viral silencing suppressor prediction using the tool VSupPred





APPENDIX IX

CLUSTAL W alignment to check orientation of the sense fragments in plasmid isolated from DH5α

```
DH5alpha Plasmid Sense Strand
CLUSTAL O(1.2.4) multiple sequence alignment


VBDHP_S_1197        CCTCTTTCCCGCCTTTTTCTTACTATGTTGTTTATGTAACAATATATTTATAAATTATTT        60
Target_S_187bp      ------------------------------------------------------------        0


VBDHP_S_1197        TTTCACAATTATAACAACTATATTATTATAATCATACTAATTAACATCACTTAACTATTT        120
Target_S_187bp      ------------------------------------------------------------        0


VBDHP_S_1197        TATACTAAAAGGAAAAAAGAAAATAATTATTTCCTTACCAAGGGCCCTGAGGAGAAGAGC        180
Target_S_187bp      ------------------------------------------------------------        0


VBDHP_S_1197        CCTGCTCATGCAACAACAAAGGATATCGGTGCATATGCAGATCAGTTCAACAAAATCTCC        240
Target_S_187bp      ---GCTCATGCAACAACAAAGGATATCGGTGCATATGCAGATCAGTTCAACAAAATCTCC        57
                       ***********************************************************


VBDHP_S_1197        AGCCATATCAACAAAAACATTCATCATCGACTACACATACCAACAATTACACATTCCATT        300
Target_S_187bp      AGCCATATCAACAAAAACATTCATCATCGACTACACATACCAACAATTACACATTCCATT        117
                    ************************************************************


VBDHP_S_1197        CGACTTCAACGGTCTAGAAGGAACAATCACATCAACCTTCAAATTTCATTACTGGGGTTC        360
Target_S_187bp      CGACTTCAACGGTCTAGAAGGAACAATCACATCAACCTTCAAATTTCATTACTGGGGTTC        177
                    ************************************************************


VBDHP_S_1197        AAAAGCAGAAAGGGTCTTGTCGTCGAGCTCGGATCCCCGAAGGGCAATTCTGCAGATCCT        420
Target_S_187bp      AAAAGCAGAA----------------------------------------------------        187
                    **********


VBDHP_S_1197        CTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGTCTTGCGAAGGATAGTGGGATTG        480
Target_S_187bp      ------------------------------------------------------------        187


VBDHP_S_1197        TGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCTTTGAAGACGTGGT        540
Target_S_187bp      ------------------------------------------------------------        187


VBDHP_S_1197        TGGAACGTCTTCTTTTTCCACGATGTTCCTCGTGGGTGGGGGTCCATCTTTGGGACCACT        600
Target_S_187bp      ------------------------------------------------------------        187


VBDHP_S_1197        GTCGGTAGAGGCATCTTGAACGATAGCCTTTCCTTTATCGCAATGATGGCATTTGTAGAA        660
Target_S_187bp      ------------------------------------------------------------        187
```

CLUSTAL W alignment to check orientation of the antisense fragments in plasmid isolated from DH5α

Sense and Antisense.txt - Notepad

File Edit Format View Help

```
DH5alpha plasmid Antisense strand
CLUSTAL O(1.2.4) multiple sequence alignment


VBDHP_AS_1272       CCCGCCGTATTGCCATGTTTGACGATCGGGGAATTCGAGCTCTAGAGCACACGACCCTTC      60
Target_AS_187bp     ------------------------------------------------------------      0


VBDHP_AS_1272       GACGACAAGACCCTTTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTGATGTGATT      120
Target_AS_187bp     --------------TTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTGATGTGATT      46
                                  *********************************************


VBDHP_AS_1272       GTTCCTTCTAGACCGTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGTAGTCGATG      180
Target_AS_187bp     GTTCCTTCTAGACCGTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGTAGTCGATG      106
                    ************************************************************


VBDHP_AS_1272       ATGAATGTTTTTGTTGATATGGCTGGAGATTTTGTTGAACTGATCTGCATATGCACCGAT      240
Target_AS_187bp     ATGAATGTTTTTGTTGATATGGCTGGAGATTTTGTTGAACTGATCTGCATATGCACCGAT      166
                    ************************************************************


VBDHP_AS_1272       ATCCTTTGTTGTTGCATGAGCAGGGCTCTTCTCCTCAAGGGTTCCGTGCTGGAACCAACT      300
Target_AS_187bp     ATCCTTTGTTGTTGCATGAGC----------------------------------------      187
                    ********************


VBDHP_AS_1272       GTAATCAATCCAAATGTAAGATCAATGATAACACAATGACATGATCTATCATGTTACCTT      360
Target_AS_187bp     ------------------------------------------------------------      187


VBDHP_AS_1272       GTTTATTCATGTTCGACTAATTCATTTAATTAATAGTCAATCCATTTAGAAGTTAATAAA      420
Target_AS_187bp     ------------------------------------------------------------      187


VBDHP_AS_1272       ACTACAAGTATTATTTAGAAATTAATAAGAATGTTGATTGAAAATAATACTATATAAAAT      480
Target_AS_187bp     ------------------------------------------------------------      187


VBDHP_AS_1272       TGATAGATCTTGCGCTTTGTTATATTAGCATTAGATTATGTTTTGTTACATTAGATTACT      540
Target_AS_187bp     ------------------------------------------------------------      187


VBDHP_AS_1272       GTTTCTATTAGTTTGATATTATTTGTTACTTTAGCTTGTTATTTAATATTTTGTTTATTG      600
Target_AS_187bp     ------------------------------------------------------------      187


VBDHP_AS_1272       ATAAATTACAAGCAGATTGGAATTTCTAACAAAATATTTATTAACTTTTAAACTAAAATA      660
Target_AS_187bp     ------------------------------------------------------------      187
```

APPENDIX X

CLUSTAL W alignment to check orientation of the sense fragments in plasmid isolated from GV3101

```
GV3101 plasmid Sense
CLUSTAL O(1.2.4) multiple sequence alignment

VBGV3101P_S_878bp      ACCCCTTTCCCGGGCCTCTTCTTACTATGTTGTTTATGTAAACAATATATTTATAAATTA     60
Target_S_187bp         ------------------------------------------------------------     0

VBGV3101P_S_878bp      TTTTTTCACAATTATAACAACTATATTATTATAATCATACTAATTAACATCACTTAACTA     120
Target_S_187bp         ------------------------------------------------------------     0

VBGV3101P_S_878bp      TTTTATACTAAAAGGAAAAAAGAAAATAATTATTTCCTTACCAAGGGCCCTGAGGAGAAG     180
Target_S_187bp         ------------------------------------------------------------     0

VBGV3101P_S_878bp      AGCCCTGCTCATGCAACAACAAAGGATATCGGTGCATATGCAGATCAGTTCAACAAAATC     240
Target_S_187bp         ------GCTCATGCAACAACAAAGGATATCGGTGCATATGCAGATCAGTTCAACAAAATC     54
                             **************************************************

VBGV3101P_S_878bp      TCCAGCCATATCAACAAAAACATTCATCATCGACTACACATACCAACAATTACACATTCC     300
Target_S_187bp         TCCAGCCATATCAACAAAAACATTCATCATCGACTACACATACCAACAATTACACATTCC     114
                       ************************************************************

VBGV3101P_S_878bp      ATTCGACTTCAACGGTCTAGAAGGAACAATCACATCAACCTTCAAATTTCATTACTGGGG     360
Target_S_187bp         ATTCGACTTCAACGGTCTAGAAGGAACAATCACATCAACCTTCAAATTTCATTACTGGGG     174
                       ************************************************************

VBGV3101P_S_878bp      TTCAAAAGCAGAAAGGGTCTTGTCGTCGAGCTCGGATCCCCGAAGGGCAATTCTGCAGAT     420
Target_S_187bp         TTCAAAAGCAGAA-------------------------------------------------     187
                       *************

VBGV3101P_S_878bp      CCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGTCTTGCGAAGGATAGTGGGA     480
Target_S_187bp         ------------------------------------------------------------     187

VBGV3101P_S_878bp      TTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCTTTGAAGACGT     540
Target_S_187bp         ------------------------------------------------------------     187

VBGV3101P_S_878bp      GGTTGGAACGTCTTCTTTTTCCACGATGTTCCTCGTGGGTGGGGGTCCATCTTTGGGACC     600
Target_S_187bp         ------------------------------------------------------------     187

VBGV3101P_S_878bp      ACTGTCGGTAGAGGCATCTTGAACGATAGCCTTTCCTTTATCGCAATGATGGCATTTGTA     660
Target_S_187bp         ------------------------------------------------------------     187
```

CLUSTAL W alignment to check orientation of the antisense fragments in plasmid isolated from GV3101

```
GV3101 plasmid Antisense
CLUSTAL O(1.2.4) multiple sequence alignment


VBGV3101P_AS_1125bp      CCCCTTTTCCACTTTGGTTTTCGTCCTGCACGATCGGGCAATTCGAGCCCTACAGCACAC   60
Target_AS_187bp          ------------------------------------------------------------   0


VBGV3101P_AS_1125bp      GACCCTTCGACGACAAGACCCTTTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTG   120
Target_AS_187bp          ----------------------TTCTGCTTTTGAACCCCAGTAATGAAATTTGAAGGTTG   38
                                               ************************************


VBGV3101P_AS_1125bp      ATGTGATTGTTCCTTCTAGACCGTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGT   180
Target_AS_187bp          ATGTGATTGTTCCTTCTAGACCGTTGAAGTCGAATGGAATGTGTAATTGTTGGTATGTGT   98
                         ************************************************************


VBGV3101P_AS_1125bp      AGTCGATGATGAATGTTTTTGTTGATATGGCTGGAGATTTTGTTGAACTGATCTGCATAT   240
Target_AS_187bp          AGTCGATGATGAATGTTTTTGTTGATATGGCTGGAGATTTTGTTGAACTGATCTGCATAT   158
                         ************************************************************


VBGV3101P_AS_1125bp      GCACCGATATCCTTTGTTGTTGCATGAGCAGGGCTCTTCTCCTCAAGGGTTCCGTGCTGG   300
Target_AS_187bp          GCACCGATATCCTTTGTTGTTGCATGAGC------------------------------   187
                         ****************************


VBGV3101P_AS_1125bp      AACCAACTGTAATCAATCCAAATGTAAGATCAATGATAACACAATGACATGATCTATCAT   360
Target_AS_187bp          ------------------------------------------------------------   187


VBGV3101P_AS_1125bp      GTTACCTTGTTTATTCATGTTCGACTAATTCATTTAATTAATAGTCAATCCATTTAGAAG   420
Target_AS_187bp          ------------------------------------------------------------   187


VBGV3101P_AS_1125bp      TTAATAAACCTACCAGTATTATTTACAAATTAATAAGAATGTTGATTGAAAATAATACTA   480
Target_AS_187bp          ------------------------------------------------------------   187


VBGV3101P_AS_1125bp      TATAAAATTGATAGATCTTGCGCTTTGTTATATTAGCATTAGATTATGTTTTGTTACATT   540
Target_AS_187bp          ------------------------------------------------------------   187


VBGV3101P_AS_1125bp      AGATTACTGTTTCTATTAGCTTGATATTATTTGTTACTTTAGCTTGTTATTTAATATTTT   600
Target_AS_187bp          ------------------------------------------------------------   187


VBGV3101P_AS_1125bp      GCTTATTGATAAATTACAAGCAGATTGGAATTTCTAACAAAATATTTATTAACTTTTAAA   660
Target_AS_187bp          ------------------------------------------------------------   187
```

APPENDIX XI

List of primers used in the study

Name	Direction	Sequence	Size (bp)	Product size (bp)
VβC1F	Forward	TTCTGCTTTTGAACCCCAGT	20	187
VβC1R	Reverse	GCTCATGCAACAACAAAGGA	20	
VLIC1	Forward	CGACGACAAGACCCTTTCTGCTTTTGAACCCCAGT	35	215
VLIC2	Reverse	GAGGAGAAGAGCCCTGCTCATGCAACAACAAAGGA	35	
VLIC3	Forward	CCAGCACGGAACCCTTGAGGAGAAGAGCCCT	31	243
VLIC4	Reverse	AGAGCACACGACCCTTCGACGACAAGACCCT	31	
VLIC5	Reverse	TCTTCTTCGTCTTACACATC	20	--
VLIC6	Reverse	AAGACCGGCAACAGGATTC	20	--
UBQ7F	Forward	CTCCACCTCGTCCTCCGTCTG	21	187
UBQ7R	Reverse	CGCCGCACTCCGCATTAAGG	20	

RNA MEDIATED RESISTANCE TO *Yellow vein mosaic virus* IN OKRA

By

KELKAR VIPUL GANESH
(2017-21-013)

ABSTRACT

Submitted in partial fulfilment of the requirement
for the degree of

Doctor of Philosophy in Agriculture

(Plant Biotechnology)

Faculty of Agriculture

Kerala Agricultural University

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF AGRICULTURE, VELLANIKKARA,
THRISSUR - 680656
KERALA, INDIA
2021

ABSTRACT

Okra (*Abelmoschus esculentus* L. Moench, Malvaceae) is one of the leading vegetable crops in hot and humid tropics. Unfortunately, this climate is conducive for many of the pests and diseases. Okra is susceptible to viruses such as *Yellow vein mosaic virus* (YVMV) and *Enation leaf curl virus* (ELCV), belonging to the genus *Begomovirus* (family Geminiviridae). Because of the favourable conditions prevailing in the coastal region, the losses in Kerala state are 60-100%, depending upon the stage of plant growth and the severity of infection. RNAi is one of the promising molecular biology approach against the viral diseases. Keeping the above facts in view, the present study "RNA mediated resistance to *Yellow vein mosaic virus* in okra" was taken up at the Centre for Plant Biotechnology and Molecular Biology, CoA, Thrissur from September 2017 to May 2021.

The high yielding and YVMV susceptible popular okra cv. Salkeerthi was selected for the development of resistance using RNAi mechanism. Total DNA was isolated from the YVMV infected plant and part of the *βC1* gene (187 bp) of the virus was amplified using primers VβC1F and VβC1R. Sequence information of PCR product has revealed that the gene is 90-95% identical with the Indian isolates. The *βC1* gene sequence was analysed using IDT software and 10 siRNAs were found at three different position (19-44, 34-59, 99-124 bp). Through Restriction Mapper, it was confirmed that the sequence selected for the preparation of sense and antisense strand, do not possess recognition sites for *Sma*I, *Hind*III and *Mau*BI restriction enzymes which are present in the pRNAiLIC vector. The output of VSupPred revealed that the fragment does not contain any Viral Suppressor Regions (VSRs), with a high prediction score (0.625).

The hairpin RNAi construct harbouring the region of *βC1* gene of β satellite of *Begomovirus* of okra was generated using pRNAi-LIC (CD3-1285) vector. The *Sma*I digested plasmid produced three fragments, vector backbone (9842 bp), Pdk intron (1641 bp) and *ccdB* gene (614 bp) and the digested plasmid was treated with dTTP. Product-1 was PCR amplified (215 bp) with VLIC1 and VLIC2 primers, using the DNA from YVMV infected plant as template. Product-2 was PCR amplified (243 bp) with VLIC3 and VLIC4 primers using product-1 as template. Product-1 and product-2 were eluted from the gel and treated with dATP. The dATP treated PCR products and

dTTP treated *Sma*I digested plasmid were mixed together and ligated by incubation at 65ºC for 5 min. followed by 22ºC for 15 min. Ligated product was successfully transformed in competent cells of *E. coli* (DH5α) and incubated on LB medium containing Kanamycin and Chloramphenicol. Colony PCR was performed, the transformation efficiency was found to be 80%. Plasmid was isolated from the positive DH5α colony and sequenced using the primers VLIC5 and VLIC6. The sequence data had shown that both sense and antisense strands are at right position and direction. Plasmid containing ihpRNA-βC1 cassette was successfully transformed into the competent cells of *Agrobacterium* (GV3101) and incubated on LB medium containing Kanamycin, Chloramphenicol and Rifampicin. Colony PCR was performed, the transformation efficiency was found to be 100%. Plasmid was isolated from the positive GV3101 colony and sequenced using the primers VLIC5 and VLIC6. Sequence data has further confirmed that both sense and antisense strands are at right position and direction.

The ihpRNA-βC1 cassette was successfully transformed into okra cv. Salkeerthi using *in planta* method of *Agrobacterium* mediated transformation. The transformation efficiency observed was 11.42% and the transformation was confirmed by the amplification of sense strand using the primers VLIC1 and VLIC5. cDNA was prepared from the total RNA isolated from transformed and control plants. siRNA synthesis was confirmed using the primers VLIC1 and VLIC5 (400bp) and Ubiquitin gene was confirmed using the primer UBQ7 (187 bp). Silencing potential of the RNA interference of *βC1* gene and the development of resistance was evaluated by keeping the 15-day old transformed and control plants along with YVMV infected plants inside containment facility, with whiteflies released into insect cage for infection. All the control plants and one transgenic plant have shown the YVMV symptoms after 10 days. Three transgenic plants were healthy with no symptoms. The present investigation was successful in the development of YVMV resistant okra plants carrying ihpRNA-βC1 using pRNAi-LIC (CD3-1285) plasmid vector. The further evaluation is needed in the coming generations for the identification of stable transgenic lines.