RNAi MEDIATED BROAD SPECTRUM RESISTANCE AGAINST BEGOMOVIRUSES OF OKRA (*Abelmoschus esculentus* L.)

Dissertation

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY
(Minor Subject: Plant Breeding and Genetics)

By

Nity Sharma
(L-2014-A-15-D)

School of Agricultural Biotechnology
College of Agriculture

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CERTIFICATE I

This is to certify that the dissertation entitled, **"RNAi mediated broad spectrum resistance against begomoviruses of okra (*Abelmoschus esculentus* L.)"** submitted for the degree of **Ph.D.** in the subject of **Agricultural Biotechnology** (Minor subject: **Plant Breeding and Genetics**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Nity Sharma (L-2014-A-15-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

_____

(Dr. Navraj Kaur Sarao)
Major Advisor
Senior Biotechnologist
School of Agricultural Biotechnology
Punjab Agricultural University
Ludhiana – 141 004

CERTIFICATE II

This is to certify that the dissertation entitled **"RNAi mediated broad spectrum resistance against begomoviruses of okra (*Abelmoschus esculentus* L.)"** submitted by **Nity Sharma (L-2014-A-15-D)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **Ph.D.** in the subject of **Agricultural Biotechnology** (Minor subject: **Plant Breeding and Genetics**) has been approved by the Student's Advisory Committee along with External Examiner after an oral examination on the same.

_____

(Dr. Navraj Kaur Sarao)
Major Advisor

_____

(Dr. Rajinder Kumar Jain)
External Examiner
Ex-Dean College of Basic Sciences & Humanities
Ex Prof. & Head, Deptt. of Mol. Biology, Biotechnology and Bioinformatics,
CCS-HAU, Hisar– 125 004

_____

(Dr. Parveen Chhuneja)
Director
School of Agricultural Biotechnology

_____

(Dr. Gurinder Kaur Sangha)
Dean, Postgraduate Studies

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ABSTRACT

Okra is an important vegetable crop of *Malvaceae* family and grown widely in tropical and subtropical areas of the world. Yellow vein, leaf curl and mosaic diseases caused by begomoviruses have emerged as most devastating viral diseases of okra. In the present investigation, RNA interference (RNAi) strategy was employed to control okra-infecting begomoviruses. For this, two hairpin RNAi constructs harbouring overlapping regions of *AC1*/*AC2* and *AC1*/*AC4* genes of DNA-A genome of begomoviruses of okra were generated. Consecutively, agroinfectious clone harbouring dimeric units of whole genome clone of okra-infecting begomoviruses was also generated. Efficacy of three constructs was checked by transient assay and plants having RNAi constructs did not show any symptoms after four weeks of agroinfilteration as compared to plants inoculated with agroinfectious clone. Further, *Agrobacterium*- mediated tissue culture dependent protocol was utilized to generate transgenic okra plants of variety Punjab 8 expressing *AC1*/*AC2*-hp and *AC1*/*AC4*-hp RNA. Hypocotyls were used as explants for transformation and a cytokinin named trans-zeatin riboside of 2mg/l concentration was used for regeneration of plantlets. Transgenic plants were assayed for resistance to okra-infecting begomoviruses using agro-infectious clone and viruliferous whiteflies. Nearly 90% resistance against begomovirus infection was observed in transgenic lines when compared with untransformed plants and the plants transformed with empty vectors. Control plants developed severe viral disease symptoms within 4 weeks. Out of eleven plants, one plant expressing *AC1*/*AC4*-hp RNAi constructs displayed appearance of milder symptoms after 6 weeks when attacked with viruliferous whiteflies. The resistant transgenic lines accumulated very low titres of viral DNA. Plants thus formed had normal phenotype with no yield penalty in greenhouse conditions.

Keywords: RNAi, Genetic transformation, *Agrobacterium*, *AC1*/*AC2* genes, *AC1*/*AC4* genes

Signature of Major Advisor **Signature of the Student**

ਖੋਜ ਪ੍ਰਬੰਧ ਦਾ ਸਿਰਲੇਖ	:	RNAi ਦੀ ਵਰਤੋਂ ਨਾਲ ਭਿੰਡੀ (*Abelmoschus esculentus* L.) ਵਿੱਚ ਬੈਗੋਮੋਵਾਇਰਸਿਸ ਵਿਰੁੱਧ ਪ੍ਰਤੀਰੋਧਕਤਾ ਦਾ ਮੁਲਾਂਕਣ
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ਸਾਰ ਅੰਸ਼

ਭਿੰਡੀ, *Malvaceae* ਪਰਿਵਾਰ ਨਾਲ ਸਬੰਧਤ ਇੱਕ ਅਹਿਮ ਸਬਜ਼ੀ ਹੈ ਅਤੇ ਸੰਸਾਰ ਦੇ ਉਸ਼ਮੀ ਅਤੇ ਉਪ-ਉਸ਼ਮੀ ਖੇਤਰਾਂ ਵਿੱਚ ਇਸਦੀ ਕਾਸ਼ਤ ਵੱਡੇ ਪੱਧਰ ਤੇ ਕੀਤੀ ਜਾਂਦੀ ਹੈ। ਬੈਗੋਮੋਵਾਇਰਸਿਸ ਕਾਰਨ ਹੋਣ ਵਾਲੇ ਪੀਲੀਆ, ਪੱਤਾ ਲਪੇਟ ਵਿਸ਼ਾਣੂੰ ਅਤੇ ਵਿਸ਼ਾਣੂੰ ਰੋਗ ਭਿੰਡਾ ਦਾ ਸਭ ਤੋਂ ਜ਼ਿਆਦਾ ਨੁਕਸਾਨ ਕਰਨ ਵਾਲੇ ਵਿਸ਼ਾਣੂੰ ਰੋਗ ਹਨ। ਮੌਜੂਦਾ ਅਧਿਐਨ ਦੌਰਾਨ, ਭਿੰਡੀ ਨੂੰ ਪ੍ਰਭਾਵਿਤ ਕਰਨ ਵਾਲੇ ਬੈਗੋਮੋਵਾਇਰਸਿਸ ਦੀ ਰੋਕਥਾਮ ਲਈ RNA ਇੰਟਰਫਿਅਰੇਂਸ (RNAi) ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਗਈ। ਇਸ ਲਈ, ਭਿੰਡੀ ਦੇ ਬੈਗੋਮੋਵਾਇਰਸਿਸ ਜੀਨੋਮ ਦੇ DNA-A ਦੇ *AC1/AC2* ਅਤੇ *AC1/AC4* ਜੀਨਾਂ ਦੇ ਓਵਰਲੈਪਿੰਗ ਖੇਤਰਾਂ ਨੂੰ ਸੰਰੱਖਿਅਤ ਕਰਨ ਵਾਲੇ ਦੇ ਦੋ ਹੇਅਪਰਿਨ RNAi ਦਾ ਵਿਕਾਸ ਕੀਤਾ ਗਿਆ। ਭਿੰਡੀ ਨੂੰ ਪ੍ਰਭਾਵਿਤ ਕਰਨ ਵਾਲੇ ਬੈਗੋਮੋਵਾਇਰਸਿਸ ਦੇ ਪੂਰੇ ਜੀਨੋਮ ਕਲੋਨ ਦੇ ਖੇਤੀ ਸੰਕਰਮਣ ਕਲੋਨ ਹਾਰਬਰਿੰਗ ਡਾਇਮੈਰਿਕ ਯੂਨਿਟਸ ਵੀ ਵਿਕਸਤ ਕੀਤੇ ਗਏ। ਟ੍ਰਾਂਜ਼ੀਐਟ ਐਸੇ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਤਿੰਨੋਂ ਸੰਰਚਨਾਵਾਂ ਦੀ ਪ੍ਰਭਾਵਸ਼ੀਲਤਾ ਦਾ ਪਤਾ ਲਗਾਇਆ ਗਿਆ ਅਤੇ ਖੇਤੀ-ਸੰਕਰਮਣ ਕਲੋਨ ਦੀ ਇਨੋਕੁਲੇਸ਼ਨ ਵਾਲੇ ਪੌਦਿਆਂ ਦੇ ਮੁਕਾਬਲੇ RNAi ਕੰਸਟ੍ਰਕਟਸ ਵਾਲੇ ਪੌਦਿਆਂ ਨੇ ਖੇਤੀ-ਸੰਕਰਮਣ ਦੇ ਚਾਰ ਹਫਤਿਆਂ ਮਗਰੋਂ ਵੀ ਕੋਈ ਲੱਛਣ ਨਹੀਂ ਦਰਸਾਏ। ਇਸ ਮਗਰੋਂ, *AC1/AC2*-hp ਅਤੇ *AC1/AC4*-hp RNA ਵਾਲੇ ਭਿੰਡੀ ਦੀ ਪੰਜਾਬ 8 ਕਿਸਮ ਦੇ ਟ੍ਰਾਂਸਜੈਨਿਕ ਪੌਦੇ ਵਿਕਸਤ ਕਰਨ ਲਈ *ਐਗਰੋਬੈਕਟੀਰੀਅਮ* ਵਾਲੇ ਟਿਸ਼ੂ ਕਲਚਰ ਉਪਰ ਅਧਾਰਤ ਪ੍ਰੋਟੋਕੋਲ ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਗਈ। ਸਥਾਨਾਂਤਰਨ ਲਈ ਹਾਈਪੋਕੋਟਇਲਸ ਨੂੰ ਐਕਸ-ਪਲਾਂਟਸ ਵਜੋਂ ਵਰਤਿਆ ਗਿਆ ਅਤੇ ਪੌਦਿਆਂ ਦੇ ਮੁੜ ਉਥਾਨ ਲਈ 2 ਮਿ.ਗ੍ਰਾ/ਲਿ. ਘਣਤਾ ਵਾਲੇ ਟ੍ਰਾਂਸ-ਜ਼ਿਆਟਿਨ ਰਾਈਬੋਸਾਈਡ ਨਾਮਕ ਸਾਈਟੋਕਾਈਨ ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਗਈ। ਖੇਤੀ-ਸੰਕਰਮਣ ਕਲੋਨ ਅਤੇ ਵਿਸ਼ਾਣੂੰ ਫੈਲਾਉਣ ਵਾਲੀਆਂ ਚਿੱਟੀਆਂ ਮੱਖੀਆਂ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਭਿੰਡੀ ਨੂੰ ਪ੍ਰਭਾਵਿਤ ਕਰਨ ਵਾਲੇ ਬੈਗੋਮੋਵਾਇਰਸਿਸ ਦਾ ਟਾਕਰਾ ਕਰਨ ਵਾਲੇ ਟ੍ਰਾਂਸਜੈਨਿਕ ਪੌਦਿਆਂ ਦਾ ਮੁਲਾਂਕਣ ਕੀਤਾ ਗਿਆ। ਬਾਕੀ ਪੌਦਿਆਂ ਦੇ ਮੁਕਾਬਲੇ ਟ੍ਰਾਂਸਜੈਨਿਕ ਲਈਨਾਂ ਵਿੱਚ ਬੈਗੋਮੋਵਾਇਰਸਿਸ ਵਿਰੁੱਧ ਲਗਭਗ 90% ਪ੍ਰਤੀਰੋਧਕਤਾ ਦਾ ਪਤਾ ਚੱਲਿਆ। ਕੰਟਰੋਲ ਪੌਦਿਆਂ ਵਿੱਚ ਚਾਰ ਹਫਤਿਆਂ ਵਿੱਚ ਵਿਸ਼ਾਣੂ ਰੋਗ ਦੇ ਲੱਛਣ ਦੇਖਣ ਨੂੰ ਮਿਲੇ। ਗਿਆਰਾਂ ਪੌਦਿਆਂ ਵਿੱਚੋਂ, AC1/AC4-hp RNAi ਵਾਲੇ ਇੱਕ ਪੌਦੇ ਨੇ ਵਿਸ਼ਾਣੂ ਰੋਗ ਫੈਲਾਉਣ ਵਾਲੀਆਂ ਚਿੱਟੀਆਂ ਮੱਖੀਆਂ ਦੇ ਹਮਲੇ ਦੇ 6 ਹਫਤਿਆਂ ਬਾਅਦ, ਰੋਗ ਦੇ ਥੋੜੇ ਜਿਹੇ ਲੱਛਣ ਦਰਸਾਏ। ਪ੍ਰਤੀਰੋਧਕ ਟ੍ਰਾਂਸਜੈਨਿਕ ਲਾਈਨਾਂ ਵਿੱਚ ਵਿਸ਼ਾਣੂ DNA ਦੇ ਟਾਈਟਰ ਦੀ ਜਜ਼ਬਤਾ ਬਹੁਤ ਘੱਟ ਸੀ। ਇਸ ਤਰ੍ਹਾਂ ਅਧਿਐਨ ਦੌਰਾਨ ਵਿਕਸਤ ਕੀਤੇ ਗਏ ਪੌਦੇ ਫਿਨੋਟਿਪਿਕ ਗੁਣਾਂ ਦੇ ਲਿਹਾਜ਼ ਨਾਲ ਆਮ ਪੌਦਿਆਂ ਵਰਗਿਆਂ ਹੀ ਸਨ ਅਤੇ ਗ੍ਰੀਨਹਾਊਸ ਹਲਾਤਾਂ ਵਿੱਚ ਇਹਨਾਂ ਦੇ ਝਾੜ ਉਪਰ ਵਿੱਚ ਕੋਈ ਕਮੀ ਨਹੀਂ ਆਈ।

ਮੁੱਖ ਸ਼ਬਦ: RNAi, ਅਨੁਵਾਂਸ਼ਿਕੀ ਸਥਾਨਾਂਤਰਨ, *ਐਗ੍ਰੋਬੈਕਟੀਰੀਅਮ*, *AC1/AC2* ਜੀਨ, *AC1/AC4* ਜੀਨ

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ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

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CHAPTER I

INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench), the most important vegetable crop of malvaceae family is grown in various parts of the tropical and subtropical parts of the world. In warm temperate zones it is grown in summers only (Asare-Bediako *et al* 2014). Okra has an origin from tropical Africa (Hussain *et al* 2006). Significant variation is found in the chromosome numbers and ploidy levels of genus *Abelmoschus*. The highest chromosome number reported for the genus are close to 200 for *Abelmoshus manihot var. caillei* and the lowest chromosome numbers reported for the same is 2n=56 for *Abelmoschus angulosus* (Ford 1938 and Siemonsma 1982). Okra crop has various nutritional, economic, medicinal and industrial values. The parts of the crop such as fruits, seeds, flowers, stem, leaves and mucilage are utilized for varied purposes. Green fruits of okra are used for cooking as a vegetable that are rich in vitamins, carbohydrates, proteins, fats and minerals; seeds contain edible oil and protein that have high nutritional importance in human diet; flowers are also eatable; being fibrous in nature mature fruit and stem is used in paper industry, leaves used as a cattle feed, pod mucilage is used for the replacement of plasma and expanding blood volume (Qhureshi 2007, Fajinmi and Fajinmi 2010, Muhammad *et al* 2012).

Worldwide production count of okra is approximately 9.62 million tones, for which India stands at first position (~ 65%) in world's okra production as 6.3 million tones of it is produced per year (Mishra *et al* 2017). The major producers of okra in India are Andhra Pradesh, West Bengal, Bihar, Gujarat and Orissa. As far as area under okra cultivation in India is concerned, Andhra Pradesh has an area of 74.25 thousand ha, West Bengal has an area of 74.60 thousand ha under cultivation followed by Bihar, Gujarat, Orissa, Jharkhand and others. In terms of okra production, it is 1.11 million tones in Andhra Pradesh, 0.86 million tonnes in West Bengal and 0.85 million tonnes in Bihar. The average productivity in India is 10.5 tonnes per hectare. Punjab produces about 0.03 million tonnes of okra from an area of 3.1 thousand ha having productivity of 10.4 tonnes per hectare. Major okra producing districts are Ludhiana (14%), Jalandhar (13%) and Amritsar (7%).

Inspite of its importance from commercial point of view, okra production is infested by various insect pests and the crop is prone to the attack of viruses, bacteria, fungus, phytoplasma and the nematodes. Okra crop is attacked by atleast 19 viruses of which major crop loss is due to okra yellow vein mosaic virus (OYVMV). OYVMV is a virus belongs to the genus begomovirus transmitted by the white fly (*Bemisia tabaci*) (Asare-Bediako *et al* 2014). In 1924, disease was first reported in India (Kulkarni 1924). Okra crop infected with OYVMV at 30, 45 and the 60 days from sowing showed predictable loss of approximately 76,

54.9 and 47.8 percent respectively (Chellaiah and Murugesan 1976). Warm climate with less or no rainfall is favorable for the multiplication of white fly (*Bemisia tabaci*) and also the incidence of the disease (Singh 1990). These viruses mostly infect dicotyledonous plants and some of the morphological symptoms found in infected plants are stunted growth. The most prominent symptoms are the networks of yellow veins on the leaf which become completely yellowish under severe conditions. Control measures were primarily focused on managing the vector with the use of insecticides. With the simultaneous problems of development of insecticide resistance, environmental concerns and low cost-benefit ratio, the use of insecticides should be reduced.

Genus begomovirus comes under the family Geminiviridae and is the major among other genera (Mastrevirus, Curtovirus and Topocuvirus) of the family. Begomoviruses consists of 2.6-2.8kb sized monopartite (single strand circular DNA-A component) or bipartite (two single strand circular component of DNA-A and DNA-B genome) genome (Venkataravanappa *et al* 2011). DNA-A component controls the functions of gene replication, gene expression, transmission and DNA-B component of the genome consists of genes that controls inter or intracellular movement of the virus and involved in symptom development. Two components share approximately 200 nucleotides non-coding sequences which contain the replication origin of virus (Taware *et al* 2010).

Monopartite begomoviruses often contain some sub-viral components in their genome known as the satellite DNAs. These satellite DNAs require helper begomoviruses for their movement and transmission between hosts. Depending upon the organization of DNA and symptoms associated with their helper begomoviruses, these satellites are of two types i.e. alpha and beta satellites (Borah and Dasgupta 2012). Both alpha (earlier known as DNA1) and beta (earlier known as DNA β) satellite DNA molecules have size of approximately 1350-1380 nucleotides which is almost half from the size of their helper begomoviruses (DNA-A & DNA-B). Recombination and pseudorecombination is very common in begomoviruses that cause devastating effects in host plant and is responsible for viral evolution that leads to emergent viral population (Venkataravanappa *et al* 2013).

Plants have a series of defense responses against viruses and viral infection affects these responses by RNA- interference. Viral proteins involved in suppression of plant's defense responses are known as RNAi suppressors. RNAi suppressor action is found in many begomoviral proteins (Voinnet 2005, Borah and Dasgupta 2012). To overcome viral infection, genetic resistance is the most powerful strategy. Genetic resistance has been reported earlier in many plants by number of ways, these are expressing viral proteins (CP, Rep and its derivatives and TrAP), expressing those proteins that are not originated from viruses but have

an anti-viral affects (toxic protein dianthin, antibodies raised against viral CP) and DNA or RNA interference (Vanderschuren *et al* 2007, Borah and Dasgupta 2012).

Viruses are hard to control because the natural host range of them are very broad and there are no source of genetic resistance to them and also conventional breeding programs show very narrow range of resistance to the viruses (Watterson 1993). In response to that, advancements of modern biotechnology can be helpful to impart resistance to viruses (Mishra *et al* 2017). Various levels of resistance were achieved via pathogen derived resistance such as expression of dsRNA of the genes of the viruses in tomato plants engineered with RNAi constructs (Shelly *et al* 2010, Ntui *et al* 2014, Ammara *et al* 2015), common bean (Bonfim *et al* 2007), legume (Nahid *et al* 2011), pepper (Medina-Hernandez *et al* 2013), tobacco (Sharma *et al* 2015) and cowpea (Kumar *et al* 2017). Therefore, RNAi-mediated post-transcriptional gene silencing (PTGS) particularly the expression of dsRNA remains an important tool to achieve high level of resistance to viruses. RNA interference (RNAi) is a homology-dependent mechanism that involves the sequence-specific degradation of cellular RNA by a complex of enzymes that are present in the genome of the plants. In plants or other eukaryotes, the mechanism of RNAi is involved in directing developmental processes and also acts as a defense against viruses, foreign nucleic acids and transposons. RNAi was first discovered in plants by the name of "post-transcriptional gene silencing" (PTGS) (Baulcombe 2000). Through RNAi, plants express a copy of a viral gene in sense and antisense orientation and show resistance upon infection with the virus. It has been found previously that RNA in duplex form was more effective than the expression of either sense or antisense RNA alone (Waterhouse *et al* 1998). The technique can be more efficiently induced using transgenes that express self-complementary hairpin (hp) RNA (Smith *et al* 2000). In the previous studies of molecular characterization of OYVMV collected from different geographical regions across the okra growing regions of Punjab revealed that *Mesta yellow vein mosaic virus* (MeYVMV) is the most prevalent strain of whitefly transmitted begomoviruses in Punjab (Kaur *et al* 2017).

In view of the above, the current study was undertaken with following objectives:

- Development of hairpin RNAi (hpRNAi) construct for resistance to begomoviruses in okra
- Genetic transformation of okra using RNAi construct and characterization of transgenics
- Development of infectious clone of begomovirus of okra in binary vector

CHAPTER II

REVIEW OF LITERATURE

Vegetables have a major role in diet, health and livelihood. Okra is an important vegetable crop of *Malvaceae* family and grown widely in tropical and subtropical areas of the world. Its origin is thought to be from Africa. It is famous in India, Pakistan, Iraq, Nigeria, Ghana and Cameroon and is found to be a rich source of Vitamin A, folic acid, carbohydrates, potassium, phosphorous and other minerals. From the production point of view, India stands first with 5.784 million tones of okra from over 0.498 mha land. Okra is grown for various purposes as its fruits are used for human consumption and dried stems are used in paper industry. Okra has also been used for replacing plasma and for expanding blood volume. Okra was previously come under the genus *Hibiscus*, section A*belmoschus* but later on section *Abelmoschus* was introduced to be a separate genus. About 50 species has been described and 8 from them are well known. Okra is an allopolyploid, having highest chromosome number of around 200 in case of *A. caillei* and lowest chromosome number of 2n = 56 in case of *A. angulosus. A. esculentus* are having a series of polyploids with chromosome numbers of 2n = 72, 108, 120, 132 and 144 (Datta and Naug 1968). Among the different species of *Abelmoschus* genus, *Abelmoschus esculentus* is the most cultivated one in Asia. Okra crop requires a long and warm climate for its normal growth and development. Two types of crops (early and late) are grown in plain areas. Crop is attacked by various insect pests, nematodes, fungi, bacteria, mycoplasma and viruses (Ali *et al* 2005). Crop pests cause yield loss of up to 20-30% and in extreme cases this yield loss accounts of about 80-90%. Cultivation of okra is seriously infested by viruses and about 19 plant viruses are known to affect its production (Brunt *et al* 1990, Swanson and Harrison 1993). Genus *begomovirus* comes under the family *Geminiviridae* and viruses of this genus become a major constraint of okra production as they affect both yield as well as quality of the crop. Begomoviruses can affect almost all stages of plant development with varied levels of losses. Several attempts had been carried out to manage virus and also the insect pests involved in its infestation. Considering it as a major problem of okra production, it is necessary to understand the nature of the begomovirus, source of infection, most affected stage of the crop, the way of virus infestation among different varieties and the management practices that can be utilized for controlling viral infection. Literature regarding the study comes under the following headings:

2.1 Begmoviruses of okra

2.1.1 Genome organization

2.1.1.1 Bipartite Genome

2.1.1.2 Monopartite Genome

2.1.1.3 Associated molecules

2.1 Begmoviruses of okra

Genus *Begomovirus* belongs to the family Geminiviridae. Geminiviruses are small arthropod-borne, plant-infecting viruses and are known to infect several plants including crops, ornamental plants and weeds (Stanley *et al* 2005). Depending upon the host range, genome structure and type of insect vector carried them, family geminiviridae is divided into four genera viz. Mastrevirus, Curtovirus, Topocuvirus and Begomovirus and when concerning about the organization of the genome, similarities among nucleotide sequence and the biological properties, family Geminiviridae is divided into nine genera viz. Becurtovirus, Begomovirus, Eragrovirus, Capulovirus, Grablovirus, Mastrevirus, Curtovirus, Topocuvirus, and Turncurtovirus. Among these genera, begomovirus is the largest genus and viral species belonging to this genus are transmitted exclusively by whitefly vector namely *Bemisia tabaci*. There are about 27 begomoviruses that are reported to infect okra. They are abundant in the regions where the insect pests carried them are prominent (Leke *et al* 2015). These begomoviruses are involved in significant crop losses in many dicotyledonous plants.

2.1.1 Genome organization

Genome of geminiviruses is circular single-stranded DNA that is encapsidated in twinned (geminate) quasi-isometric particles (Venkataravanappa *et al* 2011). Genome

organization is either of two components known as “bipartite” or a single component known as “monopartite”.

2.1.1.1 Bipartite Genome

These viruses have DNA-A and DNA-B (Figure 2.1 a & b) as two genomic components with equal size of 2.6-2.8 kb. DNA-A encodes proteins for the replication of genome and virus factors that are required to control gene expression and insect transmission between hosts. These genes are present on the viral sense and anti-sense strand. This component of genome contain five or sometimes six genes i.e. one (AV1-encodes for coat protein) or two genes (AV1 and AV2-encodes for pre-coat protein) in the viral sense strand and four genes (AC1-replication associated protein, AC2-transcriptional activator protein, AC3-replication enhancer protein and AC4-function as a suppressor of PTGS) in the complementary sense strand. DNA-B component of bipartite genome consists of 2 genes i.e. BV1 for nuclear shuttle protein (NSP) on viral sense strand and BC1 for movement protein (MP) on complementary sense strand. Both of these components are required for infection. Genes of DNA-A component are required for the viral replication while inter and intracellular movement is the activity of DNA-B component. Both components share a short ~200 nucleotide non-coding region in common known as common region (CR) which is a part of large intergenic region (IR) where replication protein gets bind for initiating replication (Borah and Dasgupta 2012). This region contain the nonanucleotide TAATATTAC sequence having origin of viral sense strand and is the replication binding region which remains conserved in all members of the family *Geminiviridae*. These bipartite viruses are widespread in new world i.e. Southern States of USA, Mexico, the Caribbean and Central and South America.

2.1.1.2 Monopartite Genome

Monopartite begomoviruses have DNA-A as only one genomic component that is the homolog of one component (DNA-A) of bipartite begomoviruses. It also has a five or sometimes six open reading frames on the viral sense and complementary strand. Genes AV1 & AV2 present on the viral sense strand and AC1, AC2, AC3 & AC4 on complementary strand have same expression as that of the DNA-A of the bipartite begomoviruses. It is reported that monopartite begomoviruses are not present truly but they are associated with another single-stranded DNA (ssDNA) molecules. These small ssDNA molecules are known as satellite DNA molecules.

2.1.1.3 Associated molecules

Monopartite begomoviruses are often associated with specialized DNA molecules known as satellite DNAs. Betasetallite (Figure 2.1d), earlier known as DNA- β is one type of

the satellite DNA molecule. Betasatellite DNA requires helper begomoviruses for their replication, transmission and some of them are involved in the modification of the symptom acuteness. Betasatellites only share nonanucleotide conserved region with the helper begomoviruses (Briddon *et al* 2001). They contain one gene encode for βC1 protein that have been shown to suppress silencing processes in the host and by this they are responsible for viral accumulation in the host plant (Leke *et al* 2013). Some of the begomoviruses-betasatellites are shown to be accumulated with the other satellite DNA molecule called alphasatellite (Figure 2.1c), earlier known as DNA-1. This type of alphasatellite contains one gene that encode for a replication initiater protein (Rep). Because of this protein, alphasetellite DNA have a capacity of autonomous replication (Venkataravanappa *et al* 2011).

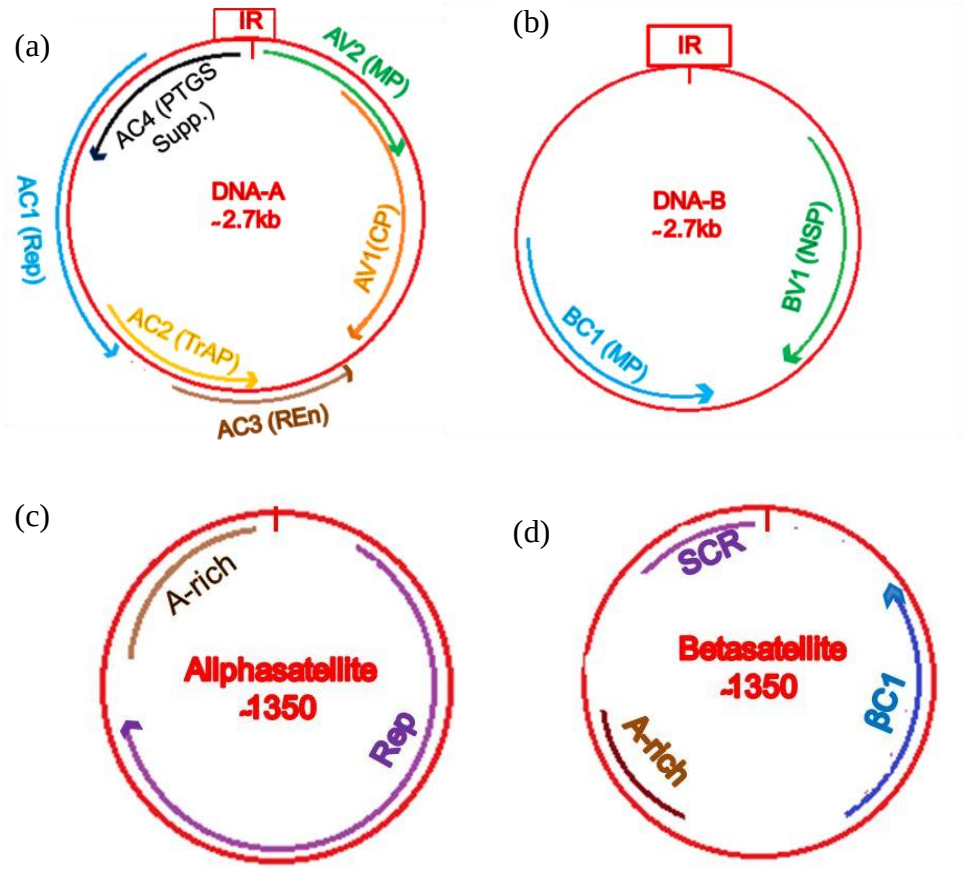


Figure 2.1: Diagrammatic representation of genomic components of begomoviruses showing encoded ORFs (a) DNA-A component shows six ORFs with intergenic region seperated the AC4 and AV2 genes (b) DNA-B component shows two ORFs with intergenic region (c) and (d) shows alpha and betasatellite DNA molecules

Alphasetellite also need a helper begomovirus for their whitefly mediated transmission and systemic infection to the plant, which shows that both alpha and beta satellite are encapsidated by helper virus's coat protein (Leke *et al* 2013). Begomovirus cause

devastating effects on the dicot plants by virtue of which green leaves of the plant shows yellow colored network and in extreme cases leaves get completely yellow or creamy in color.

2.1.2 Transmission of begomoviruses

The insect of warm climates which act as a carrier of begomovirus in okra is whitefly especially *Bemisia tabaci* that belongs to the family Aleyrodidae. Whiteflies transfer the virus in the adult stage laid their eggs on the surface of leaves which upon hatching form a scale like 'crawler' stage followed by the stage of three, sessile, immature instars. Pupa stage 'the last instar stage' become an adult (Jones *et al* 2002). Whitefly and the virus it transmits are involved in significant crop losses in varied climates. Crops which remain unprotected to whitefly are cotton, okra, cowpea, crucifers, tobacco, tomato, potato, lettuce, pea, bean, pepper respectively. Varma (1952) studied the relationship between the okra yellow vein mosaic virus and its vector and found that even a single insect is enough to transmit the virus to the host, but to reach 100 percent viral infection minimum number of flies required are 10. For the transmission of virus, female whiteflies are more efficient than male whiteflies. Various biotypes of *Bemisia tabaci* have been recognized that are similar in morphology but are nonidentical. Biotypes can be differentiated on the basis of range of host plants, host plant's adaptability to vector, phytotoxic reaction induction capability, resistance of the vector to insecticide spray and also their RAPD & AFLP patterns (Martin *et al* 2000).

2.1.3 Begomoviruses replication

From the discovery of begomoviruses, various types of works have been done to reveal the mechanism of viral DNA replication and it was found that most prominent mechanism of replication is rolling circle replication (RCR). Replication of both DNA-A and DNA-B component of virus is done by RCR. DNA-A component is independent to DNA-B for their replication but the same independence does not found in case of DNA-B. For the replication of small single stranded genome of virus, certain viral and host factors are required. Following transmission of virus by the vector, virus introduced its genome into the nucleus and initiates three-step replication process.

In the first step, by utilizing only host cellular factors, introduced viral single stranded genome is converted into double strand intermediate form or replicative form (RF) and this form of viral DNA is highly super coiled in structure. dsDNA encodes for the transcription of various viral factors and replication-related proteins such as Rep. Rep is a versatile, oligomeric protein having properties of DNA binding, nicking and ligation. Rep protein performs ATP-dependent helicase activities and is essential for the initiation and extension of RCR (Yadava *et al* 2010). Rep protein show specificity towards the replication of their

cognate genomes and by this DNA-A encoded Rep becomes able to trans-replicate DNA-B component but unable to replicate those DNA-B component that are heterologous to DNA-A (Hanley-Bowdoin *et al* 2000). For the initiation of RCR, Rep protein recognizes the common region (CR) which performs the most conserved nonameric sequence in the geminiviruses and nicks the double stranded form between the 7^{th} and 8^{th} nucleotide of the sequence. Double stranded form further act as template for the synthesis of ssDNA via RCR in combination of both viral and host cellular factors and leads to the beginning of second step known as elongation.

Phase of elongation starts from the free 3′ hydroxyl end created by Rep protein and during this phase various host proteins get adhere on the hydroxyl end and extend it in 5′ - 3′ direction. As elongation of the DNA is completed, the incipient concatenated DNA undergoes the cut and join process for the formation of single-stranded circular viral DNA. The newly formed ssDNA again go within the replication pool. At the termination step, ssDNA further involved in formation of many copies of viral ssDNA. In addition to RCR, another mode of replication known as recombination-driven mode of viral replication (RDR) is also used to some extent (Yadava *et al* 2010).

Satellite DNA molecules can be trans-replicated by helper viral genomes. Satellite conserved region is crirical for its replication which is present next to the stem loop structure TAA/GTATTAC. As similar to the DNA-A this act as a Rep binding site where Rep introduces nick for replication initiation. Alberter *et al* (2005) reported that satellite molecules also use both RCR and RDR mode of replication for their ssDNA.

2.1.4 Diversity and evolution of begomoviruses infecting okra

Begomoviruses are diverse in their distribution throughout the world. Globally, there are varying numbers of begomoviral species that infect okra crop. Only under African continent there being six different species of begomoviruses that had been reported like Okra leaf curl Cameroon virus (OLCuCMV) and Okra yellow crinkle virus (OYCrV) and also satellite molecules that were identified to infect Okra crop in Africa are Okra leaf curl Mali alphasatellite (OLCuMLA), Okra leaf curl burkina faso alphasatellite (OLCuBFA) and Okra yellow crinkle alphasatellite (OYCrA) (Leke *et al* 2015). From Asian continent there are varied number of reports solely from Indian subcontinent and reported viruses were Okra enation leaf curl virus (OELCuV, Singh, 1996; Chandran *et al* 2013, Venkataravanappa *et al* 2015), Bhindi yellow vein mosaic virus (BYVMV, Venkataravanappa *et al* 2013), Bhindi yellow vein Maharashtra, Delhi, Haryana & Madurai virus (Venkataravanappa *et al* 2013) and a new begomovirus isolate named Bhendi yellow vein Bhubhaneswar virus (BYVBV) was characterized from Bhubhaneswar, India which is suggested that the isolate is a recombinant

and its origin is supposed to be from the exchange of the genomic segments of Croton yellow vein mosaic virus, Bhendi yellow vein mosaic virus, Cotton leaf curl multan virus and Mesta yellow vein mosaic virus (Venkataravanappa *et al* 2013). Recombination is the important mechanism for the diversity of the begomoviruses.

Viruses can be identified with serological tests and hybridization methods. Now-a days OYVMV can be detected by the molecular biology tools such as Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), sequencing and the methods like Rolling Circle Amplification (RCA) in which ϕ29 polymerase is used and it acts in a sequence independent manner. Evolution of the viruses is very high but plant viruses are slow evolving as compared to the animal viruses. Various forces that drive the emergence and evolution of begomoviruses are mutation, recombination, reassortment and the selection pressure (Leke 2010). Mutation arises due to single or multinucleotide change which cause differences in disease severity, varied host range and transmission by the whitefly. Recombination is another important mechanism for the evolution of viruses. In case of recombination exchange of segments from one strand is incorporated into another strand during replication. Length of exchange fragments during recombination may vary form few nucleotides to large fragments while in case of reassortment exchange of fragments occur within the DNA-A and DNA-B component of the genome. Reassortment mainly found in the bipartite genome of begomoviruses in which both type A and B are present. Very little knowledge is there about the role of selection pressure in virus evolution. Selection of virus is on the basis of Darwin's selection theory i.e. survival of fittest (Leke 2010).

Recombination and reassortment (pseudo-recombination) are the phenomena that occur very frequently between species as well as within species and genera of geminiviruses. These two phenomena are mainly responsible for begomoviruses evolution. Mixed infection in various cases may cause the recombination and reassortment as easy events for the evolution of viruses and due to which novel strains can emerge. In the Indian subcontinent, novel strain of begomoviruses become different from the one that are causing the same disease in another area whose geography is different. For example, Venkataravenappa *et al* (2013) reported a new begomovirus isolate form Bhubhaneswar that is infecting okra showing same symptoms as observed by other known viruses and having *Croton yellow vein mosaic virus* as its closest relative. They concluded that new isolate may have its origin from the exchange of segments between *Croton yellow vein mosaic virus*, *Bhendi yellow vein mosaic virus*, *Cotton leaf curl multan virus* and *Mesta yellow vein mosaic virus* and suggest that Bhindi yellow vein Bhubhaneswar virus (BYVBV) is a recombinant. This study shows that recombination is an important and very frequent event for the occurrence of new strains of viruses and hence its evolution.

2.1.5 Economic impact of virus on crop

YVMV disease is the most destructive disease of okra. Viral disease has its impact on growth and yield of the crop. YVMV infected the okra plant at all the stages of the plant growth but destruction is more in earlier stages of growth. It was reported earlier that losses are upto 98% when disease incidence is within 20days after germination (Karri and Acharyya 2012).Under field conditions, plant shows three types of morphological symptoms. In type I, at the early stage, leaves of the young plants become yellowish and after some time yellow color of the leaves turn to brown and later it dries up. In type II, plant are not infected at earlier stages but infection started after flowering and parts of the flower and leaves gives vein clearing symptoms. As disease incidence is there after flowering so plants produce some fruits that become yellow and hard to pick from the stem. In type III, growth and fruiting of the plants are normal like that of the healthy plants but, at the end, small limited numbers of young shoots appear at the basal portion shows symptoms of vein clearing. Although, few young infected stems does not affect the yield of the plant (Sanwal *et al* 2016). In field conditions, disease is having a characteristic symptom of regular network of yellow veins which enclose islands of green tissues within the younger leaves. Infected leaves become yellowish or cream colored in severe infectious conditions. Plants shows stunted appearance and are having very limited number of deformed fruits on it. YVMV cause great loss by affecting quality and yield of the plant and the loss is as high as above 90% depends upon the age the plant (Bhagat *et al* 2001). In some cases there is a significant reduction in the height, number of fruits, flowers and overall yield but increase in the number of leaves of the infected plant as compared to the healthier one. Plant height is reduced by 19.5% and number of fruits was reduced by 34.7% and fruit weight was reduced by 80% in comparison with that of healthier plant (Khaskheli *et al* 2017). Per cent incidence of the crop can be calculated by the formula (Karri and Acharyya 2012, Khaskheli *et al* 2017):

$$\text{Per cent Disease Incidence (PDI)} = \frac{\text{No. of diseased plants}}{\text{No. of observed plants}} \times 100$$

2.2 Management practices

As okra is the most important vegetable crop in India grown for their fruits and green leaves but its production is limited by the incidence of okra yellow vein and its vector. OYVMV and its vector cause heavy losses by affecting yield and quality of the crop. Unlike fungicides and bactericides against fungal and bacterial infection, there is no viricides yet been developed against viruses. Hence, it becomes difficult to control viral infestation by some direct means but indirectly they can be managed by the vector control, by cultivation of

resistant varieties, by the control of viral replication and there are some biotechnological tools that can be exploited to control the viral vectors and virus itself (Figure 2.2).

2.2.1Vector management

Vector population can be managed by the spray of insecticides, by physical barriers or by removal of infected plants (Mishra *et al* 2017).

2.2.1.1 Insecticidal spray

Whitefly vector population can be managed by the spray of insecticides like acetamiprid, imidacloprid, triazophos, monocrotophos and metasystox. Acetamiprid, imidacloprid, triazophos and monocrotophos cause significant reduction in disease incidence and mean whitefly population (Gowdar *et al* 2007). Insecticide spray with the decrease in disease incidence and vector population density simultaneously increase the crop yield. In some cases metasystox also shows significant reduction in disease incidence (Debnath and Nath 2002). Ali *et al* (2012) check the effect of Mycotel Tracer and Imidacloprid as insecticide on disease incidence and found out of three imidacloprid was most effective against whitefly population. Moreover, overspray of insecticides is ecologically harmful.

2.2.1.2 Spray with plant extracts

Some of the plant extracts are reported that are used against the okra yellow vein mosaic virus. Chaudhary *et al* (2016) evaluate the effect of four plant extracts viz. *Azadirachta indica* (neem), *Allium sativum* (garlic), *Zingiber officinale* (ginger) and *Allium cepa* L (onion) at 5% concentration and concluded that among four plant extracts neem extract was most effective against whitefly reduction and disease incidence under field conditions. Even the leaf extracts of *Prosopis chilensis* and *Bougainvillea spectabilis* are also effective in reduction of okra yellow vein mosaic disease (Pun *et al* 1999). These extracts act as potent inhibitors of plant viruses.

2.2.2 Culture practices

Various types of culture practices are employed to control virus infestation includes roguing, disposal of the crop residues, intercropping, use of physical barriers are there but provided that they should be combined with the spray of insecticides so that they become effective (Freitas-astua *et al* 2002). Use of polyethylene films and aluminium mulches are also there in the literature but inspite of significant effects, the utilization of mulches is cost-effective when crop is grown in large areas (Freitas-Astua *et al* 2002). Ultraviolet (UV)-absorbing plastic films are also used as an insect-proof nets or as covers of the greenhouses which control the whitefly movement and especially efficient to control the begomoviral incidence. As UV-absorbing films are effective against the whitefly protection but have high

production cost and may cause increased humidity and temperature inside the greenhouses. Removal of weeds that act as an alternative hosts is the other practice for reducing viruses on the host. On the other hand, Hilje *et al* (2001) suggests that there is no need to waste resources on the elimination of weeds as these also act as reservoirs of pathogens and parasites of whitefly.

2.2.3 Breeding of resistant varieties

Developing plant varieties resistant to virus or its vector is yet another approach to control the diseases of viruses. To control the okra yellow vein mosaic virus, breeding of resistant varieties is the most utilizing and conventional approach that has used from many years and upto now varied number of cultivars have been developed. Among the cultivated forms there is no complete resistance found against the virus. Complete resistance is reported in some wild and semi-wild forms namely *A. manihot*, *A. pungens*, *A. crinitus*, *A. panduraeformis* and *A. vitifolius*. Symptomless carrier nature of viral resistance is observed in *A. manihot* ssp. *manihot*, *A. tuberculatus* and *A. cailei*. Virus resistant crops are advantageous as no extra attempts are needed for pest management and for the production of virus-free planting material. Being advantageous there are some limitations of these conventional methods.

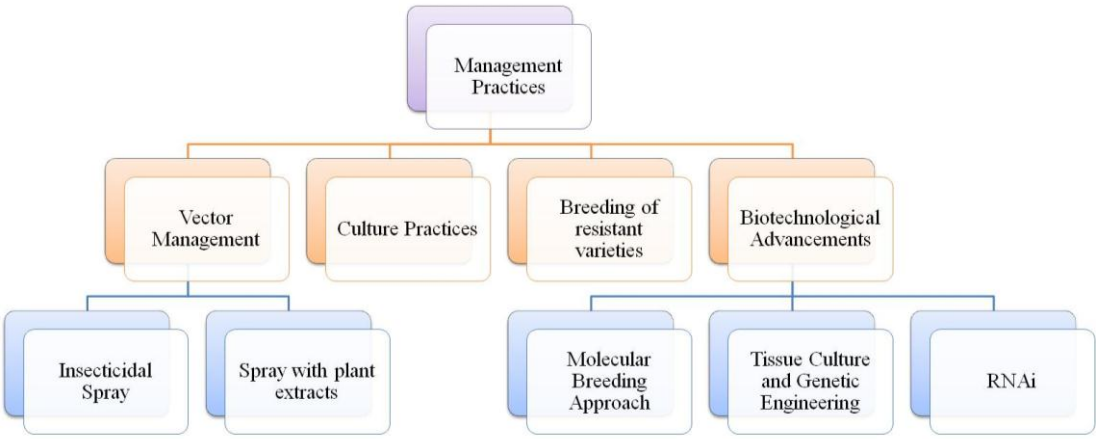


Figure 2.2: Various management practices that can be utilized to make okra crop virus-free, either with the management of vector and the management of virus itself

2.2.4 Biotechnological advancements

Utilization of biotechnological approaches in the breeding of okra is very limited; this may be due to the large genome size (polyploidy nature) and highly repetitive DNA of the okra genome. Biotechnology in case of any crop can be applied by two approaches (Mishra *et al* 2017).

2.2.4.1 Molecular breeding approach

Utilization of relevant molecular breeding strategies like Marker assisted backcrossing (MABC), Marker assisted recurrent selection (MARS), Genome-wide association studies (GWAS); development of genomic resources like molecular markers, development of mapping population, identification of molecular markers that are linked to virus resistance and their associated quantitative trait loci (QTLs) are the molecular breeding approaches that can be used for making okra, a virus-free crop (Mishra *et al* 2017). As natural resistance is very few and also various breeding programmes are not able to produce cultivars in a required time period, till date very few polymorphic molecular markers and genetic maps have been developed (Snehi *et al* 2015).

2.2.4.2 Tissue culture and genetic engineering approach

As conventional breeding programmes are difficult and time-taking and by recognizing the economic importance of okra, there is a massive scope of utilizing modern biotechnology for further improvement of it. Tissue culture and genetic engineering procedures can be utilized to make okra crop free from biotic and abiotic stresses. Regeneration protocol is prerequisite for the implementation of successful tissue culture procedures in okra and okra is known to be highly recalcitrant to the transformation and regeneration mediated by *Agrobacterium*. Due to low percentage of regeneration, excretion of mucilage and phenolic compounds from explants to medium and browning of callus, few number of regeneration protocols has been reported to date (Anisuzzaman *et al* 2010, Dhande *et al* 2012, Narendran *et al* 2013, Manickavasagam *et al* 2015). Direct shoot regeneration by taking cotyledon and cotyledonary nodal segments as explants were reported by Mangat and Roy (1986) and they reported regeneration of cotyledonary axil through callus mode (Roy and Mangat 1989). Ganesan *et al* 2007, reported plant regeneration and somatic embryogenesis of okra through suspension culture. Narendran *et al* 2013, tried to regenerate okra plantlets from cotyledon explants transformed with *Agrobacterium* but unable to do successful transformation in it, so they standardized direct multiple shoot bud regeneration protocol of genetic transformation in okra by using zygotic embryos as explants. In this new protocol, plumule portion was pricked with sterile needle for the transfer of T-DNA through *Agrobacterium* and multiple shoots were induced from this portion. Also, Manickavasagam *et al* (2015) initiate a transformation protocol using seed as an explants and show very high transformation efficiency upto 18.3 percent. These transformation protocols seem to give a great opportunity to develop transgenics of okra.

2.2.4.3 RNA-interference (RNAi)

Transgenes in various situations can silence the expression of endogeneous genes that are homologous in sequence; this led to the development of RNA-interference technology for the sequence selective silencing of the genes. RNA-interference (RNAi) is a progressively conserved gene silencing system that recognizes dsRNA (double-stranded RNA) as a signal to produce the sequence-specific degradation of mRNA which is homologous in sequence. Possibly, this is the most important advancement in biology that RNA molecules can control the expression of both exogenous and endogenous genes. RNAi technology has an application of genetic engineering of desired traits of crops which leads to the development of new varieties of crops. Depending upon the gene used, genetically engineered resistance can be achieved by two ways. These genes can be taken from pathogen itself known as pathogen derived resistance (PDR) or from another source known as non-pathogen derived resistance. In case of pathogen derived resistance, genes of the pathogen itself is utilized for making RNAi constructs. Non-pathogenic resistance involves the use of genes of host origin and the other genes that are responsible for adaptive host processes, acquired in response to attack, to obtain plants resistant to the virus (Dasgupta *et al* 2003). In case of pathogen derived resistance (PDR), resistance to a particular virus is expressed by stably transformation of plants with a transgene that is derived from the virus itself. These virus derived genes might be involved in replication, transcription and encodes for coat and pre-coat proteins. Once RNA silencing constructs having transgenes was generated and transferred to plants, it activates plant's own machinery and all RNAs homologous to the transgene were degraded, involving those RNAs that derived from infecting viruses (Lindbo *et al* 1993). Mechanism of RNAi technique against viruses involves the post-transcriptional gene silencing of viral genes by using plant's own machinery (Figure 2.3). Post transcriptional gene silencing (PTGS) involves the degradation of transcriptional product that is the mRNA formed by the gene is not able to express at the protein level and the mechanism found its name because dsRNA did not degrade the RNA that are formed from intronic region of the genome. The process of PTGS was primarily found in plants and believed to function as a component of defense mechanism against plant viruses. Afterwards, it was shown to be a resistance mechanism found in all eukaryotes involving protozoa, plants and animals. Injection of RNA in sense or antisense orientations of particular gene was able to suppress the function of the gene in sequence-dependent manner (Guo and Kemphues 1995). RNAi was first noticed as gene silencing mechanism induced by double-stranded RNAs in worms (Hannon 2002). RNAi was earlier known by the phenomenon of co-suppression, in which expression of transgene itself was suppressed with that of the genes that are homologous and present endogeneously. Co-suppression phenomenon was laterally shown to involve in either transcriptional gene

silencing (TGS) or post-transcriptional gene silencing (PTGS) mechanism (Mansoor *et al* 2006).

Development of disease resistance by RNAi

Various studies exposed the effectiveness and advantage of dsRNA-derived non-coding RNAs as a feasible tool to achieve the silencing of any gene of interest irrespective of the occurrence of an off-target activity. RNAi seems to be effective technology for resistance against viruses. Among viruses, huge number of reports shows the application of RNAi against begomoviruses (Table 2.1). Majority of reports involves the generation of intron containing hairpin RNAi constructs in which mechanism of RNAi was initiated exogeneously.

Chellappan *et al* (2004) shows that five different begomoviruses involves in the initiation of PTGS response in plants with the production of virus-specific siRNAs. Viral infection was suppressed in blackgram when RNAi constructs harbouring AC2 ORF of MYMV was transfected into it (Pooggin *et al* 2003). Praveen *et al* (2006) shows that when tomato was transformed genetically with RNAi constructs carrying replicase gene of begomoviruses in antisense orientation, they showed resistance to the tomato leaf curl viral disease. Likewise, Yang *et al* (2004) express the replicase gene in tomato plants and found that replicase protein was not essential to trigger the resistance mechanism; infact the PTGS phenomenon might be liable for the resistance. Resistance to tomato yellow leaf curl virus was achieved by expressing AV2 gene in transgenic tomato plants and distinct types of resistant phenotypes were observed in T1 generation (Zrachya *et al* 2007). Viruses of geminiviridae family is not only the source of overwhelming diseases of important crops but also act as a means to study the elementary aspects of RNA-interference (RNAi) and virus-induced gene silencing (VIGS) (Mansoor *et al* 2003, Turnage *et al* 2002).

Geminiviridae are quickly rising as overwhelming plant pathogens especially in the tropical and subtropical areas of the world. Particularly begomoviruses, largest genus of the geminivridae causes a major increase in number of viral infections. Okra yellow vein mosaic and leaf curl diseases are the economically important begomoviral diseases which causes rigorous crop damage. In spite of its agronomical importance, at present there are no effective control measures available for the management of begomoviruses of okra.

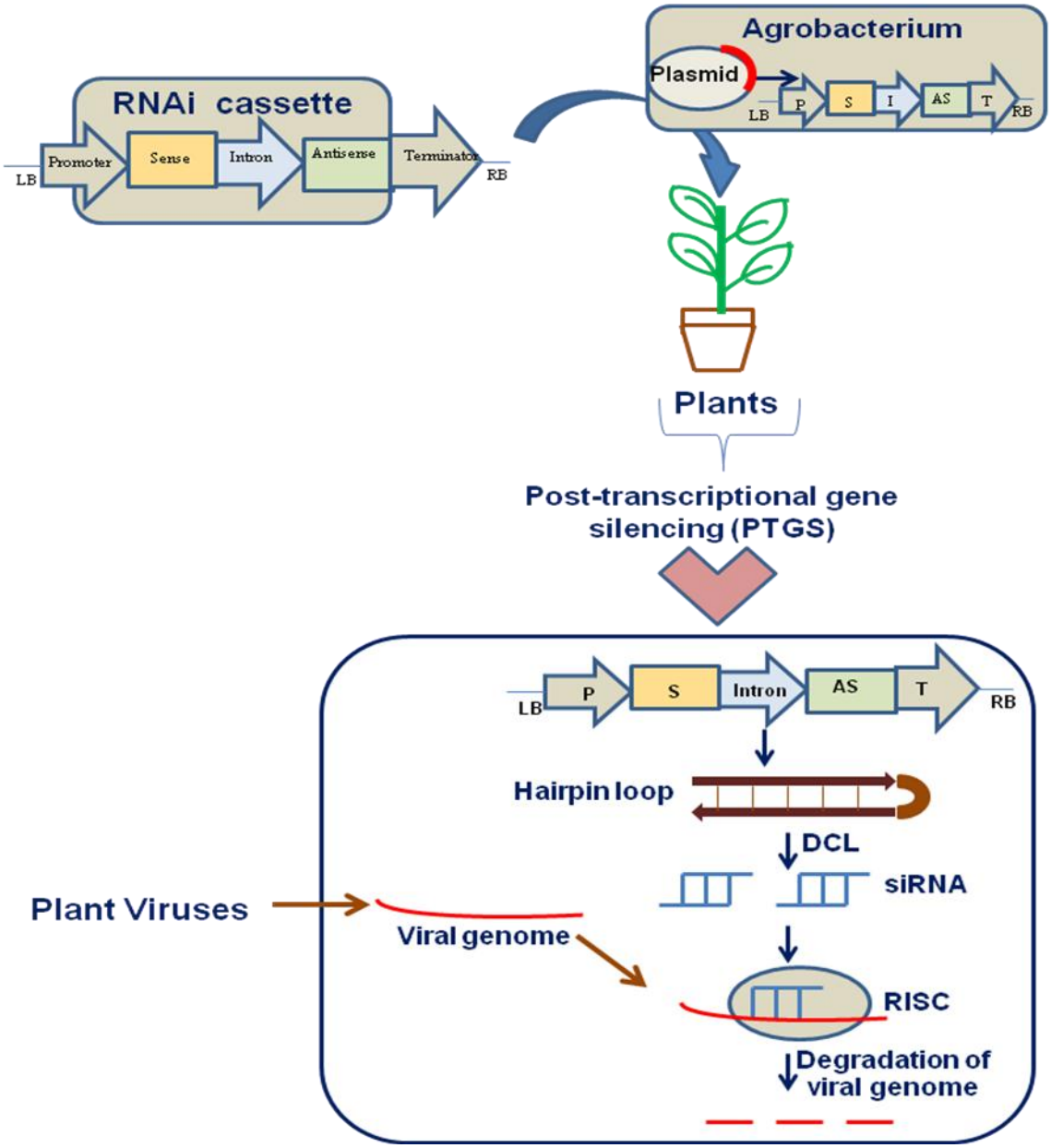


Figure 2.3: Mechanism of RNAi in plants against viruses

Table 2.1: Successful application of RNAi against plant viruses in different agricultural crops

Sr. No.	Targeted Crop	Virus	Genus of virus /Genome	Viral gene target	Target gene region(bp)	Transient expression/ Stable expression of RNAi cassette	Viral resistance produced	References
1.	Tomato	*Tomato Spotted Wilt Virus* (TSWV)	Tospovirus/ ssRNA	N (Nucleocapsid) gene	218bp	Stable expression in tobacco (model plant)	Multiple viral resistance	Jan *et al* 2002
		Turnip Mosaic Virus (TuMV)	Potyvirus/ ssRNA	CP	867bp			
2.	Tomato	*Cucumber mosaic virus* (CMV)	Cucumovirus/ RNA	CP	Full length (770bp)	Stable expression in tomato	Multiple viral resistance	Antony *et al* 2005
		Tomato leaf curl virus (ToLCV)	Begomovirus/ ssDNA	Rep	Conserved domain (~300bp)			
3.	Tomato	*Tomato yellow leaf curl virus* (TYLCV)	Begomovirus/ ssDNA	AC1	726bp	Stable expression in tomato	Single viral resistance	Fuentes *et al* 2006
4.	Common Bean	*Bean golden mosaic virus* (BGMV)	Begomovirus/ ssDNA	AC1(Rep)	421bp & 424bp	Stable expression in common bean	Single viral resistance	Bonfim *et al* 2007
5.	Rice	*Rice tungro bacilliform virus* (RTBV)	Tungrovirus/ dsDNA	ORF IV	~1326bp	Stable expression in rice	Single virus resistance	Tyagi *et al* 2008

Sr. No.	Targeted Crop	Virus	Genus of virus /Genome	Viral gene target	Target gene region(bp)	Transient expression/ Stable expression of RNAi cassette	Viral resistance produced	References
6.	Tomato	*Tomato leaf curl virus* (ToLCV)	Begomovirus/ ssDNA	PCNA endogene & AC2	-	Transient expression in tomato, tobacco, Arabidopsis & rice	Single viral resistance by VIGS	Pandey *et al* 2009
7.	Tomato	*Tomato leaf curl virus* (ToLCV)	Begomovirus/ ssDNA	AC4	200, 80, 60, 21bp	Stable expression in tomato	Single viral resistance	Shelly *et al* 2010
8.	Pulse crops	*Mungbean yellow mosaic India virus* (MYMIV)	Begomovirus/ ssDNA	AC1	566bp	Transient expression in cowpea, mungbean & black gram	Single viral resistance by antisense RNA	Haq *et al* 2010
9.	Cotton	*Cotton leaf curl virus* (CLCuMB)	Begomovirus/ ssDNA	AC1-AC4, AC1-AC2-AC3, IR	-	Transient expression in tobacco	Single viral resistance	Mubin *et al* 2011
10.	Chickpea	*Chickpea chlorotic dwarf Pakistan virus* (CpCDPKV)	Mastrevirus/ ssDNA	AC1-IR-AV2	730bp	Stable expression in tobacco (model plant)	Single viral resistance	Nahid *et al* 2011
11.	Tomato	*Potyvirus*	Potyvirus/ ssRNA	Plant translation initiation factor eIF4E	-	Stable expression in tomato	Single viral resistance	Mazier *et al* 2011
12.	Soyabean	*Alfalfa mosaic virus* (AMV) , *Bean pod mottle virus* (BPMV) *and soybean mosaic virus* (SMV)	Alfamovirus-AMV Comovirus-BPMV, Potyvirus-SMV (ssRNA)	IR	AMV-109bp dsRNA, BPMV-147bp, SMV-123bp	Stable expression in soyabean	Multiple viral resistance	Zhang *et al* 2011

Sr. No.	Targeted Crop	Virus	Genus of virus /Genome	Viral gene target	Target gene region(bp)	Transient expression/ Stable expression of RNAi cassette	Viral resistance produced	References
13.	Pepper & Tomato	*Pepper golden mosaic virus* (PepGMV) *&* *Tomato chino La Paz virus* (ToChLPV)	Begomovirus/ ssDNA	AC1-IR-AVI	AC1- 714nts IR- 326 nts AV1- 146nts (Total 1186bp)	Transient expression in tobacco (model Plant)	Multiple viral resistance	Medina-Hernandez *et al* 2013
14.	Cotton	*Cotton leaf curl Burewala virus* (CLCuBuV)	Begomovirus/ ssDNA	AV2	21bp	Stable expression in tobacco (model plant)	Single viral resistance by artificial microRNA	Ali *et al* 2013
15.	Cotton	*Cotton leaf curl virus* (CLCuV)	Begomovirus/ ssDNA	AC1	390bp	Transient expression in cotton	Single viral resistance	Yousaf *et al* 2013
16.	Tomato	*Cucumber Mosaic Virus* (CMV)	Cucumovirus/ RNA	-	1138bp	Stable expression in tomato	Mutiple resistance for 2 strains (CMV-O & CMV-Y)	Ntui *et al* 2014
17.	Chilli	*Chilli leaf curl virus* (ChiLCD)	Begomovirus/ ssDNA	AC1-AC2, AC1-AC2-$^{\beta}$C1	417bp, 610bp	Stable expression in tobacco (model plant)	Broad spectrum resistance	Sharma *et al* 2015
18.	Tomato	*Tomato Leaf Curl New Delhi Virus* (ToLCNDV)	Begomovirus/ ssDNA	AC2, AC4	-	Initially transient expression in both tomato & tobacco and after that stable expression in tobacco (model plant)	Single viral resistance by artificial trans-acting siRNA	Singh *et al* 2015

Sr. No.	Targeted Crop	Virus	Genus of virus /Genome	Viral gene target	Target gene region(bp)	Transient expression/ Stable expression of RNAi cassette	Viral resistance produced	References
19.	Tomato	*Tomato yellow leaf curl virus-oman* (TYLCV-OM)	Begomovirus/ ssDNA	AC1, IR, AV2 & AV1	AC1-175ntds, IR- 112ntds, AV2- 161 ntds, AV1-127ntds (575bp)	Initial transient expression in both tomato & tobacco and after that stable expression in tomato	Single virus resistance	Ammara *et al* 2015
20.	Cotton	*Cotton leaf curl virus* (CLCuV)	Begomovirus/ ssDNA	IR	300bp	Stable expression in cotton	Single viral resistance	Khatoon *et al* 2016
21.	Cotton	*Cotton leaf curl virus* (CLCuV)	Begomovirus/ ssDNA	$^{\beta}$C1	-	Stable expression in cotton	Single viral resistance	Sohrab *et al* 2016
22.	Cotton	*Cotton leaf curl kokhran virus-Burewala* (CLCuKoV-Bur)	Begomovirus/ssDNA	V2 & IR	240-V2	Stable expression in cotton	Stable expression in cotton	Yasmeen *et al* 2016
23.	Cowpea	*Mungbean Yellow Mosaic India Virus* (MYMIV)	Begomovirus/ ssDNA	AC2, AC4, AC2-AC4	186-AC2, 197-AC4, 383-AC2+AC4	Stable expression in cowpea	Single viral resistance	Kumar *et al* 2017
24.	Papaya	*Papaya ringspot virus* (PRSV)	Potyvirus/ ssRNA	CP	544bp	Stable expression in papaya	Single viral resistance	Jia *et al* 2017

2.3 Agroinfectious clones

Various methods such as natural field infection, inoculation of putative resistant plants with whitefly and inoculation of agroinfectious clones using young leaf or stem agroinfilteration or biolistic inoculation has been developed to check the viral gene silencing by RNAi cassettes that is being previously inoculated into the plants. Among these, natural inoculation methods are not always competent for screening of the resistance plants probably due to their non-preference by whiteflies and also non-availability of infected vector. As well, activation of vector, multiplication of the virus and its symptom development on the plants are affected by the environmental conditions that are present during and after the inoculation. Construction of agroinfectious clone provides valuable mean for the detection of resistance in plants against viruses. While unit-length DNA or cloned DNA that is having single copies of certain begomoviruses is infectious (Bonilla-Ramirez *et al* 1997) previous studies have shown that to increase the infectivity of infectious clones, they entail (partially) tandem-repeat constructs of the viral genomes (Boulton and Davies, 1988; Donson *et al* 1988; Stenger *et al* 1991), which makes the cloning process more difficult. The agroinfectious clone has been successfully constructed by various researchers from time to time. Briddon *et al* 1993 used abutting primers for the construction of agroinfectious clone; Wu *et al* 2008 and also Ferreira *et al* 2008 simplified the protocol of infectious clone construction by partially digesting the rolling circle amplified product of the viral genome. The quick and proficient method of agroinfectious clone construction of *Tomato yellow leaf curl virus* was developed by Bang *et al* 2014. Jin *et al* 2012 reported characteristic downward tomato yellow leaf curl symptoms in four to six leaf stages of tested *Solanum lycopersicum* plants at 30 days after inoculation.

2.4 Quantitative real time PCR analysis

Quantitative real-time PCR analysis is used for the consecutive amplification and quantification of required DNA molecule. The main purpose of qPCR is to quantify required molecule in a sample or compare the same between the samples. This PCR analysis is combined with the reverse transcription protocol and utilized for simultaneous amplification and gene expression analysis (Fitzgerald and McQualter 2014). After agroinfilteration of infectious clone into the RNAi plants, relative expression of target gene in control and transgenic plants will be calculated by this PCR analysis.Before the startup of PCR analysis, all the reagents are included into the reaction and during qPCR when double-stranded products were produced it leads to the emission of fluorescence. After each PCR cycle, the concentration of double-stranded product or amplicon is increased which cause the simultaneous increase in fluorescence. Increased fluorescence at every step is measured in real time. Starting concentration of DNA molecule was evaluated by calculating cycle

threshold or C^T value. Cycle threshold was measured by the number of cycles utilized to reach the set fluorescence level. For the qPCR, Syber green dye is the most usable dye for its dsDNA binding ability. Dye gets bind to dsDNA molecules formed during qPCR and upon binding they fluoresce. Dye binds to any of the double-stranded DNA and this binding of dye and given florescence is not dependent on the specific target. Use of dsDNA-binding dyes creates problems with non-specific amplification. Nowadays, dual-labeled probes are used; these probes ware designed in such a way so that it is having sequence which is complementary to some region of target DNA molecule. Some common examples of these types of probes are TaqMan and probes from Appied Biosystems. Utilization of these probes is target specific, but these are not cost-effective.

qPCR is extensively used to study gene expression. They can be applied in genotyping and for the complete quantification of the concentration of DNA or RNA for the clinical purposes. To check the expression of silenced genes in genetic engineered plants, primers related to the target gene can be synthesized and fold change in expression of the transgenic plant will be checked with respect to the control plant. Kumar *et al* (2017) synthesized primers specific to the AV2 gene of the mungbean yellow vein mosaic virus and check its fold change in expression in the tissue-culture raised RNAi plants of cowpea as compared to control. Likewise, Medina-Hernández *et al* (2013) synthesized primers that were specific to the AV1 region of pepper golden mosaic virus and check the viral load by qRT-PCR by amplifiying 104bp region in tissue-culture raised RNAi plants of tobacco and the control plants.

CHAPTER III

MATERIAL AND METHODS

The present work was conceded out with the objectives of construction of ihpRNAi (intron-containing hairpin RNAi) constructs targeting DNA-A component of okra-associated begomoviruses, transformation of okra with the ihpRNAi constructs and screening of the putative transgenic plants with agroinfectious clone at the School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana during 2015-2019. The materials and methods used in the study were as follows:

3.1 Preparation of gene constructs

3.1.1 Production of ihpRNA (intron-containing hairpin RNA) constructs

3.1.1.1 Revival and confirmation of DNA-A component of *Okra enation leaf curl virus*

3.1.1.2 Revival and confirmation of pFGC1008 (binary vector)

3.1.1.3 Amplication and cloning of target genes in pGEM-T easy vector

3.1.1.4 Confirmation of recombinant plasmids (pGEM-T) by PCR and restriction digestion

3.1.1.5 Cloning of target gene(s) fragments in sense and antisense orientation in RNAi vector

3.1.1.6 Confirmation of target genes in recombianat RNAi vectors (pFGC-*AC2*hp & pFGC-*AC4*hp) by PCR and restriction digestion

3.1.1.7 Sequencing and analysis of target gene(s) fragments in recombinant RNAi vectors

3.1.1.8 Transformation of RNAi constructs to *Agrobacterium* strain GV3101

3.1.2 Development of dimeric construct (agroinfectious clone) of DNA-A component of okra infecting begomovirus(es) in binary vector

3.1.2.1 Cloning of DNA-A component of genome of begomovirus in pUC19 vector

3.1.2.2 Preparation of partial tandem dimers (agroinfectious clone) in binary vector

3.1.3 Agroinfilteration of plants with gene constructs

3.2 Genetic transformation of okra using RNAi constructs

3.2.1 PCR analysis

3.3 Molecular characterization of okra RNAi plants

3.3.1 Viral inoculation of okra RNAi plants

3.3.1.1 Infectious clone mediated inoculation

3.3.1.2 Whitefly mediated inoculation

3.3.2 Expression analysis

3.3.2.1 Semi-quantitative PCR

3.3.2.2 Quantitative real-time PCR analysis

3.1 Preparation of gene constructs

Genes targeting the DNA-A genome of okra-associated begomoviruses were utilized to prepare the constructs. For the construction of two hairpin RNA constructs, the overlapping fragments of important genes of begomoviruses of okra were utilized. Further to check the efficacy of RNAi constructs, partial tandem dimmers of okra infecting begomoviruses were generated in binary vector. Effectiveness of three constructs was initially checked by agroinfilteration of okra plants.

3.1.1 Production of ihpRNA (intron-containing hairpin RNA) constructs

The target genes used in this study were overlapping regions of AC1/AC2 and AC1/AC4 from DNA-A component of begomoviruses associated with okra (Figure 3.1). Targeting ORFs play important role in viral DNA replication and help in suppression of plant's autoimmune response. Target genes were selected by aligning the sequences of DNA-A component of 8 isolates of okra-associated begomoviruses. Sequences were retrieved from NCBI database and aligned with the help of clustal omega programme.

***In silico* prediction of potential siRNAs from the target gene(s) fragments**

Conserved nucleotides from the target genes were ensured for the production of potential siRNAs by Jack Lin's siRNA prediction tool. Conserved regions of target genes were used as query sequence in the tool. This online tool gave the sequences of potential siRNAs found and percentage of GC content in the sequence. Each siRNA sequence was further subjected to blastn to check the similarity index with target regions of DNA-A component of okra associated begomoviruses.

3.1.1.1 Revival and confirmation of DNA-A component of *Okra enation leaf curl virus* (accession no. KP208672.1)

DNA-A component of *Okra enation leaf curl virus* (accession no. KP208672.1) was previously cloned in pJET 1.2 cloning vector (CloneJET^{TM} Thermo scientific) at *Eco*RV restriction sites and this vector has ampicillin resistance gene as a selectable marker for bacteria. Recombinant plasmid was present in *E.coli* strain DH5α in the form of glycerol stocks. A loopful of culture was streaked on the Luria Bertani agar (LBA) plates having ampicillin (50µg/ml) antibiotic. Plates were incubated overnight at 37°C. Luria broth was inoculated with the isolated colony of bacteria and incubated overnight at 37°C with continuous shaking at 250 rpm. Plasmid DNA was isolated by using alkaline lysis method with the help of QIAprep Spin Miniprep Kit from Qiagen as per manufacturer's instructions:

Isolation of plasmid DNA (Alkaline lysis method)

- Single colony was picked from a streaked culture and 1-5 ml Luria broth (LB) containing suitable antibiotic was inoculated and incubated overnight at 37°C with vigorous shaking.

- Bacterial cells were harvested by spinning the tubes at >8000rpm in a centrifuge (eppendorf) for 3 min at room temperature i.e. 15-25°C. Media was drained by inverting the tubes to remove all traces of supernatant.
- RNase A (provided with the kit) was added to 250µl of Buffer P1 (Resuspension buffer-50mM glucose, 25mM Tris (pH 8.0) and 10mM EDTA (pH 8.0). to resuspend the bacterial cells which were pelleted down and the whole suspension was transferred to 1.5ml microtube. 0.25µl of lyse blue (provided with the kit) was added in the above suspension and bacterial pellet was completely mixed by pipetting up and down.
- To the above suspension 250µl of Buffer P2 (lysis buffer-0.2N NaoH and 0.5% SDS) was added and it was thoroughly mixed by inverting the tubes 4-6 times. Vortexing was not done at this step otherwise it will lead to the shearing of the DNA. Inverting of the tubes was continued until the suspension becomes viscous and was not proceeded for more than 5min. Lyse blue added made the solution blue in color.
- 350µl of Buffer N3 (Neutralization buffer-3M potassium acetate) was added to the blue solution and thoroughly mixing was done by inverting the tubes 4-6 times. Now suspension became colorless and cloudy in appearance.
- Centrifugation was done for 10min at 13,000 rpm and after 10min the cell debris was pelleted down and left the DNA in the supernatant and this supernatant was applied to the QIAprep 2.0 spin column followed by centrifugation for 60sec at 13,000 rpm. Flow-through was discarded.
- Spin column was washed with 500µl of PB buffer (Binding buffer) and centrifuged for 60 sec at 13,000 rpm. This step was recommended to remove traces of nuclease activity when *end*A+strains were used. Flow-through was discarded.
- Spin column was again washed with 750µl of PE buffer (Wash buffer). Before using wash buffer it was recommended to add 100% ethanol to the wash buffer provided with the kit. Column was centrifuged for 60sec at 13,000 rpm and flow-through was discarded. Residual ethanol was removed by repeated centrifugation.
- Column was placed in a clean 1.5ml eppendorf tube and 35µl of EB buffer (Elution buffer) was added in the centre of the column. Incubated at room temperature for 1min and then centrifuged for 1min at 13,000 rpm.

Assessment of recombinant plasmid DNA (pJET)

Quantity and quality of recombinant plasmid DNA was checked by agarose gel electrophoresis and nanodrop spectrophometer (Thermo Fisher Scientic). For this, 0.8-2.0% (as per requirement) agarose gel was prepared by dissolving agarose powder (Electrophoresis

matrix, G Biosciences, USA) in 1X TAE buffer (Table 3.1). The mixture was heated till the solution becomes transparent and clear. It was cooled down to 60°C with constant stirring. After cooling 5 µl ethidium bromide (10mg/ml) was added to 100ml melted agarose. The agarose solution was then poured into gel mould with combs and left for about 40 min for solidification. After solidification combs were removed gently and the gel was placed in the gel tank for electrophoresis. Afterwards with the help of micropippette, samples were loaded into wells by mixing with 6X loading dye (Table 3.2) in such a way that the final concentration of loading dye becomes 1X. Along with the samples, DNA marker with known size of fragments was also loaded. After loading, the gel was subjected to electrophoresis at constant voltage of 5 V/cm for about 1hour. After electrophoresis, the gel was visualized under UV trans-illuminator and recorded with gel documentation system (Alpha Imager HP, USA).

Table 3.1: Composition of TAE Buffer

TAE Buffer (pH 8.0)	
Component	**Volume (500ml)**
Tris base (himedia)	121gm
0.5M EDTA of pH 8.0 (himedia)	50ml
Glacial Acetic Acid	28.55ml

Table 3.2: Composition of 6X loading Dye

Components	**Quantity (W/V)**
Bromophenol blue	0.25 %
Xylene Cyanol	0.25 %
Glycerol	30 %

Confirmation of DNA-A component of okra-infecting begomoviruses in recombinant plasmid (pJET)

Isolated plasmid was confirmed by PCR analysis with pJET forward and reverse sequencing primers (Table 3.5) provided with the kit. PCR was performed with reaction mixture (Table 3.3a) with the conditions as follows: initial denaturation at 94°C for 3min followed by step 2, which was denaturation at 94°C for 30sec, melting at 60°C for 30 sec and extension at 72°C for 3min and this step 2 was repeated for 25 cycles and final extension was given at 72 °C for 7min. PCR product was analyzed on 0.8% TAE agarose gel. Recombinant plasmid was further subjected to digestion with restriction enzyme *EcoR*V (Promega).

Digestion mixture was made (Table 3.3b) and was placed in thermostat (eppendorf) at 37°C for 2hours. After 2 hours of digestion reaction, products were checked on 0.8% agrose gel.

Table 3.3a: Composition of PCR reaction mixture for pJET primers

Reaction mixture (25µl)	
Component	**Volume**
DNA (500ng)	2µl
10X CoralLoad PCR Buffer / 10X PCR Buffer	2.5µl
25mM $MgCl_2$	2µl
dNTPs (10mM each)	2µl
Forward Primer (10µM)	2µl
Reverse Primer (10µM)	2µl
Taq DNA polymerase (2.5units/reaction)	0.25µl
Q-solution (5X)	5µl
RNAse-free water	7.25µl

Table 3.3b: Composition of digestion mixture for whole genome confirmation in pJET 1.2 vector

Component	**Volume (20µl)**
Plasmid (500ng/µl)	2
Buffer D (10X)	2
Acetylated BSA (10µg/µl)	0.2
EcoRV (10µg/ul) (Promega)	0.5
Sterile water	15.3

3.1.1.2 Revival and confirmation of pFGC1008 (binary vector)

Binary vector pFGC1008 (Figure 3.3) was used as plant expression vector because this vector is having intron in their T-DNA region and the targeted regions can be ligated in sense and antisense orientation in the flanking region of intron and after transfer into the plant they can make hairpin loop to initiate RNAi in the host plant. Vector was present in *E.coli* strain DH5α in the form of glycerol stocks. A loopful culture was streaked on the LBA plate having antibiotic chloramphenicol (35µg/ml). Plates were placed at 37°C overnight. Isolated colonies were used to inoculate 5ml of LB containing chloramphenicol (35µg/ml) in the 50ml

falcon tubes. Tubes were placed in incubator at 37°C overnight with vigorous shaking. Plasmid was isolated by using alkaline lysis method (section 3.1.1.1) and checked on 0.8% TAE agarose gel. Plasmid was confirmed by PCR analysis with the use of vector-specific primers (Table 3.5), first pair of primers (AS primer pair) was designed from the multiple cloning sites to the starting nucleotides of GUS-intron and second pair (BS primer pair) was designed from the ending nucleotides of GUS-intron to the multiple cloning sites of pFGC. PCR was performed and conditions for PCR were Initial denaturation at 94°C for 3min followed by step 2, which was denaturation at 94°C for 1min, melting at 55°C for 45sec and extension at 72°C for 45sec and this step 2 was repeated for 35 cycles and final extension was given at 72 °C for 7min. Plasmid was also confirmed with the help of restriction digestion with 2 pair of enzymes i.e. *Bgl*II & *Spe*I, *Xho*I & *Spe*I. Reaction mixture was prepared for the digestion of pFGC1008 (Table 3.4).

Table 3.4: Composition of reaction mixture for double-digestion of pFGC1008

Component	Volume (20µl)
pFGC1008 (500ng/µl)	2
Cut Smart Buffer (10X)	2
*Bgl*II (10,000U/ml) (NEB) / *Xho*I (20,000U/ml) (NEB)	1
*Spe*I-HF (20,000U/ml) (NEB)	1
Sterile water	14

3.1.1.3 Amplication and cloning of target genes in pGEM-T easy vector

The sequences of both the target regions were selected by aligning sequences of eight okra infecting begomovirus isolates available on NCBI database. For the purpose of alignment, clustal omega programme was utilized to obtain the conserved regions. Conserved regions were further subjected to *In silico* siRNA prediction with the help of Jack Lin's siRNA sequence finder tool. Primers were designed from the conserved regions and the restriction sites were added at the 5′end of the forward and reverse primers (Table 3.5) so that amplified products can be ligated in the plant expression vector in sense and antisense orientation. Primers SF1-*AC2* & SR1-*AC2* was for the amplication of overlapping region of AC1-*AC2* in sense-orientation and ASF1-*AC2* & ASR1-*AC2* was for amplification of overlapping region of *AC1*/*AC2* in antisense orientation. Similarly, SF2-*AC4* & SR2-*AC4* was for the amplification of *AC1*/*AC4* in sense orientation and ASF2-*AC4* & ASR2-*AC4* is for the amplification of *AC1*/*AC4* in antisense-orienation (Table 3.5).

Dilution of primers

Each primer was dissolved in 100 µl of 1x Tris EDTA (TE) buffer (himedia) and diluted further with deionized water to the working concentration of 10 µM. The primers were diluted as per following formula:

$$\mu M \text{ of oligo in } 100\mu l \text{ of solution} = \frac{OD}{10}$$

Primers (Table 3.5) were used for the amplification of the target genes (Figure 3.1) by taking recombinant pJET vector (section 3.1.1.1) as a template in such a way so that they can clone in sense and antisense orientation. PCR conditions for all the primers were remain the same. PCR reaction mixture of 50µl was prepared (Table 3.6) and the conditions were as follows: Initial denaturation at 94°C for 3min followed by 3-step cycling by denaturation at 94°C for 1 min, annealing at 62°C for 45 sec, extension at 72°C for 45 sec and number of cycles for this step was 35 and final extension was given at 72°C for 7 min. PCR was performed in the thermal cycler (Applied Biosystems) and PCR products were purified by using PCR Clean-up protocol from Macherey-Nagal (MN) as follows:

Purification of PCR products

- 100% ethanol (BRG Biomedicals) was added to the NT3 buffer (Wash buffer).

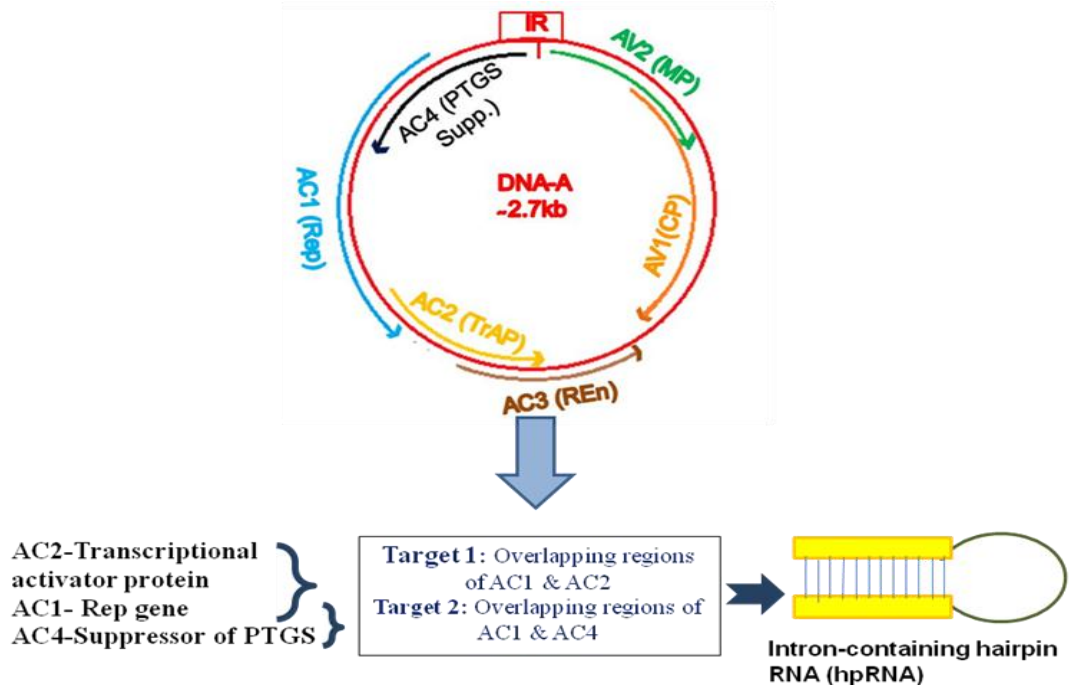


Figure 3.1: Target genes of DNA-A component of okra associated begomoviruses for RNAi

Table 3.5: List of primers used for the development of RNAi constructs

Sr. No.	Utilization of Primers	Primer code	Sequence (5'-3')	Length (bp)	Tm (◦C)
I.	**pJET specific primers**				
a.	pJET forward sequencing primer	pJET-F	5′CGACTCACTATAGGGAGAGCGGC-3′	23	66.0
b.	pJET reverse sequencing primer	pJET-R	5'AAGAACATCGATTTTCCATGGCAG-3'	24	59.3
II.	**Vector (pFGC1008) specific primers**				
a.	Sense strand confirmation	ASF	5′CAATCCCACTATCCTTCGCAAG-3′	22	55.7
		ASR	5′GACAGCAGCAGTTTCATTCAATCA-3′C	24	56.9
b.	Antisense strand confirmation	BSF	5′TGTGGAGTATTGCCAACGAAC-3′	21	55.5
		BSR	5′GTAATCAGTCTGTTAGGTTTGACCG-3′	25	55.4
III.	**Primers for the amplification of targeted regions**				
a.	Target 1 in sense orientation	SF1-AC2	5′CTA CTC GAG ATA GAG GGG ATT TGG AAC CTC CC-3′	32	63.1
		SR1-AC2	5′CTA GAG CTC AAA GGG ACT GGC AAT CCA ACA C-3′	31	63.4
b.	Target 1 in antisense orientation	ASF1-AC2	5′CTA GGA TCC AAA GGG ACT GGC AAT CCA ACA C-3′	31	63.4
		ASR1-AC2	5′CGC ACT AGT ATA GAG GGG ATT TGG AAC CTC CC-3′	32	63.6
c.	Target 2 in sense orientation	SF2-AC4	5′CAG CTC GAG CCC ACA CAA ATA GTT ATG TGG AC-3′	32	63.2
		SR2-AC4	5′CTA GAG CTC ACA GCC AAC GAC GCT TAC G-3′	28	63.8
d.	Target 2 in antisense orientation	ASF2-AC4	5′CTA GGA TCC ACA GCC AAC GAC GCT TAC G-3′	28	63.8
		ASR2-AC4	5′CGC ACT AGT CCC ACA CAA ATA GTT ATG TGG AC-3′	32	61.5

Underlined nucleotides- sites for restriction enzymes; CTC GAG-*Xho*I, GAG CTC-*Sac*I, GGA TCC-*BamH*I, ACT AGT-*Spe*I

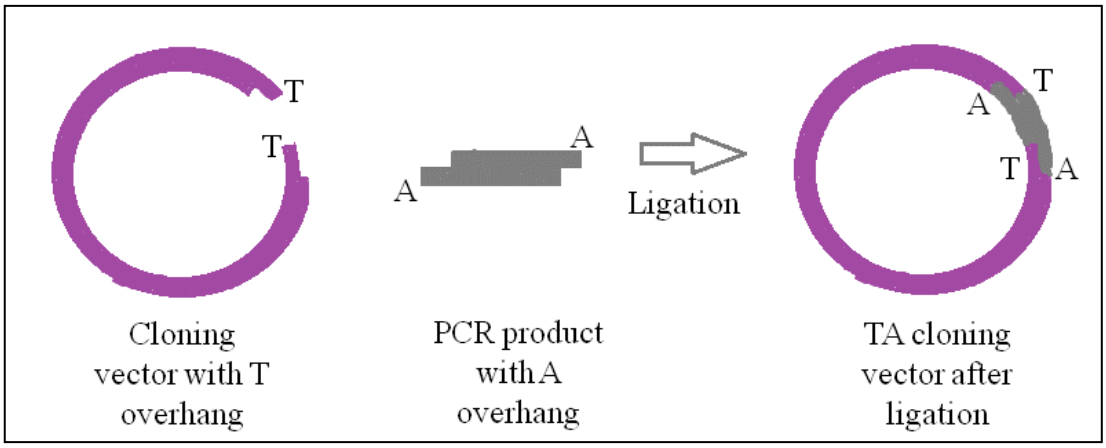
- To the 50µl of PCR reaction, double volume (100µl) of buffer NTI (binding buffer) was added and solution was mixed by pipetting up and down.
- PCR clean-up midi column (provided with the kit) was placed in the collection tube and the sample from above step was loaded into it. After loading it was centrifuged for 1 min at 11,000 rpm and flow-through was discarded.
- 700 µl of NT3 buffer (Wash buffer) was added to the column and centrifuged for 1 min at 11,000 rpm. Flow-through was discarded and step of washing was repeated. After discarding flow-through, column was placed back to the collection tube and silica membrane of the column was dried by centrifuging empty column to remove traces of ethanol.
- Collection tube at this step was discarded and column was placed to the fresh 1.5ml eppendorf tube. 25µl of Buffer NE (Elution buffer) was added to the centre of the column and incubated for 1min at room temperature followed by centrifugation at 11,000 rpm for 1min. 5µl of purified PCR products was loaded into the 2% TAE agrose gel. Above purified PCR products were further cloned into the pGEM-T easy vector as these products have A tailing and hence used for TA cloning.

Table 3.6: Composition of reaction mixture for the amplification of targeted regions

Reaction mixture (50µl)	
Component	**Volume**
DNA (500ng)	4µl
10X CoralLoad PCR Buffer / 10X PCR Buffer	5µl
25mM $MgCl_2$	4µl
dNTPs (10mM each)	4µl
Forward Primer (10µM)	4µl
Reverse Primer (10µM)	4µl
Taq DNA polymerase (2.5units/reaction)	0.5µl
Q-solution (5X)	10µl
RNAse-free water	14.5µl

Amplified products were used for ligation into the TA vector (Figure 3.2). Ligation mixture was prepared (Table 3.7) and placed in refrigerator at 4°C. For the purpose of

cloning, *E.coli* strain DH5α was used and this strain was made competent to take up the exogenous DNA (Sambrook *et al* 1989).



Schematic representation of TA cloning

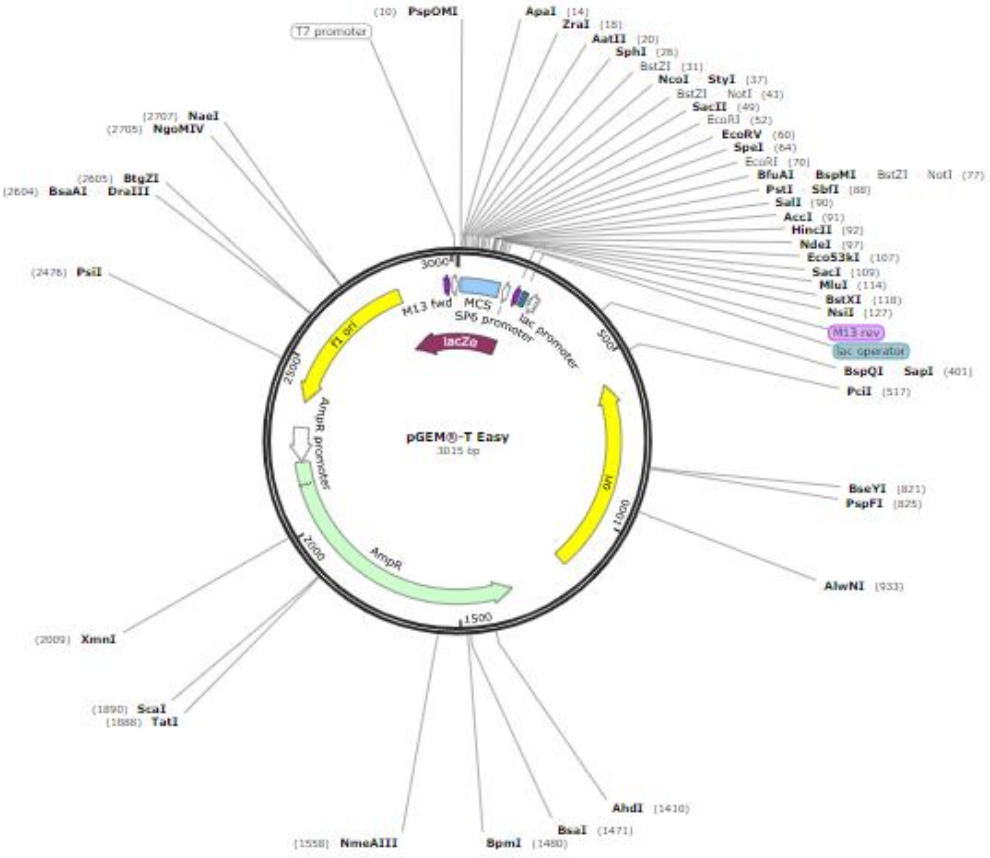


Figure 3.2: Diagrammmatic representation of TA cloning and map of pGEM-T easy vector

Table 3.7: Composition of ligation mixture for TA cloning

Ligation mixture (20µl)	
PCR product	6µl
pGEM-T easy vector (Promega)	2µl
10X ligation buffer (Promega)	4µl
T4 DNA Ligase (Promega)	1µl
RNAse-free water	7µl

Preparation of competent cells of *E.coli* strain DH5α by $CaCl_2$ method

- Single colony of the strain was used to streak Luria Bertani Agar (LBA) plates and the isolated culture was used to initiate primary culture by inoculating 5ml of Luria Broth (LB) taken in 50ml fresh falcon tube. Tubes were placed in incubator set at 37°C overnight with vigorous shaking.
- 500µl of the primary culture was used to inoculate 50ml of LB taken in 250ml flask to initiate secondary culture. Flask was placed in incubator for 3-4hours until optical density (OD) of the culture reaches 0.3-0.4. After reaching appropriate OD culture was placed on ice for 20 min by intermediate shaking with the hands.
- After 20min, culture was shifted in two 50ml falcon tubes by taking 25ml of culture in each tube. Centrifugation was done for 5min at 4,000 rpm at 4°C. After 5min, culture was again placed in ice and then 25ml of 0.1M $CaCl_2$ (autoclaved) was added to each tube and pellet formed was resuspended in the $CaCl_2$ solution and tubes were incubated for 45min on ice.
- Tubes were centrifuged for 5min at 4,000 rpm at 4°C. 1.4ml of 0.1M $CaCl_2$ with 0.6ml of 50% glycerol (autoclaved) was added to the pellet formed. Pellet was resuspended in the above mixture and 100µl of aliquots was made in 1.5ml eppendorf tubes and by immediately freezing the aliquots in liquid nitrogen, aliquots were shifted to -80°C for storage.

Transformation of recombinant plasmids (pGEM-T easy) having target gene(s) fragments

Ligation of target gene fragments with linear pGEM-T easy vector gave the recombinant circular plasmids. These plasmids were transformed to the competent cells *E.coli* strain DH5α and following reagents need to be prepared for transformation:

Preparation of X-GAL solution

Stock solution of 100mg/ml X-GAL (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was prepared by dissolving 0.5 g of X-GAL in 5 ml of dimethylsulfoxide

(DMSO). After complete suspension, it was filter sterilized by using 0.22 µm Millipore™ nylon filter. Solution was aliquot in 500µl and stored at -20°C.

Preparation of antibiotic solutions

The stock solution of antibiotics (ampicillin, chloramphenicol, rifampicin, cefotaxime, hygromycin and kanamycin sulphate) used during the study was prepared by adding required amount of salt in 2-3 drops of solvent and after dissolving final volume was scaled upto 5ml with the same (Table 3.8). Stock solutions were filter sterilized by using 0.22 µM Millipore™ nylon filter and stored at -20°C.

Preparation of IPTG (isopropyl beta-d-1-thiogalactopyranoside)

100mM of IPTG was prepared by dissolving 2.38 g of IPTG in the 8ml of distilled water and final volume was scaled upto 10ml with the same. Solution was filter sterilized with 0.22µm nylon syringe filter. Store the solution at -20°C by making aliquots of 1ml each.

Preparation of LB liquid/ agar medium

For the preparation of 1 litre LB (Luria Broth) medium, all medium components were dissolved in 800 ml of water, pH was adjusted to 7.0 and the final volume was made upto 1000 ml with distilled water (Table 3.9). For LB agar medium, liquid LB was supplemented with bacteriological agar (1.6%). Both LB-liquid and LB-agar media were autoclaved at 121°C for 30 minutes.

Table 3.8: List of antibiotics used

Antibiotic	Concentration of stock solution (mg/ml)	Solvent	Cocentration of working solution (µg/ml)
Ampicillin	50	Water	50
Chloramphenicol	35	Ethanol/Methanol	35
Rifampicin	50	Methanol	100
Hygromycin B	10	Water	400
Cefotaxime	500	Water	500

Table 3.9: Composition of LB medium

Component	Amount (per Litre)
Bacto Tryptone	10 g
Bacto Yeast extract	5 g
Sodium chloride	10 g

Aliquots of competent cells were taken from -80°C deep freezer and placed on ice for 15-20 min. Efficiency of the cells was checked by spreading them on LBA plates and colony count was done. 8µl of ligation mixture formed above (Table 3.7) was added to the competent cells of *E.coli* strain DH5α. Cells were mixed by tapping the tubes with hands. Tubes were again placed on ice for 15min. After 15min, tubes were placed in thermostat Plus (eppendorf) set at 42°C for 1min for heat-shock treatment. Tubes were placed on ice for 10min. 900µl of LB was added to the tubes and the tubes were placed in incubator for 1-2 hours at 37°C with shaking at 220rpm. After incubation tubes were centrifuged for 5min at 5,000 rpm at room temperature. Approximately 900µl of supernatant was discarded and pellet was resuspended in rest of the supernatant left in the tubes. 80µl of the suspension was spreaded on the LBA plates having ampicillin (50ug/ml), 20mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and 100mM of IPTG (isopropyl beta-d-1-thiogalactopyranoside). Plates were placed in incubator at 37°C overnight.

Colony PCR of recombinant clones having target genes

Isolated colonies were re-streaked onto fresh plates and check for the positive clones by performing colony PCR with target genes or insert specific primers (Table 3.5). PCR reaction mixture was prepared (Table 3.10**)** and PCR was performed by following conditions: Initial denaturation at 94°C for 3min, step 2 is as denaturation at 94°C for 1min, annealing at 62°C for 45sec, extension at 72°C for 45sec, repetition of step 2 for 35cycles followed by final extension at 72°C for 7min.

Table 3.10: Composition of colony PCR reaction mixture

Reaction mixture (25µl)	
Component	**Volume**
DNA (500ng)	Single colony
10X CoralLoad PCR Buffer / 10X PCR Buffer	2.5µl
25mM $MgCl_2$	2µl
dNTPs (10mM each)	2µl
Forward Primer (10µM)	2µl
Reverse Primer (10µM)	2µl
Taq DNA polymerase (2.5units/reaction)	0.25µl
Q-solution (5X)	5µl
RNAse-free water	7.25µl

3.1.1.4 Confirmation of recombinant plasmids (pGEM-T) by PCR and restriction digestion

Plasmid DNA was isolated from the positive clones and checked by performing PCR as discussed in colony PCR under section 3.1.1.3 (Table 3.10) . Isolated plasmids were also confirmed by restriction digestion with 2 pair of primers; *Xho*I & *Sac*I were utilized for the confirmation of cloned targets in sense orientation and *BamH*I & *Spe*I were used for the confirmation of targets in antisense orientation. Digestion reaction mixture was prepared (Table 3.11) and mixture was placed in thermostat plus (eppendorf) set at 37°C for two hours.

Table 3.11: Composition of digestion mixture for confirmation of TA cloning

Component	Volume (µl)
Plasmid (500ng/µl)	2
CutSmart Buffer (10X)	2
*Xho*I/*BamH*I(10u/µl)	1
*Sac*I, *Spe*I (10u/µl)	1
Sterile water	14
Total	20µl

3.1.1.5 Cloning of target gene(s) fragments in sense and antisense orientation in RNAi vector

Target regions in sense orientation were digested from TA cloning vector and used to ligate to the RNAi vector. Two targets in sense orientation along with RNAi vector (pFGC1008) were digested with *Xho*I & *Sac*I overnight at 37°C (Table 3.12). After overnight digestion, digested products along with undigested vectors were loaded separately on 0.8% agarose gel and bands of required length were purified from the gel with the help of Macherey-Nagal (MN) kit:

Purification of DNA fragments from agarose gel

- Before using the protocol, 100% ethanol (BRG Biomedicals) was added to the NT3 (Wash buffer).
- **Excision of DNA fragments or solubilization of gel slices**

  Clean scalpel was taken for the excision of the required DNA fragments by removing excess gel. Bands cut from the agarose gel were placed in the eppendorf tubes, to the 100mg gel slice, 100µl of the buffer NTI (solubilization buffer) was added to the tubes. Samples were incubated at 50°C with intermediate vortexing. Incubation was done until the gel slice was completely dissolved.

- **Binding of the DNA**

  NucleoSpin gel column (provided with the kit) was placed in the collection tube and the sample from above step was loaded into it. After loading it was centrifuged for 1 min at 11,000 rpm and flow-through was discarded.

- **Silica membrane washing**

  700 µl of NT3 buffer (Wash buffer) was added to the column and centrifuged for 1 min at 11,000 rpm. Flow-through was discarded and step of washing was repeated. After discarding flow-through, column was placed back to the collection tube and silica membrane of the column was dried by centrifugating empty column to remove traces of ethanol.

- **DNA elution**

  Collection tube at this step was discarded and column was placed to the fresh 1.5ml eppendorf tube. 25µl of NE buffer (Elution buffer) was added to the centre of the column and incubated for 1min at room temperature followed by centrifugation at 11,000 rpm for 1min. Eluted DNA thus formed was used for further ligation purpose.

Cloning of target gene(s) in RNAi vector in sense orientation

Purified fragments of 385bp of target1 and 342bp of target2 in sense orientation were used to ligate in eluted pFGC1008 of ~10,882bp size. Ligation reaction was prepared (Table 3.13) in such a way so that each of the targets in sense orientation ligated with the RNAi vector at the same restriction enzyme sites. Ligated products were transformed in competent cells of *E.coli* strain DH5α (Section 3.1.1.3). Colonies appeared were restreaked on Luria agar plates having chloramphenicol antibiotic (35µg/ml) and checked for positive clones by colony PCR with vector-specific primers (ASF & ASR) (Table 3.5). Colony PCR was performed as described in section 3.1.1.3 (Table 3.10) with the following PCR conditions: Step 1 was initial denaturation at 94°C for 5min, step 2 was denaturation at 94°C for 2min, annealing at 55°C for 45sec, extension at 72°C for 45sec followed by repeatition of step 2 for 35 cycles, step 3 was final extension at 72°C for 7min. Recombinant RNAi vectors (pFGC-*AC2*(s) & pFGC-*AC4*(s) having target gene fragments in sense orientation were isolated from positive clones and confirmed by PCR (Table 3.10) and also by restriction digestion. Digestion was done with the same enzymes that were used for the ligation of sense strands in RNAi vector i.e. double-digestion with *Xho*I & *Sac*I (Table 3.12).

Cloning of target gene(s) in recombinant RNAi vector in antisense orientation

Confirmed recombinant RNAi vectors (pFGC-*AC2*(s) & pFGC-*AC4*(s) that were having target gene fragments were double-digested separately with *BamH*I & *Spe*I at 37°C for

overnight. Double-digestion reaction was prepared (Table 3.12) with the *BamH*I & *Spe*I. Overnight digested products were loaded on the 0.8% agarose gel along with the uncut vector. Required bands were purified from the gel under UV by using Macherey-Nagal (MN) kit (as discussed above). Purified gene fragments in antisense orientation from recombianant pGEM-T were ligated in recombinant pFGC-*AC2*(s) & pFGC-*AC4*(s) (Table 3.13). Ligated products (8ul) were used for tranformation into competent cells of *E.coli* strain DH5α (section 3.1.1.3). Colonies appeared were restreaked on the Luria agar plates having chloramphenicol antibiotic (35µg/ml). Colonies were checked by colony PCR with vector-specific primers (BSF & BSR) (Table 3.5).

3.1.1.6 Confirmation of target genes in recombinant RNAi vectors (pFGC-*AC2*hp & pFGC-*AC4*hp) by PCR and restriction digestion

Recombinant plasmids having target gene fragments in sense and antisense orientation were isolated (section 3.1.1.1) and confirmed by the PCR with both gene and vector-specific primers (Table 3.5). Plasmids were also confirmed by double-digestion with two pair of enzymes i.e. *Xho*I & *Sac*I and *BamH*I & *Spe*I respectively. Two RNAi constructs (pFGC-*AC2*hp & pFGC-*AC4*hp) having two targets in sense and antisense orientation at the flanking region of GUS-intron were prepared (Figure 3.3).

3.1.1.7 Sequencing and analysis of target gene(s) fragments in recombinant RNAi vectors

Constructs were sent for the sequencing from Eurofins Genomics India Pvt. Ltd. to confirm the sequence and integration of the gene fragments into the RNAi vector. Bidirectional sequencing was done with vector-specific primers (Table 3.5) to confirm the sequence of gene fragments in sense and antisense orientation separately. Sequence was retrived and used to found percent similarity. The retrieved sequences were characterized through various online and offline Bioinformatics tools.

The partial forward and reverse sequences were first screened for vector contamination using Vec Screen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) software. The trimmed sequences (both forward and reverse) were assembled using Bioedit Sequence Alignment Editor Software (version 5.09) and saved as a single contig file. The contig file was used as query nucleotide sequence and was screened against the available online GenBank nucleotide sequence database using Basic Local Alignment Search Tool (BLAST) program to run as nucleotide blast (BLASTn). Query nucleotide in FASTA format was uploaded in the window. Program selection optimized for highly similar sequences (megablast) and query nucleotide sequence was clicked to BLAST with algorithm parameters were kept at default. Identities having query coverage of 98-99%, E-value of 0.0 or less were selected.

Table 3.12: Composition of digestion mixture

Component	Volume
Cloning vector (500ng)/pFGC 1008	5µl
10X CutSmart Buffer (NEB)	4µl
*Xho*I (NEB)	1µl
*Sac*I (NEB)	1µl
Nuclease free water	39µl
Total	**50µl**

Table 3.13: Composition of ligation reaction

Component	Volume
Sense fragment (385bp & 342bp)	6µl
pFGC1008	2µl
5x ligation buffer (invitrogen)	4µl
T4 DNA ligase (invitrogen)	1µl

3.1.1.8 Transformation of RNAi constructs to *Agrobacterium* strain GV3101

To check the expression of RNAi constructs in the okra plant, these constructs were transformed in *Rhizobium radiobacter* (*Agrobacterium tumefaciens* syn. *Agrobacterium radiobacter*) strain GV3101. For this GV3101 strain was made competent by $CaCl_2$ method (Xu and Li 2008).

Preparation of competent cells of *Agrobacterium*

- Culture of *Agrobacterium* strain GV3101 was streaked on YEP agar plates (Table 3.14) having Rifampicin antibiotic (100µg/ml) and plates were placed at 28°C for 48-72 hours until single colonies were there on the plate. A single colony of *Agrobacterium* GV3101 was inoculated in 5 ml YEP broth (Table 3.14) taken in 50ml falcon tube and tubes were incubated at 28°C overnight with shaking (~250 rpm) for making primary culture.
- 2 ml of primary culture was added to 50 ml YEP media in 250 ml screw capped conical flask and incubated for 3 hours with constant shaking until OD_{600}= 0.5~1.0.
- As soon as the OD_{600} reached 0.5, the culture was chilled on ice for 5 minutes and then transferred to 50 ml falcon tube and then centrifuged at 3000 x g for 5 minutes at 4°C and the supernatant was discarded.
- The cells were then incubated on ice for 10 min.

- Pellet formed was resuspended with 10 ml of 20 mM ice-cold $CaCl_2$ followed by centrifugation at 3000 x g for 5 min at 4°C.
- The pellet was finally resuspensed in 1 ml $CaCl_2$ (20 mM chilled CaCl2) with 0.4ml of 100% glycerol.

100µl of the cells were aliquoted in 1.5 ml pre-chilled eppendorf tubes and transferred immediately to liquid N_2 for quick freezing and then stored at -80°C until use. Competent cells prepared were used for the transformation of constructs to GV3101 strain of *Agrobacterium*. Transformation of *Agrobacterium* competent cells was done by freeze-thaw method.

Table 3.14: Composition of YEP Broth and YEP agar medium

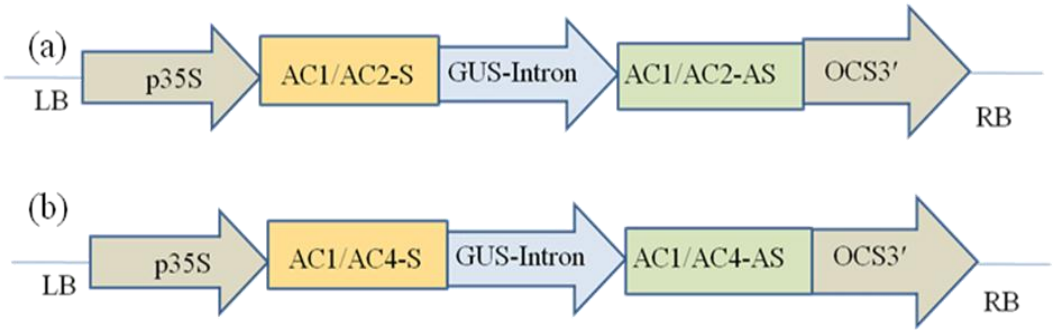
Components	**Amount**
Yep Broth modified (himedia)	2.5g
Distilled water	1000ml
For the preparation of YEP agar medium, 15g of agar (himedia) was added	

The competent cells were transformed with the two recombinant pFGC plasmids (pFGC-*AC2*hp & pFGC-*AC4*hp) along with non-recombinant plasmid as follows:

- Competent cells were taken out from -80° C deep freezer and then kept on ice for thawing for 20 min and about 1 µg of plasmid DNA was added to the competent cells and mix gently by tapping the tubes with hands.
- Tubes were then immediately put in liquid N_2 for 5 min for freezing. After that the tubes were immediately put at 37°C for 15 min for heat shock.
- After incubation at 37°C, 1 ml of YEP medium (without any antibiotic) was added to the tubes.
- Transformed cells were allowed to grow for 2 and half hours at 28°C with continuous shaking (220 rpm) in a shaker.
- Cells were pelleted down by centrifugation at 12,000 rpm for 2 min. Supernatant was discarded but approximately 150µl supernatant was retained and cells were dispensed in it. 50µl of cells were spreaded on YEPA plates containing Chloramphenicol (35µg/ml), rifampicin (100µg/ml).The plates were incubated at 28°C for 3-4 days.

Colonies appeared were restreaked on the fresh YEP agar plates having same antibiotics as used before. Colony PCR was performed with gene-specific primers (Table 3.5) with following PCR conditions: Step 1 was initial denaturation at 94°C for 5min, step 2 was

denaturation at 94°C for 2min, annealing at 55°C for 45sec, extension at 72°C for 45sec followed by repeatition of step 2 for 35 cycles, step 3 was final extension at 72°C for 7min. Plasmid DNA was isolated from positive clones and retransformed into the competent cells of *E.coli* stain DH5α (Section 3.1.1.3) for the confirmation of recombinant plasmids. Recombinant plasmid DNA were isolated from positive clones and reconfirmed by PCR performed with the help of gene-specific primers (Table 3.5).



Schematic diagram showing T-DNA region of two ihpRNA constructs

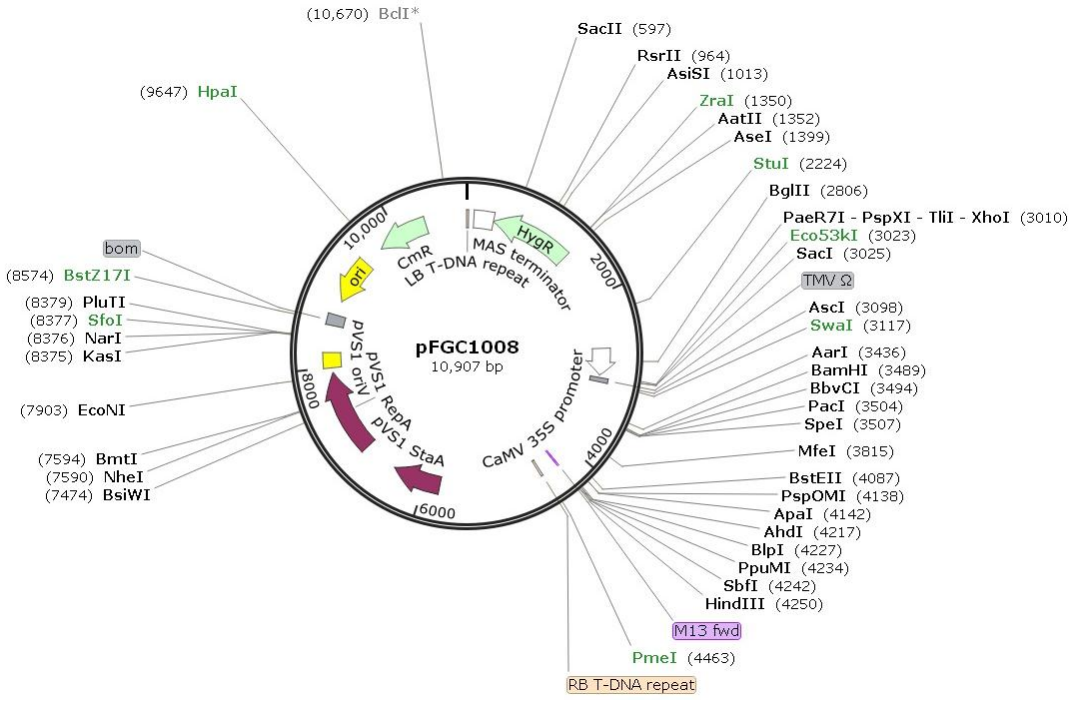


Figure 3.3: Schematic representation of T-DNA region of ihpRNA constructs and map of RNAi vector pFGC1008

3.1.2 Development of partial tandem dimers (agroinfectious clone) of DNA-A component of okra infecting begomovirus(es) in binary vector

For the purpose of analysis of plants transformed with the RNAi constructs, dimeric clone of DNA-A component of begomovirus of okra was developed. Before making dimeric clone, DNA-A genome was cloned in pUC19 vector (Figure 3.4) and this DNA-A was sub-cloned in pFGC1008.

3.1.2.1 Cloning of DNA-A component of genome of begomovirus in pUC19 vector

Leaves having characteristics symptoms of begomoviruses on okra plants were procured from Department of Vegetable Sciences, Punjab Agricultural University, Ludhiana. DNA was isolated from these infected leaf samples according to CTAB method. Since, okra possess large amount of mucilage that hinders DNA isolation. Mucilage often binds to other secondary metabolites such as phenolics, tannins and alkaloids and co-precipitates with DNA during isolation. Contamination of mucilage and other secondary metabolites not only makes the DNA unmanageable during pipetting but also hinders further downstream applications such as the polymerase chain reaction (PCR) or other enzymatic reactions since they inhibit Taq Polymerase activity (Fang *et al* 1992) and interfere directly or indirectly with the enzymatic reactions (Weishing *et al* 1995). Therefore, modified cetyl trimethyl ammonium bromide (CTAB) method given by Ghosh *et al* 2009 was used for DNA extraction from okra as follows:

Isolation of genomic DNA from leaves of okra plants by modified CTAB (Cetyl Trimethyl Ammonium Bromide) method

1. Frozen leaf tissues (1g) were ground to a fine powder with liquid nitrogen using mortar and pestle and finely grinded powder was transferred to well labelled 2.0 ml centrifuge tubes.
2. 800 µl of pre-warmed (65°C) extraction buffer (Table 3.15) was added and mixed thoroughly. Tubes were then incubated at 65°C for 30 min. During incubation, the contents were mixed four to five times by inverting the tubes gently.
3. 800 µl of phenol: chloroform: iso-amylalcohol (25:24: 1) was added to the tubes and put on the shaker for 15-20 minutes. Mixture was then centrifuged in a microcentrifuge (eppendorf) at 13,000 rpm for 10 minutes at room temperature.
4. The upper aqueous phase was then transferred to fresh tubes. About two- thirds volume of chilled iso-propanol was added and mixed well by inverting the tubes. The samples were then refrigerated at -20°C for 2 hours, centrifuged at 13,000 rpm for 10 minutes to make pellet. The supernatant was discarded.

5. A double volume of absolute ethanol (100%) was added to the tubes to precipitate DNA and was mixed gently by inverting the tubes three to four times.
6. These tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C, the supernatant was decanted

   and the pellet was retained.
7. The DNA pellet was washed with 300µl 70% ethanol and centrifuged at 13,000 rpm for 3 minutes.
8. The pellet was then air-dried and finally the purified DNA pellet was dissolved in 50µl of TE buffer.

Table 3.15: Composition of CTAB and TE buffer

Composition of CTAB buffer		
Components	**Stock concentration**	**Final concentration**
Tris- HCl	1M	100 mM
EDTA, pH 8.0	0.5M	10 mM
NaCl	2M	1.4M
CTAB	-	2%
β-marcaptoethanol	-	2%
Composition of TE buffer		
Components	**Stock concentration**	**Final concentration**
Tris HCl (pH 8)	1M	50mM
EDTA	0.5M	1mM

Quantification of extracted DNA

Quantification of nucleic acids was performed by using NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The optical surface of the spectrophotometer (Thermo Scientific NanoDrop™ 1000) system was cleaned by putting 1-2µl deionised water on the lower optical surface. The lever arm was closed and tapped few times to clean the upper optical surface and both the optical surfaces were wiped with tissue paper by lifting the lever. The NanoDrop software was clicked to open and "Nucleic acid" module was selected. Spectrophotometer was initialized by placing 1µl clean water onto the lower optic surface and selecting "Initialize" in the NanoDrop software. A "Blank" (Water and TE buffer in which DNA sample was dissolved) measurement was performed by loading 1µl of TE buffer. Measurement of the nucleic acid sample was done by loading 1µl of sample and selecting "Measure". Pure nucleic acid typically yield a 260/280 ratio of ~1.80 for DNA.

PCR detection of begomoviruses

For the partial characterization of begomovius(es) associated with okra, PCR was carried out on extracted DNA of okra using primers specific for virus coat protein gene (Table 3.16) (Wyatt and Brown 1996). PCR positive samples were subjected to RCA (Rolling Circle Amplification).

Amplification of single stranded circular DNA by Rolling Circle amplification:

Amplification was done by using TempliPhi 100 Amplification Kit (GE Healthcare Life Sciences illustra) and the protocol was as follows:

- 1 µl of the isolated DNA was added to 5 µl of sample buffer in a PCR tube. The sample was heated to 95°C for three minutes in thermal cycler to denature the DNA.
- Sample was cooled and combined with 5 µl of reaction buffer and 0.2 µl of enzyme mix (Phi29 DNA polymerase) and incubated at 30°C for 4–18 hours.
- At the end of the incubation, the ϕ29 DNA polymerase was inactivated by heating at 65°C for 10 minutes. The amplified product was stored at -20°C until use. The RCA product hence formed was quantified on 1% agarose gel containing ethidium bromide (10mg/ml).

RCA products were used for the digestion with the different restriction enzymes to find the single cutter enzyme to yield ~ 2.7kb genome of begomoviruses (Table 3.17). Digestion reaction was prepared (Table 3.18) for 2 hours at 37°C in waterbath (Thermo Fisher Scientific) except in case of *Swa*I enzyme reaction was set at 25°C. After digestion, samples were loaded on 1% TAE gel and the RCA products that yield~ 2.7kb was used further for cloning. Overnight digestion was set with the enzyme that cut the RCA product along with the circular cloning vector named pUC19. Overnight digested products were loaded on the 1% gel along with uncut plasmid and RCA product. Required bands were purified from the gel as described in section 3.1.1.5. Product of ~2.7kb eluted from gel was used for cloning in pUC19 vector that was digested with the same enzyme as that of RCA product and eluted from the gel. Ligation reaction was prepared (Table 3.19) and 8µl of ligated product was used for cloning in competent cells of *E.coli* strain DH5α (Section 3.1.1.3). Blue-white screening was done and white colonies were restreaked on the fresh Luria agar with ampicilin plate. Colonies were checked by PCR with the pair of degenerate primers (Table 3.16) and plasmid was isolated from positive clones and checked by PCR and digestion reaction. Enzyme used in digestion reaction was same as that was used for the digestion of RCA product.

3.1.2.2 Preparation of dimeric construct (agroinfectious clone) in binary vector

Pair of outward primers was designed from the sequences of begomoviruses from NCBI database (Table 3.16). *SacI* restriction site was added in the 5′end of forward primer and *Asc*I site was added in 5′end of the reverse primer. The PCR reaction was prepared and conditions were as follows: step 1 involved the initial denaturation at 94°C for 3min, step 2 was denaturation at 94°C for 1min, annealing at 64°C for 1min, extension at 72°C for 3min and the step 2 was repeated for 30 cycles followed by final extension at 72°C for 10min. PCR product was loaded on 1% TAE agarose gel and ~2.7kb fragment was gel purified and used to ligate it to pGEM-T easy vector. Ligated product was cloned in competent cells of *E.coli*. This fragment was digested from pGEM-T easy vector and cloned into binary vector pFGC1008 as *Sac*I/*Asc*I insert resulting pFGC-M. Further, 2.7kb *Sac*I fragment from pUC19 was recloned in pFGC-M to form a complete dimer of DNA-A named pFGC-2M. Cloning was confirmed by PCR with vector-specific primers (ASF-ASR) (Table 3.5). Dimeric clone formed was transformed to *Agrobacterium* strain GV3101 as discussed in section 3.1.1.8). Schematic representation of construction of tandem dimmers of DNA-A component of okra associated begomoviruses is described in Figure 3.5.

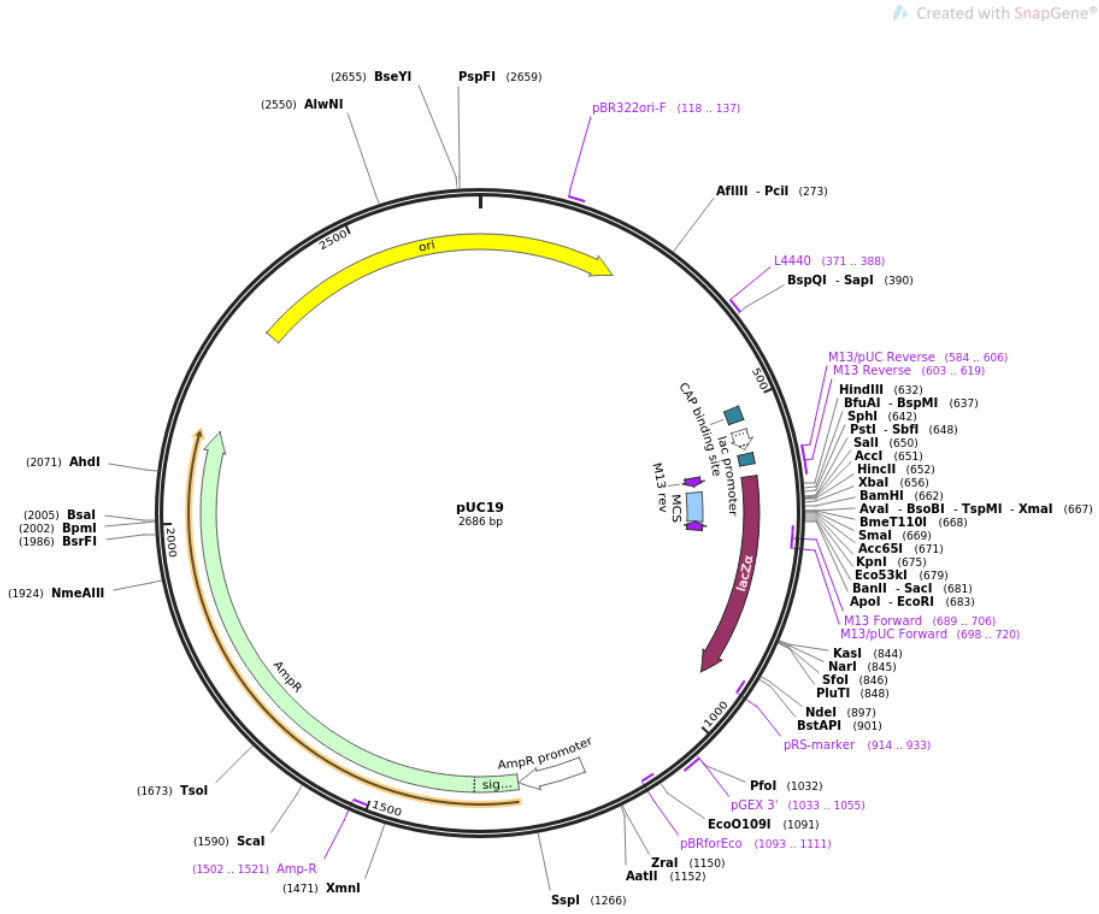


Figure 3.4: Map of pUC19

Table 3.16: List of primers used for the development of dimeric construct

Utilization of primers	Primer code	Sequence (5′-3′end)
Confirmaton of DNA-A	AV 494	5′GCCYATRTAYAGRAAGCCMAG3′
	AC 1048	5′GGRTTDGARGCATGHGTACATG3′
For the construction of dimeric clones	IFC-F	5′AATTGGCGCGCCACGGCTACTGTAAAGAA3′
	IFC-R	5′ CCAAGGATCCGGCTCGTTGTCATAAACATT GA 3′
Clone confirmation in pUC19	m13-F	5′GTAAAACGACGGCCAG3′
	M13-R	5′CAGGAAACAGCTATGAC3′
Where H is for A/C/T, R for G/A, Yfor C/T, M for A/C, D for G/A/T and N for A/T/G/C and underlined nucleotides are the sites of restriction enzymes		

Table 3.17: List of enzymes used for the digestion of RCA products

Enzymes (NEB)	Concentration
*Xho*I	20,000U/ml
*Sac*I	20,000U/ml
*Asc*I	10,000U/ml
*BamH*I	20,000U/ml
*Swa*I	10,000U/ml
*Spe*I-HF	20,000U/ml

Table 3.18: Composition of digestion mixture used for the digestion of RCA products

Component	Volume (20µl)
RCA product (1000ng/µl)	3µl
CutSmart Buffer (10X)	5µl
Enzymes (List of Table 15)	1µl
Sterile water	11µl

Table 3.19: Composition of ligation reaction for the cloning of RCA digested product

Component	Volume (20µl)
RCA digested product (~2.7kb)	6µl
pUC19 vector	2µl
5x ligation buffer (invitrogen)	4µl
T4 DNA ligase (invitrogen)	1µl

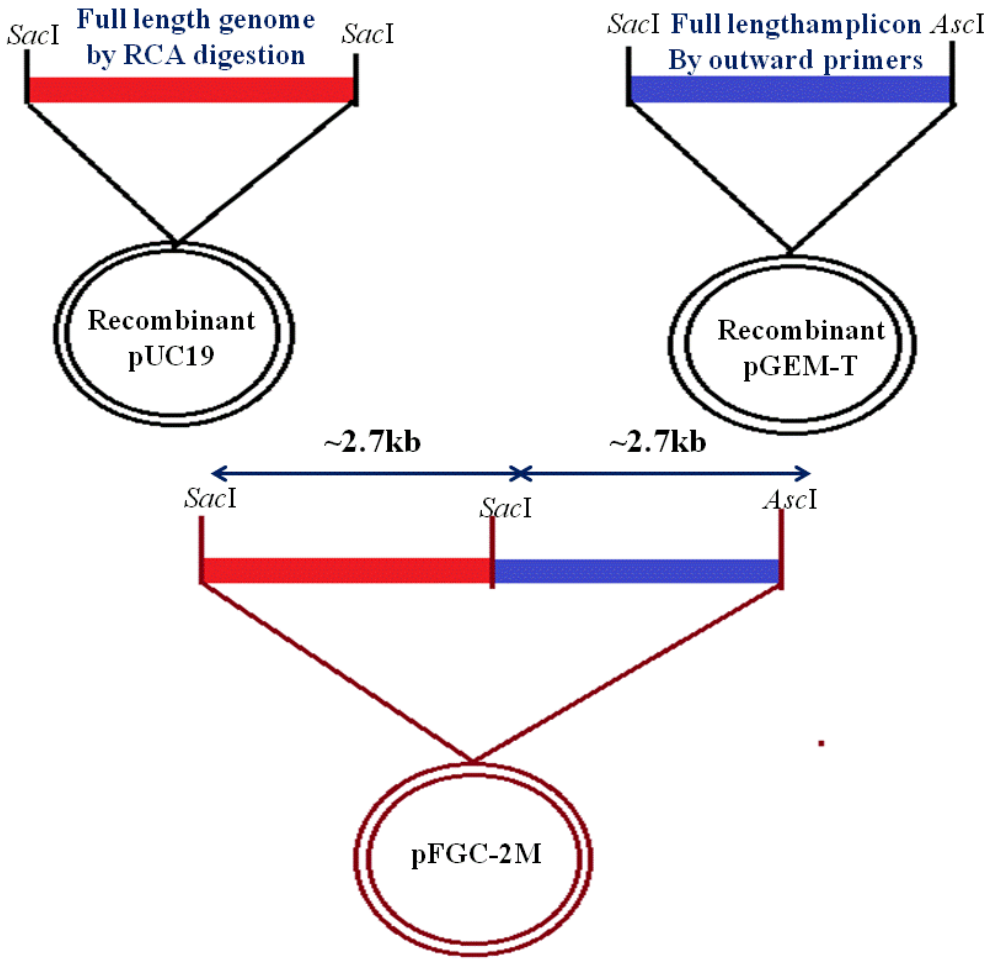


Figure 3.5: Construction of tandem dimers of DNA-A component of okra associated begomoviruses

3.1.3 Agroinfilteration of plants with gene constructs

Agrobacterium clones having recombinant, non-recombinant RNAi vectors and the agroinfectious clone of DNA-A of okra-associated begomoviruses were inoculated separatly in YEP broth containing antibiotics (35µg/ml chloremphenicol and 100µg/ml rifampicin) and grown overnight at 28°C to reach an OD_{600} = 0.6. Cells were then pelleted down by centrifugation at 4,000rpm for 10min and resuspended in an equal volume of activation buffer (Table 3.20). The resuspended cells were kept at 28°C for 1 hour with shaking at 90 rpm, with the help of syringe, suspension was used to infiltrate the bottom surface of young leaves of four weeks-old okra plants of variety Punjab 8. Infilteration was done by creating vaccum with the finger on the adaxial surface and application of infilterate on the abaxial surface. In such a way four sets of plants were made i.e. first set was inoculated with RNAi construct 1, second was inoculated with RNAi construct 2, third set was inoculated with dimeric clone of

begomoviruses and fourth was inoculated as negative control with non-recombinant plasmid for the purpose of mock inoculation. First and second set was further inoculated with the dimeric clone after 3 days of first inoculation to check the production of siRNA. Plants of all sets were maintained in green house for symptom evaluation for next 35 days.

Table 3.20: Composition of Activation Buffer

Components (Stock concentration)	Volume (10ml)	Final concentration
1M MES/KOH (pH 5.6)	100µl	10mM
10mM $MgCl_2$	10ml	10mM
200mM Acetosyringone	10µl	200µM

3.2 Genetic transformation of okra using RNAi (ihpRNA) constructs

For the purpose of ensuring stable expression of RNAi constructs, begomovirus susceptible variety of okra was chosen. Seeds of this variety (Punjab 8) were procured from Department of Vegetable Sciences, Punjab Agricultural University, Ludhiana. Genetic transformation of okra variety was done by two methods:

I. *Agrobacterium*-mediated genetic transformation using hypocotyls and cotyledonary leaves as explants.

II. *Agrobacterium*-mediated genetic transformation using embryos as explants (Narendran *et al* 2013).

Genetic transformation of okra by using embryo and hypocotyls as explants

- **Seed sterilization and germination**

Seeds of okra variety Punjab 8 were procured from Department of Vegetable Sciences, Punjab Agricultural University, Ludhiana. Seeds were washed in running tap water and then surface sterilized in strong disinfectant (0.1% weight/volume $HgCl_2$ in distilled water) for 30 min. Seeds were washed thrice in autoclaved distilled water and left overnight in sterile water for imbibition. Next day embryos from imbibed seeds were isolated aseptically and washed three times in sterile water. Washed embryos were dried on filter paper and placed on sterile water soaked filter paper.

- **Raising of explants**

  - **Raising of hypocotyls**

Isolated embryos were blot dried on sterile filter paper and placed on MSK medium for regeneration (Table 3.21). Kanamycin concentration was varied from 25ppm to 50ppm.

After 5 days, hypocotyls (1mm) just below the cotyledonary leaves were excised from the plant and used for transformation with *Agrobacterium* GV3101 containing constructs.

- **Raising of embryos**

Isolated embryos were prepared for inoculation by piercing 2-3 times at the plumule portion with the help of sterile needle.

- **Co- Cultivation of explants with *Agrobacterium* strain GV3101 containing RNAi constructs**

Two RNAi constructs transformed in *Agrobacterium* GV3101 were inoculated in 20 ml YEP broth containing chloramphenicol (35µg/ml) and rifampicin (100ug/ml) seperately, placed on incubator shaker (New Brunswick Innova 44 Incubator shaker) at 28°C until optical density at 600nm of 0.5 was reached. After reaching required optical density, cells were pelleted down by centrifugation at 4,000rpm for 10min at room temperature. Pellets formed were resuspended in 20 ml of liquid medium containing MS salts (Murashige and Skoog 1962) with 3% sucrose. 200µM of acetosyringone was added to the above suspension and again placed on rotary shaker for 1 hour at 28°C. Suspensions were used to inoculate explants. Explants were dipped in *Agrobacterium* suspension for 15 min followed by drying on autoclaved filter paper and transferred to the MSBI medium (Table 3.21) for co-cultivation. These plates were incubated for 2 days at 26±2°C. Four experiments with both constructs were performed individually.

- **Selection and regeneration**

After 2 days of co-cultivation, explants were washed with MSL medium (Table 3.21) for the removal of excess of *Agrobacterium* followed by dring on sterile filter paper. Embryos were excised from the plumule portion and hypocotyls directly were placed on MSBII medium (Table 3.21) for regeneration. Multiple green shoots regenerated on selection medium were transferred to the fresh MSBII medium. Sub culturing of regenerated shoots were performed after every 21 days i.e. in the interval of 3 weeks with 16/8hour light and dark at 26±2°C. Conditions of photoperiod and temperature were maintained same at all the incubation steps. Regenerated shoots were transferred to MSBIII medium (Table 3.21) for elongation and sub cultured after 21 days.

- **Rooting**

Elongated shoots that were grown well were transferred to MSBIV medium (Table 3.21) for rooting. Incubation was performed under 16/8 hour light and dark period at 26±2°C. Responsive plants were sub cultured on MSBIV medium for the development of tertiary roots.

- **Hardening and transfer to greenhouse**

Plants that form true roots were hardened in cotton dipped in sterile water. Sterile water dipped cotton was placed in jar, after proper washing of roots plants were transferred to jar and covered with polybags for 3 days under the same conditions of light and temperature as given in the above steps. Rooted plants were transplanted in sterile potting mixture placed in foam cups. When plants were established in cups, they were transferred to large pots and shifted to the greenhouse.

Table 3.21: Details of medium used for culturing

Medium (pH 5.8)	Composition
MSBI	MS salts (Murashige and Skoog 1962) + B5 vitamins (Gamborg *et al*., 1968) + Zeatin (2mg/l) + 0.8% agar + 3% sucrose
MSL	MS salts + 3 % sucrose + cefotaxime (500 mg/l)
MSBII	MS salts + B5 vitamins + Zeatin (2 mg/l) + 0.8 % agar + 3 % sucrose + hygromycin (25 mg/l) + cefotaxime (500 mg/l)
MSBIII	MS salts + B5 vitamins + 0.8 % agar + 3 % sucrose + hygromycin (25 mg/l) + cefotaxime (500 mg/l)
MSBIV	MS salts + B5 vitamins + 0.8 % agar + 3 % sucrose + cefotaxime (500 mg/l)
MSK	MS salts + 3 % sucrose + Kanamycin (30 mg/l) + 0.8% agar

3.2.1 PCR analysis

Tissue culture raised plants were subjected to DNA isolation (section 3.1.2.1). Isolated DNA was used for PCR analysis by using gene-specific & vector-specific primers (Table 3.5). Reaction conditions and reaction cycle used were same as discussed earlier (Section 3.1.1.2 & 3.1.1.3). Plants that show required bands with both gene-specific and vector-specific primers were proceded further for molecular characterization.

3.3 Molecular characterization of okra RNAi plants

Plants that form true roots and transferred to green house were maintained as putative transgenics and they were characterized firstly with the PCR and then bioassayed by agroinfilteration of leaves of okra with agroinfectious clone and also infestation with viruliferous whiteflies. Symptomatic and asymptomatic plants were analyzed by semi-quantitative PCR and quantitative real-time PCR.

3.3.1 Viral inoculation of okra RNAi plants

Plants whose DNA shows bands with gene or vector-specific primers, they were subjected to bioassay by two ways:

3.3.1.1 Infectious clone mediated inoculation

Agroinfectious clone that shows symptom was used to infilterate the transgenic plants. Primary culture of *Agrobacterium* having infectious clone was raised and from the primary culture, secondary culture was raised in 250ml flask. Culture was pelleted down and pellet was re-suspended in activation buffer (Table 3.20). At four-sixth leaf stage, the PCR positive plants, mock plants along with the untransformed plants were infilterate with the agroinfectious clone. Plants were observed upto the symptom appearance in the control plant. Symptomatic and non-symptomatic plants were analyzed further for the expression analysis.

3.3.1.2 Whitefly mediated inoculation

Plants shows begomoviral symptoms in the experimental field of Department of vegetable sciences were used for DNA isolation and checked with the help of RCA and then with the begomoviral specific primers. Whiteflies were procured from the experimental field of Department of vegetable sciences. 8-10 whiteflies were feeded onto the infected plant for acquisition period of ~12 hours. These whiteflies were supposed to be viruliferous and then applied on the plants that were used for agroinfilteration. Plants were observed upto the days when proper symptoms were appeared in the control plant. Both symptomatic and asymptomatic plants were analyzed further.

3.3.2 Expression analysis

Plants that were bioassayed were utilized for expression analysis. Total RNA was isolated from all the samples and cDNA was synthesized and confirmed. Fold change in expression of begomovirus- specific gene was checked by relative quantification method in control and the transgenic plants.

Isolation of total RNA from okra RNAi plants

Total RNA was isolated from all the plants that were bioassayed. Trizol method of RNA isolation was with the RNAiso takara kit which was as follows:

- 0.1% DEPC (Diethyl pyrocarbonate) water was prepared and autoclaved. Before starting RNA isolation, all the material (pestle, mortar, eppendorfs, tips) was properly dipped overnight in autoclaved DEPC water. After overnight treatment all material was autoclaved and dried.
- Leaf tissues were freshly obtained from PCR confirmed putative transgenic plants along with the control plants having viral symptoms. Tissues were immediately placed in liquid N_2.
- Tissues were homogenized individually in liquid N_2 and placed in DEPC treated microtubes. 900µl of RNAiso Plus (Takara) was added to the above and homogenate was kept at room temperature for 5 min followed by centrifugation at maximum speed for 5 min at 4°C.

- Supernatent was transferred to the fresh DEPC treated microtubes. 900µlof chloroform was added to the above and was vigorously vortexed. Solution was kept at room temperature for 5 minutes followed by centrifugation at maximum speed for 15 min at 4°C.
- Three layers were formed from this. From the three separated layers, the top most layer was the clear liquid having RNA, middle was semi-solid having DNA into that and the third layer was red in color containing less amount of DNA and the organic solvent was having polysaccharides, proteins, fatty acids, cell debris. Upper layer was transferred to the new tube.
- Equal the volume of upper layer, chilled isopropanol was added. Solution was kept at room temperature for 5min followed by centrifugation at maximum speed for 10min at 4°C. Supernatent was discarded.
- RNA pellet was washed with an equal volume of 70% ethanol (BRG Biomedicals). Centrifugation was done at 10,000 rpm for 5min at 4°C. Supernatent was discarded and the precipitate was kept for air drying. After complete drying, pellet was resuspended in nuclease free water.

Quantification of RNA on gel

For the quantification of isolated RNA on the gel, 10X MOPS buffer (0.2M) of pH 7 was prepared (Table 3.22). All the components were added to the 800ml of distilled H_2O. pH 7 was adjusted with NaOH and distilled water was added upto 1litre volume. Buffer prepared was stored in dark bottle and autoclaved.

Gel preparation

Gel was prepared by adding agarose into the solution of MOPS & DEPC water followed by boiling of the solution and cooled it upto the temperature 60°C. After cooling the gel, formaldehyde was added and gel was poured into the casting tray having combs in the required places (Table 3.23). After solidification, the gel was placed in the tank which was previously rinsed with 0.1% DEPC water. Tank was filled with buffer containing 90% DEPC water (0.1%) and 10% MOPS buffer.

Table 3.22: Composition of MOPS buffer

Component	Concentration in 1L	Mass in 1L
MOPS 3-(N-Morpholino) propanesulfonic acid) free acid	0.2M	41.86g
Sodium acetate	0.05M	4.1g
NA_2EDTA	0.01M	3.72g

Table 3.23: Composition of MOPS gel

Component	Mass&Volume/100ml
Agarose	2.5g
0.1% DEPC water	90ml
MOPS buffer	10ml
Formaldehyde	1.78ml

Sample loading

For the loading of samples into the gel, RNA samples were prepared by mixing isolated RNA samples with the equal amount of RNA loading dye. Samples prepared were loaded into the gel. Gel was run at 3 to 5 V/cm current. After the tracking dye reached upto a level, gel was visualized under UVP Gel Doc-It Imager (Analytik Jena).

3.3.2.1 Semi-quantitative PCR

After RNA quantification on gel, semi-quantitative PCR analysis was done with reference and target genes. Reference gene named β-tubulin was used and pre-coat protein gene (*AV2*) of begomovirsuses of okra was used as target gene for semi-quantitative analysis. Primers for gene *AV2* were designed by alignment of sequences of begomoviruses of okra from NCBI database. Conserved portion of pre-coat protein was utilized for designing degenerate primers. Primers were synthesized from Eurofins Genomics India Pvt. Ltd. Target gene was amplified and semi-quantitative analysis was done as follows:

cDNA synthesis from RNA

cDNA was synthesized by using cDNA synthesis kit from Applied Biosystems. Reaction mixture was prepared (Table 3.24) with PCR conditions were Step1 at 25°C for 10min, Step 2 at 37°C for 120min and Step 3 at 85°C for 5 min. Synthesized cDNA was quantified with nanodrop^{TM} 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). After quantification cDNA was normalized with nuclease-free water and 50ng was used for confirmation with reference and target gene-specific primers. PCR reaction was performed (Table 3.25) and PCR cycles were as follows: step 1 was intial denaturation at 94°C for 1min, 25cycles for step2 of denaturation at 94°C for 30sec, annealing at 62°C for 30sec and extension at 72°C for 30 sec followed by step 3 of final extension at 72°C for 5min. PCR samples were loaded into the 2.5% agarose gel and gel was visualized under UVP GelDoc-It Imager (Analytik Jena).

Table 3.24: Compositon of reaction mixture for cDNA synthesis

Component	Volume
Reverse Transcriptase (RT) Buffer (10X)	2µl
dNTP Mix (100mM)	0.8µl
RT Random Primers (10X)	2µl
Multiscribe Reverse Transcriptase	1µl
Nuclease free water	3.2µl
RNase inhibitor	1µl

Table 3.25: Composition of PCR reaction mixture for cDNA confirmation

Component	Volume (12ul)
cDNA (50ng)	4µl
Forward primer (5uM)	0.75µl
Reverse primer (5uM)	0.75µl
PCR master mix (Takara)	3.5µl
$MgCl_2$	0.5µl
dH_2O	2.5µl

3.3.2.2 Quantitative real-time PCR analysis

qRT-PCR was performed in duplicates using cDNA from transgenic, non-transgenic (control) and mock plants. PCR reaction mix (Table 3.26) was prepared with the following program: Step I was the initial denaturation at 95°C for 3 min, step II was denaturation at 95°C for 10sec, annealing at 62°C for 30sec, extension at 72°C for 30sec followed by repeatition of 2nd step for 40 cycles, Step III was melting at 60°C for 30sec. Real time PCR was performed in the LightCycler 96 (Roche). $\Delta\Delta C_T$ method was used to calculate the relative changes in gene expression which was determined from real-time quantitative PCR experiment using $2^{-\Delta\Delta C}{}_T$ (Livak and Schmittgen 2001). According to $\Delta\Delta C_T$ method:

$\Delta C_T = \Delta C_T$target - ΔC_Treference i.e. $\Delta C_T = \Delta C_T$ AV2 - ΔC_T β Tublin

$\Delta\Delta C_T = \Delta C_T$test sample - ΔC_Tcalibrator sample i.e. $\Delta\Delta C_T = \Delta C_T$ Target - ΔC_T Control

And $2^{-\Delta\Delta C}{}_T$ represents the fold change in gene expression relative to untreated control.

Table 3.26: Composition of PCR reaction mixture for quantitative real time PCR analysis

Component	Volume (10ul)
cDNA (50ng)	2µl
Forward primer (10uM)	0.5µl
Reverse primer (10uM)	0.5µl
Syber green (Applied Biosystems)	5µl
H_2O	2.5µl

CHAPTER IV

RESULTS AND DISCUSSION

The current study was undertaken with the main objectives of development of RNAi constructs against begomoviruses of okra, its transfer to okra variety Punjab 8 and characterization of putative RNAi plants through the construction of agroinfectious clones or with the help of viruliferous whiteflies. The results of the above objectives are discussed here in the following headings:

4.1 Gene constructs

4.1.1 Generation of ihpRNA (intron-containing hairpin RNA) constructs

4.1.1.1 Revival and confirmation of DNA-A component of *Okra enation leaf curl virus* in pJET vector

4.1.1.2 Revival and confirmation of pFGC1008 (binary vector)

4.1.1.3 Amplification and cloning of target gene(s) fragments in pGEM-T easy vector (TA cloning)

4.1.1.4 Confirmation of target gene(s) fragments in recombinant plasmids (pGEM-T)

4.1.1.5 Cloning of target gene(s) fragments in sense and antisense orientation in RNAi vector

4.1.1.6 Confirmation of target genes in recombinant RNAi vectors (pFGC-*AC2*hp & pFGC-*AC4*hp)

4.1.1.7 Sequencing and analysis of target gene(s) fragments in recombinant RNAi vectors

4.1.1.8 Transformation of pFGC-*AC2*hp & pFGC-*AC4*hp to *Agrobacterium* GV3101

4.1.2 Development of dimeric construct of DNA-A component of okra infecting begomovirus(es) in binary vector

4.1.2.1 Cloning of DNA-A genome of begomovirus in pUC19 vector

4.1.2.2 Enrichment of begomoviral DNA through Rolling circle amplification (RCA)

4.1.2.3 Detection of begomoviral DNA by restriction digestion of RCA product with various endonucleases

4.1.2.4 Amplication of DNA-A component by outward primers

4.1.2.5 Preparation of dimeric construct in binary vector

4.1.2.6 Confirmation of dimeric construct in binary vector by restriction endonucleases

4.1.2.7 Transfer of dimeric construct (pFGC-2M) from *E.coli* DH5α cells to *Agrobacterium strain* GV3101

4.1.3 Transient expression of gene constructs by agroinfilteration of okra leaves

4.2 Genetic transformation of Okra using RNAi (ihpRNA) constructs

4.2.1 Genetic transformation of okra using hypocotyls and cotyledonary leaves as explants

4.2.2 Genetic transformation of okra using zygotic embryos as explants

4.2.3 PCR analysis of putative transformants

4.3 Bioassay and molecular characterization of okra RNAi plants

4.3.1 Bioassay of okra RNAi plants

4.3.1.1 Infectious clone mediated inocultion

4.3.1.2 Whiteflies mediated inoculation

4.3.2 Expression analysis

4.3.2.1 Semi-quantitative PCR analysis

4.3.2.2 Quantitative real time PCR analysis

4.1 Gene constructs

RNAi constructs harbouring overlapping regions of gene(s) fragments of DNA-A component of okra-associated begomoviruses were prepared by cloning of target gene fragments in RNAi vector pFGC1008 in sense and antisense orientation at the flanking region of GUS-intron. This leads to the formation of intron-containing hairpin RNA constructs. Partial tandem dimeric construct of DNA-A component of begomoviruses infecting okra was also developed by cloning of dimers of DNA-A component prepared by ligation of restricted product and PCR product of DNA-A in binary vector pFGC1008. Efficiency of gene constructs was ensured by agroinfilteration of okra leaves.

4.1.1 Generation of ihpRNA (intron-containing hairpin RNA) constructs

For the generation of RNAi constructs, firstly target genes i.e. overlapping regions of *AC1*/*AC2* & *AC1*/*AC4* were selected by aligning the sequences of DNA-A component of okra associating begomoviruses of 8 virus isolates (Figure 4.1) prevailing in different states of India. Sequences of the 8 isolates were fetched from NCBI database and were aligned with

the help of Clustal omega tool (Figure 4.2 a & b). From the alignment results, the nucleotides that were conserved in the overlapping regions of three ORFs of begomoviruses were fetched (Figure 4.3 a & b). From this conserved region, sequence of 385 bp was selected for target gene fragment 1 which was starting from the nucleotide position 1375 to 1759 and target gene fragment 2 was of 342 bp that was starting from position 1866 to 2208 nucleotide position in DNA-A genome of begomoviruses of okra. Before designing the primers, these conserved regions of 385 and 342 bp were used as query sequences to check the production of siRNAs. siRNAs production and their GC content were ensured by Jack Lin's siRNA prediction tool (Table 4.1a &b). For each of the targets, a total of 25 siRNAs were shown by this tool.

Fuentes *et al* (2006) and Bonfim *et al* (2007), targeted AC1 gene of 726bp & 421bp to impart resistance in tomato against tomato yellow leaf curl virus through RNAi. Shelly *et al* (2010) used AC4 gene with varying length from 21bp to 200bp, they made different RNAi constructs and concluded that minimum length for the target for RNAi should be 200bp, in their case targets below 200bp increased the off-targets. Likewise, Sharma *et al* (2015) used AC1-AC2 & AC1-AC2- βC1 genes of 417 & 610bp respectively to find resistance against *chilli leaf curl virus* and successfully showed broad spectrum resistance against begomovirus of chilli. Kumar *et al* (2017) used AC2, AC4 & AC2-AC4 genes of 186,197 & 383bp respectively against mungbean yellow mosaic Indian virus of cowpea and found stable resistance through these genes in cowpea. In the present study also, overlapping regions of AC1-AC2 & AC1-AC4 of 385bp & 342bp (Figure 4.3 a & b) length respectively were selected.

```
MYVMVLudhiana       ACCGGATGGCCGCGCGATTTTTTTTTAGTGGTGGGTCCAGAACGCACGACTATGCAGAC-      59
OelcvLudhiana       ACCGGATGGCCGCGCGAT-TTTTTTTAGTGGTGGGTCCAGAACGCACGACGATGCAGACT      59
OelcvSurat          ACCGGATGGCCGCGCGAT-TTTTTTTAGTGGTGGGTCCAGAACGCACGACGATGCAGACT      59
BYVMVHimachal       ACCGGATGGCCGCGC-GATTTTTTTTAGTGGTGGGTCCAGAACGCACGACGATGCAGAC-      58
BYVMVNewDelhi       ACCGGATGGCCGCGC-GATTTTTTTAAGTGGTGGGTCCAGAACGCACGACGATGCAGAC-      58
BYVMVPhalaghat      ACCGGATGGCCGCGC-GATTTTTTTAAGTGGTGGGTCCAGAACGCACGACTATGCAGAC-      58
BYVMVKerala         ACCGGATGGCCGCGC-GATTTTTTTAAGTGGTGGGTCCAGAACGCACGACGATGCAGAC-      58
BYVMVChelur         ACCGGATGGCCGCGC-GATTTTTTTAAGTGGTGGGTCCAGAACGCACGACGATGCAGAC-      58
                    ***************    ****** * ********************** ********

MYVMVLudhiana       TCAAAGCTTAGATAACGCTCCTTTCGCTATAAGTACTTGCGCACTAAGTTTCAAATTGAA      119
OelcvLudhiana       T-AAAGCTTAGATAACGCTCCTTTCGCTATAAGTACTTGCGCACTAAGTTTCAAATTGAA      118
OelcvSurat          C-AAAGCTTAGATAACGCTCCTTTCGCTATAAGTACTTGCGCACTAAGTTTCAAATTGAA      118
BYVMVHimachal       TCAAAGCTTAGATAACGCTCCTTTGGCTATAAGTACTTGCGCACTAAGTTTAAATTCAAA      118
BYVMVNewDelhi       TCAAAGCTTAGATAACGCTCCTTCGACTATAAGTACGTGCGCACTAAGTTTCAATTCAAA      118
BYVMVPhalaghat      TCAAAGCTTAGATAACGCTCCTTCGGCTATAAGTACGTGCGCACTAAGTTTCAATTCAAA      118
BYVMVKerala         TCAAAGCTTAGATAACGCTCCTTCGACTATAAGTACGTGCGCACTAAGTTTCAATTCAAA      118
BYVMVChelur         TCAAAGCTTAGATAACGCTCCTTCGACTATAAGTACGTGCGCACTAAGTTTCAATTCAAA      118
                    **********************   ********** ************** ** *  **

MYVMVLudhiana       ACATGTGGGATCCATTGTTAAACGAGTTCCCTGAGACGGTTCACGGGTTTCGTTGCATGC      179
OelcvLudhiana       ACATGTGGGATCCATTGTTAAACGAGTTCCCTGAGACGGTTCACGGGTTTCGTTGCATGC      178
OelcvSurat          ACATGTGGGATCCATTGTTAAACGAGTTCCCCGAGACGGTTCACGGGTTTCGTTGCATGC      178
BYVMVHimachal       ACATGTGGGATCCACTATTAAACGAGTTCCCGGATACGGTTCACGGGTTTCGTTGTATGC      178
BYVMVNewDelhi       AAATGTGGGATCCACTATTAAACGAATTTCCGGATACGGTGCACGGGTTTCGTTGTATGC      178
BYVMVPhalaghat      AAATGTGGGATCCACTATTAAACGAATTTCCGGATACGGTTCACGGGTTTCGTTGTATGC      178
BYVMVKerala         AAATGTGGGATCCACTATTAAACGAGTTCCCGGATACGGTTCACGGTTTTCGTTGTATGC      178
BYVMVChelur         AAATGTGGGATCCACTATTAAACGAGTTCCCGGATACGGTTCACGGTTTTCGTTGTATGC      178
                    * ************ * ******** ** ** ** ***** ***** ******** ****

MYVMVLudhiana       TTGCTATTAAATATCTTCAACAACTGTCTGAGGAATACTCTCCTGATACGGTTGGGTACG      239
OelcvLudhiana       TTGCTATTAAATATCTTCAACAACTGTCTGAGGAATACTCTCCTGATACGGTTGGGTACG      238
OelcvSurat          TTGCTATTAAATATCTTCAACAACTGTCTGAGGAATACTCTCCTGATACGGTTGGGTACG      238
BYVMVHimachal       TATCTGTAAAATATTTGCAACTTTTGTCGCAGGATTATTCTCCAGATACGCTTGGGTACG      238
BYVMVNewDelhi       TTTCTGTCAAATATTTGCAACTTTTGTCGCAGGATTATTCTCCAGATACCCTTGGTTACG      238
BYVMVPhalaghat      TATCTGTAAAATATTTGCAACTTTTGTCGCAGGATTATTCTCCAGATACGCTTGGGTACG      238
BYVMVKerala         TTTCTGTGAAATATTTGCAACTTTTGTCGCAGGAGTATTCACCAGATACGCTTGGTTACG      238
BYVMVChelur         TATCTGTGAAATATTTGCAACTTTTGTCGCAGGAGTATTCTCCAGATACGCTTGGTTACG      238
                    *  ** * ****** * ****   ****  **** ** ** ** *****  **** ****

MYVMVLudhiana       ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      299
OelcvLudhiana       ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      298
OelcvSurat          ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      298
BYVMVHimachal       AGTTAATACGGGATTTAATTTGTATTTTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVNewDelhi       AGTTAATACGTGATTTAATCTGTATTCTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVPhalaghat      AGTTAATACGGGATTTAATTCGTATTTTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVKerala         ATTTAATACGGGATTTAATTTGTATTGTCCGTTCTCGTAATTATGTCGAAGCGAGCTGCA      298
BYVMVChelur         AGTTAATACGGGATTTAATTTGTATTGTCCGTTCTCGTAATTATGTCGAAGCGAGCTGCA      298
                    *  **** ** *** ****   **** * ** *   * ***************  ****

MYVMVLudhiana       GATATCGTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      359
OelcvLudhiana       GATATCTTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      358
OelcvSurat          GATATCGTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      358
BYVMVHimachal       GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVNewDelhi       GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVPhalaghat      GATATCGTCATTTCTACGCCCACGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVKerala         GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCT      358
BYVMVChelur         GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
                    ****** ********** *** ******* ** ** ** ** ********** ******

MYVMVLudhiana       TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCGCAAAATCACGAATGTGGGCCAAC      419
OelcvLudhiana       TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCACAAAATCACGAATGTGGGCCAAC      418
OelcvSurat          TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCACAAAATCACGAATGTGGGCCAAC      418
BYVMVHimachal       TACACCAGCCGTGCTGCTGCCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVNewDelhi       TACACCAGCCGTGCTGCTGCCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVPhalaghat      TACACCAGCCGTGCTGCTGCCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVKerala         TACACCAGCCGTGCTGCTGCCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVChelur         TACACCAGCCGTGCTGCTGCCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
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MYVMVLudhiana       AGACCCATGTACCGGAAGCCCAGAATGTACAGGATATACAGAAGCCCTGATGTTCCTAGA      479
OelcvLudhiana       AGACCCATGTACCGGAAGCCCAGAATGTACAGGATATACAGAAGCCCTGATGTTCCTAGA      478
OelcvSurat          AGACCCATGTACCGGAAGCCCAGAATGTACAGGATGTACAGAAGCCCTGATGTTCCTAGA      478
BYVMVHimachal       AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCACGG      478
BYVMVNewDelhi       AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVPhalaghat      AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVKerala         AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVChelur         AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
                    ** ** *** ** **** ************ **** ******** ** ********  *

MYVMVLudhiana       GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      539
OelcvLudhiana       GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      538
OelcvSurat          GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      538
BYVMVHimachal       GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGTTCATATT      538
BYVMVNewDelhi       GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGATCATATT      538
BYVMVPhalaghat      GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGTTCATATT      538
BYVMVKerala         GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTTGTCCATATT      538
BYVMVChelur         GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTTGTCCATATT      538
                    ******** ** ** ******** ***** *****  *     ***** * *  ** **

MYVMVLudhiana       GGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATTGGGCTGACCCATCGAGTAGGG      599
OelcvLudhiana       GGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATTGGGCTGACCCATCGAGTAGGG      598
OelcvSurat          GGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATGGGGCTGACCCATCGAGTAGGG      598
BYVMVHimachal       GGTAAGGTAATGTGTATTTCGGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
BYVMVNewDelhi       GGTAAGGTAATGTGTATTTCGGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
BYVMVPhalaghat      GGTAAGGTAATGTGTATTTCGGATGTTACGCGTGGAGTCGGTTTGACCCATCGGATAGGT      598
BYVMVKerala         GGTAAGGTAATGTGTATTTCTGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
BYVMVChelur         GGTAAGGTAATGTGTATTTCTGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
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MYVMVLudhiana       AAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGATATGGATGGATGAGAATATT      659
OelcvLudhiana       AAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGATATGGATGGATGAGAATATT      658
OelcvSurat          AAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGATATGGATGGATGAGAATATT      658
BYVMVHimachal       AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAAATATGGATGGACGAGAACATC      658
BYVMVNewDelhi       AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
BYVMVPhalaghat      AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
BYVMVKerala         AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
BYVMVChelur         AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
                    ** ******** ** ****** * *** ** * ** ** ********** ***** **

MYVMVLudhiana       AAGACTAAGAACCATACGAACACCGTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACA      719
OelcvLudhiana       AAGACTAAGAACCATACGAACACCGTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACA      718
OelcvSurat          AAGACTAAGAACCATACGAACACCGTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACA      718
BYVMVHimachal       AAGACCAAGAACCATACGAATTCGGTGATGTTTTTCCTTGTTCGTGATCGACGACCGGTA      718
BYVMVNewDelhi       AAGACCAAGAACCATACGAATTCGGTGATGTTTTTCCTTGTTCGTGATCGACGACCGGTA      718
BYVMVPhalaghat      AGGACGAAGGACCATACGAATTCGGTGATGTTTTTCCTTGGTCGTGATCGACGACCGGCA      718
BYVMVKerala         AAGACCAAGAACCATACGAATTCGGTGATGTTTTTCCTTGTTCGTGATCGACGACCGACA      718
BYVMVChelur         AAGACCAAGAACCATACGAATTCGGTGATGTTTTTCCTTGTTCGTGATCGACGACCGGTA      718
                    * *** *** **********  * ** *******   * *   * **  * ** **   *

MYVMVLudhiana       GGCACCCCCTACGATTTCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCT      779
OelcvLudhiana       GGCACCCCCTACGATTTCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCT      778
OelcvSurat          GGCACCCCCTACGATTTCCAGCAAGTGTTCAATGTTTATGACAACGAGCCTTCTACGGCT      778
BYVMVHimachal       GATAAACCACAAGATTTTGGTGAAGTATTTAATATGTTTGATAACGAGCCTAGTACGGCG      778
BYVMVNewDelhi       GATAAACCACAGGATTTTGGTGAGGTATTTAACATGTTTGACAACGAACCCAGTACGGCT      778
BYVMVPhalaghat      GATAAACCACAAGATTTTGGTGAAGTATTTAATATGTTTGATAATGAGCCCAGTACGGCG      778
BYVMVKerala         GATAAACCACAAGATTTTGGTGAAGTATTTAATATGTTTGATAACGAGCCCAGTACGGCC      778
BYVMVChelur         GATAAACCACAAGATTTTGGTGAAGTATTTAATATGTTTGATAATGAGCCCAGTACGGCG      778
                    *  *  **  * *****     * ** ** **  * * *** ** ** **   ******

MYVMVLudhiana       ACTGTAAAGAACGACGAGCCTGATCGATTCCAGGTTTTGAGGAGGTTTCAGGCGACAGTT      839
OelcvLudhiana       ACTGTAAAGAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTTCAGGCGACAGTT      838
OelcvSurat          ACTGTAAAGAACGACCAGCGTGATCGATTCCAGGTTTTGAGGAGGTTTCAGGCGACAGTT      838
BYVMVHimachal       ACCGTGAAGAACATGCATAGGGACCGGTACCAGGTGTTGAGGAAATGGCATGCAACCGTT      838
BYVMVNewDelhi       ACCGTGAAGAATAGTCATAGGGATCGTTACCAGGTGTTGAGGAAATGGCATGCAACCGTC      838
BYVMVPhalaghat      ACTGTGAAGAACATGCATAGGGATCGTTATCAGGTTTTGAGGAAATGGCATGCAACCGTT      838
BYVMVKerala         ACCGTTAAGAACATGCATAGGGATCGGTACCAGGTGTTGAGGAAATGGCATGCAACCGTT      838
BYVMVChelur         ACCGTGAAGAACATGCATAGGGATCGTTACCAGGTGTTGAGGAAATGGCATGCAACCGTT      838
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MYVMVLudhiana       ACAGGAGGACAGTATGCTTGTAAGGAACAAGTTCCAATTAGGAAATTCTATCGTGTTAAC      899
OelcvLudhiana       ACAGGAGGACAGTATGCTTGTAAGGAACAAGTTCCAATTAGGAAATTCTATCGTGTTAAC      898
OelcvSurat          ACAGGAGGACAGTATGCTTGTAAGGAACAAGTTCCAATTAGGAAATTCTATCGTGTTAAC      898
BYVMVHimachal       ACTGGTGGACAATATGCGAGTAAGGAGCACGCTTTGGTCAAGAAGTTTGTTAGGGTTAAC      898
BYVMVNewDelhi       ACGGGTGGTCAATATGCGAGTAAGGAACAGGCGTTGGTCAAGAAGTTTGTCAAGGTTAAC      898
BYVMVPhalaghat      ACTGGTGGTCAATATGCAGAGAGGGAACTGGCGTTGGTTAGGAAATTTGTCAGGGTTAAC      898
BYVMVKerala         ACTGGTGGTCAATATGCATCGAAGGAACAGGCGTTGGTTAAGAAGTTTATCAGGGTTAAC      898
BYVMVChelur         ACTGGTGGGCAAGATGCAGCGAAGGAACAGGCGTTGGTTAAGAAATTTGTTAGGGTTAAC      898
                    ** ** ** **  ****    * *** *  *      * * *** **       ******

MYVMVLudhiana       AATTACGTGGTGTATAATCACCAGGAAGCTGGGAAGTATGAAAATCACACTGAGAATGCT      959
OelcvLudhiana       AATTACGTGGTGTATAATCACCAGGAAGCTGGGAAGTATGAAAATCACACTGAGAATGCT      958
OelcvSurat          AATTACGTGGTGTATAATCACCAGGAAGCTGGGAAGTATGAAAATCACACTGAGAATGCT      958
BYVMVHimachal       AACTACGTTGTTTACAACCAGCAGGAAGCAGGAAAATACGAGAATCACACCGAGAATGCA      958
BYVMVNewDelhi       AATTATGTTGTTTACAATCAACAGGAAGCAGGAAAATACGAGAATCATACGGAGAATGCG      958
BYVMVPhalaghat      AATTATGTTGTTTACAACCAGCAGGAGGCAGGGAAATACGAGACTCACACCGAGAATGCA      958
BYVMVKerala         AATTATGTTGTTTACAACCAGCAGGAGGCAGGAAAATACGAGAATCACACCGAGAATGCA      958
BYVMVChelur         AATTATGTTGTTTACAACCAGCAGGAGGCAGGAAAATACGAGAATCATACCGAGAATGCG      958
                    ** ** ** ** ** ** ** ***** ** ** ** ** ** * *** ** ********

MYVMVLudhiana       TTGTTGTTGTATATGGCATGTACTCATGCCTCTAACCCTGTGTATGCTACTTTGAAAGTT      1019
OelcvLudhiana       TTGTTGTTGTATATGGCATGTACTCATGCCTCTAACCCTGTGTATGCTACTTTGAAAGTT      1018
OelcvSurat          TTGTTGTTGTATATGGCATGTACTCATGCCTCTAACCCTGTGTATGCTACTTTGAAAGTT      1018
BYVMVHimachal       TTGATGCTTTACATGGCTTGTACTCATGCTAGCAACCCAGTGTATGCTACTCTTAAGATT      1018
BYVMVNewDelhi       TTGATGCTTTATATGGCTTGTACCCATGCTAGTAACCCAGTGTATGCTACGCTTAAGATT      1018
BYVMVPhalaghat      TTGATGCTTTATATGGCTTGTACCCATGCTAGTAACCCAGTGTATGCTACGCTTAAGATT      1018
BYVMVKerala         TTGATGCTTTATATGGCTTGTACTCATGCTAGTAACCCAGTTTATGCTACGCTTAAGATT      1018
BYVMVChelur         TTGATGCTTTATATGGCTTGTACCCATGCTAGCAACCCAGTTTATGCTACGCTTAAGATT      1018
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MYVMVLudhiana       AGGAGTTACTTCTACGATTCTGTAACGAATTAATATTAATAAAGATCGAATTTTATATCT      1079
OelcvLudhiana       AGGAGTTACTTCTACGATTCTGTAACGAATTAATATTAATAAAGATCGAATTTTATATCT      1078
OelcvSurat          AGGAGTTACTTCTACGATTCTGTAACGAATTAATATTAATAAAGATCTAATTTTATATCT      1078
BYVMVHimachal       AGAATATATTTTTATGACTCTGTAACGAACTAATATTAATAAAGTTTAAATTGTATATCT      1078
BYVMVNewDelhi       CGTATTTATTTTTATGACTCTGTAACGAATTGAAATTAATAAAGATTGAATTTTATATCT      1078
BYVMVPhalaghat      CGGATTTATTTTTATGACTCTGTGACGAACTAATATTAATAAAGTTTGAATTTTATATCT      1078
BYVMVKerala         CGGATATATTTTTATGACTCTGTAACGAATTGATATTAATAAAGTTTAAATTGTATATCT      1078
BYVMVChelur         CGGATATATTTTTATGACTCTGTAACGAATTGAAATTAATAAAGTTTGAATTTTATATCT      1078
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MYVMVLudhiana       GAATATTGATCCACATACATTGTTTGTTGAATTACATTGTACAATACGTGTTCTACAGCC      1139
OelcvLudhiana       GAATATTGATCCACATACATTGTTTGTTGAATTACATTGTACAATACGTGTTCTACAGCC      1138
OelcvSurat          GAATATTGATCCACATACATTGTTTGTTGAATTACATTGTACAATACGTGTTCTACAGCC      1138
BYVMVHimachal       GAATATTGGTCTACATACATTGTTTTATTAATTACATTGTACAATACATGTTCAACGGCT      1138
BYVMVNewDelhi       GAATATTGGTCTACATACATTGTTTGATTAATTACATTGTACAAAACATGTTCGACAGCT      1138
BYVMVPhalaghat      GAATATTGGTCTACATACATTGTTTGATTAATTACATTGTACAATACATGTTCAACGGCT      1138
BYVMVKerala         GAATATTGGTCTACATACATTGTTTGATTAATTACATTGTACAATACATGTTCAACGGCT      1138
BYVMVChelur         GAATATTGGTCTACATACATTGTTTGATTAATTACATTGTACAATACATGTTCGACAGCT      1138
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MYVMVLudhiana      TTAATTACTAAATTAATTGAGATTACACCTAGATTGTTGAGATACTTGAGTACTTGGGTT      1199
OelcvLudhiana      TTAATTACTAAATTAATTGAGATTACACCTAGATTGTTGAGATACTTGAGGACTTGGGTT      1198
OelcvSurat         TTAATTACTAAATTAATTGAGATTACACCTAGATTGTTGAGATACTTGAGGACTTGGGTT      1198
BYVMVHimachal      TTAATAACTAAATTAATTGAGATTACACCTAGATTGTTGAGATATTTGAGGACTTGGGTT      1198
BYVMVNewDelhi      TTAATAACTAAATTAATTGAAACTACACCTAGATTGTTGAGATATTTGAGGACTTGGGTT      1198
BYVMVPhalaghat     TTAATAACTAAATTAAGTGAGATTACACCTAGATTGTTGAGATACTTGAGTACTTGGTTT      1198
BYVMVKerala        TTAATAACTAAATTAATTGAGATTACACCTAGATTGTTGAGATGTTTGAGGACTTGGTTT      1198
BYVMVChelur        TTAATAACTAAATTAATTGAAGCTACACCTAGATTGTTGAGATATTTGAGGACTTGGGTT      1198
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MYVMVLudhiana      TTGAATACCCTTAAGAAAAGACCAGTCTGAGGGTGTAAGGTCGTCCAGACCCGGAAGGTC      1259
OelcvLudhiana      TTGAATACCCTTAAGAAAAGACCAGTCTGAGGGTGTAAGGTCGTCCAGACCCGGAAGGTC      1258
OelcvSurat         TTGAATACCCTTAAGAAAAGACCAGTCTGAGGGTGTAAGGTCGTCCAGACCCGGAAGGTC      1258
BYVMVHimachal      TTGAATACCCTTAAGAAAAGACCAGTCGGAGGGTGTAAGGTCGTCCAGATTCGGAAGGTT      1258
BYVMVNewDelhi      TTGAATACCCTTAAGAAAAGACCAGTCTGAGGGTGTAAGGTCGTCCAGATTCGGAAGGTT      1258
BYVMVPhalaghat     TTGAATACCCTTAAGAAAAGACCAGTCGGAGGGTGTAAGGTCGTCCAGATTCGGAAGGTT      1258
BYVMVKerala        TTGAATACCCTTAAGAAAAGACCAGTCGGAGGGTGTAAGGTCGTCCAGATTCGGAAGGTT      1258
BYVMVChelur        TTGAATACCCTTAAGAAAAGACCAGTCGGAGGGTGTAAGGTCGTCCAGATTCGGAAGGTT      1258
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MYVMVLudhiana      AGAAAACACTTGTGCACTCCCAGAGCTCTCCGAAGGTTGTAGTTGAATTGGATCCTGATT       1319
OelcvLudhiana      AGAAAACACTTGTGCACTCCCAGAGCTCTCCGAAGGTTGTAGTTGAATTGGATCCTGATT       1318
OelcvSurat         AGAAAACACTTGTGCACTCCCAGAGCTCTCCGAAGGTTGTAGTTGAATTGGATCCTGATT       1318
BYVMVHimachal      AGAAAACACTTGTGTATTCCCAGAGCTTTCCGTAGGTTGTAGTTGAAATGGATCCTGATT       1318
BYVMVNewDelhi      AGAAAACACTTGTGTATTCCCAGAGCTTTCCGTAGGTTGTAGTTGAAATGGATCCTGAGT       1318
BYVMVPhalaghat     AGAAAACACTTGTGAATTCCCAGAGCTTTCCGTAGGTTGTAGTTGAAATGGATCCTGAGT       1318
BYVMVKerala        AGAAAACACTTGTGAATTCCCAGAGCTTTCCGTAGGTTGTAGTTGAAATGGATCCTGAGT       1318
BYVMVChelur        AGAAAACACTTGTGTATTCCCAGAGCTTTCCGTAGGTTGTAGTTGAAACGGATCCTGAGT       1318
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MYVMVLudhiana      TTTATTATGTCCATGTTCGTCGTGAATGGACGGTCGTCGTGGCTGAGGATCTTGAAATAG       1379
OelcvLudhiana      TTTATTATGTCCATGTTCGTCGTGAATGGACGGTCGTCGTGGCTGAGGATCTTGAAATAG       1378
OelcvSurat         TTTATTATGTCCATGTTCGTCGTGAATGGACGGTCGTCGTGGCTGAGGATCTTGAAATAG       1378
BYVMVHimachal      GTTATTATGTCCATGTTCGTCGTGAATGGACGGTTGTCGTGGTTGAGGATCTTGAAATAG       1378
BYVMVNewDelhi      GTTATTATGTCCATGTTCGTCGTGAATGGACGGTGGTCGTGGTTGAGGATCTTGAAATAG       1378
BYVMVPhalaghat     GTTATTATGTCCATGTTCGTCGTGAATGGACGGTTGTCGTGGTTGAGGATCTTGAAATAG       1378
BYVMVKerala        GTTATTATGTCCATGTTCGTCGTGAATGGACGGTTGTCGTGGTTGAGGATCTTGAAATAG       1378
BYVMVChelur        GTTATTATGTCCATGTTCGTCGTGAATGGACGGTTGTCGTGGTTTAGGATCTTGAAATAG       1378
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MYVMVLudhiana      AGGGGATTTGGAACCTCCCAGATAAAGACGCCATTCCTTGTATGAGCTGCAGTGATGCGT       1439
OelcvLudhiana      AGGGGATTTGGAACCTCCCAGATAAAGACGCCATTCCTTGTATGAGCTGCAGTGATGCGT       1438
OelcvSurat         AGGGGATTTGGAACCTCCCAGATAAAGACGCCATTCCTTGTATGAGCTGCAGTGATGCGT       1438
BYVMVHimachal      AGGGGATTTGGAACCTCCCAGATATAGACGCCATTCTTTGCCTGAGCTGCAGTGATGCGT       1438
BYVMVNewDelhi      AGGGGATTTGGAACCTCCCAGATATAGACGCCATTCTTTGCTTGAGCTGCAGTGATGCGT       1438
BYVMVPhalaghat     AGGGGATTTGGAACCTCCCAGATATAGACGCCATTCTTTGCTTGAGCTGCAGTGATGCGT       1438
BYVMVKerala        AGGGGATTTGGAACCTCCCAGATATAGACGCCATTCTTTGCTTGAGCTGCAGTGATGCGT       1438
BYVMVChelur        AGGGGATTTGGAACCTCCCAGATATAGACGCCATTCTTTGCTTGAGCTGCAGTGATGCGT       1438
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MYVMVLudhiana      TCCCCTGTGCGAGAATCCATGGTTGAAGCAGTTAATGGATAGATAATAAGAACACCCGCA       1499
OelcvLudhiana      TCCCCTGTGCGAGAATCCATGGTTGAAGCAGTTAATGGATAGATAATAAGAACACCCGCA       1498
OelcvSurat         TCCCCTGTGCGAGAATCCATGGTTGAAGCAGTTAATTGATAGATAATAAGAACACCCGCA       1498
BYVMVHimachal      TCCCCTGTGCGAGAATCCATGGTTGTGGCAGTTAATTGAAAGATAATAAGAACACCCGCA       1498
BYVMVNewDelhi      GCCCCTGTGCGAGAATCCATGGTTGTGGCAGTTGATAGAGAGATAATAAGAACACCCGCA       1498
BYVMVPhalaghat     TCCCCTGTGCGAGAATCCATGGTTGTGGCAGTTGATAGAGAGATAATAAGAACACCCGCA       1498
BYVMVKerala        TCCCCTGTGCGAGAATCCATGGTTGTGGCAGTTGATAGAGAGATAATAAGAACACCCGCA       1498
BYVMVChelur        TCCCCTGTGCGAGAATCCATGGTTGTGGCAGTTGATAGAGAGATAATAAGAACACCCGCA       1498
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MYVMVLudhiana      TTCA**AGATCT**ACTCTCTTCCTCCTGTGCGCTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1559
OelcvLudhiana      TTCAAGATCTACTCTCTTCCTCCTGTGCGCTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1558
OelcvSurat         TTCAAGATCTACTCTCTTCCTCCTGTGCGCTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1558
BYVMVHimachal      TTCAAGATCTACTCTCCTCCTCCTGTTGCGTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1558
BYVMVNewDelhi      TTCAAGATCTACTCTCCTCCTCCTGATGCGTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1558
BYVMVPhalaghat     TTCAAGATCTACTCTCCTCCTCCTGATGCGTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1558
BYVMVKerala        TTCAAGATCTACTCTCCTCCTCCTGATGCGTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1558
BYVMVChelur        TTCAAGATCTACTCTCCTCCTCCTGATGCGTCTCTTCGCTTCCCTGTGCTGTACTTTGAT       1558
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MYVMVLudhiana      TGGTACCTGAGTACAATGGTCCTTCAAGGGTGACGAAGATCGCATTTTTCACTGCCCAGT       1619
OelcvLudhiana      TGGTACCTGAGTACAATGGTCCTTCAAGGGTGACGAAGATCGCATTTTTCACTGCCCAGT       1618
OelcvSurat         TGGTACCTGAGTACAATGGTCCTTCAAGGGTGACGAAGATCGCATTTTTCACTGCCCAGT       1618
BYVMVHimachal      TGGTACCTGAGTACAGTGGTTCTTCGAGGGTGATGAAGATCGCATTCTTGACTGCCCAGT       1618
BYVMVNewDelhi      TGGTACCCGAGTACAACGGTTCGGTGAGAAAGACGAATGCTGCATTTTTTAAAGCCCACG       1618
BYVMVPhalaghat     TGGTACCCGAGTACAACGGTTCGGTGAGAAAGACGAATGCTGCATTTTTTAAAGCCCACG       1618
BYVMVKerala        TGGTACCCGAGTACAACGGTTCGGTGAGAAAGACGAATGCTGCATTTTTTAAAGCCCACG       1618
BYVMVChelur        TGGTACCCGAGTACAACGGTTCGGTGAGAAAGACGAATGCTGCATTTTTTAAAGCCCACG       1618
                    ******* *******  *** *    **   ** ***    ***** ** *  *****

MYVMVLudhiana      TCTTTAGTGCTGAGTTCTTATCCTCGTCTAAGAACTCTCTATAACTGCTGTTGGGACCAG       1679
OelcvLudhiana      TCTTTAGTGCTGAGTTCTTATCCTCGTCTAAGAACTCTTTATAACTGCTGTTGGGACCAG       1678
OelcvSurat         TCTTTATTGCTGAGTTCTTATCCTCGTCTAAGAACTCTTTATAACTGCTGTTGGGACCAG       1678
BYVMVHimachal      TCTTTAGTGCGTTGTTCTTTTCCTCGTCCAGGAATTCTTTATAACTGCTGTTGGGACCAG       1678
BYVMVNewDelhi      CTTTCAAAGCTGAGTTCTTTTCCTCGTCCAAGAATTCTTTATAGCTTGCGTTGGGGCCTG       1678
BYVMVPhalaghat     CTTTCAAAGCTGAGTTCTTTTCCTCGTCCAAGAACTCTTTATAGCTTGCGTTGGGGCCTG       1678
BYVMVKerala        CTTTCAAAGCTGAGTTCTTTTCCTCGTCCAAGAACTCTTTATAGCTTGCGTTGGGGCCTG       1678
BYVMVChelur        CTTTCAAAGCTGAGTTCTTTTCCTCGTCCAAGAATTCTTTATAGCTTGCGTTGGGGCCTG       1678
                     ** *  **   ****** ******** * *** *** **** **   ****** ** *
MYVMVLudhiana      GATTGCAGAGGAAGATTGTTGGTATGCCGCCTTTAATTTGAACTGGCTTTCCGTACTTTG       1739
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OelcvLudhiana       GATTGCAGAGGAAGATTGTTGGTATGCCGCCTTTAATTTGAACTGGCTTTCCGTATTTTG       1738
OelcvSurat          GATTGCAGAGGAAGATTGTTGGTATGCCGCCTTTAATTTGAACTGGCTTTCCGTACTTTG       1738
BYVMVHimachal       GATTGCAGAGGAAGATTGTTGGTATTCCGCCTTTAATTTGAACTGGCTTCCCGTACTTTG       1738
BYVMVNewDelhi       GATTGCAGAGGAAGATTGTTGGAATGCCGCCTTTAATTTGAACTGGCTTCCCGTACTTGG       1738
BYVMVPhalaghat      GATTGCAGAGGAAGATTGTTGGAATGCCGCCTTTAATTTGAACTGGCTTCCCGTACTTGG       1738
BYVMVKerala         GATTGCAGAGGAAGATTGTTGGAATGCCGCCTTTAATTTGAACTGGCTTCCCGTACTTGG       1738
BYVMVChelur         GATTGCAGAGGAAGATTGTTGGAATGCCGCCTTTAATTTGAACTGGCTTCCCGTACTTGG       1738
                    ********************** ** ************************ ***** ** *

MYVMVLudhiana       TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAACTCTTTAAAGTGTTTGAGGGAGTGGG       1799
OelcvLudhiana       TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAACTCTTTAAAGTGTTTGAGGAAGTGGG       1798
OelcvSurat          TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAATTCTTTAAAGTGTTTGAGGAAGTGGG       1798
BYVMVHimachal       TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAACTCTTTAAAGTGCTTGAGGAAGTGCG       1798
BYVMVNewDelhi       TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAATTCTTTAAAGTGTTTCAGATAATGCG       1798
BYVMVPhalaghat      TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAATTCTTTAAAGTGTTTCAGATAATGCG       1798
BYVMVKerala         TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAATTCTTTAAAGTGTTTCAGATAATGCG       1798
BYVMVChelur         TGTTGGATTGCCAGTCCCTTTGGGCCCCCATGAATTCTTTAAAGTGTTTCAGATAATGCG       1798
                    ********************************** *********** ** **  * ** *

MYVMVLudhiana       GATCTACGTCATCAATGACGTTATACCAGGCCTCGTTACTGTAGACCTTTGGACTAAGGT       1859
OelcvLudhiana       GATCTACGTCATCAATGACGTTATACCAGGCCTCGTTACTGTAGACCTTTGGACTAAGGT       1858
OelcvSurat          GATCTACGTCATCAATGACGTTATACCAGGCCTCGTTACTGTAGACCTTTGGACTAAGGT       1858
BYVMVHimachal       GATCAACGTCATCAATGACGTTATACCAAGCGTCGTTACTGTATACCTTTGGACTTAGAT       1858
BYVMVNewDelhi       GGTCAACATCATCTATAATGTTGAACCACGCATCGTTTGAATACACTTTAGGGCTTAGAT       1858
BYVMVPhalaghat      GGTCAACATCATCTATAATGTTGAACCACGCATCGTTTGAATACACTTTAGGGCTTAGAT       1858
BYVMVKerala         GGTCAACATCATCTATAATGTTGAACCACGCATCGTTGGAATACACTTTAGGGCTTAGAT       1858
BYVMVChelur         GGTCAACATCATCTATAATGTTGAACCACGCATCGTTGAAATACACTTTAGGGCTTAGAT       1858
                    * ** ** ***** ** * ***  **** ** *****    ** ** ** ** ** ** *

MYVMVLudhiana       CTAGATGCCCACACAAATAGTTATGTGGACCCAGTGATCTAGCCCACATCGTCTTCCCGG       1919
OelcvLudhiana       CTAGATGCCCACACAAATAGTTATGTGGACCCAGTGATCTAGCCCACATCGTCTTCCCGG       1918
OelcvSurat          CTAGATGCCCACACAAATAGTTATGTGGACCCAGTGATCTAGCCCACATCGTCTTCCCGG       1918
BYVMVHimachal       CTAAATGCCCACACAAATAGTTATGTGGGCCTAAAGACCTAGCCCACATTGTTTTCCCAG       1918
BYVMVNewDelhi       CTAGGTGCCCACACAAATAGTTATGTGGGCCTAATAACCTAGCCCACATCGTTTTGCCCG       1918
BYVMVPhalaghat      CTAGGTGCCCACACAAATAGTTATGTGGGCCTAATGACCAGCCACATCGTGTCTTCCCGG       1918
BYVMVKerala         CTAGGTGCCCACACAAATAGTTATGTGGGCCTAATGACCTAGCCCACATCGTTTTGCCCG       1918
BYVMVChelur         CTAGGTGCCCACACAAATAGTTATGTGGGCCTAATGACCTAGCCCACATCGTTTTGCCCG       1918
                    ***  ********************** ** *   * *   * **    ** ** ** * *

MYVMVLudhiana       TACGACTATCTCCCTCAATCACTATACTTTGTGGTCTATGTGGCCGCGCAGCGGCGTCGA       1979
OelcvLudhiana       TACGACTATCTCCCTCAATCACTATACTTTGTGGTCTATGTGGCCGCGCAGCGGCGTCGA       1978
OelcvSurat          TACGACTATCTCCCTCAATCACTATACTTTGTGGTCTATGTGGCCGCGCAGCGGCGTCGA       1978
BYVMVHimachal       TACGACTGTCACCCTCAATTACTATACTTTGAGGTCTCAGGGGCCGCGCAGCGGCGTCGA       1978
BYVMVNewDelhi       TACGACTGTCTCCCTCTAAGACTAAACTCAAAGGACGCAGAGGCCGCGCAGCGGCGTCCA       1978
BYVMVPhalaghat      TACGACTGTCTCCCTCTAATACTAAACTCAAAGGTCTCAGAGGCCGCGCAGCGGCGTCCA       1978
BYVMVKerala         TACGACTGTCTCCCTCTAAGACTAAACTCAAAGGACGCAGAGGCCGCGCAGCGGCGTCCA       1978
BYVMVChelur         TACGACTGTCTCCCTCTAAGACTAAACTCAAAGGACGCAGAGGCCGCGCAGCGGCGTCCA       1978
                    ******* ** ***** *  **** ***    ** *   * ***************** *

MYVMVLudhiana       CAACGTTCTCGGCAGCCCATTCCTCAAGTTCTTCCGGAACTTGATCAAAAGAAGAAGAAG       2039
OelcvLudhiana       CAACGTTCTCGGCAGCCCATTCCTCAAGTTCTTCCGGAACTTGATCAAAAGAAGAAGAAG       2038
OelcvSurat          CAACGTTCTCGGCAGCCCATTCCTCAAGTTCTTCCGGAACTTGATCAAAAGAAGAAGAAG       2038
BYVMVHimachal       CAACATTCTCAGACACCCACTCTTCAAGTTCTTCTGGAACTTGATCGAAAGAAGAAGAGG       2038
BYVMVNewDelhi       TGACGTTTTCCGATACCCATTCCTCAAGTTCTTCTGGAACTTGATCGAAAGAAGAAGATA       2038
BYVMVPhalaghat      TGACGTTTTCCGATACCCATTCCTCTAGTTCTTCTGGAACTTGATCGAAAGAAGAAGATA       2038
BYVMVKerala         TGACGTTTTCCGATACCCATTCCTCAAGTTCTTCTGGAACTTGATCGAAAGAAGAAGATA       2038
BYVMVChelur         TGACGTTTTCCGATACCCATTCCTCAAGTTCATCTGGAACTTGATCGAAAGAAGAAGATA       2038
                      ** ** ** *   **** ** ** ***** ** *********** ***********

MYVMVLudhiana       AAAAAGGAGAAACATAAACCTCCACAGGAGGTGTAAAAATCCTATCTAAATTAGCATTTA       2099
OelcvLudhiana       AAAAAGGAGAAACATAAACCTCCACAGGAGGTGTAAAAATCCTATCTAAATTAGCATTTA       2098
OelcvSurat          AAAAAGGAGAAACATAAACCTCCACAGGAGGTGTAAAAATCCTATCTAAATTAGCATTTA       2098
BYVMVHimachal       AAAAAGGAGAAACATAAGGAGCTGGAGGCTCCTGAAAGATCCTGTCTAGATTTGCATTTA       2098
BYVMVNewDelhi       AAAAAGGAGAAACATAAACCTCCAAGGGAGGTGTAAAAATCCTATCTAAATTGGCATTTA       2098
BYVMVPhalaghat      AAAAAGGAGAAACATAAACCTCCAAGGGAGGTGTAAAAATCCTATCTAAATTGGCATTTA       2098
BYVMVKerala         AAAAAGGAGAAACATAAACCTCCAAGGGAGGTGTAAAAATCCTATCTAAATTGGCATTTA       2098
BYVMVChelur         AAAAAGGAGAAACATAAACCTCCAAGGGAGGTGTAAAAATCCTATCTAAATTGGCATTTA       2098
                    *****************    *    **      *** ***** **** *** *******
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MYVMVLudhiana       AATTATGGAATTGTAATACATAATCTTTTGGAGCTAACTCCTTAATGACTCTAAGAGCCT       2159
OelcvLudhiana       AATTATGGAATTGTAATACATAATCTTTTGGAGCTAACTCCTTAATGACTCTAAGAGCCT       2158
OelcvSurat          AATTATGGAATTGTAATACATAATCTTTTGGAGCTAACTCCTTAATGACTCTAAGAGCCT       2158
BYVMVHimachal       AATTATGAAATTGTAGTACAAAATCTTTAGGAGCTAGTTCCTTAATGACTCTAAGAGACT       2158
BYVMVNewDelhi       AATTATGAAATTGTAATACATAATCCTTAGGTGCTAACTCCCTAATGACTCTAAGAGCCT       2158
BYVMVPhalaghat      AATTATGAAATTGTAATACATAATCCTTAGGTGCTAACTCCCTAATGACTCTAAGAGCCT       2158
BYVMVKerala         AATTATGAAATTGTAATACATAATCCTTAGGTGCTAACTCCCTAATGACTCTAAGAGCCT       2158
BYVMVChelur         AATTATGAAATTGTAATACATAATCCTTAGGTGCTAACTCCCTAATGACTCTAAGAGCCT       2158
                    ******* ******* **** **** ** ** ****  *** *************** **

MYVMVLudhiana       CTGACTTACTGCCTGCGTTAAGCGCTGCGGCGTAAGCGTCGTTGGCTGTCTGTTGTCCCC       2219
OelcvLudhiana       CTGACTTACTGCCTGCGTTAAGCGCTGCGGCGTAAGCGTCGTTGGCTGTCTGTTGTCCCC       2218
OelcvSurat          CTGACTTACTGCCTGCGTTAAGCGCTGCGGCGTAAGCGTCGTTGGCTGTCTGTTGTCCCC       2218
BYVMVHimachal       CTGACTTACTTCCCGCGTTAAGTGCTGCGGCGTAAGCGTCGTTGGCTGTTTGTTGCCCTC       2218
BYVMVNewDelhi       CTGACTTACTGCCTGCGTTAAGTGCTGCTGCGTAAGCGTCGTTGGCTGTTTGCTGTCCCC       2218
BYVMVPhalaghat      CTGACTTACTGCCTGCGTTAAGTGCTGCGGCGTAAGCGTCGTTGGCTGTTTGCTGTCCCC       2218
BYVMVKerala         CTGACTTACTGCCTGCGTTAAGTGCTGCGGCGTAAGCGTCGTTGGCTGTTTGCTGTCCCC       2218
BYVMVChelur         CTGACTTACTGCCTGCGTTAAGTGCTGCGGCGTAAGCGTCGTTGGCTGTTTGCTGTCCCC       2218
                    ********** ** ******** ***** ******************** ** ** ** *

MYVMVLudhiana       CTCTAGCAGATCGTCCGTCGATCTGAAACTCTCCCCACTCGAGAGTGTCCCCGTCCTTGT       2279
OelcvLudhiana       CTCTAGCAGATCGTCCGTCGATCTGAAACTCTCCCCACTCGAGAGTGTCCCCGTCCTTGT       2278
OelcvSurat          CTCTAGCAGATCGTCCGTCGATCTGAAACTCTCCCCACTCGAGAGTGTCCCCGTCCTTGT       2278
BYVMVHimachal       CTCTTGCTGATCTTCCGTCGATCTGAAATTCGCCCCAGTCGAGAATGTCCCCGTCCTTAG       2278
BYVMVNewDelhi       CTCTTGCAGATCGTCCATCGATCTGAAACTCTCCCCATTCGAGGGTGTCTCCGTCCTTGT       2278
BYVMVPhalaghat      CTCTTGCAGATCGTCCATCGATCTGAAACTCTCCCCATTCGAGGGTGTCTCCGTCCTTGT       2278
BYVMVKerala         CTCTTGCAGATCGTCCATCGATCTGAAACTCTCCCCATTCGAGGGTGTCTCCGTCCTTGT       2278
BYVMVChelur         CTCTTGCAGATCGTCCATCGATCTGAAACTCTCCCCATTCGAGGGTGTCTCCGTCCTTGT       2278
                    **** ** **** *** *********** ** ***** *****  **** ********

MYVMVLudhiana       CGATGTAGGATTTGACGTCGGAGCTGGATTTAGCTCCCTGTATGTTCGGATGGAAATGTG       2339
OelcvLudhiana       CGATGTAGGATTTGACGTCGGAGCTGGATTTAGCTCCCTGTATGTTCGGATGGAAATGTG       2338
OelcvSurat          CGATGTAGGATTTGACGTCGGAGCTGGATTTAGCTCCCTGTATGTTCGGATGGAAATGTG       2338
BYVMVHimachal       CGATGTAGGACTTGACGTCGGAGCTGGATTTAGCTCCCTGAATGTTTGGATGGAAATGTG       2338
BYVMVNewDelhi       CGATGTAGGACTTGACATCTGAGCTTGATTTAGCTCCCTGAATGTTCGGATGGAAATGTA       2338
BYVMVPhalaghat      CGATGTAGGACTTGACATCTGAGCTTGATTTAGCTCCCTGAATGTTTGGATGGAAATGTG       2338
BYVMVKerala         CGATGTAGGACTTGACATCTGAGCTTGATTTAGCTCCCTGAATGTTCGGATGGAAATGTA       2338
BYVMVChelur         CGATGTAGGACTTGACATCTGAGCTTGATTTAGCTCCCTGAATGTTCGGATGGAAATGTA       2338
                    ********** ***** ** ***** ************** ***** ************

MYVMVLudhiana       CTGACCTGCTTGGGGAGACCAGGTCGAAGAATCGCTGATTCTGGCACTTGTATTTCCCCT       2399
OelcvLudhiana       CTGACCTGCTTGGGGAGACCAGGTCGAAGAATCGCTGATTCTGGCACTTGTATTTCCCCT       2398
OelcvSurat          CTGACCTGCTTGGGGAGACCAGGTCGAAGAATCGCTGATTCTGGCACTTGTATTTCCCCT       2398
BYVMVHimachal       CTGACCTGGTTGGGGATATGAGGTTGAAGAATCGGTTATTTTTGCATTGGTATTTGCCCT       2398
BYVMVNewDelhi       CTGACCTGCTTGGGGAGACCAGGTCGAAGAATCGCTGATTCTGGCACTTGTATTTGCCCT       2398
BYVMVPhalaghat      CTGACCTAGTTGGGGAGGTGAGGTCGAAGAATCTATTGTTCCTGCACTGGAACTTTCCTT       2398
BYVMVKerala         CTGACCTGCTTGGGGAAACCAGGTCGAAGAATCGCTGATTCTGGCACTTGTATTTGCCCT       2398
BYVMVChelur         CTGACCTGCTTGGGGAGACCAGGTCGAAGAATCGCTGATTCTGGCACTTGTATTTGCCCT       2398
                    *******  *******    **** ********  *  **   *** * * * ** ** *

MYVMVLudhiana       CGAACTGGATGAGCACGTGCAGATGAGGTTCCCCATTTTCATGAAGTTCTCTGCAGATTT       2459
OelcvLudhiana       CGAACTGGATGAGCACGTGCAGATGAGGTTCCCCATTTTCATGAAGTTCTCTGCAGATTT       2458
OelcvSurat          CGAACTGGATGAGCACGTGCAGATGAGGTTCCCCATTTTCATGAAGTTCTCTGCAGATTT       2458
BYVMVHimachal       CGAACTGGATGAGCACGTGAAGATGAGGTTCCCCATTTTCATGAAACTCTCTGCAGATTC       2458
BYVMVNewDelhi       CGAACTGGATGAGCACGTGCAGATGAGGTTCCCCATTCTCGTGAAGCTCTCTGCAGATTC       2458
BYVMVPhalaghat      CGAACTGGATGAGAACATGCAAGTGAGGATTCCCATCTTCATGAAGTTCTCTGCAGATTC       2458
BYVMVKerala         CGAACTGGATGAGCACGTGCAGATGAGGTTCCCCATTCTCGTGAAGCTCTCTGCAGATCT       2458
BYVMVChelur         CGAACTGGATGAGCACGTGGAGATGAGGTTCCCCATTCTCGTGAAGCTCTCTGCAGATCT       2458
                    ************* ** ** *  ***** * *****  ** ****  ***********
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Figure 4.1: Multiple sequence alignment of DNA-A component of 8 okra-associated begomoviral isolates by Clustal omega tool

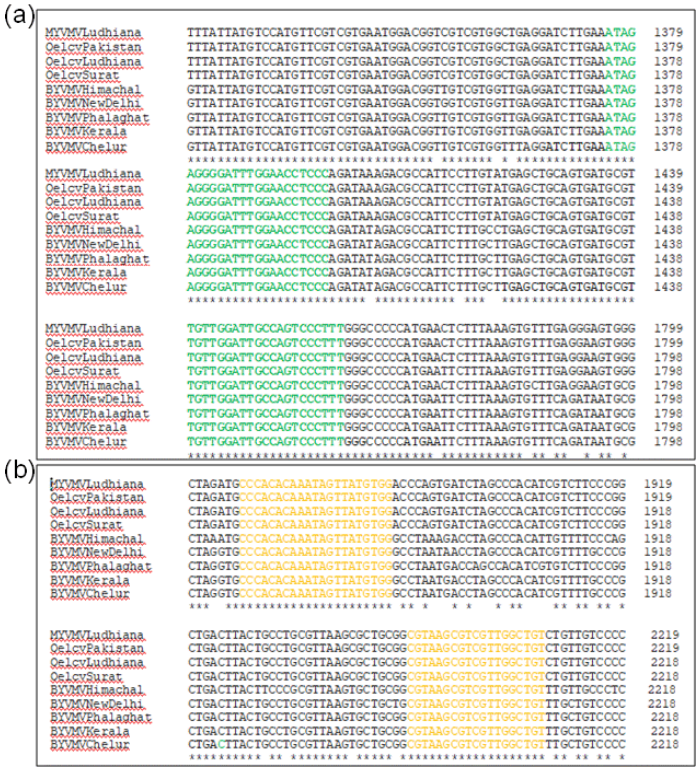

Figure 4.2: Multiple sequence alignment of target gene(s) fragments from DNA-A component of 8 okra-associated begomoviruses by clustal omega tool

(a) Multiple sequence alignment for the overlapping region of *AC1/AC2*

(b) Multiple sequence alignment for the overlapping region of *AC1/AC4*

(a)

TARGET 1 (385) 1375-1759

ATAGAGGGGATTTGGAACCTCCCAGATAAAGACGCCATTCCTTGTATGAGCTGCAGT
GATGCGTTCCCCTGTGCGAGAATCCATGGTTGAAGCAGTTAATGGATAGATAATAAG
AACACCCGCATTCAAGATCTACTCTCTTCCTCCTGTGCGCTCTCTTCGCTTCCCTGTG
CTGTACTTTGATTGGTACCTGAGTACAATGGTCCTTCAAGGGTGACGAAGATCGCAT
TTTTCACTGCCCAGTTCTTTAGTGCTGAGTTCTTATCCTCGTCTAAGAACTCTTTATA
ACTGCTGTTGGGACCAGGATTGCAGAGGAAGATTGTTGGTATGCCGCCTTTAATTTG
AACTGGCTTTCCGTATTTTGTGTTGGATTGCCAGTCCCTTT

(b)

TARGET 2 (342) 1866-2208

CCCACACAAATAGTTATGTGGACCCAGTGATCTAGCCCACATCGTCTTCCCGGTACG
ACTATCTCCCTCAATCACTATACTTTGTGGTCTATGTGGCCGCGCAGCGGCGTCGAC
AACGTTCTCGGCAGCCCATTCCTCAAGTTCTTCCGGAACTTGATCAAAAGAAGAAGA
AGAAAAAGGAGAAACATAAACCTCCACAGGAGGTGTAAAAATCCTATCTAAATTAG
CATTTAAATTATGGAATTGTAATACATAATCTTTTGGAGCTAACTCCTTAATGACTCT
AAGAGCCTCTGACTTACTGCCTGCGTTAAGCGCTGCGGCGTAAGCGTCGTTGGCTGT

Figure 4.3: Target gene(s) fragments of DNA-A component of begomoviruses infecting okra

(a) 385bp region of overlapping *AC1/AC2* gene(s) of DNA-A component

(b) 342bp region of overlapping *AC1/AC2* gene(s) of DNA-A component

Table 4.1(a): Potential siRNAs of overlapping region of *AC1*/*AC2* predicted through Jack Lin's siRNA prediction tool

Found siRNA sequence	Starting position (bp)	GC content (%)
AAGGGTGACGAAGATCGCATT	210	59%
AAAGACGCCATTCCTTGTATG	28	47%
AACACCCGCATTCAAGATCTA	115	53%
AATTTGAACTGGCTTTCCGTA	339	47%
AATCCATGGTTGAAGCAGTTA	78	47%
AACTCTTTATAACTGCTGTTG	277	35%
AAGAACTCTTTATAACTGCTG	274	35%
GATTGTTGGTATGCCGCCTTT	318	53%
TATGAGCTGCAGTGATGCGTT	45	59%
CAGATAAAGACGCCATTCCTT	23	47%
GAATCCATGGTTGAAGCAGTT	77	47%
TACCTGAGTACAATGGTCCTT	188	53%
GATAATAAGAACACCCGCATT	106	41%
TATCCTCGTCTAAGAACTCTT	263	47%
GAACTCTTTATAACTGCTGTT	276	35%
CAGGATTGCAGAGGAAGATTG	302	47%
TACAATGGTCCTTCAAGGGTG	196	53%
CAGAGGAAGATTGTTGGTATG	319	41%
CAGTTCTTTAGTGCTGAGTTC	241	41%
GAACTGGCTTTCCGTATTTTG	344	41%
GATTGCAGAGGAAGATTGTTG	305	41%
GATTGGTACCTGAGTACAATG	182	41%
TAGTGCTGAGTTCTTATCCTC	249	47%
CATTCAAGATCTACTCTCTTC	123	35%
GAAGCAGTTAATGGATAGATA	89	35%

Table 4.1(b): Potential siRNAs of overlapping region of *AC1*/*AC4* predicted through Jack Lin's siRNA prediction tool

Found siRNA sequence	Starting position (bp)	GC content (%)
AATCCTATCTAAATTAGCATT	211	29%
AATTGTAATACATAATCTTTT	242	18%
AATAGTTATGTGGACCCAGTG	9	47%
AAGTTCTTCCGGAACTTGATC	139	47%
AACGTTCTCGGCAGCCCATTC	115	65%
AAAAGGAGAAACATAAACCTC	175	35%
AAGCGCTGCGGCGTAAGCGTC	313	76%
CAGCCCATTCCTCAAGTTCTT	126	53%
GACTCTAAGAGCCTCTGACTT	280	53%
CAACGTTCTCGGCAGCCCATT	114	65%
GAATTGTAATACATAATCTTT	241	18%
TAGCATTTAAATTATGGAATT	225	24%
CATAAACCTCCACAGGAGGTG	186	53%
GATCTAGCCCACATCGTCTTC	29	53%
TAAACCTCCACAGGAGGTGTA	188	59%
GAGCCTCTGACTTACTGCCTG	288	59%
GAGCTAACTCCTTAATGACTC	264	41%
GACTATCTCCCTCAATCACTA	57	47%
TACTGCCTGCGTTAAGCGCTG	300	65%
TAATGACTCTAAGAGCCTCTG	276	47%
CACTATACTTTGTGGTCTATG	73	35%
CAATCACTATACTTTGTGGTC	69	35%
GAGGTGTAAAAATCCTATCTA	201	35%
CATTTAAATTATGGAATTGTA	228	18%
TAAATTAGCATTTAAATTATG	220	12%

4.1.1.1 Revival and confirmation of DNA-A component of *Okra enation leaf curl virus* in pJET vector

Okra leaf samples infected with begomoviruses was taken and whole genome of it was previously cloned in pJET vector. This clone was sequenced and submitted in NCBI database with accession number KP208672.1. pJET vector was cloned in DH5α strain of *E.coli*. Vector was revived from DH5α strain and presence of DNA-A component of begomovirus(es) was confirmed by pJET forward and reverse sequencing primers (provided with the kit) and also with the help of restriction enzyme assay (Figure 4.4). For the restriction digestion, *EcoR*V enzyme was used. This enzyme was previously used to clone the DNA-A genome in the multiple cloning sites of pJET vector.

4.1.1.2 Revival and confirmation of pFGC1008 (binary vector)

RNAi vector pFGC1008 was used as a binary vector for viral gene silencing. Mohanpuria *et al* 2010 used this vector for the silencing of caffeine synthase gene in *Camellia sinensis* L. They observed 61% and 67% reduction in caffeine and theobromine contents. In the present study this vector was used for the silencing of three genes of begomoviruses. Vector was first transformed and multiplied into *E.coli* DH5α competent cells. Vector was then isolated and confirmed by the PCR with the help of pFGC 1008 specific primers as well as with restriction. PCR was performed with AS primers giving 200bp amplicon size and BS primers giving 550bp amplicon size. pFGC 1008 has multiple cloning sites in its T-DNA portion and out of them *Bgl*II which was present at position 2806, *Xho*I at 3010 and *Spe*I at 3507 were used for its confirmation. Restriction double digestion was performed with two pairs of enzymes i.e. with *Bgl*II & *Spe*I and with *Xho*I & *Spe*I. This double digestion gives 700bp fragment with first pair and 500 bp fragments were obtained from pFGC with second pair of enzymes (Figure 4.5).

4.1.1.3 Amplification and cloning of target gene(s) fragments in pGEM-T easy vector (TA cloning)

Two targets were amplified with the primer pairs having restriction sites specific to the RNAi vector at their 5′end. Restriction sites were added in such a way so that the amplified products will clone in sense and antisense orientation in the binary vector. Products from PCR were run on 2.5% gel (Figure 4.6) with the negative control in the subsequent wells. Amplified products were purified with the help of Macherey-Nagal (MN) kit. After purification, products were ligated to pGEM-T easy vector by TA cloning. pGEM-T easy vector (Promega, WJ, USA) is a linear vector having multiple cloning sites and 5′ terminal thiamidine (T)-overhang at both ends, these T-overhangs were used for the ligation of the 3′

terminal adenine (A)-overhang of the double stranded PCR amplified products. Ligated products were transformed to DH5α strain of *E.coli* and confirmed by adding X-gal to the medium. X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactoside) is a substrate of LacZ gene which is present in the pGEM-T easy vector and by using substrate it imparts blue-color. Amplified products were ligated in between the LacZ gene and hence interrupt the LacZ portion of the gene and gives white color. Transformed cells gave white color and blue color was shown by untransformed one. White colonies were picked and subcultured. Ten of subcultured colonies from each target were checked for having gene of interest through colony PCR with the help of insert specific primers. All colonies checked from target one and four colonies from target two showed positive results with colony PCR (Figure 4.7).

4.1.1.4 Confirmation of target gene(s) fragments in recombinant plasmids (pGEM-T)

Positive clones were used for the isolation of plasmids having gene of interest. Plasmids were quantified and confirmed with PCR and restriction digestion with the same enzymes whose sites were added at the 5′end of the primers. Isolated plasmids shown 385bp amplicon size for target one and 342bp for target two in PCR analysis and restriction enzymes cleave fragments of same size for each target (Figure 4.8).

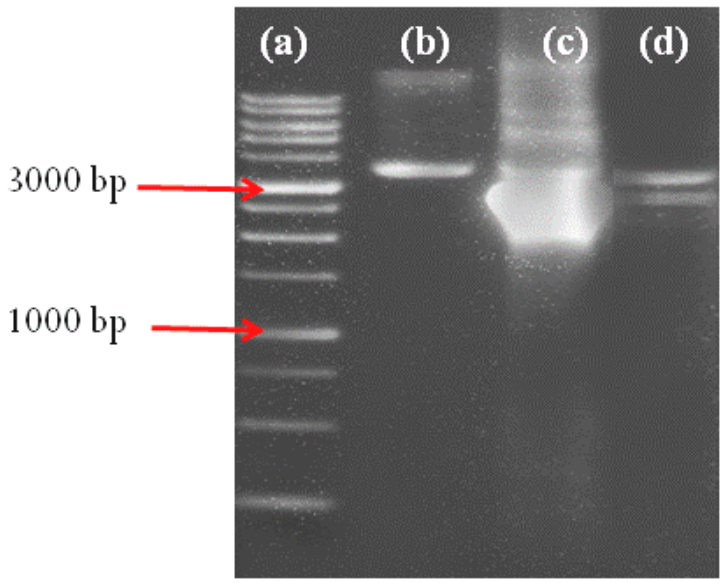

Figure 4.4: Confirmation of DNA-A component in pJET vector on 0.8% agarose gel

(a) 1kb DNA ladder (b) Recombinant pJET having DNA-A component (c) Amplicon of DNA-A component with pJET primers (d) Restriction digestion of recombinant pJET with *EcoR*V

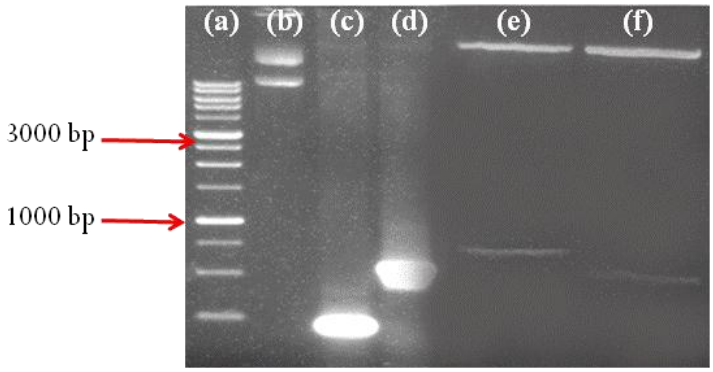

Figure 4.5: Confirmation of pFGC1008 RNAi (a) 1kb DNA ladder (b) RNAi vector pFGC1008 (c) Amplicon from pFGC with AS primers (d) Amplicon from pFGC with BS primers (e) Double digestion of pFGC with *Bgl*II & *Spe*I (f) Double digestion of pFGC with *Xho*I & *Spe*I

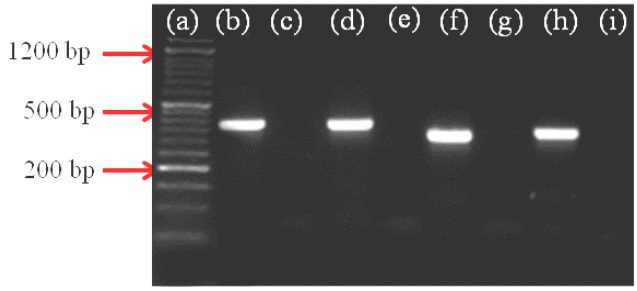

Figure 4.6: Amplification of target gene(s) fragments from DNA-A component in sense and antisense orientation

(a) 50bp DNA ladder (b) & (d) Amplicon of target 1 (overlapping region of *AC1*/*AC2*) in sense and antisense orientation (f) & (h) Amplicon of target 2 (overlapping region of *AC1*/*AC4*) in sense and antisense orientation (c), (e), (g) & (i) Negative controls

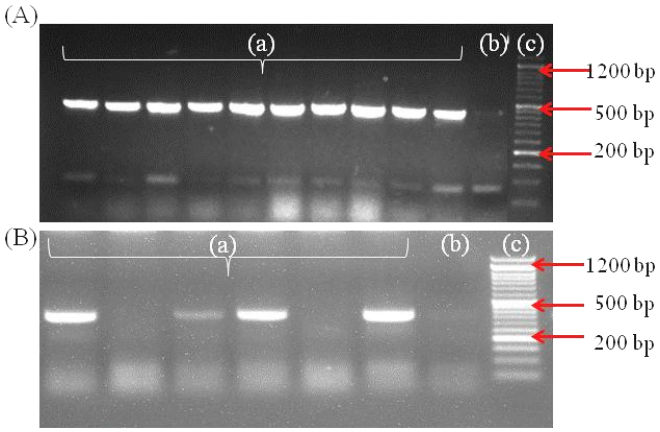

Figure 4.7: Confirmation of of targets gene(s) fragments in pGEM-T easy vector by Colony PCR

(A) Confirmation of overlapping region of *AC1*/*AC2* (a) Positive clones for target 1 (b) Negative control (c) 50bp DNA ladder

(B) Confirmation of overlapping region of *AC1*/*AC4* (a) Positive clones of target 2 (b) Negative control (c) 50bp DNA ladder

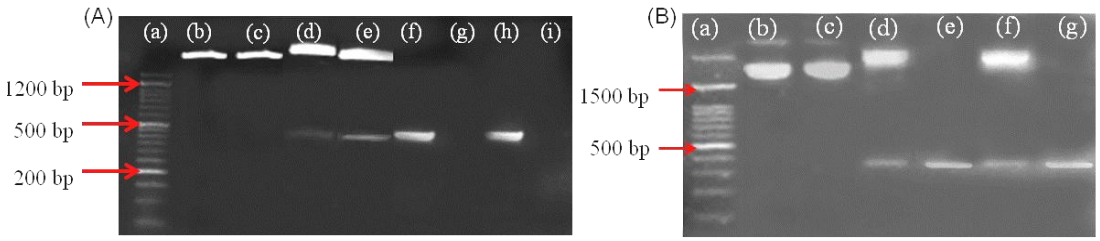

Figure 4.8: Confirmation of recombinant pGEM-T easy vector having target gene(s) fragments with PCR and restriction digestion

(A) Confirmation of target 1 in recombinant pGEM-T easy vector (a) 50bp DNA ladder (b) & (c) Recombinant vectors having target 1 (d) Double digestion with *Xho*I & *Sac*I (e) Double digestion with *BamH*I & *Spe*I (f) & (h) Amplicons of target 1 with sense and antisense-specific primers (g) & (i) Negative controls

(B) Confirmation of recombinant pGEM-T having target 2 (a) 50bp DNA ladder (b) & (c) Recombinant having target 2 (d) Double digestion with *Xho*I & *Sac*I (e) & (h) Amplicons of target 2 with sense and antisense specific primers (f) Double Digestion with *BamH*I & *Spe*I

4.1.1.5 Cloning of target gene(s) fragments in sense and antisense orientation in RNAi vector

Sense strands were double-digested from the pGEM-T easy vector by *Xho*I & *Sac*I. Overnight digestion reaction was performed by treating the cloning vector (having targets in sense orientation) and the RNAi vector with the same restriction enzymes. Digested cloning vector and RNAi vector was run individually in different gels i.e cloning vector on 2.5% and RNAi vector on 0.8% agarose gel (Figure 4.9) depending upon the size of the vectors. These digested products were ligated at 16°C for 24 hours with the help of T_4 DNA ligase (Invitrogen). T_4 DNA ligase helps in the joining of compatible ends with each other by making phosphodiester bond with the help of ATP present in the buffer supplied with it. In the above case, both cloning and RNAi vector cleaves with *Xho*I & *Sac*I and the insert eluted from the cloning vector used to ligate with RNAi vector having same compatible ends.

Cloning of target gene(s) fragments in RNAi vector in sense orientation

Ligated products were used for the transformation of DH5α competent cells and the colonies formed were subcultured and ten colonies from each plate were confirmed for having required insert by colony PCR with insert-specific primers. Three colonies from target 1 and six colonies from target 2 were shown positive results with colony PCR (Figure 4.10). Plasmids were isolated from positive clones and they were quantified by taking 2 clones per transformation. Recombinant plasmids were checked for having required inserts with insert-specific primers and also by restriction digestion with same enzymes that were used for

cloning. Recombinant plasmids gave 385bp amplicon size with target 1 specific primers and 342bp with target 2 specific primers (Figure 4.11 A). Restriction digestion gave same fragment size as achieved by PCR analysis and show that the isolated plasmids are the recombinant one having target 1 and 2 in sense orientation at the T-DNA portion within the left and right border repeat (Figure 4.11 B).

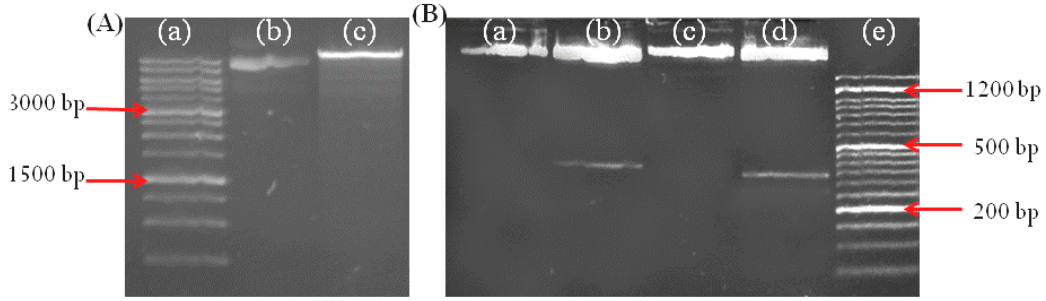

Figure 4.9: Overnight digestion of pGEM-T and RNAi vector with *Xho*I & *Sac*I endonucleases

(A) Digestion of RNAi vector (a) 1kb DNA ladder (b) Undigested pFGC1008 (c) Digestion with *Xho*I & *Sac*I

(B) Digestion of recombinant pGEM-T having target gene(s) in sense orientation (a) Undigested recombinant pGEM-T having target 1 (b) Double digestion of pGEM-T having target 1 (c) Undigested recombinant pGEM-T having target 2 (d) Double digestion of pGEM-T having target 2 (e) 1kb DNA ladder

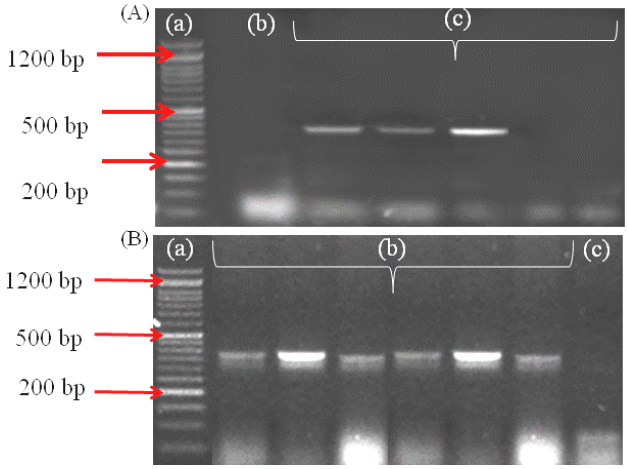

Figure 4.10: Confirmation of recombinant RNAi vector (pFGC-*AC2*(s) & pFGC-*AC4*(s) by colony PCR

(A) Confirmation of target 1 in pFGC-*AC2*(s) (a) 50 bp DNA ladder (b) Negative control (c) Amplicons of target 1 with sense orientation specific primers

(B) Confirmation of target 2 in pFGC-*AC4* (a) 50 bp DNA ladder (b) Amplicons of target 2 with sense orientation specific primers (c) Negative control

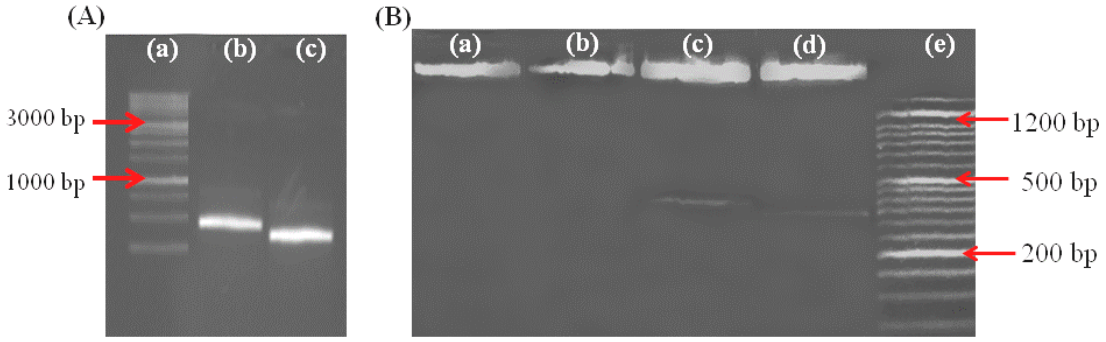

Figure 4.11: Confirmation of recombinant RNAi vectors (pFGC-*AC2*(s) & pFGC-*AC4*(s)

(A) Confirmation with gene-specific primers (a) 1kb DNA ladder (b) Amplicon of target 1 with sense orientation specific primers (c) Amplicon of target 2 with sense primers

(B) Confirmation with restriction digestion (a) Undigested pFGC-*AC2*(s) (b) Undigested pFGC-*AC* (c) Double-digestion of pFGC-*AC2*(s) with *Xho*I & *Sac*I (d) Double-digestion of pFGC-*AC4* with *Xho*I & *Sac*I (e) 50 bp DNA ladder

Cloning of target gene(s) fragments in antisense orientation in recombinant RNAi vectors (pFGC-*AC2*(s) & pFGC-*AC4*(s)

The recombinant RNAi vectors (pFGC-*AC2*(s) & pFGC-*AC4*(s) were having target gene(s) fragments in sense orientation and further the recombinant vectors were overnight digested with *BamH*I & *Spe*I so that gene(s) fragments in antisense orientation be cloned at the T-DNA portion of the flanking region of GUS-intron. Recombinant pGEM-T vectors having two targets gene(s) fragments in antisense orientation were overnight digested with the same restriction enzymes with which recombinant RNAi vectors were digested (Figure 4.12). After the digestion, compatible ends were generated and they were ligated to each other with T_4 DNA ligase (Invitrogen) which used phosphate group of ATP present in the ligase buffer for the formation of phosphodiester bonds. Ligated products were used to transform to the DH5α competent cells. Colonies were subcultured and ten of the colonies were confirmed for the presence of insert by insert-specific primers. Seven from target 1 and five colonies from target 2 shown positive results with colony PCR (Figure 4.13).

4.1.1.6 Confirmation of target genes in recombinant RNAi vectors (pFGC-*AC2*hp & pFGC-*AC4*hp)

Recombinant plasmids were isolated from positive clones and were quantified on 0.8% agarose gel (Figure 4.14). These plasmids were confirmed by PCR and restriction digestion. PCR was performed by both insert and vector-specific primers (Figure 4.14 A & B). Insert-specific primers gave 385bp amplicon size for target 1 and 342 bp for target 2 (Figure 4.14 A). Whereas, vector-specific primers were differentially designed for both sense and antisense strands, therefore the size of amplicons were also different for both strands and

also for both the targets. For target 1, 597 & 938bp amplicon was obtained for sense and antisense strands and 554 & 895bp amplicon was there for target 2 for sense and antisense strands (Figure 4.14 B). Restriction digestion was done by two pairs of enzymes i.e. *Xho*I & *Sac*I and *BamH*I & *Spe*I. Restriction digestion gave 385bp fragment for target 1 and 342bp fragment for target 2 (Figure 4.15). These enzymes cleave the sites in the recombinant vectors where the inserts were ligated to the vectors. Resulting recombinant RNAi constructs (pFGC-*AC2*hp & pFGC-*AC4*hp) were having both target gene(s) fragments in sense and antisense orientation at the flanking region of GUS-intron of their T-DNA portion (Figure 4.16).

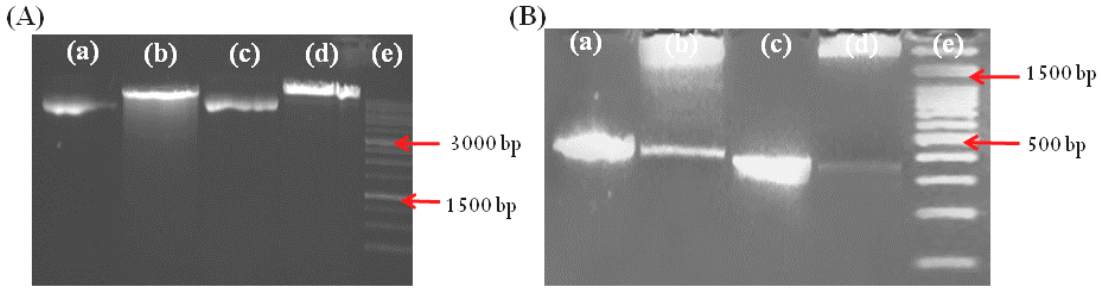

Figure 4.12: Confirmation of recombinant RNAi vectors (pFGC-*AC2*(s) & pFGC-*AC4*(s) and recombinant pGEM-T vectors with *BamH*I & *Spe*I endonucleases

(A) Confirmation of recombinant RNAi vectors (a) & (c) Undigested pFGC-*AC2*(s) and undigested pFGC-*AC4*(s) (b) Digested pFGC-*AC2* (d) & (e) pFGC-*AC4*(s) digested fragments (e) 1kb DNA ladder

(B) Confirmation of recombinant pGEM-T (a) Amplicon of target 1 with sense orientation specific primers (b) Digested recombinant pGEM-T having target 1 (c) Amplicon of target 2 with sense orientation specific primers (d) Digested recombinant pGEM-T having target 2 (e) 50bp DNA ladder

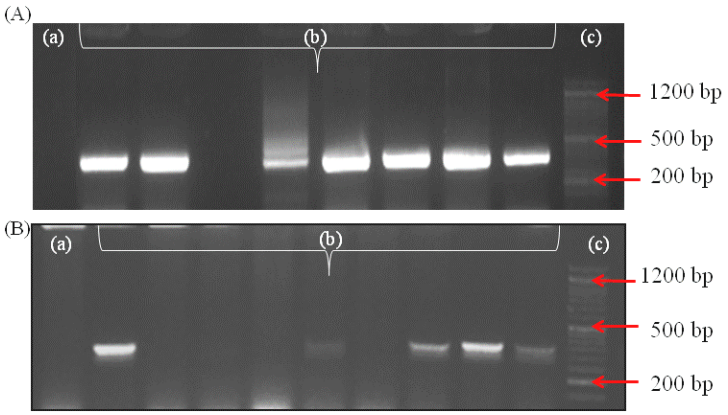

Figure 4.13: Confirmation of RNAi constructs by colony PCR with gene-specific primers

(A) Confirmation of RNAi construct 1 (a) Negative control (b) Amplicons with sense and antisense specific primers (c) 50bp DNA ladder

(B) Confirmation of RNAi construct 2 (a) Negative control (b) Amplicons with sense and antisense specifc primers (c) 50bp DNA ladder

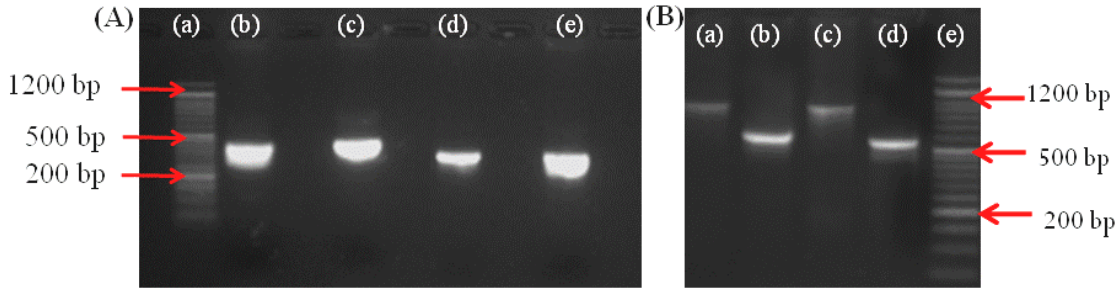

Figure 4.14: (A) Confirmation of recombinant RNAi vectors with gene-specific primers

(a) 50bp DNA ladder (b) & (c) Confirmation of target 1 in pFGC-*AC2*hp in sense and antisense orientation (d) & (e) Confirmation of target 2 in pFGC-*AC4*hp in sense and antisense orientation

(B) Confirmation of recombinant RNAi constructs with vector-specific primers

(a) & (b) Confirmation of target 2 in pFGC-*AC2*hp with AS & BS primers (c) & (d) Confirmation of target 2 in pFGC-*AC4*hp with AS & BS primers (e) 50bp DNA ladder

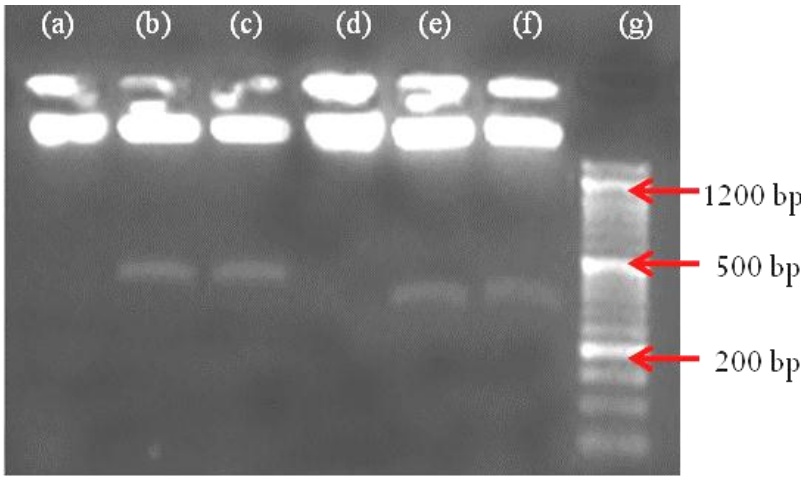

Figure 4.15: Confirmation of target gene(s) fragments in pFGC-*AC2*hp & pFGC-*AC4*hp by using restriction endonucleases (a) & (e) Undigested pFGC-*AC2*hp and undigested pFGC-*AC4*hp (b) & (e) Confirmation of target 1 in pFGC-*AC2*hp and target 2 in pFGC-*AC4*hp with *Xho*I & *Sac*I endonucleases (c) & (f) Confimation of target 1 in pFGC-*AC2*hp and target 2 in pFGC-*AC4*hp with *BamH*I & *Spe*I endonucleases (g) 50bp DNA ladder

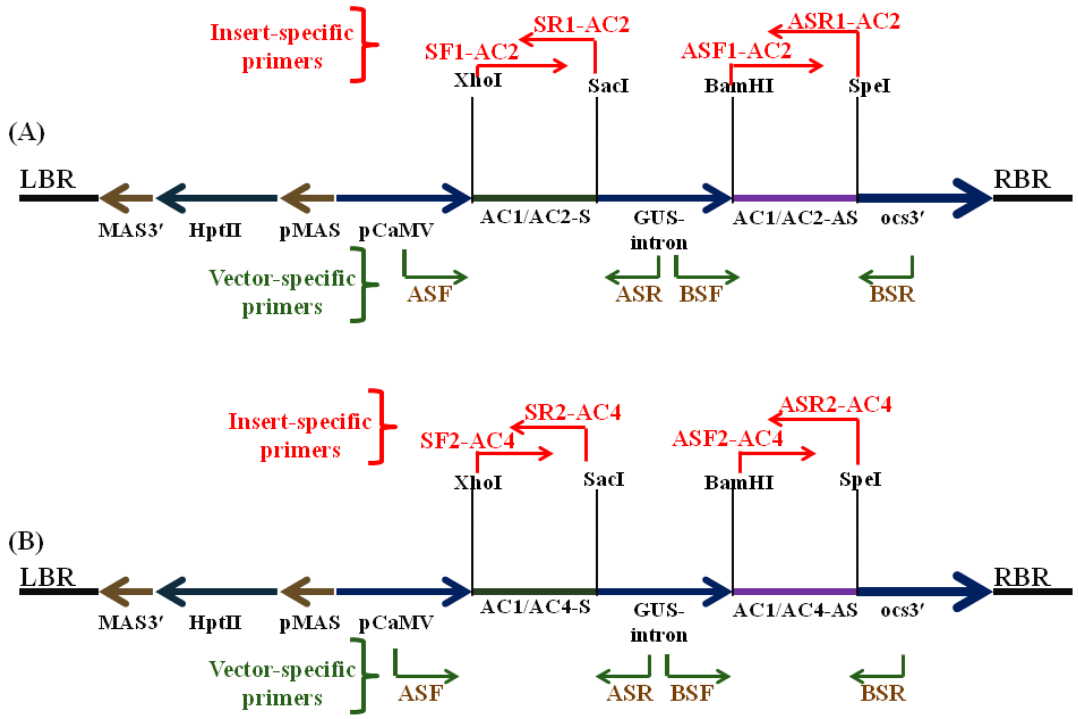

Figure 4.16: Schematic representation of T-DNA region of RNAi constructs (pFGC-*AC2*hp & pFGC-*AC4*hp)

(A) & (B) pFGC-*AC2*hp RNA and pFGC-*AC4* RNA constructs respectively

LBR is left border repeat joined by promoter (pMAS) and terminator of mannopine synthase gene (MAS3′) that involves in the transcription of hygromycin resistance gene (HptII) present in between them, followed by cauliflower mosaic virus promoter (pCaMV) and octopine synthase terminator (ocs3′) that transcribes the overlapping region of *AC1/AC2* or *AC1/AC4* in sense and antisense orientation present at the flanking region of GUS-intron, RBR is right border repeat present at 3′end of the octopine synthase terminator

4.1.1.7 Sequencing and analysis of target gene(s) fragments in recombinant RNAi vectors

Recombinant RNAi vectors were outsourced for bi-directional sequencing with vector-specific primers. Contigs of partial forward and reverse sequence were generated by Bioedit sequence alignment editor (Figure 4.17). These contigs were used as query sequence for blastn to find similarity index with the DNA-A genome of begomoviruses of okra. Contig of pFGC-*AC2*hp with both AS and BS primers gave 97.9% and 98.24% identity with *Okra enation leaf curl virus* isolate India: Ludhiana (Accession no. KP208672.1). Contig of pFGC-*AC4*hp with AS and BS primers gave 99.7% and 98.88% identity with *Okra enation leaf curl virus* isolate clone 5.2, complete genome (Accession no. MK084766.1) (Figure 4.18).

(A)

>Contig-pFGC-AC2 with AS primers

TAGAGGGGATTTGGAACCTCCCAGATAAAGACGCCATTCCTTGTATGAGCGTGAAAAAAAAGTGATGCG
TTCCCCTGTGCGAGAATCCATGGTTGAAGCAGTTAATGGATAGATAATAAGAACACCCGCATTCAAGATC
TACTCTCTTCCTCCTGTGCGCTCTCTTCGCTTCCCTGTGCTGTACTTTGATTGGTACCTGAGTACAATGGT
CCTTCAAGGGTGACGAAGATCGCATTTTTCACTGCCCAGTTCTTTAGTGCTGAGTTCTTATCCTCGTCTAA
GAACTCTTTATAACTGCTGTTGGGACCAGGATTGCAGAGGAAGATTGTTGGTATGCCGCCTTTAATTTGA
ACTGGCTTTCCGTATTTTGTGTTGGATTGCCAGTCCCTTTGAACTCCATTTTGACAGTTTTTTTTTGGTAC
AGATGACAAGAGGCAACAGTACAAACAACAAACAACATTACAATTACATTTACAATTACCATGGGGCGC
GCCCAATCGATGATTAATCCCCCAGAGAACAGCCGGC

>Contig-pFGC-AC2 with BS primers

GGCCGTTCGCAGGTGCCGGGAAATATTTCGCGCCACTGGGCGGAAGCAACGCGTAAACTCGACCCGAC
GCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGCTCACACCGATACCATCAGCGATCTCTTTGAT
GGGGATCCAAAGGGACTGGCAATCCAACACAAAACTACGGAAAGCCAGTTCAAATTAAAGGTCGGCAT
ACCAACAATCTTCCTCTGCAATCCTGGTCCCAACAGCAGTTATAAAGAGTTCTTAGACGAGGATAAGAA
CTCAGCACTAAAGAACTGGGCAGTGAAAAATGCGATCTTCGTCACCCTTGAAGGACCATTGTACTCAGG
TACCAATCAAAGTACAGCACAGGGAAGCGAAGAGAGCGCACAGGAGGAAGAGAGTAGATCTTGAATG
CGGGTGTTCTTATTATCTATCCATTAACTGCTTCAACCATGGATTCTCGCACAGGGGAACGCATCACTGCA
GCTCATACAAGGAATGGCGTCTTTATCTGCCACGTTCCAAATCCCCTCTATACTAATCCCGACAGGGCCTG
CTTTAATGAGATATGCGAGACGCCTATGATCGCATGATATTTGCTTTCAATTCTGTTGTGCACGTTGTAAA
AAACCTGAGCATGTGTAGCTCAGATCCTTACCGCCGGTTTCGGTTCATTCTAATGAATATATCACCCGTTA
CTATCGTATTTTTATGAATAATATTCTCCGTTCAATTTACTGATTGTACCCTACTACTTATATGTACAATATTA
AAATGAAAACAATATATTGTGCTGAATAGGTTTATAGCGACATCTATGATAGAGCGCCACAATAACAAAC
AATTGCGTTTTATTATACAAATCCAATTAAAAAGCCACCCTGAAT

(B)

>Contig-pFGC-AC4 with AS primers

CCTCCTTAGGGAGTTCATTTTCATTTGGAGAGGACACGCTCGAGCCCACACAAATAGTTATGTGGACCCA
GTGATCTACCCCACATCGTCTTCCCGGTACGACTATCTCCCTCAATCACTATACTTTGTGGTCTATGTGGCC
GCGCAGCGGCGTCGACAACGTTCTCGGCAGCCCATTCCTCAAGTTCTTCCGGAACTTGATCAAAAGAAG
AAGAAGAAAAAGGAGAAACATAAACCTCCACAGGAGGTGTAAAAATCCTATCTAAATTAGCATTTAAAT
TATGGAATTGTAATACATAATCTTTTGGAGCTAACTCCTTAATGACTCTAAGAGCCTCTGACTTACTGCCT
GCGTTAAGCGCTGCGGCGTAAGCGTCGTTGGCTGTGAGCTCTATTTTTACAACAATTACCAACAACAAC
AAACAACAAACAACATTACAATTACATTTACAATTACCATGGGGCGCGCCCAATCGATGATTTAAATCCC
CCAGATGAACATGGCATCGTGGTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTG
GTTTCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAACGGGGAAACTCAGCA
AGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAATAC

>Contig-pFGC-AC4 with BS primers

GGGGCGCCTTTTTAATTGGATTTGTATAATAAAACGCAATTGTTTGTTATTGTGGCGCTCTATCATAGATGT
CGCTATAAACCTATTCAGCACAATATATTGTTTTCATTTTAATATTGTACATATAAGTAGTAGGGTACAATCA
GTAAATTGAACGGAGAATATTATTCATAAAAATACGATAGTAACGGGTGATATATTCATTAGAATGAACCG
AAACCGGCGGTAAGGATCTGAGCTACACATGCTCAGGTTTTTTACAACGTGCACAACAGAATTGAAAGC
AAATATCATGCGATCATAGGCGTCTCGCATATCTCATTAAAGCAGGACTCTAGGGACTAGTCCCACACAA
ATAGTTATGTGGACCCAGTGATCTAGCCCACATCGTCTTCCCGGTACGACTATCTCCCTCAATCACTATAC
TTTGTGGTCTATGTGGCCGCGCAGCGGCGTCGACAACGTTCTCGGCAGCCCATTCCTCAAGTTCTTCCGG
AACTTGATCAAAAGAAGAAGAAGAAAAAGGAGAAACATAAACCTCCACAGGAGGTGTAAAAATCCTAT
CTAAATTAGCATTTAAATTATGGAATTGTAATACATAATCTTTTGGAGCTAACTCCTTAATGACTCTAAGAG
CCTCTGACTTACTGCCTGCGTTAAGCGCTGCGGCGTAAGCGTCGTTGGCTGTGGATCCCCATCAAAGAG
ATCGCTGATGGTATCGGTGTGAGCGTCGCAGAACATTACATTGACGCA

Figure 4.17: Contigs of forward and reverse sequence of RNAi constructs formed by Bioedit sequence alignment editor

(A) Contigs of pFGC-*AC2*hp with partial sequence obtained by AS and BS primers

(B) Contigs of pFGC-*AC4*hp with partial sequence obtained by AS and BS primers

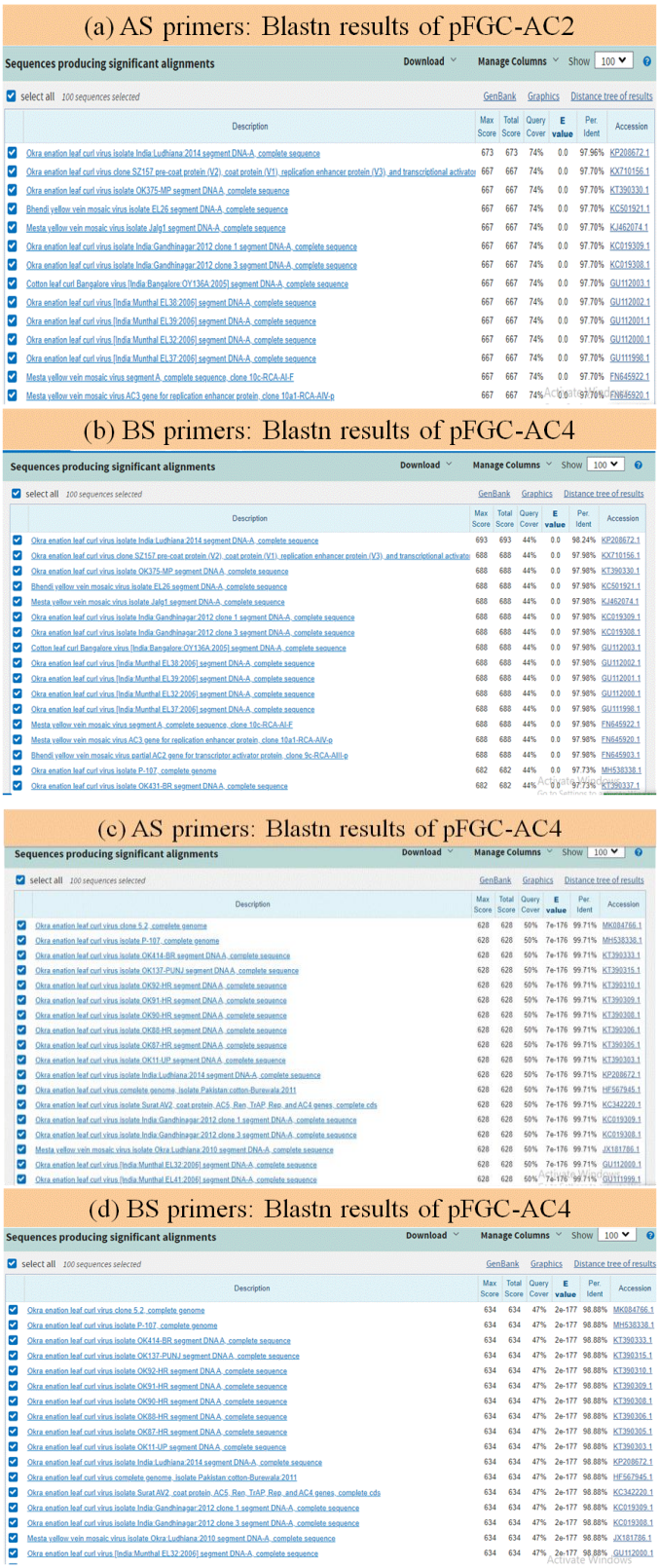

Figure 4.18: Blastn results of RNAi constructs with vector-specific primers

(a) Blastn results of pFGC-*AC2*hp with AS primers (b) Blastn results of pFGC-*AC2*hp with BS primers

(c) Blastn results of pFGC-*AC4*hp with AS primers (d) Blastn results of pFGC-*AC4*hp with BS primers

4.1.1.8 Transformation of pFGC-*AC2*hp & pFGC-*AC4*hp to *Agrobacterium* GV3101

Recombinant pFGC-AC2 & pFGC-AC4 were transformed from *E.coli* to *Agrobacterium tumifaciens*. As *Agrobacterium* is known to be natural genetic engineer and due to its property to transfer its genes naturally to the plants; various strains are artificially synthesized by replacing its T-DNA region with the region of interest (Sheikh *et al* 2014). GV3101 strain is hypervirulent strain and having rifampicin resistance gene in their genome, so for the transformation of *Agrobacterium* with RNAi constructs, positive clones were grown on YEP medium containing both chloramphenicol and rifampicin antibiotics. Colonies produced were confirmed by colony PCR. All the colonies that were checked for having insert gave positive results in the colony PCR (Figure 4.19). Two plasmids for each construct were isolated from positive clones. *Agrobacterium* maintains the plasmids in low copy number, therefore, the quantity of isolated plasmids is less to do molecular analysis, due to this the isolated plasmids were back transformed to *E.coli* to confirm the presence of right plasmid in the *Agrobacterium* strain and further molecular analysis was done with the plasmids that were isolated from *E.coli* (Figure 4.20).

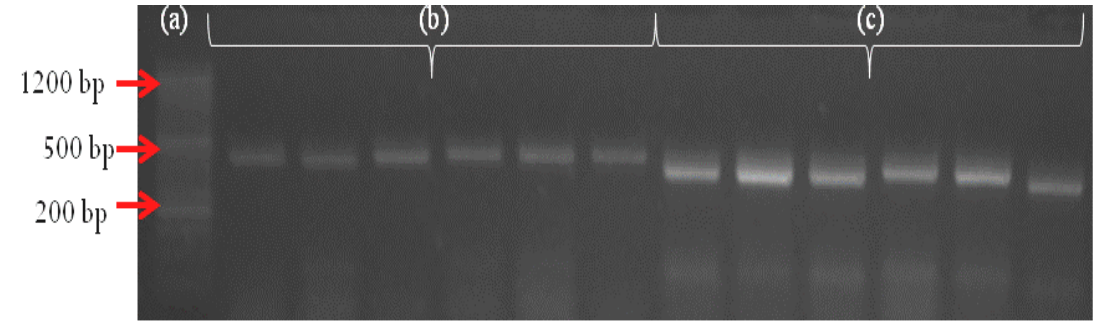

Figure 4.19: Confirmation of RNAi constructs in agroclones by colony PCR

(a) 50bp DNA ladder (b) Confirmation of construct 1 with sense and antisense specific primers (c) Confirmation of construct 2 with sense and antisense specific primers

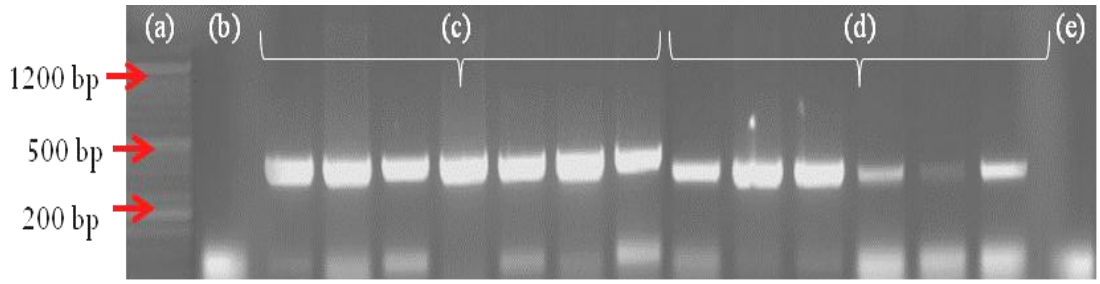

Figure 4.20: Confirmation of RNAi constructs transferred from *Agrobacterium* to *E.coli*

(a) 50bp DNA ladder (b) Negative control (c) Confirmation of construct 1 with insert-specific primers (d) Confirmation of construct 2 with insert-specific primers (e) Negative control

4.1.2 Development of dimeric construct of DNA-A component of okra infecting begomovirus(es) in binary vector

Begomovirus is a genus of plant viruses whose genome is made up of 4 sepearate genomes from which two are large of approximately 2.6-2.8kb in size and two are of small size having size of approximately 1.5-1.7kb. For the proper symptom appearance of viruses on the host plant, all the four genomes are required, but large genome named DNA-A can also impart symptoms as they are having ORFs that form replication mediated proteins. Grimsley and Basaro (1987) reported that for the successful agroinfilteration of virus to produce symptoms into the host plant, it is essential to have cloned viral DNA of more than unit length. Therefore, it becomes necessary to construct viral dimeric or partial dimeric clones that harbor at least two origin of replications. The unit genome component will be released by replication and/or less efficiently by recombination. The constructs can be transferred to binary vectors and by *Agrobacterium* they are used to deliver to plants by agro-inoculations (Stenger *et al* 1991). Agro-infectious clone of more than one unit length was prepared in binary vector by the mixture of RCA and PCR based technique (Kumar *et al* 2017). Clone of the most prevalent virus was prepared in binary vector pFGC1008.

4.1.2.1 Cloning of DNA-A genome of begomovirus in pUC19 vector

Leaves having characteristic symptoms of yellow patches, yellowing of veins and vein clearing were used for DNA isolation (Figure 4.21). DNA isolation was done by modified CTAB method. In this method, concentration of NaCl used was more to reduce mucilage in the downstream steps of isolation. After isolation, it was quantified on 0.8% agarose gel (Figure 4.22). All DNA samples gave sharp bands of high-molecular DNA on agarose gel and gave concentration of above 2000ng/µl on nanodrop spectrophotometer.

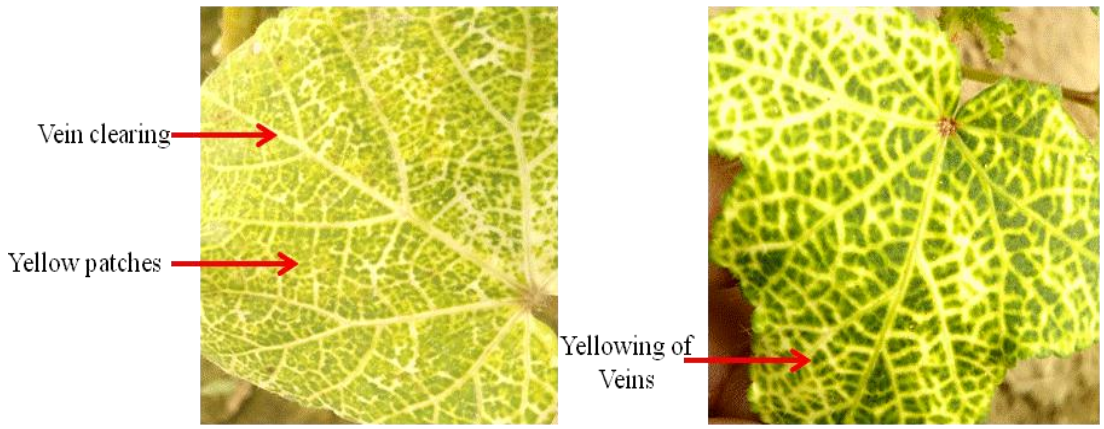

Figure 4.21: Okra leaves infected with begomovirus(es)

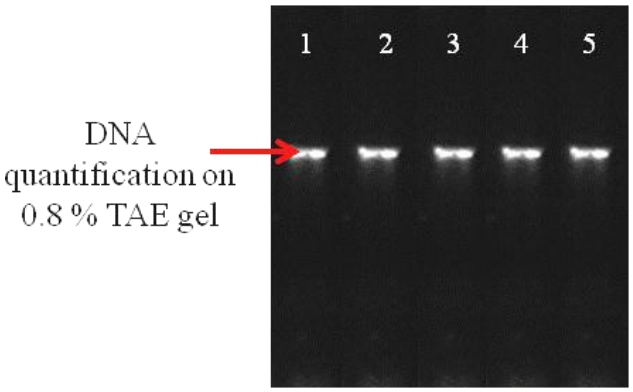

Figure 4.22: DNA quantification of samples infected with begomovirus(es) of okra

PCR detection of begomoviruses

Presence of begomoviral DNA in the infected samples was checked by using begomoviral specific degenerate primers. All infected samples show required amplicon size with the primers specific to viral coat protein (Figure 4.23).

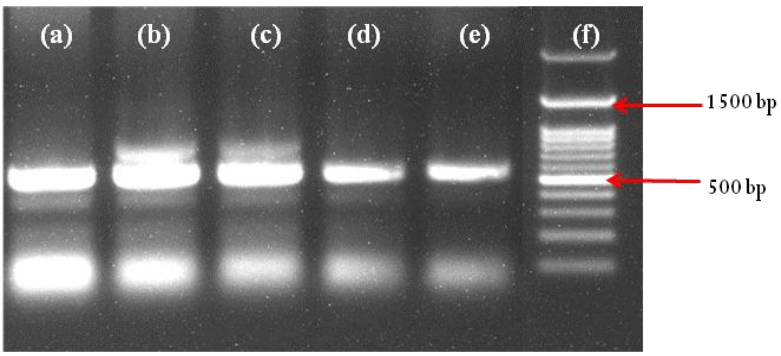

Figure 4.23: Confirmation of begomoviral DNA by begomoviral specific degenerate primers

(a) to (e) Infected samples show required amplicon size (f) 100bp DNA ladder

4.1.2.2 Enrichment of begomoviral DNA through Rolling circle amplification (RCA)

Rolling-circle amplification (RCA) was done on DNA samples that were isolated from okra leaves infected with begomovirus(es) (Figure 4.24). As genome of begomoviruses is circular, so we used TempliPhi kit of DNA amplification which make use of DNA polymerase of ø29 bacteriophage for the exponential amplification of circular DNA templates of single or double stranded form by rolling circle amplification. This method utilized picogram quantities of DNA as the starting material (Dean *et al* 2001). DNA sample was mixed with the sample buffer that contains random hexamer primers that leads DNA synthesis arbitrarily, these primers attached to the DNA at numerous sites and then the mixture was heated at 95°C to denature the DNA. Reaction mixture thus added provides salts and the mixture of dNTPs which adjusted the pH and also involves the DNA synthesis from

the site of primer with the help of polymerase present in the enzyme mixture. When the polymerase reaches to the primer at the end, displacement synthesis of the strand occurs. The strand that displaced was single stranded and was free to anneal with more hexamer primers. The process lasts to about 4-18 hours at 30°C and results in high-molecular weight circular double-stranded DNA template from picogram quantities of DNA (Dean *et al* 2001).

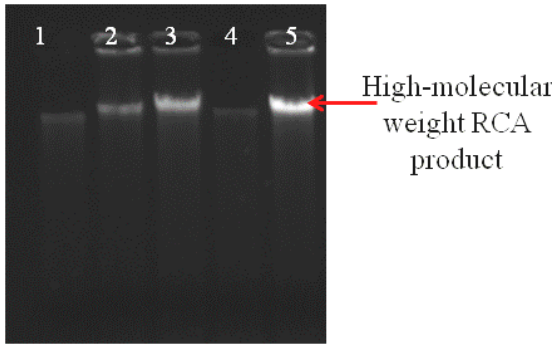

Figure 4.24: High molecular weight viral DNA amplified through RCA

4.1.2.3 Detection of begomoviral DNA by restriction digestion of RCA product with various endonucleases

RCA products were digested by six enzymes to yield the unit length genome of begomoviruses. These enzymes were used for overnight digestion and products were run on the gel. Only one enzyme (*Sac*I) yields the 2.6-2.7 kb genome of virus (Figure 4.25). This band of required size was eluted from the gel and used to ligate to pUC19 cloning vector. pUC19 vector has the restriction site of same enzyme by which RCA was digested. pUC19 and the RCA product were overnight digested, ligated and then the recombinant pUC19 was used to transform *E.coli* DH5α competent cells. Colony PCR was done to check the presence of whole genome of begomovirus. Three colonies show positive results with colony PCR (Figure 4.26). Recombinant plasmids were isolated from positive clones and were quantified and confirmed by PCR (Figure 4.27).

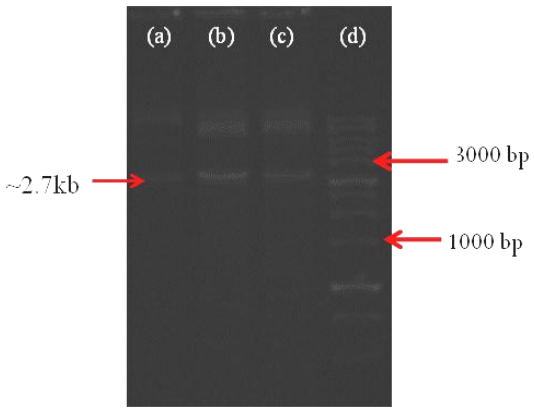

Figure 4.25: Overnight digestion of RCA products with *Sac*I enzyme (a) to (c) Digestion of distinct RCA products (d) 1kb DNA ladder

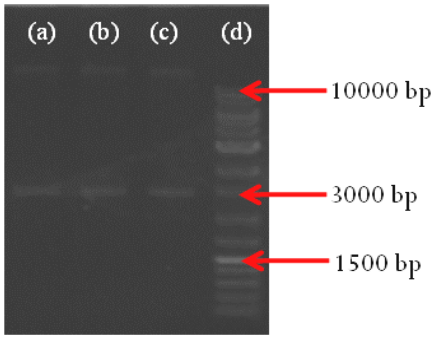

Figure 4.26: Confirmation of recombinant pUC19 by colony PCR (a) to (c) Clones confirmed with m13 primers (d) 1kb DNA ladder

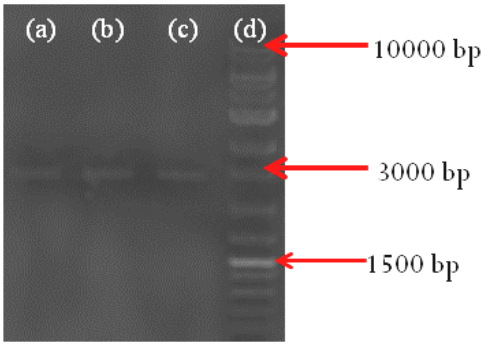

Figure 4.27: Confirmation of recombinant pUC19 by PCR analysis

(a) to (c) Confirmation of recombinant plasmid with m13 primers (d) 1kb DNA ladder

4.1.2.4 Amplication of DNA-A component by outward primers

Outward primers were designed from the conserved region of coat-protein gene of begomoviruses. Sequences of coat-protein genes were retrived from NCBI database and used to align with the help of clustal omega tool (Figure 4.28). The conserved region was used to design outward primers. These primers were having *Sac*I restriction site at its 5′end of forward primer and *Asc*I restriction site at the 5′end of reverse primer. Briddon *et al* 1993 used outward or abutting primers for making dimeric clone of *African cassava mosaic virus*, which is a begomovirus. They successfully agro-infilterate the clone to tobacco plant and in the presense of DNA-B, symptoms were appeared on the *Nicotiana benthemania* plants. Outward primers having *Sac*I and *Asc*I at their ends were applied on recombinant pUC19 vector that is having *Sac*I digested RCA product. PCR was run on 0.8% agarose gel and 2.6-2.8 kb amplicon size (Figure 4.29) was achieved. Amplified products were purified and used to ligate with pGEM-T easy vector to form recombinant pGEM-T and transformed to *E.coli* DH5α competent cells. They were confirmed by colony PCR and colonies that were positive in colony PCR were used for plasmid isolation. Isolated recombinant plasmids were quantified and confirmed by restriction digestion. Restriction digestion was done by *Sac*I and *Asc*I, because sites of these enzymes were added at 5′end of the outward primers. Double-digestion gave 2.6-2.7kb fragment of whole genome of begomoviruses.

```
MYVMVLudhiana       ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      299
OelcvLudhiana       ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      298
OelcvSurat          ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      298
BYVMVHimachal       AGTTAATACGGGATTTAATTTGTATTTTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVNewDelhi       AGTTAATACGTGATTTAATCTGTATTCTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVPhalaghat      AGTTAATACGGGATTTAATTCGTATTTTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVKerala         ATTTAATACGGGATTTAATTTGTATTGTCCGTTCTCGTAATTATGTCGAAGCGAGCTGCA      298
BYVMVChelur         AGTTAATACGGGATTTAATTTGTATTGTCCGTTCTCGTAATTATGTCGAAGCGAGCTGCA      298
                    *  **** ** *** ****   **** * ** *   * ***************  ****

MYVMVLudhiana       GATATCGTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      359
OelcvLudhiana       GATATCTTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      358
OelcvSurat          GATATCGTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      358
BYVMVHimachal       GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVNewDelhi       GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVPhalaghat      GATATCGTCATTTCTACGCCCACGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVKerala         GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCT      358
BYVMVChelur         GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
                    ****** ********** *** ******* ** ** ** ** ********** ******

MYVMVLudhiana       TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCGCAAAATCACGAATGTGGGCCAAC      419
OelcvLudhiana       TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCACAAAATCACGAATGTGGGCCAAC      418
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BYVMVHimachal       TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVNewDelhi       TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVPhalaghat      TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVKerala         TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVChelur         TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
                    **  * * ****** * ******** ********** *****  **     *** * ***

MYVMVLudhiana       AGACCCATGTACCGGAAGCCCAGAATGTACAGGATATACAGAAGCCCTGATGTTCCTAGA      479
OelcvLudhiana       AGACCCATGTACCGGAAGCCCAGAATGTACAGGATATACAGAAGCCCTGATGTTCCTAGA      478
OelcvSurat          AGACCCATGTACCGGAAGCCCAGAATGTACAGGATGTACAGAAGCCCTGATGTTCCTAGA      478
BYVMVHimachal       AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCACGG      478
BYVMVNewDelhi       AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVPhalaghat      AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVKerala         AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVChelur         AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
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MYVMVLudhiana       GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      539
OelcvLudhiana       GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      538
OelcvSurat          GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      538
BYVMVHimachal       GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGTTCATATT      538
BYVMVNewDelhi       GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGATCATATT      538
BYVMVPhalaghat      GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGTTCATATT      538
BYVMVKerala         GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTTGTCCATATT      538
BYVMVChelur         GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTTGTCCATATT      538
                    ******** ** ** ******** ***** *****  *     ***** * *  ** **

MYVMVLudhiana       GGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATTGGGCTGACCCATCGAGTAGGG      599
OelcvLudhiana       GGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATTGGGCTGACCCATCGAGTAGGG      598
OelcvSurat          GGTAAGGTTATCTGTCTATCTGATGTTACTAGGGGTATGGGGCTGACCCATCGAGTAGGG      598
BYVMVHimachal       GGTAAGGTAATGTGTATTTCGGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
BYVMVNewDelhi       GGTAAGGTAATGTGTATTTCGGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
BYVMVPhalaghat      GGTAAGGTAATGTGTATTTCGGATGTTACGCGTGGAGTCGGTTTGACCCATCGGATAGGT      598
BYVMVKerala         GGTAAGGTAATGTGTATTTCTGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
BYVMVChelur         GGTAAGGTAATGTGTATTTCTGATGTTACGCGTGGAGTCGGTTTGACCCATCGTATAGGT      598
                    ******** ** *** * ** ********  * **  * **  **********  ****

MYVMVLudhiana       AAACGTTTTTGCGTGAAGTCAT000TGTATTTTGTTGGCAAGATATGGATGGAGAATATT      659
OelcvLudhiana       AAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGATATGGATGGATGAGAATATT      658
OelcvSurat          AAACGTTTTTGCGTGAAGTCATTGTATTTTGTTGGCAAGATATGGATGGATGAGAATATT      658
BYVMVHimachal       AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAAATATGGATGGACGAGAACATC      658
BYVMVNewDelhi       AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
BYVMVPhalaghat      AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
BYVMVKerala         AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
BYVMVChelur         AAGCGTTTTTGTGTCAAGTCAGTTTATGTTTTAGGTAAGATATGGATGGACGAGAACATC      658
                    ** ******** ** ****** * *** ** * ** ** ********** ***** **

MYVMVLudhiana       AAGACTAAGAACCATACGAACACCGTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACA      719
OelcvLudhiana       AAGACTAAGAACCATACGAACACCGTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACA      718
OelcvSurat          AAGACTAAGAACCATACGAACACCGTTATGTTTTGGATCGTGAGAGACAGGCGTCCTACA      718
BYVMVHimachal       AAGACCAAGAACCATACGAATTCGGTGATGTTTTTCCTTGTTCGTGATCGACGACCGGTA      718
BYVMVNewDelhi       AAGACCAAGAACCATACGAATTCGGTGATGTTTTTCCTTGTTCGTGATCGACGACCGGTA      718
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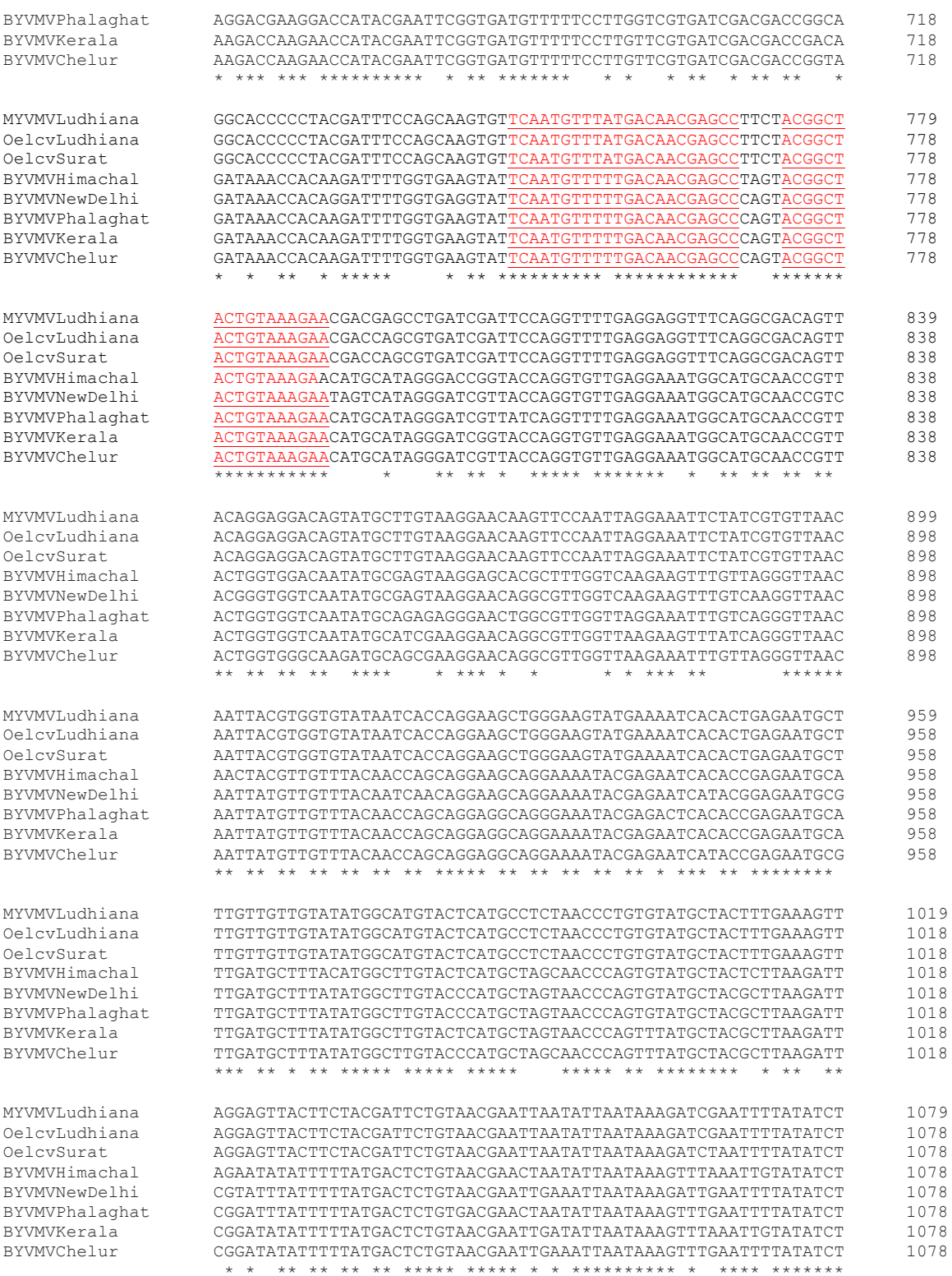

Figure 4.28: Multiple sequence alignment of coat-protein region of begomoviruses of okra.

Underlined colored sequences were utilized for the designing of outward primers

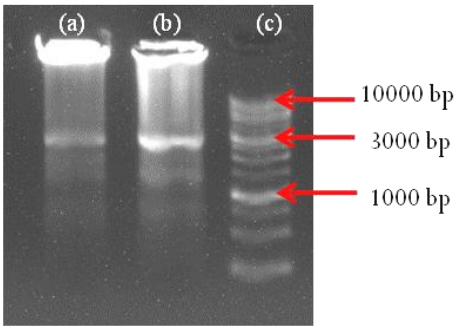

Figure 4.29: Amplification of full length (2.6-2.7kb) by using outward primers

(a) to (b) Full length genome with outward primers (c) 1kb DNA ladder

4.1.2.5 Preparation of dimeric construct in binary vector

Amplicons of DNA-A component of the genome were cleaved from recombinant pGEM-T by double-digestion with *Sac*I and *Asc*I. Overnight double-digestion of pFGC1008 and recombinant pGEM-T was done. 3kb and 2.7kb fragments were cleaved from recombinant pGEM-T and one linear fragment was shown from pFGC1008 after double-digestion (Figure 4.30).

Subcloning of full length amplicon of otward primers in binary vector

Digested products (2.7kb from pGEM-T and linearized pFGC) were eluted from the gel and used to ligate with each other followed by transformation to DH5α competent cells. Plasmids were isolated from clones, quantified and confirmed by restriction digestion. After double-digestion of recombinant pFGC1008, 2.7kb fragment was cleaved from it (Figure 4.31). This pFGC (pFGC-M) is having monomeric unit of begomovirus in between its *Sac*I and *AscI* site.

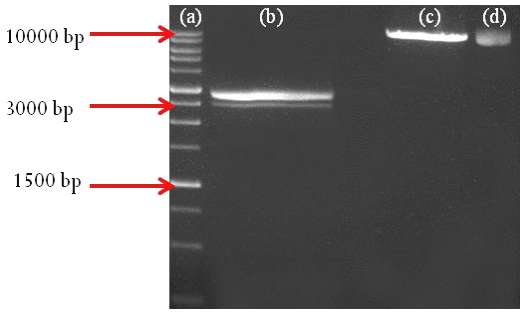

Figure 4.30: Full length released from recombinant pGEM-T using *Sac*I and *Asc*I endonucleases

(a) 1kb DNA ladder (b) Full length of viral DNA released from agarose gel (c) Linearised pFGC1008 (d) Undigested pFGC1008

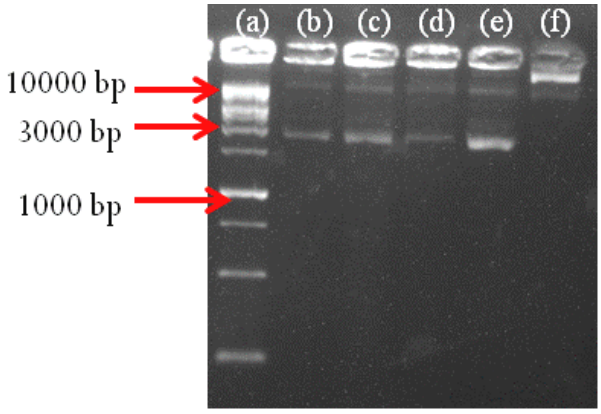

Figure 4.31: Full length released from recombinant pFGC (pFGC-M) by using *Sac*I and *Asc*I endonucleases (a) 1kb DNA ladder (b) to (e) 2.7kb fragment cleaved from recombinant pFGC (f) Undigested pFGC-M

Subcloning of full length genome of pUC19 to pFGC-M

Recombinant Binary vector (pFGC-M) and recombinant pUC19 was overnight digested with *Sac*I, as both have same restriction site. After digestion, circular binary vector becomes linear and pUC19 eluted 2.6-2.8kb RCA digested product (Figure 4.32). Product from pUC19 was eluted from gel and used to ligate with pFGC-M and transformed to competent cells of *E.coli* DH5α.

4.1.2.6 Confirmation of dimeric construct in binary vector by restriction endonucleases

Colonies were picked and isolated plasmids were quantified and confirmed by restriction double-digestion with both *Sac*I and *Asc*I. Double-digestion gave two fragments, from which one was of approximately 5.4kb and other was of 2.7kb respectively (Figure 4.33). This dimeric construct was having more than unit length i.e. pFGC-2M (Figure 4.34).

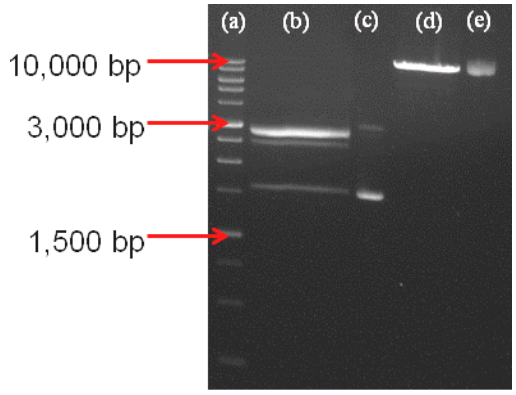

Figure 4.32: Full length released from recombinant pUC19 with *Sac*I restriction endonuclease (a) 1kb DNA ladder (b) Released full length from agarose gel (c) Undigested pUC19 (d) linearized pFGC-M (e) Undigested pFGC-M

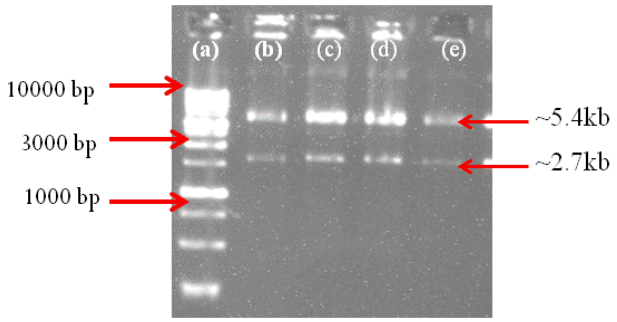

Figure 4.33: Confirmation of dimeric construct (pFGC-2M) by using *Sac*I and *Asc*I restriction endonucleases (a) 1kb DNA ladder (b) to (e) 5.4kb dimer construct and 2.7kb full length genome released from agarose gel

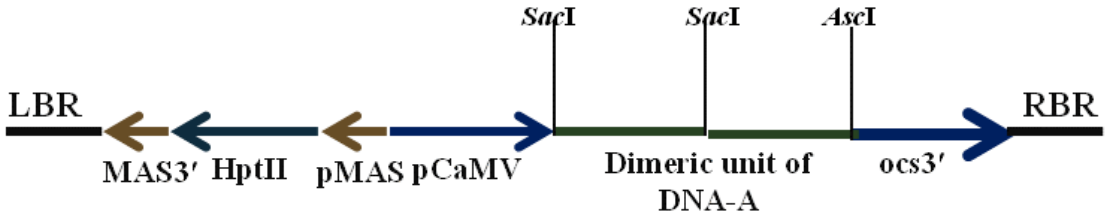

Figure 4.34: Diagrammatic representation of T-DNA region of binary vector having dimeric unit of DNA-A component of begomoviruses infecting okra

4.1.2.7 Transfer of dimeric construct (pFGC-2M) from *E.coli* DH5α cells to *Agrobacterium strain* GV3101

Dimeric construct was transferred from *E.coli* to *Agrobacterium tumifaciems* by freeze-thaw method. Recombinant pFGC-2M was confirmed by colony PCR (Figure 4.35) and plasmid was isolated from positive clones and checked on gel, but it was not visualized on the gel, because of low quantity of the isolated plasmid; therefore the isolated plasmid was back transformed to *E.coli* and quantified it. Isolated plasmids gave same confirmation as shown in *E. coli* before transferring them to *Agrobacterium* (Figure 4.36).

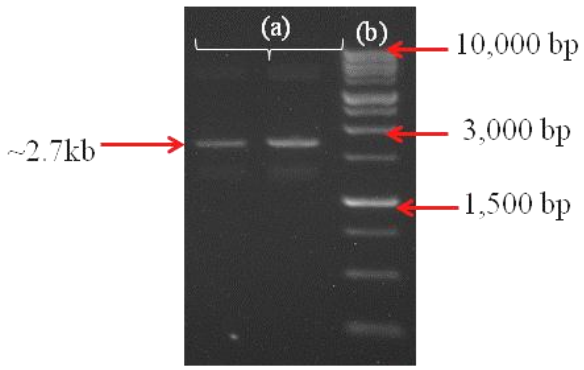

Figure 4.35: Confirmation of agroclones by colony PCR using full length primers of DNA-A component (a) to (b) Positive agroclones (c) 1kb DNA ladder

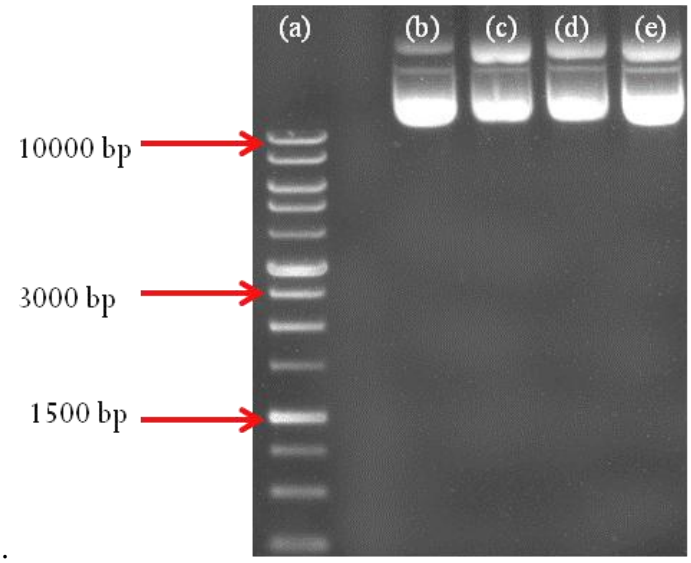

Figure 4.36: Quantification of pFGC-2M in *E.coli*

(a) 1kb DNA ladder (b) to (e) Quantification of dimeric constructs in agarose gel

4.1.3 Transient expression of gene constructs by agroinfilteration of okra leaves

Seeds of okra variety Punjab 8 were sown in polybags. Plants were divided in four sets in correspond to each construct and one is for mock inoculation i.e. empty vector (Figure 4.37). Plants were agroinfiltered with each construct individually and after 3 days plants of RNAi constructs and mock inoculations were agroinfiltered with infectious clone. Agroinfilteration was done by creating vaccum with the help of finger on the adaxial surface and mouth of the syringe on the abaxial surface (Figure 4.38). Symptom appearance was observed for 35 days from the day of agroinfilteration. Control plants that were agroinfiltered with infectious clone and the plants that were agroinfiltered with both empty vector and infectious clone shows symptoms of yellow mosaic on the leaves, but these symptoms were not observed in the plants that were agroinfiltered with RNAi vector and the infectious clone (Figure 4.39). Mubin *et al* 2011 ensured the transient expression of RNAi constructs against *Cotton leaf curl virus* in tobacco plants. In transient assays, all RNAi constructs significantly reduced the replication of the virus in inoculated tissues. Likewise, Medina-Hernandez *et al* 2013 checked transiently the expression of RNAi constructs harbouring the ORFs of *Pepper golden mosaic virus* (PepGMV) & *Tomato chino La Paz virus* (ToChLPV) in tobacco and found multiple virus resistance in the plants agroinfiltered with the similar constructs.

Figure 4.37: Four sets of plants raised upto four-leaf stage

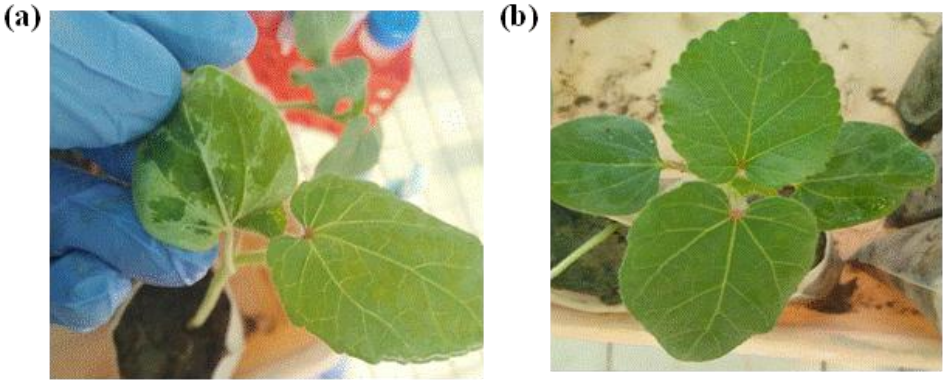

Figure 4.38: Agroinfilteration of okra leaves

(a) Leaves agroinfiltered with syringe (b) Leaves after agroinfilteration

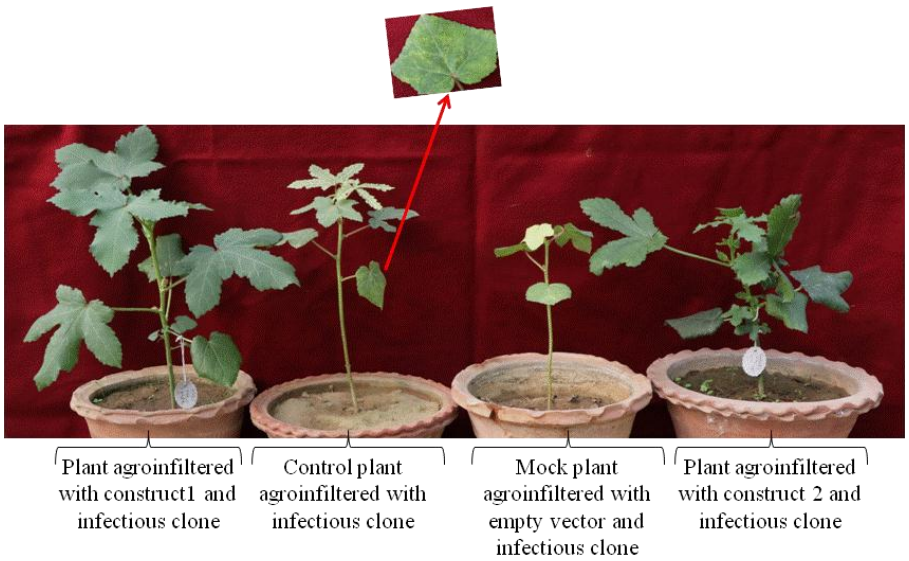

Figure 4.39: Plants agroinfiltered with infectious clone and RNAi constructs

4.2 Genetic transformation of Okra using RNAi (ihpRNA) constructs

Okra variety Punjab 8 was used for the stable expression of RNAi constructs. Tissue culture based *Agrobacterium* mediated genetic transformation method was used. Two methods were utilized based on the explants used and the transformation protocol.

4.2.1 Genetic transformation of okra using hypocotyls and cotyledonary leaves as explants

Seeds of okra plant were surface sterilized with 0.1% mercuric chloride (Figure 4.40a). Mercuric chloride is a strong disinfectant, as okra seeds are having hard seed coat, so the treatment with mercuric chloride was given for 30min. After treatment, seeds were washed to remove traces of disinfectant, otherwise they were intrupt with the germination of seeds. Seeds were dipped in autoclaved distilled water for overnight (Figure 4.40b). Embryos were isolated aseptically and placed on filter paper soaked in distilled water for the purpose of germination (Figure 4.40c). Seeds that were directly placed on media were consumed time to germinate and for the appearance of hypocotyls it took at least 20 days. To reduce time of germination, embryos were isolated aseptically from seeds followed by placement of isolated embryos on MS media for germination (Figure 4.40d).

After 6 days, hypocotyls and cotyledonary leaves were excised from the growing seeds (Figure 4.40e) and used for the transformation with *Agrobacterium* having RNAi constructs (Figure 4.40f). *Agrobacterium* is a natural genetic engineer for dicots. When wound occurred in plant part of dicots, it releases various phenol compounds of which acetosyringone activates some genes of Ti plasmid present in *Agrobacterium tumifaciens* (Glevin 2003). This compound is not secreted by monocot plants; hence for the transfer of recombinant *Agrobacterium*, there will be a need for the addition of synthetic acetosyringone to the medium for the initiation of genes of Ti plasmid for the transfer of T-DNA region of the binary vector to the monocot plants. In the present study 100µM of acetosyringone was adding into the medium and also to the inoculation broth (Manickavasagam *et al* 2015). After co-cultivation with *Agrobacterium*, hypocotyls and cotyledonary leaves were transferred to callus induction media containing BAP (0.5mg/l) and NAA (2mg/l) (Kabir *et al* 2008) and induced callus was placed on regeneration media containing BAP (2mg/l) + IAA (0.1 mg/l) and BAP (2.0 mg/l) + NAA (0.5 mg/l) (Kabir *et al* 2008). In the present study regeneration was not achieved from the induced callus, therefore trans-zeatin riboside, a cytokinin in different concentrations (0.5mg/l - 2mg/l) was used for direct shoot regeneration (Narendran *et al* 2013). Trans-zeatin riboside of 2mg/l concentration was the best for direct shoot regeneration.

After co-cultivation, hypocotyls (Figure 4.40g) and cotyledonary leaves were placed on selection media containing hygromycin (50mg/l) and cefotaxime (500mg/l). Hygromycin

antibiotic resistant gene was present in the T-DNA portion of the RNAi vector, therefore the only explants that transformed with the RNAi constructs were able to grow on the selection media. After 3 weeks, direct shoot regeneration was initiated only in the transformants in which hypocotyls were used as explants and the leaves does not respond to the regeneration media (Figure 4.40 h & i). After shoot regeneration, they were excised from rest of the part and placed on fresh media upto the emergence of multiple shoots (Figure 4.40j). Multiple shoots were transferred to rooting media and subcultured upto the formation of secondary roots (Figure 4.40k). Plantlets with well-developed roots were transferred to sterile potting mixture having cocopeat: vermiculite: perlite in the ratio of 2:1:1 (Figure 4.40l). Plantlets survived were transferred to the large pots in green house (Figure 4.40m).

4.2.2 Genetic transformation of okra using zygotic embryos as explants

Seeds of okra variety Punjab 8 were used for the transformation with RNAi constructs (Figure 4.41a). Seeds were surface sterilized with disinfectant and soaked overnight in distilled water (Figure 4.41b). Embryos were isolated from the seeds in aseptic conditions and placed overnight on filter paper (Figure 4.41c). These embryos were pricked 2-3times with sterile needle at their plumule portion. Pricking was done to create wound from where the *Agrobacterium* transfers its T-DNA portion. Co-cultivation period was given for two days after that the plumule portions from growing seeds were excised for direct shoot regeneration (Figure 4.41d & e). Plumule portion was placed on selection media (Figure 4.41f). For the regeneration to occur from the growing part, trans-zeatin riboside (2mg/l) was utilized as growth hormone. Shoots were regenerated from plumule portion (Figure 4.41 g & h). Tertiary roots were developed from multiple shoots (Figure 4.41i). Plantlets were acclimatized on wet cotton, as the tissue culture raised plants are dependent on the invitro sources, therefore for the survival of the plantlets it is necessary to acclimatize with cotton soaked in sterile distilled water (Figure 4.41j). Survived plants were transferred to potting mixture containing cocopeat: vermiculite: perlite in the ratio of 2:1:1 (Figure 4.41k). Plants that survived in the potting mixture were transferred to large pots for further molecular analysis (Figure 4.41l). Narendran *et al* 2013 utilized zygotic embryos of okra as explants for transformation of *cry1AC* gene and found maximum transformation efficiency of 3.38%.

Figure 4.40: Steps for the transformation of RNAi constructs to okra by using hypocotyls as explants

(a) Seeds placed in petri plate (b) Seeds soaked overnight in distilled water (c) Isolated embryos placed on filter paper (d) Embryos placed on regeneration media (e) Seedlings arised from okra seeds (f) Cotyledonary leaves and hypocotyls excised for co-cultivation (g) Hypocotyls on co-cultivation media (h) Shoot regeneration from hypocotyls (i) Only transformants regenerate from hypocotyls (j) Multiple shoots regeneration (k) Well developed roots (l) Plantlet survived on potting mixture (m) Survived plantlet transferred to large pot

Figure 4.41: Steps for the transformation of RNAi constructs to okra by using zygotic embryos as explants

(a) Seeds of okra variety Punjab 8 (b) Seeds soaked overnight in distilled water (c) Isolated embryos placed on filter paper (d) Embryos on co-cultivation media after pricking (e) Embryos after 2 days of co-cultivation (f) Excised plumule portion placed on selection media (g) Direct shoot regeneration on selection media (h) Shoot elongation on selection media (i) Well-developed roots appeared on selection media (j) Hardening of the plantlet (k) Plantlet survival on potting mixture (l) Survived plants transferred to large pot

In the present study, six transformation experiments were done for each construct. Out of six experiments, four experiments were conducted by using hypocotyls as explants and two were performed by using embryos as explants. When hypocotyls were useds, a total of 613 hypocotyls were co-cultivated with *Agrobacterium* harbouring RNAi construct 1 and 542 hypocotyls were used for *Agrobacterium* co-cultivation containing RNAi construct 2. 129 from 613 and 115 from 542 hypocotyls respond to the selection media and form shoots from it and number of shoots that transferred to the rooting media was 50 & 48 for both the constructs (Table 4.2 a & b). Likewise when embryos were used as explants, 250 embryos per construct were pricked and used for co-cultivation. 48 and 50 embryos were responded to the selection media and involves in direct shoot regeneration. 23 and 26 shoots were placed on rooting media (Table 4.3 a & b).

4.2.3 PCR analysis of putative transformants

Tissue culture raised plants that were transferred to large pots were used for DNA isolation from their leaves (Figure 4.42) and checked for the presence of transgene in their genome with the help of gene-specific and vector-specific primers. When hypocotyls were used as explants, a total of eleven plants have shown required amplicon size with both insert and vector-specific primers (Figure 4.43 & 4.44). Overall transformation efficiency was 0.978 % for construct 1 and 0.906 % for construct 2 (Table 4.2 a & b). RNAi plants (Figure 4.45) thus formed were utilized further for characterization. In the present study, when embryos were utilized as explants, the putative transformants did not show the required amplicon size and hence no transformants were achieved with the same. There may be two reasons for no transformants when zygotic embryos were used as explants: 1) the effect of genotype 2) lesser number of starting material i.e number of explants.

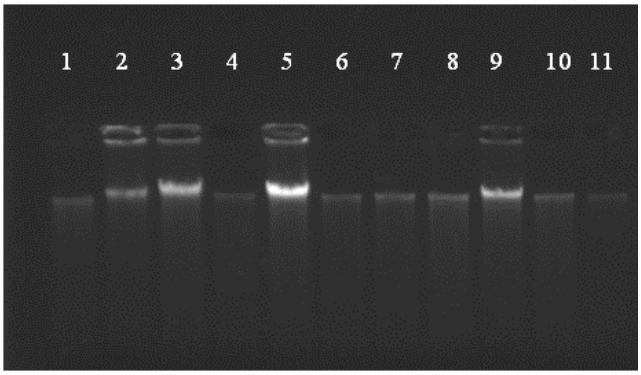

Figure 4.42: DNA quantification of putative transformants

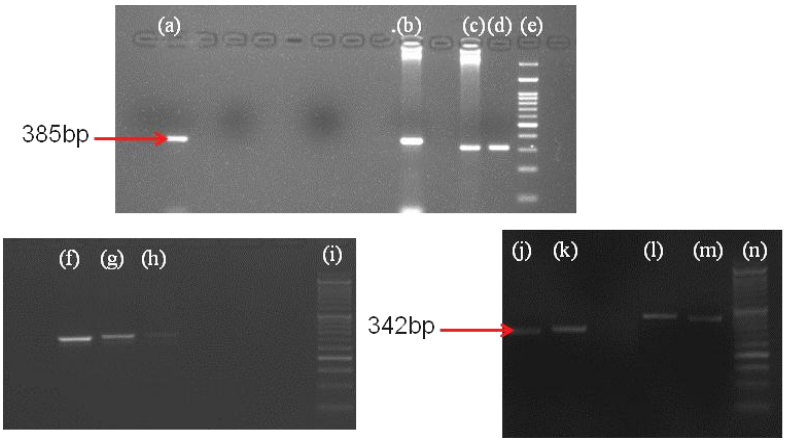

Figure 4.43: Confirmation of putative transformants with gene-specific primers

(a), (b), (g), (h), (l) & (m) – PCR positive plants having construct 1 (c), (d), (f), (j) & (k) – PCR positive plants having construct 2 (e), (i) & (n) 50bp DNA ladder, Empty lanes shows the PCR negative tissue culture raised plants

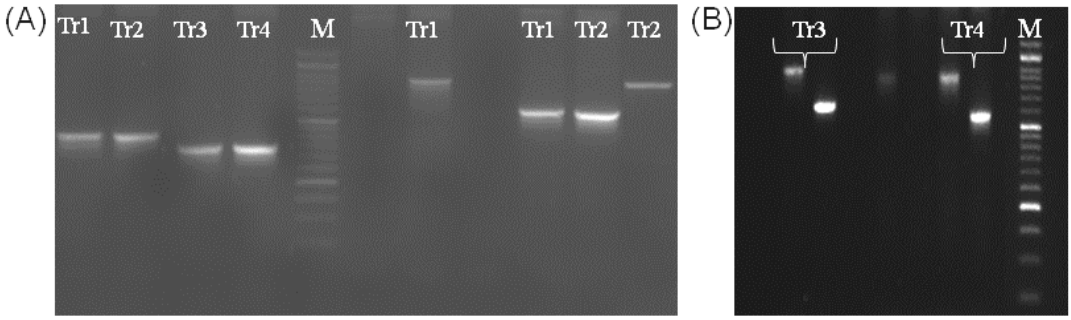

Figure 4.44: Confirmation of transformants with vector-specific primers

Table 4.2(a): Details of transformation experiments performed for construct 1 by using hypocotyls as explants

Experiment No.	No. of explants inoculated in *Agrobacterium* GV3101 suspension	No. of explants that formed shoots on selection media	No. of shoots rooted on selection media	No. of PCR positive plants	Transformation efficiency (%)
1	124	44	12	1	0.80
2	174	33	10	2	1.14
3	162	30	13	2	1.23
4	153	22	15	1	0.65
Total	**613**	**129**	**50**	**6**	**0.978**

Table 4.2(b): Details of transformation experiments performed for construct 2 by using hyocotyls as explants

Experiment No.	No. of explants inoculated in Agrobacterium GV3101 suspension	No. of explants that formed shoots on selection media	No. of shoots rooted on selection media	No. of PCR positive plants	Transformation efficiency (%)
1	184	41	15	2	1.08
2	102	20	10	1	0.98
3	122	24	12	1	0.819
4	134	30	11	1	0.746
Total	**542**	**115**	**48**	**5**	**0.906**

Table 4.3(a): Details of transformation experiments performed for construct 1 by using embryos as explants

Experiment No.	No. of embryos inoculated in Agrobacterium suspension	No. of shoots regenerated on selection media	No. of shoots rooted on selection media
1	100	20	11
2	150	28	12
Total	250	48	23

Table 4.3(b): Details of transformation experiments performed for construct 1 by using embryos as explants

Experiment No.	No. of embryos inoculated in Agrobacterium suspension	No. of shoots regenerated on selection media	No. of shoots rooted on selection media
1	125	21	10
2	125	29	16
Total	250	50	26

Figure 4.45: Transgenic plants in large earthen pots

4.3 Bioassay and molecular characterization of okra RNAi plants

4.3.1 Bioassay of okra RNAi plants

RNAi plants that were PCR positive along with control were used for viral inoculation with the infectious clone or by feeding with viruliferous whiteflies.

4.3.1.1 Infectious clone mediated inocultion

Out of eleven plants that show positive PCR results, five were used for inoculation with whiteflies along with untransformed plants. Leaves of the plants were agroinfiltered with agroinfectious clone whose expression was checked earlier by transient assay (Figure 4.46). Plants were observed for 35 days for the appearance of symptoms. No symptoms were appeared in transgenic plants as compared to the untransformed plants and mock inoculated plant that show mosaic like symptoms in the infiltered leaves (Figure 4.47). Nahid *et al* 2011, inoculated RNAi plants of tobacco with infectious clone and found no viral DNA accumulation in transgenic plants as compared to non-transgenic plants that show severe symptoms. Similarly, Kumar *et al* 2017, inoculated RNAi plants of cowpea with agroinfectious clone and found no symptoms in transgenic plants as compared to non-transgenic one. Symptoms were observed for 10 weeks and there were no symptoms in RNAi plants produced by tissue culture dependent genetic transformation method.

Figure 4.46: Agroinfilteration of RNAi plants with infectious clone

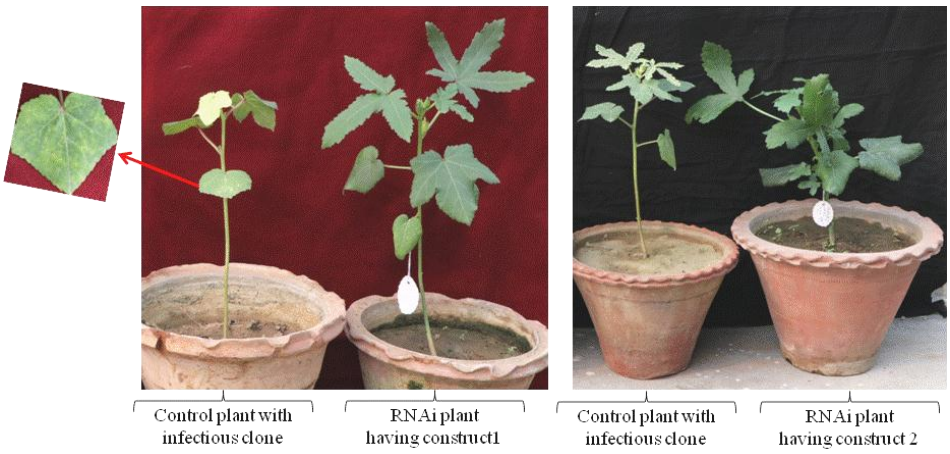

Figure 4.47: RNAi and untransformed plants after 35 days of agroinfilteration with agroinfectious clone

4.3.1.2 Whiteflies mediated inoculation

Whiteflies being a vector of begomoviruses were used to make viruliferous. Plants infected with begomoviruses of okra were used to feeding with whiteflies. Plants were placed in an insect proof cage (Figure 4.48). 9-10 whiteflies were inoculated on the infected plant for a acquisition period of 12 hours. After 12 hours of acquisition, these flies were inoculated on the RNAi plants along with untransformed plants. Symptoms were observed for 35 days and RNAi plants do not show any symptoms as compared to control (Figure 4.49). All the plants that were used for bioassay were utilized for expression analysis with control plant and the mock inoculated plant. Untransformed plants were used as control and the expression of coat-protein gene was checked with respect to that control. Both infectious clone and viruliferous whiteflies have begomoviruses of okra and produce characteristic symptoms of vein chlorosis, yellow patches and vein clearing. Khatoon *et al* 2016, inoculated RNAi plants of cotton with viruliferous whiteflies and found no symptoms in transgenic plants even after 90 days of inoculation.

Figure 4.48: RNAi plants along with untransformed plants placed in insect-proof net house

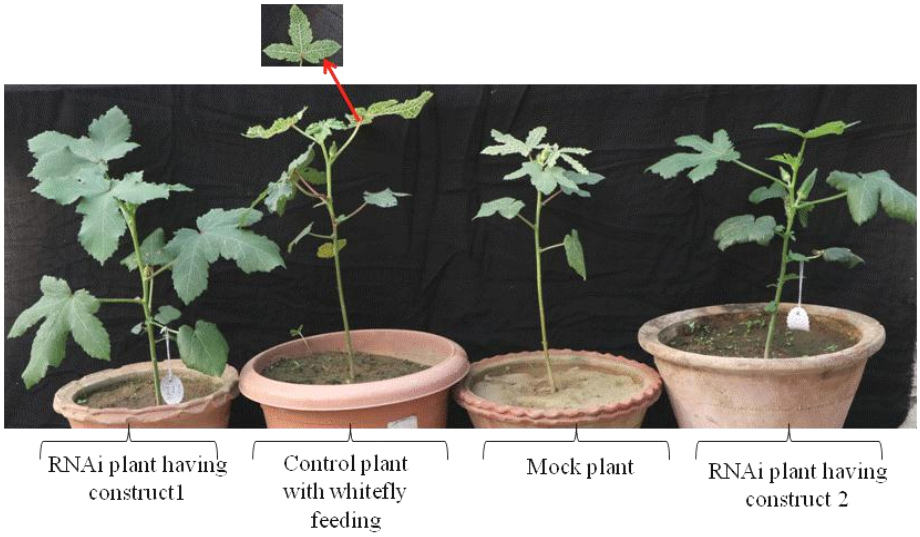

Figure 4.49: RNAi and untransformed plants after 35 days of inoculation with viruliferous whiteflies

4.3.2 Expression analysis

To check the expression of viral genes in RNAi plants, expression analysis was done in semi-quantitative and quantitative manner. RNA was isolated from the leaves of plants that were positive with both gene and vector-specific primers along with untransformed and mock inoculated plants. RNA was isolated according to protocol given with trizol plus reagent. Isolated RNA was quantified on 2.5% MOPS & formaldehyde gel (Figure 4.50).

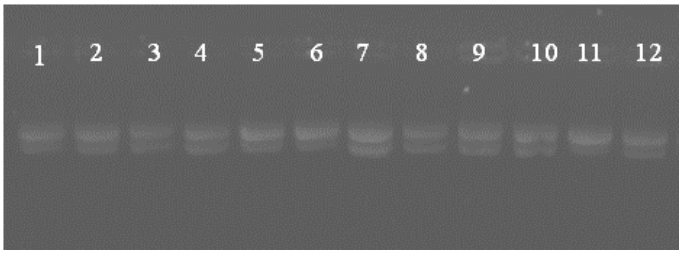

Figure 4.50: Quantification of isolated RNA for expression analysis
Lane 1 to 12 – RNA isolated from transformants with control

4.3.2.1 Semi-quantitative PCR analysis

For the expression analysis, two pairs of primers were used i.e. primers for the amplification of reference gene (β-tubulin- internal control) and primers for the amplification of target gene. As whiteflies can attack by varied species of begomoviruses of okra so we align the sequences of *AV2* gene of DNA-A component of 8 different begomoviral isolates associated with okra and degenerate primers were designed from them. Degenerate primers were designed to check the broad spectrum resistance in RNAi plants (Figure 4.51). *AV2* gene specific primers were utilized to check their expression in the plants challenged with infectious clone and viruliferous whiteflies. Kumar *et al* 2017, utilized *AV2* gene of begomoviruses for semi-quantitative analysis and quantitative analysis of RNAi plants and found very less viral titer in RNAi plants of cowpea.

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MYVMVLudhiana       ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      299
OelcvLudhiana       ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      298
OelcvSurat          ATCTAATTCGCGATCTAATTTCTATTTTACGTTGTAGGAATTATGTCGAAGCGTCCTGCC      298
BYVMVHimachal       AGTTAATACGGGATTTAATTTGTATTTTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVNewDelhi       AGTTAATACGTGATTTAATCTGTATTCTACGCTCCC-GTAATTATGTCGAAGCGAGCTGCC     298
BYVMVPhalaghat      AGTTAATACGGGATTTAATTCGTATTTTACGCTCCCGTAATTATGTCGAAGCGAGCTGCC      298
BYVMVKerala         ATTTAATACGGGATTTAATTTGTATTGTCCGTTCTCGTAATTATGTCGAAGCGAGCTGCA      298
BYVMVChelur         AGTTAATACGGGATTTAATTTGTATTGTCCGTTCTCGTAATTATGTCGAAGCGAGCTGCA      298
                    *  **** ** *** ****   **** * ** *   * ***************  ****

MYVMVLudhiana       GATATCGTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      359
OelcvLudhiana       GATATCTTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      358
OelcvSurat          GATATCGTCATTTCTACCCCCGCGTCGAAGGTGCGTCGTCGACTGAACTTCGACAGCCCG      358
BYVMVHimachal       GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVNewDelhi       GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVPhalaghat      GATATCGTCATTTCTACGCCCACGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
BYVMVKerala         GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCT      358
BYVMVChelur         GATATCGTCATTTCTACGCCCGCGTCGAAAGTACGCCGGCGTCTGAACTTCGGCAGCCCA      358
                    ****** ********** *** ******* ** ** ** ** ********** ******

MYVMVLudhiana       TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCGCAAAATCACGAATGTGGGCCAAC      419
OelcvLudhiana       TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCACAAAATCACGAATGTGGGCCAAC      418
OelcvSurat          TATTCAACCCGTGCAGTTGCCCCCACTGTCCGCGTCACAAAATCACGAATGTGGGCCAAC      418
BYVMVHimachal       TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVNewDelhi       TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVPhalaghat      TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVKerala         TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
BYVMVChelur         TACACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAACAACAGGCATGGACAAAC      418
                    **  * * ****** * ******** ********** *****  **     *** * ***

MYVMVLudhiana       AGACCCATGTACCGGAAGCCCAGAATGTACAGGATATACAGAAGCCCTGATGTTCCTAGA      479
OelcvLudhiana       AGACCCATGTACCGGAAGCCCAGAATGTACAGGATATACAGAAGCCCTGATGTTCCTAGA      478
OelcvSurat          AGACCCATGTACCGGAAGCCCAGAATGTACAGGATGTACAGAAGCCCTGATGTTCCTAGA      478
BYVMVHimachal       AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCACGG      478
BYVMVNewDelhi       AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVPhalaghat      AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVKerala         AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
BYVMVChelur         AGGCCTATGAACAGGAAACCCAGAATGTACCGGATGTACAGAAGTCCGGATGTTCCAAGG      478
                    ** ** *** ** **** ************ **** ******** ** ********  *

MYVMVLudhiana       GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      539
OelcvLudhiana       GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      538
OelcvSurat          GGATGTGAAGGCCCATGTAAGGTGCAGTCTTTTGATGCGAAGAACGATATTGGTCACATG      538
BYVMVHimachal       GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGTTCATATT      538
BYVMVNewDelhi       GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGATCATATT      538
BYVMVPhalaghat      GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTCGTTCATATT      538
BYVMVKerala         GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTTGTCCATATT      538
BYVMVChelur         GGATGTGAGGGTCCCTGTAAGGTACAGTCGTTTGAATCTCGACACGATGTTGTCCATATT      538
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Figure 4.51: Multiple sequence alignment of region of pre-coat protein gene (*AV2* gene) of begomoviruses of okra by clustal omega tool

cDNA was synthesized by reverse transcriptase (RT) kit from Applied Biosystems. This kit was having reverse transcriptase enzyme that form DNA by taking RNA as a template. These RT kit has oligo dt that prime with the poly-A tail of RNA. After annealing, RT enzyme start synthesizing DNA which is complementary to the RNA strand, hence named cDNA (complementary DNA). This DNA form loop at the end and start synthesizing second strand of DNA. cDNA formed was quantified with nanodrop^{TM} spectrophotometer and confirmed by semi-quantitative analysis with β-tubulin and *AV2* gene (Figure 4.52). Through semi-quantitative analysis β-tubulin gene was amplified in all the samples and region of *AV2* gene was amplified in six samples depending upon the symptom appearance on the leaves of the plants.

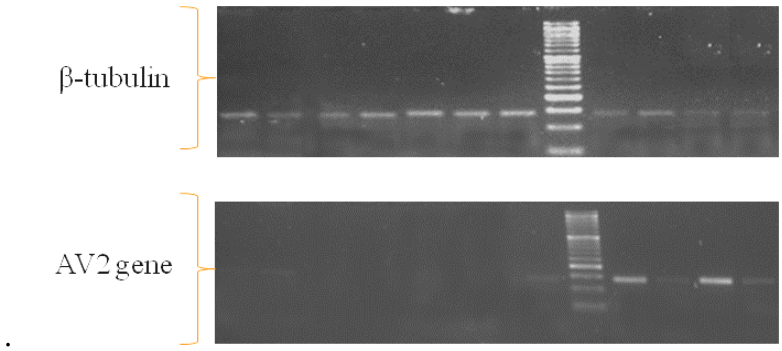

Figure 4.52: cDNA confirmation with β-tubulin and *AV2* gene specific primers

4.3.2.2 Quantitative real time PCR analysis

qPCR normalization was done by relative quantification method by calculating $2^{-\Delta\Delta C}{}_T$ value. In case of relative quantification, a gene or set of genes can utilize as reference. These genes are supposed to have stable expression across the varied experimental groups. The expression of required or target gene can be evaluated in relation to the reference gene that is stable. Then the C_T values of target from qPCR analysis were compared C_T values of reference gene. This comparison gives the total number of target molecules in the samples. In the present study, relative quantification method was performed by using β-tubulin as a reference gene and compares the expression of *AV2* gene (target gene) with it. Data was analyzed and $2^{-\Delta\Delta C}{}_T$ value was calculated by keeping value 1 for the control (Table 4.4 a & b) (Livak and Schmittgen 2001). As compared to control, the expression of *AV2* gene was lesser in the RNAi plants. A real time PCR analysis shows very less level of viral DNA accumulation in RNAi plants. Out of 11 plants, the expression of *AV2* gene in RNAi plants Tr2AC4 and Tr4AC4 was high when fed with viruliferous whiteflies and infectious clone respectively (Figure 4.53).

Table 4.4(a): Fold change in expression of *AV2* gene in control and RNAi plants inoculated with viruliferous whiteflies

Samples ID	$2^{-\Delta\Delta C_T}$
Feeding with whitefly	1
Mock Inoculation	0.995025
Tr1AC2	0.110698
Tr2AC2	0.153489
Tr3AC2	0.139816
Tr1AC4	0.224503
Tr2AC4	0.436423
Tr3AC4	0.202345

Table 4.4(b): Fold change in expression of *AV2* gene in control and RNAi plants inoculated with infectious clone

Sample ID	$2^{-\Delta\Delta C_T}$
Agroinfilteration with infectious clone	1
Mock inoculation	0.985186
Tr4AC2	0.102879
Tr5AC2	0.114562
Tr6AC2	0.196789
Tr4AC4	0.302234
Tr5AC4	0.223432

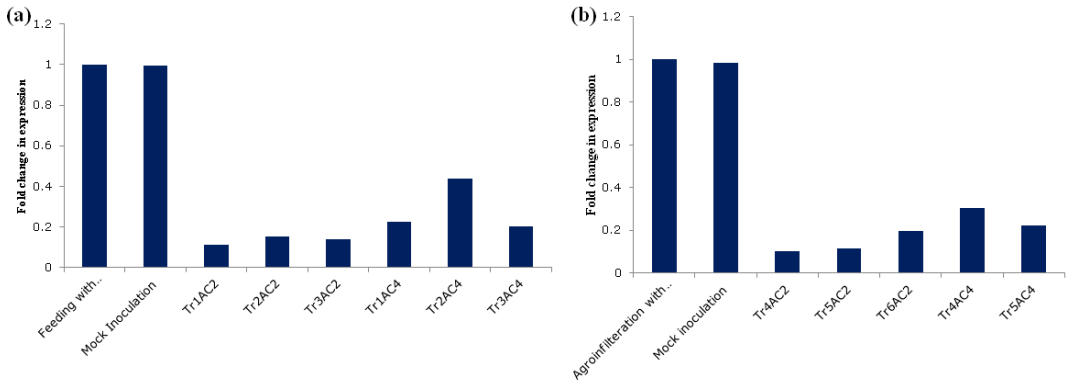

Figure 4.53: Fold change in expression of pre-coat protein gene (*AV2*) in control and RNAi plants

(A) Fold change in expression of *AV2* gene in plants fed with whiteflies
(B) Fold change in expression of *AV2* gene in agroinfiltered plants
Tr1AC2 to Tr6AC2- Okra transgenic plants having pFGC-AC2hp construct
Tr1AC4 to Tr5AC4- Okra transgenic plants having pFGC-AC4hp construct

CHAPTER V

SUMMARY

Diseases caused by begomoviruses are the serious obstacle for the production of most of the vegetable crops in India. In okra, it caused vein chlorosis, vein clearing, leaf thickening, malformed fruits and arrested growth of the plants. Begomovirus is a species of the virus that comes under the family Geminiviridae. This virus is exclusively carried by whitefly (*Bemisia tabaci*). Begomoviruses have single or monopartite genome (DNA-A) of about 2.7kb in size, but most of the time they have bipartite genomes (DNA-A & DNA-B) of about 2.7kb in size. DNA-A genome of begomovirus codes for the proteins that are involved in replication, transcription and encapsidation of the viral particles and DNA-B genome codes for proteins related to the movement of the viral particles in the plant. Satellite DNA molecules are associated with many monopartite begomoviruses that involved in enhancement of symptom developed by helper genomes. These satellite DNA molecules are alpha and beta satellites.

Various biotechnological advancements are available; out of them RNAi seems to be most powerful in the control of viral diseases of plants including the diseases caused by begomoviruses. RNAi response in plants is triggered by the double-stranded RNA (dsRNA) transferred exogeneously into the plants. These dsRNA are then cleaved into small interfering RNAs (siRNAs) by Dicer-like proteins (DCLs) and form a complex named RNA Induced Silencing Complex (RISC). The intron containing hairpin RNAi constructs directs the expression of dsRNA and effectively induced sequence specific degradation of target RNA by developing siRNAs in the plants.

Application of RNAi for viral gene silencing in okra exclusively depends on the accessibility of consistant transformation system. Okra is a recalcitrant crop to the genetic manipulations. Until now, only few researchers become able to successfully transform okra crop. So, the present study was conducted to produce virus-resistance okra with standardized protocol of its genetic manipulations. This objective was achieved in four experiments which were done from January 2015 to August 2019 in the Molecular Biology and tissue culture laboratory of School of Agricultural Biotechnology, PAU, Ludhiana.

To confer broad spectrum resistance against begomoviruses of okra, conserved sequences were fetched from the multiple sequence alignment of sequences of DNA-A component of eight isolates of begomoviruses of okra. The sequences that were conserved within the overlapping regions of *AC1*& *AC2* (385bp) and the overlapping regions of *AC1* & *AC4* (342bp) from alignment were chosen as the targets of RNAi. *AC1* gene encodes for replication mediated proteins, *AC2* encodes for transcription activator protein and *AC4* encodes for the protein responsible for the suppression of PTGS response of the plants. The

selected regions were confirmed for siRNA production by JackLin's siRNA prediction tool. siRNA prediction tool gave 25 potential siRNA sequences for each target with percentage of GC content present in the sequence. These regions were then utilized to design primers having restriction sites specific to the binary vector (pFGC1008) at their 5′end. Primers were synthesized and used for the amplification of selected region by using DNA-A component of *Okra enation leaf curl virus* (accession no. KP208672.1) as a template. Selected regions were amplified in both sense and antisense orientations. Amplified products were then purified seperately and cloned to the pGEM-T easy vector (cloning vector) by TA cloning and transferred to the *E.coli* DH5α competent cells. Colony PCR was performed to check the colonies obtained by TA cloning. Recombinant pGEM-T easy vectors were then isolated from the positive clones and confirmed with the help of PCR and restriction digestion analysis for the presence of selected regions at their multiple cloning sites. Target gene(s) fragments from recombinant pGEM-T vector were then cloned in RNAi vector. For this, recombinant pGEM-T easy vectors having targets in sense orientation along with the RNAi vector were overnight digested with *Xho*I & *Sac*I restriction endonucleases. Digested fragments of targeted regions and RNAi vector were eluted from the agarose gel and ligated with each other and transformed in competent cells of *E.coli* strain DH5α. Colonies obtained were checked by colony PCR with gene-specific primers. Recombinant RNAi vectors (pFGC-*AC2*(s) & pFGC-*AC4*(s) having two target gene(s) fragments in sense orientation were isolated from positive clones and reconfirmed by PCR with gene-specific primers and by double-digestion with *Xho*I & *Sac*I restriction endonucleases. Confirmed pFGC-*AC2*(s) & pFGC-*AC4*(s) were further digested for overnight along with recombinant pGEM-T having two targets in antisense orientation with *BamH*I & *Spe*I restriction endonucleases. Digested fragments were ligated with each other and transformed to competent cells of *E.coli* DH5α. Colonies obtained were confirmed by colony PCR with gene-specific primers. RNAi constructs (pFGC-*AC2*hp & pFGC-*AC4*hp) were isolated from positive clones and reconfirmed by PCR with both gene-specific and vector-specific primers and also by restriction digestion with two pairs of endonucleases i.e. *Xho*I & *Sac*I and *BamH*I & *Spe*I respectively. Resulting RNAi constructs further transformed to *Agrobacterium* strain GV3101.

For screening of RNAi plants, dimeric construct of DNA-A component of begomoviruses of okra was prepared. For the construction of agroinfectious clone, five okra leaf samples infected with begomoviruses were procured from the experimental field of Department of Vegetable Sciences. DNA was isolated from the infected samples with the help of modified CTAB method and checked for the presence of viral genome by begomoviral specific primers (*AV*/*AC*). All infected samples showed the presence of begomoviral DNA. Viral DNA was further enriched by rolling circle amplification (RCA) of DNA samples

isolated from infected plants. High molecular weight RCA products were digested with various restriction endonucleases to release full length genome of the begomovirus(es). Out of six endonucleases used, only *Sac*I endonuclease cleaves the full length genome of the virus. Digested products of 2.6-2.8kb size was eluted from agarose gel and ligated with pUC19 vector digested with the same restriction enzyme (*Sac*I). Restriction site of *Sac*I enzyme is present at the multiple cloning sites of pUC19. Ligated products were transformed to competent cells of E.coli strain DH5α. Colonies appeared were checked for the presence of full length genome by colony PCR with m13 primers. Recombinant pUC19 vectors were isolated from positive clones and reconfirmed by PCR with m13 prmers. Further, a pair of outward primers was designed with the multiple sequence alignment of sequences of DNA-A component of eight isolates of begomoviruses of okra. Restriction sites of *Asc*I and *Sac*I was added at the 5′end of forward primer and reverse primer. These outward primers were designed from coat-protein region of DNA-A genome of okra associated begomoviruses. Outward primers were utilized for the amplification of full length genome of DNA-A component of okra by taking recombinant pUC19 vector as a template. Amplified products were ligated with pGEM-T easy vector and transformed to *E.coli* DH5α competent cells. Recombinant pGEM-T easy vector were isolated from the positive clones checked by colony PCR. Full length genome was eluted from recombinant pGEM-T easy vector and cloned into binary vector (pFGC1008) at *Asc*I and *Sac*I site and leads to the formation of recombinant pFGC1008 (pFGC-M) having monomeric unit of DNA-A genome. *Sac*I digested RCA product was also subcloned from pUC19 to pFGC-M and the resulting binary vector (pFGC-2M) was having two monomeric units of full length genome of begomovirus of okra.

Efficacy of three constructs was checked by transient assay. Plants were arranged in four sets. Three sets for each of the constructs and one for the mock inoculation. At 4^{th}- 6^{th} leaf stage, leaves of two sets of plants were agroinfiltered with RNAi constructs and one with empty vector and fourth set was infiltered with dimeric constructs (agroinfectious clone). 3 days post-infilteration, plants agroinfiltered with RNAi constructs and empty vector were infiltered with agroinfectious clone. Plants of RNAi construct did not show any symptoms as compare to the control and mock-inoculated plant.

RNAi constructs were then genetically transformed to okra variety Punjab 8 through tissue culture. Two protocols were utilized for its transformation. These protocols were depending upon the type of explants used for transformation. Hypocotyls and zygotic embryos were used for tissue culture of okra. 0.5 O.D. of *Agrobacterium* was used for the inoculation of explants in suspension. Two days of co-cultivation period was given for both the explants. After co-cultivation, explants were placed on selection media and grown

plantlets were characterized both morphologically and genotypically. An eleven tissue-culture raised plants were shown positive results with PCR and overall transformation efficiency was 0.9% when hypocotyls were used as explants. Transgenic plants were similar in appearance to the wild plant. Due to genotype effects, no transformants were found when zygotic embryos were used as explants. Out of eleven RNAi plants, five plants were screened by inoculation with agroinfectious clone and six plants were used to inoculate with viruliferous whiteflies. Expression of begomoviral pre-coat protein gene (*AV2)* was checked by semi-quantitative PCR analysis. Fold-change in expression was calculated by quantitative real-time PCR with *AV2* gene specific primers. Data analysis shows that very low expression of AV2 gene was found in okra RNAi plants as compared to control plant and mock-inoculated plant. In future, this study will give an opportunity to researchers to genetically manipulate okra crop for different traits and also produce RNAi plants of different varieties of okra to make them virus-resistant.

REFERENCES

Alberter B, Rezaian M A and Jeske H (2005) Replicative intermediates of Tomato leaf curl virus and its satellite DNAs. *Virol J* **331**: 441-48.

Ali S, Khan M A, Rasheed A H S and Iftikhars Y (2005) Correlation of environmental conditions with okra yellow vein mosaic virus and *Bemisia tabaci* population density. *Int J Agrl Biol* **7**: 142-44.

Ali M I, Khan M A, Rashid A, Ehetisham-ul-haq M, Javed T and Sajid M (2012) Epidemiology of okra yellow vein mosaic virus (OYVMV) and its management through tracer, mycotal and imidacloprid. *Amer J Plant Sci* **3**: 1741-45.

Ali I, Amin I, Briddon R W and Mansoor S (2013) Artificial microRNA-mediated resistance against the monopartite begomovirus Cotton leaf curl Burewala virus. *Virol J* **10**: 231.

Ammara U, Mansoor S, Saeed M, Amin I, Briddon R W and Al-Sadi A M (2015) RNA interference-based resistance in transgenic tomato plants against tomato yellow leaf curl virus-Oman (TYLCV-OM) and its associated betasatellite. *Virol J* **12**: 38.

Anisuzzaman M, Kabir A H, Sarker K, Jarin S and Alam M F (2010) Micropropagation of *Abelmoschus esculentus* L. (Moench.) for disease free plantlets through meristem culture *Arch. Phytopathology Plant Protect* **43**(5): 460-46.

Antony G, Mishra AK and Praveen S (2005) A single chimeric transgene derived from two distinct viruses for multiple virus reslstance. *J Plant Biochem Biotech* 14: 101-105.

Asare-Bediako E, Vander Puije E C, Taah K J, Abole E A and Baidoo A (2014) Prevalence of okra mosaic and leaf curl diseases and *Podagrica* spp. Damage of okra (*Albelmoschus esculentus*) plants. *Int J Curr Res Aca Rev* **6**: 260-71.

Bang B, Lee J, Kim S, Park J, Nguyen T T and Seo Y S (2014) A Rapid and efficient method for construction of an infectious clone of *Tomato yellow leaf curl virus. Plant Pathol J* **30**: 310-15.

Baulcombe D C (2000) Unwinding RNA silencing. *Science* **290**: 1108.

Bhagat A P, Yadav B P and Prasad Y (2001) Rate of dissemination of okra yellow vein mosaic virus in three cultivars of okra. *Ind Phytopathol* **54**: 488-89.

Bonfim K, Faria J C, Nogueira E O P L, Mendes E A and Aragao A J L (2007) RNAi-mediated resistance to bean golden mosaic virus in genetically engineered common bean (*Phaseolus vulgaris*). *Mol Pl Microbe Interact* **20**: 717-26.

Bonilla-Ramirez G M, Guevara-Gonzalez R G, Garzon-Tiznado J A, Ascencio-Ibanez J T, Torres-Pacheco I, Rivera-Bustamante R F (1997) Analysis of the infectivity of monomeric clones of pepper huasteco virus. *J Gen Virol* **78**: 947-51.

Borah B K and Dasgupta I (2012) Begomovirus research in India: A critical appraisal and the way ahead. *J Biosci* **37**: 791-06.

Boulton M I and Davies J W (1988) Specificity of Agrobacterium-mediated delivery of maize streak virus DNA to members of the Gramineae. Annual report, AFRC Institute of Plant Science Research and John Innes Institute.

Briddon R W, Prescott A G, Lunness P, Chamberlin L C L and Markham P G (1993) Rapid production of full-length, infectious geminivirus clones by abutting primer PCR (AbP-PCR). *J Virol Methods* **43**: 7-20.

Briddon R W, Mansoor S, Bedford I D, Pinner M S, Saunders K, Stanley J, Zafar Y, Malik K and Markham P G (2001) Identification of DNA components required for induction of cotton leaf curl disease. *Virology* **285**: 234-43.

Brunt A, Crabtree K and Gibbs A (1990) Viruses of tropical plants. Wallingford: CAB International.

Chandran S A, Packialakshmi R M, Subhalakshmi K, Prakash C, Poovannan K and Prabhu A N (2013) First report of an alpha satellite associated with okra enation leaf curl virus. *Virus Genes* **46**: 585-587.

Chaudhary A, Khan M A and Riaz K (2016) Spatio-temporal pattern of okra yellow vein mosaic virus and its vector in relation to epidemiological factors. *J Plant Pathol Microbiol* **7**: 6.

Chellaiah S and Murugesan S (1976) Estimation of loss due to yellow vein mosaic disease in Bhendi. *Madras Agric J* **63**: 169-70.

Chellappan P, Masona M V, Vanitharani R, Taylor N J and Fauquet C M (2004) Broad spectrum resistance to ssDNA viruses associated with transgeneinduced gene silencing in cassava. *Plant Mol Biol* **56**: 601-11.

Dasgupta I, Malathi V G and Mukherjee S K (2003) Genetic engineering for virus resistance. *Curr Sci* **84**: 341-54.

Datta P C, and Naug A (1968) A few strains of *Abelmoschus esculentus* (L.) Moench their karyological study in relation to phylogeny and organ development. *Beitr. Biol Pflanzen* **45**: 113-26.

Dean F B, Nelson J R, Giesler T L and Lasken R S (2001) rapid amplification of plasmid and phage dna using phi29 dna polymerase and multiply-primed rolling circle amplification *Genome Res* **11**(6): 1095–99.

Debnath S and Nath P S (2003) Performance of okra varieties in relation to yield and tolerance to YVMV. *Ann Pl Protec Sci* **11**: 400-401.

Dhande G A, Patil V M, Raut R V, Rajput J C and Ingle A G (2012) Regeneration of okra (*Abelmoschus esculentus* L.) via apical shoot culture system. *Afr J Biotechnol.* **11**: 15226-30.

Donson J, Gunn H V,Woolston C J, Pinner M S, Boulton M I, Mullineaux P M and Davies J W (1988) Agrobacterium-mediated infectivity of cloned digitaria streak virus DNA. *Virology* **162**: 248-50.

Fajinmi A A and Fajinmi O B (2010) Incidence of okra mosaic virus at different growth stages of okra plants (*Abelmoschus esculentus* (L.) Moench) under tropical condition. *J Gen Mol Virol* **2**: 28-31.

Fang G, Hammer S and Grumet R (1992) A quick and inexpensive method for removing polysaccharides from plant genomic DNA. *BioTechniques* **13**: 52-56.

Ferreira P T O, Lemos T O, Nagata T and Inoue-Nagata A K (2008) One-step cloning approach for construction of agroinfectious begomovirus clones. *J Virol Methods* **147**: 351-54.

Fitzgerald T L and McQualter R B (2014) The quantitative real-time polymerase chain reaction for the analysis of plant gene expression. *Methods Mol Biol* **1099**: 97-115.

Ford C E (1938) A contribution to a cytogenetical survey of the malvaceae. *Genetica* **20:** 431-52.

Freitas-Astúa J, Purcifull D E, Polston J E and Hiebert E (2002) Traditional and transgenic strategies for controlling tomato-infecting begomoviruses. *Fitopatol Bras* **27**: 437.

Fuentes Alejandro, Ramos Pedro L, Fiallo Elvira, Callard Danay, Sanchez Yadira, Peral Rudy, Rodrıguez Raidel and Pujol Merardo (2006) Intron–hairpin RNA derived from replication associated protein C1 gene confers immunity to Tomato Yellow Leaf Curl Virus infection in transgenic tomato plants. *Transgenic Res* **15**: 291-304.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. *Exp Cell Res* **50**: 151-58.

Ganesan M, Chandrasekar R, Ranjitha B D and Jayabalan N (2007) Somatic embryogenesis and plant regeneration of *Abelmoschus esculentus* through suspension culture. *Biol plantarum* **51**: 414-20.

Ghosh R, Paul S, Ghosh S K and Roy A (2009) An improved method of DNA isolation suitable for PCR-based detection of begomoviruses from jute and other mucilaginous plants. *J Virol Methods* **159**: 34-39.

Glevin S B (2003) Agrobacterium-Mediated Plant Transformation: the Biology behind the "Gene Jockeying Tool". *Microbiol Mol Biol Rev* **67**: 16-37.

Gowdar S B, Babu H N R and Reddy N A (2007) Efficacy of insecticides on okra yellow vein mosaic virus and whitefly vector, *Bemisia tabaci (*Guenn.). *Ann Pl Protec Sci* **15**: 116-19.

Grimsley N and Bisaro D (1987) Agroinfection. In: Hohn T and Schell J (ed) *Plant DNA Infectious Agents*. pp. 87-107. Springer, Vienna.

Guo S and Kamphues K J (1995) par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically disturbed. *Cell* **81**: 611-20.

Hamilton R I (1980) In: Viruses. Plant Disease: An Advanced Treatise. Academic Press, NY. **5**: 279.

Hanley-Bowdoin L, Settlage S B, Orozco B M, Nagar S and Robertson D (2000) Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. *Crit Rev Biochem Mol Biol* **35**: 105-40.

Hannon G J (2002) RNA interference. *Nature* **418**: 244-251.

Haq Q M I, Ali Arif and Malathi V G (2010) Engineering resistance against mungbean yellow mosaic india virus using antisense RNA. *Indian J Virol* **21**: 82-85.

Henry R J and Furtado A (Eds.) (2014) Cereal Genomics: Methods and Protocols, Methods in Molecular Biology, vol.1099, DOI 10.1007/978-1-62703-715-0_9© Springer Science+Business Media New York.

Hilje L, Costa H S and Stansly P A (2001) Cultural practices for managing *Bemisia tabaci* and associated viral diseases. *Crop Prot* **20**: 801-12.

http: //www.ncbi.nlm.nih.gov/tools/vecscreen/

Hussain S, Sajid M, Amin N, Alam S and Iqbal Z (2006) Response of okra (*Abelmoschus esculentus*) cultivars to different sowing times. *J Agric Biol Sci* **1**: 1-8.

Irfan Ali, Imran Amin, Rob W Briddon and Shahid Mansoor (2013) Artificial microRNA-mediated resistance against the monopartite begomovirus Cotton leaf curl Burewala virus. *Virol J* **10**: 231.

Jan F J, Fagoaga C,. Pang S Z and Gonsalves D (2002) A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. *J Gen Virol* **81**: 2103-09.

Jia R, Zhao H, Huang J, Kong H, Zhang Y, Guo J, Huang Q, Guo Y, Wei1 Q, Zuo J, Zhu Y J, Peng M and Guo A (2017) Use of RNAi technology to develop a PRSV-resistant transgenic papaya. *Sci Rep* **7**: 12636.

Jin M, Li C, Shi Y, Ryabov E, Huang J, Wu Z, Fan Z and Hong Y (2012) A single amino acid change in a geminiviral Rep protein differentiates between triggering a plant defence response and initiating viral DNA replication. *J Gen Virol* **89**: 2636-41.

Jones D R (2002) Plant viruses transmitted by whiteflies. *Eur J Plant Pathol* **109**: 195-219.

Kabir A H, Sarker K K, Sharmin S A, Islam M S and Alam M F (2008) Callus induction and plantlet regeneration in *Abelmoschus esculentus* (L.) Moench. *J Agric Technol* **4**: 193-204.

Karri S R and Acharyya P (2012) Incidence of yellow vein mosaic virus disease of okra [*Abelmoschus esculentus* (L.) Moench] under summer and rainy environments. *Int J Curr Res* **4**: 8-21.

Kaur G, Sarao N K, Sharma A and Khan I (2017) Molecular identification and characterization of alphasatellites associated with Okra enation leaf curl virus. *Curr J Appl Sci Technol* **23**: 1-9.

Khaskheli M I, Jiskani M M, Goswami S P, Poussio G B and Khaskheli M A (2017) Effect of okra yellow vein mosaic virus (OYVMV) on plant growth and yield. *J Basic Appl Sci* **13**: 1-7.

Khatoon S, Kumar A, Sarin N B and Khan J A (2016) RNAi-mediated resistance against Cotton leaf curl disease in elite Indian cotton (Gossypium hirsutum) cultivar Narasimha. *Virus Genes* **52**: 530–-37.

Kulkarni G S (1924) *Mosaic and other related diseases of crops in the Bombay Presidency*. Poona Agricultural College Magazine **6**: 12.

Kumar S, Tanti B, Patil BL, Mukherjee SK, Sahoo L (2017) RNAi-derived transgenic resistance to Mungbean yellow mosaic India virus in cowpea. *PLoS ONE* **12**: e0186786.

Leke W N (2010) *Molecular epidemiology of begomoviruses that infect vegetable crops in southwestern Cameroon.* Doctoral Thesis of Swedish University of Agricultural Sciences Uppsala. ISSN 1652-6880.

Leke W N, Sattarb M N, Nganed E B, Ngevec J M, Kvarnheden A and Browne J K (2013) Molecular characterization of begomoviruses and DNA satellites associated with okra leaf curl disease in Cameroon. *Virus Res* **174**: 116-25.

Leke W N, Mignouna D B, Brown J K and Kvarnheden A (2015) Begomovirus disease complex: emerging threat to vegetable production systems of West and Central Africa. *Agric Food Security* **4**: 1.

Lindbo J A, Rosales L S, Proebsting W M and Dougherty W G (1993) Induction of a highly specific antiviral state in transgenic plants: implications for the regulation of gene expression and viral resistance. *Plant Cell* **5**: 1749-59.

Livak K J and Schmittgen T D (2001) Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods* **25**: 402-08.

Mangat B S and Roy M K (1986) Tissue culture and plant regeneration of okra (*Abelmoschus esculentus*). *Plant Sci* **47**: 57-61.

Manickavasagam M, Subramanyam K, Ishwarya Iujb, Elayaraja D, and Ganapathi A (2015) Assessment of factors influencing the tissue cultureindependent Agrobacterium-mediated in planta genetic transformation of okra [*Abelmoschus esculentus* (L.) Moench]. *Plant Cell Tissue Organ Cult* **123**: 309-20.

Mansoor S, Briddon R W, Zafar Y and Stanley J (2003) Gemini virus disease complexes: an emerging threat. *Tr Plant Sci* **8**: 128-34.

Mansoor S, Zafar Y and Briddon R W (2006) Gemini virus disease complexes: the threat is spreading. *Trends Plant Sci* **11**: 209-12.

Martin J H, Mifsud D and Rapisarda C (2000) The whiteflies (Hemiptera: Aleyrodidae) of Europe and the Mediterranean basin. *Bull Entomol Res* **90**: 407-48.

Mazier M, Flamain F, Nicolaı¨ M, Sarnette V and Caranta C (2011) Knock-Down of Both eIF4E1 and eIF4E2 Genes Confers Broad-Spectrum Resistance against Potyviruses in Tomato. *PLoS ONE* **6**(12): e29595.

Medina- Hernandez D, Rivera-Bustamante R F, Tenllado F and Holguin-Pena R J (2013) Effects and effectiveness of two RNAi constructs for resistance to pepper golden mosaic virus in *Nicotiana benthamiana* Plants. *Viruses* **5**: 2931-45.

Mishra G P, Singh B, Seth T, Singh A K, Halder J, Krishnan N, Tiwari S K and Singh P M (2017) Biotechnological Advancements and Begomovirus Management in Okra (Abelmoschus esculentus L.): Status and Perspectives. *Front Plant Sci* doi: 10.3389/fpls.2017.00360.

Mohanpuria P, Kumar V, Ahuja P S and Yadav S K (2010) Agrobacterium-Mediated Silencing of Caffeine Synthesis through Root Transformation in *Camellia sinensis* L. *Mol Biotechnol* doi10.1007/s12033-010-9364-4.

Mubin M, Hussain M, Briddon R W and Mansoor S (2011) Selection of target sequences as well as sequence identity determine the outcome of RNAi approach for resistance against cotton leaf curl geminivirus complex. *Virol J* **8**: 122.

Muhammad I A, Muhammad A K, Abdul R, Muhammad E, Muhammad T J and Muhammad S (2012) Epidemiology of okra yellow vein mosaic virus (OYVMV) and its management through tracer, mycotal and imidacloprid. *Am J Pl Sci* **3**: 1741-45.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**: 473-97.

Nahid N, Amin I, Briddon R W and Mansoor S (2011) RNA interference-based resistance against a legume mastrevirus. *Virol J* **8**: 499.

Narendran M, Deole S G, Harkude S, Shirale D, Nanote A, Bihani P, Parimi S, Char B R and Zehr U B (2013) Efficient genetic transformation of okra (*Abelmoschus esculentus* L. Moench) and generation of insect-resistant transgenic plants expressing the cry1Ac gene. *Plant Cell Rep* **32**: 1191-98.

Ntui V O, Kong K, Azadi P, Khan R S, Chin D P, Igawa T, Mii M and Nakamura I (2014) RNAi-mediated resistance to cucumber mosaic virus (CMV) in genetically engineered tomato. Am *J Pl Sci* **5**: 554-72.

Pandey P, Choudhury N R and Mukherjee S K (2009) A geminiviral amplicon (VA) derived from *Tomato leaf curl virus* (ToLCV) can replicate in a wide variety of plant species and also acts as a VIGS vector. *Virol J* **6**: 152.

Pooggin M, Shivaprasad P V, Veluthambi K and Hohn T (2003) RNAi targeting of DNA virus in plants. *Nat Biotechnol* **21**: 131-32.

Prakasha T L, Patil M S and Benagi V I (2010) Survey for bhendi yellow vein mosaic disease in parts of Karnataka. *Karnataka J Agric Sci* **23**: 658-59.

Praveen S, Anil M and Ginny A (2006) Viral suppression in transgenic plants expressing chimeric transgene from *Tomato Leaf Curl Virus* and *Cucumber Mosaic Virus*. *Plant Cell Tissue Organ Cult* **84**: 49-55.

Praveen, S., Anil, M and Ginny A (2006) Viral suppression in transgenic plants expressing chimeric transgene from *Tomato Leaf Curl Virus* and *Cucumber Mosaic Virus*. *Plant cell tissue and organ culture* **84**: 49-55.

Pun K B, Doraiswamy S and Arajan R J (1999) Screening of plant species for the presence of antiviral principles against okra yellow vein mosaic virus. *Indian Phytopath* **52**: 221-23.

Qhureshi Z (2007) *Breeding investigation in bhendi (Abelmoschus esculentus (L.) Moench)*. M.Sc. Thesis, University of Agriculture Sciences, GKVK, Bangalore, India.

Roy M K and Mangat B S (1989) Regeneration of plants from callus tissue of okra (*Abelmoschus esculentus*). *Plant Sci* **60**: 77-81.

Sambrook J, Fritsch E F and Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., *Cold Spring Harbor Laboratory Press*, New York, USA.

Sanwal S K, Venkataravanappa V and Singh B (2016) Resistance to bhendi yellow vein mosaic disease: a review. *Indian J Agric Sci* **86**: 835-43.

Sharma V K, Basu S and Chakraborty S (2015) RNAi mediated broad-spectrum transgenic resistance in *Nicotiana benthamiana* to chilli-infecting begomoviruses. *Pl Cell Rep* **34**: 1389-99.

Sheikh A H, Raghuram B, Lippold L E, Scheel D, Lee J and Sinha A K (2014) Agroinfilteration by Cytokinin-Producing *Agrobacterium* sp. Strain GV3101 Primes Defense Responses in *Nicotiana tabacum*. *Mol Plant Microbe Interact* **27**: 1175-85.

Shelly P, Ramesh S V, Mishra A K, Koundal V and Palukaitis P (2010) Silencing potential of viral derived RNAi constructs in tomato leaf curl virus-AC4 gene suppression in tomato. *Trans Res* **19**: 45-55.

Siemonsma J S (1982) West African okra, morphological and cytological indications for the existence of a natural amphiploid of *Abelmoschus esculentus* (L) Moench and *A. Manihot* (L) Medikus. *Euphytica* **31**: 241-52.

Singh A, Taneja J, Dasgupta I and Mukherjee S K (2015) Development of plants resistant to tomato geminiviruses using artificial trans-acting small interfering RNA. *Mol Plant Pathol* **16**: 724-34.

Singh J S (1990) Etiology and epidemiology of whitefly transmitted virus disease of okra. *Pl Dis Res* **5:** 64-70.

Singh J S (1996) Assessment of losses in okra due to enation leaf curl virus. *Indian J Virol* **12**: 51-53.

Smith N A, S P S, Wang M-B, Stoutjesdijk P A, Green A G and Waterhouse P M (2000) Total silencing by intron-spliced hairpin RNAs. *Nature* **407**: 319-20.

Snehi S K, Raj S K, Prasad V and Singh V (2015) Recent research findings related to management strategies of begomoviruses. *J Plant Pathol Micro* **6**: 6.

Sohrab S S, Kamal M A, Ilah A, Husen A, Bhattacharya P S and Rana D (2016) Development of Cotton leaf curl virus resistant transgenic cotton using antisense ßC1 gene. *Saudi J Biol Sci* **23**: 358-62.

Stanley J, Bisaro D M, Briddon R W, Brown J K, Fauquet C M, Harrison B D, Rybicki E P and Stenger D C (2005) Geminiviridae. In: Fauquet C M, Mayo M A, Maniloff J, Desselberger U and Ball L A (Eds.) *Virus Taxonomy, VIIIth Report of the ICTV* London: Elsevier/Academic Press; **2005**: 301-326.

Stenger D C, Revington G N, Stevenson M C, Bisaro D M (1991) Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. *Proc Natl Acad Sci* U.S.A. **88**: 8029-33.

Swanson M M and Harrison B D (1993) Serological relationships and epitope profiles of isolates of okra leaf curl geminivirus from Africa and the Middle East. *Biochimie* **75**: 707-11.

Taware S D, Thengane R J, Harke S N and Taware A S (2010) Genome sequence analysis of DNA-A from *Okra yellow vein mosaic virus* isolates. *Int J Curr Res* **8**: 097-107.

Turnage M A, Muangsan N, Peele C G and Robertson D (2002) Geminivirus-based vectors for gene silencing in *Arabidopsis*. *Plant J* **30**: 107-14.

Tyagi H, Rajasubramaniam S, Rajam M V and Dasgupta I (2008) RNA-interference in rice against Rice tungro bacilliform virus results in its decreased accumulation in inoculated rice plants. *Transgenic Res* **17**: 897-904.

Vanderschuren H, Stupak M, Futterer J, Gruissem W and Zhang P (2007) Engineering resistance towards geminiviruses – review and perspectives. *Pl Biotech J* **5:** 207-20.

Varma P M (1952) Studies on the relationship of bhindi yellow vein mosaic virus and its vector, the white fly (*Bemisia tabaci*). *Indian J Agric Sci* **22**: 75-91.

Venkataravanappa V, Reddy C N L, Swaranalatha P, Lalali S, Briddon R W and Reddy M K (2011) Diversity and phylogeography of begomovirus associated beta satellites of okra in India. *Virol J* **8**: 555.

Venkataravanappa V, Reddy C N L, Jalali S and Reddy M K (2013) Molecular characterization of a new species of begomovirus associated with yellow vein mosaic of bhendi (Okra) in Bhubhaneswar, India. *Eur J Plant Pathol* **136**: 811-22.

Venkataravanappa V, Reddy C N L, Jalali S, Briddon R W and Reddy M K (2015) Molecular identification and biological characterization of a begomovirus associated withokra enation leaf curl disease in India. *Eur J Plant Pathol* **141** (2): 217-35.

Vionnet O (2005) Induction and suppression of RNA silencing: insights from viral infections. *Nature* **6**: 206-20.

Waterhouse P, Graham MW and Wang M-B (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Nat Acad Sci USA* **95:** 13959-64.

Watterson J C (1993) Development and breeding of resistance to pepper and tomato viruses. In: Kyle M M (Ed.) *Resistance to Viral Disease of Vegetables*, Timber Press, Portland, pp. 80-110.

Weishing K, Nybom H, Wolff K and Meyer W (1995) DNA isolation and purification. In: *DNA Fingerprinting in Plants and Fungi*, pp. 44-59, CRC Press, Boca Raton, Florida.

Wu C, Lai , Lin N S, Hsu Y H, Tsai H T, Liao J Y and Hu C C (2008) A simplified method of constructing infectious clones of begomovirus employing limited restriction enzyme digestion of products of rolling circle amplification. *J Virol Methods* **147**: 355-59.

Wyatt S D and Brown J K (1996) Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathol* **86**: 1288-93.

Xu R and Li Q Q (2008) Protocol: Streamline cloning of genes into binary vectors in *Agrobacterium* via the Gateway® TOPO vector system. *Plant Methods* **4**: 4-10.

Yadava P, Suyal G and Mukherjee S K (2010) Begomovirus DNA replication and pathogenicity. *Curr Sci.* **98**: 3.

Yang Y, Sherwood T A, Patte C P, Hiebert E and Polston J E (2004) Use of *Tomato yellow leaf curl virus* (TYLCV) *Rep* gene sequences to engineer TYLCV resistance in tomato. *Phytopathol* **94**: 490-96.

Yasmeen A, Kiani S, Butt A, Rao A Q, Akram F, Ahmad A, Nasir I A, Tayyab Husnain, Shahid Mansoor, Imran Amin, Shaheen Aftab, Muhammad Zubair, Muhammad Nouman Tahir, Sohail Akhtar, Jodi Scheffler, Brian Scheffler (2016) Amplicon-Based RNA Interference Targeting V2 Gene of Cotton Leaf Curl Kokhran Virus-Burewala Strain Can Provide Resistance in Transgenic Cotton Plants. *Mol Biotechnol* **58**: 807-20.

Yousaf S, Rasool G, Amin I, Mansoor S and Saeed M (2013) Interference of a synthetic Rep protein to develop resistance against cotton leaf curl disease. *Int J Agric Biol* **15**: 6.

Zhang X, Sato S, Ye X, Dorrance A E, Morris T J, Clemente T E and Qu F (2011) Robust RNAi-based resistance to mixed infection of three viruses in soybean plants expressing separate short hairpins from a single. *Transgene* **101**: 11.

Zrachya A, Glick E, Levy Y, Arazi T, Citovsky V and Gafni Y (2007) Suppressor of RNA silencing encoded by *Tomato yellow leaf curl virus*-Israel. *Virology* **358**: 159-65.

VITA

Name of the student	**:**	Nity Sharma
Father's name	**:**	Mr. Jagmohan Sharma
Mother's name	**:**	Mrs. Suman Lata
Nationality	**:**	Indian
Date of birth	**:**	2nd October, 1989
Permanent home address	**:**	H. No. 80, New GK Estate, Chandigarh Road, Mundian Kalan, Ludhiana, Punjab

EDUCATIONAL QUALIFICATIOIN

Bachelor degree	**:**	B.Sc. Biotechnology
University and year of award	**:**	Guru Nanak Dev University, Amritsar 2010
%age of marks	**:**	76.0%
Master's degree	**:**	M.Sc. Biotechnology
University and year of award	**:**	HP University, Shimla 2012
%age of marks	**:**	71.2%
Title of Master's Thesis	**:**	Protease production from agrowaste by solid state fermentation
Ph.D.	**:**	Ph.D. (Agricultural Biotechnology)
University and year	**:**	Punjab Agricultural University, Ludhiana, Punjab, 2020
OCPA	**:**	8.05/10.00
Title of the Dissertation	**:**	RNAi mediated broad spectrum resistance against begomoviruses of okra (*Abelmoschus esculentus* L.)
Awards/Distinctions/Fellowships	**:**	▪ ICAR – Senior Research Fellowship