EVALUATION OF EXPRESSION CLONES FOR RECOMBINANT COAT PROTEIN OF CUCUMBER MOSAIC VIRUS INFECTING BANANA

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(2019-11-034)

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THESIS

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2022

DECLARATION

I, Sowmya R (2019-11-034) hereby declare that the thesis entitled **"Evaluation of expression clones for recombinant coat protein of Cucumber mosaic virus infecting banana"** is a bonafide record of research work done by me during the course of research and that the thesis has not been previously formed for the award of any degree, diploma, fellowship or other similar title, of any other University or society.

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CERTIFICATE

Certified that this thesis entitled **"Evaluation of expression clones for recombinant coat protein of Cucumber mosaic virus infecting banana"** is a record of research work done independently by **Ms. Sowmya R.** (2019-11-034) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the advisory committee of **Ms. Sowmya R. (2019-11-034)**, a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Pathology**, agree that this thesis entitled **"Evaluation of expression clones for recombinant coat protein of Cucumber mosaic virus infecting banana"** may be submitted by Ms. Sowmya R. in partial fulfillment of the requirement for the degree.

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Sowmya R.

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LIST OF ABBREVIATIONS

%	:	per cent
Aº	:	angstrom (10^{-10})
bp	:	base pair(s)
cDNA	:	Complementary deoxyribonucleic acid
CMV	:	Cucumber mosaic virus
CP	:	Coat protein
Da	:	Dalton(s)
DAC	:	Direct antigen coating
DMSO	:	Dimethly sulfoxide
dNTPs	:	deoxy Nucleotide triphosphates
E. coli	:	*Escherichia coli*
ELISA	:	Enzyme linked immuno- sorbent assay
G	:	gram
h	:	hour(s)
His-	:	Histidine-tagged
IC-RT-PCR	:	Immune capture reverse transcriptase polymerase chain reaction
IgG	:	Immunoglobulin G
IPTG	:	Isopropyl β-D-1- thiogalactopyranoside
K	:	kilo (10^{3})
L	:	litre
LB	:	Luria Broth
m	:	milli (10^{-3})
M	:	Molar
Min	:	minute(s)
MP	:	Movement protein
Ni^{2+}-NTA	:	Nickel-nitrilotriacetic acid
nm	:	nano metre
nt	:	nucleotide(s)
°C	:	Degree Celsius
OD	:	Optical density

ORF	:	Open reading frame
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase chain reaction
RdRp	:	RNA-dependent RNA polymerase
RNA	:	Ribonucleic acid
rpm	:	revolutions per minute
RT-PCR	:	Reverse transcriptase polymerase chain reaction
S	:	second(s)
SDS	:	Sodium dodecyl sulphate
spp.	:	Species (plural)
TAS	:	Triple antibody sandwitch
UV	:	Ultraviolet
v/v	:	volume/volume
V	:	Volts
w/v	:	weight/volume
α	:	Alpha
β	:	Beta
µ	:	micro (10^{-6})

Introduction

1. INTRODUCTION

Banana (*Musa paradisiaca*), one of the world's most important fruit crops, plays a critical role in the income security of many tropical and subtropical countries. It is a major fruit crop in India. Banana is threatened by various biotic problems like pests and diseases. In recent years, due to an increase in the area of banana cultivation and due to climate change, there is an increased occurrence of pest and diseases causing significant reduction in yield. Some diseases particularly virus diseases are considered as major threat in banana cultivation due to abundance of insect vectors, easily available collateral host and vegetative method of propagation. There are many different viruses reported to infect banana worldwide. The economically important viruses infecting banana are Banana bunchy top virus, Banana bract mosaic virus, Banana streak mosaic virus, Banana mild mosaic virus and Cucumber mosaic virus (Gambley and Thomas, 2001; KAU, 2016).

Banana viruses are primarily transmitted through vegetative planting materials such as suckers and have direct effect on production by decreasing plant growth and yield (Vishnoi *et al.*, 2013). Since viral diseases cannot be controlled with chemicals, early detection is the only useful method for its control. Using virus free planting material is the most important measure to avoid viral diseases in banana. Growing tissue culture bananas is very popular among banana growers in Kerala. Therefore, virus indexing at early stage is an inevitable practice to ensure the quality of tissue culture plants.

Cucumber mosaic virus disease is an emerging disease in Kerala, with leaf distortion, stunting of plant and yield loss up to 40-100 per cent (Estelitta *et al*., 1996). Cucumber mosaic virus is the type member of the genus *Cucumovirus* in the family *Bromoviridae* having small icosahedral symmetry with a diameter of 29 nm and single-stranded positive-sense RNA as genome. The genome consists of three single-stranded positive-sense RNAs, named RNA 1 (3000 nucleotides), RNA 2 (3700 nucleotides) and RNA 3 (2200 nucleotides). The RNA3 contains a fourth strand of RNA, called RNA 4 (1,000 nucleotides) known as sub genomic RNA (Zitter

and Murphy, 2009; Jacquemond, 2012). The CMV infects over 1000 species of hosts and has broadest host range which is readily transmitted by several species of aphids including *Aphis gossypii, Rhopalosiphum prunifolium* and *Myzus persicae* in a non-persistent manner.

CMV causes variable symptoms from mild chlorosis to severe chlorotic streaks on leaf lamina depending on the pathogen strain varietal susceptibility and the weather conditions. In case of severe infection, there is severe damage and poses a great threat to banana cultivation (Niblett *et al*., 1994). Early diagnosis is necessary to ensure the use of virus-free planting material so as to avoid viral diseases of banana. Many methods have been developed to detect CMV such as biological, serological, molecular techniques like, RT- PCR and DAC- ELISA.

Antiserum production is a necessary prerequisite for serological detection of the virus. In earlier periods, this was done using purified virus, but it was often a cumbersome procedure in terms of purity and concentration of the final product. Contamination of antigen by plant proteins or other viral proteins in case of mixed infections and presence of inhibitory compounds are also a drawback of this method. Coat protein region of CMV in banana is sufficient to provide a reliable antigen for virus detection. Hence, recombinant coat protein based antiserum is an effective alternative to purified virus in antiserum production.

Hence, the present study was undertaken to produce recombinant coat protein of Cucumber mosaic virus in *Escherichia coli* with the project entitled "Evaluation of expression clones for recombinant coat protein of Cucumber mosaic virus infecting banana". This study was carried out according to the objectives enlisted below:

- Confirmation of recombinant pET28a for CMV coat protein gene
- Over expression of recombinant coat protein gene in pET28a expression vector
- Development of molecular clones of CMV coat protein gene

- Over expression of recombinant coat protein gene in pET32a expression vector

Review of Literature

2. REVIEW OF LITERATURE

Banana is a major fruit crop cultivated all over the world and it is threatened by various biotic stresses such as pests and diseases. In recent years, due to the increase in banana cultivation area and climate change, the frequency of pests and diseases had increased significantly, causing significant reduction in yield. Several diseases, especially viral diseases, are considered as a major threat to banana cultivation in and around the world (Magnaye and Valmayor, 1995). The viral diseases of banana are Banana bunchy top, Banana bract mosaic, Infectious chlorosis, Banana streak mosaic and Banana mild mosaic (Diekmann and Putter, 1996) diseases. Infectious chlorosis is an important viral disease of banana which leads to reduction in yield by 40 to 100 per cent (Estelitta *et al*., 1996). The disease caused by Cucumber mosaicvirus belonging to the genus *Cucumovirus* and family *Bromoviridae* (Smith *et al*., 2000). The virus is commonly transmitted by more than 60 species of aphids (especially green peach aphid, *Myzus persicae*) in a non-persistent manner (Zitter and Murphy, 2009).

2.1 HISTORY AND DISTRIBUTION

Cucumber mosaic disease is distributed worldwide and it has been called by various names: Leaf yellowing, heart rot, banana mosaic, infectious chlorosis and virus sheath rot. It was first reported as a cucurbits disease by Doolittle (Michigan) and Jagger (New York) in 1916. Cucumber mosaic was first described by M.J.P. Magee in Australia in 1930 (Magee, 1940) and later reported from many countries in most areas of banana cultivation.

In Australia, banana mosaic disease considered as an endemic disease and not actively controlled (Jones, 1991). In Taiwan, banana mosaic disease was first observed in 1959, but it causes severe damage in small outbreaks when intermediate hosts of CMV are grown on adjacent plantations. In the Philippines, banana mosaic disease is widespread and severe outbreaks have been reported in Cavendish and Saba bananas, particularly in Southern Cotabato. In Indonesia and Thailand, banana mosaic disease is recognized as a minor disease and does not cause major losses in banana cultivation (Magnaye and Valmayor, 1995). Cucumber mosaic virus has also been

reported from banana in North America, New Zealand, Europe, Africa, Southeast Asia, Iran and India (Roossinck *et al.*, 1999; Lockhart and Jones, 2000; Thomas *et al*., 2000; Nematollahi *et al*., 2012).

In India, Cucumber mosaic virus in banana was first reported in 1943 in Maharashtra (Kamat and Patel, 1951). Later it was described by Mali and Rajegore in 1980 based on serology. Then, association of CMV with the banana mosaic disease was reported by Srivastava *et al.* (1995) based on immuno/nucleic acid probe assays. Cucumber mosaic virus in bananas had also been reported from Gujarat, Tamil Nadu, Karnataka, Andhra Pradesh, Uttar Pradesh and Kerala (Joshi and Joshi, 1976; Mohan and Lakshmanan, 1988; Estelitta *et al.,* 1996; Ramesh, 2009; Khan *et al.,*2011; Ali *et al.,* 2012).

In Kerala, infectious chlorosis caused by Cucumber mosaic virus in banana was observed in all districts (Thiruvananthapuram, Kollam, Kottayam, Ernakulam, Thrissur, Palakkad and Malappuram) with bunch weight reduction of 54, 45 and 62 per cent in the commercial varieties Nendran, Palayankodan and Karpooravalli, respectively (Estelitta *et al.,*1996). The disease is noticed in banana varieties such as Nendran, Amritsagar, Grand Naine, Peykunnan, Kosthabontha, Mottapoovan, Madhuraga, Karpooravalli, Bhimkol, Palayankodan, Dhakhinsagar, Rasthali and *Musa ornata* in Kerala (KAU, 2016; Mujtaba, 2017).

2.2 DISEASE CYCLE AND EPIDEMIOLOGY

Cucumber mosaic virus is geographically wide spread with broad host range, including many agricultural and horticultural crops, infecting more than 1200 plant species in tropical and subtropical regions of the world (Edwardson and Christie, 1991). The disease is considered as an endemic disease of vegetables, particularly tomato, pepper and cucurbits in Spain (Jorda *et al*., 1992; Luis-Arteaga *et al*., 1998). The virus survives during winter in reservoir hosts, including perennial weeds like wild cucumber, bur cucumber, Jimsonweed, pokeweed, wild cherry, pigweed, milkweeds, white cockle, and horsenettle. The virus also survives in the seeds of at least 19 plant species including wild cucumber, chickweed, gourds, muskmelon and

garden squash (Zitterand Murphy, 2009).

Primary infection occurs by mechanical transmission, especially in green houses, on worker's hands and pruning knives, by using infected seeds and also by feeding infected cucumber beetles. Secondary spread of the disease in a crop usually by non-persistent aphid vectors (Kaper and Waterworth, 1981). Cucumber mosaic virus is transmitted by more than 60 species of aphids, as well as cucumber beetles. The cycle is complete when host is infected, usually by aphid feeding, but sometimes from infected weed seeds (Laviña *et al*., 1996; Palukaitis and Garcia-Arenal,2003).

In banana cultivated fields, primary infection of CMV occurs from the planting of infected suckers and secondary spread of the disease occurs through the aphid vectors in non- persistent manner (Bird and Wellman, 1962). Cucumber mosaic virus infecting banana is highly transmitted by several species of aphids. The two most common aphids, with the widest host range, are the cotton aphid, *Aphis gossypii*, and the corn aphid, *Rhopalosiphum maidis*. Transmission by *Myzus persicae*, *Macrosiphum pisi* and *Rhopalosiphum prunifoliae* has also been recorded (Magnaye and Valmayor, 1995).

Studies of banana mosaic disease outbreaks in Australia revealed that these outbreaks occurred on plantations close to the cultivation of cucurbits, tomatoes and other vegetables. This has led to the suggestion that the main spread of mosaic disease is by aphid spreading from vegetable crops to banana plants, not between banana plants. (Jones, 1991). Subsequent evidence from Taiwan was consistent with this view. Infection is less common in banana fields surrounded by banana or rice plants than in fields near vegetable crops (Gowen, 1995). Shukla and Govind(2000) performed a detailed study to establish virus-vector relationship of CMV aphid infecting banana. It was showed that a short pre acquisition fasting (2 h) and acquisition threshold (2 min) for *A. pisum* increased the efficiency of the aphid and that a single aphid could induce infection with low percentage and the percentage of infection increased with an increase in the aphid population.

2.3 SYMPTOMATOLOGY

The disease is characterised by typical mosaic symptoms on cucumber leaves, including plant stunting and fruit yield reduction. Mottle or mosaic symptom is also observed on fruits. In some cucumber varieties, a few days after infection with CMV, a rapid and complete wilting of mature plants was observed. In pepper plants, CMV infection causes mosaic and necrotic symptoms. In field and greenhouse peppers, mosaic symptoms would fade over time and the leaves turn dull green, brittle and leathery. Systemically, infected leaves become narrow and remain small and the plant appears to be stunted due to the shortened internodes. Infected plants will usually produce the same number of fruit as the uninfected ones, but the fruit will be small and are of less marketable quality. The fruit has a wrinkled, bumpy appearance, pale to yellowish green colour and often ripens irregularly. Sometimes, the fruit develops sunken lesions with necrotic centres (Conti *et al*., 1979; Green and Kim, 1991).

In cucurbits, CMV infection causes stunting due to drastic reduction in leaf size and stem internodes along with yellow foliar mosaic. When the virus becomes systemic, the leaves curl downward, develop a mosaic pattern and remain small. The flowers are unusually developed and have greenish petals. Infected fruits are often distorted and discoloured, remain small and when severely infected, produce a negligible amount of seeds in cucurbits. In celery, CMV infection causes the young petioles to curl downward, which gives plants opened or flattened look. The leaves may grow thin and interveinal areas may become dark green and thick, making the leaves to appear crinkled. Plants infected with CMV when young may develop elongated brown to translucent sunken spots on their petioles (Zitter and Murphy, 2009).

In tomato CMV infection causes filiformity or the development of thin, shoe string-likeleaves. In the early stages of the disease, foliage will turn yellow and plants will become bushy and stunted with mottled leaves. The foliar symptoms may be transitory, meaning that the bottom and top-most leaves will be affected but middle leaves will remain asymptomatic. Severely affected plants often do not produce fruit and if formed remains small, become mottled and has delayed maturity. In greenhouse tomatoes, leaf chlorosis and mottling is easily observed, along with plant stunting. In

field tomatoes, if plants are infected at early stages, the mosaic symptoms can be slightly masked by leaf deformation and develops upward cupping or rolling of leaves (Pratap *et al*., 2012).

In zucchini squash, symptoms of CMV are severe, including mosaic, yellow spots and leaf distortions. Sometimes, infected plants remain stunted and fruit setting is drastically reduced and fruits become deformed with pinpoint depressions. In watermelon, severe CMV infection causes dark necrotic lesions on fruits and leaf deformation (Maria and Deusdedith, 2016).

2.3.1 Symptoms of Cucumber mosaic virus infection in banana

Cucumber mosaic virus causes yellow mosaic and leaf stripe disease in infected banana plants. Severe infection is a great threat to banana cultivation (Niblett *et al*., 1994). The virus causes chlorosis, mosaic and heart rot in banana and has been found in most banana growing areas of the world. The infection presents variable symptoms ranging from mild chlorosis to severe chlorotic streaks on leaf lamina depending on the pathogen strain and the weather conditions. Light yellow streaks runnning parallel to leaf veins giving a leaf striped appearance. The streaks run usually from midrib to edge of the blade. Leaf size reduction and malformation of leaves are also observed. Chlorosis occurs on newly formed leaves which is called as infectious chlorosis and characterised by conspicuous inter-veinal chlorosis of the leaves. In severe cases, this is accompanied by rotting of heart leaf and leaf sheaths (heart rot), progress into the pseudostem and eventually plant dies. Sometimes, diseased plants do not reach maturity. Due to repeated use of suckers from infected plants, the disease spreads leads to gradual decrease in yield and quality (Magnaye and Valmayor, 1995; Dheepa and Paranjothi, 2010).

Leaf deformation and curling are occasionally observed in infected banana plants. Sometimes, mosaic symptoms are also observed on the fruit of infected plants. In general, symptoms are more severe in winter when temperature drop below 24 °C in tropical and subtropical regions. Symptoms are more pronounced including necrosis of emerging leaves and internal tissues of pseudostem, when banana plants

are infected with virulent strains of the virus. Fruit may have mosaic symptoms with bunches having malformed fruit or no fruit. Plant death may occur in very severe cases especially when the plant get infected with virulent strain soon after planting (Sivaprasad *et al*., 2016; Tripathi *et al*., 2016).

The most characteristic symptoms of CMV infected banana in Kerala are leaf discolouration in patches, parallel chlorotic streaks appearing on young leaves giving a striped appearance to them. As the disease progresses, leaves emerge distorted, margins become irregularly wavy, often with patches of necrotic tissues along with reduced leaf lamina. In severe cases, rotted areas are found throughout the leaf sheath and pseudostem and affected plants produce small bunches (KAU, 2016).

Visible symptoms of CMV disease in banana have been studied and observed in banana varieties such as Nendran, Amritsagar, Karpooravalli, and Grand Naine in Kerala. Initially, symptoms appear on leaves showing small spindle shaped chlorotic lesions with yellow margins and central remains green, resulting in mild mosaic streaks. The chlorotic mosaic streaks were found to initiate at midrib and extended towards the margin of leaf lamina running parallel to the veins. Later, the midrib of leaves turned purplish-black colour. The leaves showed severe upper curling at four months after planting. After six months of planting, the plants revealed severe leaf distortion along with reduced leaf lamina. The bunch of infected plants were with abnormally elongated 'S' shaped peduncle and produced a less number of hands and fingers (Mujtaba, 2017).

2.4 GENOME ORGANIZATION

Cucumber mosaic virus is a type member of the genus *Cucumovirus* in the family *Bromoviridae*. The plant viruses of the family Bromoviridae have small icosahedral, single-stranded positive-sense RNA. There are five genera in this family: bromovirus, cucumovirus, ilarvirus, alfamovirus and oleavirus. The size of the virions of the first three genera is 26-35 nm in diameter. In these three genera, the two genomic RNA 1 and RNA 2 are encapsidated individually into independent particles, while the other two RNA 3 and subgenomic RNA 4 are encapsidated together.

Cucumoviruses have isometric particles 29 nm containing four molecules of positive-sense, single-stranded RNA's like RNA 1, RNA 2, RNA 3 and RNA 4 are encapsidated in three different particles. RNA 1 (3.3 kb in length), RNA 2 (3.0 kb) and RNA 3 (2.2 kb) are required for infectivity. RNA 4 (1.0 kb) is a monocistronic RNA which is packaged with the genomic RNA 3 and containing the gene for coat protein. This small RNA is a satellite RNA which can be associated with special symptom expression (Roossinck, 2001).

CMV virions are icosahedral particles with a diameter of 29 nm and composed of 180 subunits of single coat protein and 18 per cent RNA. The genome consists of three single- stranded positive-sense RNAs, named RNA 1 (3000 nucleotides), RNA 2 (3700 nucleotides), and RNA 3 (2200 nucleotides). The RNA 3 contains a fourth strand of RNA, called RNA 4 (1,000 nucleotides) known as subgenomic RNA, which consists of a separate RNA strand produced during replication (Zitter and Murphy, 2009; Jacquemond, 2012).

RNA 1 is monocistronic and codes for 1a protein, which is required for the replication of the viral genome. Protein 1a has two functional domains: the N-terminal and the C-terminal domains. The N-terminal domain has methyltransferase activity to add cap structure to the 5'- terminus of genomic and subgenomic RNA, while the C-terminal domain of protein 1a is a putative helicase whose function is to "unwind" the double stranded RNA that develops during viral replication (Rozanov *et al*., 1992; Kadaré and Haenni, 1997). RNA 2 codes for large 2a protein and small 2b protein. Protein 2a is required for CMV replication and has characteristic RdRp motifs. The small 2b protein is expressed from the ORF over- lapping the 3' terminal part of ORF 2a. The 2b protein interferes with the host RNA interfering pathway i.e. host's post-transcriptional gene silencing mechanism (Ding *et al*., 1994; Ishihama and Barbier, 1994; O'Reilly and Kao, 1998). Bicistronic RNA 3 encodes the 3a or movement protein of the virus, and Coat protein. Although the first ORF of each bicistronic RNA was expressed from the genomic RNA, the second ORFs were expressed from the subgenomic RNA 4A (protein 2b) and RNA 4 (CP) (Fig 2.1). Protein 3a is required for virus cell - to - cell movement. The coat protein is the only protein associated with virus particles and is the sole determinant of aphid vector

transmission (Zitter and Murphy, 2009; Jacquemond, 2012).

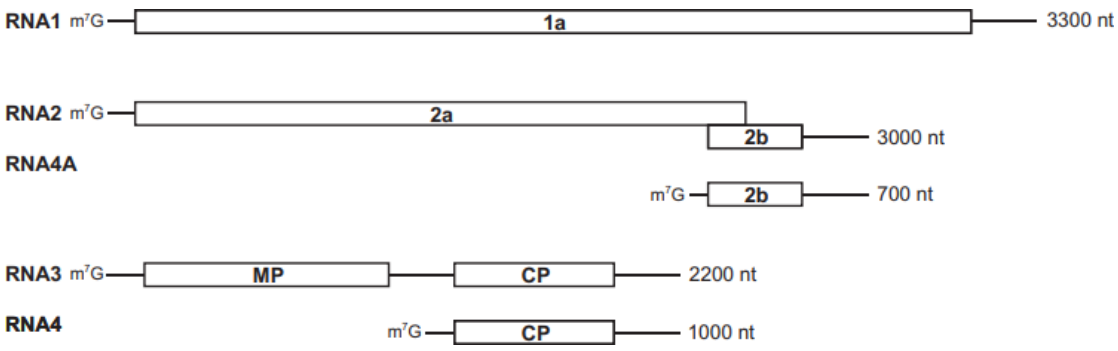


Fig. 2.1 Genome organization of CMV

2.5 DETECTION OF VIRUS

Virus diseases cannot be controlled with chemicals. So early detection is the only useful method for virus disease management. Virus diagnosis is necessary to use virus-free seeds and virus-free planting material to avoid viral diseases of crops (Niblett *et al*., 1994).

Various methods for the detection and identification of plant viruses include biological assays involving the interaction of the virus with the host or its vectors (symptomatology and transmission tests), electron microscopy, serological tests such as precipitation tests, ELISA and immunoblotting. Viral nucleic acid-based techniques such as dot-blot hybridization assays, polymerase chain reaction and other molecular means. The availability of these diagnostic methods provides greater flexibility, increased sensitivity and specificity for rapid diagnosis of viral diseases (Webster *et al*., 2004; Makkouk and Kumari, 2006; Lopez *et al*., 2009; Kolte andTewari, 2011).

The diagnostic techniques like RT-PCR and ELISA have been used for detection of CMV isolates along with biological characterisation, serology and electron microscopy in chrysanthemum (Srivastava *et al*., 1992), sugarcane (Hema *et*

al., 2003), geranium (Verma *et al*., 2004), gladiolus, pepper and vanilla (Madhubala *et al*., 2005), carnation (Raikhy *et al.*, 2007), tomato (Lin *et al*., 2010), anthurium (Miura *et al*., 2013), cucumber (Koolivand *et al.*, 2017) and banana (Mujtaba, 2017).

2.5.1 Serological diagnosis

Many serological methods have been reported including ELISA, it has been successfully used for the large scale detection of plant viruses (Clark and Adams, 1977). Serological detection systems use specific antibody developed in animals in respond to antigens for detection of viruses. It is relatively in expensive, sensitive, rapid, simple, reliable and suitable for the testing of many samples at the same time (Clark, 1981; Torrance, 1998; Makkouk and Kumari, 2006; Vemulapati *et al.*, 2014). Specific antiserum has been developed against the target virus and it has been employed for the detection of a lot of viruses including Citrus tristeza virus, Potato leaf roll virus, Potato virus X, and Potato virus Y, CMV (Torrance, 1998; Sun *et al*., 2001; El-Araby *et al*., 2009; Eni *et al*., 2010).

CMV isolates in various crops were detected by using TAS-ELISA and IC-RT-PCR (Yu *et al*., 2005). Garden sage (*Salvia* spp.) infected with CMV was detected by DAC-ELISA with specific polyclonal antiserum and alkaline phosphatase-linked secondary antibodies. The mean absorbance at 405 nm for positive and negative controls were recorded. Infected samples showed four times the value of negative control which proved the presence of CMV in *Salvia* spp. (Ali *et al*., 2012). For identification of CMV virus, ELISA by using polyclonal antibody was successfully used in 10 crops viz. tomato, pea, watermelon, tobacco, broad bean, soybean, squash, eggplant, cucumber and lettuce. Among these, the highest and the lowest CMV infection was associated with watermelon (62.44 per cent) and lettuce (Zero per cent), respectively (Hosseinzadeh *et al*., 2012). Pandey (2015) standardised DIBA and ELISA with expressed CMV-CP and CMV infected plants using the antibodies raised against the virus, with optimum titre values at 1: 100 dilution of antigen and 1: 1000 dilution of primary and secondary antibody. CMV infected chilli plants were tested and confirmed the presence of virus by DAC-ELISA (Vinodhini *et al.*, 2020).

Kiranmai *et al.* (1996) have demonstrated potential applicability of DAC-ELISA in large scale indexing of banana for CMV infection. Aglave *et al*. (2007) standardised DAC- ELISA for the detection of CMV in Ardhapuri variety of banana. The virus was immunogenic and antiserum with titre of 1:6000 was raised and used in DAS-ELISA. DAC-ELISA was standardised with antiserum dilution of 1:4000; with alkaline phosphatase enzyme labelled IgG (1:10,000) and antigen dilution of 1:10 and 1:100 was optimum for the detection of CMV in leaf, sucker and plants.

2.5.2 Molecular diagnosis

Molecular techniques can be applied for diagnosis of many viral diseases and most commonly used techniques in the laboratory due to its high accuracy and sensitivity. Polymerase chain reaction is a molecular technique that is effectively used for molecular diagnosis of plant viral diseases (Schaad and Frederick, 2002; Makkouk and Kumari, 2006). The Reverse transcription polymerase chain reaction technique is sensitive, specific and used to detect RNA viruses that require a reverse transcriptase enzyme to be added to the reverse transcription step before the conventional PCR reaction (Webster *et al*., 2004; Lopez *et al*., 2009).

RT-PCR has been developed and used to detect many potato viruses such as PVX, PLRV and PVS in potato stem and seed (Ham, 2003; Peiman and Xie, 2006; Peter *et al*., 2009; Drygin *et al*., 2012). It was used for quarantine purposes to detect plant RNA viruses. This technique can detect viruses including Cucumber vein yellowing virus, Cucurbit yellow stunting disorder virus, Potato aucuba mosaic virus, Potato yellow dwarf virus, Tomato chlorosis virus and Cucumber mosaic virus (Lee *et al*., 2011).

Reverse transcriptase polymerase chain reaction is widely used to detect CMV isolates infecting various crops. RT-PCR was performed using the total nucleic acid from infected leaf samples and specific primers to detect CMV in Chrysanthemum species (Srivastava *et al*., 1992). RT-PCR using primers complementary to the conserved CMV-RNA 3 sequence has been successfully applied to detect CMV

isolates infecting tobacco, tomato and cucumber plants (De Blas *et al*., 1994). RT-PCR was performed for specific detection of CMV genome in crude extracts of both healthy and infected gladiolus tissue using primers from the conserved CMV RNA 3 sequence (Raj *et al*., 1998). RT-PCR has been used to detect and identify cucumoviruses in various crops (Choi *et al.*, 1999).

Niimi *et al*. (2003) detected CMV infecting lilium species by RT-PCR. Viral CP gene was amplified using RT-PCR to detect CMV infection in vanilla plants (Madhubala *et al*., 2005). RT-PCR and restriction fragment length polymorphism analysis has been used for detection of CMV virus infecting tomato (Sudhakar *et al*., 2006). Zein and Miyatake (2009) detected CMV infected chrysanthemum cultivars by amplifying the viral coat protein region (~650 bp) using CMV-CP specific primers in RT-PCR. Ali and Kobayashi (2010) detected seed-borne infection and seed transmission frequency of CMV in pepper by RT-PCR technique. The occurrence of CMV was confirmed by RT-PCR with 670 bp CMV-CP gene amplification from total RNA recorded for the first time in sweet potato (Opiyo *et al*., 2010).

Ali *et al*. (2012) performed RT- PCR using CMV coat protein gene specific primers in Salvia spp. resulted in the amplification of ~650 bp fragment in the infected sample, indicating that the presence of the CMV strain. RT-PCR was performed to identify the CMV virus in *Anthurium* spp. (Miura *et al*., 2013). El-Borollosy and Hassan (2014) detected CMV infected cucumber isolates by amplifying the viral coat protein region (~657 bp) by RT-PCR. The association of CMV with severe eggplant (*Solanum melongena L.*) mosaic disease collected from Lucknow and Kanpur, India was detected and confirmed by RT-PCR using coat protein gene specific primers (Kumar *et al.*, 2014). Shetti *et al*. (2014) obtained 657 bp CMV-CP amplicon by RT-PCR from infected cucumber plant samples.

RT-PCR assay was developed for detection of CMV isolates by amplifying 750 bp CMV-CP gene from infected banana plants (Hu *et al*., 1995). RT-PCR was used to identify CMV isolates that belongs to sub group1 from banana plants infected with infectious chlorosis disease (Singh *et al*., 1995). Khan *et al*. (2012) reported an association of CMV with banana mosaic disease by RT-PCR with amplification of

657 bp CMV-CP gene. RT-PCR has been used for detection of CMV virus by gene specific primers from CMV infected banana plants (Prema *et al*., 2012). Basavaraj *et al.* (2017) performed RT-PCR using CMV coat protein gene specific primers resulting in amplification of ~650 bp fragment from infected banana plants. RT-PCR technique has been used for molecular detection of CMV virus infecting banana plants from Kerala resulting in amplification of ~750 bp coat protein gene in the infected banana plants (Mujtaba, 2017; Antony, 2019)

2.6 RECOMBINANT COAT PROTEIN AND ITS APPLICATIONS

Recombinant coat protein had a wide range of applications in research, diagnostics and therapy. Recombinant coat protein had been used for raising antiserum against plant viruses, development of drugs and vaccines against human and animal diseases, production of toxins, enzymes and also for structural study of proteins (Pavlou and Reichert, 2004; Belsham and Bøtner, 2015). Different strains of *Escherichia coli* and expression vectors are the most important production system for recombinant coat protein (Yin *et al*., 2007).

Agarwal *et al*. (2009) expressed recombinant coat protein gene of Papaya ring spot virus (PRSV) in *Escherichia coli* strain TB1 as expression host. PRSV-CP gene cloned into pGEMT Easy vector and further subcloned into expression vector pMAL-C2X for production of polyclonal antibodies. Gulati-Sakhuja *et al*. (2009) cloned Pelargonium zonate spot virus coat protein gene into bacterial expression vector PTriEX-4EK/LIC and transformed into *Escherichia coli* Rosetta2 (DE3) PLacI cells for production of polyclonal antiserum against recombinant coat protein of PZSV which is used for detection of virus using serological method.

Lin *et al*. (2010) cloned CMV-CP gene infecting tomato into pET32a expression vector and transformed into *Escherichia coli* BL21 (DE3) expression host for recombinant coat protein production. Bonning *et al.* (2014) used the coat protein of Luteovirus, an aphid vectored plant virus, fused with spider derived insect-specific toxin that acts within the hemocel for the control of aphid pest. Aphids feed on recombinant coat protein-toxin fusion, later they showed signs of neurotoxin-induced

paralysis, finally to their death. Luteo viral coat protein- insect neurotoxin fusions represent a promising strategy for transgenic control of aphids.

Paula *et al.* (2016) used recombinant insulin-like proteins isolated from *Moringa oleifera* leaves for the production of new drugs to treat diabetes mellitus. Amino acid sequence of insulin-like protein obtained from *Moringa oleifera* leaves were cloned the gene encoding this protein using RT-PCR and other techniques. The recombinant protein, tagged with 6 histidine residues, was expressed in *Escherichia* coli BL21 (DE3), followed by purification using affinity chromatography on a nickel-nitrilotriacetic acid column. This recombinant polypeptide-p significantly reduced the plasma glucose levels of induced diabetic mice, 4 h after intravenous administration of a 1 mg/kg dose. These recombinant insulin-like proteins helped for the production and release of new drugs to treat diabetes mellitus.

Dilip *et al.* (2020) cloned coat protein gene of Banana bract mosaic virus infecting banana into expression vectors, pRSET-C and pGEX-4T-2 and the construct was transformed into BL21 (DE3) pLysS cells for over expression. 34 kDa and 60 kDa coat protein were obtained from pRSET-C/BBrMV-CP and pGEX/BBrMV-CP clones respectively for raising polyclonal antiserum to detect the virus.

2.7 PROTEIN EXPRESSION AND PURIFICATION

E. coli BL21 (DE3) pLysS cells are widely used for protein expression which contains T_7 RNA Polymerase gene, under the control of LacUV 5 promotor. BL21 (DE3) pLysS also includes one plasmid containing the gene expressing the lysozyme, resulting in decomposition of the enzyme T_7 polymerases before induction. The lysozyme can be affected in low levels and there is no adverse effect due to the very high expression of T_7 polymerases after induction. T_7 promoter system is also present in expression vector which is used for recombinant coat protein expression. This system induced by IPTG for protein expression studies (Rosano and Ceccarelli, 2014; Hayat *et al*., 2018).

2.7.1 Protein expression and purification for scientific purposes

Jacob and Usha (2002) cloned Cardamom mosaic virus (CdMV) coat protein gene intopProEX HTb expression vector and transformed into *E. coli* BL21 cells for production of recombinant coat protein of CdMV. The over expressed CP was purified by affinity chromatography under denaturing conditions. The expressed CP was confirmed by SDS-PAGE and positive reaction with anti CdMV IgG antibody in Western blotting. Carnation etched ring virus coat protein gene was cloned into the pET28a expression vector and transformed into *Escherichia coli* BL21 (DE3) pLysS expression host for fusion protein production to raise antiserum (Raikhy *et al*., 2007).

Rostami *et al.* (2014) conducted experiment to study expression of Cucumber mosaic virus coat protein gene and assembly of Virus like particles in cells of *E. coli* expressing the CMV-CP gene under transmission electron microscope. CMV- CP gene was cloned into pTZ57R/T vector and further sub cloned to the expression vector pET21a and that construct was transformed to *E. coli* strain Rosetta cells for over expression. Sequencing was done by Marcrogen, Inc. to confirm the correct insertion of the CP gene in the vector. After inducing by isopropyl thiogalactosides, the molecular weight of the expressed protein was determined by SDS-PAGE and CMV-CP was detected by Western blotting by a CMV specific polyclonal antibody and conjugate. The protein extracted from the CP producing clone was studied undera JEOL 100-CXII transmission electron microscope with 100000× magnification at an acceleration voltage of 100 kV. The results showed that the CP gene was expressed in the prokaryotic system successfully and was assembled into the CMV like particle.

Gulati *et al.* (2016) used recombinant coat protein to study the structural characterisation of Tobacco Streak Virus (TSV). Tobacco streak virus-CP gene was cloned to pRSET- C vector and the construct was transformed into *E. coli* BL2 (DE3) cells for over expression. The protein was purified using isopycnic density gradient ultra-centrifugation method. Purity of the proteins obtained was examined by 12 per cent SDS- PAGE. The purified TSV-CP was crystallised in two district forms and their structures were determined at resolutions of 2.4 Aº and 2.1 Aº respectively. Dimers of CP with swapped C-terminal arms were observed in both crystal forms. The C-arm was found to be flexible and responsible for polymorphic and pleomorphic

nature of TSV capsids.

Sabharwal (2017) cloned Pepper vein banding virus (PVBV) coat protein gene into pET20b vector to study the structure and function of PVBV-CP. Later PVBV-CP was sub cloned into pRSET- C vector and the construct was transformed into *E. coli* BL21 (DE3) pLysS cells for over expression. The over expressed CP was examined by SDS- PAGE. PVBV- CP was purified using sucrose density gradient centrifugation.

2.7.2 Protein expression and purification for antiserum production

Expression of viral coat proteins in *E. coli* followed by purification and production of polyclonal antisera has been reported for many plant viruses. These antisera have been successfully used to detect plant viruses.

Grapevine leafroll associated closterovirus-3 coat protein gene was cloned into the pRSET-C expression vector. The recombinant plasmid was transformed in *E. coli* BL21 (DE3)and expressed the coat protein by adding with IPTG. The expressed coat protein was purified by Ni-NTA affinity chromatography and used to raise polyclonal antiserum in rabbit which is effectively used in serological assays to detect GLRaV-3 in grapevine (Ling *et al*., 2000).

Sugarcane streak mosaic virus (SCSMV), belong to Potyviridae, causes mosaic disease of sugarcane. The coat protein gene of Andhra Pradesh isolate of SCSMV (SCSMV-AP) was cloned on pRSET- A vector and expressed in *E. coli*. BL21 pLysS, the recombinant coat protein was used to raise high quality antiserum. The CP antiserum was used to develop an IC- RT-PCR based assay for the detection and discrimination of SCSMV isolates in AP. The sensitivity of the IC-RT-PCR was compared with DAC-ELISA and DIBA. It was found to be more sensitive and thus used to detect the virus in sugarcane (Hema *et al*., 2003). Abou-Jawdah *et al.* (2004) cloned recombinant coat protein gene of Prune dwarf virus belongs to genus Ilarvirus into pGEMT vector, later sub cloned into pRSET and pQE-30 expression vectors. pRSET construct was transformed into *Epicurian coli* BL21-Gold cells and pQE30 construct was transformed into *E. Coli* M15 cells for production of high

quality antiserum. Later, antiserum was standardised through Dot blot immunoassays, Indirect ELISA and DAS-ELISA.

Hamdayanty *et al.* (2016) used *Escherichia coli* BL21 (DE3) and *Rosetta-gami* (DE3) pLysS as expression host for antiserum production. Sugarcane streak mosaic virus coat protein was cloned into pET28a expression vector and transformed into above expression host for production of high quality antiserum against SCSMV. Papaya ringspot virus coat protein gene was cloned into pRSET- B and transformed into *E.coli* DH5α cells for over expression. The expressed recombinant PRSV-CP was purified using Ni-NTA His-tag protein purification kit. Polyclonal antiserum was raised in six month old female New Zealand white rabbit which is used for detection of the PRSV (Valekunja *et al.,* 2016).

Sugarcane mosaic virus (SCMV) coat protein gene was inserted into pET28a expression vector and over expressed in *Escherichia coli* BL21 (DE3) to produce recombinant protein. The recombinant fused protein with a molecular size of 44 kDa was expressed in an insoluble fraction. Purification of the recombinant protein was done using an affinity Ni-NTAresin, followed by SDS-PAGE separation, resulted in a high purity of the protein and used to raise the polyclonal antiserum in rabbit. This antiserum was successfully used to detect and diagnose SCMV infection (Darsono *et al*., 2018). Shibaei *et al*. (2018) cloned recombinant coat protein gene of Grapevine fan leaf virus into pET28a bacterial expression vector and the construct was transformed into *E. coli* strain BL21 (DE3) cells for the production of polyclonal antibodies which is used for immuno detection of virus.

Sweet potato feathery mottle virus (SPFMV) coat protein gene was cloned into TA cloning vector and then transformed into *E. coli* DH5α cells. Positive clones were sub cloned into pET28a expression vector and transformed into *E. coli* BL21 DE3 cells for over expression.The expressed coat protein (SPFMV CP) was purified using Ni-NTA resin anity chromatography. Purified protein was immunized into two New Zealand white rabbits for polyclonal antibody production. Antiserum was produced with 1:6000 dilution and used for serological detection of virus (Thangaraj and Jayakrishnan, 2021).

2.7.3 Protein expression and purification of plant viruses infecting banana for antiserum production

Wanitchakorn *et al*. (1997) cloned Banana bunchy top virus coat protein into pMAL-c2 expression vector and transformed into *E. coli* strain DH5α cells. Coat protein with 20 kDa molecular size protein was expressed. Fusion product was purified and used for production of polyclonal antiserum in a 10 week-old female New Zealand white rabbit. Rodoni *et al*. (1999) cloned coat protein gene of Banana bract mosaic virus into T-tailed vector pGEM-T (Promega). Further sub cloned into pProEX-1 expression vector and transformed into *E. coli* M15 cells for over expression. Approximately 40 kDa fusion protein was produced and used to raise high-titre BBrMV-specific polyclonal antiserum for serological assays.

Selvarajan *et al*. (2016) cloned viral-associated protein (VAP) gene of Banana streak virus into pTZ57R/T plasmid. Then sub cloned into the 50 terminal 69 His-tagged pCold vector I and transformed into *Escherichia coli* BL21 (DE3) for prokaryotic expression. The expressed 15 kDa rVAP was purified using Ni-NTA Purification System Kit. VAP expressed in bacteria was used to produce polyclonal antibodies in rabbits. This was the first report on the production of polyclonal antiserum against VAP of BSV.

Arumugam *et al*. (2017) cloned the Banana bunchy top virus coat protein gene into T-tailed vector pTZ57R/T by T/A cloning method. Then, further sub cloned into pET28a (+) expression vector and transformed into *E. coli* BL21 cells. The cells were induced by adding 1mM IPTG and expressed 19.5 kDa coat protein. The expressed protein was purified through Ni- column chromatography and used to raise antiserum to detect BBTV.

Dilip (2020) cloned coat protein gene of Banana bunchy top virus into expression vectors, pRSET-C, pET32a and pGEX-4T-2. The construct was transformed into *E. coli* strain BL21 (DE3) pLysS and *Rosetta* (DE3) pLysS cells for over expression. 37 kDa fusion protein was over expressed from pET/BBTV-CP clone. The recombinant CP (r-CP) fused with His-tag was purified from *Escherichia*

coli using Ni^{2+}-NTA affinity column chromatography and used as an antigen for the production of polyclonal antiserum to detect the virus. The antiserum had a titre of 1:128,000 when evaluated by DAC-ELISA with r-CP.

Koh *et al.* (2020) Banana bunchy top virus coat protein gene was cloned into pEXP5- NT/TOPO expression vector and transformed into *E. coli* BL21StarTM (DE3) pLysS strain for over expression. The expressed fusion protein pEXP5-NT/ TOPO-BBTVCP was purified using Ni-NTA gravity flow chromatography kit (Invitrogen). Purified recombinant BBTV coat protein was used as antigen for production of polyclonal antiserum in rabbit.

2.7.4 Protein expression and purification of Cucumber mosaic virus for antiserum production

Khan *et al*. (2012) cloned Cucumber mosaic virus coat protein gene infecting banana into pGEMT-Easy vector, further sub cloned into pQE-30 expression vector and the construct was transformed into *E. coli* M15 cells for over expression. The recombinantCP fused with His-tag was purified from *Escherichia coli* using Ni-NTA resin and used as an antigen for the production of polyclonal antisera. This antiserum was used to develop ELISA and IC-RT-PCR based assay for the detection of CMV isolates. The antiserum had a titre of 1:8000 when tested with r-CP.

Kim *et al*. (2016) CMV-CP gene infecting Lilium was cloned into pGEM-T Easy vector, later sub cloned into pET21d (+) expression vector and the construct was transformed into *E. coli* BL21 (DE3) cells followed by purification through Ni-NTA affinity column chromatography. Purified CMV recombinant CP was used for antiserum production. The virus infected lily samples were tested using the polyclonal antisera by ELISA and Western blotting assays.

Koolivand *et al*. (2017) cloned coat protein gene of CMV infecting Cucumber into pTZ57R/T vector, further sub cloned into pET21a expression vector and the construct was transformed into *E. coli* strain Rosetta cells for over expression. Expression of CMV- CP was examined in SDS- PAGE and confirmed through Western blotting. Expressed protein was purified using T_7- Tag affinity purification

kit and used for raising polyclonal antiserum in mice. The raised antiserum was used as serological assay for virus indexing.

Antony (2019) cloned coat protein gene of Cucumber mosaic virus into expression vectors, pRSET-C and pET28a and the construct was transformed into BL21 (DE3) pLysS cells for over expression to raise polyclonal antiserum.

Material and Methods

3. MATERIALS AND METHODS

The present study on "Evaluation of expression clones for recombinant coat protein of Cucumber mosaic virus infecting banana" was conducted at Division of Plant Pathology, Banana Research Station, Kannara and Department of Plant Pathology, College of Agriculture,Vellanikkara during the academic year 2019-2022. A detailed description of materials used and methodologies followed in this research project is mentioned in this section.

3.1 EVALUATION OF EXISTING RECOMBINANT EXPRESSION CLONES FOR CMV COAT PROTEIN GENE

Two recombinant clones of CMV-CP (pRSET-C and pET28a) were produced by previous workers. The present study was initiated with the evaluation of the existing recombinant clones of CMV-CP which involved plasmid isolation, restriction digestionand confirmation.

3.1.1 Isolation of recombinant plasmids pRSET-C/CMV-CP and pET28a/CMV- CP from *E. coli* DH5α

The recombinant plasmids were isolated from *E. coli* DH5α cells by alkali lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981).

Table 3.1: Composition of reagents for plasmid isolation

Reagents	Composition
Resuspension solution (Solution 1)	25 mM Tris- pH 8.0 (pH was adjusted with Con. HCl)10 mM EDTA - pH 8.0 50 mM glucose Solution 1 was prepared, sterilised and stored at 4 ℃
Lysis solution (Solution 2) (freshly prepared)	1 per cent sodium dodecyl sulphate 0.2 N sodium hydroxide
Neutralising solution (Solution 3)	3 M potassium acetate- pH 5.5 (pH was adjusted with glacial acetic acid)

Single colony of *E. coli* DH5α/pET28a/ CMV- CP from subcultured plate was inoculated into 5 ml of LB broth/kanamycin (25 μg/ml) and incubated at 37 °C, at

180 rpm, overnight. The culture was transferred into 2 ml microcentrifuge tubes and centrifuged at 10,000 rpm for 5 min at 4 °C. The bacterial pellet was resuspended in 150 µl of solution 1 (Table 3.1) along with 100 µg/ml RNase and vortexed. The tubes were incubated at room temperature for 5 min. Then, 150 µl of freshly prepared solution 2 (Table 3.1) was added and mixed gently by inverting the tubes by hand. The tubes were incubated on ice for 5 min. Then, 200 µl of solution 3 (Table 3.1) was added and mixed gently by inverting the tubes by hand. The tubes were incubated on ice for 5 min. The tubes were then centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant (500 µl) was collected in 2 ml microcentrifuge tube without disturbing the pellet. Isopropanol (500 µl) was added and mixed well by hand. Then the tubes were incubated at 4 °C for 10 min for precipitation and centrifuged at 10,000 rpm for 15 min at 4 °C. Supernatant was discarded and pellet was washed with 100 µl of absolute ethanol. Then it was centrifuged at 10,000 rpm for 2 min and the supernatant was discarded. Then, the pellet was air dried until the ethanol evaporated. Later, the pET28a/ CMV- CP plasmid was dissolved in 25 µl of sterile distilled water.

3.1.2 Confirmation of recombinant expression clones through restriction digestion

The recombinant plasmids pRSET-C and pET28a were subjected to restriction digestion using *Nhe*1 and *Bam*H1 restriction enzymes. The reagents for restriction digestion are mentioned in table 3.2.

Table 3.2: Composition of reagents for restriction digestion

Reagents	Quantity of plasmid (µl)	
	pRSET-C / CMV-CP	**pET28a / CMV-CP**
Plasmid	3.0	3.0
*Nhe*1	0.4	0.4
*Bam*H1	0.4	0.4
Tango buffer	1.0	1.0
Water	5.2	5.2
Total	10.0	10.0

The reaction mixture was centrifuged at 10,000 rpm for 1 min and incubated at 37 °C for 4 h.

3.1.2.1 Agarose gel electrophoresis

The digested plasmids were electrophoresed at 0.8 per cent agarose gel at 65 V.

Procedure of agarose gel electrophoresis

The gel casting tray was prepared by placing a comb on a gel tray about 1inch from one end of the tray. Agarose (0.8 g) was added to 100 ml of 1X TAE buffer in a conical flask and dissolved using a microwave oven until the solution a clear. The solution was allowed to cool about 42 – 45 ℃. When the agarose gel became cool, 2.0 µl of ethidium bromide was added and poured that warm gel solution into the gel casting tray. Then the gel was allowed to solidify for 30-40 min at room temperature. Running buffer (1X TAE Buffer) was prepared from 50X TAE buffer using distilled water and poured into gel electrophoresis chamber. To run the gel, after gel solidification, the comb was removed gently and the gel tray was placed in the electrophoresis chamber and covered the gel tray with running buffer. Then, the samples were loaded with 6X loading dye (Thermo fisher scientific) into each well of the gel along with 1 kb DNA ladder (Thermo fisher scientific). The electrophoresis was performed at 65 V, until the dye reached $3/4^{th}$ of the gel length. The gel image was documented by BioRad Gel Doc Ez Imager.

3.1.3 Transformation of recombinant pET28a expression vector into *E. coli* DH5α cells

3.1.3.1 Preparation of competent *E. coli* DH5α cells

The competent cells were prepared to transform the recombinant plasmid into *E. coli* DH5α cells by following the protocol given by Mandel and Higa (1970).

Five to ten single bacterial colonies from subcultured plate were inoculated into 100 ml LB broth and incubated the culture at 180 rpm at 37 ºC for 4 h with vigorous agitation to obtain optimum OD. The culture was transferred into ice cold 50 ml oakridge tube and incubated on ice for 20 min to arrest the growth. Then, the culture was centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in sterile ice- cold 0.1 M $CaCl_2$ fused (10 ml) and

incubated on ice for 20 min. Then, the culture was centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in sterile ice cold 0.1 M $CaCl_2$ fused (2 ml) and incubated on ice for 10 min. Then, the bacterial suspension was transferred into 1.5 ml micro centrifuge tube (100 µl each).

3.1.3.2. Transformation

Transformation of recombinant plasmid pET28a/CMV- CP in to competent *E. coli* strain DH5α cells was performed by following the standard protocol (Sambrook and Russell, 2001).

The recombinant plasmid (4 µl) was transformed into competent cells (100 µl), mixed gently and incubated on ice for 40 min. The tube was rapidly taken from ice and a heat shock was given at 42 °C exactly for 90 S without shaking and immediately placed back on ice for 5 min. Then, the LB broth (250 µl) was added to the culture and the tube was inverted twice to mix the contents. Later, the transformed culture was incubated at 37 °C at 180 rpm for one h. Then, the culture was centrifuged at 10,000 rpm for two min at 4 °C and the bacterial pellet was dissolved in 100 µl of supernatant. Then, the transformants of *E. coli* DH5 α/pET28a/ CMV-CP were plated on LB- agar/ kanamycin (25 µg/ml) medium by the spread plate method.

3.1.4. Confirmation of cloning pET28a/CMV- CP through restriction digestion

3.1.4.1. Isolation of recombinant plasmid pET28a/CMV- CP for restriction digestion

The recombinant plasmid was isolated from *E. coli* DH5α cells as given in section 3.1.1.

3.1.4.1.a. Agarose gel electrophoresis

The isolated plasmid was analysed on 0.8 per cent agarose as given in section 3.1.2.1.

3.1.4.2. Confirmation of recombinant plasmid through restriction digestion

The recombinant plasmid pET28a/CMV- CP was subjected to restriction digestion using restriction enzymes. The reagents for restriction digestion are mentioned in table 3.3.

Table 3.3. Composition of reagents for restriction

Reagents	Quantity (µl)
Plasmid	3.0
*Nhe*1	0.4
*Bam*H1	0.4
Tango buffer	1.0
Water	5.2
Total	10

The mixture was centrifuged at 10,000 rpm for one min and incubated at 37 °C for four h.

3.1.4.2.a. Agarose gel electrophoresis

The digested plasmid was analysed on 0.8 per cent agarose as given in section 3.1.2.1.

3.2. OVER EXPRESSION OF RECOMBINANT COAT PROTEIN GENE IN pET28a EXPRESSION VECTOR

The recombinant pET28a/CMV-CP plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS cells and induced by IPTG for over expression of CMV coat protein gene.

3.2.1. Transformation of plasmid (pET28a/CMV-CP) into *E. coli* BL21

3.2.1.1. Preparation of competent *E. coli* BL21 cells

The competent cells were prepared to transform the recombinant plasmid into *E. coli* BL21 cells by following the protocol given by Mandel and Higa (1970).

Five to ten single bacterial colonies from subcultured plate were inoculated into

100 ml LB broth/chloramphenicol (34 µg/ml) and incubated the culture at 180 rpm at 37 ºC for 4 h with vigorous agitation to obtain optimum OD value. The culture was transferred to ice cold 50 ml oakridge tube and incubated on ice for 20 min to arrest the growth. Then, the culture was centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in sterile ice- cold 0.1 M $CaCl_2$ fused (10 ml) and incubated on ice for 20 min. Then, the culture was centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in sterile ice cold 0.1 M $CaCl_2$ fused (2 ml) and incubated on ice for 10 min. Then, the bacterial suspension was transferred into 1.5 ml micro centrifuge tube (150 µl each)

3.2.1.2. Transformation

Transformation of recombinant plasmid (pET28a/CMV- CP) in to competent *E. coli* BL21 (DE3) pLysS cells was performed by following the standard protocol (Sambrook and Russell, 2001).

The recombinant plasmid (4 µl) was transformed in to competent cells (150 µl), mixed gently and incubated on ice for 40 min. The tube was rapidly taken from ice and a heat shock was given at 42 °C exactly for 90 S without shaking and immediately placed back on ice for 5 min. Then, 250 µl of LB broth/chloramphenicol (34 µg/ml) was added to the culture and the tube was inverted twice to mix the contents. Later, the transformed culture was incubated at 37 °C at 180 rpm for one h. Then, the culture was centrifuged at 10,000 rpm for two min at 4 °C and the bacterial pellet was dissolved in 100 µl of supernatant. Then the transformants of *E. coli* BL21 (DE3) pLysS /pET28a/ CMV- CP culture were plated on LB- agar/ kanamycin (25 µg/ml) /chloramphenicol (34 µg/ml) medium by the spread plate method.

3.2.2. Confirmation of cloning of pET28a/CMV- CP into *E. coli* BL21

3.2.2.1. Confirmation of cloning through colony PCR

The recombinant clones were analysed for the presence of insert by colony PCR using CMV-CP specific primers reported by Cherian *et al*. (2004) and are mentiond in table 3.4. Randomly selected single white colonies were subcultured on LB agar

plate containing kanamycin (25 µg/ml) and chloramphenicol (34 µg/ml). Then, single colonies were selected from subcultured plates and resuspended in 20 µl of sterilised distilled water. Then, the cell suspension was heated at 98 °C for two min in a thermocycler and centrifuged at 10,000 rpm for 2 min. The supernatant (2 µl) was used as template for PCR reaction.

Table 3.4. CMV- coat protein gene specific primer and its sequence

Primer	Sequence (5'- 3')	Length (bp)
CMV- FP	CATCGACCATGGACAAATCTGAATCAAC	28
CMV- RP	CTCTCCATGGCGTTTAGTGACTTCAGCAG	29

The PCR reaction mixture is given in table 3.5.

Table 3.5: Composition of reaction mixture for PCR amplification of CMV-CP gene

Reagents	Quantity
Nuclease-free water	20.0 µl
Taq buffer A with 10 mM $MgCl_2$	2.5 µl
10 mM dNTP set	0.25 µl each
CMV FP (10 mM)	0.1 µl
CMV RP (10 mM)	0.1 µl
3 U/µl *Taq* Polymerase	0.2 µl
Template	2.0 µl

After the preparation of reaction mixture, the PCR tubes were short spinned and placedin thermocycler (Eppendorf Mastercycler Gradient) for PCR amplification.

The programme for colony PCR

The entire programme for colony PCR is given in table 3.6.

Table 3.6. The thermal profile for colony PCR

Step	Temperature (°C)	Time period
Initial denaturation	94	3 min
Denaturation	94	30 S
Annealing	61	45 S
Elongation	72	1:30 min
Final elongation	72	10 min
Hold	4	5 min

(30 cycles of denaturation, annealing and elongation)

3.2.2.2. Agarose gel electrophoresis

The amplified PCR product was analysed on one per cent agarose as given in section 3.1.2.1.

3.2.3. Standardisation of over expression of coat protein from recombinant pET28a/BL21 cells

Single *E. coli* BL21 (DE3) pLysS /pET28a/CMV-CP cell was inoculated into 50 ml of LB/kanamycin (25 µg/ml)/chloramphenicol (34 µg/ml) medium and incubated at 37 °C, at 180 rpm, overnight. Next day, 5 ml of primary culture was transferred into 35 ml of LBbroth/kanamycin (25 µg/ml)/chloramphenicol (34 µg/ml) medium and incubated at 37 °C, at 180 rpm for four h. From the incubated culture, 2 ml was taken as uninduced and 3 ml was taken for induction. Three milliliters culture was induced with 0.3 mM, 0.5 mM and 1 mM IPTG. Then, induced and uninduced culture were incubated at 20 °C, 25 °C, 30 °C and 37 °C at 180 rpm in shaker incubator for 4 h, 6 h, 10 h, 12 h, 14 h and 16 h. Later, each vial was centrifuged at 8000 rpm for 15 min at 4 °C and the supernatant was discarded. The bacterialcell pellet was lysed using Tractor buffer (Protein purification kit)/Sonicator and the expression of coat protein was evaluated in Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS- PAGE).

3.2.3a. Lysis of bacterial cell by tractor buffer

Bacterial cells were lysed by using Tractor buffer as per the manufacturer's protocol (Takara) mentioned below.

Tractor buffer (2 ml) was added to 100 mg of cell pellet. Then, the pellet was resuspended by a pipette and 1 µl of DNase was added to the resuspended pellet and mixed gently. Then, the cell suspension was incubated on ice for 15 min and centrifuged for 20 min at 10,000 rpm at 4 °C. The supernatant was collected and used as the protein sample for SDS-PAGE.

3.2.3b. Lysis of bacterial cell by sonication

Bacterial cells were lysed by Ultrasonic Probe Sonicator (Lark) as per the standard protocol (Feliu *et al.*, 1998).

The pellet obtained was dissolved in 200 µl sterile distilled water and lysed by ultrasonic probe sonicator in a thermoflask containing ice cubes. Sonication was performed for one minute cycle with 2 S pulse on and 3 S pulse off mode at 30 per cent amplitude. The sonicated sample was taken as protein sample for SDS-PAGE and stored at -80 °C.

Fig: 3.1 Ultrasonic Probe Sonicator

3.2.4. SDS-PAGE

The electrophoresis unit was assembled such that, the glass plates were clamped to the unit along with the spacers placed in-between them at two vertical edges. Resolving gel was poured first in between the plates and allowed to solidify. In order

to avoid oxidation, immediately after the casting of resolving gel, 70 per cent ethanol (2-3 drops) was added to the casting tray. Then, stacking gel was poured on top of resolving gel, immediately placed the comb by avoiding air bubbles. Allowed to solidify for 30 min. Tris-glycine-SDS running buffer (800 ml - 1X) was poured into the unit so that the buffer connects the two electrodes and the comb was removed from the stacking gel carefully and placed the glass plates into the SDS gel running unit.

Sample preparation: The lysed sample (20 µl) was mixed with 30 µl of the sample loading buffer and the tubes were heated at 95 ℃ for 5 min in a water bath. Immediately after heat treatment, 50 µl of sample was loaded including 10 µl of low range protein marker (Takara) into each well created by comb in the stacking gel (Smith, 1984).

The gel was run at 70 V, until the dye moved out of the gel. Later, the gel was taken out with care using spatula into a plastic tray containing distilled water. Then, the water was removed from the tray. Later, staining solution (100 ml) was added to the gel and incubated overnight in shaker at room temperature. Next day, 50 ml of de-staining solution was added to the tray and kept in shaker at room temperature until distinct bands were visible. The gel was removed from the de-staining solution and the gel image was documented by BioRadGel Doc Ez Imager.

3.2.5. Western blotting

SDS- PAGE for western blotting was performed as mention in section 3.2.4

Filter papers and nitrocellulose membrane used for immunoblotting of coat protein were soaked in transfer buffer. The immunoblotting set was prepared by placing a nitrocellulose membrane on top of SDS-PAGE gel and the nitrocellulose membrane and SDS-PAGE gel were sandwiched between filter papers (Fig 3.2.). The immunoblotting set was kept for blotting (3 h) at 80 mA, in a transfer apparatus in the presence of transfer buffer. After immunoblotting, the nitrocellulose membrane was stained with 0.1 per cent (w/v) Ponceau- S dye. Protein bands were visualised on the nitrocellulose membrane in the presence of

Ponceau-S dye and marked the protein ladder with a ball point pen. The dye was washed with tap water until the dye disappeared.

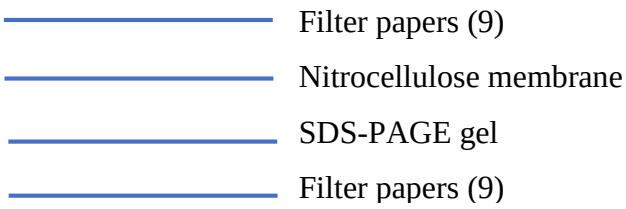


Fig. 3.2. Arrangement of nitrocellulose membrane, filter paper and SDS- PAGE in transfer apparatus

3.2.5.1. Detection of antigen

Nitrocellulose membrane was incubated in blocking buffer (20 ml) for one hour at room temperature with shaking (<100 rpm). The membrane was washed thrice with 1X PBS buffer along with shaking for 5 min each to remove excess blocking buffer. Primary (1°) antibody diluent buffer (5 ml) was added on to the nitrocellulose membrane and incubated for 1 h 30 min at room temperature along with shaking. The membrane was washed thrice with 1X PBS buffer along with shaking for 5 min each to remove excess buffer. Secondary (2°) antibody diluent buffer (5 ml) was added on to the NC membrane and incubated for one hour at room temperature along with shaking. The membrane was washed thrice with 1X PBS buffer along with shaking for 5 min each to remove excess buffer. Then, 5 µl of 30 per cent H_2O_2 and 2 ml DAB (0.5 mg/ml) were added on to the membrane and incubated for 5 min in the dark for colour development.

3.3. DEVELOPMENT OF MOLECULAR CLONES OF CMV COAT PROTEIN GENE

For development of molecular clones of CMV coat protein gene, infected leaf samples of banana showing typical symptoms were collected, tested for CMV infection by serological and molecular techniques, ligated to pGEMT-easy vector and transformed into *E.coli* DH5α cells.

3.3.1. Collection of CMV infected samples

Banana leaves showing typical symptoms of Cucumber mosaic virus (KAU, 2016; Mujtaba, 2017) were collected from the net house and field of Banana Research Station, Kannara,Thrissur, Kerala.

3.3.2. Preliminary assay by Enzyme Linked Immunosorbent Assay (ELISA)

Infected leaf samples of banana showing typical symptoms, along with healthy control were tested for CMV infection by Direct Antigen Coating- Enzyme Linked Immunosorbent Assay (DAC-ELISA) using polyclonal CMV antiserum purchased from the National Research Centre for Banana, Trichy, Tamil Nadu by following standard protocol (Clark and Adams, 1977)**.**

Antigen was isolated from one gram of infected and healthy banana leaf samples by grinding in 5 ml of coating buffer (1X) using sterile pre-chilled mortar and pestle. The samples were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 5000 rpm for 10 min at 4 °C.100 µl of supernatant (sample) was loaded into wells of ELISA microtiter plate (96 wells, Tarson Products Pvt. Ltd.) containing 2 per cent PVP dissolved in 100 µl of 1X coating buffer and incubated at 37 °C for one h. The plate was washed with three changes of PBS-T buffer, to remove the sap and unbound antigen at 3 min interval for each wash. Then, 200 µl of blocking buffer was added to each well and the plate was incubated at 37 °C for one h. The plate was washed with three changes of PBS-T buffer, the plate was loaded with 200 µl of primary antibody (1:1000 v/v), covered the plate with aluminium foil and incubated at 4 °C, overnight.

Next day, the plate was washed with three changes of PBS- T buffer. Then 200 µl of anti- rabbit alkaline phosphatase conjugate (Sigma Aldrich USA, 1:10000 v/v) as secondary antibodysolution was added to each well and incubated at 37 °C for 2 h. The plate was washed with three changes of PBS- T buffer and 200 µl of *p*- nitrophenyl phosphate (PNPP) substrate solution was added to each well in the microtiter plate. The plate was covered and incubated in dark at room temperature for 30 min for

colour development. The absorbance was measured at 405 nm in an ELISA plate reader. The absorbance values of infected samples and healthy samples were compared.

3.3.3. Isolation of total RNA from CMV infected leaves

Young leaf samples of virus infected banana plants showing high absorbance value in DAC-ELISA were taken for RNA isolation. Total RNA was extracted using QIAGEN^{R} RNeasy^{R} Plant Mini Kit and TRIzol based method (Sigma Aldrich). All the materials used for RNA isolation were soaked in 0.2 per cent Diethyl pyrocarbonate (DEPC) treated water, overnight and autoclaved.

3.3.3.1. RNA isolation by TRIzol based method (Sigma Aldrich)

The infected leaf samples (100 mg) were taken and ground to fine powder in liquid nitrogen using DEPC treated pre chilled mortar and pestle. TRIzol reagent (1 ml) was added to the samples and homogenised. The suspension was transferred into DEPC treated 1.5 ml microcentrifuge tubes, vortexed immediately and incubated at room temperature for 5 min. Then, it was centrifuged at 12,000 rpm at 4 ℃ for 5 min. The supernatant was transferred into new DEPC treated 1.5 ml microcentrifuged tubes and incubated at room temperature for 3 min to allow complete dissociation of nucleoproteins complex. Then, 200 µl of chloroform was added and mixed by inverting 3 to 5 times and incubated at room temperature for 3 min. Then, it was centrifuged at 12,000 rpm for 15 min at 4 ℃. The mixture separates into a lower red phenol-chloroform, an interphase and colourless upper aqueous phase. The aqueous phase containing RNA was transferred to a new DEPC treated 1.5 ml microcentrifuge tubes by angling the tube at 45° and pipetted out the solution. Then 500 µl of isopropanol was added to the aqueous phase and incubated for 10 min at room temperature. Centrifugation at 12,000 rpm at 4 ℃ was done for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 75 per cent ethanol. Then, it was centrifuged at 8,000 rpm at 4 ℃ for 5 min. The supernatant was discarded and the RNA pellet was air dried for 10 min. Then, the pellet was resuspended in 20 µl of RNase-free water and stored at – 80 °C for further studies.

3.3.3.2. RNA isolation using QIAGEN^{R} RNeasy^{R} Plant Mini Kit (cat. Nos.74903 and 74904)

The infected leaf samples (100 mg) were taken and ground to fine powder in liquid nitrogen using DEPC treated pre chilled mortar and pestle. 450 µl of RLT buffer (10 µl of β- mercaptoethanol was added to 1 ml RLT buffer just before use) was added to 100 mg tissue and mixed thoroughly. Then, the samples were transferred into precooled DEPC treated 1.5 ml microcentrifuge tubes and vortexed vigorously. The lysate was transferred in to QIAShredder spin column (lilac) placed in a 2 ml collection tube and centrifuged for 2 min at 10,000 rpm. The supernatant was transferred to a new 1.5 ml microcentrifuge tube (DEPC treated) without disturbing the cell-debris pellet. Then, 500 µl of absolute ethanol was added to the cleared lysate and mixed immediately by pipetting. Then, transferred the samples in to the RNeasy Mini spin column (pink) in a 2 ml collection tube (supplied) and centrifuged for one min at 10,000 rpm. The flow through was discarded. Then, 700 µl of RW1 buffer was added to the RNeasy spin column and centrifuged at 10,000 rpm for one min. The flow through was discarded. Then, 500 µl of RPE buffer was added to the RNeasy spin column and centrifuged for one min at 10,000 rpm. The flow through was discarded. Again, 500 µl of RPE buffer was added to the RNeasy spin column and centrifuged for 2 min at 10,000 rpm. Then, the RNeasy spin column was placed in a new 2 ml collection tube (supplied) and centrifuged at 10,000 rpm for one min to dry the membrane. Then, the RNeasy spin column was placed in a new 1.5 ml microcentrifuge tube (supplied along with the kit). RNase-free water (30 µl) was added directly to the spin column membrane and centrifuged for one min at 10,000 rpm to elute the RNA. Isolated RNA was stored at -80 °C for further studies.

3.3.3.2a. Agarose gel electrophoresis

The isolated RNA was electrophoresed in 0.8 per cent agarose gel at 65 V as per section 3.1.2.1.

3.3.4. Synthesis of first strand complimentary DNA (c-DNA)

Isolated RNA was used to synthesis the c-DNA by Thermo scientific Revert Aid First Strand c-DNA synthesis Kit (Cat. No. K1621) as per the manufacturer's instructions. The reagents used for c-DNA synthesis are given in table 3.7.

Table 3.7. Reagents used for c-DNA synthesis

Reagents	Quantity
50-100 ng Template RNA	5 µl
5 µM Oligo $(dT)_{18}$ primer	1 µl
5X Reaction buffer	4 µl
20 U/µl Ribolock RNase inhibitor	1 µl
10 mM dNTP mix	2 µl
200 U/µl RevertAid M-MuLV RT	1 µl
Nuclease-free water	6 µl
Total	20 µl

The reagents were mixed gently in PCR tubes and centrifuged at 8000 rpm for 2 min. Then, the reaction mixture was incubated at 42 ℃ for 60 min followed by termination at 70 ℃ for 5 min in a thermocycler. Later, the c-DNA was stored at -20 ℃ for PCR amplification.

3.3.4.1. Quantification of c-DNA

Quantification of c-DNA was carried out using Nano drop 2000/2000cc (Thermo scientific). The software icon in the desktop was selected and opened. The sampling arm was opened and 1 µl blank (*i.e.*, distilled water) was loaded onto the lower measurement pedestal and sampling arm was closed, after which the measure was selected in order to calibrate the software. Then 1 µl each RNA sample was loaded onto the lower measurement pedestal and sampling arm was closed, selected the measure. Then, the concentration of the c-DNA samples were recorded. The quality of c-DNA was determined by analysing the absorbance value at the $A_{260/280}$

3.3.5. Polymerase chain reaction (PCR) amplification for detection of CMV coat protein gene

CMV- coat protein specific primer reported by Cherian *et al*. (2004) was used in PCR to detect CMV coat protein gene and the c-DNA synthesised from the isolated RNA was used as template for PCR amplification as per section 3.2.2.1.

3.3.5.1. Agarose gel glectrophoresis

The PCR product was analysed on 1 per cent agarose as given in section 3.1.2.1.

3.3.6. PCR product purification

The CMV- CP amplicon obtained through the amplification of c-DNA using CMV-CP specific primer was purified using NucleoSpin Gel and PCR Clean-up Kit (REF 740609.10). The PCR product (100 µl) was mixed with 200 µl of NTI buffer. NucleoSpin Gel and PCR Clean-up column was placed into a collection tube (2 ml) and the sample was loaded up to 700 µl and centrifuged at 11,000 rpm for one min. The flow through was discarded and placed the column back into the collection tube. NT3 buffer (700 µl) was added to the NucleoSpin Gel and PCR Clean-up column and centrifuged at 11000 rpm for one min. The flow through was discarded and placed the column back into the collection tube. Then, centrifuged at 11,000 rpm for one min to remove NT3 buffer completely. NucleoSpin Gel and PCR Clean-up column was opened and incubated at 70 °C for 5 min to remove the traces of ethanol. Then, NucleoSpin Gel and PCR Clean-up column was placed into a new 1.5 ml microcentrifuged tube. NE buffer (15 µl) was added to the centre of the column and incubated at room temperature for 2 min. Centrifuged for one min at 11,000 rpm to elute the PCR product. Again, 10 µl of NE buffer was added to the centre of the column and incubated at room temperature for 2 min. Then, it was centrifuged for one min at 11,000 rpm to elute the large amount of PCR product. The column was discarded and the purified PCR product was stored at -20 °C.

The purified PCR product was analysed on one per cent agarose (section 3.1.1.1) and quantified using nanodrop (section 3.3.4.1). The purified PCR products along with CMV-CP specific forward and reverse primers (Cherian *et al*., 2004) were sent to Agri genome, Kakkanad, Kochi for sequencing. The sequence obtained was further analysed and homology of the best aligned sequence of the samples were compared with CMV- CP sample, using BLASTn analysis.

3.3.7. Molecular cloning

3.3.7.1. Ligation

The purified PCR product was ligated in pGEM- T Easy vector as per the manufacturer's protocol (Promega Corporation, USA) mentioned below;

Table 3.8: Composition of ligation mixture

Reagents	Quantity (µl)
2X Rapid ligation buffer	5.0
pGEM- T Easy vector (50 ng)	1.0
Purified PCR product	3.0
T4 DNA ligase (3weiss units/µl)	1.0
Total	10

The ligation mixture was centrifuged at 8,000 rpm for two min and incubated at 22 °C for 2 h. Then, the ligated mixture was stored at -20 °C for transformation.

3.3.7.2. Preparation of competent cells

The competent cells were prepared using $Mgcl_2$ and $CaCl_2$ to transform the recombinant plasmid into *E. coli* DH5α cells by following the protocol given by Mandel and Higa (1970) with some modifications.

The single colony of *E. coli* DH5α cell from plate was inoculated to 25 ml LB broth and incubated at 37 °C at 180 rpm for two h to obtain optimum OD value.Then the cells were transferred to pre chilled and sterile oakridge tube. The cell suspension was centrifuged at 5,000 rpm for 10 min at 4 °C and the supernatant was discarded. Then the cells were resuspended in 6.25 ml ice-cold and sterile 100 mM $Mgcl_2$ solution by gentle swirling while keeping on ice. The fully resuspended cells were kept on ice for 5 min. The cell suspension was centrifuged at 4,000 rpm for 10 min at 4 °C and the supernatant was discarded. Then the cells were resuspended in 1.25 ml of ice-cold and sterile 100 mM $CaCl_2$ solution by gentle swirling while keeping on ice. The fully resuspended cells were kept on ice for 20 min. The cell suspension was centrifuged at 4,000 rpm for 10 min at 4 °C and the supernatant was discarded. Then the cells were resuspended in 850 µl of ice-cold and sterile 100 mM $CaCl_2$ solution by gentle swirling while keeping on ice. 150 µl of sterile absolute

glycerol was added into the $CaCl_2$ treated cells and mixed by gentle swirling while keeping on ice. Then, the competent cells were pipetted as aliquots of 100 µl each into 1.5 ml microcentrifuge tube. The cells were snap-frozen in liquid nitrogen and stored at -80 °C deep freezer for future use.

3.3.7.3. Transformation

Transformation of recombinant plasmid (pGEMT/CMV- CP) in to competent *E. coli* strain DH5α cells was performed by following the standard protocol (Sambrook and Russell, 2001).

The ligated product (10 µl) was added to 100 µl of competent cells, the contents were mixed gently and kept on ice for 30 min. The tube was rapidly taken from ice and a heat shock was given at 42 °C exactly for 90 S in a water bath without shaking and immediately placed back on ice for 5 min. Then, 250 µl of LB broth was added to the cells and mixed by inverting the tubes twice. Then, the transformed cells were incubated at 37 °C at 180 rpm for one hour.

3.3.7.4. Blue- white screening of recombinants (Hanahan *et al*., 1985)

The transformed cells (100 µl) were plated on LB-agar/ampicillin (50 µg/ml) plates overlaid with X-gal (40 µl) and IPTG (40 µl), incubated at 37 °C, overnight. Later, blue and white colonies were observed. Individually selected each white colony and streaked on LB-agar/ampicillin (50 µg/ml) plates and incubated at 37 °C, overnight.

3.3.8. Confirmation of cloning (pGEM- T/CMV- CP) through colony PCR

The recombinant clones were analysed for the presence of insert by colony PCR using pGEMT-Easy plasmid primers T7 and SP6 (Promega Corporation, USA). Single white colonies were selected from subcultured plate and resuspended in 20 µl of steriled distilled water. Then, the cell suspension was heated at 98 °C for two min in a thermocycler and centrifuged at 10,000 rpm for 2 min. The supernatant (2 µl) was used as template for PCR. ThePCR reaction mixture is given in table 3.9.

Table 3.9: Composition of reaction mixture for colony PCR

Reagents	Quantity
Nuclease-free water	20.0 µl
Taq buffer A with 10 mM $MgCl_2$	2.5 µl
10 mM dNTP set	0.25 µl each
Primers (10 mM) Forward primer T_7 Reverse primer SP_6	 0.1 µl 0.1 µl
3 U/µl *Taq* Polymerase	0.2 µl
Template	2.0 µl

After the preparation of reaction mixture, the PCR tubes were centrifuged at 8,000 rpm for 2 min and placed in a thermocycler (Eppendorf Mastercycler Gradient) for PCR reaction.

The programme for colony PCR is given in table 3.10.

Table 3.10: The thermal profile for PCR

Step	Temperature (°C)	Time period
Initial denaturation	94	2 min
Denaturation	94	45 S
Annealing	55	1 min
Elongation	72	2 min
Final elongation	72	10 min
Hold	4	10 min

(30 cycles of denaturation, annealing and elongation)

3.3.8.1. Agarose gel electrophoresis

The amplified PCR product was analysed on one per cent agarose as given in section 3.1.2.1.

3.3.9. Sequence analysis

3.3.9.1. Isolation of pGEM- T/CMV- CP plasmid for sequence analysis

The recombinant plasmid was isolated from *E. coli* DH5α cells by alkali lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981) as given in

section 3.1.3.1.

3.3.9.1a. Agarose Gel Electrophorosis: The isolated plasmid was analysed in 0.8 per cent agarose as given in section 3.1.2.1.

3.3.9.2. Sequencing of recombinant plasmid

The pGEM- T/CMV- CP plasmid, which was confirmed through colony PCR was sent to Agri genome, Kakkanad, Kochi for sequencing. The sequence obtained was further analysed and homology of the best aligned sequence of the sample was compared with CMV- CP sample, using BLASTn analysis.

3.4 OVER EXPRESSION OF RECOMBINANT COAT PROTEIN GENE IN pET32a EXPRESSION VECTOR

As over expression of recombinant coat protein gene in pET28a was not satisfactory, another expression system with pET32a/CMV-CP was developed using *E. coli* BL21 (DE3) pLysS expression host. For that a set of new primer was designed along with recognition sites of restriction enzymes. CMV-CP gene was amplified using designed primer and cloned into *E. coli* BL21 (DE3) pLysS /pET32a expression system. The expression system was induced by IPTG for over expression of recombinant coat protein gene.

3.4.1 Primer designing and standardisation of annealing temperature

3.4.1.1 Designing of CMV- CP gene specific primer

The new primer was designed manually along with recognition sites of restriction enzymes for cloning of CMV-CP gene into pET32a expression vector. The complete coding region of the CMV-CP gene was identified by ExpASy Translate tool using reference isolate MF280290.1 for CMV. Forward primer was designed from the 5’ end of the CMV-CP gene. Reverse compliment of the sequence from the 3’ end of the gene was selected as the reverse primer. *Eco*R1 and *Xho*1 restriction

enzyme site in the pET32a vector sequence were selected and inserted at the 5' end of the forward primer and reverse primer for cloning (Table 3.11). It was also evaluated for frame shift mutation while translation of the protein due to cloning in the sites selected (Thompson *et al*., 1994).

Table 3.11. Designed primers of CMV CP for pET32a expression vector

Primer	Sequence (5'- 3')
CMV- FP (30 bp)	5' GGG **GAA TTC** ATG GAC AAA TCT GAA TCA ACC 3'
CMV- RP (29 bp)	5' CCC **CTC GAG** AAC TGG GAG CAC CCC AGA TG 3'

- *Eco*R1 recognition site (GAATTC)
- *Xho*1 recognition site (CTCGAG)

The designed primer was sent to Eurofins Genomics India Pvt. Ltd., Whitefield, Bangalore for synthesis.

3.4.1.2 Standardisation of annealing temperature for designed primer

The standardisation of annealing temperature of designed primer was determined by gradient PCR. The c-DNA synthesised from the isolated RNA was used as template for PCR amplification. The composition of PCR reaction mixture using the designed primer set is given in table 3.12.

Table 3.12. Composition of reaction mixture for gradient PCR

Reagents	Quantity
Nuclease-free water	20.0 µl
Taq buffer A with 10 mM $MgCl_2$	2.5 µl
10 mM dNTP set	0.25 µl each
Designed primers (10 pmol) Forward primer Reverse primer	 0.1 µl 0.1 µl
3 U/µl *Taq* Polymerase	0.2 µl
Template	2.0 µl

After the preparation of reaction mixture, the PCR tubes were centrifuged at 8,000 rpm for 2 min and placed in a thermocycler (Eppendorf Mastercycler Gradient) for gradient PCR using programme set at an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 S, annealing temperatures *viz*. 52.5 °C, 53 °C, 54.3 °C, 55 °C, 56.5 °C, 57 °C, 57.5 °C and 58.3 °C for one minute, elongation at 72 °C for 1 min 30 S and final elongation at 72 °C for 10 min. Among the range of temperature, temperature showing maximum amplification at 750 bp was selected as annealing temperature of the primer.

3.4.2 PCR amplication of CMV- CP gene using designed primer for protein expression studies

The recombinant pGEM-T Easy vector was used as template for PCR reaction. Phusion DNA polymerase was the enzyme used to amplify the CMV-CP gene during PCR. The PCR reaction mixture by using designed primer was given (Table 3.13).

Table 3.13. Composition of reaction mixture for PCR

Reagents	Quantity
Nuclease-free water	34 µl
1x HF buffer	10 µl
10 mM dNTP set	1 µl each
Designed primers (10 pmol) Forward primer Reverse primer	 0.1 µl 0.1 µl
6 % DMSO	1.5 µl
2 U Phusion DNA polymerase	0.5 µl
50 ng template	2.0 µl

After the preparation of reaction mixture, the PCR tubes were centrifuged at 8,000 rpm for 2 min and placed in thermocycler (Eppendorf Mastercycler Gradient) for PCR.

Table 3.14: The thermal profile for PCR

Step	Temperature (°C)	Time period
Initial denaturation	98	1 min
*Denaturation	98	30 S
*Annealing	55	30 S
*Elongation	72	35 S
Final elongation	72	10 min
Hold	4	5 min

(30 cycles of denaturation, annealing and elongation)

3.4.2.1 Agarose gel electrophoresis

The amplified PCR product was analysed in one per cent agarose as given in section 3.1.2.1.

3.4.3. PCR product purification

The CMV- CP amplicon obtained through the amplification of c-DNA using CMV-CP specific primer was purified using GeneiPure™ Quick PCR Purification Kit (Cat. No. 117310). The total volume of PCR product was made up to 100 µl with binding buffer. Then, 500 µl of binding buffer was added to 100 µl of PCR product and mixed thoroughly using a pipette. GeneiPure™ column was placed into collection tube (2 ml) and loaded the sample. Then, it was centrifuged at 10,000 rpm for one min. The flow through was discarded and placed the GeneiPure™ column back in the collection tube. The GeneiPure™ column was washed with 500 µl of wash buffer 1 and centrifuged at 10,000 rpm for one min. The flow through was discarded and placed the GeneiPure™ column back in the collection tube. Diluted 150 µl of wash buffer 2 with 600 µl of absolute ethanol (just before use). The GeneiPure™ column was washed with 700 µl of diluted wash buffer 2 and centrifuged at 10,000 rpm for one min. The flow through was discarded and placed the GeneiPure™ column back in the collection tube. Centrifuged at 10,000 rpm for two min to remove traces of wash buffer. The collection tube was discarded. GeneiPure™ column was opened and placed into a new 1.5 ml microcentrifuge tube and incubated at 70 °C for 5 min to remove the traces of ethanol. Then, 15 µl of elution buffer was added to the centre of

the column and incubated at room temperature for 2 min. Then, it was centrifuged for one min at 11,000 rpm to elute the PCR product. Again, 10 µl of elution buffer was added to the centre of the column and incubated at room temperature for 2 min. Centrifuged for one min at 11,000 rpm to elute the PCR product. The column was discarded and the purified PCR product was stored at -20 °C. Later, the purified PCR product was analysed in one per cent agarose (section 3.1.2.1) and quantified using nanodrop (section 3.3.4.1).

3.4.3a Agarose gel electrophoresis

The purified PCR product was analysed in one per cent agarose as given in section 3.1.2.1.

3.4.4. Cloning of coat protein gene to expression vector

3.4.4.1. Restriction digestion

The purified PCR product and pET32a expression vector were subjected to restriction digestion using *Eco*R1 and *Xho*1 restriction enzymes. The reagents used for restriction digestion of CMV- CP amplicon and expression vectors are enlisted below.

Table 3.15. Reagents used for restriction digestion of PCR product

Reagents	**Quantity** (µl)
Purified PCR product	7.0
0.5 U/µl *Eco*R1	0.5
0.5 U/µl *Xho*1	0.5
2X Tango buffer	2.0
Sterile water	NIL
Total volume	10.0

Table 3.16. Reagents used for restriction digestion of expression vector

Reagents	**Quantity** (µl)
pET32a plasmid	5.0
0.5 U/µl *Eco*R1	0.5
0.5 U/µl *Xho*1	0.5
2X Tango buffer	2.0
Sterile water	2.0
Total volume	10.0

The restriction digestion mixture was centrifuged at 8,000 rpm for 2 min and incubated at 37 °C for 4 h.

3.4.4.1a. Agarose gel electrophoresis

The digested PCR product and digested plasmid were analysed in one per cent agarose (section 3.1.2.1) and quantified using Nanodrop (section 3.3.4.1).

3.4.4.2. Digested PCR product purification

The CMV- CP amplicon obtained through the amplification of c-DNA using CMV-CP specific primer was purified using NucleoSpin Gel and PCR Clean-up Kit (REF 740609.10) as per section 3.4.3.

3.4.4.2a. Agarose gel electrophoresis

The digested purified PCR product was analysed in one per cent agarose as given in section 3.1.2.1.

3.4.4.3. Ligation of CMV- CP gene with expression vector

A molar ratio of 1:10 vector to insert was followed for ligation. Molar ratio (1:10) was calculated using the formula:

$$\frac{\text{ng vector} \times \text{size of insert} \times 10}{\text{size of the vector} \times 1} = \text{ng of the insert}$$

Table 3.17. Composition of ligation mixture

Reagents	Quantity (µl)
T4 DNA ligase (3 weiss units/µl)	1.0
1X Ligase buffer	1.0
Digested PCR product	7.0
Vector (pET32a)	1.0
Total volume	10.0

Ligation mixture was centrifuged at 8,000 rpm for 2 min and incubated at 16 °C, overnight.

3.4.4.4. Preparation of competent cells

The competent cells were prepared to transform the recombinant plasmid into *E. coli* DH5α cells by following the protocol given by Mandel and Higa (1970) as per section 3.3.7.2.

3.4.4.5. Transformation

Transformation of ligated plasmid (pET32a/CMV- CP) in to competent *E. coli* strain DH5α cells and selection was performed by following the standard protocol (Sambrook and Russell, 2001).

The ligated product (10 µl) was added to the 100 µl of competent cells, the contents were mixed gently and kept on ice for 30 min. The tube is rapidly taken from ice and a heat shock was given at 42 °C exactly for 90 S in a water bath without shaking and immediately placed back on ice for 5 min. Then, 250 µl of LB broth was added to the cells and mixed by inverting the tubes twice. Then, the transformed cells were incubated at 37 °C at 180 rpm for one h and centrifuged at 10,000 rpm for 2 min. Then, the bacterial pellet was dissolved in 100 µl of supernatant and plated on to LB agar supplemented with 50 µg/ml ampicillin.

3.4.5. Confirmation of cloning (pET32a/CMV- CP)

3.4.5.1. Confirmation of cloning through colony PCR

The recombinant clones were analysed for the presence of insert by colony PCR

using CMV-CP specific primers as given in section 3.2.2.

3.4.5.1a. Agarose gel electrophoresis

The amplified PCR product was analysed in one per cent agarose as given in section 3.1.2.1.

3.4.6. Sequence analysis

3.4.6.1. Isolation of pET32a/CMV- CP plasmid for sequence analysis

The recombinant plasmid was isolated from *E. coli* DH5α cells by alkali lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981) as given in section 3.1.3.1.

3.4.6.1a. Agarose gel electrophoresis

The isolated plasmid was analysed in 0.8 per cent agarose as given in section 3.1.2.1.

3.4.6.2. Sequencing of recombinant plasmid

The pET32a/CMV- CP plasmid, which was confirmed through colony PCR was sent to Agri genome, Kakkanad, Kochi for sequencing. The sequence obtained was further analysed and homology of the best aligned sequence of the sample was compared with CMV- CP sample, using BLASTn. The CMV- CP clone in recombinant plasmid, was selected for protein expression analysis as it has shown maximum homology with NCBI deposited samples.

3.4.7. Over expression of recombinant coat protein in pET32a/CMV-CP

3.4.7.1. Transformation of pET32a/CMV-CP plasmid into *E. coli* BL21 cells

3.4.7.1a. Preparation of competent cells

The competent cells were prepared to transform the recombinant plasmid into *E. coli* BL21 (DE3) pLysS cells by following the protocol given by Mandel and Higa (1970) as given in section 3.3.7.2.

3.4.7.1b. Transformation

Transformation of ligated plasmid (pET32a/CMV- CP) in to competent *E. coli* strain BL21 (DE3) pLysS cells and selection was performed following the standard protocol (Sambrook and Russell, 2001) as given in section 3.2.1.2.

3.4.8. Confirmation of cloning (pET32a/CMV- CP)

3.4.8.1. Confirmation of cloning through colony PCR

The recombinant clones were analysed for the presence of insert by colony PCR using CMV-CP specific primers as given in section 3.2.2.

3.4.8.1a. Agarose gel electrophoresis

The amplified PCR product was analysed in one per cent agarose as given in section 3.1.2.1.

3.4.9. Standardisation of expression of coat protein from recombinant plasmid

Single *E. coli* BL21 (DE3) pLysS /pET32a/CMV-CP cell was inoculated into 25 ml of LB/ampicillin (50 µg/ml)/chloramphenicol (34 µg/ml) medium and incubated at 37 °C, at 180 rpm, overnight. After 12 h, 1 ml of primary culture was transferred into 5 ml of LB broth/ampicillin (50 µg/ml)/chloramphenicol (34 µg/ml) medium and incubated at 37 °C, at 180 rpm for 4 h. From the incubated culture, 3 ml was taken as uninduced and 3 ml was taken for induction. Then, 3 ml culture was induced with 0.3 mM IPTG. The induced and uninduced cultures were incubated at 37 °C for 3 h, 4 h, 5 h and 20 °C for 16 h at 180 rpm shaker incubator. Later, each vial was centrifuged at 8,000 rpm for 15 min at 4 °C and the supernatant was discarded. The bacterial cell pellet was lysed by using tractor buffer (section 3.2.3a.)/ sonicator (section 3.2.3b.) and the expression of coat protein was evaluated as given in section 3.2.4.

Results

4. RESULTS

The research on “Evaluation of expression clones for recombinant coat protein of Cucumber mosaic virus infecting banana” was conducted during the period 2019-2022. The results of various studies carried during this research are described below.

4.1 EVALUATION OF EXISTING RECOMBINANT EXPRESSION CLONES FORCMV COAT PROTEIN GENE

The existing recombinant expression clones of CMV coat protein in *E. coli* DH5α were evaluated by plasmid isolation and restriction digestion. The recombinant plasmids pRSET-C/CMV-CP (3 kb) and pET28a/CMV-CP (5 kb) were isolated from *E. coli* DH5α cells. For confirmation of CMV coat protein gene restriction digestion was carried out using *Nhe*1 and *Bam*H1 restriction enzymes. Insert having 750 bp CMV-CP gene was released from pET28a plasmid. But, there was no any release of CMV-CP gene from pRSET-C plasmid (Plate 1A). Hence, the further research was carried out by this confirmed recombinant pET28a/CMV-CP expression vector.

The confirmed pET28a expression vector along with CMV-CP insert was transformed into *E. coli* DH5α cells to amplify the clone. White colonies were observed on LB agar supplemented with kanamycin (Plate 1B). Randomly selected individual transformed colonies from transformed plate was subcultured on LB agar plate supplemented with kanamycin.The recombinant pET28a plasmid was reisolated from randomly selected DH5α cells by alkali lysis method. The recombinant pET28a plasmid having 5 kb molecular size was observed on 0.8 per cent agarose (Plate 2A). Later, it was subjected to restriction digestion using *Nhe*1 and *Bam*H1 restriction enzymes for the confirmation of CMV coat protein gene. Insert having 750 bp CMV-CP gene was released from pET28a (5 kb) plasmid as given in plate 2B.

4.2 OVER EXPRESSION OF CMV-CP GENE IN pET28a EXPRESSION VECTOR

The recombinant pET28a/CMV-CP plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS cells for overexpression of protein. White colonies were observed on LB agar supplemented with kanamycin/chloramphenicol (Plate 3A and 3B). Presence of recombinant clone in transformed colonies were confirmed by colony PCR using specific CMV-CP primers as mention in section 3.2.2. CMV-CP gene got amplified at 750 bp from transformed BL21 (DE3) pLysS cells as given in plate 4.

The *E. coli* BL21 (DE3) pLysS cells harboring pET28a/CMV-CP were grown in LB broth until reached the log phase and induced with different concentration of IPTG. Induced culture along with uninduced culture were incubated at varying temperature and time. The incubated bacterial culture was lysed using tractor buffer (Protein purification kit)/Sonicator. The cell lysate of induced and uninduced culture were loaded on to 12 per cent SDS-PAGE for separation and to understand the protein profile. Standardisation of over expression of coat protein at different concentration of IPTG and incubated at varying temperature and time was given in table 4.1.

Table 4.1. Standardisation of overexpression of coat protein

IPTG Concentration	**Temperature**	**Time**	**Cell lysis**	**Result**
0.3 mM	20 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
	25 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
	30 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
	37 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression

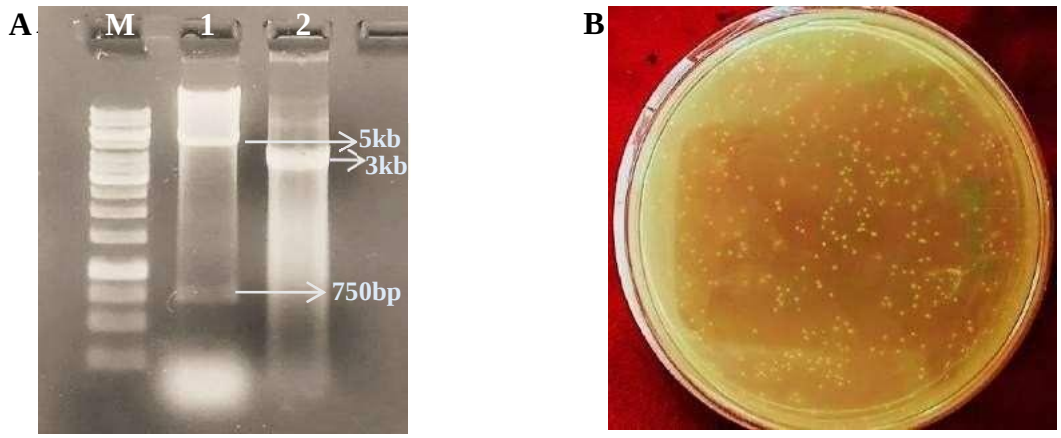


Plate 1. Confirmation of recombinant clones: A. Confirmation of recombinant clones by restriction digestion. M: 1 kb DNA ladder, Lane 1: pET28a/CMV-CP, Lane 2: pRSET-C/CMV- CP. The insert was released from Lane 1 clone. **B.** Plate with transformed DH5α/pET28a/CMV-CP cells on LB agar supplemented with kanamycin

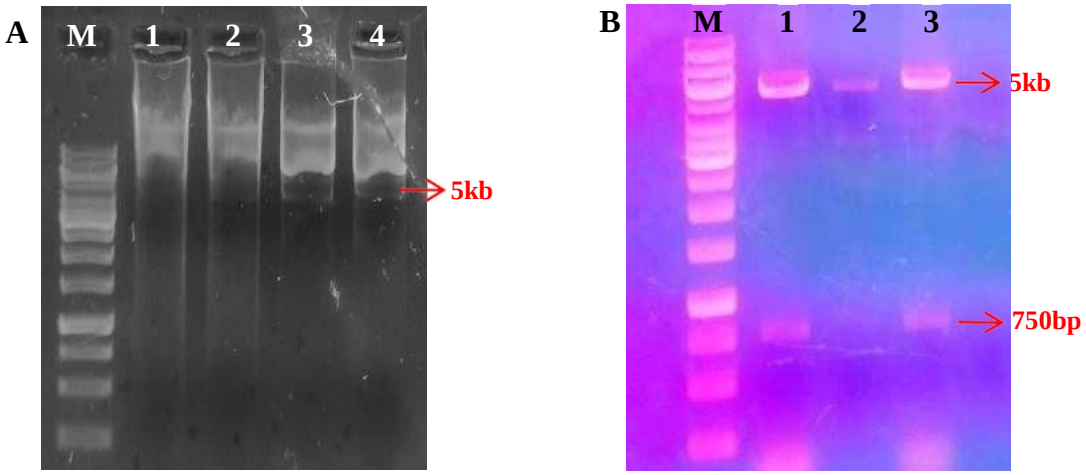


Plate 2. Confirmation of recombinant pET28a plasmid: A. Plasmid isolation from randomly selected transformed white colonies. M: 1 kb DNA ladder, Lane 1-4: pET28a/CMV-CP. **B.** Confirmation of recombinant clone by restriction digestion M: 1kb DNA ladder, Lane1-3: pET28a/CMV-CP. The insert was released from Lane 1 and Lane 3 clone

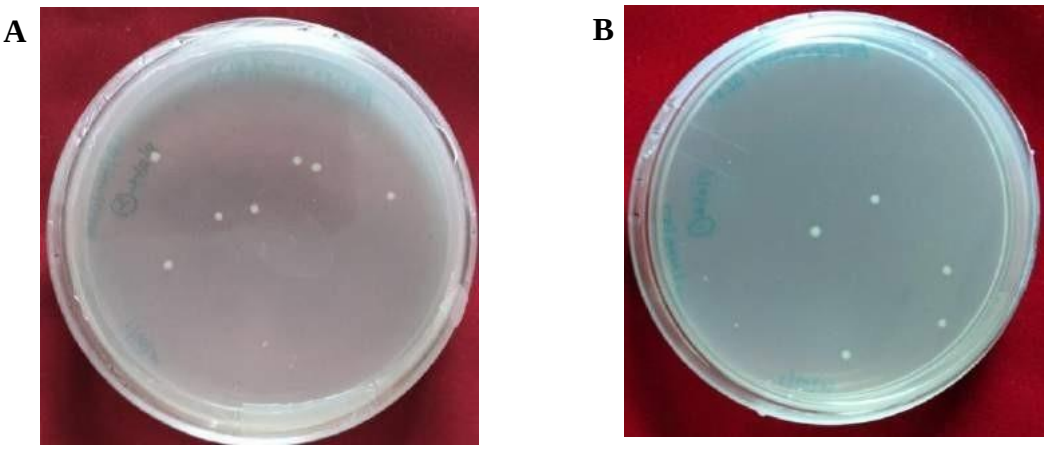


Plate 3. **Screening of transformants: 3A**, **3B** Plates with transformed BL21 pLysS /pET28a/CMV-CP cells on LB agar supplemented with kanamycin/chloramphenicol

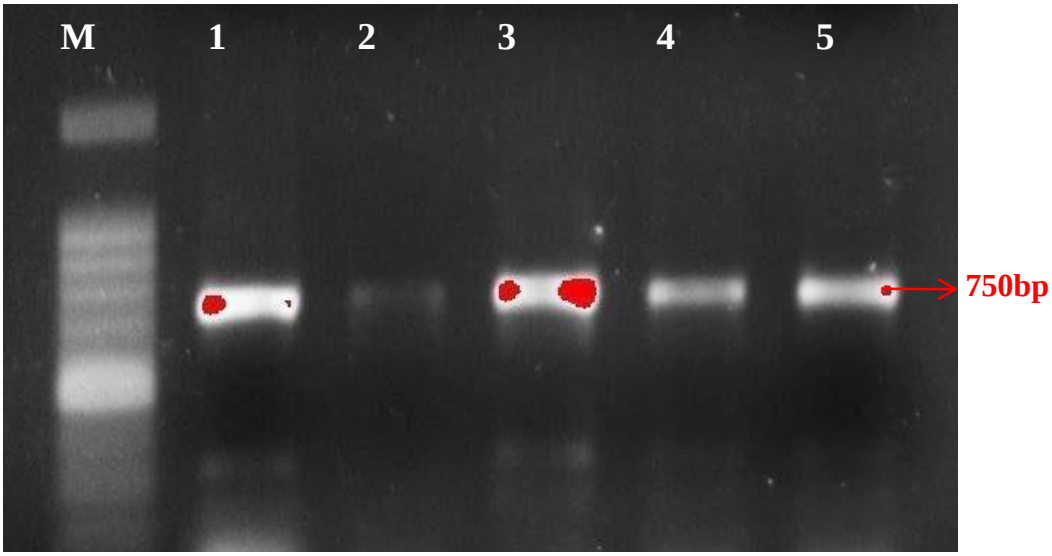


Plate 4. Confirmation of pET28a/CMV-CP clone by colony PCR: M: 100bp DNA ladder, Lane 1-5: randomly selected transformed colonies from plate 3A & 3B. CMV-CP gene got amplified at 750 bp from transformed BL21 pLysS cells

0.5 mM	20 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
	25 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
	30 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
	37 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
1 mM	**20 ℃**	4h 6h 8h 10h 12h 14h	Tractor buffer/ Sonicator	No expression
		16h	**Tractor buffer**	**Expression**
		16h	Sonicator	No expression
	25 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
	30 ℃	4h 6h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression
		8h	Sonicator	No expression
		8h	**Tractor buffer**	**Expression**
	37 ℃	4h 6h 8h 10h 12h 14h 16h	Tractor buffer/ Sonicator	No expression

In the SDS gel, expression was observed at 25 kDa corresponding to the CMV-CP in the culture induced by 1 mM IPTG at 30 °C for 8 h and 20 °C for 16 h incubation period in which the cell lysis was done using tractor buffer (Plate 5A and 5B). Confirmation of the coat protein was done by Western blotting. SDS-PAGE was run with suspected samples for Western blotting (Plate 6A) and the gel was blotted on

nitrocellulose membrane for confirmation (Plate 6B) as mentioned in section 3.2.5. There was no band observed corresponding to 25 kDa on nitrocellulose membrane after addition of primary and secondary antibody (Plate 6C).

As the production of recombinant coat protein was not successful in recombinant pET28a expression clone, experiments were conducted to develop a new recombinant expression clone using another expression vector pET32a.

4.3 DEVELOPMENT OF MOLECULAR CLONES OF CMV COAT PROTEIN GENE

Development of molecular clone of CMV coat protein gene is the first step of recombinant coat protein production. Development of molecular clone includes RNA isolation from infected samples, PCR amplification using CMV-CP specific primers, Ligation to pGEMT-easy vector, cloning to *E.coli* DH5α cells and its confirmation.

4.3.1 Collection of Cucumber mosaic virus infected banana samples

Cucumber mosaic virus infected banana leaf samples were collected from the net house and field of Banana Research Station, Kannara, Thrissur, Kerala, based on characteristic symptoms. The characteristic symptoms in different banana varieties were parallel chlorotic streaks and irregular wavy leaf margins (Namarai, AA), small spindle chlorotic lesions with yellow margins (Amrit sagar, AAA) and upper leaf curling, chlorotic mosaic symptoms and stunting of plants (Nendran, AAB) (Plate 7).

4.3.2. Preliminary assay by DAC-ELISA

Infected leaf samples of banana showing typical symptoms, along with healthy control (Nendran) were tested for CMV infection by DAC-ELISA using specific antiserum purchased from the National Research Centre for Banana, Trichy, Tamil Nadu. The plant extracts were coated on to ELISA plate as per the protocol mentioned in methods 3.3.2. Absorbance value at 405 nm more than twice that of negative control was considered positive (Plate 8). Absorbance values of test samples were mentioned in table 4.1.

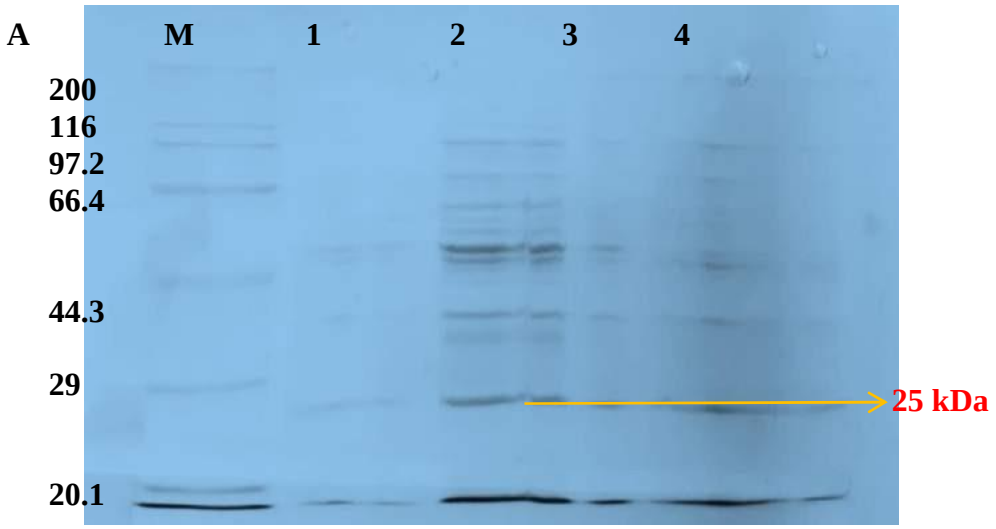


Plate 5A. Standardisation of overexpression in BL21/pET28a/CMV-CP: M: Protein molecular weight marker (kDa), Lane 1 and 2: 30 ℃, 8 h, uninduced and induced BL21/pET28a/CMV-CP cells, Lane 3 and 4: 30 ℃, 4 h and 6 h, induced BL21/pET28a/CMV-CP cells. Slight expression was observed at 25 kDa corresponding to CMV- CP

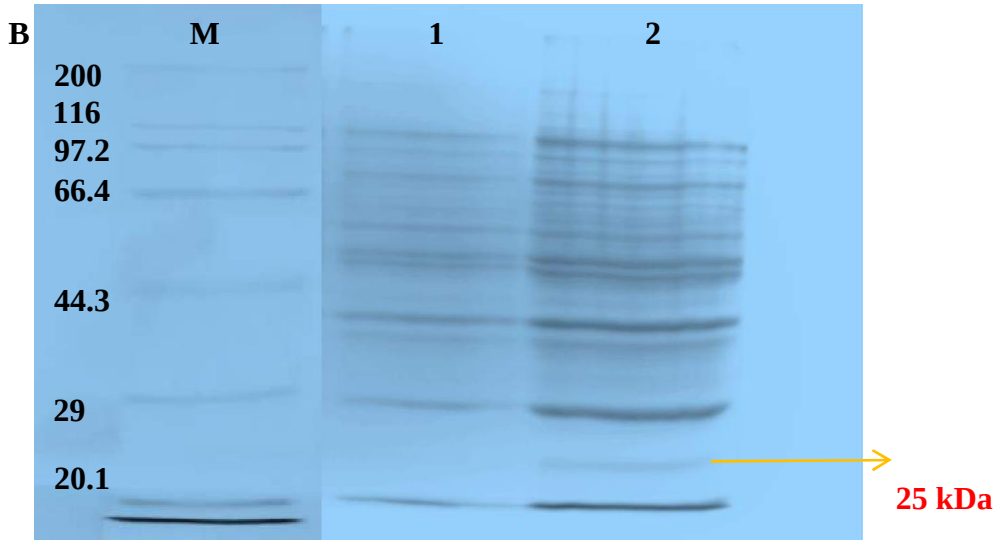


Plate 5B. Standardisation of overexpression in BL21/pET28a/CMV-CP: M: Protein molecular weight marker (kDa), Lane 1 and 2: 20 ℃, 16 h, uninduced and induced BL21/pET28a/CMV-CP cells by 1 mM IPTG.
Slight expression was observed at 25 kDa corresponding to CMV-CP in Lane 2

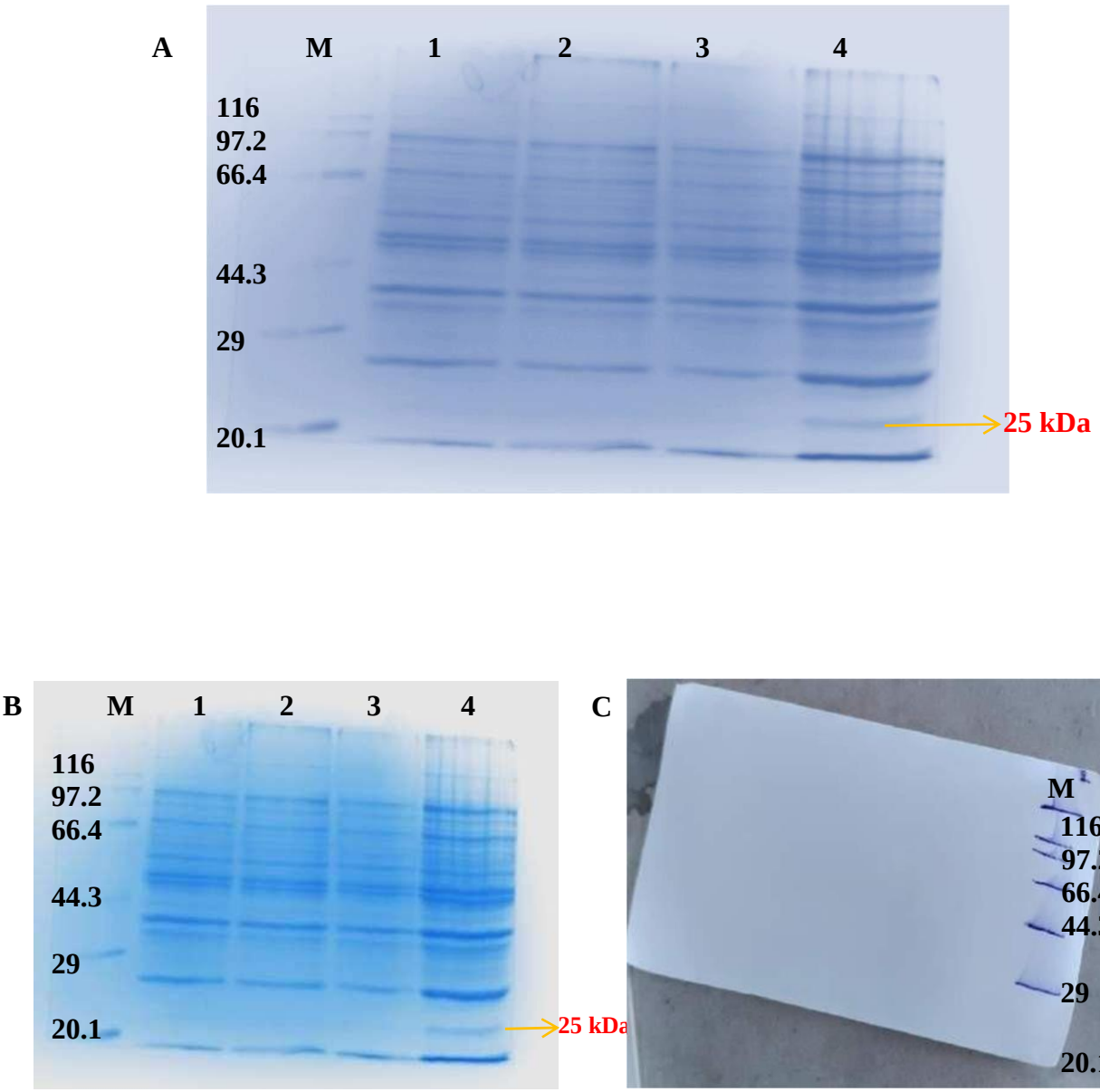


Plate 6. Over expression of pET28a/CMV-CP and confirmation: A. Recombinant CMV-CP overexpressed in BL21/pET28a/CMV-CP. M: Protein molecular weight marker (kDa), Lane 1 and 2: 30 ℃, 8 h, uninduced and induced culture, Lane 3 and 4: 20 ℃, 16 h, uninduced and induced culture. Slight expression was observed at 25 kDa corresponding to CMV-CP in Lane 4. **B.** Blotting the SDS-PAGE gel on nitrocellulose membrane. M: Protein molecular weight marker (kDa), Lane 1 and 2: 30 ℃, 8 h, uninduced and induced culture, Lane 3 and 4: 20 ℃, 16 h, uninduced and induced culture. **C.** Confirmation of over expression by western blotting. M: Protein molecular weight marker (kDa). No bands corresponding to 25 kDa was observed on nitrocellulose membrane

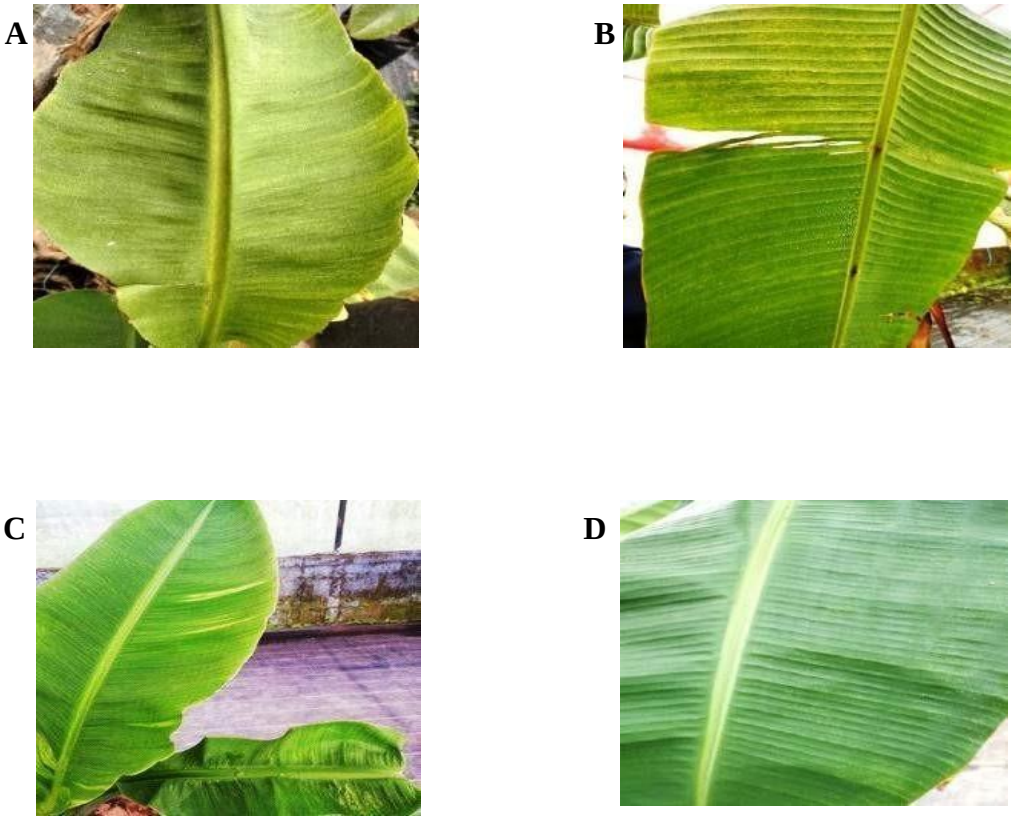


Plate 7. Symptoms of CMV infection in banana leaves of different varieties: A. Namrai, **B.** Amrit Sagar, **C.** Nendran and **D.** Nendran healthy (control)

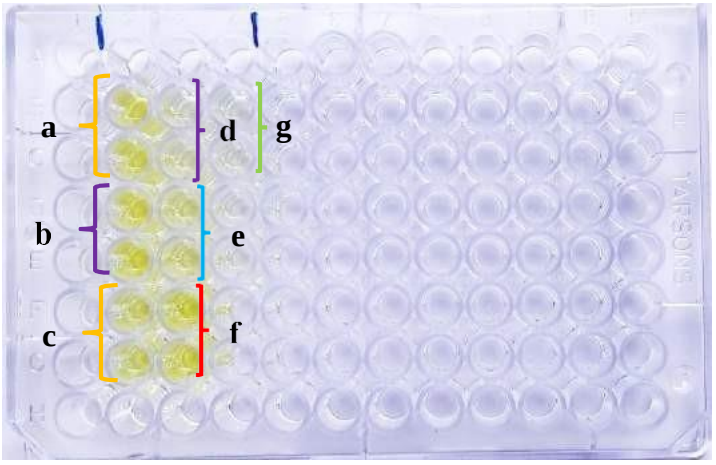


Plate 8. Detection of CMV infection by DAC-ELISA: a. KANM-1, **b.** KAAS-2, **c.** KANM-2, **d.** KAAS-2, **e.** KAND-1 **f.** NDRNS-4, **g.** KANH (control)

Table 4.2. Absorbance values of test samples through DAC-ELISA

Sample	Variety	Absorbance at 405 nm
KANM-1	Namrai	3.142
KAAS-1	Amrit Sagar	2.431
KANM-2	Namrai	1.192
KAAS-2	Amrit Sagar	2.547
KAND-1	Nendran	0.283
NDRNS-4	Nendran	**3.286**
KANH (Negative control)	Nendran	0.181

NDRNS-4 showed the highest (3.286) absorbance value at 405nm compared with negative control (0.181) followed by KANM-1 (3.142) and KAAS-2 (2.547) in DAC- ELISA. Hence, NDRNS-4, KANM-1 and KAAS-2 were selected for molecular detection using RT-PCR.

4.3.3. Total RNA isolation and RT-PCR

Total RNA was isolated from CMV infected leaf samples that revealed showed maximum intensity of infection in DAC-ELISA along with negative control by using two methods, *viz*. TRIzol reagent and RNeasy plant mini kit. RNA isolation using RNeasy plant mini kit provided the highest quality of RNA when assessed by gel electrophoresis and Nanodrop compared to TRIzol reagent method. A thick double band 25S and 18S RNA were observed in 0.8 per cent agarose without any DNA contamination (Plate 9). The quantity of isolated RNA by RNeasy plant mini kit was checked by Nanodrop and the results obtained are mentioned in table 4.3.

Table 4.3. Quantity of isolated RNA from CMV infected samples

Sample	A_{260}/A_{280} ratio	Concentration (ng/ µl)
NDRNS-4	1.98	90.2
KANM-1	1.98	86.6
KAAS-2	1.96	84.3

Maximum concentration of isolated RNA was observed from the samples NDRNS- 4 followed by KANM-1 and KAAS-2. Isolated RNA samples were incubated with Reverse transcriptase enzyme at appropriate conditions to produce c-

DNA as mentioned in section 3.3.4. The concentration of c-DNA synthesised were checked by using Nanodrop and the results obtained are given in table 4.4.

Table 4.4. Quantity of c-DNA synthesised from isolated RNA

Sample	A_{260}/A_{280} ratio	Concentration (ng/ µl)
NDRNS-4	1.78	3725.3
KANM-1	1.73	2643.8
KAAS-2	1.71	2430.2

Maximum concentration of c-DNA was observed from the samples NDRNS-4 followed by KANM-1 and KAAS-2. The PCR was performed with the synthesised c-DNA samples to amplify the CMV-CP gene using specific CMV-CP primers. An amplicon of 750 bp was obtained after RT-PCR (Plate 10).

The amplified PCR products were purified using NucleoSpin Gel and PCR Clean- up Kit. A pure amplicon having 750 bp molecular size was obtained after purification of PCR product as given in plate 11A and sent for sequencing. The pre-processed sequences obtained after sequencing were subjected to nucleotide BLAST analysis and identified as CP gene of CMV having 99.29 per cent identity with the sequences deposited in NCBI data base. Since, Nendran variety is the most common banana variety growing in Kerala and have high concentration of cDNA, the purified PCR product of NDRNS- 4 was taken for further studies.

4.3.4. Molecular cloning of CMV-CP/pGEM-T Easy vector

The purified PCR product of NDRNS- 4 was ligated to pGEM-T Easy vector and cloned to *E. coli*. DH5α cells by transformation. The clone was selected by blue-white screening on LB agar plate supplemented with ampicillin/X-gal/IPTG (Plate 11B). Randomly selected white colonies were streaked on LB agar plate supplemented with ampicillin (Plate 12). The subcultured white colonies were subjected to colony PCR usingT7 and SP6 primers for the confirmation of CMV-CP gene. Out of nine colonies selected, seven were positive clones that gave an amplicon of 750 bp as given in Plate 13A. pGEMT/ CMV-CP plasmid was isolated from positive clones by alkali lysis method.The recombinant pGEMT plasmid having 3 kb

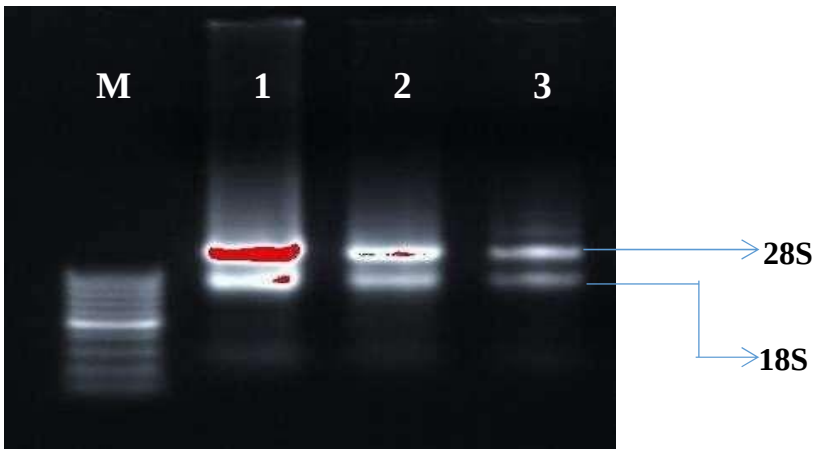


Plate 9. Total RNA isolation: Total RNA isolated from the infected banana leaves electrophoretically separated on 0.8 % agarose gel. M: 100 bp DNA ladder, Lane 1: NDRNS-4, Lane2: KANM1, Lane3: KAAS2

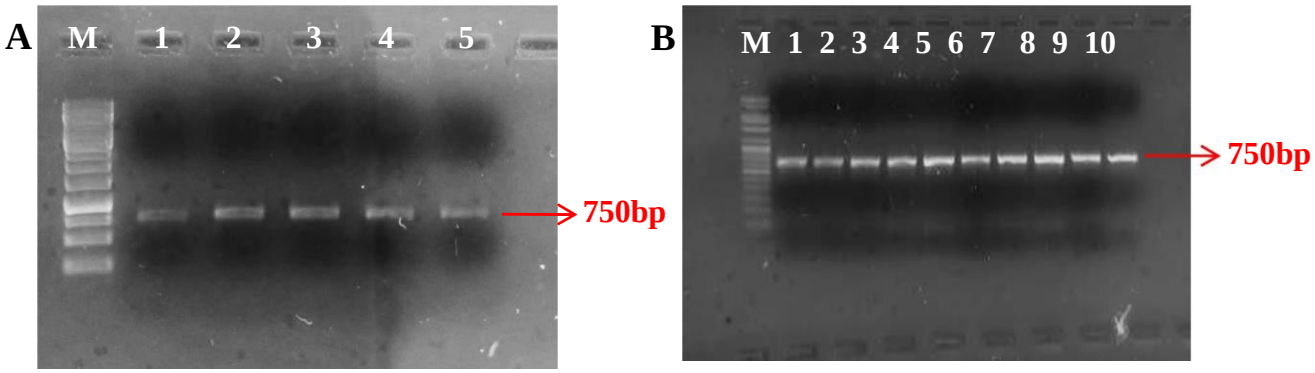


Plate 10. RT-PCR: Reverse transcriptase- PCR using CMV-CP specific primers to obtain 750 bp ampicon. **A.** M: 1 kb DNA ladder, Lane1, 2: KANM1. Lane3-5: KAAS2. **B.** M: 100 bp DNA ladder, Lane 1-10: NDRNS-4

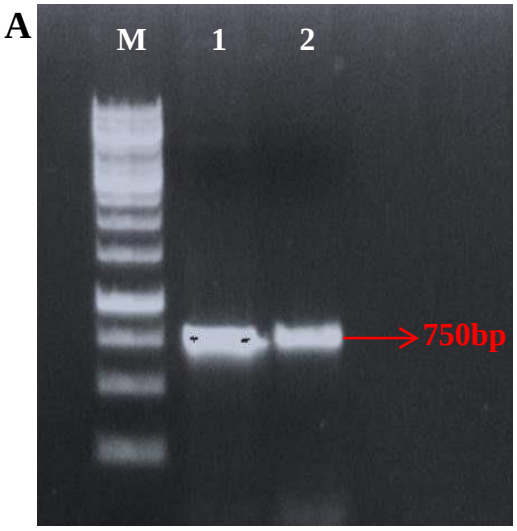
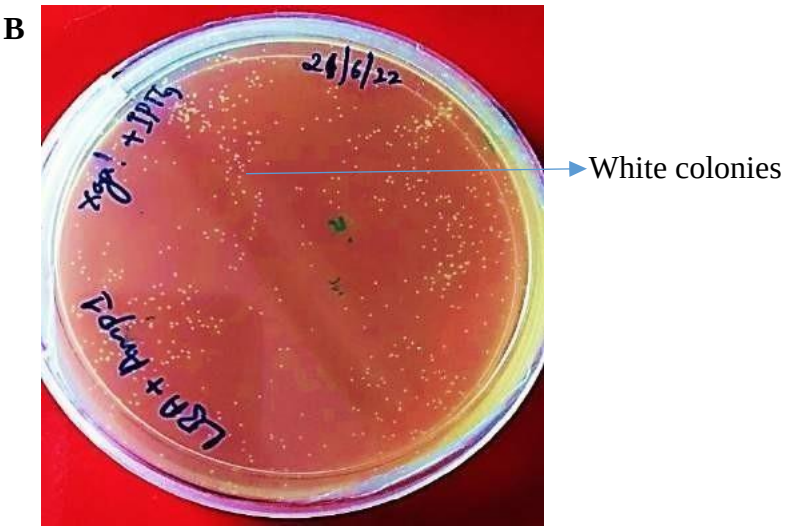


Plate 11. Molecular cloning: A Purification of PCR product**:** M: 1 kb DNA ladder, Lane 1, 2: NDRNS-4



B. Blue white screening of transformants: Plate B with transformed DH5α/ pGEMT/ CMV-CP cells on LB agar supplemented with ampicillin/X-gal/IPTG

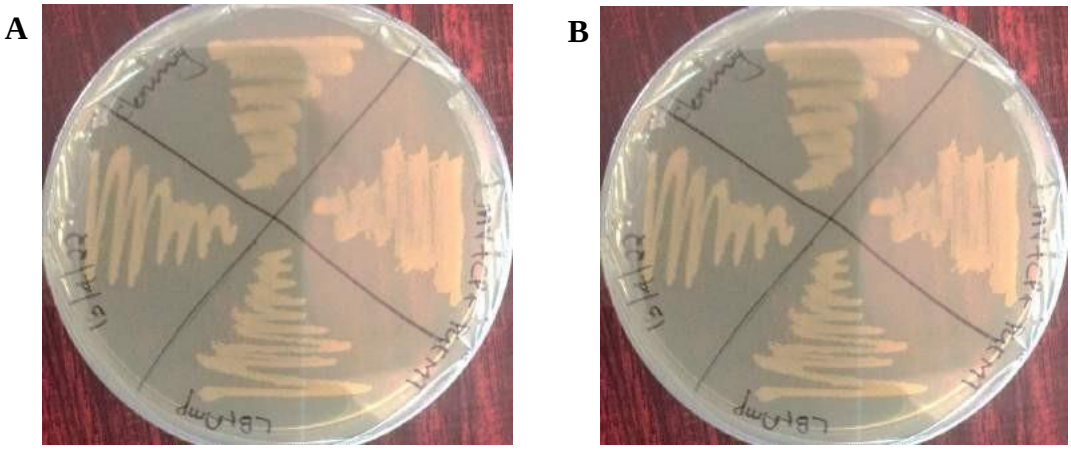


Plate 12. Selection of recombinant clones: A, B Plates with subcultured DH5α /pGEM-T/CMV-CP cells on LB agar supplemented with ampicillin

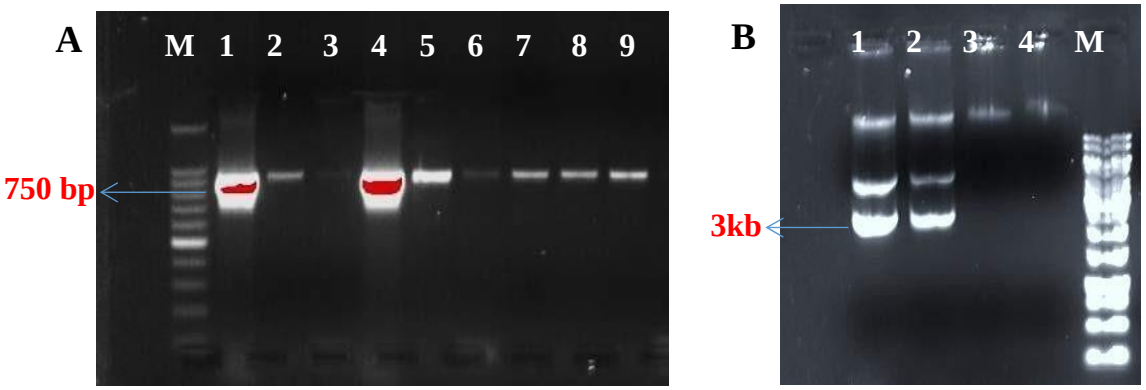
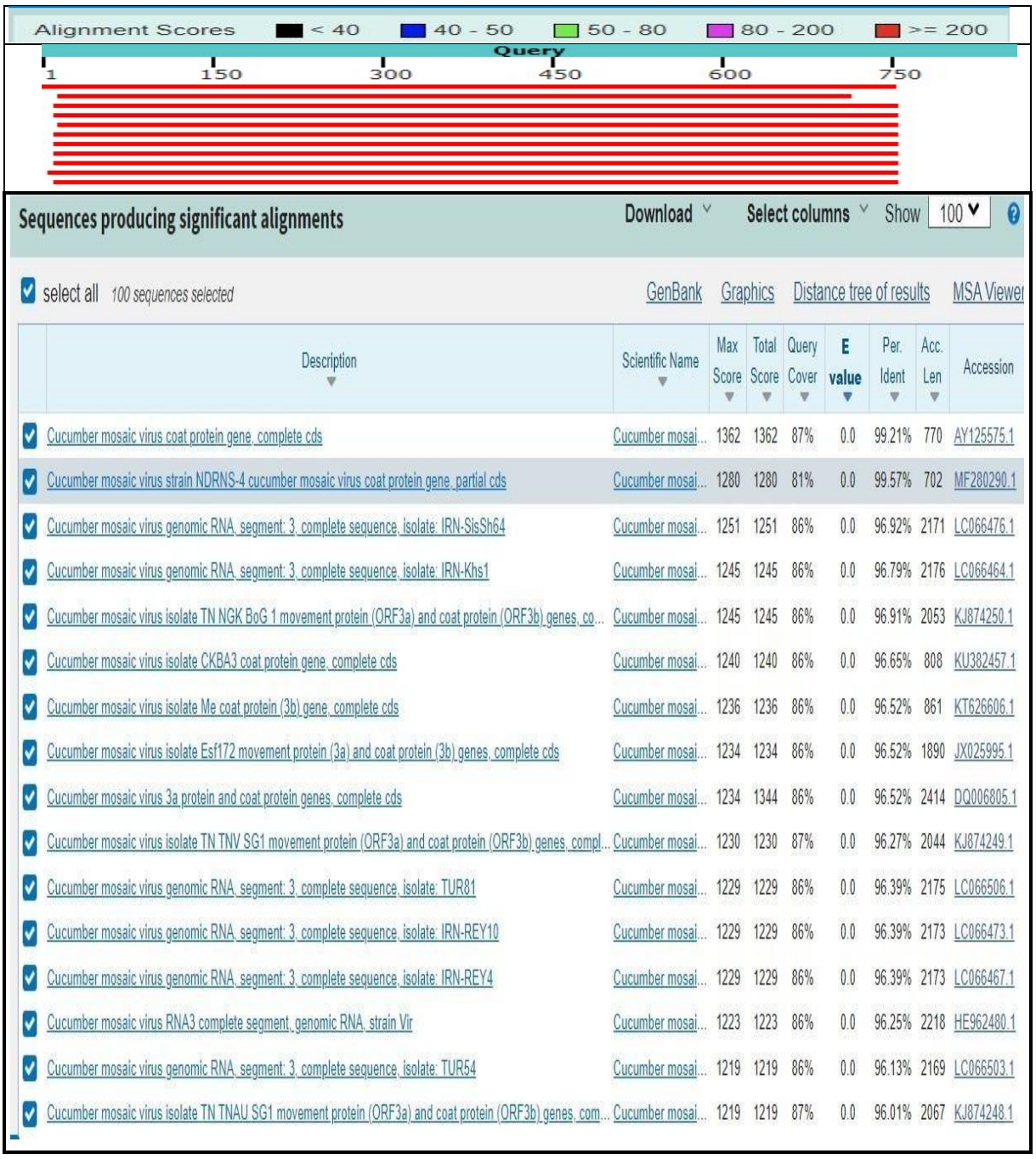


Plate 13. Confirmation of recombinant pGEM-T easy plasmid: A Confirmation of pGEM-T easy/CMV-CP clone by colony PCR M: 100 bp DNA ladder, Lane 1-9: randomly selected transformed colonies from plate 11A & B. CMV-CP gene got amplified at 750 bp from transformed DH5α cells. Samples 1,2,4,5,7,8,9 are positive clones. **B.** Plasmid isolation from positive DH5α clones M: 1 kb DNA ladder, Lane 1&2: pGEM-T easy/CMV-CP plasmid



Sequences producing significant alignments

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select all 100 sequences selected

GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cucumber mosaic virus coat protein gene, complete cds	Cucumber mosai...	1362	1362	87%	0.0	99.21%	770	AY125575.1
Cucumber mosaic virus strain NDRNS-4 cucumber mosaic virus coat protein gene, partial cds	Cucumber mosai...	1280	1280	81%	0.0	99.57%	702	MF280290.1
Cucumber mosaic virus genomic RNA, segment: 3, complete sequence, isolate: IRN-SisSh64	Cucumber mosai...	1251	1251	86%	0.0	96.92%	2171	LC066476.1
Cucumber mosaic virus genomic RNA, segment: 3, complete sequence, isolate: IRN-Khs1	Cucumber mosai...	1245	1245	86%	0.0	96.79%	2176	LC066464.1
Cucumber mosaic virus isolate TN NGK BoG 1 movement protein (ORF3a) and coat protein (ORF3b) genes, co...	Cucumber mosai...	1245	1245	86%	0.0	96.91%	2053	KJ874250.1
Cucumber mosaic virus isolate CKBA3 coat protein gene, complete cds	Cucumber mosai...	1240	1240	86%	0.0	96.65%	808	KU382457.1
Cucumber mosaic virus isolate Me coat protein (3b) gene, complete cds	Cucumber mosai...	1236	1236	86%	0.0	96.52%	861	KT626606.1
Cucumber mosaic virus isolate Esf172 movement protein (3a) and coat protein (3b) genes, complete cds	Cucumber mosai...	1234	1234	86%	0.0	96.52%	1890	JX025995.1
Cucumber mosaic virus 3a protein and coat protein genes, complete cds	Cucumber mosai...	1234	1344	86%	0.0	96.52%	2414	DQ006805.1
Cucumber mosaic virus isolate TN TNV SG1 movement protein (ORF3a) and coat protein (ORF3b) genes, compl...	Cucumber mosai...	1230	1230	87%	0.0	96.27%	2044	KJ874249.1
Cucumber mosaic virus genomic RNA, segment: 3, complete sequence, isolate: TUR81	Cucumber mosai...	1229	1229	86%	0.0	96.39%	2175	LC066506.1
Cucumber mosaic virus genomic RNA, segment: 3, complete sequence, isolate: IRN-REY10	Cucumber mosai...	1229	1229	86%	0.0	96.39%	2173	LC066473.1
Cucumber mosaic virus genomic RNA, segment: 3, complete sequence, isolate: IRN-REY4	Cucumber mosai...	1229	1229	86%	0.0	96.39%	2173	LC066467.1
Cucumber mosaic virus RNA3 complete segment, genomic RNA, strain Vir	Cucumber mosai...	1223	1223	86%	0.0	96.25%	2218	HE962480.1
Cucumber mosaic virus genomic RNA, segment: 3, complete sequence, isolate: TUR54	Cucumber mosai...	1219	1219	86%	0.0	96.13%	2169	LC066503.1
Cucumber mosaic virus isolate TN TNAU SG1 movement protein (ORF3a) and coat protein (ORF3b) genes, com...	Cucumber mosai...	1219	1219	87%	0.0	96.01%	2067	KJ874248.1

Fig 4.1. NCBI BLAST analysis to confirm the CMV-CP gene in pGEM-T/CMV-CP plasmid: Graphical summary and descriptions of CMV-CP gene with NCBI deposited samples in pGEM-T/CMV-CP plasmid

molecular size was observed on 0.8 per cent agarose as given in plate 13B. Then, the recombinant plasmid was sent for sequencing for further confirmation. The sequences were subjected to nucleotide BLAST analysis and identified as CP gene of CMV (Fig 4.1) having 99.57 per cent identity with the sequences deposited in the NCBI data base.

4.4. OVER EXPRESSION OF RECOMBINANT COAT PROTEIN GENE IN pET32a EXPRESSION VECTOR

As over expression of recombinant coat protein gene in pET28a was not satisfactory, another expression system with pET32a/CMV-CP was developed using *E. coli* BL21 (DE3) pLysS expression host. Different steps in development of new expression clone were primer designing, PCR amplification, ligation to pET32a expression vector, cloning to *E. coli* DH5α and BL21 cells and its confirmation. The expression system was induced by IPTG for overexpression of recombinant coat protein gene.

4.4.1. Primer designing and validation of designed primer for detection of CMV

New primer was designed along with recognition sites for restriction enzymes *Eco*R1 and *Xho*1. Annealing temperature was standardised by gradient PCR as mentioned in section 3.4.1.2. Highest intensity of amplification was observed at 55 °C followed by 54.3 °C, 56.5 °C, 57 °C, 57.5 °C and 58.3 °C. The lowest intensity was observed at 53 °C and 52.5 °C. Annealing temperature of designed CMV- CP primer was selected as 55 °C for PCR amplification (Plate14). The PCR was performed with pGEM- T/ CMV- CP plasmid as template using designed primer and phusion DNA polymerase for detection of CMV. An amplicon of 750 bp CMV-CP was obtained after PCR as given in Plate 15A. The amplified PCR product was purified using GeneiPure^{TM} Quick PCR Purification Kit. A pure amplicon having 750 bp molecular size was obtained after purification of PCR product as given in Plate 15B.

4.4.2. Molecular cloning of CMV-CP/pET32a expression vector

The purified PCR product of NDRNS- 4 was cloned to pET32a expression vector as per mentioned in section 3.4.4. For blunt cloning, restriction digestion site of *EcoR1* was added to the 5' end and *Xho1* was added to the 3' end of the CMV-CP gene using designed primer by PCR. Both purified CMV-CP amplicon and pET32a expression vector were subjected to restriction digestion by *Eco*R1 and *Xho*1 enzymes to develop blunt ends for cloning (Plate 16A). The digested CMV-CP amplicon was inserted to double digested pET32a vector and later, transformed into *E. coli*. DH5α cells. White colonies were observed on LB agar supplemented with ampicillin (Plate 16B). Randomly selected individual colonies from transformed plate was sub cultured on LB agar plate supplemented with ampicillin (Plate 17).

The recombinant pET32a plasmid was isolated from randomly selected DH5α cells by alkali lysis method. Out of eight colonies selected, two were positive clones that gave exactly 6 kb pET32a plasmid by isolation as given in Plate 18A. The subcultured 4^{th} and 8^{th} colony having pET32aplasmid were subjected to colony PCR using specific CMV- CP primers for the confirmation of CMV-CP gene. CMV-CP gene having 750 bp was amplified from both positive colonies (Plate 18B). Later, the isolated recombinant plasmid was sent for sequencing for further confirmation. The sequences obtained after sequencing were subjected to nucleotide BLAST analysis and identified as CP gene of CMV (Fig 4.2) having 98.87 per cent identity with the sequences deposited in the NCBI data base.

4.4.3. Over expression of recombinant coat protein in pET32a BL21 cells

The recombinant pET32a/CMV-CP plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS cells for overexpression of protein. White colonies were observedon LB agar supplemented with ampicillin/chloramphenicol (Plate 19). Randomly selected individual colonies from transformed plate were sub cultured on LB agar plate supplemented with ampicillin/chloramphenicol (Plate 20). The recombinant pET32a plasmid was isolated from randomly selected *E. coli* BL21 (DE3) pLysS cells by alkali lysis method. Out of twelve colonies selected, eight were positive clones that gave exactly 6 kb pET32a plasmid by isolation (Plate 21A). The

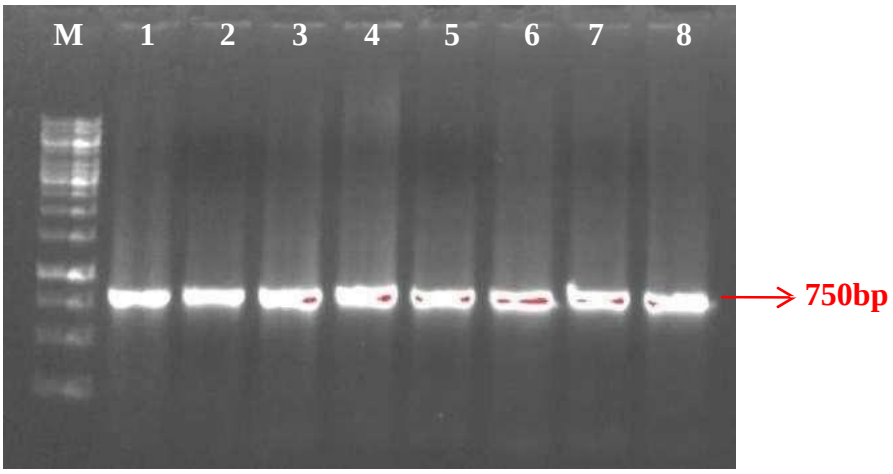


Plate 14. Standardisation of annealing temperature for CMV-CP designed primer using gradient PCR: M: 1 kb DNA ladder, Lane1-8: Amplification of annealing temperatures 52.5 ℃, 53 ℃, 54.3 ℃, 55 ℃, 56.5 ℃, 57 ℃, 57.5 ℃ and 58.3 ℃. Highest intensity of amplification was observed at 55 °C

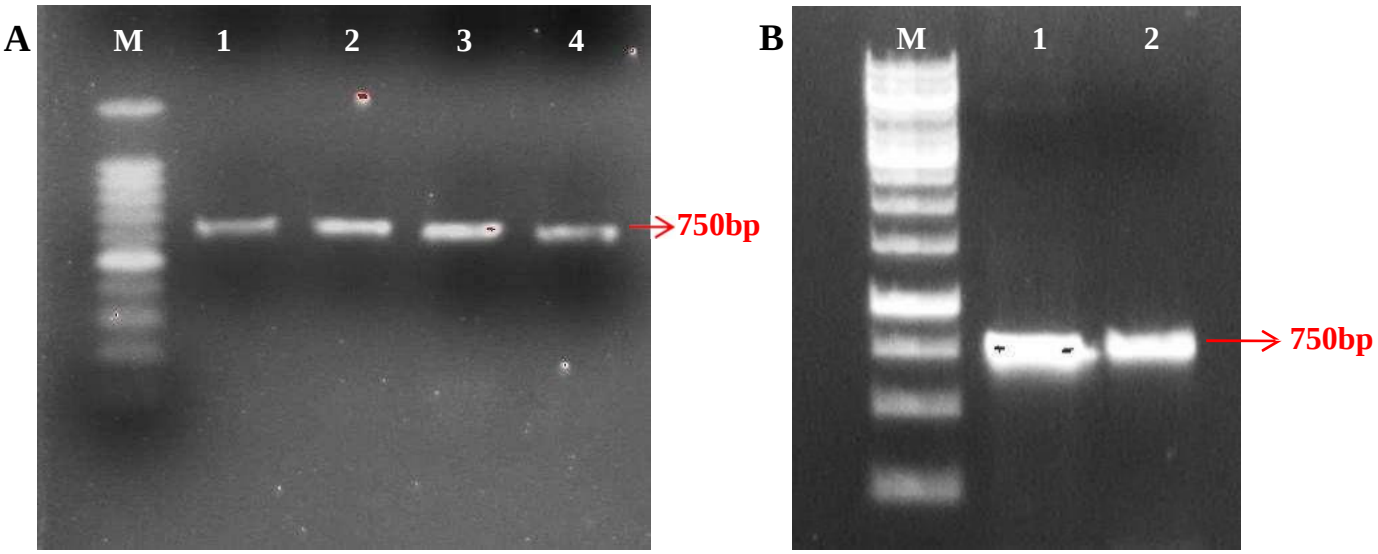


Plate 15. PCR amplification of CMV-CP using designed primer: A. PCR amplification usingCMV-CP designed primers to obtain 750 bp ampicon. M: 100 bp DNA ladder, Lane 1-4: pGEMT/CMV-CP Plasmid. **B.** Purification of PCR product**:** M: 1 kb DNA ladder, Lane 1, 2: CMV-CP amplicon

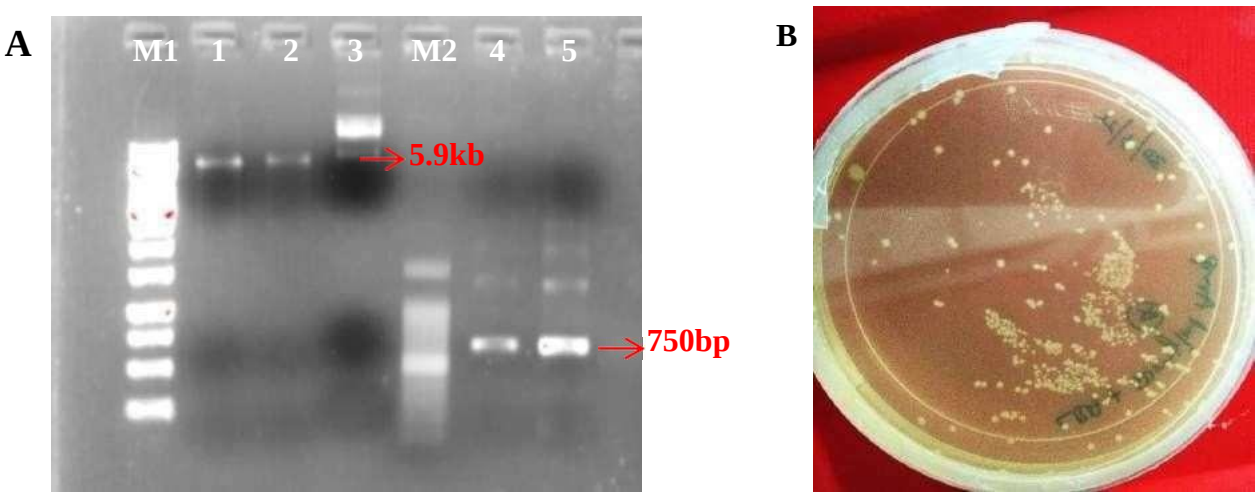


Plate 16. Molecular cloning: A. Restriction digestion of purified PCR product and pET32a plasmid using *EcoR1* and *Xho1* enzymes**:** M1: 1 kb DNA ladder, Lane 1, 2: Double digested pET32a plasmid, Lane3: Undigested pET32a plasmid. M2: 100 bp DNA ladder, Lane 4, 5: Doubledigested purified PCR product. **B.** Screening of transformants: Plate-B with transformed DH5α/pET32a/CMV-CP cells on LB agar supplemented with ampicillin

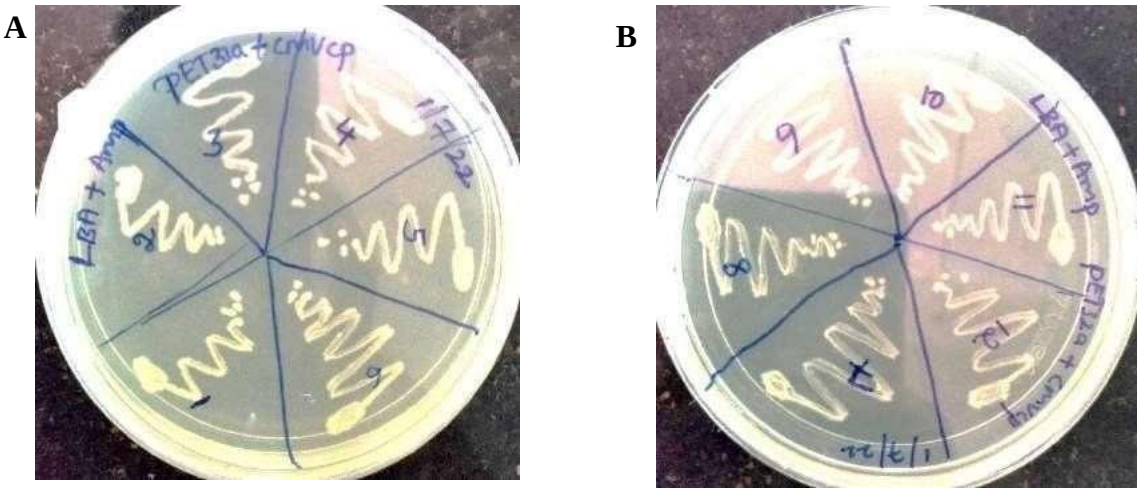


Plate 17. Selection of recombinant clones: A, B Plates with subcultured DH5α /pET32a/CMV-CP cells on LB agar supplemented with ampicillin

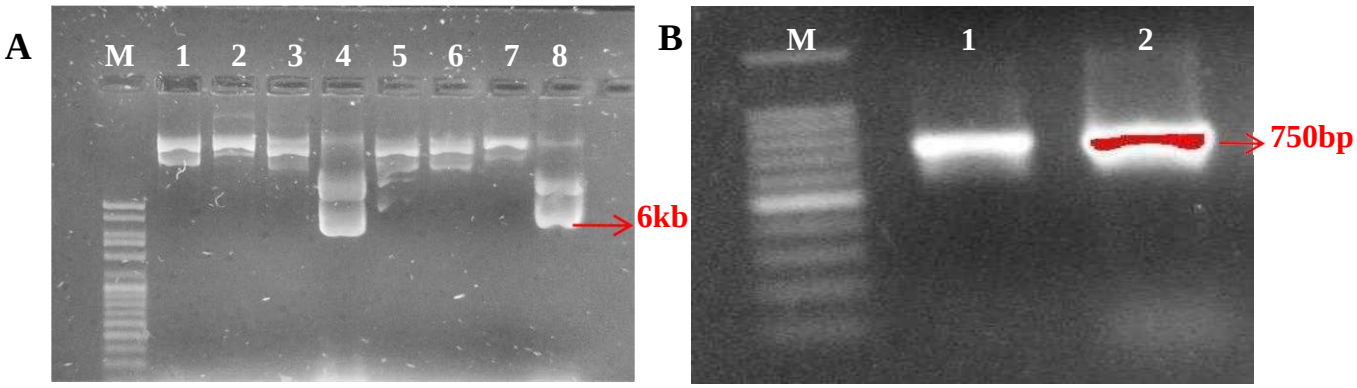


Plate 18. Confirmation of recombinant pET32a plasmid: A Plasmid isolation from randomly selected and subcultured DH5α clones M: 1k b DNA ladder, Lane 1-8: Plasmid isolation from subcultured DH5α cells. Lane 4&8 having 6 kb pET32a/CMV-CP plasmid. **B.** Confirmation of pET32a/CMV-CP clone by colony PCR M: 100 bp DNA ladder, Lane1&2: 4th and 8th colony from plate 17A & B having pET32a plasmid. CMV-CP gene got amplified at 750 bp from 4th and8th DH5α cells. Samples 4 and 8 are positive clones

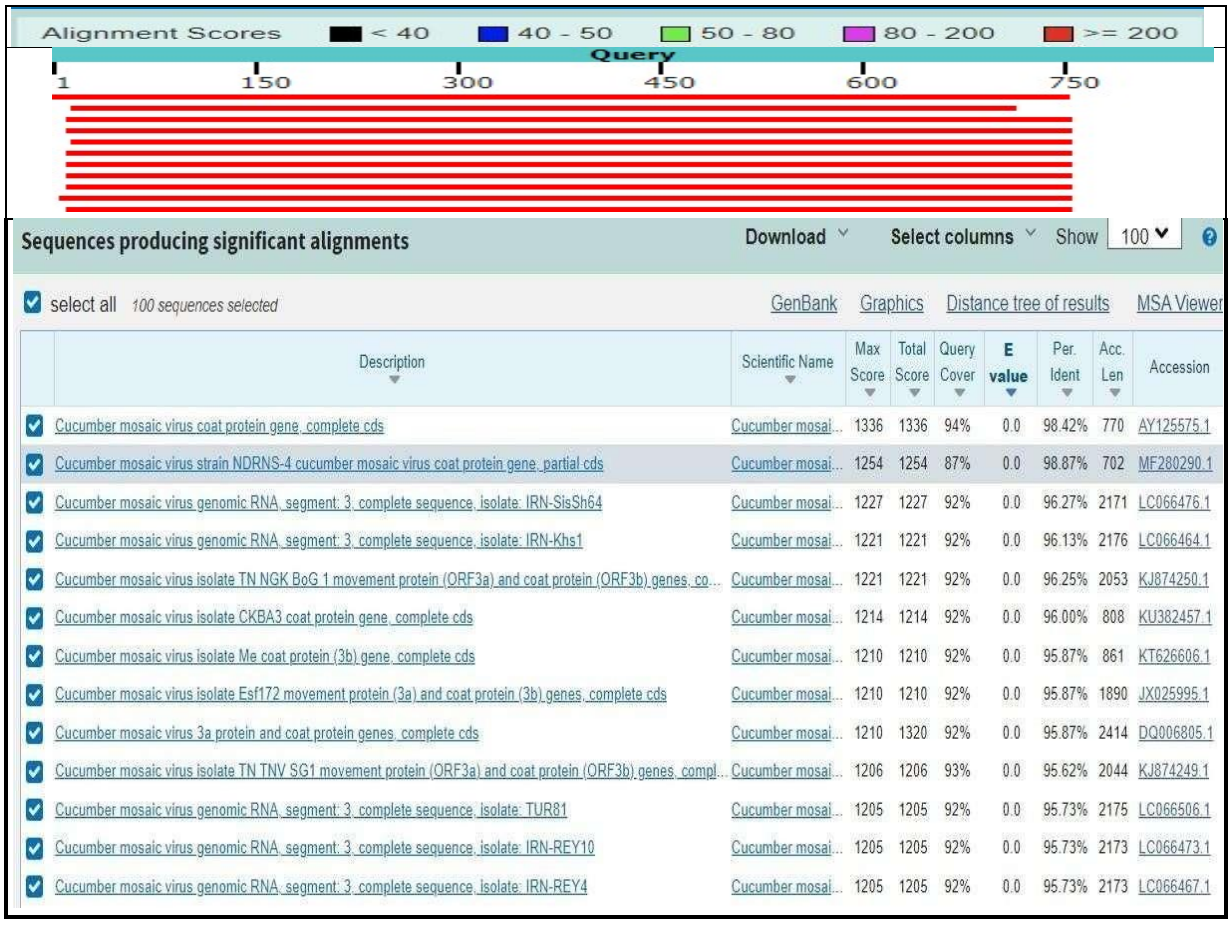
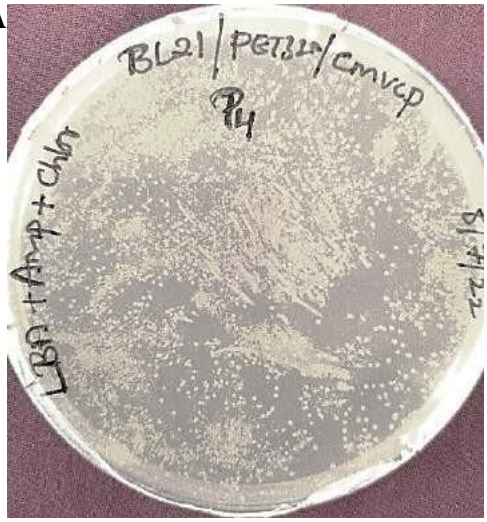


Fig 4.2. NCBI BLAST analysis to confirm the CMV-CP gene in pET32a/CMV-CP plasmid: Graphical summary and descriptions of CMV-CP gene with NCBI deposited samples in pET32a/CMV-CP plasmid

A



B

Plate 19. **Screening of transformants: A**, **B** Plates with transformed BL21 pLysS /pET32a/CMV-CP cells on LB agar supplemented with ampicillin/chloramphenicol

A

B

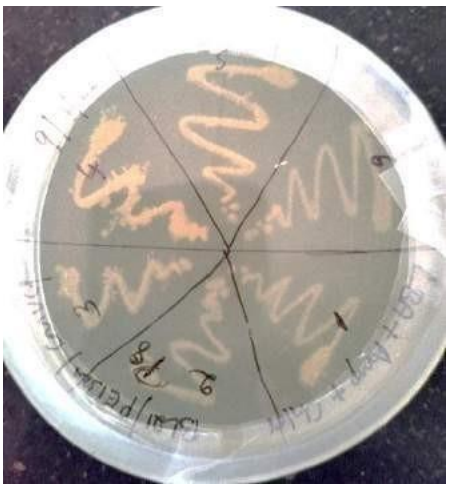


Plate 20. Selection of recombinant clones: A, B Plates with subcultured BL21 pLysS /pET32a/CMV-CP cells on LB agar supplemented with ampicillin/chloramphenicol

subcultured eight white colonies which were having pET32a plasmid were subjected to colony PCR by using specific CMV-CP primers for the confirmation of CMV-CP gene. CMV-CP gene having 750 bp was amplified from all eight positive colonies (Plate 21B).

The *E. coli* BL21 (DE3) pLysS cells harbouring pET32a/CMV-CP was grown in LB broth until reached the log phase and induced with 0.3 mM IPTG. Induced culture along with uninduced culture were incubated at 37 °C for 3 h, 4 h, 5 h and 20 °C for 16 h (Table. 4.5). The incubated bacterial culture were lysed using tractor buffe**r** (Protein purification kit)/sonicator. The cell lysate of induced and uninduced culture were loaded on to 12 per cent SDS-PAGE for separation and to understand the protein profile. In the gel, expression was noticed at 25 kDa corresponding to the CMV-CP in the induced fraction of incubated culture at 37 °C for 4 h (Plate 22).

Table 4.5. Standardisation of overexpression of coat protein in pET32a expression vector

IPTG Concentration	Temperature	Time	Cell lysis	Result
0.3 mM	**37 ℃**	3 h	Tractor buffer/Sonicator	No expression
		4 h	**Sonicator**	**Expression**
		4 h	Tractor buffer	No expression
		5 h	Tractor buffer/Sonicator	No expression
	20 ℃	16 h	Tractor buffer/Sonicator	No expression

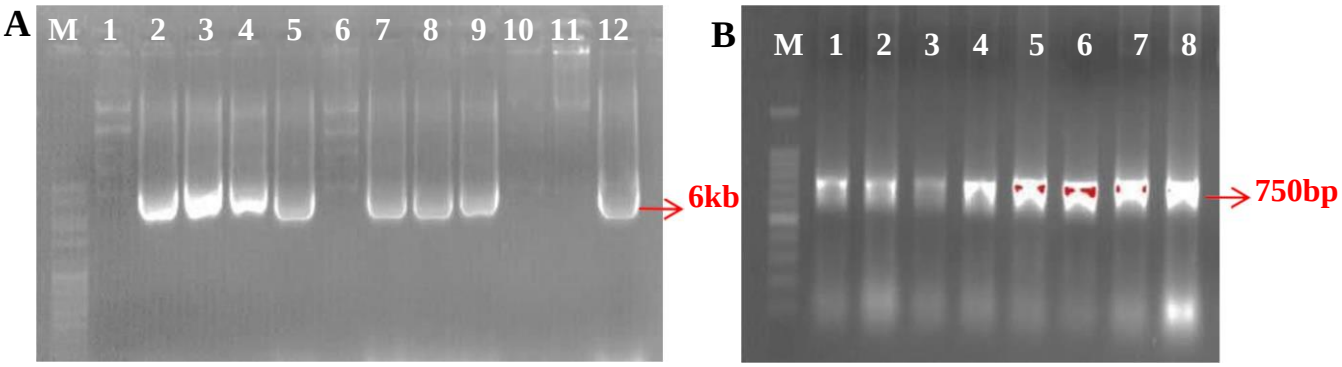


Plate 21. Confirmation of recombinant pET32a plasmid: A. Plasmid isolation from randomly selected and subcultured DH5α clones M: 1 kb DNA ladder, Lane 1-12: Plasmid isolation from subcultured DH5α cells. Lane 2,3,4,5,7,8,9 &12 having 6 kb pET32a/CMV-CP plasmid. **B.** Confirmation of pET32a/CMV-CP clone by colony PCR M: 100 bp DNA ladder, Lane1-8: subcultured colonies from plate 21A & B having pET32a plasmid. CMV-CP gene got amplifiedat 750 bp from DH5α cells

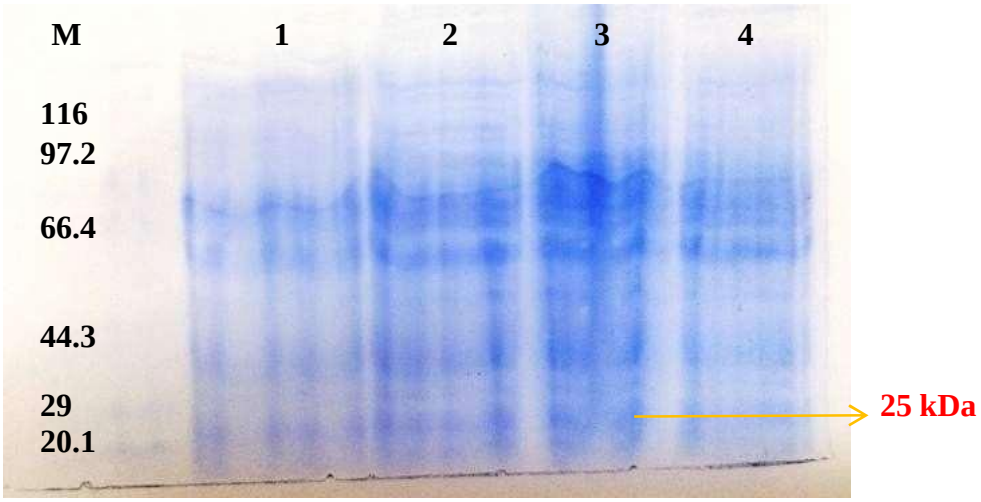


Plate 22. Standardisation of overexpression in BL21/pET32a/CMV-CP: M: Protein molecular weight marker (kDa), Lane 1and 2: 37 ℃, 3 h, uninduced and induced BL21/pET28a/CMV-CP cells, Lane 3 and 4: 37 ℃, 4 h induced and uninduced BL21/pET28a/CMV-CP cells. Slight expression was observed at 25 kDa corresponding to CMV-CP in Lane 3 induced culture

Discussion

5. DISCUSSION

Banana is a major fruit crop cultivated all over the world and it is threatened by various biological problems such as pests and diseases. In recent years, due to the increase in banana cultivating area and climate change, the frequency of pests and diseases had increased significantly, causing significant reduction in yield of banana cultivation. Several diseases, especially viral diseases, are considered a major threat to banana cultivation around the world (Magnaye and Valmayor, 1995). The viruses known to infect banana are Banana bunchy top virus, Banana bract mosaic virus, Cucumber mosaic virus, Banana streak mosaic virus and Banana mild mosaic virus (Gambley and Thomas, 2001; KAU, 2016). Cucumber mosaic virus disease is an important and emerging viral disease of banana in Kerala which leads to reduction in yieldby 40 to 100 per cent (Estelitta *et al.,* 1996).

In banana fields, primary infection of CMV occurs from planting of infected suckers and secondary spread of disease occurs through the aphid vectors in non-persistent manner (Zitter and Murphy, 2009). Due to repeated use of infected suckers from plants, the disease spreads and leads to gradual decrease in yield and quality (Magnaye and Valmayor, 1995; Dheepa and Paranjothi, 2010). According to Niblett *et al*. (1994) the most effective strategy for control of CMV in banana is using virus-free plants for banana cultivation. Many methods have been developed to detect plant viruses, such as microscopic observations, serological techniques, molecular methods, etc. (Webster *et al.,* 2004; Makkouk and Kumari, 2006; Lopez *et al.,* 2009). Among these, serological techniques especially ELISA with high quality antiserum has been used to index banana plants for CMV infection (Hu *et al*., 1995).

The present study entitled “Evaluation of expression clones for recombinant coat proteinof Cucumber mosaic virus infecting banana” was carried out with the objective to over express CMV coat protein using existing expression clones in *Escherichia coli* and to develop new expression clones for recombinant coat protein of CMV infecting banana, which can be utilized for producing high quality antiserum for the detection of CMV infecting banana.

The outline of present study is,

- Evaluation of existing recombinant expression clones for CMV coat protein gene
- Over expression of recombinant CMV coat protein gene in pET28a expression vector
- Development of molecular clones of CMV coat protein gene
- Over expression of recombinant CMV coat protein gene in pET32a expression vector

5.1. EVALUATION OF EXISTING RECOMBINANT EXPRESSION CLONES FOR CMV COAT PROTEIN GENE

Two expression clones of CMV-CP gene were developed by Antony (2019) using expression vectors pRSET-C and pET28a. The present study was carried out using available recombinant pRSET-C and pET28a expression clones. Recombinant pRSET-C and pET28a plasmids were isolated from *E. coli* strain DH5α cells by alkali lysis method. Restriction digestion was carried out to confirm the presence of CMV coat protein gene in the existing expression vectors. On digestion with *Nhe*1 and *Bam*H1 enzymes, the insert was released only from pET28a expression vector. The confirmed pET28a expression vector was taken forward for over expression of CMV coat protein.

The expression vectors pET28a and pRSET-C vectors have T_7 promoter system which is induced by IPTG for recombinant coat protein production and multiple cloning site for the incorporation of foreign gene. The cloning/expression region of the coding strand is transcribed by T7 RNA polymerase. The pET28a vector carries an N-terminal His- Tag/thrombin/T7 Tag sequence and C-terminal His-Tag sequence for purification of protein. It also contains kanamycin resistance gene for screening. The pRSET-C vector carries cleavable 6XHis-Tag for high level protein expression and contains ampicillin resistance gene for screening. The f1 origin present in both the vectors is oriented so that infection with helper phage produces virions containing

single-stranded DNA corresponding to the coding strand. Therefore, single stranded sequencing is performed using the T7 terminator primer (Sørensen and Mortensen, 2005; Rosano and Ceccarelli, 2014).

Use of pRSET-C expression vector for recombinant coat protein production was reported by earlier workers. It was used for Sugarcane streak mosaic virus by Hema *et al*. (2003), CMV infecting cucumber by Pandey (2015), Tobacco streak mosaic virus by Gulati *et al*. (2016), Pepper vein banding virus by Sabharwal (2017) and Banana bract mosaic virus and Banana bunchy top virus by Dilip (2020).

Similarly, the use of pET expression vector for recombinant coat protein production of plant viruses were reported in Carnation etched ring virus (Raikhy *et al.,* 2007), Banana bract mosaic virus (Selvarajan *et al.,* 2016) and Cucumber mosaic virus infecting cucumber (Koolivand *et al.,* 2017),

5.2. OVER EXPRESSION OF CMV-CP GENE IN pET28a EXPRESSION VECTOR

In the present study, *E. coli* strain BL21 (DE3) pLysS was selected as expression host for production of recombinant protein. *E. coli* strain denoted as DE3, lack 1on and ompT proteases and also carries the T_7 RNA polymerase gene in its chromosome under the control of lacUV- 5 promoter. Such strains produce protein from target genes cloned in pET expression vectors on induction with IPTG. *E. coli* strain along with pLysS plasmid encodes T7 lysozyme gene, which inhibits the leaky expression of T7 RNA polymerase prior to induction (Sørensen and Mortensen, 2005; Rosano and Ceccarelli, 2014).

The recombinant pET28a/CMV-CP plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS cells for over expression of protein. Use of *E. coli* BL21 (DE3) pLysS expression host was reported earlier by different workers, Raikhy *et al*. (2007) for Carnation etched ring virus coat protein gene, Selvarajan *et al.* (2016) for BBrMV coat protein gene, Koolivand *et al*. (2017) for CMV-CP coat protein gene. *E. coli* BL21 (DE3) pLysS/pET28a/CMV-CP was induced by Isopropyl β-d-1-thiogalactopyranoside (IPTG) which is an inducer for *E. coli* and pET expression

system. IPTG is the structural analogue of lactose that triggers transcription on binding to the Lac repressor. Therefore, it is used in recombinant protein production. Expression of target gene in expression vector was initiated by T_7 RNA polymerase which was encoded by bacterial genome in DE3 prophage. IPTG was added to the expression system, which triggers the release of LacI from lac-operator and allows RNA polymerase to initiate T_7 transcription in bacterial host. Then, T_7 RNA polymerase is able to activate the T_7 promoter on the expression vector and transcribes the recombinant CMV- CP gene in presence of IPTG (Sørensen and Mortensen, 2005; Rosano and Ceccarelli, 2014) (Fig 5.1).

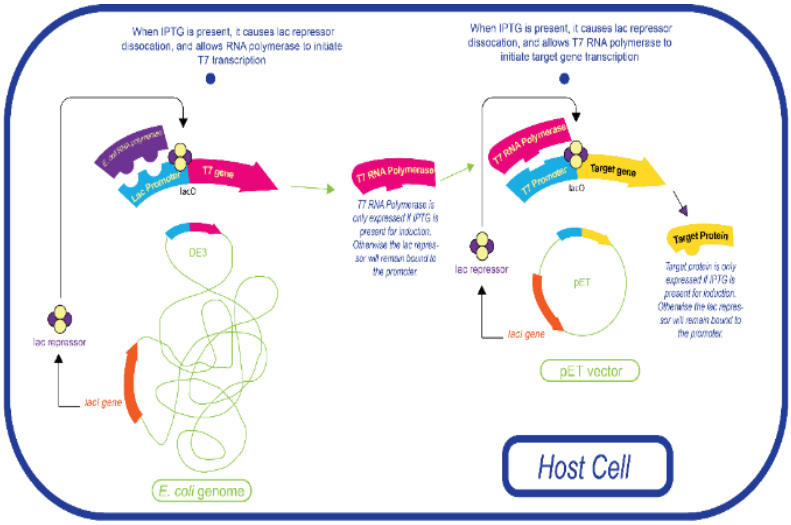


Fig 5.1. Overexpression of target gene in *E. coli* BL21 (DE3) pLysS and pET expression system

In the present study, *E. coli* BL21 (DE3) pLysS / pET28a/CMV-CP cells were inducedwith 0.3 mM, 0.5 mM and 1 mM IPTG and incubated at varying temperature and time. The incubated bacterial culture were lysed by chemical method using Tractor buffer which is from protein purification kit (Takara) and by mechanical method using Ultrasonic probe Sonicator (Lark). Usually, protein is produced inside the bacterial expression system. Sonication is performed by a sonication probe which applies high-frequency ultrasonic energy (generally >20 kHz) to the samples. The energy released from agitation, which disrupts the cell membrane that causes

ultimately cell lysis. Heat energy will be generated during sonication, so that the samples were placed on ice inside a glass beaker (Feliu *et al.,* 1998). Kim *et al*. (2016) induced *E. coli* BL21 (DE3)/pET21d/CMV-CP with 1 mM IPTG for expression of 26 kDa coat protein. Shibaei *et al*., (2018) induced *E. coli* BL21 (DE3)/ pET28a/Grape vine fan leaf virus coat protein expression system with 1 mM IPTG, incubated at 37 °C for 7 h and expressed 42 kDa coat protein.

The cell lysate was loaded on to 12 per cent SDS-PAGE for evaluation of over expression. A slight expression of 25 kDa CMV coat protein was observed in 1 mM IPTG induction, incubated at 30 °C, 8 h and 20 °C, 16 h in which the cells were lysed by using tractor buffer. Confirmation of the CMV coat protein was done by Western blotting. Western blotting enables the electrophoretic transfer of proteins from SDS gel onto nitrocellulose membrane. Later, the immobilised proteins were detected by antibody- antigen interaction (Towbin *et al*., 1979). Detection of CMV-CP by western blotting using polyclonal anti-CMV-CP antibody was reported by Lin *et al*. (2010); Khan *et al.* (2012); Rostami *et al*. (2014) and Antony (2019).

In the gel, even though a slight expression of CMV-CP was observed, no band was observed in Western blotting. The attempts to overexpress CMV-CP in pET28a was not successful. Later, experiments were conducted to develop new expression clone using pET32a expression vector.

5.3. DEVELOPMENT OF MOLECULAR CLONES OF CMV COAT PROTEIN GENE

Cucumber mosaic virus infected banana leaves were collected from net house and field. Parallel chlorotic streaks on leaves along with irregular wavy leaf margins were noticed in the samples from field as characteristic symptoms of Cucumber mosaic virus in Namarai variety. Small spindle shaped chlorotic lesions on young leaves with yellow margins were noticed as characteristic symptoms of Cucumber mosaic virus in Amrit sagar. Upper leaf curling, stunted plants along with parallel chlorotic streaks on leaves were also noticed in Nendran. Similar symptoms for CMV infection in banana were observed and recorded by various authors (Estellita *et al.*,

1996; Sivaprasad *et al*., 2016; Tripathi *et al*., 2016; Mujtaba, 2017; Antony, 2019).

Preliminary screening of CMV infected leaf samples was conducted by DAC-ELISA. Each samples along with control were tested in the laboratory using specific polyclonal CMVantiserum purchased from the National Research Centre for Banana, Trichy, Tamil Nadu. Generally, absorbance value at 405 nm more than twice that of negative control was considered positive. Out of 7 samples, NDRNS-4 showed higher absorbance value at 405 nm compared with negative control followed by KANM-1 and KAAS-2 in ELISA reader. DAC-ELISA is one of the most widely used serological tests for virus detection. The amount of virus present is proportionalto the amount of enzyme-labelled antibody to the antigen in the test solution. Among the serological techniques, DAC-ELISA is a simple, sensitive and adaptable laboratory technique used to detect the presence of CMV in banana samples (Mujtaba, 2017). In the present study, CMV infected samples with higher absorbance value at 405 nm, *viz.* NDRNS-4, KANM- 1 and KAAS-2 were selected for molecular cloning.

For molecular cloning, total RNA was isolated from CMV infected samples showing more absorbance in DAC-ELISA. According to Ghangal *et al*. (2009), isolation of good quality RNA is required for molecular experiments. Young leaves of infected samples are more suitable than older ones for isolating good quality RNA as they contain less amount of phenolics and tannins. In the present study, total RNA was isolated from infected young leaves by two methods *viz.* use of TRIzol reagent and RNeasy plant minikit. RNA isolation using RNeasy plant minikit provided good quality of RNA without any DNA and protein contamination.

The total RNA isolated from infected samples were converted into cDNA using Revert Aid First Strand c-DNA synthesis Kit. The synthesised cDNA was further utilised in PCR using specific CMV primers for the detection of CMV from infected samples. CMV coat protein gene got amplified at 750 bp from CMV infected samples by RT-PCR. Hu *et al*. (1995); Cherian *et al*. (2004) and Khan *et al*. (2012) also successfully amplified 750 bp CMV-CP gene from CMV infected samples. Similar results were also reported by Mujtaba (2017) and Antony (2019) from CMV infected banana samples from Kerala. The CMV amplicons of 750 bp were sequenced and

subjected to nucleotide BLAST and identified that CP gene of CMV was having 99.29 per cent similarity with the sequences deposited in NCBI data base. Similar results were reported by Cherian *et al*. (2004) and Mujtaba (2017). Since, Nendran variety is commonly growing in Kerala and NDRNS-4 amplicon was having highest cDNA concentration, PCR product of NDRNS- 4 was taken for further studies.

In the present study, the CMV- CP amplicon of NDRNS-4 was purified using NucleoSpin Gel and PCR Clean-up Kit and ligated to pGEM- T easy vector by T- A cloning for maintaining the gene. During PCR reaction, *Taq* DNA polymerase enzyme adds A at the 3'end of the product. This is made use of direct cloning of PCR products in vector containing T overhang like pGEM-T easy vector (Promega). Later, the ligated vector was transformed into *E. coli*. DH5α cells for molecular cloning. *E. coli*. Strain DH5α have high cell density and plasmid transformation with exogenous DNA is fast and easy (Rosano andCeccarelli, 2014).

The transformed colonies were selected based on blue-white screening, a technique used for the detection of successful transformants in molecular cloning. *E. coli*. has Lac complex which consists of lac-I and lac Z gene. The lac Z gene produces the enzyme β- galactosidase, which can break down a derivative of lactose called X-gal. This produces a blue dye which colours the colony blue. In *E. coli,* the expression of Lac Z gene is regulatedby the Lac I repressor protein which binds to the promoter and prevents transcription until lactose analogue is added. IPTG is a lactose analogue, which binds to the Lac I repressor protein and allows transcription of Lac I gene. Generally, pGEM-T vector encodes the N- terminal alpha-peptide of beta-galactosidase. The multiple cloning site in this vector was located within Lac Z gene. Cloning an insert into this site, will disrupt the transcription of Lac Z gene and recombinants will be unable to break down X-gal. When the recombinants are allowed to grow in medium containing X-gal and IPTG, the non- recombinant colonies will appear blue, where as the recombinants colonies will be white.

For the confirmation of CMV- CP gene in the recombinant white colonies, colony PCR was performed followed by plasmid isolation and sequencing. The insert was ligated to pGEM-T vector between T_7 and Sp6 promoter regions. Hence, the

universal specific primers, *viz*. T_7 and Sp6 primers were used to amplify 750 bp CMV-CP gene through colony PCR. The recombinant plasmid was sent for sequencing for further confirmation. The sequences were subjected to nucleotide BLAST and identified as CP gene of CMV having 99.57 per cent similarity with the sequences deposited in the NCBI data base. Similarly, Cherian *et al*. (2004); Mujtaba (2017) and Antony (2019) had cloned CMV-CP gene of Cucumber mosaic virus infecting banana to pGEM- T vector and confirmed by colony PCR and sequencing.

5.4. OVER EXPRESSION OF RECOMBINANT COAT PROTEIN GENE IN pET32a EXPRESSION VECTOR

The pET32a vector system includes T_7 promoter which is induced by IPTG for high level expression and multiple cloning site for inserting foreign gene. Cloning site carries cleavable N-terminal His-Tag/Trx-Tag and S-Tag sequences and C-terminal His-Tag sequence for detection and purification. It also contains ampicillin resistance gene for screening. Expression system requires host strain lysogenised by a DE3 phage fragment encoding the T7 RNA polymerase under the control of the IPTG inducible Lac UV5 promoter. The cloning/expression region of the coding strand is transcribed by T7 RNA polymerase (Sørensen and Mortensen, 2005; Rosano and Ceccarelli, 2014).

A new primer set was designed to amplify CMV-CP gene along with restriction sites selected from pET32a expression vector and also for cloning of CMV-CP gene into pET32a expression vector. Restriction sites were added to forward and reverse primers. The complete coding region of the CMV-CP gene was identified by ExpASy Translate tool using reference CMV isolate from NCBI database. Forward primer was designed from the 5' end of the CMV-CP gene which contains start codon. Reverse complement of the sequence from the 3' end of the gene which contains stop codon was selected as the reverse primer. *Eco*R1 and *Xho*1 restriction enzyme site in the pET32a vector sequence were selected and inserted at the 5' end of the forward primer and reverse primer for cloning. Linker sequences were also added on the 5' ends of the primers, for proper restriction digestion. Triplets of guanidine and cytosine was added as linker sequences in both the primers to tally the total GC

content as well as conserve the overall protein translation frame. Similarly, Antony (2019) designed new primers to amplify CMV-CP gene infecting banana along with recognition sites of *Bam*H1 and *Eco*R1 on forward and reverse primer, respectively for expression studies of CMV coat protein. Dilip (2020) designed primers to amplify BBTV-CP infecting banana based on the sequences available in the NCBI data base, along with recognition sites responsible for *Eco*RV and *Bam*H1 were added to forward and reverse primer, respectively and cloned BBTV-CP into pET32a expression vector.

In the present study, the annealing temperature of designed CMV-CP primer was standardised by gradient PCR and selected as 55 °C for PCR amplification. The CMV- CP gene having 750 bp was amplified using designed primer and *Phusion* DNA polymerase enzyme for protein expression studies. *Phusion* DNA polymerase is a high fidelity DNA polymerase enzyme isolated from *Pyrococcus furiosus* for high performing PCR. Compared to *Taq* DNA polymerase, *phusion* DNA polymerase is having high thermostability and 3' to 5' exonuclease proof reading activities. Consequently, *phusion* DNA polymerase generated PCR amplicons will have fewer errors and produces high yield than *Taq* generated PCR fragments (Verkuil *et al*., 2008). Similarly, Lundberg *et al*. (1991);Verkuil *et al*. (2008); Bryksin and Matsumura (2010); Norholm (2010); Gulati *et al*. (2016) and Sabharwal (2017) amplified DNA fragments using high fidelity *phusion* DNA polymerase enzyme. Antony (2019) standardised the annealing temperature of CMV- CP designed primers and amplified 750 bp amplicon using *phusion* DNA polymerase. Dilip (2020) also standardised the annealing temperature of designed primers and amplified Banana bract mosaic virus and Banana bunchy top virus coat protein gene using high fidelity *phusion* DNA polymerase for molecular cloning.

CMV-CP amplicon and pET32a expression vector were digested using *Eco*R1 and *Xho*1 restriction enzymes to develop blunt ends for cloning. Digested CMV-CP amplicon was inserted to double digested pET32a vector by using T4 DNA ligase enzyme and later, transformed into *E. coli*. DH5α cells. Since the gene responsible for blue-white screening is absent in pET32a expression vector, positive clones were selected and confirmed by colony PCR using CMV- CP specific primers followed

by plasmid isolation and sequencing. Lin *et al.* (2010) cloned CMV-CP gene infecting tomato into pET32a expression vector for recombinant coat protein production. Antony (2019) cloned CMV- CP gene infecting banana into pET28a expression vector at *Nhe*1 and *Bam*H1 site and confirmed by colony PCR followed by plasmid isolation and sequencing. Dilip (2020) cloned coat protein gene of Banana bunchy top virus into pET32a expression vector at *Eco*RV and *Bam*H1 site and confirmed the recombination by colony PCR, plasmid isolation and sequencing.

The recombinant pET32a/CMV-CP plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS cells for over expression of protein and induced with 0.3 mM IPTG and incubated at 37 °C for 3 h, 4 h, 5 h and 20 °C for 16 h. The incubated bacterial culture were lysed using tractor buffer (Protein purification kit) / Sonicator. The cell lysate were loaded on to 12 per cent SDS-PAGE for evaluation of over expression. In the gel, a slight expression was noticed at 25 kDa corresponding to the CMV-CP in the induced fraction of incubated culture at 37 °C for 4 h in which the cells were lysed by using Sonicator. Lin *et al.* (2010) induced *E. coli* BL21 (DE3) pLysS/pET32a/CMV-CP with 1 mM IPTG and incubated at 37 °C for 7 h. Later, cells were lysed using by Sonicator and successfully expressed recombinant coat protein to raise antiserum. Dilip (2020) induced *E. coli* BL21 (DE3) pLysS with recombinant expression vectors pET32a/BBTV-CP, pRSET-C/BBTV-CP, pGEX-4T-2/BBTV-CP by adding IPTG and successfully produced 37 kDa recombinant coat protein from pET32a/BBTV-CP which was used for antiserum production. The recombinant coat protein production from pRSET-C and pGEX-4T-2 expression clones in *E. coli* BL21 (DE3) pLysS were not satisfactory. In the present study as the expression of CMV coat protein was not satisfactory in both pET28a/CMV-CP and pET32a/CMV-CP BL21 expression clones, further research is required to improve the over expression of coat protein.

Summary

6. SUMMARY

The present study entitled “Evaluation of expression clones for recombinant coat protein of Cucumber mosaic virus infecting banana” was carried out to evaluate recombinant expression clones for recombinant coat protein of CMV infecting banana, which can be utilized for producing high quality antiserum for the detection of CMV infecting banana. The study was conducted using existing facilities at Banana Research Station, Kannara and College of Agriculture, Vellanikkara during the academic year 2019-2022.

Evaluation of the existing recombinant pET28a and pRSET-C expression clones in *E coli.* DH5α cells was the first objective of the study. For that, recombinant pET28a/CMV-CP and pRSET-C/CMV-CP plasmids were isolated and restriction digestion was carried out using *Nhe*1 and *Bam*H1 restriction enzymes. The insert having 750 bp CMV-CP gene was released from pET28a expression vector only. The confirmed pET28a expression vector along with CMV-CP insert was transformed to *E. coli* DH5α cells to amplify the clone. The recombinant pET28a/CMV-CP plasmid was again isolated from randomly selected DH5α cells by alkali lysis method and confirmed by restriction digestion using *Nhe*1 and *Bam*H1 for CMV coat protein gene.

Over expression of recombinant pET28a expression vector, the second objective of the project, the recombinant pET28a/CMV-CP expression vector was transformed into expression host *E. coli* BL21 pLysS cells. The transformed BL21 pLysS cells were confirmed by colony PCR using specific CMV-CP primers for CMV coat protein gene. The *E. coli* BL21 pLysS cells harbouring pET28a/CMV-CP were grown in LB broth and induced with 0.3 mM, 0.5 mM and 1 mM IPTG. Induced culture along with uninduced culture were incubated at varying temperatures (20 °C, 25 °C, 30 °C and 37 °C) and time (4 h, 6 h, 8 h, 10 h, 12 h, 14 h and 16 h) for standardising the over expression. The incubated bacterial cells were lysed by tractor buffer/Sonicator. The cell lysate of induced and uninduced culture were loaded on to 12 per cent SDS-PAGE for evaluating the overexpression of coat protein. Later, confirmation of the coat protein was done by Western blotting.

As the production of recombinant coat protein was not successful in pET28a expression clone, experiments were conducted to develop new expression clone using expression vector pET32a. For that, molecular clone of CMV coat protein was developed which was the third objective of the project. Infected leaf samples of banana showing typical symptoms, along with healthy control were collected from the net house and field of Banana Research Station, Kannara, Thrissur. Preliminary assay was conducted by DAC-ELISA using specific anti-CMV-CP polyclonal antiserum. Isolate namely, NDRNS-4, KANM-1 and KAAS- 2 showed maximum absorbance at 405 nm and hence selected for molecular detection of CMVcoat protein. The total RNA was isolated from CMV infected leaf samples, the quality was checked in agarose gel and quantified by Nanodrop. Isolated RNA samples were converted into c-DNA using Revert Aid First strand c-DNA synthesis kit. The synthesised c-DNA was used as template for PCR to amplify 750 bp of CMV-CP gene using CMV-CP specific primers.

The PCR product was purified using NucleoSpin Gel and PCR Clean-up Kit. The purified PCR product of NDRNS-4 was cloned to pGEM- T Easy vector by TA cloning for maintaining the gene and later transformed into *E. coli* DH5α cells. Positive clones were selected based on blue- white screening, in which white colonies were considered as recombinants and blue colonies as non-recombinants. Individually selected each white colony and subcultured and later confirmed the ligation through colony PCR using T7 and Sp6 specific universal primers. The recombinant plasmid was isolated by alkali lysis method and confirmed by sequencing.

Over expression of recombinant CMV coat protein gene in pET32a expression vector was the last objective of the project. For that, CMV coat protein specific forward and reverse primers were designed for cloning along with recognition sites of restriction enzymes *EcoR1* and *Xho1*. The annealing temperature was standardised as 55℃ using gradient PCR. The coat protein gene of CMV was amplified using designed primers and high fidelity *phusion* DNA polymerase enzyme. The resulted amplicon contained recognition sites of both restriction enzymes *EcoR1* and *Xho1*. For molecular cloning, the amplicon was restricted by *EcoR1* and *Xho1* enzymes. The product was ligated to double digested pET32a expression vector and later

transformed into *E. coli* DH5α cells. Individually selected each positive colony and subcultured and later, confirmed the ligation through colony PCR using CMV-CP specific primers. The recombinant plasmid was isolated by alkali lysis method and pET32a/ CMV- CP clone was confirmed by sequencing.

The recombinant pET32a/CMV-CP plasmid was transformed into expression host *E. coli* BL21 (DE3) pLysS cells for over expression of coat protein and the transformed BL21 pLysS cells were confirmed by colony PCR using specific CMV-CP primers for CMV coat protein gene followed by plasmid isolation. The *E. coli* BL21 pLysS cells harbouring pET32a/CMV-CP were grown in LB broth and induced with 0.3 mM IPTG. Induced culture along with uninduced culture were incubated at 37 °C for 3 h, 4 h, 5 h and 20 °C for 16 h. The incubated bacterial cells were lysed by Tractor buffer/Sonicator. The cell lysate of induced and uninduced culture were loaded on to 12 per cent SDS-PAGE for evaluating the over expression of coat protein. Expression was observed on gel at 25 kDa corresponding to the CMV coat protein.

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Appendix I

Composition of buffers and dyes used in gel electrophoresis

1. 50X TAE buffer (pH 8.0)

2 M Tris base - 24.2 g

1 M Glacial acetic acid - 5.7 ml

0.5 M EDTA (pH 8.0) [18.6 g EDTA was weighed, dissolved using NaOH pellets, adjusted the pH 8.0 and made upthe volume to100 ml] - 10 ml

Distilled water - made upto100 ml

2. Ethidium bromide Stock

The preparation of the ethidium bromide (EtBr) stock solution was carried out by dissolving 10 mg of EtBr in 1 ml of sterile distilled water and stored in amber coloured bottle.

Composition of reagents used in 12 per cent SDS-PAGE

1. Resolving gel

Sterile distilled water - 3.5 ml

30 % Acrylamide-bisacrylamide - 4.25 ml

1.5 M Tris (pH 8.8) - 2.5 ml

10 % SDS -150 µl

10 % Ammonium per sulphate (APS) - 150 µl (freshly prepared)

Tetramethylethylenediamine (TEMED) – 30 µl

2. Stacking gel

Sterile distilled water - 2.7 ml

30 % Acrylamide-bisacrylamide - 0.67 ml

0.5 M Tris (pH 6.8) - 0.5 ml

10 % SDS - 40 µl

10 % Ammonium per sulphate (APS) - 40 µl (freshly prepared)

Tetramethylethylenediamine (TEMED) – 10 µl

3. **Acrylamide-Bisacrylamide solution (30 %)**

Acrylamide-bisacrylamide (30 per cent) was prepared by dissolving 29.2 g of acrylamide and 0.8 g of bisacrylamide (light sensitive) in 100 ml sterile distilled water and stored in brown colour bottle at 4 ℃.

4. **Running buffer pH 8.3 (10X)**

Tris - 30.3 g
Glycine - 144 g
SDS - 10 g
Sterile distilled water - 1000 ml

5. **Sample loading buffer**

Sterile distilled water - 3.55 ml
0.5 M Tris pH 6.8 - 1.25 ml
Glycerol - 2.5 ml
10 % SDS - 2.0 ml
0.5 % Bromophenol blue - 0.7 ml

50 µl of β-mercaptoethanol was added to 950 µl of sample loading buffer prior to use

6. **Staining solution**

Methanol - 40 ml
Acetic acid - 10 ml
Coomassie Brilliant Blue R -250 (CBB R- 250) - 0.1 g
Total volume - 100 ml

7. **Destaining solution**

Methanol - 40 ml
Acetic acid - 10 ml
Total volume - 100 ml

Composition of buffers used in Western blotting and DAC-ELISA

1. **Transfer buffer (100 ml) pH 8.3**

1X Tris- glycine buffer and 20 ml methanol

2. **Phosphate Buffered Saline (PBS) pH 7.4 (10X)**

Sodium chloride - 80.0 g

Potassium dihydrogen phosphate - 2.0 g

Disodium hydrogen phosphate - 11.6 g

Potassium chloride - 2.0 g

Distilled water - 1000 ml

3. **Wash buffer: PBS-Tween- 1X (PBST)**

10 X PBS buffer (100 ml) + 0.5 ml Tween-20 + 900 ml distilled water was used to prepare 1000 ml of PBS-T buffer

4. **0.1 %(w/v) Ponceau S staining solution**

1 g Ponceau- S, 50 ml acetic acid and made up to 1000 ml with distilled water

5. **Blocking buffer**

Spray dried milk (SDM)/PBS buffer: 5 g SDM in 100 ml PBS

6. **Primary antibody diluent buffer**

1 µl of 1° antibody (CMV specific, polyclonal antibody, 1:1000) was dissolved in 1 ml PBS- T

7. **Secondary antibody diluent buffer**

1µl of 2° antibody (Goat-anti-rabbit IgG, 1: 10,000) was dissolved in 10 ml PBS-T

8. **Substrate solution for Western blotting**

30 % H_2O_2 and 0.5 mg/ml 3, 3'-Diaminobenzidine (DAB)

9. Coating buffer pH 9.2 (10X)

Sodium carbonate - 15.9 g

Sodium bicarbonate - 29.3 g

Sodium nitrate - 2.8 g

Distilled water - 1000 ml

10. Antibody diluent buffer/ Enzyme conjugate diluent buffer

20 g of Polyvinylpyrrolidine (PVP) and 2 g of Bovine serum albumin (BSA) was added to 1000 ml of PBST

i. Primary antibody used at 1:1000 dilution
ii. Secondary antibody used at 1:10000 dilution

11. Substrate buffer pH 9.8 for DAC-ELISA

9.7 ml of diethanolamine was added to 50 ml distilled water, adjusted the pH to 9.8 with concentrated HCl and made up to 100 ml by adding distilled water. Stored in brown coloured bottle at 4 ℃.

12. Substrate buffer solution for DAC-ELISA

1 mg of para nitro phenyl phosphate (PNPP) was added in 1 ml of substrate buffer

Composition of chemicals used for Blue- white screening

1. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

20 mg of X-gal was dissolved in 1 ml of Dimethyl sulfoxide (DMSO)

2. IPTG (Isopropyl β-D-1- thiogalactopyranoside)

200 mg of IPTG was dissolved in 1 ml of steriled distilled water. Filter sterilizedand stored at -20 °C

Appendix II

Vector maps

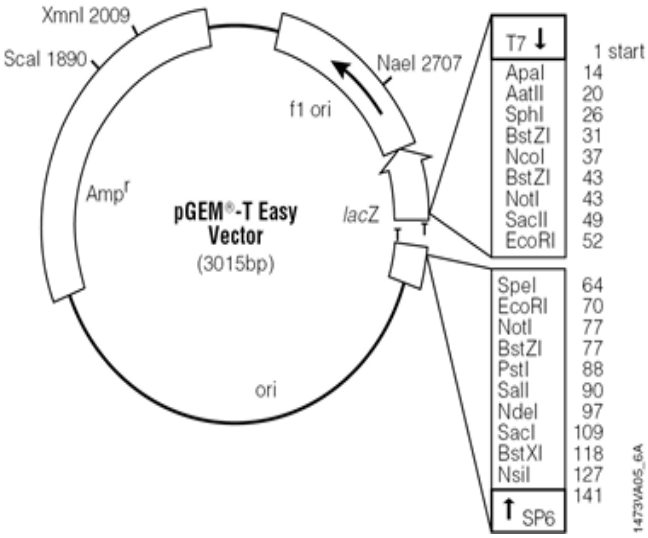

Fig 1. Vector map of pGEMT easy vector

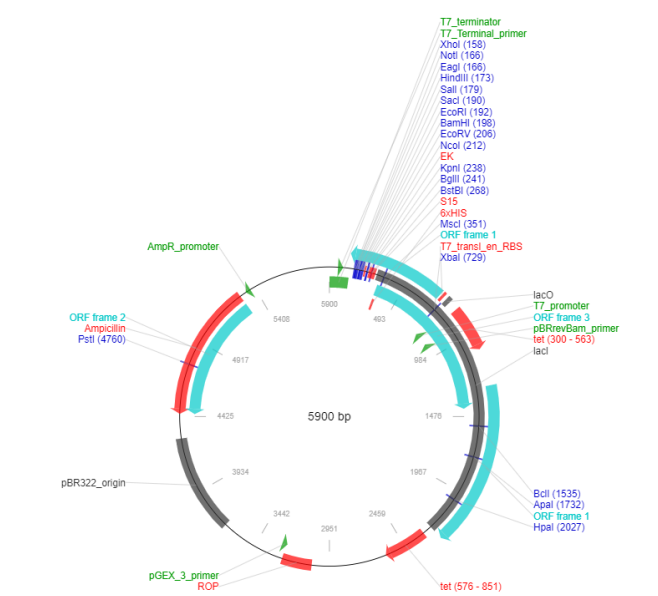

Fig 2. Vector map of pET32a vector

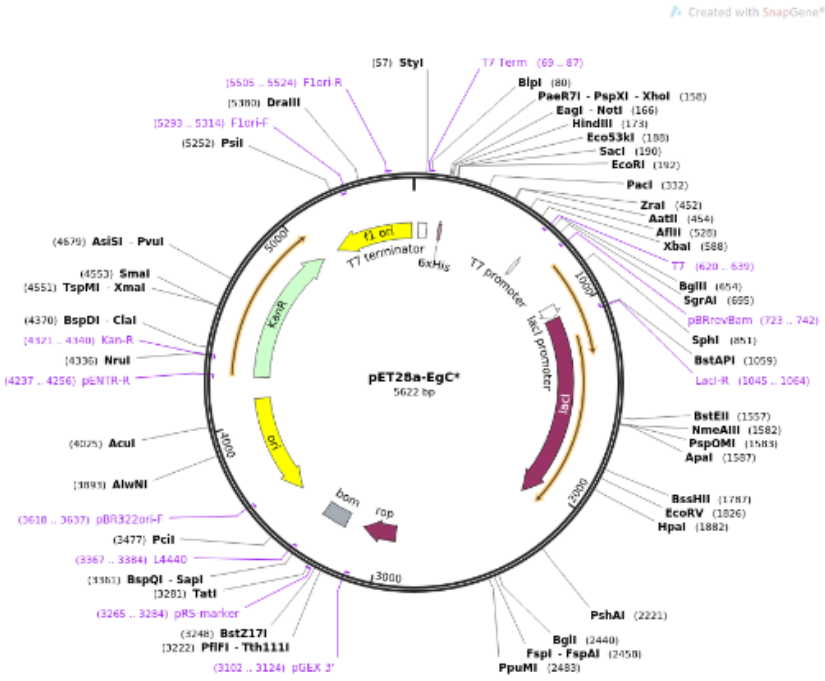

Fig 3. Vector map of pET28a vector

EVALUATION OF EXPRESSION CLONES FOR RECOMBINANT COAT PROTEIN OF CUCUMBER MOSAIC VIRUS INFECTING BANANA

By

SOWMYA R.

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ABSTRACT OF THE THESIS

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ABSTRACT

The present study was carried out to evaluate recombinant expression clones for recombinant coat protein of CMV infecting banana, which can be utilized for producing high quality antiserum for the detection of CMV infecting banana. The study was conducted using existing facilities at Banana Research Station, Kannara and College of Agriculture, Vellanikkara during the academic year 2019-2022.

The confirmation of recombinant expression clones with pRSET-C and pET28a expression vectors were carried by restriction digestion using *Nhe*1 and *Bam*H1 restriction enzymes. The insert having 750 bp CMV-CP gene was released from pET28a plasmid. The confirmed pET28a expression clone along with CMV-CP insert was transformed into *E. coli* DH5α cells to amplify the clone.

The recombinant pET28a/CMV-CP plasmid was cloned into *E. coli* BL21 pLysS cells for over expression of the protein and presence of recombinant clone was confirmed by colony PCR by using specific CMV-CP primers. The *E. coli* BL21 pLysS cells harbouring pET28a/CMV-CP was grown in LB broth until reached the log phase and induced with 0.3 mM, 0.5 mM and 1 mM IPTG. Induced culture along with uninduced culture were incubated at varying temperature and time. The incubated bacterial culture was lysed by using Tractor buffer (Protein purification kit)/Sonicator. The cell lysate of induced and uninduced culture were loaded on to 12 per cent SDS-PAGE for separation and to understand the protein profile. In the gel, even though slight expression of CMV coat protein was observed, no band was observed in Western blotting which was the confirmation test.

As production of recombinant coat protein was not successful in pET28a expression clone, experiments were conducted to develop new expression clone using expression vector pET32a. Infected leaf samples of banana showing typical symptoms, along with healthy control were tested for CMV infection by DAC-ELISA using specific antiserum purchased from the National Research Centre for Banana, Trichy, Tamil Nadu. Isolate namely, NDRNS-4, KANM-1 and KAAS-2 showed maximum absorbance at 405 nm and hence selected for molecular detection using reverse transcriptase polymerase chain reaction with CMV- CP specific primer. The PCR product was purified and CMV- CP amplicon of NDRNS-4 isolate was ligated to

pGEM- T linear plasmid vector, which was later transformed into *E. coli* DH5α cells. Positive clones were selected according to blue-white screening. Cloning *i.e*., *E.coli* DH5α/pGEM-T/CMV- CP was confirmed through colony PCR using T_7 and S_{p6} primers of that plasmid, followed by sequencing.

Coat protein specific forward (5'GGG **GAA TTC** ATG GAC AAA TCT GAA TCA ACC 3') and reverse primers(5'CCC **CTC GAG** AAC TGG GAG CAC CCC AGA TG 3') were designed along with recognition sites of restriction enzymes *Eco*R1 and *Xho*1.The annealing temperature of designed primer was standardized as 55 °C using gradient PCR. The coat protein gene of CMV was amplified at 750 bp using designed primers and phusion DNA polymerase enzyme. Expression vector as well as amplicon were subjected to ligation and the recombination in expression plasmid (pET32a/CMV- CP) was confirmed through colony PCR and followed by sequencing.

The recombinant pET32a/CMV-CP plasmid was transformed into *E. coli* BL21 pLysS cells for overexpression of the protein and presence of recombinant clone was confirmed by colony PCR by using specific CMV-CP primers. The *E. coli* BL21 pLysS cells harbouring pET32a/CMV-CP was grown in LB broth until reached the log phase and induced with 0.3 mM IPTG. Induced culture along with uninduced culture were incubated at 37 °C for 3 h, 4h, 5h and 20 °C 16 h. The incubated bacterial culture was lysed by using Tractor buffer (Protein purification kit)/Sonicator. The cell lysate of induced and uninduced culture was loaded on to 12 per cent SDS-PAGE for evaluating the over expression of coat protein. Expression was observed on gel at 25 kDa corresponding to the CMV coat protein.