RNAi Mediated Resistance to *Cucumber mosaic virus* (CMV) in Black pepper (*Piper nigrum* L.)

thesis submitted to

University of Calicut

For the award of Degree of

DOCTOR OF PHILOSOPHY

(BOTANY)

By

REVATHY K.A.

ICAR-Indian Institute of Spices Research

Kozhikode- 673012, Kerala, India

भाकृअनुप - भारतीय मसाला फसल अनुसंधान संस्थान
ICAR - INDIAN INSTITUTE OF SPICES RESEARCH
(भारतीय कृषि अनुसंधान परिषद *Indian Council of Agricultural Research*)
पी. बी संख्याः *Post Bag No: 1701*, मेरिकुन्नु पोस्ट *Marikunnu Post*,
कोषिक्कोड *Kozhikode-673012*, केरल, *Kerala*, भारत *India*
(ISO 9001: 2008 Certified Institute)

A. Ishwara Bhat, Ph.D
Principal Scientist & Research Guide
Email: Ishwarabhat.a@icar.gov.in; aib65@yahoo.co.in

CERTIFICATE

This is to certify that the corrections suggested by the adjudicators are incorporated in the thesis entitled '**RNAi mediated resistance to *Cucumber mosaic virus* (CMV) in black pepper (*Piper nigrum* L.)**' submitted by **Ms. Revathy K.A.,** to University of Calicut for the award of degree of Doctor of Philosophy in Botany.

Place : Kozhikode
Date : 23rdNovember 2019

(A. Ishwara Bhat)

भाकृअनुप - भारतीय मसाला फसल अनुसंधान संस्थान
ICAR - INDIAN INSTITUTE OF SPICES RESEARCH
(भारतीय कृषि अनुसंधान परिषद *Indian Council of Agricultural Research)*
पी. बी संख्याः *Post Bag No: 1701,* मेरिकुन्नु पोस्ट *Marikunnu Post,*
कोषिक्कोड *Kozhikode-673012,* केरल, *Kerala,* भारत *India*
(ISO 9001: 2008 Certified Institute)

Dr. A. Ishwara Bhat
Principal Scientist

CERTIFICATE

This is to certify that the thesis entitled **'RNAi mediated resistance to *Cucumber mosaic virus* (CMV) in black pepper (*Piper nigrum* L.)'** submitted to the University of Calicut by **Ms. Revathy K.A.** for the award of the degree of **Doctor of Philosophy in Botany,** is a bonafide record of research work carried out by her at ICAR-Indian Institute of Spices research, Kozhikode, Kerala, under my supervision and guidance. No part of the work has formed the basis for the award of any other degree or diploma previously. The plagiarism has been checked at CHMK library, University of Calicut and the values are well within the acceptable limit.

Place: Kozhikode
Date : 1st March 2019

A. Ishwara Bhat

पी ए बी एक्स PABX: 0495-2731410/2731753/2731345 निदेशक का कार्यालय Director's Office: 0495-2730294 परियोजना समन्वयक Project Coordinator: 0495-2731794, एटिक ATIC: 0495 - 2730704. आई आई एस आर प्रयोगिक प्रक्षेत्र, पेरुवण्णामुषि ISSR Experimental Farm, Peruvannamuzhi: 0496 2249371 कृषि विज्ञान केन्द्र Krishi Vigyan Kendra, पेरुवण्णामुषि Peruvannamuzhi: 0496-2666041, तार Grams: RESEARCH CALICUT. फैक्स Fax: 0091-495-2731187 ई-मेल Email: mail@spices.res.in वेबसाईट Website: www.spices.res.in

DECLARATION

I hereby declare that the thesis entitled 'RNAi mediated resistance to *Cucumber mosaic virus* (CMV) in black pepper (*Piper nigrum* L.)' submitted to University of Calicut by me for the award of the degree of Doctor of Philosophy in Botany is a record of bonafide research work done by me at the Division of Crop Protection, ICAR–Indian Institute of Spices Research (IISR), Kozhikode, Kerala, under the supervision and guidance of Dr. A. Ishwara Bhat, Principal Scientist, Division of Crop Protection, ICAR–IISR, Kozhikode, Kerala. This thesis or part of it has not formed the basis for the award of any other degree or diploma previously. The plagiarism has been checked at CHMK library, University of Calicut and the values are well within the acceptable limit. All sources of help received by me during the period of this study have been duly acknowledged.

Place : Kozhikode **Revathy K.A.**

Date : 1st March 2019

Dedicated to Amma, Appa, Visu, Sreeni

ACKNOWLEDGEMENT

The success and final outcome of this research required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my thesis. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

First and foremost, I respect and thank my guide Dr A. Ishwara Bhat, Principal Scientist, Division of Crop Protection, ICAR–Indian Institute of Spices Research (IISR) for introducing me to the gorgeous and inquisitive world of virus research, transgenics and gene silencing. His transcendence, allegiance, diligence and zeal for the "concealed" had an everlasting effect and taught me many lessons of life.

I express my sincere thanks to Department of Science and Technology, Government of India, for providing necessary financial support through WOS–A during my doctoral research that buttressed me to continue and complete my work comfortably. I also thank Department of Biotechnology, Government of India for the funding during initial years of my research at IISR.

I am indebted to Dr. K. Nirmal Babu, Director, ICAR–IISR, for providing the lab facilities of the institute. I remember with pleasure and gratitude, the inspiration, encouragement and support, given by him for the successful completion of my research work.

I express my deepest sense of gratitude to Dr. Santhosh J. Eapen, Head, Divison of Crop Protection, ICAR–IISR for the persistent support, approachable nature and special concern showered on me during the tenure of my research.

I extend my heartfelt thanks to Dr. M. Anandaraj, former Director, ICAR–IISR, for giving me the wonderful opportunity to conduct research, for his encouragement and constructive criticisms.

I am grateful to Dr. Johnson K. George, Principal Scientist, ICAR–IISR, Dr R. Susheela Bhai, Principal Scientist, ICAR–IISR, Dr. Rashid Pervez, Principal Scientist, ICAR–Indian Agricultural Research Institute and Dr. C. Sarathambal, Scientist, ICAR–IISR for their pleasant gesture, friendly nature and constant support.

My profound thanks to Dr. R. Ramakrishnan Nair, Principal scientist, ICAR–IISR, for his valuable advice in black pepper regeneration.

I express my appreciation to Dr. J. Rema, Head in Charge, Division of Crop Improvement and Biotechnology, ICAR–IISR, Dr. C.K. Thankamani, Head in Charge, Division of Crop Production and Postharvest Technology, ICAR–IISR, Dr. S. Devasahayam, former Head, Division of Crop Protection, ICAR–IISR, Dr. B. Sasikumar, former Head, Division of Crop Improvement and Biotechnology, ICAR–IISR, Dr. T. John Zachariah, former Head, Division of Crop Production and Postharvest Technology, ICAR–IISR and, other scientists at ICAR–IISR, Dr. K. Kandiannan, Dr. K. V. Saji, Dr. N.K. Leela, Dr. K.S. Krishnamurthy and Dr. P. Uma Devi for their suggestions and support.

I am thankful to Dr E.Jayashree, HRD Secretary, ICAR–IISR for her concern, timely help and constant encouragement during the period. I also express my sincere thanks to other members of HRD, PME and Administration of ICAR–IISR for all the help rendered.

I have immense pleasure in thanking 'Mr A. Sudhakaran, Artist cum Photographer' (Technical Officer), ICAR–IISR, for devoting his valuable time assisting me in photography, designing posters, cover page and other artistic helps. His patience and valuable suggestions during thesis writing is highly appreciated.

I would also like to thank Mr K. Jayarajan, Assistant Chief Technical Officer, ICAR–IISR, for his timely help, especially with statistical analysis; Dr. Sushama Devi, former Senior Technical Officer and Mr. P Ramesh Kumar, Chief Technical Officer, ICAR–IISR, for their help in accessing the library services.

I am thankful to Mrs P.K. Chandravally, Technical Officer, ICAR–IISR, Mr. K.P. Premachandran, Senior Technical Assistant, ICAR–IISR and Mrs. C.M. Kamalam, Skilled Support Staff, ICAR–IISR for all the help rendered.

I acknowledge the help and support extended by the staff of DISC, ICAR–IISR for access to internet and bioinformatics tools.

I greatly acknowledge all the members of my Doctoroal Committee, especially Dr. P.R. Manish Kumar, Professor, Dept. of Biotechnology, University of Calicut; for their sincere appreciation and valuable suggestions. I also thank Dr. T. Makesh Kumar, Principal Scientist, ICAR–Central Tuber Crops Research Institute, for conducting my coursework viva voce. I immensely thank Dr. M. Manamohan, Principal Scientist, ICAR–Indian Institute of Horticultural Research for his suggestions in hairpin construct preparation.

I owe a huge debt of gratitude to my friends Kuttan, Prameela, and Aswathy, for their mental support and concern throughout the course of my study. I am indebted to my friend Nisthar for always rendering a helping hand during the plant tissue culture tasks. I also thank Dr. Ann Jasmine for proofreading the thesis. A great thanks goes to my senior Dr. Siljo Abraham and my junior Anju for all the timely help.

I thank all my friends and labmates Sugumaran, Divya, Vipin, Jisha, Vishnu, Jithesh, Prashina, Subila, Ahammed Mujtaba, Karthika, Rosana, Pamitha, Deepthi, Bijitha, Shina, Deeshma, Vinitha, Suraby, Agisha, Krishna, Deepa, Santhi, and Neema, for making my research life livelier.

I express my warm thanks to all my teachers who have ever taught me; specially Dr. S. Jayasree, Associate Professor, Coordinator, Dept. of Biotechnology and Bioinformatics, Mercy College, Palakkad; Sr. V.P. Shaini, Mrs Saritha Ravindran, Late Dr. N. Abitha Devi, former Head and, Dr. S.R. Madhan Shankar, Associate Professor, Head, Dept. of Biotechnology, PSG College of Arts and Science Coimbatore, for their support and encouragement.

Last but certainly not the least; with deep love, I am forever indebted and obliged to my family for their encouragement, inspiration, patience and eternal support for completing the research work. I thank my parents and brothers for their endless love, inseparable support and prayers that refilled my zeal and energy at every wearing moments in life.

I am forever grateful to God Almighty to the greatest extent for all the blessings and for being an invisible inspiration throughout, which has given me the strength to believe in my passion and pursue my dreams. I could never accomplish this goal without the faith I have in God.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

α	Alpha
B	Beta
%	Percentage
/	Per
°C	Degree centigrade
µ	Micro
µg	Microgram
µL	Microliter
µM	Micromolar
A_{260}	Absorbance at 260 nm
AGO	Argonaute
BA	6–Benzyl adenine
BLAST	Basic local alignment search tool
bp	Base pairs
cDNA	Complementary DNA
chv	Chromosomal virulence
cm	Centimeter (s)
conc.	Concentration
CP	Coat protein
CPMR	Coat protein mediated resistance
Ct	Cycle threshold
CTAB	Cetyl trimethyl ammonium bromide
C–terminal	Carboxy terminal
cv.	Cultivar
DCL	Dicer like
dCTP	Deoxycytidine triphosphate
DEAE	Diethylaminoethyl
DIG	Digoxigenin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid

F	Forward
g	Gram or relative centrifugal force
GFP	Green fluorescent protein
GUS	Glucouronidase
h	Hour (s)
ha	Hectare area (s)
hpt	Hygromycin phosphotransferase
ihpRNA	Intron hairpin RNA
IPTG	Isopropyl β–D–1–thiogalactopyranoside
IR	Intergenic region
kb	Kilobase (s)
kbp	Kilobase pair
kDa	Kilodalton (s)
Kg	Kilogram (s)
L	Liter
LB	Luria Bertani
M	Molar
m	Meter
mg	Milligram (s)
min	Minute (s)
miRNA	Micro RNA
mL	Milliliter (s)
mM	Millimolar
mm	Millimeter
MP	Movement protein
mRNA	Messenger RNA
MS	Murashige and Skoog
MSA	Multiple sequence alignment
MW	Molecular weight
n	Number
NAA	Naphthalene acetic acid
NCBI	National Centre for Biological Information
Ng	Nanogram (s)
nm	Nanometer
nos	Nopaline synthase

NPTII	Neomycin phosphotransferase II
nt	Nucleotide
N–terminal	Amino terminal
OCS	Octopine synthase
OD	Optical density
ORF	Open Reading Frame
ori	origin
PCR	Polymerase chain reaction
PDR	Pathogen derived resistance
PEG	Polyethylene glycol
PGR	Plant growth regulator
pH	$-\log_{10}[H]$
pmol	Pico mole
PTGS	Post transcriptional gene silencing
PVP	Polyvinylpyrrolidone
q–PCR	Real time quantitative PCR
R	Reverse
RCF	Relative centrifugal force
rDNA	Recombinant DNA
RdRp	RNA dependent RNA polymerase
rep	Replicase
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RPA	Recombinase polymerase assay
rpm	Revolutions per minute
RT	Reverse transcription
S	Svedberg coefficient
s	Second (s)
satRNA	Satellite RNA
SDS	Sodium dodecyl sulphate
SH	Schenk and Hildebrandt
siRNA	Small interfering RNA
sRNA	Small RNA

TAE	Tris amino ethane
T–DNA	Transfer DNA
TE	Tris–EDTA
TGS	Transcriptional gene silencing
Ti	Tumor inducing
Tm	Melting temperature
U	Unit (s)
UTR	Untranslated
UV	Ultraviolet
V	Volts
v/v	Volume by volume
VIGS	Virus induced gene silencing
vir	Virulence
VRTP	Virus resistant transgenic plants
w/v	Weight by volume
WPM	Woody plant medium
X–gal	X–galactopyranoside
X–gluc	5–bromo–4–chloro–3–indolyl β–D–glucuronic acid

VIRUS SPECIES AND ACRONYMS

ACLSV	*Apple chlorotic leaf spot virus*
ACMV	*African cassava mosaic virus*
BCMV	*Bean common mosaic virus*
BGMV	*Bean golden mosaic virus*
CaMV	*Cauliflower mosaic virus*
CBSUV	*Cassava brown streak Uganda virus*
CBSV	*Cassava brown streak virus*
CMV	*Cucumber mosaic virus*
CoYMV	*Commelina yellow mottle virus*
CPMV	*Cow pea mosaic virus*
CsVMV	*Cassava vein mosaic virus*
CTV	*Citrus tristeza virus*
CymRSV	*Cymbidium ring spot virus*
GFLV	*Grapevine fan leaf virus*
GINV	*Grapevine berry inner necrosis virus*
GRSV	*Groundnut ring spot virus*
MYMV	*Mungbean yellow mosaic virus*
PLRV	*Potato leaf roll virus*
PPV	*Plum pox virus*
PRSV	*Papaya ring spot virus*
PSV	*Peanut stunt virus*
PVX	*Potato virus X*
PYMoV	*Piper yellow mottle virus*
RTBV	*Rice tungro bacilliform virus*
ScBV	*Sugarcane bacilliform virus*
SCMV	*Sugarcane mosaic virus*
TAV	*Tomato aspermy virus*
TCSV	*Tomato chlorotic spot virus*
TLCV	*Tomato leaf curl virus*
TMV	*Tobacco mosaic virus*
ToCV	*Tomato chlorosis virus*
ToMV	*Tomato mosaic virus*
TRV	*Tobacco rattle virus*
TSV	*Tobacco streak virus*
TSWV	*Tomato spotted wilt virus*
WMV	*Watermelon mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*

INTRODUCTION

Black pepper (*Piper nigrum* L.) commonly known as the 'King of spices' and 'black gold' is an economically important spice crop renowned for its culinary and medicinal properties. Black pepper is a perennial woody flowering vine that belongs to the genus *Piper* in the Piperaceae family. The berries or the mature dried fruit of this plant has a characteristic pungency, aroma and flavor that make it a very important constituent of different cuisines worldwide. This is due to the presence of an alkaloid called piperine which is responsible for the pungency of black pepper (Govindarajan and Stahl, 1977). Besides, the medicinal and antimicrobial properties of the berries make it the one of the most valuable spice worldwide and an inevitable constituent in many traditional Ayurvedic drugs (Ravindran, 2000). The spice is also used in perfumery and was an essential ingredient in embalming mixture used by ancient Egyptians. Betelvine (*Piper betle* L.) and Indian long pepper (*Piper longum* L.) are two other important species of the genus *Piper,* with notable medicinal properties.

Origin of black pepper is believed to be in the tropical evergreen forests of Western Ghats of India and eventually the crop was introduced to other Asian, South East Asian, Latin American and African countries (Ravindran, 2000) and currently this spice crop is cultivated in more than 44 countries of the world (FAOSTAT, 2018) covering a total area of 4,58,731 ha (IPC, 2017). Among these nations, India has the largest (1,34,280 ha) share of 29%, of the total global area under black pepper cultivation, followed by Indonesia with 26%, Vietnam with 24% and Sri Lanka with 8%. Vietnam is the largest producer of pepper accounting for 39% of world's black pepper production, followed by Indonesia with an average production of 15%, Brazil with 13% and India with an average production of 11% (IPC, 2017). As per the 2017 records the total black pepper production in India was 57,000 metric tonnes from 1,34,280 ha (IPC, 2017). In India, the South Western regions, spanning the states of Kerala and Karnataka are the major growers of black pepper with an annual contribution of 55 and 34 percent respectively. Limited volumes of black pepper are also cultivated in

Tamil Nadu (3%) and other states like Assam, Meghalaya, Tripura, and West Bengal (Shashidhara *et al*., 2009; Spices Board, India, 2018).

Although India has the largest area of black pepper cultivation, the production is often low due to various biotic and abiotic stresses. The abiotic stresses mainly include the fluctuations in the global climate, non–availability of water, nutritional deficiency, and scarcity of labour. The biotic stresses are often, the diseases caused by pests and microbes which include fungi, nematodes, viruses, oomycetes and phytoplasma (Savary *et al*., 2012). Among the different biotic stresses, the major diseases of black pepper are, the foot rot caused by the oomycete (*Phytophthora capsici*), slow decline caused by nematodes (mainly *Radopholus similis)* , stunted disease caused by viruses [namely *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV)] and anthracnose caused by fungus (*Colletotrichum gloeosporioides*) (Anandaraj and Sarma, 1995). Amongst these, the diseases caused by the fungi and nematodes can be successfully controlled using chemicals whereas, those that are caused by the viruses and the phytoplasmal pathogens are very difficult to control. Viruses and the phytoplasmal pathogens do cause huge economical loss to the black pepper production and also affect the quality of the product (Philip *et al*., 1992).

Based on the severity and crop loss, stunted disease caused by the viruses, is considered the third major production constraint of black pepper and cause significant crop loss due to their wide spread nature (Sarma *et al*., 2001). The disease was first spotted in 1975 in a black pepper nursery in Neriamangalam of Idukki in Kerala (Pailey *et al*., 1981) and then mosaicism in black pepper was observed in Tamil Nadu (Prakasam *et al*., 1990). Subsequently, the disease was reported from most of the pepper plantations across South India. In a survey conducted in 2004 in India, high incidence and severity of the disease was reported from black pepper plantations located especially at high altitudes such as Idukki (29.4%) and Wayanad (45.4%) districts of Kerala and, from Kodagu (14.9%) and Hassan (5.2%) districts of Karnataka (Bhat *et al*., 2005a). Association of PYMoV with the stunted disease has been reported from Malaysia, Thailand, Philippines (Lockhart *et al.,* 1997), India (Bhat *et al*., 2003) and Sri

Lanka (de Silva *et al.,* 2002). PYMoV belongs to the genus *Badnavirus* and family Caulimoviridae and its genome comprises of double stranded DNA. Typical symptoms of the disease include leaf distortion, chlorotic mottling, vein clearing and ultimately the reduction in plant vigour and fruit quality. Vegetative propagation and different species of mealy bugs spread the virus (Bhat *et al*., 2003; Bhat *et al*., 2005b).

Cucumber mosaic virus (CMV) (genus: *Cucumovirus*, family: Bromoviridae) is a tripartite positive sense RNA virus which is known to be the most devastating plant pathogen with the broadest host range infecting more than 1200 species of plants worldwide (Edwardson and Christie, 1991). Association of CMV with the stunted disease has been reported from India (Sarma *et al*., 2001), Sri Lanka (de Silva *et al*., 2001) and Brazil (Eiras *et al*., 2004). Diseased plants show mild to severe mottling, mosaicism, yellowing, malformed leaves and in cases of high severity, the leaves become extremely narrowed and sickled. Ultimately, the whole plant becomes stunted leading to reduced vigor and low productivity. The virus is mainly spread through infected stem cuttings and more than 80 species of aphids are known to spread CMV in a non–persistent manner (Palukaitis *et al*., 1992).

Currently there are no known varieties or cultivars of black pepper resistant to CMV and no chemicals are available to control the virus. The use of virus free planting materials of elite genotypes is the management strategy adopted till now. Due to the lack of virus resistant varieties in any of the black pepper growing countries, developing genetic resistance is the best alternative strategy for which thorough understanding of the complete genome sequence of CMV isolate from black pepper is essential. Though many CMV strains have been reported worldwide, limited information is available about the black pepper isolate of CMV. So far, complete genome sequence information of one black pepper isolate from China is available in the NCBI GenBank. However, CMV is known to undergo reassortment and recombination leading to the evolution of new strains of this virus (Kim *et al*., 2014; Nouri *et al*., 2014). From India, only

the coat protein (CP) sequence of black pepper isolate is reported till date (Bhat *et al*., 2005c).

Genetically engineered resistance against CMV was initially developed in tobacco plants using the CP of CMV as transgene (Cuozzo *et al*., 1988). Later, coat protein mediated resistance (CPMR) of varying levels has been demonstrated in different hosts including cucumber, tomato, muskmelon, squash and pepper using a number of different constructs (Prins *et al*., 2008). The mechanism of resistance also varied in different hosts depending upon the viruses studied and the constructs used. One of the mechanism of resistance is the protein mediated where the complete protein encoded by the transgene acts as the effector (Prins *et al*., 2008). The other mechanism of resistance is the RNA mediated where the transgene transcribed RNA acts as the effector. This mechanism, also known as RNA interference (RNAi) or post–transcriptional gene silencing (PTGS) is the predominant one and is highly sequence specific (Lindbo *et al*., 1993). Recent studies show that, transgenes in the form of hairpin structures, where sense and antisense strands of the same sequence are separated by an intron, will trigger RNA silencing and confer systemic resistance against viral genomes (Simon–Mateo and Garcia, 2011). The mechanism of RNAi has been successfully used for conferring resistance to pathogens in crops and also for improving the quality traits of crops. However, the effectiveness of the mechanism depends on the sequence similarity between the transgene and the viral genome. Thus sequence polymorphism in the target gene limits the application of RNAi to a great extent (Gordon and Waterhouse, 2007).

An important prospect of disease management is the use of resistant varieties for which a reliable screening technique is a prerequisite. Among the various factors that make the resistance/susceptibility studies difficult are the involvement of vector, the efficiency of transmission, acquisition period, persistence and semi–persistence nature of viruses, as well as the host–vector virus interactions. However these problems will easily be overcome by artificial transmission of viruses through grafting. Further infectious clones of CMV are unavailable and sap transmission of CMV to black pepper is not successful till

date. Hence, there is a need for an efficient and reliable method of transmission of CMV from infected to healthy black pepper plants that can be used for the screening of transgenic and non–transgenic black pepper plants, for CMV resistance.

Taking into consideration, the importance of black pepper, it's export value and the threat posed by CMV to the production of black pepper, the current study was formulated with the following objectives,

1. Complete genome sequencing of black pepper isolate of CMV, phylogenetic analyses with CMV isolates reported worldwide from subgroups I and II, and nucleotide diversity studies of different genes.
2. To select the most suitable region of all the five genes (1a, 2a, 2b, 3a and 3b) of CMV from black pepper for dsRNA synthesis, with maximum specificity and minimum off targets and, to identify the common siRNAs that will be synthesized from these regions.
3. To prepare hairpin gene constructs using CMV 3b/2b sequences in suitable plant transformation vector.
4. *Agrobacterium* mediated transformation of black pepper somatic embryos using the viral constructs prepared, plantlet regeneration and screening of the putative transformants.
5. Development of cleft grafting method for challenge inoculation of CMV in black pepper.

CONTENTS

REVIEW OF LITERATURE

India, the 'Land of Spices' cultivates different spice crops and the diversity of spices is attributed to the differing climatic conditions ranging from tropical to temperate, that prevails in the country. Almost all spice crops are grown in the country and at least one spice is grown in different states and Union territories. Among the different spices, black pepper is the most widely cultivated that substantially contributed to the economic growth of India and other countries. Although the country dominated in black pepper production for centuries, its position started dwindling during the recent years. The setback in production mainly owe to the devastation caused by biotic and abiotic factors including the diseases, pests and also high production costs. The situation beckons proper disease management strategies that require thorough understanding of the mechanism of diseases.

The literature appertaining to the various aspects of *Cucumber mosaic virus* (CMV) in black pepper (*Piper nigrum* L.) *viz.* black pepper and its importance, threatening diseases of black pepper, stunted disease caused by CMV, genetic transformation of plants, engineered resistance to CMV and RNAi mediated resistance to CMV in plants, was briefly outlined in this chapter under the following titles.

2.1 Black pepper

Black pepper (*Piper nigrum* L.) is the most commercially important spice crop belonging to the genus *Piper* in the family Piperaceae. The mature dried berries of black pepper are the product of commerce that have a characteristic pungency, aroma and flavour making it a well–known culinary spice. The use of black pepper dates back to the times of King Solomon (BC 1015 – BC 66) and became popular throughout the world changing the status of nations and people, both culturally and economically. Due to its importance in the international trade, black pepper is renowned as 'King of Spices' (Mathew *et al*., 2001; Srinivasan, 2007). Prediction is that by the year 2020, the global demand of black pepper will jump colossally to nearly 2,80,000 metric tonnes and by 2050 this will further escalate to 3,60,000 metric tonnes (Nair, 2011).

2.1.1 Origin, botanical description and distribution of black pepper

The earliest of the records of *Piper* species of India was in the *Hortus Indicus Malabaricus* published by Rheede, (1688). Following that Linnaeus, (1753) introduced the *Piper* genus in his book *Species Plantarum* along with 1000 other species, among which, about 110 species were of Indian origin (Purseglove *et al*., 1981). Among these 1000 species, black pepper is the most widely cultivated ascribed to its economic value (Bhat *et al*., 1995). Cultivation of black pepper is mainly limited to the South East Asian countries, *viz*. India, Vietnam, Thailand, Malaysia, Sri Lanka and Brazil. Vietnam accounts for the highest black pepper production of 39% followed by Indonesia (15%). Brazil holds the third position with 13% and India has now come down to the fourth position accounting for 11% of the world's production of black pepper (IPC, 2017).

Black pepper is one among the earliest perennial climber that originated in the humid, tropical evergreen forests of Western Ghats of India (Ravindran, 2000). As the black pepper grow over the living or non–living supports, orthotropic and plagiotropic branching can be seen. While the orthotropic branches are straight, grows upward and monopodial, the plagiotropic fruiting branches are sympodial and grows laterally. Black pepper has dorsiventral leaves with prominent veins, which are nearly 20 cm long and 15 cm broad that varies with the variety/cultivar. The fruiting branches bear the spikes which are actually the inflorescence comprising of five to hundred minute flowers in the fleshy, ovate and copular bracts of the spikes. The berries are mono seeded with tiny embryo having a small endosperm and copious perisperm (Ravindran, 2000). Predominantly cultivated types of black pepper are the monoecious plants (where male and female flowers are seen in the same spike), however, complete male or female plants are also found.

Black pepper is grown as a mono–crop as well as inter crop. Sufficient humidity and rainfall of Western Ghats make it ideal for black pepper cultivation. It is also known to survive in regions at high altitudes and tolerates varied environmental conditions which is the major reason for its interspecies diversity (Howard, 1973). The adaptability of black pepper is such that it thrives between

20°C North and South latitude, and thrives even up to 1500 m above the sea level along with coffee, tea and cardamom plantations. The crop can grow in a wide range of temperature, ranging from 10–40°C and soil pH from 4.5–6.5, though red laterite soils are more preferable (Sivaraman *et al*., 1999). An annual rainfall of 125–200 cm is perfect for the cultivation of black pepper.

2.1.2 Varieties of black pepper and cytology

In Kerala, nearly 75 varieties of black pepper are cultivated and the most popular cultivar among them is Karimunda. It bears berries regularly and prolifically, produces medium sized berries of good quality and quantity (Ravindran and Nair, 1984). Among the other varieties Narayakodi is popular in Central Kerala, Neelamundi is common in Idukki, Balancotta and Kuthiravally, the alternately bearing ones, are seen in Kozhikode and Idukki. Kalluvally is popular in North Kerala, Kottanadan is mainly cultivated in South Kerala, Malligesara and Aimpiriyan in Wayanad and Uddagiri (Karnataka). Among these Kottanadan and Aimpiriyan are known for their highest oleoresin content of 17.8% and 15.7% respectively that adds to the commercial value of these varieties.

Besides these varieties, nineteen improved varieties of black pepper have been released jointly by the Pepper Research Station, Panniyur, Kerala, India and ICAR–Indian Institute of Spices Research, Kozhikode, Kerala, India. These are Panniyur–1 to Panniyur–8, Sreekara, Subhakara, Pournami, Panchami, Arka Coorg Excel, Vijay, PLD–2, IISR–Thevam, IISR–Shakthi, IISR–Girimunda and IISR–Malabar Excel. The Pepper Research Station released the hybrid varieties Panniyur–1, Panniyur–3, and Panniyur–6; and ICAR–IISR released the hybrid varieties, IISR–Girimunda and IISR–Malabar Excel. Panniyur–5, Panniyur–7 and IISR Shakthi were developed through open pollinated progeny selection from Perumkodi, Kuthiravalli and Perambramundi respectively (Source: http://www.aicrps.res.in).

Though cultivated black pepper have their chromosomal composition 2n=52 (Mathew, 1958), polyploidy have been reported in the genus *Piper*. However, the basic chromosome number of the genus *Piper* was reported as X=12, 14, 16 by Darlington and Wylie, (1961). A heteromorphic bivalent was

identified in *P. longum* male plants and a natural triploid with significant chromosome variations from 2n=52 to 2n=104 have been identified in the variety Vadakan (Nair *et al*., 1993) and its progenies showed abnormality in growth, production and yield.

2.1.3 Economic importance of black pepper

Black pepper is the most widely used culinary spice besides being used as an ingredient in food processing and perfumery (Philip *et al*., 1992). The odour and pungency of black pepper is an important quality parameter. The odour or flavor is due to the essential oil content which is a mixture of β–caryphyllene, limonene and β–pinene. Besides, the volatile oil consists of more than eighty types of compounds including aldehydes, terpene alcohols, monoterpenoids and diterpenoids (Tripathi *et al*., 1996). The pungency is attributed to the alkaloid piperine present in black pepper (Govindarajan and Stahl, 1977).

Secondary metabolites extracted from black pepper was proved to have a protective action against the infections caused by the microbes, insects and animals (Lupina and Crrips, 1987). Antimicrobial activity of black pepper against different pathogens is reported previously (Dorman and Deans 2000; Umit *et al*., 2009). Insecticidal properties of piperamides extricated from the black pepper have also been reported (Scott *et al*., 2008; Boff *et al*., 2006).

Black pepper is also known for its amazing medicinal properties. Healing properties of black pepper in the treatment of ailments like fever, cold, gastric problems and colic disorder has been reported (Parmar *et al*., 1997; Kumar *et al*., 2007). Further, there are studies showing the use of black pepper for treating pulmonary diseases (Ravindran, 2000). The β–caryophyllene and nerolidol which are important sesquiterpenes present in the essential oil, have anesthetic property (Santra *et al*., 2005).

The viability of black pepper seeds are low, yield few progenies that are heterogenous and show sterility in further generations (Atal and Banga, 1962). Alternative methods of black pepper proliferation are the micropropagation of shoot tips from mature vines (Philip *et al*., 1992), from seedlings (Mathew and Rao, 1984), and from micropylar region of mature seeds through cyclic

secondary somatic embryogenesis (Mathew and Rao, 1984). Though these methods can be used for multiplication of black pepper, the propagation of black pepper is mainly done through stem cuttings due to uncertainty in seed production (Nair and Dutta, 2006).

2.2 Threatening diseases of black pepper

The production of black pepper is limited by various biotic constraints that include the fungal, viral and phytoplasma diseases besides nutritional deficiency and lack of labor. The problems caused by the pests and diseases are identified as major reason for low productivity in black pepper (Sarma and Anandaraj, 1997).

2.2.1 Foot rot of black pepper

The oomycete *Phytophthora capsici* infects all parts of black pepper including leaves, stems and roots causing the disease 'foot rot' leading to wilting and sudden death of plants. The infection can be either soil–borne or air–borne. The major symptoms of infection are the appearance of dark spots on leaves that extend promptly, causing defoliation. The black lesions that appear on the infected stems lead to blight. Finally the root system starts rotting, yellowing, drying and eventually, the plant dies (Sarma and Anandaraj, 1997).

2.2.2 Fusarium wilt in black pepper

The fungus *Fusarium solani f. sp. Piperis.* is the causative agent of *Fusarium* wilt. The major symptoms of infestation are root rot starting from the stem or root system that advances to the yellowing of foliage and flaccidity that leads to the death of the plants in a short period. Infection of the roots result in the destruction of the feeder root that make the leaves lose their turgidity, which becomes flaccid, yellow and falls off before maturity. Infection in the stem also leads to yellowing with lesions appearing around the nodes. The infested plants either produce no shoots or produce weak shoots with retarded growth thus reducing the economic life of the crop to almost 12 years and curtailing the crop yield to half (Duarte and Albuquerque, 1991).

2.2.3 Slow decline of black pepper

The disease also known as 'slow wilt/yellow disease' is caused by the burrowing nematode and the Root–knot nematode, *Radopholus similis* and *Meloidogyne incognita* respectively. The root infestation by the nematodes causes formation of galls, necrotic lesions, rotting and complete devastation of the feeder roots. This results in poor growth, interveinal chlorosis and yellowing of leaves leading to the death of the plants in 3–4 years (Anandaraj *et al*., 1996).

2.2.4 Anthracnose disease of black pepper

The disease also called as 'Pollu' (indicating hollow fruits), and 'black berry disease' in Indonesia and Malaysia, is caused by the fungus *Colletotrichum gloeosporioides*. The symptoms of the disease are the appearance of lesions on leaves and spikes that ultimately turn black and the berries also become blackened before maturity. Small black spots gradually spread, forming larger blights covering the entire leaf area. The fruit becomes hollow and finally drops off (Anandaraj and Sarma, 1995).

2.2.5 Phyllody in black pepper

The disease caused by Phytoplasma (Sarma *et al*. 1988), was first reported from Wayanad district of Kerala (Sarma and Anandaraj, 1992). Also known as 'antholysis' (Remila and Neelakandan, 1994), the phytoplasma associated with Phyllody was detected by PCR and, based on the 16S rDNA sequence identity it was classified as a member of aster group (Bhat *et al*., 2006). The major symptoms are the conversion of spikes and flowers to leaf–like structures that make it appear like a witch's broom. Smaller leaves with yellowing and chlorosis are other symptoms of the disease.

2.3 Stunted disease of black pepper

In India, the disease was first identified in 1975 from Idukki district of Kerala in a government black pepper Nursery at Neriamangalam (Pailey *et al*., 1981) and then in 1978 from the Wayanad district of Kerala in a place called Pulpally (Sarma *et al*., 1992). Later, the disease was reported from Tamil Nadu (Prakasam *et al*., 1990). Subsequently, the disease was reported from all the

pepper growing regions of South India. It is also called 'mosaic disease in India (Prakasam *et al*., 1990), 'stunted disease' in Indonesia (Sitepu and Kasim, 1991), 'little leaf disease' in Sri Lanka, and 'wrinkled leaf disease' in Malaysia (Kueh and Sim, 1992). In 1991, 'stunted disease' was decided to be used as a common terminology to denote all the viral diseases, by the International workshop on black pepper diseases, held at Lampung, Indonesia (Wahid *et al*., 1992). In India, high incidence and severity of the disease was reported from black pepper plantations located at high altitudes, such as Idukki (29% incidence) and Wayanad (45% incidence) districts of Kerala and, Hassan (5.2% incidence) and Kodagu district (14.9%) of Karnataka (Bhat *et al*., 2005a). The stunted disease is the third major productivity constraint of the crop in India (Bhat *et al*., 2005c) and typical symptoms include stunting of plant, mottling and mosaic on leaves, reduction of spike length and poor filling of spikes leading to yield reduction (Figure 2.1). The disease is mainly spread through infected stem cuttings, insect vectors and plants which are infected but look symptomless (Bhat *et al*., 2012).

2.3.1 Causal viruses

Studies indicated that a Badnavirus and a closterovirus–like flexuous virus are major causes of stunted disease (Eng *et al*., 1993). The Badnavirus associated with the stunted disease was named as *Piper yellow mottle virus* and electron microscopy of partially purified extracts of leaves from affected samples from Thailand, showed non–enveloped bacilliform virions (Lockhart *et al*., 1997). *Cucumber mosaic virus* was also identified from the diseased plants in Sri Lanka by immunosorbent electron microscopy of the purified extract of leaves (de Silva *et al*., 2001). Subsequently, the association of these two viruses with the disease was reported from South East Asian countries (Lockhart *et al*., 1997), India (Sarma *et al*., 2001; Bhat *et al*., 2005b) and Sri Lanka (de Silva *et al*., 2002).

2.3.1.1 Piper yellow mottle virus (PYMoV)

Piper yellow mottle virus (genus: *Badnavirus*; family: caulimoviridae) is a pararetrovirus similar to the other badnaviruses with circular, relaxed 7.5–8.0 kb double stranded DNA molecules contained inside non enveloped bacilliform

particles of size 28–32 nm in width and 115–135 nm in length (Lockhart, 1990; Qu *et al*., 1991; Bouhida *et al*., 1993). Association of PYMoV with the stunted disease has been reported in India, Sri Lanka, Indonesia, Malaysia, Thailand, Philippines and Brazil (Lockhart *et al*., 1997; deSilva *et al*., 2002; Hareesh and Bhat, 2008). Besides black pepper, the virus infects eight other species of the genus *Piper* including *Piper betle* and *Piper longum*. The virus is systemic; its distribution is irregular and can be detected from several tissues like phloem, epidermis and mesophyll (Lockhart *et al*., 1997). The spread of the virus is mainly through vegetative means by stem cuttings and by means of insects like mealy bug (*Planococcuscitri* and *Ferrisiavirgata*) and black pepper lace bug (*Diconocoris distant*) (Lockhart *et al*., 1997; deSilva *et al*., 2002; Hareesh and Bhat, 2008). The genome of PYMoV consists of four Open Reading Frames (ORFs). ORF III is highly conserved among the species and encodes for a 218.6 kDa polyprotein that is cleaved by the aspartic protease to form four functional proteins; coat protein, movement protein, aspartic protease and a replicase that in turn comprises of a reverse transcriptase and ribonuclease H. ORFs, I, II and IV encodes for putative proteins with 15.7, 17.1 and 17.9 kDa respectively (Deeshma and Bhat, 2014).

2.4 *Cucumber mosaic virus* (CMV)

Cucumber mosaic virus (genus: *Cucumovirus*; family: Bromoviridae), first reported concurrently by Doolittle, (1916) and Jagger, (1916), is known to be the most devastating plant pathogen with the broadest host range of 1200 species of plants worldwide (Edwardson and Christie, 1991). Infection is systemic, and mainly affects the newer cells and tissues formed after the infection. Once infected, the virus concentration increases for many days, later starts declining and becomes static or cause the death of the plants (Agrios, 1978). The virus is highly unstable, cannot tolerate temperatures above 70°C for 10 min (Francki *et al*., 1979). The virus spreads through sap of the infected plant (Francki *et al*., 1979), infected stem cuttings and more than 80 species of aphids in 33 genera are known to spread CMV in a non–persistent manner (Palukaitis *et al*., 1992). *Aphis gossypii* and *Myzus persicae* are important among them. The preference of

CMV towards different species of insects also varies and affects the process of transmission by insects (Francki *et al.*, 1979). CMV is also known to be transmitted through seeds as well as some weeds (Singh *et al.*, 1995).

Figure 2.1 Healthy and virus infected black pepper vines, a) healthy black pepper vines, **b) and c)** virus infected black pepper vines showing mosaicism, mottling and stunting.

2.4.1 CMV genome and its organization

CMV is a tripartite positive sense RNA virus with three single stranded RNA molecules designated as RNA1, RNA2 and RNA3 enclosed in separate isometric particles of size 28 nm as revealed by electron microscopy (Peden and Symons, 1973; Bhat *et al*., 2004). The virions have a T3 icosahedral symmetry of size approx. 29 nm diameters. The capsid is made up of single protein of 24 kDa present in 180 copies. RNA1 is monocistronic coding for 1a gene. RNA2 and RNA3 are dicistronic coding for 2a, 2b and 3a, 3b genes respectively. The C–terminal of RNA1a is homologous to viral helicases (Gorbalenya *et al*., 1989) and the N–terminal region of 1a protein has putative methyltransferase domain (Mikhail *et al*., 1992). Open reading frames 1a and 2a are the viral components of replicase (Palukaitis *et al*., 1992). There are reports showing that the 1a and 2a protein have elicitor functions for defense responses in plants (Kim *et al*., 1997; Diveki *et al*., 2004; Kang *et al*., 2012). Another study revealed that mutations in 1a protein in a pepper isolate of CMV can have an effect on the resistance responses to CMV (Kang *et al*., 2012). The 2b gene is expressed from subgenomic RNA4A. Besides its role in long distance movement (Ding *et al*., 1996; Ji and Ding, 2001; Soards *et al*., 2002; Wang *et al*., 2004), it acts as suppressor of post transcriptional gene silencing (Beclin *et al*., 1998; Brigneti *et al*., 1998; Li *et al*., 1999) and aphid transmission (Ziebel *et al*., 2011). The 2b protein suppresses the RNA silencing by inhibiting the cleavage of Argonaute1 (AGO) protein in the RNA induced silencing complex (RISC) assembling (Zhang *et al*., 2006). Further, the infectious clones of bean isolate of CMV were constructed and RNA2 was shown as the specific determinant for systemic infection (Thompson *et al*., 2015). Recently, it was shown that the 2b protein recruited the endogenous 30S ribosomal subunit protein S11 by direct interaction, thereby facilitating the gene silencing suppressor activity (Wang *et al*., 2017). The 3a gene encodes the movement protein (MP) and 3b, the coat protein (CP) expressed through subgenomic RNA (RNA4). MP is involved in cell to cell movement of the virus and CP helps in encapsidation of viral nucleic acid and affects symptom expression (Shintaku *et al*., 1992; Suzuki *et al*., 1995). Previous studies showed RNA3 as the genetic determinant for symptom expression (Mossop and Francki, 1977; Rao

and Francki, 1982). Mutation in the amino acid at position 129 of CP of CMV was reported to affect the expression of symptoms (Mochizuki and Ohki, 2011). In a more recent study, the CMV CP was confirmed to be the genetic determinant for mosaic symptom expression in tobacco plants and that the interaction of the CP with a chloroplast protein ferredoxin I influenced the development of chlorotic symptoms on tobacco leaves (Qiu *et al*., 2018).

Based on the serological data, the virus is closely related to *Tomato aspermy virus* (TSV) and *Peanut stunt virus* (PSV). Based on serology, symptomology, host range data, peptide mapping of CP and nucleic acid hybridization, CMV strains has been classified into subgroups I and II (Roossinck *et al*., 1999). Subgroup I is further classified into IA and IB based on the 3' UTR of RNA3 (Roossinck, 2001). Subgroup IA and II is seen worldwide whereas IB is mainly restricted to Asian countries (Roossinck, 2002). Many studies have reported the occurrence of reassortants and recombinants between these subgroups (Kim *et al*., 2014; Nouri *et al*., 2014).

2.4.2 CMV in black pepper

The virus along with PYMoV, causes stunted disease in black pepper. Typical symptoms include stunting of plant, mottling and mosaic on leaves, reduction of spike length and poor filling of spikes leading to reduction in yield. From black pepper, CMV could be readily transmitted to different Solanaceous and Cucurbitaceous plants but not the other way round (Sarma *et al*., 2001; de Silva *et al*., 2002). Association of CMV with the stunted disease in black pepper has also been reported from Sri Lanka (de Silva *et al*., 2001; de Silva *et al*., 2002) and Brazil (Eiras *et al*., 2004). Besides black pepper, CMV infects other related species such as Indian long pepper and betelvine (Hareesh *et al*., 2006). Currently, there are no varieties or cultivars of black pepper that are resistant to CMV and no chemicals are available to control the virus. The use of virus free planting materials of elite genotypes is the best management strategy that is adopted to minimize the disease incidence. Due to the lack of virus resistant varieties in any of the country growing black pepper, developing genetic resistance is the best alternative, for which sequence information of CMV from black pepper isolate is essential. The population genetics of CMV have been

studied by many researchers based on Recombinase polymerase assay (RPA), partial genomic sequences and serological analyses (Fraile *et al*., 1997; Lin *et al*., 2004; Davino *et al*., 2012) but there are evidences for recombination and reassortments leading to the evolution of new CMV strains (Roossinck, 2002; Lin *et al*., 2004; Jacquemond, 2012). Thus, complete genome sequencing of black pepper isolate of CMV and thorough analyses will help in the detailed understanding of the genetic structure of CMV populations. This will also help in devising the most suitable genes for transgene construct preparation for providing resistance to CMV.

2.5 Genetic transformation of plants: an overview

Viruses pose an important threat to plants, reducing the quality and quantity of the crops worldwide. The economic relevance of viruses is such that the viruses come second in the list of the most important plant pathogens, first being the fungus. Traditional methods to control plant viruses include, the early screening and removal of the virus infected plants, breeding for resistance, crop rotation, cross protection and/or control of the viral vectors using chemicals, but all these methods are only partially successful in eradicating the viruses (Hull, 2014). Though there was considerable improvement in crop characteristics due to conventional breeding, the ever increasing demand for crop yield and quality due to the exponential growth in population and nutritional requirements, calls for a better strategy for vanquishing the curbs on crop productivity. Besides, conventional plant breeding is constrained by various factors such as absence of important traits like pathogen resistance in the breeding populations, barriers to gene transfers that exist between the species due to deleterious gene linkages, and the complications in producing fertile progenies. Also, the long reproductive cycles of some plant species like conifers make the traditional plant breeding more time consuming. These barriers can easily be tackled through genetic engineering, whereby desirable genes are introduced into the plants that will confer novel phenotypes like improving quality, yield or make them resistant to pathogens, which is one of the indispensable intentions of genetic engineering.

The first transgenic plant was produced in 1983, (Herrera–Estrella, 1983) and the technology saw a major leap in later 1980's due to the advancement in

the chimeric gene constructs (Bevan *et al*., 1983; Fraley *et al*., 1983), plant transformation vectors (Hoekema *et al*., 1983; Bevan, 1984), DNA delivery systems (Hernalsteens *et al*., 1980; Draper *et al*., 1982; Krens *et al*., 1982; Fromm *et al*., 1985; Sanford *et al*., 1987) and efficient plant regeneration systems (Zambryski *et al*., 1983; Paszkowski *et al*., 1984; Shimamoto *et al*., 1989; Gordon–Kamm *et al*., 1990).

Some of the indispensable components for the production of transgenic plant are:

(1) Development of suitable gene constructs in plant transformation vectors
(2) An efficient transformation technique for delivering the gene constructs into the plants
(3) Dependable plant regeneration method in tissue culture
(4) Selection and amplification of the transformed plants
(5) Characterization of transgenic plants at the molecular and genetic level and
(6) Analyses of the transgenic plants.

2.5.1 Development of gene constructs in plant transformation vectors

Initially, the transgenes were introduced into the T–DNA regions of the Ti plasmids of *Agrobacterium tumefaciens* by complex genetic alterations (Garfinkel *et al*., 1981; Zambrbyski *et al*., 1983; Fraley *et al*., 1983). The Ti plasmids were huge, stringent, lacked *ori* site that function in *E. coli* and, suitable restriction enzyme sites for cloning were absent. Also, the oncogenes that cause tumor in the plants had to be removed from the Ti plasmids for the production of transgenic plants without any abnormality. Further, the unwanted opine synthase genes need to be removed as they have no role in the transformation procedure. A breakthrough in this regard was the generation of binary Ti vectors where the T–DNA and the vir region resided on the separate plasmids (Hoekema *et al*., 1983; de Framond *et al*., 1983). In this vector system, the modified Ti plasmids in the *Agrobacterium* served the functions of the vir gene that helped in the infection process and the desired gene was integrated into the T–DNA of the vector construct. The binary vector system also had the origins of replication that worked both in *E. coli* and *Agrobacterium* along with the marker genes that

facilitated the selection of transformants. The most commonly used marker genes are the ones coding for kanamycin, tetracycline, gentamycin and streptomycin. Some of the commonly used binary vectors are pBI121, pGreen, pBIN19, pCGN, pJJ series and pPZP series. Confirmation of the gene of interest inserted into the binary vector can be done by PCR, restriction digestion and sequencing.

Primarily, the most commonly used binary vector was the pBin19 (Bevan, 1984). The binary vector, pBI121 has the pBin19 background with the addition of a marker gene. Besides, the GUS gene in the vector can be easily replaced with the gene of interest (Chowrira *et al*., 1995; Charity *et al*., 1999; Mundembe *et al*., 2009).The β–GUS present in the pBIN series facilitated the identification of transformants by the histochemical GUS staining within a short period after the transformation (Svabova *et al*., 2005; Agarwal and Kanwar, 2007). In another study, a bifunctional marker gene was placed under the control of a double *Cassava vein mosaic virus* (CsVMV) promoter and then introduced into the T–DNA region of pBIN19 derived binary vector (Dhekney *et al*., 2008). The binary vector system pBI121 carrying the GUS reporter gene under the control of CaMV 35S promoter was used for the *Agrobacterium* mediated transformation of tomato (Ashakiran *et al*., 2011). The binary vector pROK2 was used for the transformation of eggplant (Pratap *et al*., 2011) and tomato (Pratap *et al*., 2012). The binary vector pCAMBIA1301 harboring the GUS gene and the hygromycin phosphotransferase gene coding for hygromycin resistance, both under the control of CaMV 35S promoter was used for the transformation of tobacco (Pathi *et al*., 2013). The pCAMBIA2301 vector system which contained the neomycin phosphotransferase gene (nptII) in the T–DNA along with the β–glucuronidase reporter gene, with an intron interrupting the coding sequence, driven by the CaMV 35S promoter was used for the transformation of cassava (Nyaboga *et al*., 2015). In a recent report, the CP gene of CMV was introduced in between the CaMV 35S promoter and nptII marker gene of the binary vector pBI121and was used for the transformation of *N. tabacum* plants (Dubey *et al*., 2015). The binary vector system pBIN AR with the CaMV 35S promoter, OCS terminator and nptII

gene, was used for the transformation of sunflower plants (Singareddy *et al*., 2018).

2.5.2 Transformation techniques for delivering transgenes into plants

Transformation techniques can be broadly classified into direct and indirect gene transfer methods. Both these methods are widely used with the aim of producing maximum number of plants with high genetic stability; and the method that can be used for the transformation of any explant, of any genotype is highly preferred. The direct or vector less gene transfer methods include, physical transfer methods, chemical methods and DNA imbibitions by cells, tissues and organs. The direct methods include biolistic bombardment, electroporation, microinjection, DNA transfer *via* pollen, silicon carbide mediated transformation, liposome mediated, shoot apex method and vacuum infiltration (Rao *et al*., 2009). The chemical transfer methods include, polyethylene glycol (PEG) mediated transfer, calcium phosphate co–precipitation, the dimethyl sulfoxide (DMSO) technique and the diethyl amino ethane (DEAE) dextran procedure. The random and unpredictable DNA integration patterns make the direct methods undesirable for many transformation experiments (Hadi *et al*., 1996).

The indirect methods comprise of the use of vectors, *Agrobacterium tumefaciens* Ti plasmid vectors and viral vectors. Among these, the most popular methods are the *Agrobacterium* mediated transformation and biolistic bombardment (Potrykus, 1991; Dai *et al*., 2001). Earlier, *Agrobacterium* mediated transformation was mainly used for the transformation of dicotyledonous species but of late both these methods are widely used for the transformation of both monocotyledonous and dicotyledonous plants (Altpeter *et al*., 2005). There are reports indicating that biolistic method is preferred for the transformation of monocotyledonous species (Kothari *et al*., 2010), however, *Agrobacterium* mediated transformation was considered the best method for genetic transformation of plants (Meyers *et al*., 2010). Recently, Agrolistics, a combination of *Agrobacterium* mediated transformation and biolistics have also been reported (Mohanty *et al*., 2016).

2.5.2.1 Direct transfer methods

Direct transfer methods like the biolistic bombardment were mainly used for cereals that are not susceptible to the *Agrobacterium* mediated transformation (Vasil and Vasil, 1999; Vasil, 2005). Biolistic bombardment mainly involves the use of a gold microcarrier that is coated with the gene of interest which is delivered into the target cells or tissues at high velocity (Sanford *et al*., 1987; Finer and McMullen, 1990; Sanford, 2000). For a successful transformation by biolistic bombardment, factors like pressure of helium gas used for the transfer, distance between the macro carrier and the target tissue, the DNA concentration, the osmotic treatment given, and the age of the explant needs to be optimized (Vain *et al*., 1993; Ingram *et al*., 1999; Parveez 2000; Vidal *et al*., 2003; Wagiran, 2003; Ivic-Haymes and Smigocki, 2005; Indurker *et al*., 2007). Similar observations were made in another study where the success was independent on the genotype of the explants but depended on all other factors mentioned earlier (Sailaja *et al*., 2008). Microprojectile mediated transformation method was used for the transformation of *Cucumis melo* L. with the CMV CP (Gonsalves *et al*., 1994). Recently, transformation of sugarcane has been reported by the use of particle bombardment where the transgene construct was co–precipitated with the tungsten microprojectiles (Zhang *et al*., 2015; Yao *et al*., 2017).

2.5.3 *Agrobacterium* mediated transformation

The use of *Agrobacterium* as a means of indirect transfer of genetic material started as early as 1980s (de Block *et al*., 1984) and was used widely due to the several advantages of this method over the other direct transfer methods, which include the ability to transfer larger segments of DNA, defined and stable integration of the transgenes, single copy of integration and lack of co–suppression and instability related problems making it more preferable for the breeders (Smith and Hood, 1995; Dai *et al*., 2001; Gelvin, 2005). The only problem associated with *Agrobacterium* mediated transformation was that earlier its use was restricted to the dicots but, this was later solved with the use of acetosyringone and supervirulent *Agrobacterium* strains. Thus, monocots and cereal crops could be easily transformed by using the embryogenic tissues as

explants (Hiei *et al*., 1994; Komari and Kubo, 1999). Further, it was reported that the transformation efficiency was higher for *Agrobacterium* mediated transformation compared to that by particle bombardment in wheat (He *et al*., 2010).The difficulties in the development of gene delivering techniques and the absence of a successful regeneration protocol hinder the success of genetic transformation (Mariashibu *et al*., 2013). Recently, a new method of transformation was reported, the '*In planta'* method where the explants are directly infected with the *Agrobacterial* cultures without involving plant tissue culture or regeneration. This reduces the time, labor costs and also the somaclonal variations arising in the plants regenerated by tissue culture (Mayavan *et al*., 2013; Kalbande and Patil, 2016). *Agrobacterium* injection, vacuum infiltration, gene transfer *via* pollen, floral dip and spray methods are the various *in planta* methods widely used (Niazian *et al*., 2017).

2.5.3.1 Agrobacterium as a natural genetic engineer

Agrobacterium is a gram negative soil borne phytopathogen that causes the crown gall disease mainly in dicotyledonous plants and in fewer monocot species (Nester *et al*., 1984). *Agrobacterium* mainly infects the wounds in the plant and the chemicals released by the wounded tissue, activate the virulence (vir) genes initiating the process of infection. Upon infection, the bacterium transfers a set of genes called the Transfer–DNA (T–DNA), residing in the tumor inducing (Ti) plasmid present in the bacterium, into the plant genome. The genes mainly present in the T–DNA include the oncogenes that cause tumor in plants (Gaudin *et al*., 1994) and opine synthesis genes that are responsible for the synthesis of opines. Opines are derivatives of amino acid used by the *Agrobacterium* as a sole source of nitrogen. The other bacterial chromosomal virulence genes (chv) and the virulence genes in Ti plasmid aid in the synthesis and transport of the T–DNA into the host cell. Many other factors are also known to assist the transformation process especially during the intacellular and nuclear transport, and the integration of the T–DNA (Tzfira and Citovsky, 2002; Gelvin, 2003; McCullen and Binns, 2006).

2.5.3.2 Agrobacterium strains used for transformation

The biology and biotechnology of the different *Agrobacterium* strains have been extensively studied during the past few decades (Nester *et al*., 1984) which led to the formulation of many new strains of *Agrobacteria*, recombinant plasmids and protocols of transformation. The *Agrobacterium* strains in which the gene of interest is incorporated into the native T–DNA of Ti–plasmid, are the most common recombinant vectors used for the efficient transformation of many plant species (Draper *et al*., 1988). Majority of the transformation experiments were focused on the use of *Agrobacterium tumefaciens* as the genetic engineer (Chan *et al*., 1993; Hiei *et al*., 1994; Dong *et al*., 1996; Cheng *et al*., 1997; Arencibia *et al*., 1998; de La Riva *et al*., 1998), however, *Agrobacterium rhizogenes* have also been used in few transformation studies (Otani *et al*., 1993; Van de Velde *et al*., 2003).

Among the different strains developed; EHA101, EHA105, LBA4404 and AGL1 are the most commonly used ones. EHA101 is a derivative of A281, carries the plasmid pEHA101 and is supervirulent. The strain AGL1 carries the disarmed Ti plasmid, pTiBo542 and has the chromosomal background of C58 (Lazo *et al*., 1991). EHA105 is another supervirulent strain developed from the EHA101 and carries the plasmid pEHA105. This plasmid is derived from the plasmid pTiBo542 (Hood *et al*., 1993). The four strains, LBA4404, C58, EHA105 and AGL1 were used for the transformation of lavandin and their efficiencies were compared (Dronne *et al*., 1999). The strain LBA4404 was used for the transformation of *Euphorbia pulcherrima* (Dronne *et al*., 1999) and carries the TiAch5 chromosomal structure and the modified Ti plasmid pAL4404 (Hellens *et al*., 2000). Many transformation studies were carried out with the strain EHA105 (Khawale *et al*., 2006; Li *et al*., 2006; Firoozabady, 2006; Nandakumar *et al*., 2007; Petri *et al*., 2008; Dutta *et al*., 2008; Dhekney *et al*., 2008; Vidal *et al*., 2010). The *Agrobacterial* strain EHA105 was used for the genetic transformation of vanilla (Retheesh and Bhat, 2011). The same strain was used for the transformation of black pepper in a similar study (Jiby and Bhat, 2011).

2.5.4 A reliable plant regeneration system by tissue culture

Regeneration of transformed tissues into plantlets is the most strenuous task in production of transgenic plants, so an efficient system for *in vitro*

regeneration is needed. Selection of suitable explants is the primary step for the initiation of *in vitro* regeneration system. Previous studies report the use of various explants like apical meristem of shoots (McCabe *et al*., 1988; Christou *et al*., 1990; Bidney *et al*., 1992; Brar *et al*., 1994). Embryogenic callus as explants for transformation had an advantage that the number of chimeric plants regenerated from these tissues was less compared to that regenerated from explants like cotyledons (Polito *et al*., 1989; Gonzalez *et al*., 1998). Actively dividing embryogenic tissues were found to be good explants for the production of transgenic crops (Finer and McMullen, 1990; Parrott *et al*., 1994; Wang *et al*., 1998; Cheng *et al*., 2004; Leelavathi *et al*., 2004; Salaj *et al*., 2009). Transformation of monocot plants like rice, wheat, maize, sorghum and grass were successfully accomplished by regeneration of the induced calli (Hiei *et al*., 1994; Xia *et al*., 1999; Zhao *et al*., 2000; Li *et al*., 2000; Kusano *et al*., 2003). In many other transformation studies, the commonly used explants were the zygotic embryos and mature somatic embryos (Jones and Van Staden, 1995; 2001). In case of the friable embryogenic callus there were fewer escapes or non–transformed plants, which was not the case with other explants like shoots (Li *et al*., 1996; Zhang *et al*., 2000). Some other explants commonly used for transgenic studies include the cotyledon (Taskin *et al*., 1999; Orczyk *et al*., 2000; Lopez *et al*., 2000; Agarwal and Kanwar, 2007) and leaves (Liu and Bao, 2003; Kenel *et al*., 2010). Earlier, it was reported that active vegetative cells were more susceptible to *Agrobacterium* mediated transformation (Gelvin, 2003), and that the embryogenic tissues show the same genetic background with the cultivar from which it was derived (Li *et al*., 2003). Henceforth, there were many reports on the use of embryogenic clumps as explants for transformation (Leelavathi *et al*., 2004; Salaj *et al*., 2009). Besides, they can be easily cultured and have good proliferation and regeneration capabilities (Xing *et al*., 2008). Finally, there are reports where stem (Kotsuka and Tada, 2008), and callus tissues (Ozawa, 2009; Danilova *et al*., 2009) have been used as explants for transformation. Somatic embryogenesis was considered as a better propagation system due to the less time needed for establishment of cultures and regeneration of plantlets with high genetic uniformity (Kothari *et al*., 2010).

Thus, for both the dicotyledonous and monocotyledonous plants, the explants most commonly used for *Agrobacterium* mediated transformation were the embryogenic callus (Delbreil *et al*., 1993; Nakano *et al*., 1994; Cheng *et al*., 1997; Moran *et al*., 1998; Yamamoto *et al*., 2000; Zhai and Liu, 2003). The embryogenic calli derived from the citrus was highly stable during the various stages of tissue culture like multiplication, conversion and regeneration (Li *et al*., 2003). The embryogenic cultures initiated from the leaves of *in vitro* grown plants were used for the transformation of muscandine grape and transformed plantlets were recovered (Dhekney *et al*., 2008). Similarly, leaf disc explants were used for the transformation of *Nicotiana benthamiana* and plantlets were successfully regenerated from the transformed leaf discs (Srivastava *et al*., 2008). *Agrobacterium* mediated transformation of olive tissues was successfully done using the embryogenic mass as explants (Torreblanca *et al*., 2009; 2010). In another study, the premature zygotic embryos were found to be good explants for biolistic method of transformation of papaya (Kung *et al*., 2010). The somatic embryos induced from leaves were used for the *Agrobacterium* mediated transformation of *Quercus robur* and stable transformants were regenerated (Vidal *et al*., 2010). Further, in a similar study, somatic embryos were used for the *Agrobacterium* mediated transformation of Norway spruce, however, only transgenic callus was recovered (Pavingerova *et al*., 2011). Somatic embryos developed from the cotyledon explants were used for the *Agrobacterium* mediated transformation of tomato and the efficiency of transformation was found to be 70% (Ashakiran *et al*., 2011). Protocorm like bodies developed from the shoot tips of vanilla were used as explants for *Agrobacterium* mediated transformation and transformed tissues were regenerated (Retheesh and Bhat, 2011). Regeneration of plantlets was reported in a different study where cotyledon explants were used for the *Agrobacterium* mediated transformation of eggplant (Pratap *et al*., 2011). The leaf disc explants were used for the transformation of tomato and successful regeneration of 30% shoots was observed (Pratap *et al*., 2012). A highly efficient protocol for *Agrobacterium* mediated transformation of tobacco somatic embryos initiated from leaf discs was developed and, high frequency regeneration with 95% transformation

efficiency was obtained (Pathi *et al*., 2013). In a recent report, friable embryogenic calli derived from the somatic embryos induced on axillary buds and immature leaf lobes were used for the transformation of cassava and, plantlets were regenerated with higher transformation efficiency (Nyaboga *et al*., 2015). The *Agrobacterium* mediated transformation of sunflower was carried out using the split cotyledons and the average frequency of shoot formation was found to be 3.3% (Singareddy *et al*., 2018).

2.5.5 Tissue culture of black pepper

For *in vitro* studies done using black pepper, the MS (Murashige and Skooge, 1962) medium was the most commonly used one for the micropropagation of black pepper. Fungal and endogenous bacterial contamination was the main problem arising in tissue culture system (Raj Mohan, 1985; Fitchet, 1990). The fungal contamination arising in tissue culture of black pepper were controlled by giving the explants, a wash with the fungicides like Bavistin (carbendazim) and Fytolan (copper oxychloride) before the surface sterilization of the explants (Nazeem *et al*., 1992). The growth and development of the *in vitro* plants were affected by the antibiotics used for controlling the contamination (Meyer *et al*., 1992).

Clonal propagation through shoot tips from mature vines of black pepper has been previously reported (Philip *et al*., 1992; Joseph *et al*., 1996; Nazeem *et al*., 2004). Similar studies used Gamborg B5 and Woody plant medium (WPM) instead of MS medium for the micropropagation of black pepper (Bhat *et al*., 1995; Babu *et al*., 1997). The growth hormone most preferred in tissue culture of black pepper was benzyl adenine (BA) alone or in combination with some auxins that favor multiple shoot formation. Once the plants were developed *in vitro*, basal medium free of hormones can be used for rooting of the plantlets (Rema *et al*., 1995). The hormonal combination of 3.0 mg/L BA with 1.0 mg/L kinetin resulted in multiple shoot induction (Babu *et al*., 1997). Another severe problem faced in tissue culture of black pepper was the existence of phenolic compounds especially during the initiation stage of culture establishment that causes browning. This was then controlled by the use of activated charcoal at a concentration of 2 g/L of the culture medium (Madhusudhanan and Rahiman,

2000). Further, an important breakthrough in tissue culture of black pepper was the development of an efficient protocol for a high frequency regeneration of plantlets from the cyclic secondary somatic embryos of black pepper. These cyclic secondary embryos arose from the primary somatic embryos, induced on the micropylar tissues of germinating seeds (Nair and Dutta, 2003; 2006). The hormone BA was used for the induction of callus and, its regeneration was reported from the petiole explants with the maximum regeneration of shoots (92%) noted at 0.5 mg/L of BA (Ahmad *et al*., 2010).

2.5.6 *Agrobacterium* mediated transformation of black pepper

There are very few reports on transformation of black pepper. Transformation of the leaf and cotyledon explants of black pepper using the LBA4404 strain of *Agrobacterium* carrying the plasmid PGA 472 with nptII selectable marker was attempted and visible callus was observed only in the cotyledon explants under the selection pressure of kanamycin. Confirmation of the transformed calli was done by selection using kanamycin. Only the transformed calli proliferated in the presence of kanamycin and no proliferation was observed in the control calli in kanamycin (Sasikumar and Veluthambi, 1996).

Agrobacterium mediated transformation of the leaf, stem and petiole explants of black pepper axenic seedlings was reported using strain LBA4404 carrying the plasmids pMOG23 and pTOK47. After two days of co–cultivation, the explants were cultured in MS medium with the antibiotics cefotaxime and carbenicillin at 100 and 500 mg/L concentration respectively for arresting the bacterial growth. The medium contained another antibiotic, kanamycin at a concentration of 75 mg/L that served as selective agent for transformed cells. After the elimination of *Agrobacteria*, transformed shoots were selected at a higher kanamycin concentration of 75 mg/L and were analyzed for the expression of GUS by staining the cells with X–gluc. The transformed cells showed blue color on staining, but the nptII gene was not detected by PCR (Sim *et al*., 1998).

An efficient protocol for the production of transgenic black pepper was developed using *Agrobacterium* mediated transformation of somatic embryos

derived from the micropylar regions of mature berries. After co–culturing, *Agrobacterium* was killed using cefotaxime (100 µg/mL). Transformed embryos were selected by increasing concentrations of kanamycin (25 µg/mL to 50 µg/mL) and finally selection was done at a concentration of 100 µg/mL. The selected transformants were regenerated, hardened and screened for the presence of transgene by PCR, dot blot and Southern blotting techniques (Jiby and Bhat, 2011).

An efficient protocol for black pepper multiplication was developed using *in vitro* germinated seedlings as explants. *Agrobacterium* mediated transformation of black pepper shoots regenerated from the above explants was carried out using EHA105 harboring pCAMBIA1301 with GUS as the reporter gene and hpt II as the antibiotic resistance gene. Infection with the bacterium was allowed for 15 min followed by two days of co–cultivation. PCR and histochemical GUS assay were used for the preliminary screening of the transgene (Maju and Soniya, 2012).

2.5.7 Selection and regeneration of transformants

Antibiotics mainly used during the transformation are:

a) for killing the *Agrobacteria* and
b) for the selective growth of the transformants.

The β–lactam antibiotics like the carbenicillin and cefotaxime with minimum level of toxic effects on the plant cells are mainly used for killing the *Agrobacteria* after co–culturing (Mathias and Boyd, 1986; Tang *et al*., 2000; Alsheikh *et al*., 2002; Leelavathi *et al*., 2004; Han *et al*., 2007). There are reports showing that these antibiotics have beneficial effect on the growth of the plants since the breakdown products mimic the phytohormones and alter the levels of auxins and cytokinins that favor regeneration (Barik *et al*., 2005). On contrary to this, cefotaxime and carbenicillin at different concentrations adversely affected the shoot induction as shown by many researchers (Li *et al*., 2007; Oz *et al*., 2009). However, the beneficial effects of the antibiotics also were further reported in a study (Oz *et al*., 2009).

The major selective agents used for screening transformants are the antibiotic resistance genes that include kanamycin (Moran *et al*., 1998; Zhai and Liu, 2003; Duque *et al*., 2004), hygromycin (Bull *et al*., 2009; Pathi *et al*., 2013) and paromomycin (Petri *et al*., 2008). Among these, simplicity, efficiency and eco–friendly nature of kanamycin makes it the most preferred selective agent used in transformation studies (Flavell *et al*., 1992; Zhang *et al*., 2001; Barik *et al*., 2005; Dutta *et al*., 2008; Srivastava and Raj, 2008; Sharma *et al*., 2009; Gao *et al*., 2010; Vidal *et al*., 2010; Ashakiran *et al*., 2011; Pavingerova *et al*., 2011; Retheesh and Bhat, 2011; Pratap *et al*., 2011; Pratap *et al*., 2012; Nyaboga *et al*., 2015; Dubey *et al*., 2015; Yao *et al*., 2017; Singareddy *et al*., 2018).

2.5.7.1 Kanamycin for selection of black pepper transformants

Earlier studies reported that, optimum kanamycin concentration for selection of transformed calli of black pepper was found to be 50 µg/mL and higher concentration beyond this inhibited the callus formation completely (Sasikumar and Veluthambi, 1994). However, kanamycin concentration upto 150 µg/mL was found to be safe for the selection of the transformed calli in black pepper (Sasikumar and Veluthambi, 1996). In the study conducted by Jiby and Bhat, (2011), a step wise increase in the concentration of kanamycin from 25 µg/mL to 50 µg/mL and finally 100 µg/mL was found to be suitable for the selection of transformants of black pepper. In the study conducted by Maju and Soniya, (2012); hygromycin was the antibiotic used for the selection of transformants at a concentration of 20 µg/mL.

2.5.8 Screening of transgenic plants

2.5.8.1 GUS assay

GUS gene coding for the enzyme β–glucuronidase is the most commonly used reporter gene. The enzyme catalyses the conversion of the chromogenic substrate X–gluc (5–bromo–4–chloro–3–indolyl β–D–glucuronic acid) to 5,5'–dibromo 4,4'–dichloro indigo, which is an insoluble blue colored product. The production of blue color is an indication that the cells are transformed as the native plant cells do not have any GUS activity. This method can be performed easily, is sensitive, reliable and inexpensive as the detection is done visually without the aid of any sophisticated instruments (Jefferson, 1987; Jefferson *et al*.,

1987). The use of GUS genes for screening transformants has been reported by many researchers in many plant species. Some recent reports were in Japanese apricot (Gao *et al*., 2010), *Quercus robur* (Vidal *et al*., 2010), tomato (Ashakiran *et al*., 2011), Norway spruce (Pavingerova *et al*., 2011), vanilla (Retheesh and Bhat, 2011), black pepper (Jiby and Bhat, 2011), tobacco (Pathi *et al*., 2013) and cassava (Nyaboga *et al*., 2015).

2.5.8.2 Polymerase chain reaction assay

Initial screening for the presence of the transgene can be done by the polymerase chain reaction (PCR) which is a fast and sensitive method although the sensitivity and the reliability varies (Hiei *et al*., 1994; Shekhawat *et al*., 2008; Yang *et al*., 2010). Though the method is not fool–proof and there are reports on false positives (Sarria *et al*., 2000; Yang *et al*., 2010), PCR can be used as a cost–effective and fast method for the preliminary screening of the transformants and useful and initial data can be obtained. The contamination of the cells with the *Agrobacterium* carrying the transgene may result in false positives in PCR (Shekhawat *et al*., 2008). Many studies related to transgenic crop production have used PCR as the basic technique for detection of the transgene in the transformants of *Nicotiana benthamiana* (Srivastava and Raj, 2008), *Quercus robur* (Vidal *et al*., 2010), eggplant (Pratap *et al*., 2011), Norway spruce (Pavingerova *et al*., 2011), vanilla (Retheesh and Bhat, 2011), black pepper (Jiby and Bhat, 2011), tomato (Pratap *et al*., 2012), tobacco (Pathi *et al*., 2013), cassava (Nyaboga *et al*., 2015), *Nicotiana tabacum* (Dubey *et al*., 2015), and sunflower (Singareddy *et al*., 2018).

2.5.8.3 Southern and northern hybridization

As popular tools used for the confirmation of transgenicity, the Southern hybridization confirms the presence of transgenic DNA, while northern hybridization helps in detecting the transcript production from the transgene. Though these methods are tedious, they are important confirmatory tests and in most of the transgenic experiments, the testing of transgenic plants by PCR is usually followed by the Southern and northern hybridization. The confirmation of *Tobacco mosaic virus* (TMV) CP in the transgenic *Capsicum annuum* was done

by Southern and northern hybridization. Southern hybridization was performed on randomly selected plants screened by PCR, using the (P32)–dCTP labeled TMV CP gene as probe. Southern hybridization revealed a single copy insertion and northern hybridization revealed the production of 657 bp transcript in these plants (Lee *et al*., 2004). The copy number of the transgene in the T0 transgenic plants of *Nicotiana benthamiana* were confirmed by both Southern and northern hybridization using the probe for the CP gene of CMV (Srivastava and Raj, 2008). Southern hybridization confirmed the presence of transgene in all the five randomly selected plants and northern detected the presence of transcript only in the transgenic plants and no signal was seen in the non–transformed controls. Southern hybridization of the total DNA using the nptII and GFP specific probes revealed single gene insertions in 15 transgenic calli lines and multiple insertions in three lines of *Hevea brasiliensis* (Leclercq *et al*., 2010). The true transgenics of vanilla were confirmed by the Southern and northern hybridization using the probe specific for CMV CP (Retheesh and Bhat, 2011). Southern hybridization using the DIG–labeled DNA probe specific to the GUS gene confirmed the true transgenicity in all the PCR positive plants with insertions at different locations (Nyaboga *et al*., 2015). Southern hybridization of total DNA restricted with *Hind*III and hybridized with the α–32P dCTP radiolabeled CP specific probe confirmed the transgenicity in tobacco plants (Dubey *et al*., 2015). Southern hybridization confirmed the presence of transgene in thirteen transgenic lines of sugarcane carrying the *Sugarcane mosaic virus* (SCMV) CP gene (Yao *et al*., 2017). In the transgenic study conducted in sunflower the presence of the *Tobacco streak virus* (TSV) CP gene was confirmed by Southern and northern hybridization (Singareddy *et al*., 2018).

2.5.9 Challenge inoculation for evaluating the transgenic plants

Understanding of resistance in transgenic plants is equally important to the development of these plants by transformation. Evaluation of resistance for a particular virus involves the successful transmission of virus to the host plants. This can be done by mechanical inoculation through sap transmission, grafting or transmitting the virus through vectors like aphids. In a previous study, challenge inoculation in transgenic tomato against the *Tomato leaf curl virus*

(TLCV) was done by aphid transmission using whitefly (Praveen *et al*., 2005). Further, resistance in transgenic tobacco against the *Tobacco rattle virus* (TRV), was tested by introducing the virus by challenge inoculation through foliar rub–inoculation and transmission of virus by nematodes (Vassilakos *et al*., 2008). In transgenic tobacco plants carrying the inverted repeats of *Grapevine fan leaf virus* (GFLV), challenge inoculation of the virus was done by sap transmission using the abrasive carborandum (Jardak–Jamoussi *et al*., 2009). In the case of black pepper, sap transmission of CMV from black pepper to *Chenopodium* and *Nicotiana* was found to be successful but not to black pepper itself, the major limiting factors being the woody nature of black pepper and a harder and hydrophobic leaf lamina. The presence of oxidants, phenols and other inhibitors and low viral concentrations also affect sap transmission (Sarma *et al*., 2001; Akhtar and Haq, 2003). Transmission of CMV to black pepper was attempted by sap inoculation, aphid transmission, grafting and results show that CMV transmission from black pepper to *N. glutinosa* was successful but not to black pepper itself, due to the presence of phenolic compounds and the relatively low concentration of virus in the crude sap. Inoculation of the virus by aphid transmission was also not successful. Fifty percent of the plants inoculated by grafting got established and all these showed symptoms but the presence of CMV was not confirmed (de Silva *et al*., 2002). Further, 100% success in grafting and transmission of *Mungbean yellow mosaic virus* (MYMV) was reported in mungbean (Akhtar and Haq, 2003). Transmission efficiency of *Cassava brown streak virus* to cassava by chip bud grafting was found to be 70–100% successful (Wagaba *et al*., 2013). The various factors that make the resistance/susceptibility studies (by aphid transmission) laborious are the involvement of vector, the lower efficiency of transmission, acquisition period, persistence and semi–persistence nature of viruses, as well as host–vector–virus interactions (Wagaba *et al*., 2013).

2.6 Engineered resistance for diseases in plants

Plant viruses are one among the major constraints for agricultural production worldwide (Kang *et al*., 2005). The management strategies for

diseases pertaining to plant viruses are mainly based on preventive measures due to the lack of proper therapeutic treatments and the absence of resistance genes. Thus engineered genetic resistance in the plants is the best method to combat viruses. Genes of any type/function including pathogen derived genes, defense related genes, genes coding for antimicrobial peptides, from any source including bacteria, plants, animals or human beings can be successfully made to express in plant systems. When the genes used to confer resistance were taken from the pathogens, the concept was termed as pathogen derived resistance (PDR) as initially described by Sanford and Johnston, (1985). The first transgenic plant produced using this approach was tobacco, that expressed the CP gene of *Tobacco mosaic virus* (Abel *et al*., 1986).

Engineering virus resistance in plants is one of the major applications of transgenic technology that ensure the production of safe and long–lasting virus resistant crops which were otherwise impossible by the conventional plant breeding techniques (Fuchs and Gonsalves, 2007). Starting from the *Agrobacterium* mediated transformation of plants in the 1980's it has been widely used against the viruses of all genera and also the viriods (Lin *et al*., 2007; Prins *et al*., 2008; Schwind *et al*., 2009). Subsequently, different viral gene constructs were used to confer resistance and the mechanism of resistance also differed in different hosts depending upon the viruses studied and the constructs used (Morroni *et al*., 2008). One of the mechanism of resistance is protein mediated where the complete protein encoded by the transgene acts as the effector (Register and Beach, 1988; Wu *et al*., 1990; Osbourn *et al*., 1990; Asurmendi *et al*., 2007; Bendahmane *et al*., 2007). The other mechanism of resistance is RNA mediated where the transgene transcribed RNA acts as the effector. This mechanism, also known as 'RNA interference (RNAi)' and 'post transcriptional gene silencing (PTGS)' is the predominant one and is highly sequence specific (Lindbo *et al*., 1993).

2.6.1 Coat protein mediated resistance (CPMR)

In this strategy the complete virus CP is expressed in transgenic plants. The tobacco plants carrying CP gene of *Tobacco mosaic virus* (TMV) were

resistant not only to TMV (Abel *et al*., 1986), but also to *Tobamo* viruses whose coat protein was closely related to TMV (Masuta *et al*., 1998). Similarly potato plants expressing the CP of *Potato virus X* (PVX) showed resistance to PVX (Hemenway *et al*., 1988). A commercially cultivated crop based on PDR is the transgenic papaya developed using the CP gene of *Papaya ringspot virus* (PRSV) HA 5–1 by particle bombardment (Fitch *et al*., 1990). These transgenic plants showed resistance to the virus under glasshouse (Fitch *et al*., 1992) and in field conditions (Lius *et al*., 1997). Subsequently, the commercialized transgenic papaya varieties were developed (Tennant *et al*., 2001; Tripathi *et al*., 2007) and the high level of resistance was correlated to the presence of high copy number of transgene insertions (Kung *et al*., 2010). Transgenic tobacco plants expressing the CP genes of *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV) and *Groudnut ring spot virus* (GRSV) showed resistance to all these three viruses (Prins *et al*., 1995). Transgenic summer squash carrying the CP gene of *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV) showing resistance to both single and multiple infection by these viruses, were produced and commercialized in United States (Tricoli *et al*., 1995; Clough and Hamm, 1995; Fuchs and Gonsalves, 1995; Fuchs *et al*., 1998; Gaba *et al*., 2004; Klas *et al*., 2006; Johnson *et al*., 2007). Transgenic plum carrying the CP gene of *Plum pox virus* (PPV) were found to be highly resistant to the virus (Scorza *et al*., 2007). In the field study conducted in transgenic sugarcane lines transformed with the *Sugarcane mosaic virus* (SCMV) CP, the transgenic plants were highly resistant to SCMV and showed increased cane production. The stability of the transgene was also confirmed in vegetatively propagated generations (Yao *et al*., 2017). Recently, sunflower was transformed with the CP gene of *Tobacco streak virus* (TSV) *via Agrobacterium* and on challenge inoculation by sap transfer, one transgenic line showed streak disease resistance (Singareddy *et al*., 2018).

2.6.1.1 Coat protein mediated resistance to CMV

Transgenic squash lines have been developed using the coat protein genes from CMV, ZYMV and WMV and showed resistance to all these viruses (Tricoli *et al*., 1995; Fuchs *et al*., 1998). Similarly, CP mediated strategy was

widely used against CMV and promising results were seen in mainly six plant species that includes cucumber, tobacco, tomato, melon, squash and pepper (Morroni *et al*., 2008). Coat protein mediated resistance to CMV subgroup IB was shown in *Nicotiana benthamiana* plants (Srivastava and Raj, 2008). Transgenic chilli plants resistant to CMV pathotype, CMVP1 were produced *via A. tumefaciens* transfected with the CP of a different strain CMVP0 (Lee *et al*., 2009). Similarly transgenic eggplants carrying the CP of CMV showed significant degree of resistance to the virus (Pratap *et al*., 2011). Coat protein mediated resistance to CMV subgroup IB was also manifested in tomato plants (Pratap *et al*., 2012). Further, CPMR to Gladiolus isolate of CMV subgroup IA was demonstrated in *Nicotiana tobacum* (Dubey *et al*., 2015). Transgenic tomato and pepper plants carrying the CMV CP gene was reported to be deregulated in China (James, 2009; National Academics of Sciences and Medicine, 2016).

The mechanism of resistance mediated by coat protein is attributed to the fact that the transgene on transcription produces high levels of CP that coat the viral RNAs which are nascent and not assembled, making it unavailable for translation, thereby conferring resistance (Morroni *et al*., 2008; Lindbo and Falk, 2017).

2.6.2 Replicase (Rep) protein mediated resistance

The use of RNA dependent RNA polymerases (RdRps) as transgenes to confer resistance to viruses was first demonstrated in tobacco plants using the modified RdRp of TMV (Golemboski *et al*., 1990). Resistance to *Cymbidium ring spot virus* was shown in transgenic tobacco plants carrying the full length replicase gene of the virus (Rubino *et al*., 1993). Resistance in transgenic tobacco plants using the complete rep gene was manifested against the *Cow pea mosaic virus* (Sijen *et al*., 1995). The full length rep gene was cloned into potato plants for resistance against *Potato leaf roll virus* (Thomas *et al*., 2000).

There is always a dilemma regarding the mechanism of resistance imparted by replicase protein which was found to be strain specific and protein mediated, from the studies conducted separately by Carr *et al*. (1992) and Donson *et al*. (1993). The concurrence of both resistance phenomenons, of

protein mediated and RNA mediated was later proposed (Tenllado *et al*., 1995; 1996). On contrary, the mechanism of resistance was solely attributed to the RNA mediated in a resistance study against TMV (Marano and Baulcombe, 1998). Finally, it was concluded that initial resistance imparted was RNA mediated, which is further enhanced by a more active protein mediated resistance and thus both the mechanisms are supposed to coexist.

2.6.2.1 Replicase mediated resistance to CMV

Tobacco plants transformed with the defective 2a gene of subgroup IA CMV strain was found to be resistant to both the virion and RNA of CMV (Anderson *et al*., 1992). The presence of the defective RNA transcript (Anderson *et al*., 1992) and protein (Carr *et al*., 1994) was confirmed in the resistant transgenic lines. The same construct provided resistance against both the subgroup IA and IB strains of CMV in tomato (Gal–On *et al*., 1998). Similar studies were conducted with the same construct in tobacco plants and different levels of resistance were found (Hellwald and Palukaitis, 1994; Wintermantel and Zaitlin, 2000). Further, the rep region from RNA1 was used as transgene and tobacco plants with 0–100 % resistance were obtained (Canto and Palukaitis, 1998).

2.6.3 Movement protein (MP) mediated resistance

The use of dysfunctional or mutant MP as transgene was found to confer broader resistance as compared to the CP and rep mediated resistance. This kind of resistance was demonstrated against the TMV and, the transgenic plants showed delay in viral infection and appearance of symptoms (Lapidot *et al*., 1993; Malyshenko *et al*., 1993). The resistance conferred in the above case is due to a competition between the viral MP and the dysfunctional MP transgene for binding to the sites of plasmodesmata. Resistance conferred was due to the complete blocking of the cell–to–cell and systemic spread of the virus, in another experiment (Seppanen *et al*., 1997). Transgenic *Nicotiana occidentalis* plants expressing the P50 MP and *Apple chlorotic leaf spot virus* (ACLSV) deletion mutants showed resistance to *Grapevine berry inner necrosis virus* (GINV)

(Yoshikawa *et al*., 2006). Besides these findings, the actual mechanism of MP mediated resistance still remains ambiguous.

2.6.4 Satellite RNA mediated resistance

Satellite RNAs are parasites of plant viruses and their replication and encapsidation is dependent on the associated helper virus (Roossinck *et al*., 1992). Tobacco plants carrying the partial or multiple copies of sat–RNAs showed delay in symptom appearance on challenge inoculation with CMV (Baulcombe *et al*., 1986). The symptom attenuation was due to the competition between the sat–RNA and the helper virus genome for the replication components (Collmer and Howell, 1992; Wu and Kaper, 1995). Similarly transgenic tobacco, tomato and hot pepper plants resistant to CMV were developed (Tousch *et al*., 1994; McGarvey *et al*., 1994; Kim *et al*., 1997; Cillo *et al*., 2004).

2.6.5 RNA interference (RNAi) and virus resistance

Among the different strategies used for PDR, CP mediated resistance was found to be highly successful in combating the viral infections in plants. However, there are certain disadvantages in using the CP and other viral proteins as transgene that include lack of specificity and, varying levels of resistance that can be easily vanquished by higher dose of the inoculums. Further, the mechanisms underlying the CPMR is not clearly inferred in many cases (Prins *et al*., 2008). Moreover, the studies on CPMR indicate that they actually operate through RNA mediators rather than the proteins expressed from the viral transgene. Initially, this highly sequence specific mechanism was called the RNA mediated virus resistance or post transcriptional gene silencing (PTGS) but later, the term RNA silencing was coined referring to RNA mediated transcriptional gene silencing (TGS) and RNA interference (RNAi) (Baulcombe, 2004).

2.6.5.1 Mechanism of RNAi

RNAi is the phenomenon of post transcriptional down regulation or degradation of endogenous genes due to the expression of transgene mRNA that share high sequence similarity. This mechanism provides resistance to any

organism from aberrant, excess, unwanted or foreign RNA. In case of viral transgenes, RNAi provides resistance to viruses and is referred to as virus induced gene silencing (VIGS) (Napoli *et al*., 1990; vand der Krol *et al*., 1990; Dougherty *et al*., 1995). This mechanism was attributed as the sole reason for the anomalous phenotypes where transgenic lines with low levels of transgene mRNA were highly resistant to viruses and *vice–versa* (Dougherty *et al*., 1995). In some cases, there is delayed resistance or recovery where infection by a virus causes the silencing of an initially active homologous transgene (Lindbo *et al*., 1993; Dougherty and Parks, 1995; English *et al*., 1996; Guo and Garcia, 1997; Baulcombe, 2002; Cerrutti, 2003). The process evolved as a natural resistance mechanism in plants against the invading DNA viruses (Kjemtrup *et al*., 1998) or RNA (Smyth, 1999).The RNAi based PDR strategy aims on the pre–activation of the silencing mechanism present in plants by the expression of transgene that can synthesize dsRNA on processing or by directly applying the dsRNA specific to an invading virus (Pooggin, 2017).

In RNAi, double stranded RNA (dsRNA) acts as the trigger (Fire *et al*., 1998; Hamilton and Baulcombe, 1999) and secondary RNA specific dsRNA precursors are further generated by a complex of cellular factors, also involved in this process. These include components like the translation elongation factor (Zou *et al*., 1998), enzymes like RNase (Ketting *et al*., 1999), RNA dependent RNA polymerase (RdRp) (Cogoni and Macino, 1999; Mourrain *et al*., 2000) and RNA helicases (Dalmay *et al*., 2001).

These dsRNA molecules are processed by specific Dicer like (DCL) enzyme in RNase III–type into short interfering RNAs (siRNAs) of size 21–24 nucleotides (nt) (Bernstein *et al*., 2001). The siRNAs also known as guide RNAs are then loaded onto RNA–induced silencing complexes (RISCs) and these effector complexes with Argonaute (AGO) protein then guide the degradation of homologous RNA molecules (Hammond *et al*., 2001), thereby causing the repression of translation of these molecules (Denli and Hanon, 2003; Tijsterman *et al*., 2002). The DCL, AGO and RdRp enzymes have been encoded by multigene families in plants and possess varied functions of plant development, physiology,

stress responses and virus, viral and non–viral pathogens, transgenes and transposons (Zvereva and Pooggin, 2012; Borges and Martienssen, 2015).

2.6.5.2 siRNAs and miRNAs

There are reports suggesting that virus resistance in plants can be contributed not only by the autonomous 21–22 nt siRNAs (Leibman *et al*., 2011) but also by the mobile 24 nt siRNAs (Zhao and Song, 2014). The small RNAs present in plants are of two types, microRNAs (miRNAs) and small interfering RNAs (siRNAs). The miRNAs are produced by DCLs from the gene transcripts that form hairpin like structures on themselves, whereas the siRNAs are produced by DCLs from dsRNA precursors generated in RdRp dependent or RdRp independent manner from viruses, multiple genes/genomic loci. The AGOs are involved in sorting both these RNAs based on the size and nucleotide identity of the 5' end and forms the RISCs thereby mediating RNAi by mRNA cleavage or DNA methylation both directed by these sRNAs (Fang and Qi, 2016). The miRNA mediated resistance was non–transmissible through grafting (Zhang *et al*., 2011). However, the mobile nature of 24–nt siRNAs make it possible to graft the non–transgenic scions on transgenic rootstocks, which is a very important antiviral strategy in case of woody perennial plants (Cirilli *et al*., 2016).

2.6.5.3 Intron hairpin RNA (ihpRNA) construct preparation

Though it was found that multiple copies of the transgene enhanced the induction of RNA silencing (Swaney *et al*., 1995), initially, RNAi in transgenic plants was made functional by the use of single copy of the transgene in positive polarity (Elmayan and Vaucheret, 1996). Later, high efficiency was observed especially when transgene was arranged as inverted repeats capable of forming dsRNA on transcription (Stam *et al*., 1998) and the resistance conferred in the plants using such constructs was higher compared to the plants with single copy of the transgene (Waterhouse *et al*., 1998). The hairpin RNAs were initially separated by a spacer (Smith *et al*., 2000; Kalantidis *et al*., 2002) but later, the efficiency of the phenomenon was further increased by the replacement of spacer with intron in between the inverted repeats of the transgene. Further, the intron spliced hairpin RNAs were found to be stronger triggers for RNA silencing

(Smith *et al*., 2000; Wesley *et al*., 2001; Kerchen *et al*., 2004). There are studies showing that the siRNAs are mainly generated from the viral hairpin sequences and not from the intron (Montes *et al*., 2014; Fuentes *et al*., 2016).

Though the transgene sequences of 23–60 nt can induce gene silencing and provide resistance against the viruses (Thomas *et al*., 2001), effective gene silencing usually occurs when the transgenes and the viral genome share sequence similarity of more than 100 nt (Pang *et al*., 1997; Jan *et al*., 2000). Though there are reports on the use of hairpin constructs of up to 2.5 kbp (Liu *et al*., 2007), the ideal size of RNAi construct for providing effective resistance against plant viruses are found to be 300–800 nt (Simon–Mateo and Garcia, 2011). However, longer sequences in turn may result in off–targets leading to the siRNA directed silencing of host genes sharing sequence similarity that may hinder the plant development. Usually, GC–rich target sequences and AU–poor hairpin arms are not usually preferred because the former forms secondary structures hindering the RNA induced silencing complex (RISC) access and latter hinders the formation of RISCs (Blevins *et al*., 2011; Rajeswaran *et al*., 2014). Hairpin constructs with sequences from different viruses may confer broader resistance (Arif *et al*., 2012; Chung *et al*., 2013). Recent studies show that RNAi constructs of different lengths are successful in conferring resistance to viruses (Cillo and Palukaitis, 2014).

Several methods have been investigated for the successful generation of ihpRNA constructs. The traditional vectors include the pHANNIBAL and pHELLSGATE into which the PCR products of the desired gene can be inserted by cloning or Gateway directed recombination system and used directly for RNA silencing in plants (Helliwell and Waterhouse, 2003). Further, a method was devised for directly amplifying the intron hairpin RNA from the genomic DNA (Xiao *et al*., 2006). Overlap PCR extension method was also used for the hairpin construct preparation (Yan *et al*., 2009). A similar strategy was used in the hairpin construct preparation that can easily be inserted into the plant transformation vectors by TA cloning (Chen *et al*., 2009). A vector named pRNAi–LIC was developed that makes the construction of intron hairpin RNA constructs more easy, with one step cloning and without background ligations (Xu *et al*.,

2010). A novel, efficient and simple restriction–ligation approach called the Golden Gate cloning method, for the construction of hairpin RNAi vectors containing intron was devised using a vector pRNAi–GG. The utility of the developed construct in silencing many marker genes, both endogenous and exogenous in nature was also demonstrated (Yan *et al*., 2012). An intron–containing hairpin RNA was assembled by a simple and single step of PCR, restriction, ligation and transformation using the tomato phytoene desaturase gene (Manamohan *et al*., 2013).

2.6.6 RNAi mediated resistance to viruses in plants

Main focus of RNA silencing of viral transgenes was on RNA viruses of positive sense but the mechanism was also found to be effective against negative stranded RNA viruses like the tospoviruses (Pang *et al*., 1996; Prins *et al*., 1997). Vulnerability of single stranded DNA viruses like the Gemini viruses towards RNAi was less compared to the effectiveness of the mechanism towards the RNA viruses (Noris *et al*., 2004; Ribeiro *et al*., 2007). However, in some cases the mechanism was found to induce strong resistance to DNA viruses due to the coexistence of transcriptional gene silencing induced by these viruses along with the PTGS (Buchmann *et al*., 2009; Rodriguez–Negrete *et al*., 2009; Zhang *et al*., 2011).

Among the various PDR approaches, VIGS or PTGS remains the most powerful and promising technology so far and was proved beneficial for the production of several virus resistant crops. This can be made potentially useful for engineering resistant plants in the developing countries where economy largely sustain on agriculture (Reddy *et al*., 2009). In a study against the *African cassava mosaic virus* (ACMV), integration of the AC1 gene from the virus in cassava provided PTGS mediated resistance not only to the homologous strain of the virus but also induced cross–protection against two other heterologous geminiviruses infecting cassava (Chellappan *et al*., 2004). Production of transgenic cassava was accomplished using intron hairpin RNA from ACMV DNA–A and siRNAs specific to the transgene were detected (Vanderschuren *et al*., 2007). The presence of these siRNAs was then correlated with the

attenuation of symptoms seen in cassava on inoculation with the virus. Production of transgenic common bean plants resistant to *Bean golden mosaic virus* was accomplished using intron hairpin construct targeting AC1 sequence of the virus (Bonfim *et al*., 2007). And, transgene specific siRNAs were detected in both challenged and non–challenged plants. In *Nicotiana benthamiana* plants RNAi mediated resistance was demonstrated against *Cassava brown streak disease* caused by two different viruses; *Cassava brown streak virus* and *Cassava brown streak Uganda virus* (Patil *et al*., 2011).

2.6.6.1 RNAi mediated resistance to CMV

Tobacco plants transformed with inverted repeats of CMV CP and 1445 bp spacer were found to be resistant to CMV and the level of resistance was correlated to the presence of siRNAs (Kalantidis *et al*., 2002). Transgenic *Nicotiana benthamiana* plants carrying the inverted repeats of CMV RNA2 or CP sequences were produced and resistance to CMV was found to be 50–75% in these plants, whereas it was 30% when a similar construct of shorter nucleotide sequence was used (Chen *et al*., 2004). Transgenic *Arabidopsis* plants were developed using an inverted repeat construct targeting a 21 bp sequence of 2b gene (Qu *et al*., 2007) and 61% resistance to CMV was demonstrated. When the CMV 3' UTR derived hairpin RNA was used as the transgene construct, delayed resistance to the virus was seen in the transgenic tobacco plants that expressed the 3' UTR derived siRNAs (Duan *et al*., 2008). In another experiment, transgenic tobacoo lines were produced by *Agrobacterium* mediated transformation using the partial sequences of both TMV MP and CMV rep protein inverted repeats. Resistance to these viruses was demonstrated in three transgenic lines that remained stable even in the T4 progeny (Hu *et al*., 2011). In a recent study, tomato plants harboring the hairpin construct comprising of 1138 bp rep gene of CMV–O strain showed different levels of resistance to the virus and transgenic specific siRNAs were detected in the resistant plants (Ntui *et al*., 2014).

2.6.7 Concerns regarding RNAi and future prospects

The durability of the resistance mechanism is dependent on the abilities of the viruses to combat RNAi and there are reports showing that suppressor

protein encoded by the viruses are capable of overcoming the RNAi. These include the 2b gene of CMV, the P1/HCPro of Potyviruses, p19 of Tombusviruses, p25 of PVX, CP of Carmoviruses, p20, p23 and CP of *Citrus tristeza virus* (Brigneti *et al*., 1998; Voinnet *et al*., 1999; Kasschau *et al*., 2003; Qu *et al*., 2005; Hassani *et al*., 2009).

Later, the miRNA transgenes that code for 21–22 nt miRNA–like species, were designed (Niu *et al*., 2006), but reports show that viruses evade this type of resistance by mutating the short target sequence (Simon–Mateo and Garcia, 2006; Martinez *et al*., 2012). The main concern regarding RNAi is that they cause off–target effects as majority of plant transformation vectors are based on CaMV 35S promoter (Fuentes *et al*., 2016). Recently, an attractive alternative to RNAi was modulated which involved the delivery of dsRNA specific to viruses thereby providing protection. These dsRNAs were loaded on clay nanosheets and sprayed on the leaves and virus protection was conferred for at least twenty days (Mitter *et al*., 2017). The close interaction of the fungal and insect pathogens of plants may lead to the uptake of dsRNA or siRNAs from the host plant cells thereby providing RNA silencing mediated resistance. Thus, anti–fungal and anti–insect pathogens based on host induced gene silencing are being developed (Mitter *et al*., 2017).

CONTENTS

MATERIALS AND METHODS

The present investigation on 'RNAi mediated resistance to *Cucumber mosaic virus* (CMV) in black pepper (*Piper nigrum* L.)' was carried out at ICAR–Indian Institute of Spices Research (IISR), Kozhikode, Kerala, India.

3.1 Complete genome sequencing of black pepper isolate of CMV

3.1.1 Plant material

Black pepper naturally infected with CMV showing typical symptoms, collected from Kodagu District, Karnataka, India and maintained at the ICAR–IISR was used for the study. The infected and healthy plants of black pepper were identified by the presence or absence of symptoms and reverse transcription polymerase chain reaction (RT–PCR) using the CMV coat protein (CP) specific primers AIB 1 and AIB 2 (Table 3.1).

Table 3.1 Primers used for the screening of *Cucumber mosaic virus* infected black pepper plants

Primer	Sequence (5'.....3')	Tm °C	Product size	Remarks
AIB 1	ATGGACAAATCTGAATCAAC	56	650 bp	Amplifies the complete CP gene of CMV from black pepper
AIB 2	TCAAACTGGGAGCACCC			

Those plants that showed positive amplification with the CMV CP specific primers, served as mother plants for complete genome sequencing and the plants that tested negative were used as healthy controls. Both the infected and healthy black pepper plants were vegetatively propagated by stem cuttings in insect proof greenhouse. The flow chart showing the different steps involved in complete genome sequencing and analyses is depicted in Figure 3.1.

3.1.2 RNA extraction

Total RNA was extracted from 50 mg leaf tissue by modified acid guanidium thiocyanate phenol chloroform method (Siju *et al.*, 2007). Fifty milligrams of fresh leaf tissue was ground in 500 µL of acid guanidium thiocyanate having 0.1% (v/v) β–mercaptoethanol and 0.5% sodium sulphite.

The extract was transferred to a microcentrifuge tube and 50 µL of 2.0 M sodium acetate pH 4.0, 500 µL of water–saturated phenol and 100 µL of chloroform:isoamyl alcohol was added sequentially with thorough mixing at each step. This was incubated in ice for 15 min after shaking the tube for 15 s. The homogenate was then subjected to centrifugation at a speed of 13,000 rpm at 4°C for 15 min. The supernatant was collected and mixed with equal volume of isopropanol and incubated further for 1 h at –80°C and then centrifuged for 15 min at 13,000 rpm at 4°C. The pellet was washed with 70% ethanol, air–dried and diluted in 50 µL of RNase free water.

3.1.3 RT–PCR for amplifying the complete genome

RT–PCR was done as per the protocol of Siju *et al.* (2007) using the primers designed (Table 3.2) based on multiple sequence alignment of subgroup I CMV isolates, available in GenBank. The specificity of the primers designed was checked by the BLASTn programme available in NCBI (www.Ncbi.nlm.nih.gov), the primer complementarities and secondary structure formations were analyzed through the tool 'OligoCalc' (http://www.northwestern.edu/biotools/oligocalc.html). The schematic diagram of the location of primers designed, is shown in Figure 3.2. The reagent composition of RT–PCR is given in Table 3.3.

Table 3.2 Primers used for complete genome amplification of *Cucumber mosaic virus* from black pepper

Primer and Orientation		Tm °C	Sequence (5'.....3')	Product size (kb)
RNA1	AIB203 (F)	52	GTTTATTTACAAGAGCGTACG	1.7
	AIB246 (R)		CTTCTTTGGAATCCGACGAT	
	AIB245 (F)	49	GATTGCTATGAGGTGTTACA	1.7
	AIB263 (R)		TGGTCTCCTTTDRGAGRCC	
RNA2	AIB 204 (R) *	56	CCATCTCAAGGGATGAAATC	1.6
	AIB 235 (F) **	49	ATGATAAAATCTGATGTGAAACC	1.6
RNA3	AIB 282 (F)	56	GTAATCTTACCACTGTGTGT	1.2
	AIB 208 (R)		GTGGAGAAGCATCCATGAA	
	AIB 237 (F) **	50	CTATAGTGTCCTGTGTGAGTT	1.2

***AIB203 was used as forward primer**
****AIB263 was used as reverse primer**

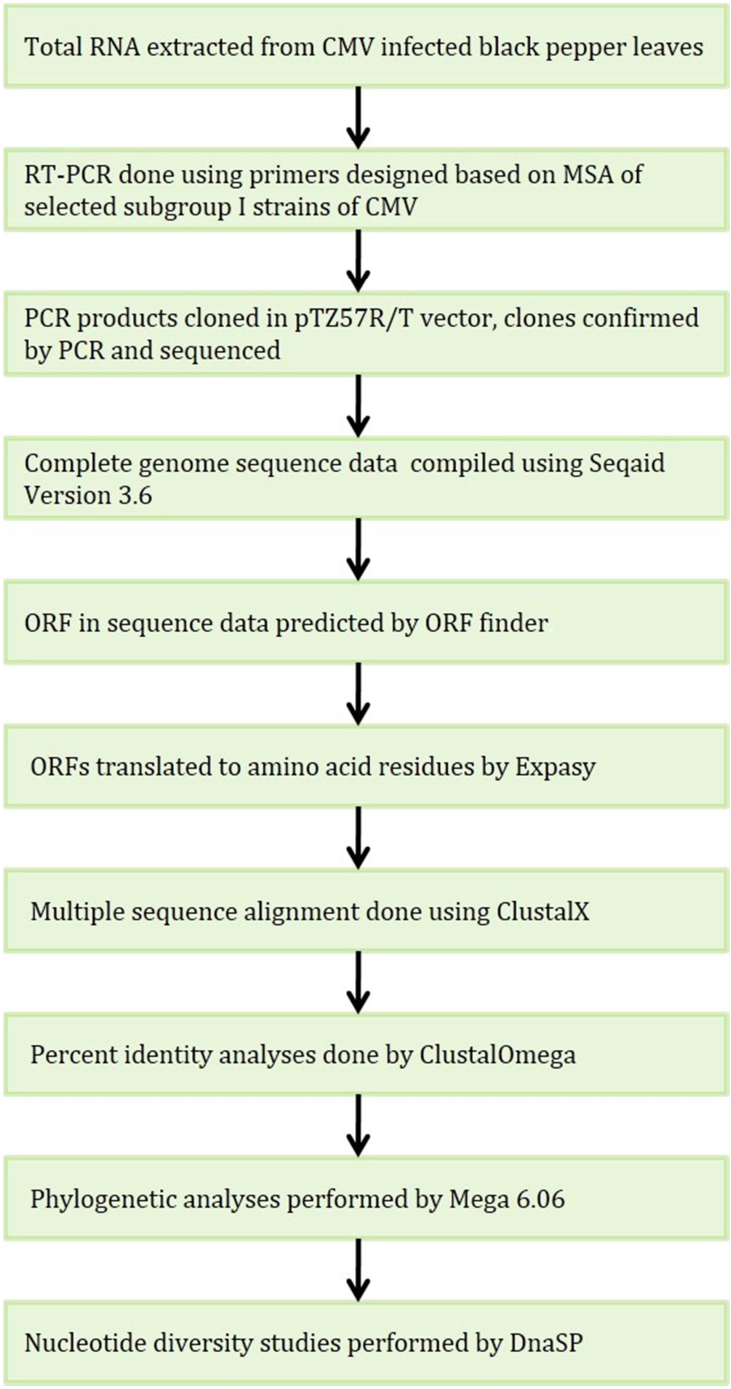

Figure 3.1 Flowchart of the different steps involved in complete genome sequencing and analyses of black pepper isolate of *Cucumber mosaic virus*.

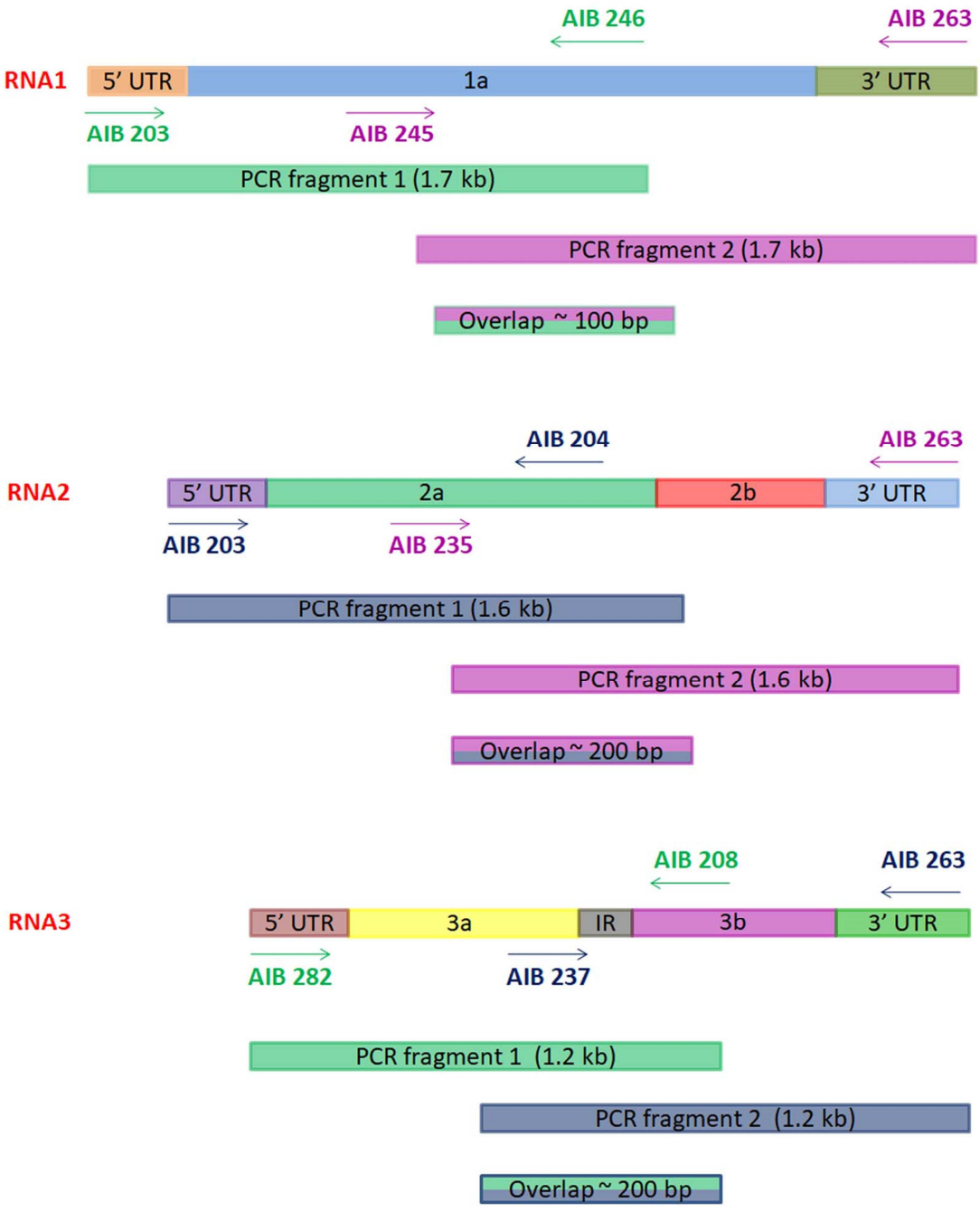

Figure 3.2 Schematic diagram of, the location of primers designed for amplifying the three RNAs of *Cucumber mosaic virus*, the products expected and overlapping regions covered by the primers.

Table 3.3 Reagent composition and concentration for RT–PCR

Reagent	Volume (µL)
10x Assay buffer with $MgCl_2$	5.0
DTT (10 mM)	5.0
dNTP mix (375 µM)	1.0
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
RNasin (10 U)	0.5
RevertAid RT (20 U)	1.0
Taq DNA polymerase (1.5 U)	1.0
Template RNA	1.0
Deionised water	33.5
Total	**50**

Prior to the addition of RNA template to the reaction mixture, it was heated to 80°C for 10 min and rapidly cooled in ice for 3 min to make the RNA linear. Single step, RT–PCR was carried out in thermo cycler as per the thermal profile given in Table 3.4.

Table 3.4 Steps and conditions of thermal cycling for RT–PCR

Step	Temperature (°C)	Duration (min)	Cycle
cDNA synthesis	42	45	1
Initial denaturation	94	5	1
Denaturation	94	1	35
Annealing	Specific T_m	1	
Extension	72	1	
Final extension	72	20	1
Hold	4	~	1

The amplified products after RT–PCR were then seperated by agarose gel electrophoresis using 1x TAE buffer using 0.8% agarose gels and visualized in gel documentation system, under UV light. The size of the PCR products was determined using a standard molecular weight ladder. The specific products obtained were extracted from the gel using GeneJET gel extraction kit following the manufacturer's protocol.

3.1.4 Gel extraction and purification of PCR products

The amplified PCR products were purified from the agarose gel using GeneJET gel extraction kit. The PCR bands were cut from the gel using a sterile scalpel, taking care to extract minimum gel volume without disturbing the DNA band. This was transferred to a 1.5 mL microcentrifuge tube, binding buffer was added in the 1:1 ratio (weight:volume), and incubated at 60°C for 10 min. The tube was shaken intermittently by inverting and, finally vortexed to dissolve the gel completely. To the solubilised gel, 2 volumes (w/v of the gel) of 100% isopropanol was added, mixed by inverting the tube and 800 µL of this gel solution was transferred to the GeneJET purification column with collection tube. The column was then centrifuged at 13,000 rpm for 1 min and the flow–through was discarded. This was repeated for the remaining solubilised gel solution. To the column, 700 µL of diluted wash buffer (provided in kit) was then added and centrifuged for 1 min at 13,000 rpm. Again the flow–through was discarded. The centrifugation step was repeated to remove the residual wash buffer completely. The GeneJET purification column was then transferred to a sterile 1.5 mL microcentrifuge tube and 50 µL of elution buffer was added to the centre of the column membrane. The column was then incubated for 2 min followed by centrifugation at 13,000 rpm for 1 min. The purified DNA was quantified and directly used for ligating with the vector.

3.1.5 Quantification of DNA

The purified DNA in the tube was quantified using the Biophotometer plus. The optical density (absorbance) of 1 µL of the eluted DNA samples and the control (sterile water or elution buffer in which the DNA was dissolved) were measured at 260 nm (A_{260}). The concentration of DNA samples in µg/mL were directly noted from the spectrophotometer. The quality of the purified DNA samples was then analyzed by agarose gel electrophoresis. The purified product was mixed with the 6x loading dye (in the ratio 1:3), and was loaded onto the gel. The approximate concentration of the DNA was determined using the pBR322 (50 ng/µL) loaded in the gel.

3.1.6 Ligation, transformation and cloning

For ligation, transformation and cloning the InsTAclone PCR cloning kit was used. The quantified PCR products were initially ligated to the vector pTZ57R/T (Figure 3.3) available in the kit. The different components and their composition used for ligation are given in Table 3.5. The ligation mixture was initially incubated at 22°C for 1 h and further incubated overnight at 4°C for increasing the efficiency of transformation.

The quantity of the PCR products required for ligation was calculated as per the formula,

Nanogram of insert = {nanogram of vector x insert size (kb)} / vector size (kb)}

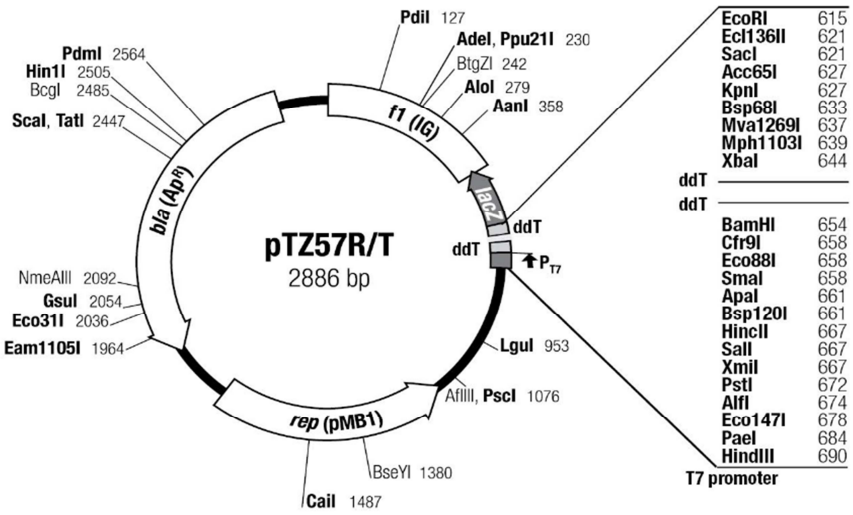

Figure 3.3 Map of the cloning vector pTZ57R/T, showing the TA region and multiple cloning sites with the different restriction enzymes including *Bam*HI, *Nsi*I and *Sac*I used for the hairpin vector construct preparation in the study.

Table 3.5 Different components used for the ligation of PCR products with pTZ57R/T vector

Component	Volume (µL)
Linearised vector, pTZ57R/T (55 ng/µL)	1.5
Ligation buffer (5x)	3.0
T4 DNA ligase (5U/µL)	0.5
Insert DNA (50 ng/ µL)	9.0
Nuclease free water	1.0
Total	**15**

3.1.6.1 Preparation of competent cells of E. coli DH5α

E. coli strain DH5α cells were freshly streaked on Luria–Bertani (LB) agar plate without any antibiotic. A single colony from the overnight grown culture was then inoculated into 2 mL C–medium (provided in the kit) and incubated at 37°C overnight with intermittent shaking at 150 rpm. The following day, 150 µL from the overnight grown culture was inoculated in 1.5 mL of prewarmed C–medium in a 2.0 mL microcentrifuge tube. This was incubated at 37°C for 20 min at 150 rpm. Meanwhile, the T–solution was prepared by mixing 250 µL each of T–solution A and T–solution B in a separate 1.5 mL microcentrifuge tube (for two transformations) and kept on ice. After 20 min, the inoculated bacterial culture was centrifuged at 8,000 rpm at room temperature for 1 min. The pellet collected was resuspended in 300 µL of prepared T–solution and incubated on ice for 5 min. The resuspended solution was centrifuged for 1 min at room temperature, the supernatant was discarded and the pellet obtained was resuspended in 120 µL of remaining T–solution. This was incubated on ice for another 5 min and transformation was done using this competent cell mixture.

3.1.6.2 Transformation

After incubation, 50 µL of the competent cell mixture was slowly mixed with 2.5 µL of the ligation mixture taken in chilled PCR tubes. From the competent cell mixture 20 µL was mixed with 0.5 µL of control DNA2 (vector pTZ57R/T DNA with a control PCR fragment inserted into it), to be used as control for transformation. After 5 min of incubation, all the mixtures were plated on individual pre–warmed LB agar plates with ampicillin (50 µg/mL), IPTG (isopropyl β–D–1–thiogalactopyranoside) (0.8 mM) and X–Gal (5–bromo–4–chloro–3–indolyl–β–D–galactopyranoside) (0.8 mM). The plates were incubated at 37°C overnight.

3.1.6.3 Master plate preparation

The next day the white recombinant colonies were individually streaked onto a fresh LB agar plate with ampicillin (50 µg/mL) and further incubated

overnight at 37°C. This streaked grid plate formed the master plate from which the colonies, numbered consecutively, were taken for screening of the positive recombinants by colony PCR and plasmid isolation.

3.1.7 Screening of recombinants

3.1.7.1 Colony PCR

For initial screening of the recombinant clones, colony PCR was performed. From the master plate prepared, a small portion of each bacterial colony was suspended in 50 µL of 10 mM of EDTA in a microcentrifuge tube and the mixture was boiled for cell lysis for 5 min. The cell lysate was then centrifuged for 30 min at room temperature at 10,000 rpm. From the supernatant, 1 µL was taken for the colony PCR with the specific primers for the specific clones designed. PCR reaction mixture was prepared as per Table 3.6.

Table 3.6 Reagent composition and concentration for PCR

Reagent	Quantity used (µL)
10x assay buffer with $MgCl_2$	2.5
dNTP mix (100 µM)	0.5
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Taq DNA polymerase (1.5 U)	1.0
Template DNA	2.0
Sterile deionised water	18.0
Total	**25.0**

The programme for the PCR, consisted of initial denaturation, and the 35 cycles of denaturation, annealing and extension and, was set in the thermo cycler. The details of the different steps of PCR, with the temperature and time for each step is given in the Table 3.7 and PCR products were analyzed by electrophoresis on 0.8% agarose gel with ethidium bromide. The agarose was run using the buffer 1X TAE for 30–40 min at 100 V. Once the run was over, the products were visualized under the UV light in gel documentation system. Along

with the PCR products 1 kb ladder was added to determine the size of the products.

Table 3.7 Steps and conditions of thermal cycling for PCR

Step	Temperature (°C)	Duration (min)	Cycle
Initial denaturation	94	5	1
Denaturation	94	1	
Annealing	Specific T_m	1	35
Extension	72	1	
Final extension	72	20	1
Hold	4	~	1

3.1.8 Plasmid DNA isolation

For plasmid DNA isolation, two colonies from the positive clones were selected, inoculated to 2 mL LB broth with ampicillin (50 µg/mL) and placed in an incubator shaker overnight at 37°C at 150 rpm. From the overnight grown culture, plasmid DNA isolation was done using GeneJET Plasmid Miniprep Kit. The bacterial culture (2 mL) grown overnight was centrifuged for 1 min at 13,000 rpm at room temperature in a 2 mL microcentrifuge tube and to the pellet formed 250 µL of resuspension solution was added and mixed thoroughly. To this bacterial suspension, 250 µL of lysis solution was added and the tube was inverted 4–6 times for proper mixing. To this solution 350 µL of neutralization solution was added and mixed by inverting the tubes quickly and thoroughly. This mixture was then centrifuged for 1 min at 13,000 rpm at room temperature and the filtrate was transferred to a GeneJET spin column available in the kit and again centrifuged for 1 min at 13,000 rpm at room temperature and the flow through was discarded. The column was then washed twice by centrifugation for 1 min at 13,000 rpm at room temperature, using the wash buffer provided in the kit. Each time, the flow through was discarded and finally the empty column was centrifuged to remove the residual ethanol. The GeneJET spin column was then placed in a sterile 1.5 mL microcentrifuge tube and elution buffer (50 µL) was added to the centre of the column. After incubating the tube for 2 min, the

plasmid DNA was eluted from the column by centrifuging the tube assembly for 1 min at 13,000 rpm at room temperature. The eluted plasmid DNA collected in the microcentrifuge tube was quantified using the Biophotometer plus and stored at –20°C for further use.

3.1.9 Confirmation of the clones by PCR

PCR was performed to confirm the presence of the insert in the purified plasmid DNA. For testing the presence of insert, forward and reverse primers specific to the insert were used. PCR reaction was performed using 0.5 µL of the plasmid (110–115 ng/µL) as template in a 25 µL reaction as given in the section 3.1.7. PCR products were checked by gel electrophoresis using 0.8% agarose gels.

3.1.10 Sequencing and analyses

Plasmids isolated from positive clones were sequenced using the universal M13 forward and T7 reverse primers. At least two clones were sequenced for each amplicon and each amplicon was sequenced from both strands to get the consensus sequence. Sequences were assembled with Seqaid Version 3.6 (Peltola *et al*., 1984). ORFs in the sequence data were predicted by ORF finder (www.ncbi.nlm.nih.gov/projects/gorf) and translated into amino acid residues using Expasy tool (Gasteiger *et al*., 2003). The analyses were carried out using the complete genome of 27 isolates of CMV belonging to subgroup I and II and one outgroup (*Peanut stunt virus*), retrieved from NCBI database (Table 3.8) in the form of nucleotide and deduced amino acid sequences. Sequences were aligned using clustalX (Thompson *et al*., 1997), BioEdit sequence alignment editor version 7.0.5.3 (Hall, 1999), and percentage identity was calculated using Clustal Omega (Sievers *et al*., 2011). Phylogenetic analyses were performed using the maximum likelihood method (Juke Cantor model for nucleotide and Poisson model for deduced amino acid; with 1000 non–parametric bootstrap replicates) in MEGA 6.06 (Tamura *et al*., 2013). The number of segregation sites and ratio of dN/dS for different genes was estimated using DnaSP version 5.10 (Librado and Rozas, 2009).

3.2 Designing of potential siRNAs *in silico* for various target genes of CMV subgroup IB

The 1a, 2a, 2b, 3a and 3b gene sequences of black pepper isolate of CMV were subjected to an online software 'dsCheck', (http://dsCheck. RNAi.jp/) to select the specific region that will yield dsRNA. The flowchart of the steps involved in designing of potential siRNAs from the different genes is given in Figure 3.4.

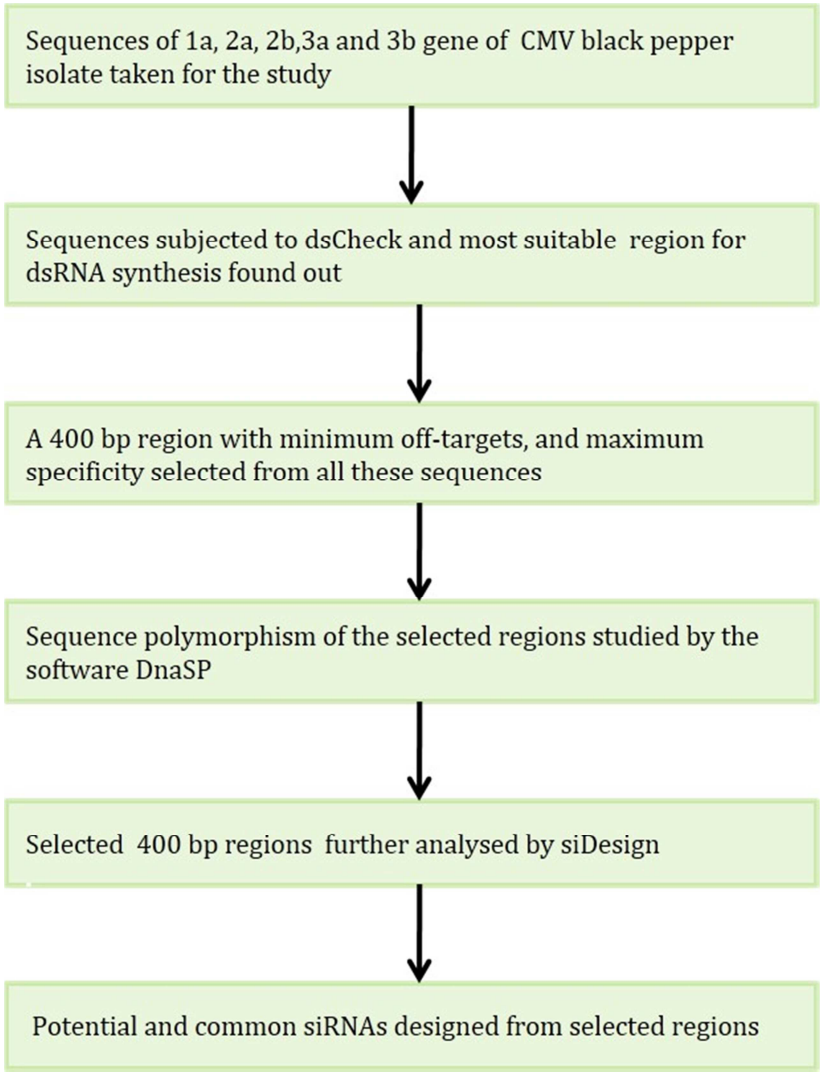

Figure 3.4 Flowchart of the different steps involved in designing of common siRNAs from the different genes of black pepper isolate of *Cucumber mosaic virus*.

A 400 bp off–target minimized region was selected by the software from all the five genes. Corresponding sequences of 13 isolates of CMV subgroup IB strains for which complete genome sequence information was available were retrieved from NCBI (Table 3.8), and sequence polymorphism of these regions was then studied using the software Dnasp version 5.10 (Librado *et al*., 2009). The selected dsRNA sequences were subjected to another online software 'siDESIGN' center (http://www.dharmacon.com), for identifying the potential and common siRNAs present in these regions.

Table 3.8 Details of isolates of *Cucumber mosaic virus* and their GenBank accession numbers used in the study

No:	Isolate	Host	Country	Subg–roup	GenBank Accession Number		
					RNA1	**RNA2**	**RNA3**
1	Black pepper (BP)	*Piper nigrum*	India	IB	KU947029 (Present study)	KU947030 (Present study)	KU947031 (Present study)
2	WN1	*Piper nigrum*	China	IB	KT004542	KT004543	KT004544
3	KO	*Capsicum annuum*	India	IB	KM272277	KM27278	KM272275
4	ND	*Solanum lycopersicum*	India	IB	GU111227	GU111228	GU111229
5	CLW2	*Cucumis sativus*	Malaysia	IB	JN054636	JN54637	JN054635
6	HM3	*Lycopersicon esculentum*	Egypt	IB	KT921314	KT921315	No Data
7	IX	*Lycopersicon esculentum*	Philippines	IB	U20220	U20218	U20219
8	IA	No data	Indonesia	IB	AB042292	AB042293	AB42294
9	Nt9	*Lycopersicon esculentum*	Taiwan	IB	D28778	D28779	D28780
10	SD	*Nicotiana tabacum*	China	IB	AF071551	D86330	AB008777
11	Tfn	*Solanum lycopersicum*	Italy	IB	Y16924	Y16925	Y16926
12	Pl– 1	*Solanum lycopersicum*	Spain	IB	AM183114	AM183115	AM183116
13	Vir	*Capsicum annuum*	North Italy	IB	HE962478	HE962479	HE962480
14	CMV209	*Glycine soja*	South Korea	IB	KJ400002	KJ400003	KJ400004
15	Lil	*Lilium longiflorum*	India	IA	AJ879490	AJ865382	AJ831578
16	Fny	*Cucumis melo*	USA	IA	D00356	D00355	D10538
17	Mf	*Melandrium firmum*	Korea	IA	AJ276479	AJ276480	AJ276481
18	Leg	Legume	Japan	IA	D16403	D16406	D16405
19	I17F	*Solanum lycopersicum*	France	IA	HE793683	HE793684	Y18137
20	Rs	*Raphanus sativus*	Hungary	IA	AJS11988	AJS17801	AJS17802
21	Ri– 8	*Lycopersicon esculentum*	Spain	IA	AM183117	AM183118	AM183119

22	Pal	*Cuccumis sativus*	India	II	HE650150	HE613667	HE583224
23	Q	*Capsicum annuum*	Australia	II	X02733	X00985	M21464
24	Tag	*Tagetes erecta*	China	II	EU665000	EU665001	EU665002
25	LS	*Lactuca saligna*	USA	II	AF416899	AF416900	AF127976
26	Trk7	*Trifolium repens*	Hungary	II	AJ007933	AJ007934	L15336
27	TN	*Solanum lycopersicum*	Japan	II	AJ007933	AB176848	AB176847
28	R	*Ranunculus asiaticus*	France	II	HE793685	HE793686	Y18138
29	BX	*Pinellia ternate*	China	III	DQ399548	DQ399549	DQ399550
30	PSV	*Vigna unguiculata*	China	Outgroup	NC_002038	NC_002039	NC_002040

3.3 Hairpin construct preparation

3.3.1 CMV 3b (coat protein) hairpin construct preparation

3.3.1.1 Amplification of sense, antisense and intron genes

CMV 3b gene of 337 bp starting from the 5' end was selected for the preparation of hairpin construct. The schematic diagram of the 3b hairpin construct preparation in the cloning vector pTZ57R/T is shown in the Figure 3.5. The primers used for preparation of hairpin construct are given in the Table 3.9. The CMV 3b gene in the sense and antisense orientation was first amplified from the clone having the complete 657 bp CP gene, using the primers AIB 211 and AIB 212 for sense; AIB 212 and AIB 213 for antisense. The sense fragment was amplified using forward primer flanked with the restriction enzyme *Bam*H1 and reverse primer flanked with *Nsi*1. The antisense fragment was amplified by using the forward primer flanked with *Sac*1 and reverse primer with *Nsi*1. Phytoene desaturase sixth intron from tomato was amplified using the primers, both flanked with *Nsi*1 from the clone having this gene, already available. All the PCR reactions were performed as per the programme given in section 3.1.7 using 0.5 µL of the plasmid. PCR products were purified from the gel using the GeneJET gel extraction kit as illustrated in the section 3.1.4.

Table 3.9 Primers used for 3b hairpin construct preparation in cloning vector pTZ57R/T and its confirmation

Primer	Sequence	Tm	Product size (bp)	Remarks

AIB 211 AIB 212	AGGAGGGGATCCATGGACAAATCTGAATCAA GGGGGCATGCATGATTAACTCAATTTGAAT	60	349	Amplifies CMV 3b sense construct
AIB 213	AGAAGGGAGCTCATGGACAAATCTGAATCAA	60	349 with AIB 212	Amplifies CMV 3b antisense construct
AIB 214 AIB 215	GGCGGCATGCATCTGCAAATTATATCAAAGA AGGAGGATGCATGTGAGC TAATCACGAGTAA	60	236	For amplifying intron
AIB 190	CAGGAAACAGCTATGAC	47	850 with AIB 214	Amplifies region from intron to M13 reverse
AIB 1	ATGGACAAATCTGAATCAAC	56	950 (single primer)	For amplifying the hairpin construct

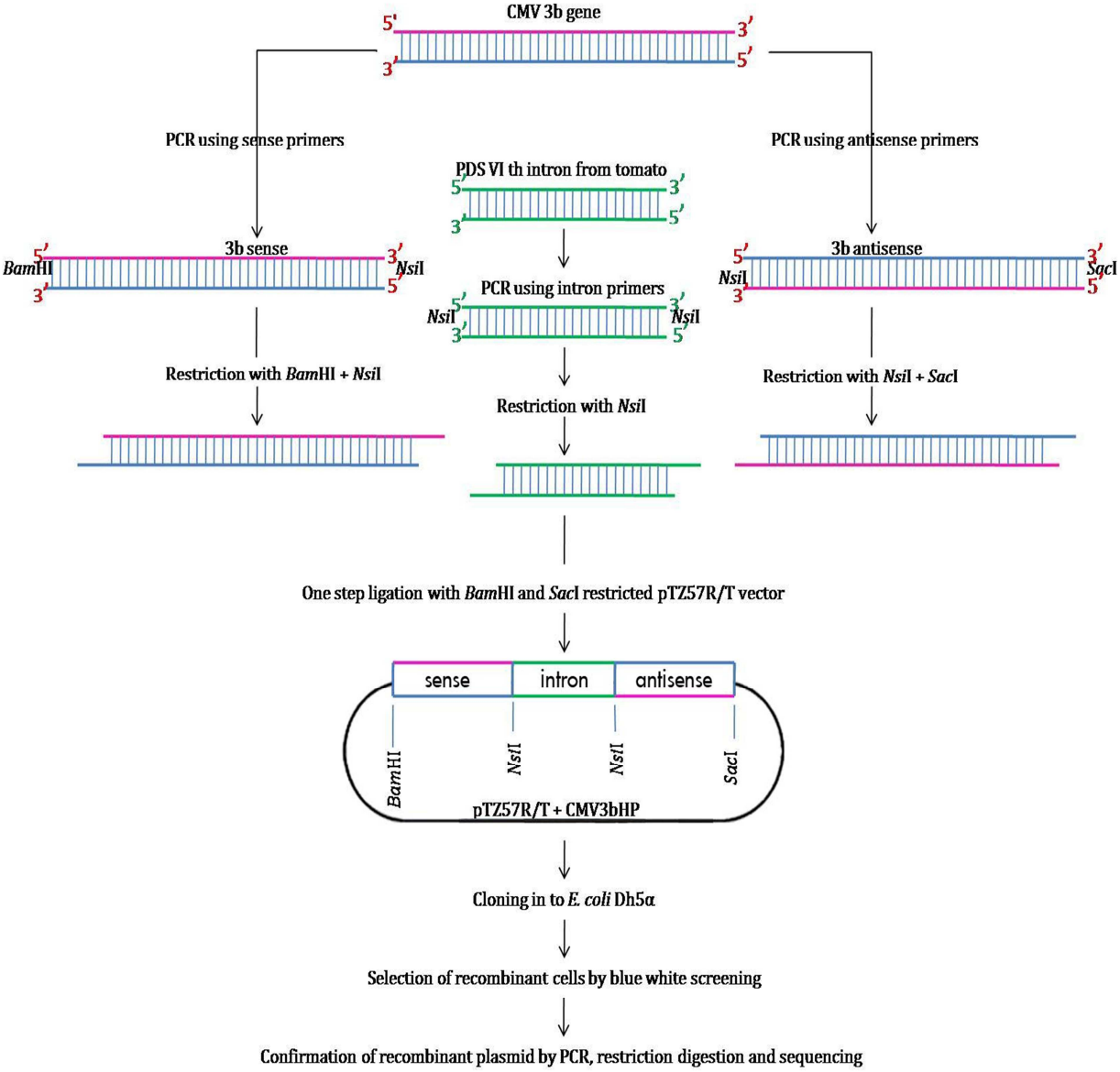

Figure 3.5 Schematic diagram of the different steps involved in 3b hairpin construct preparation in the cloning vector pTZ57R/T.

3.3.1.2 Restriction digestion of sense, antisense and intron PCR products

The purified sense PCR product was first restricted with *Nsi*1 for 1 h at 37°C, enzyme inactivated by incubating at 65°C for 15 min. Then *Bam*H1 was added and incubated for 45 min and the enzyme was inactivated by incubating at 80°C for 5 min. Similarly antisense PCR product was restricted with *Nsi*1 followed by *Sac*1. Intron PCR product was restricted with *Nsi*1 alone. The vector, pTZ57R/T was also restricted with *Bam*H1 and *Sac*1 sequentially. The reagent composition for restriction of PCR products and the cloning vector pTZ57R/T are given in the Table 3.10 and Table 3.11 respectively.

Table 3.10 Components used for restriction digestion of PCR products

Component	Volume (µL)
Sense/antisense/intron PCR product	10.0 (500 ng)
Enzyme buffer (10x)	2.0
Restriction enzyme (10 U/µL)	0.5
Nuclease free water	7.5
Total	**20**

Table 3.11 Components used for restriction digestion of cloning vector pTZ57R/T

Component	Volume (µL)
Plasmid DNA (250 ng/ µL)	2.0
Enzyme buffer (10x)	2.0
Restriction enzyme (10 U/µL)	0.5
Nuclease free water	15.5
Total	**20**

3.3.1.3 Ligation of sense, antisense and intron constructs with the cloning vector

The restricted sense, antisense and intron fragments along with the double restricted pTZ57R/T vector were kept for ligation overnight. The ligation reaction components and volume is shown in the Table 3.12. The ligated product was transformed into *E. coli* DH5α using InstaTA cloning kit as given in the section 3.1.6.

Table 3.12 Different components used for the ligation of sense, antisense and intron with pTZ57R/T vector

Components	Volume (µL)
Double restricted vector, pTZ57R/T (60 ng/µL)	1.0
Ligation buffer (5x)	3.0
T4 DNA ligase (5U/µL)	1.0
Restricted sense fragment (50 ng/µL)	4.0
Restricted antisense fragment (55 ng/µL)	4.0
Restricted intron (48 ng/µL)	12.0
Nuclease free water	5.0
Total	**30**

3.3.1.4 Confirmation of hairpin construct in cloning vector in E. coli

The transformed colonies were screened by colony PCR using the intron primers AIB 214 and AIB 215. The reaction setup is given in section 3.1.7. Plasmid was isolated from the positive colony confirmed by PCR using the primer pairs AIB 190/ AIB 214; and a single primer AIB 1. Plasmid was isolated from the selected colonies (plasmid isolation protocol given in section 3.1.8) and confirmed by single restriction using *Bam*H1, *Sac*1, *Nsi*1 seperately and also by double restriction using *Bam*H1 and *Sac*1. The restriction reactions carried out was as per the procedure given in section 3.3.1.2. Finally, sequencing of the plasmid was done using the M13 forward and T7 reverse primers. Similar to the complete genome sequencing, two clones were sequenced for each amplicon and each amplicon was sequenced from both strands to get the consensus sequence.

3.3.1.5 Cloning of the hairpin construct in to the binary vector pBI121

The recombinant plasmid was isolated from the transformed colony and double restricted using *Bam*H1 and *Sac*1 to release the approx.1 kb hairpin construct. The binary vector pBI121 was also restricted using *Bam*H1 and *Sac*1 to release the GUS gene of 2 kb. The hairpin construct purified from the restricted pTZ57R/T vector was ligated in to the pBI121 vector purified after removal of the GUS gene, using T_4 DNA ligase available in the InstaTA cloning kit. The ligated product was transformed in to *E. coli* DH5α using the components of the kit as shown in the section 3.1.6. The schematic picture of the hairpin construct preparation in binary vector pBI121 is shown in Figure 3.6.

Confirmation of the 3b hairpin construct in binary vector pBI121 is given in section 3.3.1.7.

3.3.1.6 Mobilization of hairpin construct in to Agrobacterium by triparental mating

Agrobacterium strain EHA105 was transformed with the recombinant plasmid by triparental mating as per the protocol of Ditta *et al*. (1980). Briefly, *E. coli* having pBI121 with the hairpin construct, *E. coli* having helper plasmid pRK2014 and *Agrobacterium* strain EHA105 was grown for 24 h, 24 h and 38 h respectively. From each culture, 20 µL was pipetted one above the other onto nitrocellulose filter placed on LB agar media without any antibiotics and incubated for 24 h. This was then plated onto minimal media with kanamycin and rifampicin at a concentration of 50 µg/mL and incubated for 48 h. The colonies grown were then streaked onto YEB media with kanamycin and rifampicin at a concentration of 50 µg/mL.

3.3.1.7 Confirmation of the hairpin construct in binary vector in E. coli and Agrobacterium

Both the *E. coli* and *Agrobacterium* colonies were screened from the corresponding master plate using intron specific forward and reverse primers (AIB 267 and AIB 268) and, the recombinant *Agrobacterium* clones were screened by PCR using the *Agrobacterium* specific primers, AIB 121/AIB 122 and AIB 258/AIB 259. Colony PCR was performed as described in the section 3.1.7. Plasmid was isolated from positive colonies as per the protocol given in the section 3.1.8 and confirmed by PCR using the intron specific reverse (AIB 267), promoter specific forward primers (AIB 108) and intron specific forward (AIB 268), nos terminator specific reverse primers (AIB 269). The schematic diagram of the location of different sets of primers used for the confirmation of the hairpin construct is shown in the Figure 3.7. Primers used for confirmation of the hairpin construct in pBI121 in *E. coli* and *Agrobacterium* are given in the Table 3.13. Confirmation of the hairpin construct was also done by restriction analyses using both the *Bam*H1 and *Sac*1 restriction enzymes as per the protocol

mentioned previously (section 3.3.1.2). Recombinant plasmids isolated from both the transformed *E. coli* and *Agrobacterium* were confirmed by sequencing.

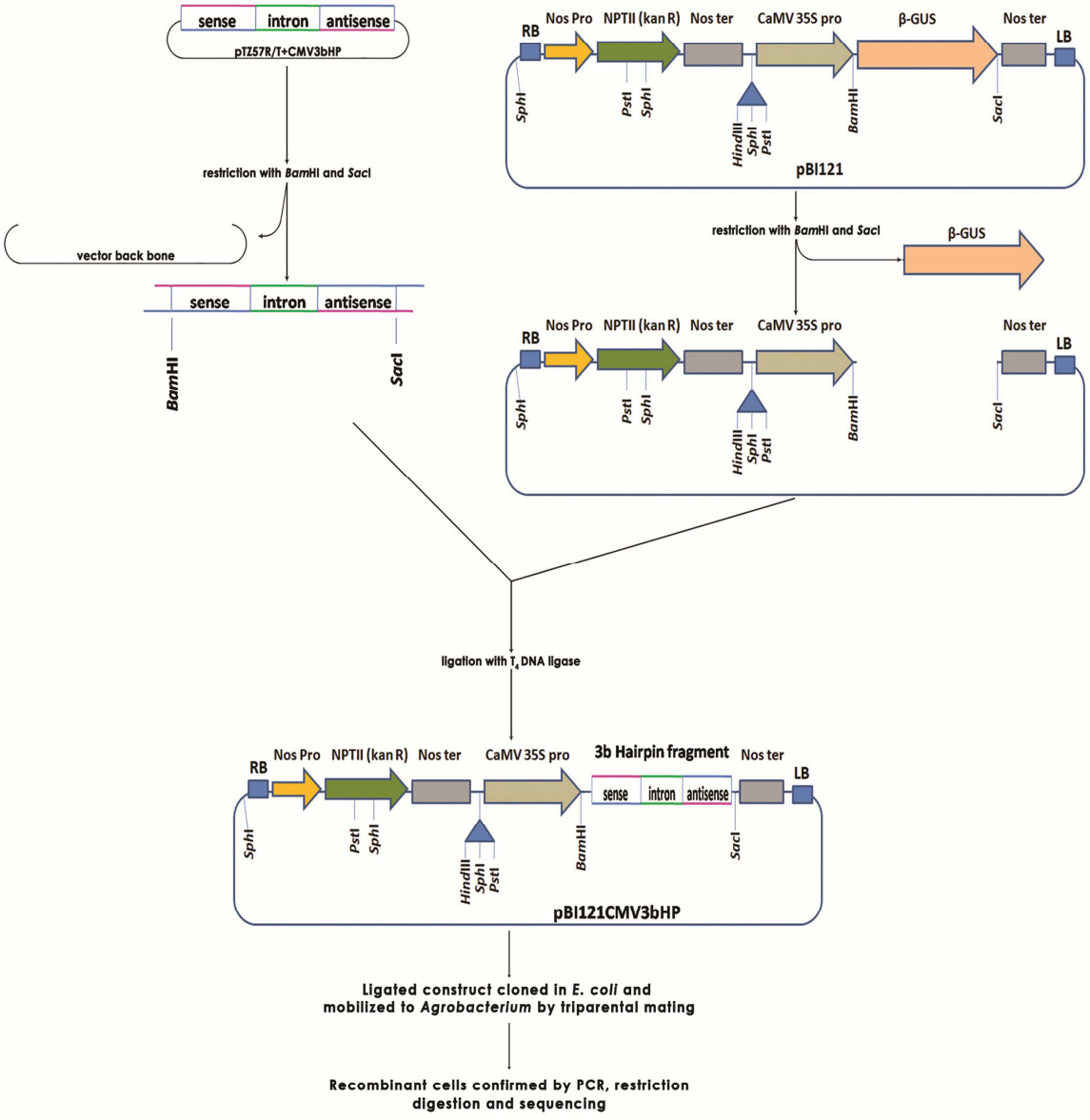

Figure 3.6 Schematic diagram of the 3b hairpin construct preparation in the binary vector pBI121.

Table 3.13 Primer used for confirmation of hairpin constructs of 3b and 2b in pBI121 in *E. coli* and *Agrobacterium*

Primer	Sequence (5'.....3')	Tm	Product size (bp)	Remarks
AIB 267 (R)	TAATCACGAGTAAATTTCTCCC	50	227	For amplifying intron of hairpin constructs in *E. coli*, *Agrobacterium* and transgenic plants, without the flanking restriction enzyme sites
AIB 268 (F)	TGCACACAAAAGGTCCAATG			
AIB 267 (R)	TAATCACGAGTAAATTTCTCCC	50	800	For confirmation of hairpin constructs in binary vector *in E. coli, Agrobacterium* and transgenic plants
AIB 108 (F)	TCCAACCACGTCTTCAAAGC			
AIB 268 (F)	TGCACACAAAAGGTCCAATG	54	900	For confirmation of hairpin constructsin binary vector *in E. coli, Agrobacterium* and transgenic plants
AIB 269 (R)	ACCGCGCGCGATAATTTAT			
AIB 121 (F)	GCGGCACGGATCACTGTA	58	948	For confirmation of transformation of *Agrobacterium*, will not amplify in *E. coli* and transgenic plants
AIB 122 (R)	CCGAATAGCCTCTCCAC			
AIB 258 (F)	CGAAACGCTGTTCGGCCTGTGG	62	898	For confirmation of the transf ormation of *Agrobacterium*, will not amplify in *E. coli* and transgenic plants
AIB 259 (R)	GTTCAGCAGGCCGGCATCCTGG			
AIB 119 (F)	CAACGTTGAAGGAGCCAC	56	955	For confirmation of the presence of transgene in recombinant vectors and transformed plants
AIB 120 (R)	ACGAGGAAGCGGTCAGC			

Figure 3.7 Schematic picture of the location of primers used for the confirmation of 3b/2b hairpin construct in the binary vector pBI121. The same primers were used for the detection of transgene and transcript in the putative transformed plantlets.

3.3.2 CMV 2b hairpin construct preparation

The flowchart showing the different steps involved in 2b hairpin construct preparation is shown in Figure 3.8. The 2b gene was first amplified from CMV infected leaves of black pepper using the primers AIB 183 and AIB 184 and was cloned in pTZ57R/T. The details of primers used are given in Table 3.14. RNA isolation and RT–PCR setup are given in the sections 3.1.2 and 3.1.3 respectively. Cloning of the 2b gene was done using the InstaTA cloning kit as per the protocol given in 3.1.6 and colonies were screened by colony PCR as given in section 3.1.7, using the primers AIB 183/ AIB 184. The hairpin construct of 2b gene was assembled from this clone using the complete 336 bp gene, following similar procedure used for 3b hairpin construct preparation. Here, the phytoene desaturase, 6th intron of size 224 bp from *Solanum lycopersicum* was sandwiched in between the 336 bp sense and antisense strands of 2b gene in pBSK vector instead of the cloning vector pTZ57R/T used for 3b hairpin construct preparation. This was then mobilized in to the binary vector pBI121, and transformed initially in to *E. coli* DH5α and finally mobilized to *Agrobacterium tumefaciens* strain EHA105 by triparental mating. The schematic picture of pBSK with the 2b hairpin construct and that of the binary vector with the construct is shown in the Figure 3.9 and Figure 3.10 respectively.

3.3.2.1 Confirmation of 2b hairpin construct in pBI121 in E. coli and Agrobacterium

Confirmation of the 2b hairpin construct in the binary vector was done by PCR with 0.5 µL of the plasmid isolated from both *E. coli* and *Agrobacterium* cells; using intron specific forward (AIB 268) and reverse primers (AIB 267), promoter specific forward (AIB 108) and intron specific reverse (AIB 267); and intron specific forward (AIB 268) and nos terminator reverse (AIB 269). PCR setup is given in section 3.1.7. Restriction digestion using two enzymes *Bam*H1 and *Sac*1 was performed for further confirmation as shown in the section 3.3.2.1. The plasmids from both *E. coli* and *Agrobacterium* were sent for sequencing. Primers used for the confirmation of the construct are given in the Table 3.13.

Figure 3.8 Flowchart of the different steps involved in 2b hairpin construct preparation in cloning vector and binary vector.

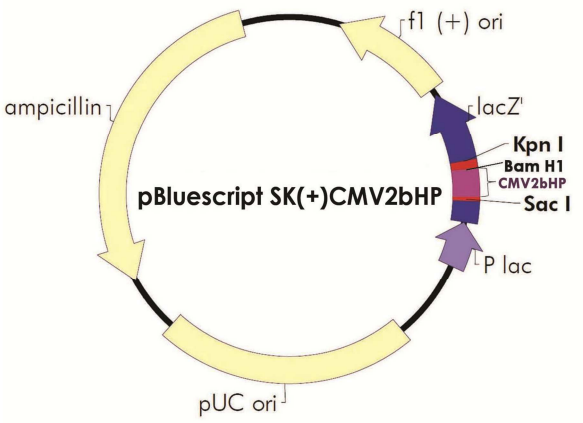

Figure 3.9 Map of the cloning vector pBluescript SK(+), showing the multiple cloning sites with the different restriction enzymes *Bam*HI and *Sac*I.

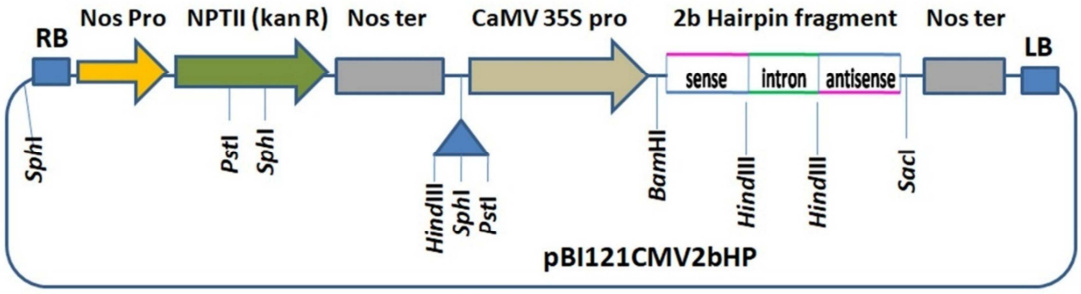

Figure 3.10 Schematic diagram of the 2bHP construct in the binary vector pBI121, showing the restriction enzymes used for cloning; *Bam*HI, *Hind*III and *Sac*I.

3.4 Production of transgenic lines of black pepper using the constructs developed

3.4.1 Somatic embryo production and establishment of embryogenic mass

Mature berries from three varieties of black pepper (Panniyur–1, IISR–Thevam and Sreekara) were used for somatic embryo production based on the protocol developed by Nair and Dutta, (2006) with slight modifications by Jiby and Bhat, (2011). The flowchart showing the different steps involved in somatic embryo production is shown in Figure 3.11.

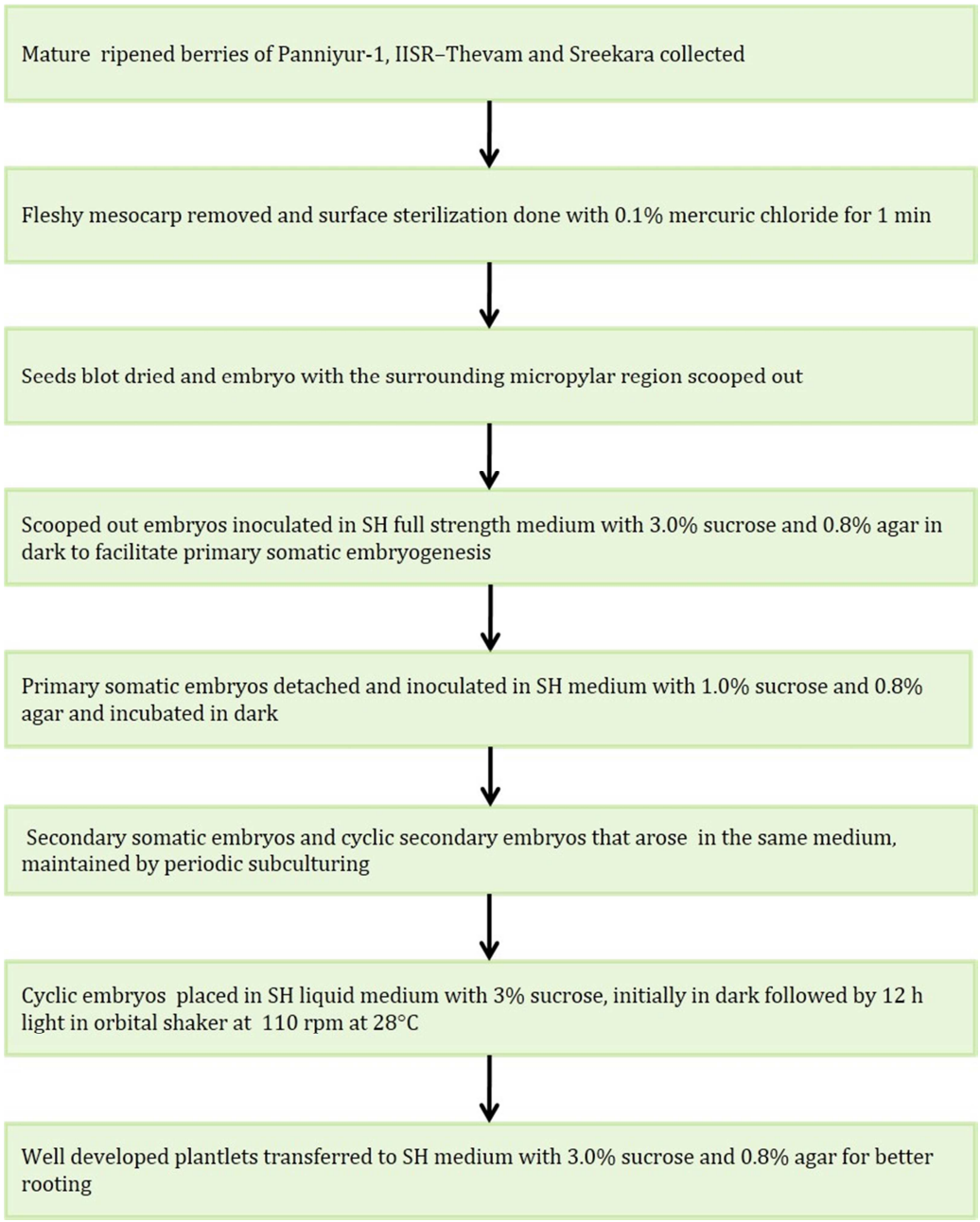

Figure 3.11 Flowchart of the different steps involved in somatic embryo production in three varieties of black pepper.

The berries were soaked overnight in tap water followed by the removal of outer mesocarp by repeated washing in distilled water. The remaining procedures were done under aseptic conditions in laminar air flow. Surface sterilization was done with 0.1% mercuric chloride for 1 min followed by repeated washing in sterile distilled water for 5–6 times. The seeds were then allowed to blot dry completely. From dried seeds embryos were scooped out

with the help of a sterile surgical blade along with surrounding micropylar tissue and cultured on full strength SH (Schenk and Hildebrandt, 1972) medium containing 3.0% (w/v) sucrose and 0.8% agar free of any growth regulators, under darkness. Primary somatic embryo clumps that emerged from the polar region of roots of germinating seedlings were carefully detached and inoculated again on SH full strength medium free of hormones with lesser concentration of sucrose (1.0%), gelled with 0.8% agar and incubated in complete darkness to promote secondary embryogenesis. The cyclic somatic embryos that arose from the secondary somatic embryos were maintained by subculturing in the fresh medium of same composition during an interval of 30 days.

3.4.2 *Agrobacterium* mediated transformation of somatic embryos

Two varieties of black pepper, Sreekara and IISR–Thevam were taken for transformation by *Agrobacterium* EHA105 having the pBI121CMV3bHP and the *Agrobacterium* EHA105 harboring pBI121CMV2bHP using the protocol of Jiby and Bhat, (2011). The flowchart showing the different steps involved in the *Agrobacterium* mediated transformation, regeneration and screening of the putative transformants is shown in Figure 3.12. Cyclic somatic embryos approx. 1g was was infected with 1/5th diluted 18–24h grown culture of the bacterium and shaken for 15 min to 1 h after which the these were co–cultured in SH medium with 1.5% sucrose and 0.8% agar (hormone free) for 48 h. The co–cultured embryogenic mass was washed with antibiotic cefotaxime to kill the bacterial cells and placed in selection medium (SH basal +1.5% sucrose +100 µg/mL cefotaxime +25 µg/mL kanamycin), for 3–5 weeks in dark.

Vigorous growing points observed were removed and placed in the same medium with a higher kanamycin concentration (50 µg/mL) for another 30 days, for further proliferation. Each growing point was considered as separate event. The proliferated embryogenic mass was transferred to basal SH (liquid) with 3% sucrose and 100 µg/mL cefotaxime (without kanamycin) for development of embryos into plantlets under dark with shaking at 110 rpm for 30 days. Medium was replaced every 10 days.

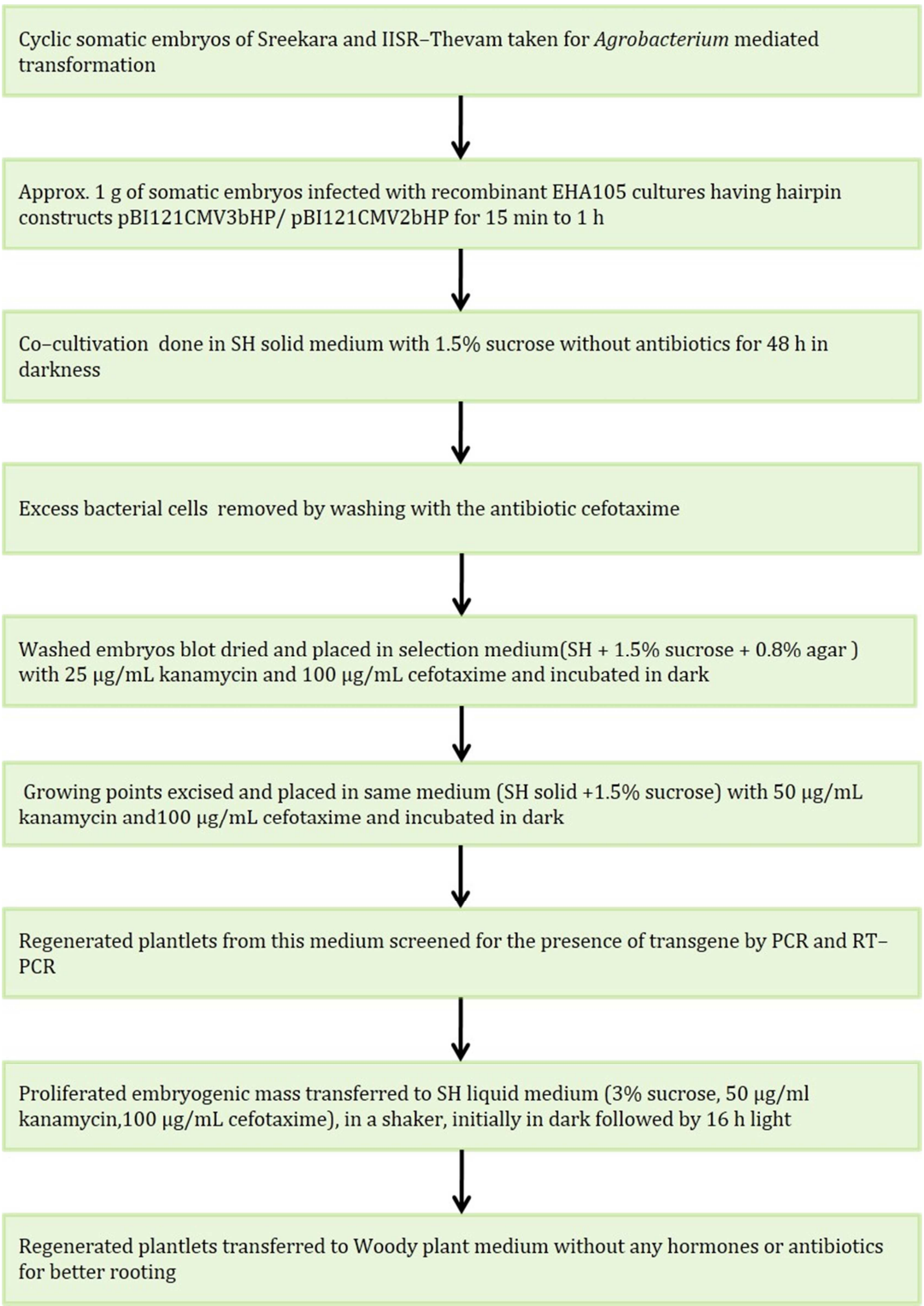

Figure 3.12 Flowchart of the different steps involved in *Agrobacterium* mediated transformation of black pepper.

3.4.3 Regeneration of plantlets from the embryogenic mass

Regeneration of plantlets from the somatic embryos was done according to the protocol of Nair and Dutta, (2006). Basal SH liquid medium with higher sucrose concentration of 3% was used for the regeneration of plantlets and about 250 mg of the embryogenic proliferates were placed in the liquid medium and incubated in dark with shaking at 110 rpm for a month. The medium was changed every 10 days. When the somatic embryos developed into plantlets, they were transferred to Woody plant medium with 3% sucrose and 0.8% agar for better rooting and shooting.

3.4.4 Screening of the putative transformants

3.4.4.1 DNA extraction and PCR

For preliminary screening of putative transformants, the plantlets that emerged from the growing points of proliferating and regenerating somatic embryos in selection media (100 µg/mL cefotaxime + 50 µg/mL kanamycin) were tested for the presence of transgene by DNA PCR. For this, total DNA was extracted from 50 mg of the regenerated plantlets of IISR–Thevam and Sreekara transformed using the pBI121CMV2bHP/ pBI121CMV3bHP constructs, as per the protocol of Hareesh and Bhat, (2008). A total of ten regenerated plantlets from ten different events of each variety and each construct were taken for the study. The DNA extraction procedure in brief consisted of the following steps. Initially 50 mg of the plantlet was weighed and placed in a chilled mortar and pestle. To this 500 µL of DNA extraction buffer was added and the sample was ground well. The extract was collected in an eppendorf tube and incubated at 65°C for 30 min. The extract was allowed to cool and to this, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed thoroughly. This was then centrifuged for 10 min at 3,200 rpm in room temperature. The supernatant was transferred to a new tube and to this 10% CTAB and equal volume of chlorofoam:isoamyl alcohol was added and mixed properly. The solution was centrifuged at 3,200 rpm for 10 min at room temperature and to the supernatant collected in a new tube, 0.1 M sodium acetate (pH 5.2) and

isopropanol in equal volume of the supernatant was added. The tube content was mixed thoroughly and incubated for 30 min in ice. The mixture after incubation was centrifuged at 12,000 rpm at 4°C for 15 min. Supernatant was discarded and to the pellet 70% ethanol was added and washed by centrifugation at 12,000 rpm for 5 min. Again, supernatant was discarded and the pellet was allowed to air dry. The pellet was dissolved in 50 µL of RNase free water and stored at –20°C for further use. The quality of the extracted DNA was analysed by 0.8% agarose gel electrophoresis and the quantity analyzed by Biophotometer plus.

For DNA PCR, three different sets of primers were used; CaMV 35S promoter specific forward (AIB 108) and intron specific reverse (AIB 267); intron specific forward (AIB 268) and nos terminator reverse (AIB 269) and, kanamycin specific forward (AIB 119) and reverse (AIB 120). The schematic diagram showing the location of primers is shown in Figure 3.7 and the list of primers used for screening is given in Table 3.13. The PCR was performed using 2 µL of the total DNA, reagent composition and the cycling conditions are given in the section 3.1.7.

3.4.4.2 RNA extraction and RT–PCR

For RT–PCR a total of five events from each variety and each construct were subjected to total RNA extraction (section 3.1.2). RT–PCR was performed using 2 µL of the total RNA using the same set of primers used for DNA PCR. The schematic diagram showing the location of primers is shown in Figure 3.7 and the list of primers used for screening is given in Table 3.13. Total RNA extraction, the reagent composition and cycling conditions for RT–PCR are given in the sections 3.1.2 and 3.1.3 respectively.

3.5 Development of cleft grafting method for challenge inoculation of CMV in black pepper

Healthy and CMV infected black pepper (Panniyur–1) cuttings maintained through vegetative propagation were used for the study. Initially, the plants were tested for the presence of CMV by RT–PCR using coat protein specific primers. Total RNA extraction and RT–PCR reaction setup are given in sections 3.1.2 and

3.1.3 respectively. The flowchart showing the different steps involved in the study is given in Figure 3.13.

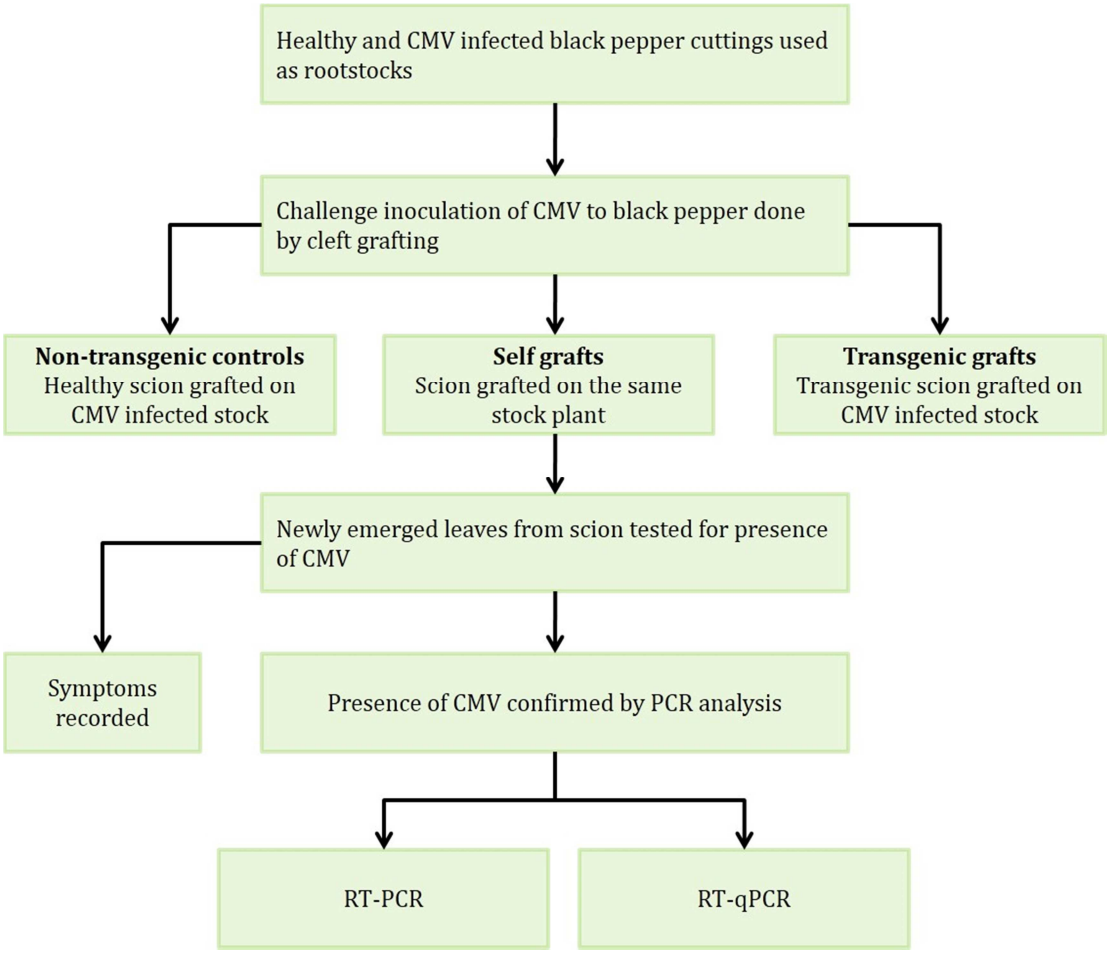

Figure 3.13 Flowchart of the different steps involved in the development of challenge inoculation method for *Cucumber mosaic virus* in black pepper by cleft grafting.

About two month old black pepper cuttings raised in pots were used as rootstocks. The apical end of the rootstock was removed using a clean scalpel. A vertical cut of 3–4 cm was made from the center of the stub and a split was formed. Scion shoot of pencil thickness having roundish shape with 2 to 3 nodes and 15–20 cm long was selected from the desired plant and defoliated prior to grafting. An equal–sized cleft, similar to that in stock was made in the base of the scion followed by the insertion of the scion into the rootstock in a way that, both the cambial tissues of stock and scion were in contact. The stock and scion were

then tied firmly with a polythene strip. The different steps involved in cleft grafting are shown in Figure 3.14. The pot was covered with a polythene bag to retain the moisture and was removed once terminal bud of the scion started to sprout. The polythene strip was further loosened so that the shoot grew normally (Kester *et al*., 2002).

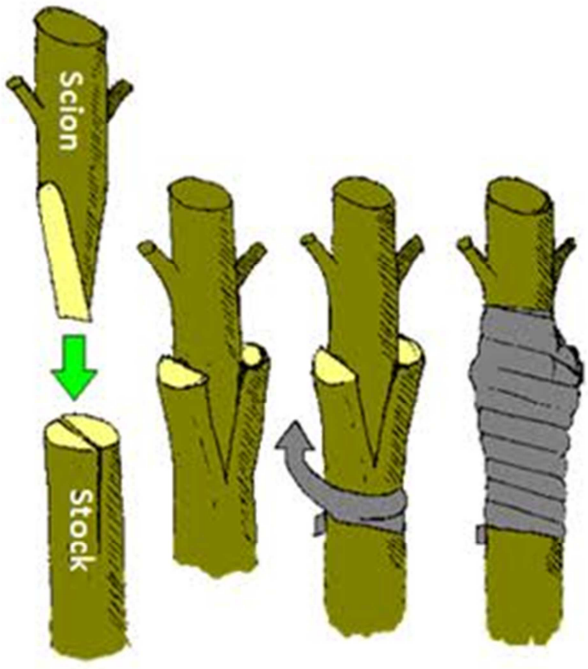

Figure 3.14 Different steps involved in the cleft grafting in black pepper.

To study the transmission efficiency of CMV in black pepper, 30 grafts were performed where scion free of CMV infection was grafted onto the infected stock (non–transgenic controls). As a control, 30 self–grafts were performed, where scion excised from a particular stock plant was grafted onto the same plant at the excised position to study the success of grafting. The symptoms in the newly emerged leaves were recorded and once the grafts established, new leaves from the scion were taken, total RNA isolated and tested for the presence of virus by RT–PCR using two sets of primers and RT–qPCR using the 2b gene specific primers. The details of the primers used for testing the presence of CMV in challenge inoculated black pepper plants is given in the Table 3.14. Total RNA extraction from the leaves, the reagent composition used and cycling conditions

for RT–PCR are as per the procedure given in the sections 3.1.2 and 3.1.3 respectively.

Table 3.14 Primers used for the screening of *Cucumber mosaic virus* in challenge inoculated plants

Primer	Sequence (5'……3')	Tm °C	Product size (bp)	Remarks
AIB 1 (F) AIB 2 (R)	ATGGACAAATCTGAATCAAC TCAAACTGGGAGCACCC	56	650	Amplifies the complete coat protein gene of CMV
AIB 183 (F) AIB 184 (R)	GTCGTCAGTGCGAATTGA TTCAAAACGCACCTTCCG	50	420	Amplifies the region from the 2a gene to the complete 2b gene of CMV
AIB 243 (F) AIB 279 (R)	GTTTATTTACAAGAGCGTACGG GAACATCATCGTTCCCTTCA	50	750	Amplifies the RNA 1 region of CMV
AIB 239 (F) AIB 240 (R)	AACGAAGGCGCAATGACAAAC AACAGTCTGAGATTTGAACGCG	56	127	Amplifies the region from the 2b gene by RT–qPCR

3.5.1 RT–qPCR study

RT–qPCR was performed using the total RNA extracted from the leaves as per the protocol given in section 3.1.2). The quality and quantity of the extracted RNA was analyzed using the Biophotometer plus. The primers used for the RT–qPCR amplification was designed from the 2b region of CMV from black pepper. The sequence specificity and complementarity were analyzed (section 3.1.3). RT–qPCR reactions were carried out in PCR tubes using the 2x QuantiFast™ SYBR Green PCR Master Mix. Total RNA from healthy black pepper was taken as the healthy control and reaction mix without the template was taken as the water control, to check the specificity of the primers designed and the contamination within the different components used. The specificity of the RT–qPCR products was checked by subjecting the amplicons to a melt curve analysis from 72°C to 95°C. The Ct value of the reactions was calculated using the Rotor–Gene Q system software. The different components

used for RT–qPCR is given in the Table 3.15 and the reaction profile used is given in Table 3.16.

Table 3.15 Reagent composition and concentration for RT–qPCR

Component	Volume (µl)
2X QuantiFast ™ SYBR Green PCR Master mix	12.5
Forward primer (1µM/µL)	1.0
Reverse primer (1µM/µL)	1.0
RevertAid Reverse transcriptase (50 U)	0.5
Total RNA	1.0
Water	9.0
Total	**25**

Table 3.16 Steps and conditions of thermal cycling for RT–qPCR

Step	Temperature (°C)	Duration	Cycle
cDNA synthesis	42	45 min	1
Initial denaturation	95	10 min	1
Denaturation	95	10 sec	35
Annealing	Specific T_m	15 sec	
Extension	72	15 sec	
Final extension	72	20 sec	1
Hold	4	~	1

To test the utility of the method, transgenic black pepper plants carrying the CP gene of CMV in the sense orientation available at ICAR–IISR (Jiby, 2011), and maintained under greenhouse conditions were grafted onto CMV infected stocks. A total of 8 transgenic lines in triplicates were subjected to cleft grafting. Once the grafts established, monitoring of the symptoms was done at regular intervals. Transmission of CMV to the scion was tested after establishment of the grafts using RT–PCR (section 3.1.3) and RT–qPCR (section 3.6.2) using the CMV 2b gene (RNA2) specific primers. The details of primers are given in Table 3.15. A plant naturally infected with CMV and one negative for CMV were used as

positive and negative controls respectively. Here, the RT–qPCR products were also verified by gel electrophoresis using 1.5% agarose.

CONTENTS

RESULTS

4.1 Complete genome sequencing of black pepper isolate of *Cucumber mosaic virus* (CMV)

4.1.1 Plant material

Among the five plants selected from the infected and healthy black pepper plants, two plants gave specific 650 bp product and three plants did not amplify in RT–PCR done using the CMV coat protein specific primers AIB 1 / AIB 2 (Figure 4.1, Table 3.1). The plants that amplified in RT–PCR were taken as the mother plants for complete genome sequencing of CMV and as the positive control in related experiments, while the plants that did not amplify were taken as the healthy controls.

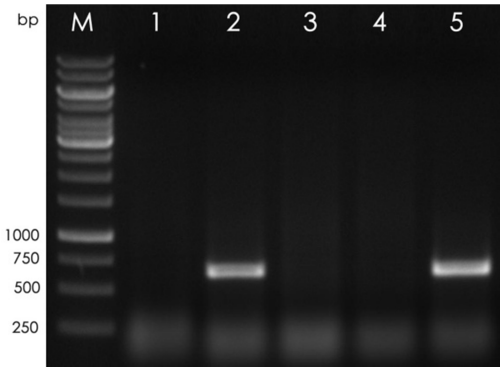

Figure 4.1 Identification of *Cucumber mosaic virus* infected and healthy black pepper plants by RT–PCR using the *Cucumber mosaic virus coat protein* specific primers AIB 1 / AIB 2. Lane M: 1 kb ladder, Lane 1–5: black pepper plants. The details of primers are given in the Table 3.1.

4.1.2 RNA extraction, RT–PCR and cloning

RNA1 of CMV was amplified using the primers AIB 203 / AIB 246 and AIB 245 / AIB 263 each giving approx. 1.7 kb product in RT–PCR (Figure 4.2a, Lane 1 and 2). The RNA2 was amplified using the primer combinations AIB 203 / AIB 204 and AIB 235 / AIB 263, each giving specific product of approx. 1.6 kb (Figure 4.2a, Lane 3 and 4). Similarly RNA3 was amplified using the primer sets AIB 282 / AIB 208 and AIB 237 / AIB 263; these on amplification gave an approx. 1.2 kb product in RT–PCR (Figure 4.2a, Lane 5 and 6). The healthy control gave no

amplification in any of the RT–PCR reaction. The specific products obtained in RT–PCR were extracted from the gel, ligated in to the cloning vector and transformed.

4.1.3 Confirmation of recombinant clones by PCR

After transformation, the selection plates (X–gal, IPTG, ampicillin) showed both transformed (white) and non–transformed (blue) colonies (Figure 4.3a). From these colonies the master plate was prepared by selectively streaking the colonies that were white in color onto Luria Bertani Agar plates having ampicillin (Figure 4.3b). The recombinant clones obtained using primers AIB 203 / AIB 246 were designated as 203246, the clones obtained using primers AIB 245 / AIB 263 were designated as 245263. Similarly, the clones of RNA2 were designated as 203204 and 235263. The RNA3 clones were designated as 282208 and 237263. Colony PCR was performed by taking four colonies from each master plate after the overnight incubation. The colony PCR of 203246 and 245263 clones gave expected amplicons of approx. 1.7 kb with corresponding primers. The clones 203204 and 235263 gave approx. 1.6 kb and the clones 282208 and 237263 gave amplicons of size approx. 1.2 kb. The amplicons of each clone from a single colony is shown in Figure 4.2b and the plasmids isolated from the corresponding clones are shown in Figure 4.2c. The presence of the insert in the plasmid was confirmed by PCR using insert specific forward and reverse primers (Figure 4.2d). The confirmed recombinant clones of CMV (203246, 245263, 203204, 235263, 282208 and 237263) were sequenced.

4.1.4 Complete genome sequence

Complete nucleotide (nt) sequence of the CMV–black pepper isolate comprised RNA1 (3349 nt), RNA2 (3049 nt) and RNA3 (2217 nt) (Table 4.1). RNA1 (Figure 4.4) has a single ORF coding for 990 aa 1a protein. RNA2 (Figure 4.5) and RNA3 (Figure 4.6) each have two ORFs. The 2a and 2b genes codes for proteins with 858 and 111 amino acids respectively. ORF 3a codes for the movement protein with 279 amino acids and ORF 3b codes for 218 amino acids

long coat protein. The amino acid sequences deduced from each ORF is shown in Figure 4.7.

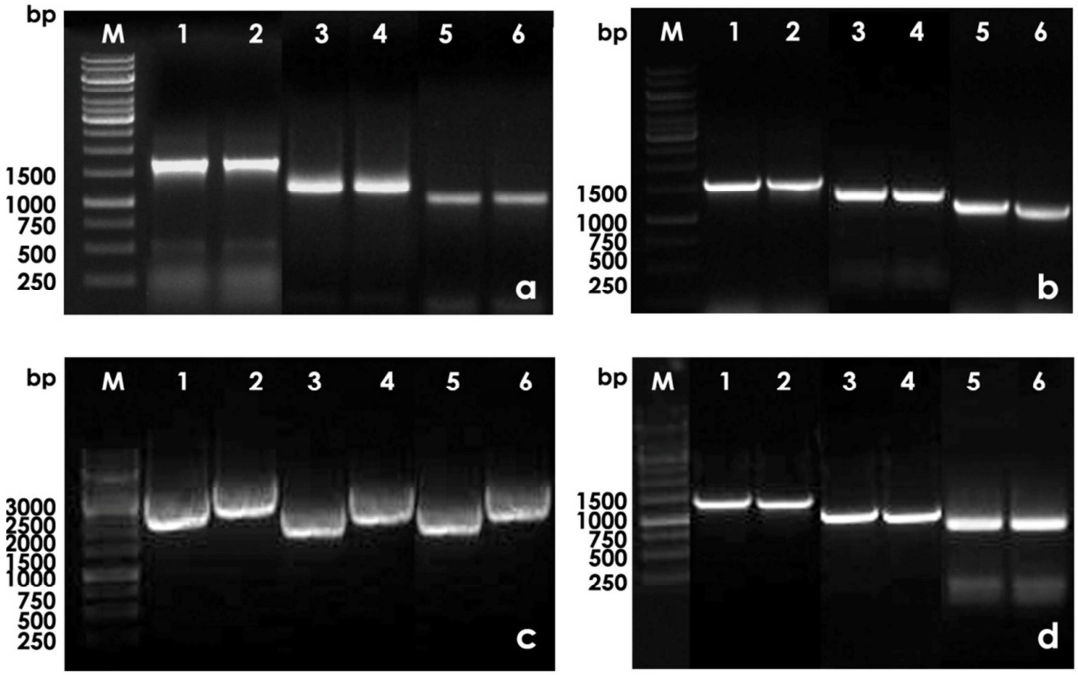

Figure 4.2 PCR analyses of three RNAs of black pepper isolate of *Cucumber mosaic virus* for complete genome sequencing, (a) RT–PCR of three RNAs. Lane 1: RT–PCR of RNA1 using AIB 203/ AIB 246, Lane 2: RT–PCR of RNA1 using AIB 235 / AIB 263, Lane 3: RT–PCR of RNA2 using AIB 282 / AIB 208, Lane 4: RT–PCR of RNA2 using AIB 235 / AIB 263, Lane 5: RT–PCR of RNA3 using AIB 282 / AIB 208 and Lane 6: RT–PCR of RNA3 using AIB 237 / AIB 263, **(b) colony PCR for screening transformants**. Lane 1: clone 203246 amplified using AIB 203 / AIB 246, Lane 2: clone 235263 amplified using AIB 235 / AIB 263, Lane 3: clone 282208 amplified using AIB 282 / AIB 208, Lane 4: clone 235263 amplified using AIB 235 / AIB 263, Lane 5: clone 282208 amplified using AIB 282 / AIB 208 and Lane 6: clone 237263 amplified using AIB 237 / AIB 263, **(c) plasmids isolated from the clones.** Lane 1: 203246, Lane 2: 235263, Lane 3: 282208, Lane 4: 235263, Lane: 5: 282208 and Lane 6: 237263 and, **(d) PCR of plasmid isolated from the respective clones using insert specific primers**. Lane 1: 203246, Lane 2: 235263, Lane 3: 282208 Lane 4: 235263, Lane 5: 282208 and Lane 6: 237263. Lane M: 1 kb ladder. In **(a)**, **(b)** and **(d)** ; Lane 1, 2: product size approx. 1.7 kb, Lane 3, 4: product size approx. 1.6 kb and Lane 5, 6: product size approx. 1.2 kb. In **(c)**, the size of the plasmid with the insert is approx. 4–3.5 kb. Details of primers are given in Table 3.2.

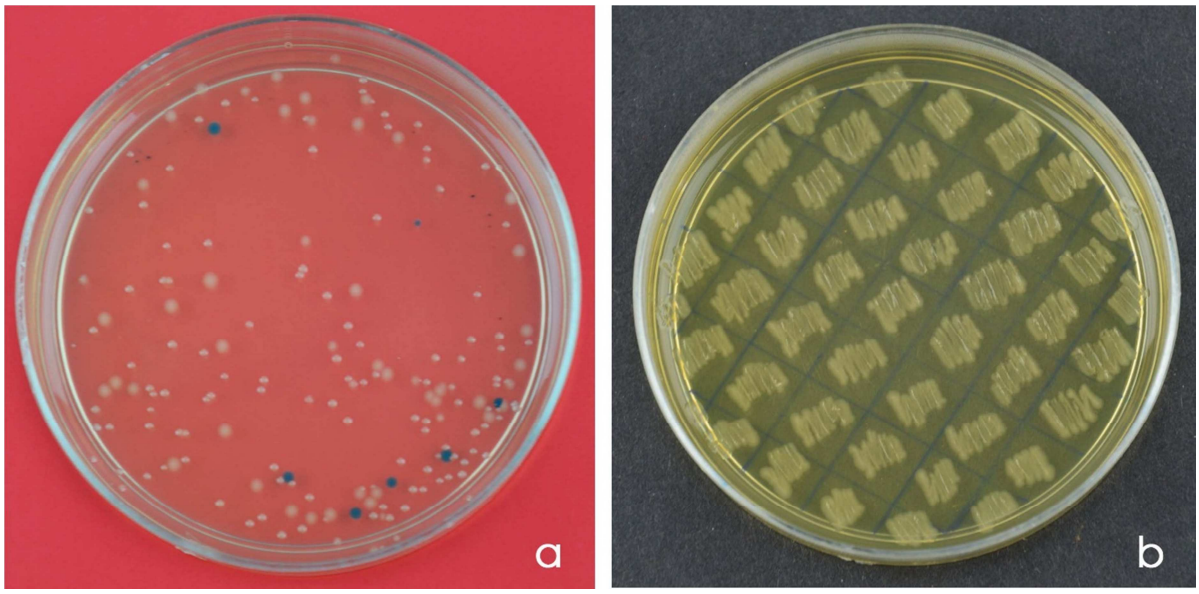

Figure 4.3 Identification of recombinant colonies by blue white screening, where both white and blue colonies were obtained on LB Agar plate with X–Gal, IPTG and ampicillin, **(a)** transformed colonies of 203246 and, **(b)** master plate preparation from the white colonies obtained on blue white screening.

Table 4.1 Complete genome sequence of black pepper isolate of *Cucumber mosaic virus*

Genome segment	Length in nucleotides			ORF	Protein encoded	Nucleotide position	Protein coding region [nt]/[aa]
	Total	**5' UTR**	**3' UTR**				
RNA1	3349	95	281	1a	Methyltransferase	96–3068	2973/990
RNA2	3049	76	302	2a	RNA polymerase	77–2653	2577/858
				2b	Suppressor protein	2412–2747	336/111
RNA3	2217	120	301	3a	Movement protein	121–960	840/279
				3b	Coat protein	1260–1916	657/218

CMV–Black pepper RNA1 3349 bases (KU947029)

GTTTATTTACAAGAGCGTACGGTTCAATCCCTGCCTCCCCTGTAAATTAACCCTTTGAAAAACCTCTTTCTTCTAAT
CTTTTCTTTGTAATTCCTATGGCGACGTCCTCGTTCAATATCAATGAATTGGTAGCCTCCCCACGGCGATAAAGGACT
ACTCGCGACCGCCCTCGTTGATAAGACAGCTCATGAGCAGCTCGAGGAGCAATTACAACATCAACGTCGGGGCCGTA
AGGTCTACATTCGGAATGTTCTGAGTGTAAAGGATTCCGAGGTCATCCGGAATCGGTATGGAGGGAAGTACGACCTC
CATCTTACCCAGCAGGAGTTTGCTCCCCACGGCCTAGCTGGTGCCCTCCGCTTGTGTGAAACTCTCGATTGTCTAGA
CTCTTTCCCTTCTTCAGGTCTGCGGCAGGACCCCGTCTTAGACTTCGGAGGAAGTTGGGTCACACATTACCTCCGCG
GACATAACGTACACTGCTGTTCCCCATGTTTGGGTGTCCGTGATAAAATGCGCCACACGGAACGTTTAATGAACATG
CGCAAGATCATCTTGAACGATCCACAACAGTTCGATGGTCGACAGCCGGATTTCTGCACTCATCCTGCTGCTGATTG
CGATGTACCAGCCCACTTCGCTATATCTATTCATGGAGGTTATGATATGGGCTTTAGAGGATTATGTGAAGCGATGA
ACGCTCACGGAACCACCATTTTGAAGGGAACGATGATGTTCGATGGTGCGATGATGTTTGACGACCAGGGCATAATA
CCTGAGCTTAATTGTCAGTGGAGGAAGATTAGGAGTGCTTTCTCCGAAACTGAAGACGTCACGTCTTTGTCGGGAAA
AATTGCGTCCACTCGCGTGCGTAAGTTTAAGACTATGGTAGCTTTCGATTTTATCAACGAGTCTACTATGTCTTATG
TTCATGATTGGAAGAATATAAAGTCATTTATGACAGACCAGACGTACTCGTATAAAGGGATGACTTATGGTATTGAA
CGCTGTACCATTAATGCGGGTATCATGACGTACAAGATCATCGGTGTGCCTGGGATGTGTCCACCCGAACTCATTCG
ACATTGCATTTGGTTCCCCTCTATTAAAGACTATGTTGGTCTGAAGATTCCCGCGTCACAGGACTTGGTTAAGTGGA
AAACAGTGCGAGTTTTAACGTCAACATTACGTGAAACTGAGGAGATTGCTATGAGGTGCTATAATGACAAGAAGAAT
TGGATGGAACAGTTTAAGGTCATCTTAGGTGTTCTTTCTGCTAAGTCTTCTACCATTGTTATCAATGGTATGTCCAT
GCAATCTGGCGAACGTATAGATATTGATGATTATCATTACATAGGATTCGCCATTCTTCTTCACACGAAAATGAAGT
ATGAACAGCTAGGGAAAATGTACGATATGTGGAACGCTTCTTTTATCTGGAAGTGGTTCGCAGCGTTGACTCGTCCG
TTGCGTGTGTTTTTCTCTCTTGCTGTTCGCACACTGTTCCCGACTTTGAGACCACGCGAGGAAAAAGAATTCCTGAT
CAAGCTCTCCACCTTCGTTACTTTTAATGAAGAGTGCTCATTTGATGGTGGAGAGGAATGGGACGTGATATCATCTG
CTGCATTTACTGCCGCGCAGGCTGTTACCGATGGGGAAGTTTTGGCTGCGCAGAAAGCCGAGAAGCTCGCTGAGAAG
CTTGCGCAACCCGTGATTGAGGTATCGGGCAGTCCTGAGACGCCATCTCCAACGTCTGATGATACTGCTGACGTTTG
TGGAAAGGAGCAAGAGGTTTCGGAACTTGACTCCCTGTCAGCTCAGACACGTTCCCCCGTCACTAGAATCGCTGAAA
GGGCTACTGCTATGTTAGAGTATGCCGCTTATGAGAAACAATTACATGACACTACGGTGTCTAATTTAAAACGCATT
TGGAACATGGCGGGTGGTGATGACAAGAAAAACTCCCTCGAAGGCAATTTGAAATTTGTCTTCGACACATATTTTAC
TGTCGACCCTATGGTGAACGTTCATTTTTCCACGGGTCGGTGGATGCATCCAGTGCCGGAGGGAGTTATTTATTCTG
TTGGTTTCAATGAGCGAGGCTTAGGCCCGAAATCTGATGGAGAGCTTTACATTGTCAATAGCGAGTGTGTGGTTTGC
AATAGTGAGTCTTTGTCTGCTGTCACGCGCTCGTTGCAAGCTCCGACTGGAACCATTAGTCAAGTCGACGGGGTCGC
GGGTTGTGGTAAAACCACAGCAATTAAATCTATTTTTGATCCGACCACCGATATGATCGTCACTGCGAATAAGAAAT
CCGCCCAAGACGTGCGTATGGCACTTTTCCAATCGTCGGACTCCAAAGAAGCTTGCACCTTCGTCCGAACAGCCGAT
TCTGTTCTGCTCAATGAATGTCCGACTGTTAGCAGAGTGTTGGTTGATGAGGTTGTGTTGTTACACTTTGGTCAATT
ATGTGCCGTTATGTCAAAATTGAAGGCCGTGAGAGCCATATGTTTCGGGGATTCAGAGCAAATTGCCTTTTCCTCTC
GAGATGCCTCGTTTGACATGCGTTTCTCTAAGATCATTCCTGATGAAACTAGTGACGCGGACACCACATTCCGTAGC
CCACAAGATGTTGTGCCGCTTGTGCGTTTAATGGCTACGAAGGCCCTTCCGAAAGGAACTCATTCAAAATATACCAG
ATGGGTTTCTCAATCTAAAGTGAAGAGATCTGTTACATCTCGTGCCGTCGCAAGTGTGACTCTGGTTGACCTGGACC
CTACTAGGTTTTATGTCACGATGACACAAGCTGATAAAGCTTCACTGATTTCAAGGGCGAAAGAGATGAAATTACCA
AAAACTTTCTGGAATGAAAGGATTAAAACTGTTCATGAGTCTCAAGGTATTTCAGAGGACCATGTCACTTTGGTAAG
ATTGAAGAGCACAAAGTGTGACCTGTTCAAAAAATTCTCTTATTGTCTCGTCGCTTTGACTAGACACAAGGTCACTT
TCCGCTACGAGTATTGTGGTATGTTAAGCGGTGATTTAATCGCCGATTGTATTGCTCGTGCTTAGCGGCTTCCCTCC
TTCGGGCGGGATCTGAGTTGGCGGTAATCTACAAACCGTCTGAGGTCACTAAACGCTTTGCGGTGAACGGGTTGTCC
ATCCAGCTTACGGCTAAAATGGTCAGTCGTGGAGAAATCCACGCCAGCAGACTTACAAGTCTCTGAGGTGCCTTTGA
AACCATCTCCTAGGTTTCTTCGGAAGGACTTCGGTCCGTGTACTTCCAGCACAAGATGCTGGTTTTAGGGTACGGC
ACCCCCCACACATTAGTGGGGCCTCTAAAAGGAGACCA

Figure 4.4 Complete nucleotide sequence of RNA1 (3349 bases) of black pepper isolate of *Cucumber mosaic virus*. The 5'UTR: 1–95 (95 bases), 1a gene: 96–3068 (2973 bases) and the 3'UTR: 3069–3349 (281 bases), are highlighted.

CMV–Black pepper RNA2 3049 bases (KU947030)

GTTTATTTACAAGAGCGTACGGTTCAACCCCTGCCTCCTCTGTAAAACTCCAAACTTTCTAGTTTTCTATCCTTCTA
TGGCTTTCCCTGCCCCCGCTTTCTCACTAGCCAATCTTTTGAATGGCAGTTACGGTGTCGACACTCCCGAGGAAGTG
GAACGCGTGCGATCTGAGCAACGCGAAGAGGCTGCTGCGGCCTGCCGTAATTACAGGCCCCTACCCGCTGTGGATGT
CAGCGAGAGTGTCACGGAGGATGCGCATTCCCTCCAGACTCCAGACGGAGCTCCCTCTGAGGAGGTGTCTGTTGAGT
CTGTCTCTTATGGTGCTGAAGATTACCTTGAAAAATCTGATGATGAGCTCCTTGTCGCTTTTGAGACGATGGTCAAA
CCCATGTGTGTCGGTCAGCTATGGTGCCCCGCGTTTAATAAATGTTTATTTATTTCCAGCATTGCTATGGCCAGAGC
TTTGTTGTTGGCACCTAGAACATCCTCCCGAACCATGAAGTGTTTTGAGGACCTAGTCGCGGCTATTTACACTAAAT
CCGATTTCTATTACGATGATGAGTGTGAAGCCGACGACGTTCAGATGGATATCTCGTCTCGCGATGTACCCGGTTAT
TCTTTCGAACCGTGGTCCCGAACGTCTGGATTTGAACCACCGCCTATCTGTGAGGCGTGTGACATGATCATGTACCA
GTGCCCGTGTTTCGATTTCAATGCTTTAAAGAAATCGTGTGCTGAGAGGACTTTCGCTGACGATTATGTTATTGAAG
GTTTAGATGGTGTTGTTGACAATGCGACTTTGTTGTCCAATTTAGGTCCATTCTTGGCGCCCGTGAAATGCCAATAT
GAGAAATGTCCAACACCAACCGTCGTGATTCCTCCGAGTTTAAATCGTGCTACAGATCGTGTTGACACCAATTTAGT
TCAATCCATTTGTGACTCGACTCTGCCCACTCATAACAACTACGACGACTCTTTTCATCAAGTGTTTGTCGAAAGTG
CTGATTATTCTATTGATCTGGACCACGTTAGACTTCGACAGTCTGATCTTATTGCAAAAATTCCAGATTCAGGGCAT
ATGATACCGGTTTTGAATACCGGGAGCGGTCACAAGAGAGTAGGTACGACGAAGGAGGTTCTTACAGCAATCAAGAA
ACGTAATGCTGACGTTCCGGAGCTAGGTGACTCCGTTAATCTGTCCAGGTTGAGTAAGGCTGTGGCTGAGAGGTTTT
TCATTTCATATATCAATGGCAGCTCTCTAGCATCCAGCAACTTTGTTAATGTCGTTAGTAACTTCCACGATTACATG
GAAAAGTGGAAGTCCTCAGGTCTTTCGTATGATGATCTCCCAGATCTTCATGCTGAAAATTTGCAGTTTTATGATCA
TATGATAAAATCTGATGTGAAACCTGTAGTGAGCGACACACTCAACATCGACAGACCGGTTCCAGCTACCATAACGT
ATCATAAGAAAGGTATAACCTCCCAGTTCTCACCGTTATTTACAGCGCTATTCGAGCGCTTCCAGAGATGCCTTCGA
GAACGTATCATTCTTCCTGTTGGAAAGATTTCATCCCTTGAGATGGCGGGATTCGATGTTAGGAATAAACACTGTCT
CGAGATCGATTTGTCTAAATTTGACAAGTCCCAAGGTGAATTTCATTTGATGATTCAGGAACATATTTTGAATGGTC
TAGGATGCCCAGCTCCGATAACCAAATGGTGGTGTGATTTCCACCGATTCTCTTACATCAGAGACCGTAGAGCTGGT
GTTGGTATGCCCATCAGTTTCCAGAGACGAACTGGTGATGCGTTTACCTATTTCGGTAACACCATAGTTACTATGGC
TGAATTCGCATGGTGTTACGATACTGACCAATTCGAAAAACTCTTGTTCTCAGGCGATGATTCTCTAGGATTTTCAT
TACTTCCCCCTGTTGGTGATCCGAGTAAATTCACAACTCTATTCAACATGGAAGCTAAGGTGATGGAACCTGCGGTA
CCATATATTTGTTCGAAGTTCTTACTCTCTGACGAGTTCGGTAATACTTTTTCCGTTCCAGATCCATTGCGTGAGAT
TCAGCGGTTAGGAACAAAGAAAATCCCCTATTCCGACAATGATGAATTCCTGTTCGCTCACTTCATGAGCTTTGTTG
ATCGATTGAAGTTTTTGGACAGAATGACTCAGTCTTGCATCGATCAGCTTTCACTCTTTTTCGAGTTGAAATACAGG
AAGTCAGGGGCTGAGGCTGCTTTAATGTTAGGTGCCTTTAAGAAATATACCGCTAATTTCCAATCCTATAAAGAGCT
CTACTATTCAGATCGTCGTCAGTGCGAATTGATCAATTCGTTTAGTTGTGTGGAGTTAAGGATTGAGCGTTCGATTT
CTACTAAGCAGCGAAAGAAGAAAGATGGAATTGAACGAAGGCGCAATGACAAACGTCGAACTCCAGCTGGCTCGCAT
GATGGAGGTGAAGAGACAGAGACGAAGGTCTCACAAGAAGAATCGACGGGGACGAGGTCACAAAAGTCCCAGCGAGA
GGGCGCGTTCAAATCTCAGACTGTTCCGTTTTCTACCATTTTTTCAGATAGATGGTTCGGAACTGGTAGGGATGTAC
CACCGTGCGCGCGCGGTGGAGCTGTCCGAGTCTGAGGCCCCTTGTTTTCCGTTACCAGCGGAAGAAGACCATGATTT
TGACGATACGGATTGGTTTGCTGGCAACGAATGGGCGGAAGGCGCGTTTTGAATCTCCCCTTCCTTTTCTCCCTCCA
GCTTTCTGAGGCGGGAGCTGAGTTGGCAGTATTGCTATAAACTGTCTGAAGTCACTAAACGCTTTGCGGTGAACGGG
TTGTCCATCCAGCTTACGGCTAAAATGGTCAGTCGTGGAGAGATCCACGCCAGTAGACTTACAAGTCTCTGAGGTGC
CTTTGAAACCATCTCCTAGGTTTCTTCGGAAGGACTCCGGTCCGTGTATCTCCAGCACACGATGCTGGTTTTAGGGT
ACGGGCACCCCCCCCACTTTGATGGGGGGTCTCTCAAAGGAGACCA

Figure 4.5 Complete nucleotide sequence of RNA2 (3049 bases) of black pepper isolate of *Cucumber mosaic virus*. The 5'UTR: 1–76 (76 bases), the 2a gene: 77–2653 (2577 bases), the 2b gene: 2412–2747 (336 bp), the overlapping region of 2a and 2b genes, and 3' UTR: 2748–3049 (302 bases), are highlighted.

CMV–Black pepper RNA3 2217 bases (KU947031)

GTAATCTTACCACTGTGTGTGTGCGTGTGTGTGTCGCGTCGTGTCGAGTCGTGTTGTCCGCACATTGAGTCGTGT
TGTCCGCACACTCTTTTATTTCAGTGTGTTAGATTCCCGAGGTATGGCTTTCCAAGGTACCAGTAGGACTTTAACTC
AACAGTCCTCAGCGGCTACGTCTGACGATCTTCAGAAGATATTATTTAGCCCTGATGCCATTAAGAAAATGGCTACT
GAGTGTGACCTAGGCCGGCATCATTGGATGCGTGCTGATAATGCGATATCAGTCCGGCCCCTCGTTCCCGAAGTAAC
CCATGGTCGTATTGCTTCCTTCTTTAAGTCTGGATATGACGTCGGTGAATTGTGCTCTAAGGGATACATGAGCGTTC
CTCAAGTGTTGTGTGCTGTTACTCGAACAGTTTCTACCGATGCTGAGGGATCTTTGAGAATTTACCTGGCTGATCTA
GGCGACAAGGAGTTGTCTCCTATAGACGGACAATGCGTTTCGTTACATAACCATGATCTCCCCGCTTTAGTGTCTTT
CCACCCGACGTTTGACTGTCCTATGGAAATTGTTGGGAACCGTAAGCGGTGTTTTGCTGTCGTCATCGAGAGACATG
GTTACGTTGGGTATACCGGCACCACAGCAAGCGTGTGTAGTAATTGGCAAGCACGATTCTCTTCTAAGAATAATAAC
TACACTCGTATCGCTGCTGAGAAGACTCTAGTACTGCCATTCAACAGATTAGCTGAGCAAACTAAACCGTCAGCCGT
CGCTCGCCTGTTGAAGTCGCAATTGAATAACATAGAATCTTCGCAATACGTTTTAACGGATTCGAAGATTAATCAAA
ATGCGCGCAGTGAGTCCGAGGATTTGGTTGTTGAAAGCCCTCCCGTCGCAATCGGGAGTTCTTCCGCGTCCCGCTCC
GAAACCTTTAGACCGCAGGTGGTTAACGGTCTTTAGCGTTTCCGTTGCGTATTAGATTATATGTATATGTGAGTCTG
CACATAATACTATATCTATAGTGTCCTGTGTGAGTTGATACAGTAGACAACTGTGACGCGATGCCGTGTTGAGAAGA
GAGCACATCTGGTTTAGTAAAACCCACATCATTAGTTTGAGGTTCAATTCCTCTTACTCCCTGATGGGTTTCTTTTA
CTTTTCATGGATGCTTCTCCACGAGATTGCGTTTCGTCTACTTATCTTAAGAGTATTGTGTTGTGTTTTTTTCTTTT
GTGTTGTAGATTGAGTCGAGTCGAGTCATGGACAAATCTGAATCAACCAGTGCTGGTCGTAACCGTCGGCGTCGTCC
GCGTCGCGGTTCCCGCTCCGCCTCCTCCTCTGCGGATGCTACATTTAGAGTCCTGTCGCAACATCTTTCGCGACTCA
TCAAGACGTTAGCAGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGTTGTAAACCTGGATAC
ACGTTCACCTCGATTACCCTGAAGCCACCAAAAATAGACCAAGGGTCTTATTATGGCAAAAGGTTGTTACTTCCTGA
TTCAGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATTTGATTCTA
CCGTGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTGTCCGTTTCCGCCATCTCTGCTATGTTTGCGGAC
GGAGCCTCACCGGTACTGGTTTATCAGTATGCTGCATCTGGCGTTCAAGCCAACAACAAATTGTTGTATGATCTTTC
AGTGATGCGCGCTGATATTGGTGATATGAGAAAGTACGCCGTGCTCGTGTATTCAAAAGACGATGCGCTCGAGACGG
ACGAACTGGTACTTCATGTCGACATTGAGCACCAACGCATTCCCACGTCTGGGGTGCTCCCAGTTTGAACTCGTGTT
TTCCAGGACCCTCCCTCCGTTTCTGTGGCGGGAGCTGAGTTGGTAGTGTTGCTATAAACTGCCTGAAGTCACTAAAC
GCTTTGCGGTGAACGGGTTGTCCATCCAGCTTACGGCTAAAATGGTCAGTCGTGGAGAAATCTGCGCCAGTAGACTT
ACAAGTCTCTGAGGTGCCTTTGAAACCATCTCCTAGGTTTCTTCGGAAGGACTTCGGTCCGTGTACTTCCAGCACAA
GATGCTGGTGTTAGGGTACGGGCATCCCCCCACTTTAGTGGGGGCCTCTAAAAGGAGACCA

Figure 4.6 Complete nucleotide sequence of RNA3 (2217 bases) of black pepper isolate of *Cucumber mosaic virus*. The 5'UTR: 1–120 (120 bases), 3a gene: 121–960 (840 bases), intergenic region: 961–1259 (299 bases), 3b gene: 1260–1916 (657 bases) and 3' UTR: 1917–2217 (301 bases), are highlighted.

1aP

MATSSFNINELVASHGDKGLLATALVDKTAHEQLEEQLQHQRRGRKVYIRNVLSVKDSEVIRNRYGGKYDLHLTQQE
FAPHGLAGALRLCETLDCLDSFPSSGLRQDPVLDFGGSWVTHYLRGHNVHCCSPCLGVRDKMRHTERLMNMRKIILN
DPQQFDGRQPDFCTHPAADCDVPAHFAISIHGGYDMGFRGLCEAMNAHGTTILKGTMMFDGAMMFDDQGIIPELNCQ
WRKIRSAFSETEDVTSLSGKIASTRVRKFKTMVAFDFINESTMSYVHDWKNIKSFMTDQTYSYKGMTYGIERCTINA
GIMTYKIIGVPGMCPPELIRHCIWFPSIKDYVGLKIPASQDLVKWKTVRVLTSTLRETEEIAMRCYNDKKNWMEQFK
VILGVLSAKSSTIVINGMSMQSGERIDIDDYHYIGFAILLHTKMKYEQLGKMYDMWNASFIWKWFAALTRPLRVFFS
LAVRTLFPTLRPREEKEFLIKLSTFVTFNEECSFDGGEEWDVISSAAFTAAQAVTDGEVLAAQKAEKLAEKLAQPVI
EVSGSPETPSPTSDDTADVCGKEQEVSELDSLSAQTRSPVTRIAERATAMLEYAAYEKQLHDTTVSNLKRIWNMAGG
DDKKNSLEGNLKFVFDTYFTVDPMVNVHFSTGRWMHPVPEGVIYSVGFNERGLGPKSDGELYIVNSECVVCNSESLS
AVTRSLQAPTGTISQVDGVAGCGKTTAIKSIFDPTTDMIVTANKKSAQDVRMALFQSSDSKEACTFVRTADSVLLNE
CPTVSRVLVDEVVLLHFGQLCAVMSKLKAVRAICFGDSEQIAFSSRDASFDMRFSKIIPDETSDADTTFRSPQDVVP
LVRLMATKALPKGTHSKYTRWVSQSKVKRSVTSRAVASVTLVDLDPTRFYVTMTQADKASLISRAKEMKLPKTFWNE
RIKTVHESQGISEDHVTLVRLKSTKCDLFKKFSYCLVALTRHKVTFRYEYCGMLSGDLIADCIARA

2aP

MAFPAPAFSLANLLNGSYGVDTPEEVERVRSEQREEAAAACRNYRPLPAVDVSESVTEDAHSLQTPDGAPSEEVSVE
SVSYGAEDYLEKSDDELLVAFETMVKPMCVGQLWCPAFNKCLFISSIAMARALLLAPRTSSRTMKCFEDLVAAIYTK
SDFYYDDECEADDVQMDISSRDVPGYSFEPWSRTSGFEPPPICEACDMIMYQCPCFDFNALKKSCAERTFADDYVIE
GLDGVVDNATLLSNLGPFLAPVKCQYEKCPTPTVVIPPSLNRATDRVDTNLVQSICDSTLPTHNNYDDSFHQVFVES
ADYSIDLDHVRLRQSDLIAKIPDSGHMIPVLNTGSGHKRVGTTKEVLTAIKKRNADVPELGDSVNLSRLSKAVAERF
FISYINGSSLASSNFVNVVSNFHDYMEKWKSSGLSYDDLPDLHAENLQFYDHMIKSDVKPVVSDTLNIDRPVPATIT
YHKKGITSQFSPLFTALFERFQRCLRERIILPVGKISSLEMAGFDVRNKHCLEIDLSKFDKSQGEFHLMIQEHILNG
LGCPAPITKWWCDFHRFSYIRDRRAGVGMPISFQRRTGDAFTYFGNTIVTMAEFAWCYDTDQFEKLLFSGDDSLGFS
LLPPVGDPSKFTTLFNMEAKVMEPAVPYICSKFLLSDEFGNTFSVPDPLREIQRLGTKKIPYSDNDEFLFAHFMSFV
DRLKFLDRMTQSCIDQLSLFFELKYRKSGAEAALMLGAFKKYTANFQSYKELYYSDRRQCELINSFSCVELRIERSI
STKQRKKKDGIERRRNDKRRTPAGSHDGGEETETKVSQEESTGTRSQKSQREGAFKSQTVPFSTIFSDRWFGTGRDV
PPCARGGAVRV

2bP

MELNEGAMTNVELQLARMMEVKRQRRRSHKKNRRGRGHKSPSERARSNLRLFRFLPFFQIDGSELVGMYHRARAVEL
SESEAPCFPLPAEEDHDFDDTDWFAGNEWAEGAF

3aP

MAFQGTSRTLTQQSSAATSDDLQKILFSPDAIKKMATECDLGRHHWMRADNAISVRPLVPEVTHGRIASFFKSGYDV
GELCSKGYMSVPQVLCAVTRTVSTDAEGSLRIYLADLGDKELSPIDGQCVSLHNHDLPALVSFHPTFDCPMEIVGNR
KRCFAVVIERHGYVGYTGTTASVCSNWQARFSSKNNNYTRIAAEKTLVLPFNRLAEQTKPSAVARLLKSQLNNIESS
QYVLTDSKINQNARSESEDLVVESPPVAIGSSSASRSETFRPQVVNGL

3bP

MDKSESTSAGRNRRRRPRRGSRSASSSADATFRVLSQHLSRLIKTLAAGRPTINHPTFVGSERCKPGYTFTSITLKPPKID
QGSYYGKRLLLPDSVTEFDKKLVSRIQIRVNPLPKFDSTVWVTVRKVPASSDLSVSAISAMFADGASPVLVYQYAASGVQ
ANNKLLYDLSVMRADIGDMRKYAVLVYSKDDALETDELVLHVDIEHQRIPTSGVLPV

Figure 4.7 Deduced amino acid sequences of 1a, 2a, 2b, 3a and 3b genes of black pepper isolate of *Cucumber mosaic virus.*

4.1.5 Multiple sequence alignment

Multiple sequence alignment of the five genes revealed nine nucleotides deletion in the methyltransferase domain of 1a gene (Figure 4.8) which was also reflected in the multiple sequence alignment of deduced amino acid sequence that showed deletion of three amino acids (Figure 4.9). This deletion was observed only in two more CMV isolates (CLW2 from Malaysia infecting cucumber and HM3 from China infecting tomato) among one hundred 1a gene sequences analyzed including the CMV isolate infecting black pepper from China and Indian long pepper (a closely related species to black pepper) from India. The size of 1a gene in these three isolates was 2973 nt whereas in all other subgroup I isolates it was 2982 nt. A ten nucleotide deletion and five amino acid deletion in the decuced sequence in the same region of *peanut stunt virus* (PSV), a distinct species under the genus *Cucumovirus* (Figure 4.8) are notable. In PSV another five nucleotide deletion compensates the frame shift made by the ten nucleotide deletion (Figure 4.8).

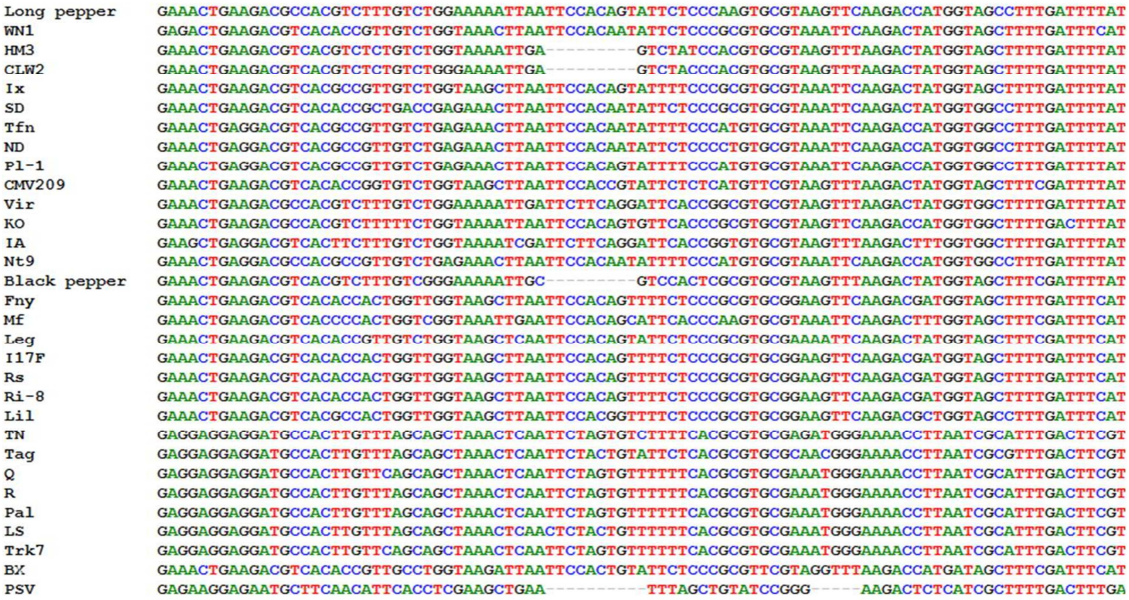

Figure 4.8 Multiple sequence alignment of a portion of *Cucumber mosaic virus* 1a nucleotide of black pepper isolate with other *Cucumber mosaic virus* strains, including black pepper (WN1 from China), Indian long pepper and outgroup *Peanut stunt virus,* showing deletion of nine bases, aligned using clustalX. The details of isolates are provided in Table 3.8.

Figure 4.9 Multiple sequence alignment of a portion of *Cucumber mosaic virus* 1a deduced amino acid of black pepper isolate with other *Cucumber mosaic virus* strains, including black pepper (WN1 from China), Indian long pepper and outgroup *Peanut stunt virus,* showing deletion of three amino acids, aligned using clustalX. The details of isolates are provided in Table 3.8.

4.1.6 Percent identity analyses

The results of percent identity analyses are summarized in Table 4.2–Table 4.5. Percent identity analysis of CMV black pepper (CMV BP) isolate revealed that all five genes at the deduced amino acid level showed maximum identity to subgroup IB strains with an identity of 91–97% (89–95% at nucleotide level) in 1a, 94–98% (91–97% at nucleotide level) in 2a, 77–95% (84–95% at nucleotide level) in 2b, 85–97% (87–97% at nucleotide level) in 3a and 95–98% (90–98% at nucleotide level) in 3b. The identity of the corresponding genes to subgroup IA, at the amino acid level were, 93% (89–90% at nucleotide level), 92–94% (90–91% at nucleotide level), 68–79% (80–85% at nucleotide level), 91–95% (88–92% at nucleotide level) and 95–96% (90–93% at nucleotide level), respectively. Among five genes, 3b gene showed maximum identity among the subgroups (80–99% at deduced amino acid level and 76–98% at nucleotide level) and 2b showed the minimum (51–95% at deduced amino acid level and 56–95% at nucleotide level). All the genes of BP isolate

showed highest identity of 95–98% with KO isolate from Karnataka India, followed by CLW2 isolate from Malaysia sharing 89–98% identity. The percent identity with black pepper isolate WN1 from China ranged from 80–96%, which is less as compared to the identity with KO isolate from India and CLW2 isolate from Malaysia. The 3′ UTR of three RNAs of the current isolate shared more similarity of 91% (Figure 4.10), compared to the 5′ UTR (52–80%). Also, the 3′ and 5′ UTR of the three RNAs of BP isolate of CMV showed highest identity of 85–97% and 82–98% to CMV subgroup IB strains. The identity of the above regions of the BP isolate to subgroup IA strains was 86–92% and 81–92%. Percent identity analyses thus suggest that the BP isolate of CMV belongs to subgroup IB.

Table: 4.2 Percent identity in the 2b (below the diagonal) and 3b (above the diagonal) genes of black pepper isolate of *Cucumber mosaic virus* at the deduced amino acid level with other isolates of subgroup I and II (done using Clustal Omega).

Strain 2b/3b	Black Pepper	IB	IA	II
Black Pepper		94–98	95–96	80–82
IB	75–95		95–99	80–83
IA	68–79	65–84		80–85
II	51–52	43–55	39–52	

Table: 4.3 Percent identity of the 2b (below the diagonal) and 3b (above the diagonal) genes of black pepper isolate of *Cucumber mosaic virus* at the nucleotide level with other isolates of subgroup I and II (done using Clustal Omega).

Strain 2b/3b	Black Pepper	IB	IA	II
Black Pepper		90–98	90–93	76–77
IB	84–95		90–96	76–78
IA	80–85	79–89		76–78
II	59–60	57–62	56–60	

Table 4.4 Percent identity of 1a (in parenthesis), 2a (below the diagonal) and 3a (above the diagonal) genes of black pepper isolate of *Cucumber mosaic virus* at the deduced amino acid level with other isolates of subgroup I and II (done using Clustal Omega).

Strain 2a/3a	Black Pepper	IB	IA	II
Black Pepper		85–97	91–95	81–82
IB	93–95 (91–97)		82–98	75–85
IA	92–95 (93)	90–95 (92–97)		78–93
II	75–76 (84–85)	73–76 (82–85)	75–80 (84–86)	

Table 4.5 Percent identity of 1a (in parenthesis), 2a (below the diagonal) and 3a (above the diagonal) genes of black pepper isolate of *Cucumber mosaic virus* at the nucleotide level with other isolates of subgroup I and II (done using Clustal Omega)**.**

Strain 2a/3a	Black Pepper	IB	IA	II
Black Pepper		87–97	88–92	77–78
IB	91–97 (89–95)		83–94	73–80
IA	90–91 (89–90)	89–93 (89–93)		73–78
II	71–72 (77–78)	71–73 (77–78)	71–73 (77)	

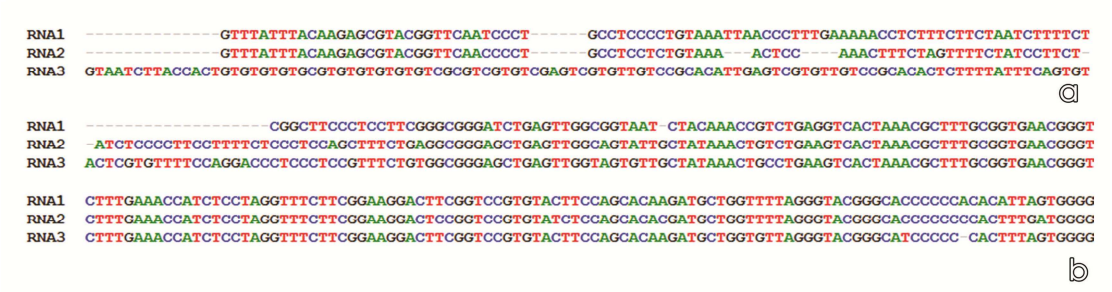

Figure 4.10 Multiple sequence alignment of the 5' UTR (a) and 3' UTR (b) of the three RNAs of black pepper isolate of *Cucumber mosaic virus* done using BioEdit, showing higher percent identity in the 3' UTR of three RNAs compared to the 5' UTR.

4.1.7 Phylogenetic analyses

The phylogenetic analyses of the three RNAs at nucleotide sequence level (Figure 4.11) and, the five genes at the nucleotide and deduced amino acid level (Figure 4.12–4.16) showed that BP isolate of CMV is clustered to subgroup IB strains. The phylogenetic trees at nucleotide and deduced amino acid were identical topologically. In all the phylogenetic trees BP isolate was clustered with KO and CLW2 strains.

4.1.8 Nucleotide diversity studies

Though all the genes of the current isolate showed haplotype diversity value of nearly one, the highest nucleotide diversity was seen in 2b gene (0.20663) while 3b gene (0.12255) had the least (Table 4.6). The ratio of non–synonymous to synonymous substitution is consistent with the above data with 3b gene (0.12) having the least but 1a gene had the highest value of 0.5. Though these values are less than one for all the genes indicating purifying selection, the value of 1a gene is nearly five times greater than the 3b gene indicating that the protein encoded by 1a gene is subjected to greater selection pressure. In this regard, among the three RNAs, protein encoded by RNA1 is highly tolerant to amino acid changes, followed by RNA2 and RNA3 is found to be the least tolerant.

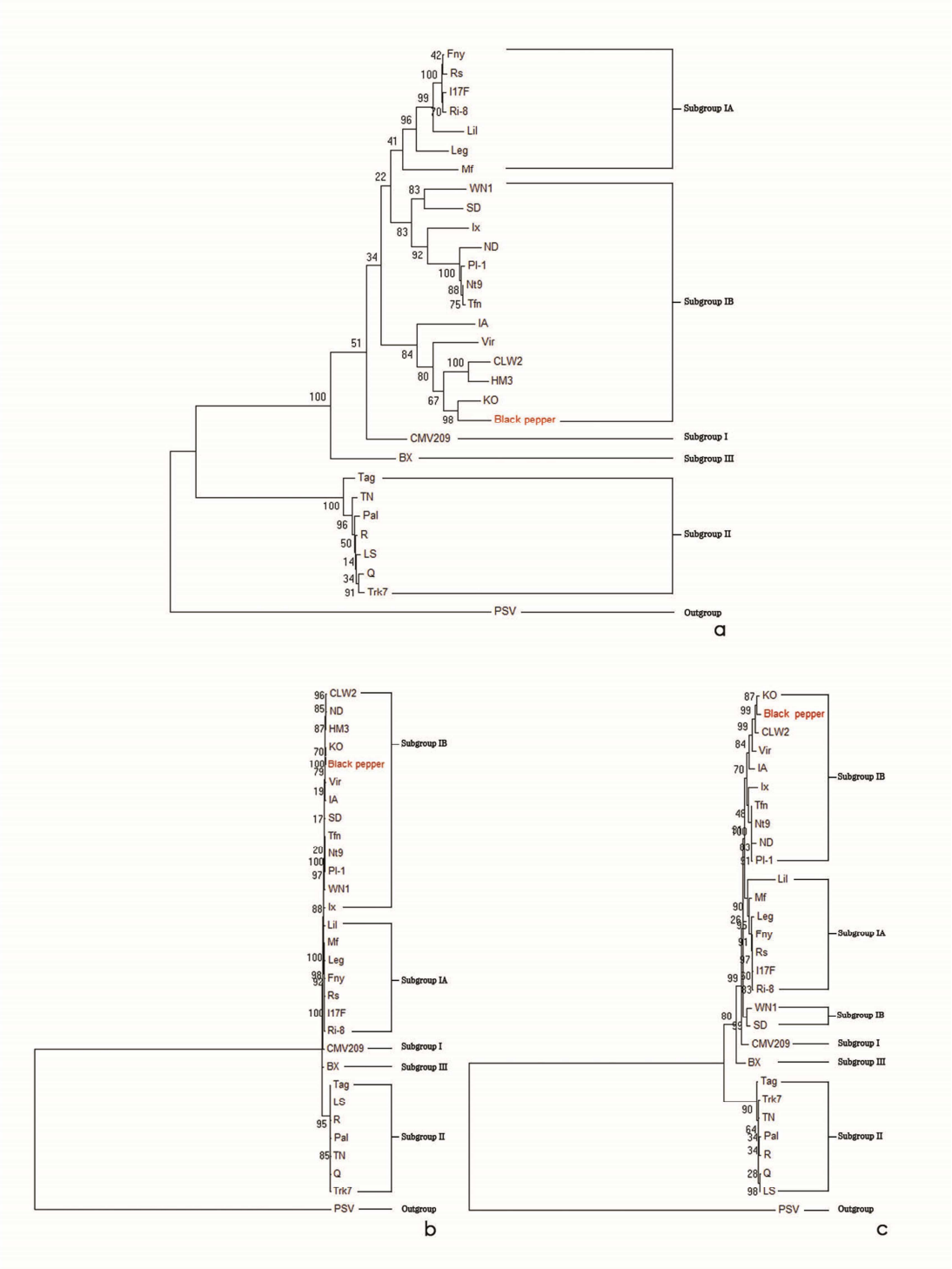

Figure 4.11 The phylogenetic trees of (a) complete RNA1, (b) complete RNA2 and (c) complete RNA3, of black pepper isolate of *Cucumber mosaic virus,* constructed using the maximum likelihood method in Mega 6.06 with 1000 bootstrap replicates, and the Jukes–Cantor model.

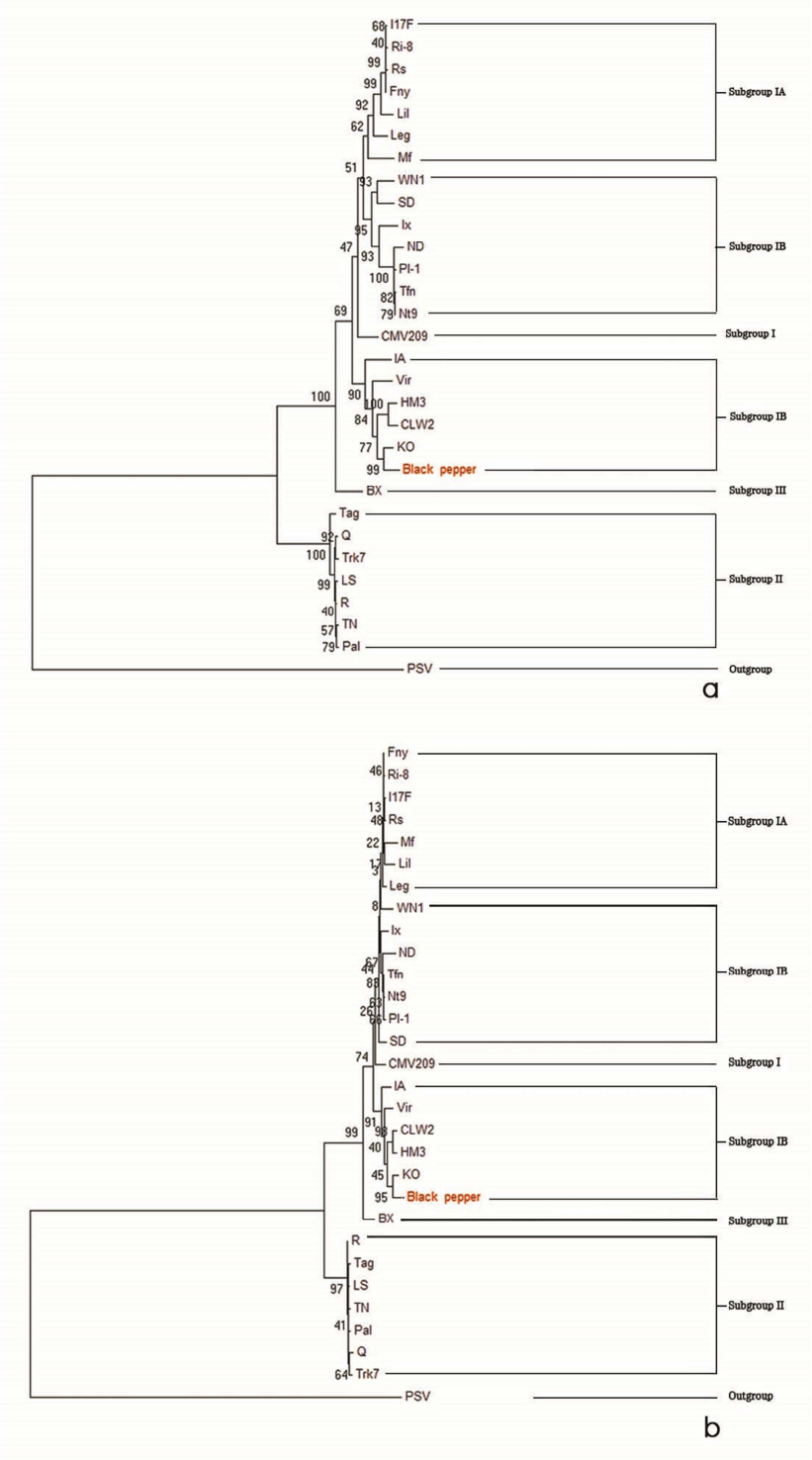

Figure 4.12 Phylogenetic trees of 1a gene of black pepper isolate of *Cucumber mosaic virus* (a) at the nucleotide level and (b) at the deduced amino acid level, constructed using maximum likelihood method in Mega 6.06 with 1000 bootstrap replicates (Jukes–Cantor model was selected for constructing nucleotide tree and Poisson model was used for constructing the amino acid tree).

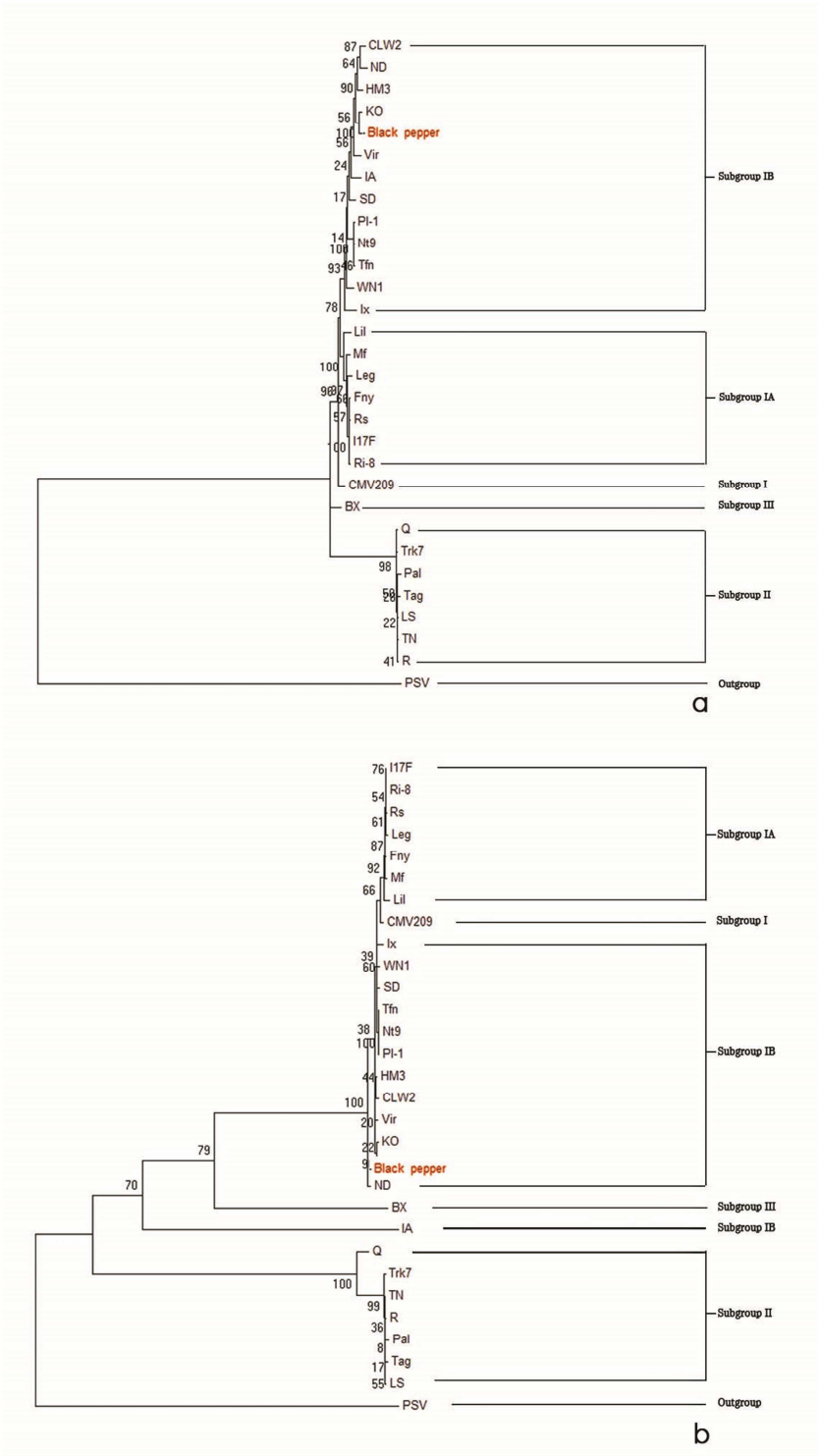

Figure 4.13 Phylogenetic trees of 2a gene of the black pepper isolate of *Cucumber mosaic virus,* (a) at the nucleotide level and (b) the deduced amino acid level constructed using the maximum likelihood method in Mega 6.06 with 1000 bootstrap replicates (Jukes–Cantor model was selected for constructing the nucleotide tree and the Poisson model for amino acid tree).

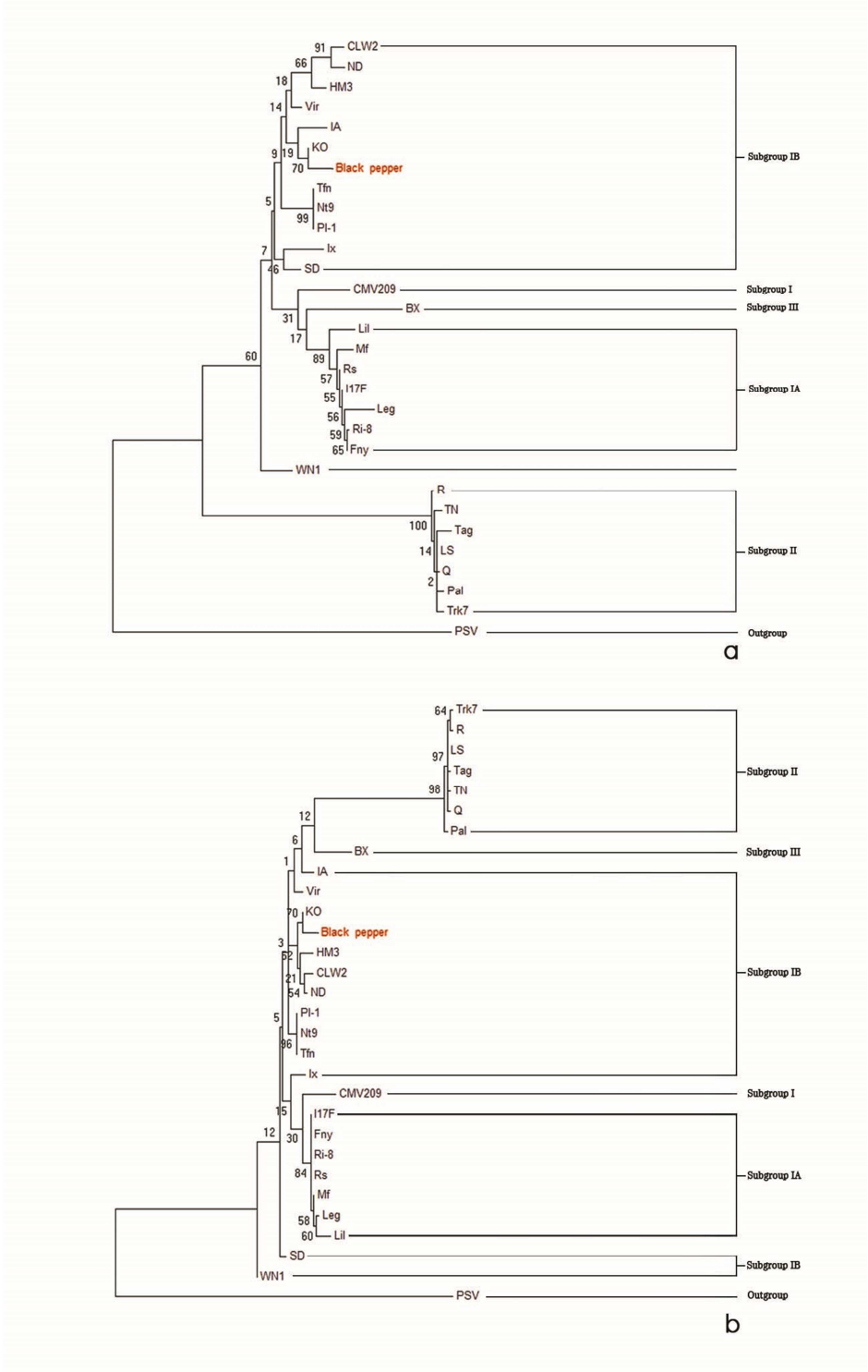

Figure 4.14 Phylogenetic trees of 2b gene of black pepper isolate of *Cucumber mosaic virus*, (a) at nucleotide level and (b) at the deduced amino acid level constructed using the maximum likelihood method in Mega 6.06 with 1000 bootstrap replicates (Jukes–Cantor model was selected for the nucleotide tree and Poisson model for amino acid tree).

Figure 4.15 Phylogenetic trees of 3a gene of black pepper isolate of *Cucumber mosaic virus* (a) at the nucleotide level and (b)at the deduced amino acid level, constructed using the maximum likelihood method in Mega 6.06 with 1000 bootstrap replicates (Jukes–Cantor model was selected for the nucleotide tree and Poisson model for amino acid tree).

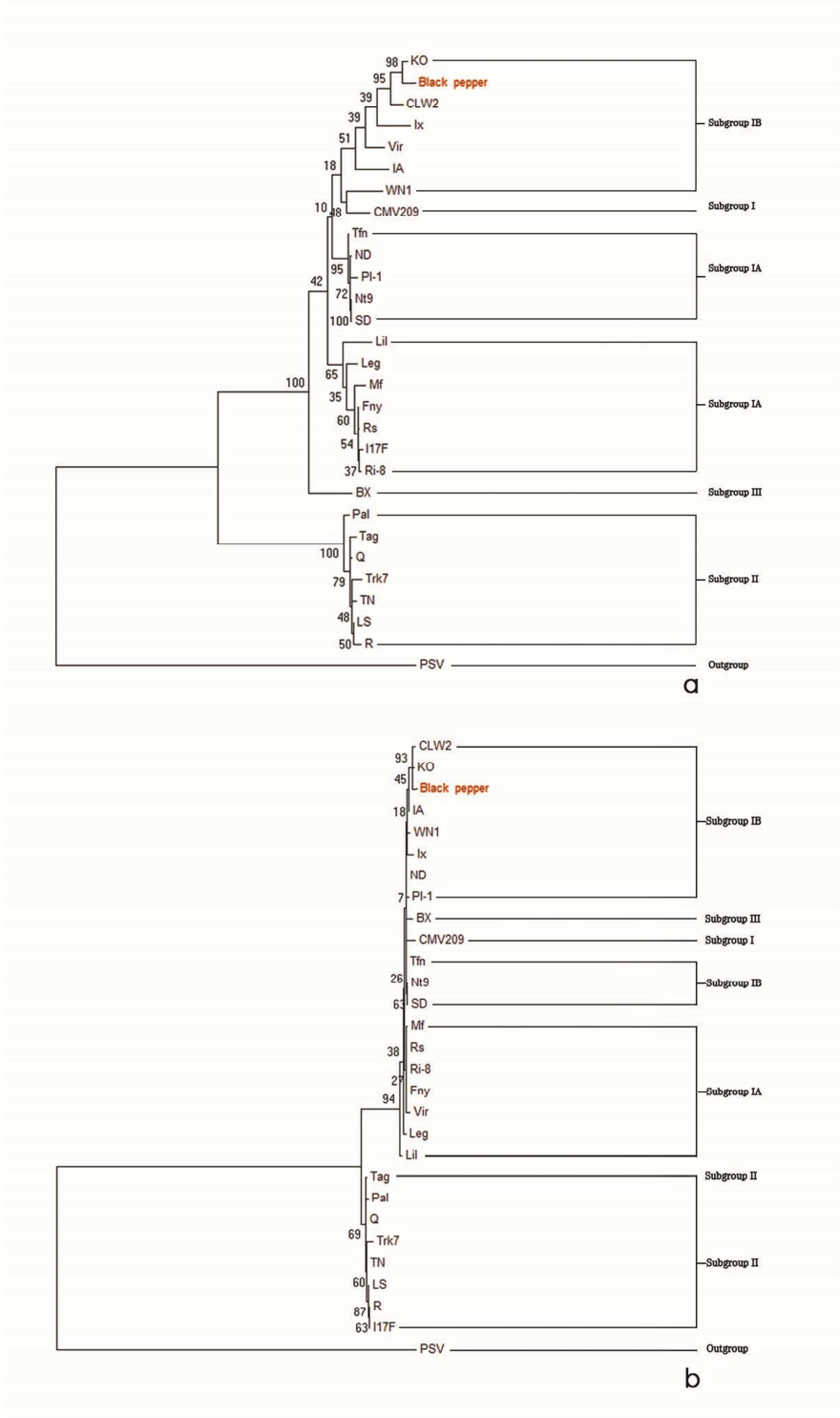

Figure 4.16 Phylogenetic trees of 3b gene of black pepper isolate of *Cucumber mosaic virus* (a) at the nucleotide level and (b) at the deduced amino acid level, constructed using the maximum likelihood method in Mega 6.06 with 1000 bootstrap replicates (Jukes–Cantor model was chosen for nucleotide tree and Poisson model for amino acid tree).

Table 4.6 Summary of nucleotide diversity studies of five genes of black pepper isolate of *Cucumber mosaic virus* with other isolates of subgroup I and II

Genes	n	S	Hd	π	nS	πS	nN	πN	ω
1a	2966	1107	1.0000	0.13643	637	0.25970	2311	0.13041	0.502
2a	2513	1086	1.0000	0.15187	576	0.366	1920	0.13651	0.372
2b	297	175	0.997	0.20663	67	0.57077	218	0.20276	0.36
3a	819	412	1.0000	0.12972	183	0.324	600	0.10554	0.33
3b	654	229	0.997	0.12255	168	0.4999	480	0.05913	0.12

n–no: of sites,
S–no: segregating sites,
π–nucleotide diversity
nS–no: of synonymous sites,
πS–synonymous substitution
nN–no: of nonsynonymous sites ,
πN–nonsynonymous substitution
ω–Ratio of πN/πS

4.2 Designing of siRNAs

The 1a, 2a, 2b, 3a and 3b open reading frame (ORF) sequences of black pepper isolate of CMV, were subjected to dsCheck (http://dsCheck.RNA.jp/) and the specific region that yields dsRNA, for each gene was selected. A 400 bp region was selected to minimize the off–targets, for all the five genes of black pepper isolate of CMV except the 2b gene where the complete 336 bp region was selected for dsRNA synthesis. Analyses of these regions by dsCheck revealed that the sequences from 2327–2702 bp of 1a gene, 600–999 bp of 2a gene, 1–337 bp of 2b gene, 92–491 bp of 3a gene and 28–427 bp of 3b gene were the regions most suitable for dsRNA synthesis (Figure 4.17). Comparison of these regions with thirteen selected CMV subgroup IB strains by DnaSP showed a sequence polymorphism of 25, 30, 32, 21 and 17 percent for 1a, 2a, 2b, 3a and 3b genes respectively (Table 4.7). Analysis by the 'siDesign' revealed many siRNAs from all the five genes of black pepper CMV which were uncommon among the different isolates, except the four designed from the 3b gene (Table 4.8). These common siRNAs can target all the subgroup IB strains used in the study (Figure 4.18).

1a (2328-2727 bp)

AGTGTTGGTTGATGAGGTTGTGTTGTTACACTTTGGTCAATTATGTGCCGTTATGTCAAAATTGAAGGCCGTGAGAG
CCATATGTTTCGGGGATTCAGAGCAAATTGCCTTTTCCTCTCGAGATGCCTCGTTTGACATGCGTTTCTCTAAGATC
ATTCCTGATGAAACTAGTGACGCGGACACCACATTCCGTAGCCCACAAGATGTTGTGCCGCTTGTGCGTTTAATGGC
TACGAAGGCCCTTCCGAAAGGAACTCATTCAAAATATACCAGATGGGTTTCTCAATCTAAAGTGAAGAGATCTGTTA
CATCTCGTGCCGTCGCAAGTGTGACTCTGGTTGACCTGGACCCTACTAGGTTTTATGTCACGATGACACAAGCTGAT
AAAGCTTCACTGATT

2a (660-1059 bp)

TGAGAGGACTTTCGCTGACGATTATGTTATTGAAGGTTTAGATGGTGTTGTTGACAATGCGACTTTGTTGTCCAATT
TAGGTCCATTCTTGGCGCCCGTGAAATGCCAATATGAGAAATGTCCAACACCAACCGTCGTGATTCCTCCGAGTTTA
AATCGTGCTACAGATCGTGTTGACACCAATTTAGTTCAATCCATTTGTGACTCGACTCTGCCCACTCATAACAACTA
CGACGACTCTTTTCATCAAGTGTTTGTCGAAAGTGCTGATTATTCTATTGATCTGGACCACGTTAGACTTCGACAGT
CTGATCTTATTGCAAAAATTCCAGATTCAGGGCATATGATACCGGTTTTGAATACCGGGAGCGGTCACAAGAGAGTA
GGTACGACGAAGGAG

2b (1-336 bp)

ATGGAATTGAACGAAGGCGCAATGACAAACGTCGAACTCCAGCTGGCTCGCATGATGGAGGTGAAGAGACAGAGACG
AAGGTCTCACAAGAAGAATCGACGGGGACGAGGTCACAAAAGTCCCAGCGAGAGGGCGCGTTCAAATCTCAGACTGT
TCCGTTTTCTACCATTTTTTCAGATAGATGGTTCGGAACTGGTAGGGATGTACCACCGTGCGCGCGCGGTGGAGCTG
TCCGAGTCTGAGGCCCCTTGTTTTCCGTTACCAGCGGAAGAAGACCATGATTTTGACGATACGGATTGGTTTGCTGG
CAACGAATGGGCGGAAGGCGCGTTTTGA

3a (172-571 bp)

CTCGTTCCCGAAGTAACCCATGGTCGTATTGCTTCCTTCTTTAAGTCTGGATATGACGTCGGTGAATTGTGCTCTAA
GGGATACATGAGCGTTCCTCAAGTGTTGTGTGCTGTTACTCGAACAGTTTCTACCGATGCTGAGGGATCTTTGAGAA
TTTACCTGGCTGATCTAGGCGACAAGGAGTTGTCTCCTATAGACGGACAATGCGTTTCGTTACATAACCATGATCTC
CCCGCTTTAGTGTCTTTCCACCCGACGTTTGACTGTCCTATGGAAATTGTTGGGAACCGTAAGCGGTGTTTTGCTGT
CGTCATCGAGAGACATGGTTACGTTGGGTATACCGGCACCACAGCAAGCGTGTGTAGTAATTGGCAAGCACGATTCT
CTTCTAAGAATAATA

3b (77-476)

AACATCTTTCGCGACTCATCAAGACGTTAGCAGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAG
CGTTGTAAACCTGGATACACGTTCACCTCGATTACCCTGAAGCCACCAAAAATAGACCAAGGGTCTTATTATGGCAA
AAGGTTGTTACTTCCTGATTCAGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTT
TGCCGAAATTTGATTCTACCGTGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTGTCCGTTTCCGCCATC
TCTGCTATGTTTGCGGACGGAGCCTCACCGGTACTGGTTTATCAGTATGCTGCATCTGGCGTTCAAGCCAACAACAA
ATTGTTGTATGATCT

Figure 4.17 Different regions of 1a, 2a, 2b, 3a and 3b genes of black pepper isolate of *Cucumber mosaic virus* chosen for dsRNA synthesis (using 'dsCheck').

Table 4.7 Region selected from different genes of black pepper isolate of *Cucumber mosaic virus* for dsRNA synthesis

Gene	Accession No:	Region Selected	Mismatch			Sequence Polymorphism (%)	Common siRNAs
			0	1	2		
1a	KU947029	2328–2726	0	1	2	25	–
2a	KU947030	660–1059	0	0	3	30	–
2b		1–337	0	3	2	32	–
3a	KU947031	172–571	0	3	2	21	–
3b		77–476	0	4	2	17	4

Table 4.8 Common siRNA and target positions in the 400 bp selected region of 3b gene of *Cucumber mosaic virus* subgroup IB strains, for dsRNA synthesis

Sense strand sequence	Start position	GC%	Score
GAGTTAATCCTTTGCCGAA	220	42	79
AGTTAATCCTTTGCCGAAA	221	37	75
GTTAATCCTTTGCCGAAAT	222	37	74
CGAGTTAATCCTTTGCCGA	219	47	68

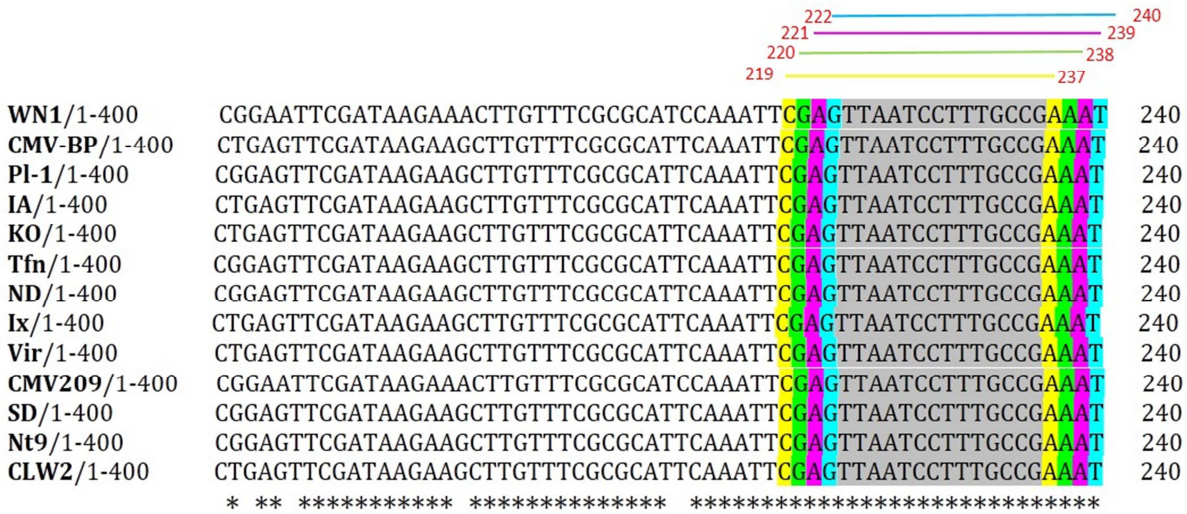

Figure 4.18 Multiple sequence alignment of the 400 bp selected region of *Cucumber mosaic virus* coat protein gene (3b) showing the common siRNAs and target positions.

4.3 Hairpin construct preparation

4.3.1 CMV 3b (coat protein) hairpin construct preparation

4.3.1.1 PCR, restriction, ligation and transformation of 3b hairpin construct in E. coli

Sequence of the CMV 3b gene and phytoene desaturase 6th intron from *Solanum lycopersicum* used for hairpin construct preparation are shown in Figure 4.19. The CMV 3b gene sense, antisense, and the intron upon amplification by PCR using the primers designed (Table 3.6), gave specific products of approx. 350, 350 and 250 bp respectively (Figure 4.20a). The sense PCR product was flanked with the restriction enzymes *Bam*H1 and *Nsi*1; the antisense product with the *Sac*1 and *Nsi*1 enzymes and the intron was flanked with the restriction enzyme *Nsi*1 on both sides. The primers were designed in such a way that the PCR products will have six base pair overhangs after the restriction enzyme sites. The PCR products were purified and approx. 500 ng of each product was subjected to restriction digestion. The 3b sense construct was sequentially restricted using the enzymes *Bam*H1 and *Nsi*1. Similarly the antisense PCR product was restricted using the enzymes *Sac*1 and *Nsi*1; the intron PCR product was restricted using the enzyme *Nsi*1. The pTZ57R/T vector was also double restricted with the enzymes *Bam*H1 and *Sac*1 and further the restricted products were run on agarose gel and purified. The restricted PCR products and the vector were of good quality as seen in the agarose gel electrophoresis under UV light in the Alpha Image analyzer. On ligation of the cloning vector pTZ57R/T with the PCR products and transformation in to *E. coli* DH5α cells, both blue and white colonies were obtained on Luria Bertani agar plate with X–gal, IPTG and ampicillin. A master plate was made by streaking the white colonies alone from blue and white colonies obtained after transformation, onto Luria Bertani agar medium with only the antibiotic ampicillin and without the X–gal and IPTG used for the blue white screening, and incubated overnight at 37°C. These colonies were then screened for the presence of the hairpin construct by colony PCR using the different sets of primers.

3b gene sequence (1-337 bp)

ATGGACAAATCTGAATCAACCAGTGCTGGTCGTAACCGTCGGCGTCGTCCGCGTCGCGGTTCCCGCTCCGCCTCCTC
CTCTGCGGATGCTACATTTAGAGTCCTGTCGCAACATCTTTCGCGACTCATCAAGACGTTAGCAGCTGGTCGTCCTA
CTATTAACCACCCAACCTTTGTGGGTAGTGAGCGTTGTAAACCTGGATACACGTTCACCTCGATTACCCTGAAGCTA
CCAAAAATAGACCAAGGGTCTTATTATGGCAAAAGGTTGTTACTTCCTGATTCAGTCACTGAGTTCATAAGAAGCTT
GTTTCGCGCATTCAAATTCAAGTTAATC

Intron sequence (227 bp)

GTGAGCTAATCACGAGTAAATTTCTCCCTCTTGTAGTTATTTTGTTAAACTTCCCTAATAAGCTGTAAAGTTGATTA
GAATTCTAAAAAAAAAAATCTGTAAAATTGATAATTCAATCACACCTATGGGACTTTACTAACCTTAAAAGAGCATAA
AAGTTCATTACTTCTTCATTGGACCTTTTGTGTGCAGCTAAAATATTAAATTCTTTGATATAATTTGCAG

2b gene sequence (1-336 bp)

ATGGAATTGAACGAAGGCGCAATGACAAACGTCGAACTCCAGCTGGCTCGCATGATGGAGGTGAAGAGACAGAGACG
AAGGTCTCACAAGAAGAATCGACGGGGACGAGGTCACAAAAGTCCCAGCGAGAGGGCGCGTTCAAATCTCAGACTGT
TCCGTTTTCTACCATTTTTTCAGATAGATGGTTCGGAACTGGTAGGGATGTACCACCGTGCGCGCGCGGTGGAGCTG
TCCGAGTCTGAGGCCCCTTGTTTTCCGTTACCAGCGGAAGAAGACCATGATTTTGACGATACGGATTGGTTTGCTGG
CAACGAATGGGCGGAAGGTGCGTTTTGA

Figure 4.19 Sequence of the *Cucumber mosaic virus* 3b gene, phytoene desaturase 6th intron of tomato and *Cucumber mosaic virus* 2b gene used for hairpin construct preparation.

4.3.1.2 Confirmation of the 3b hairpin construct in E. coli

From the master plate prepared eleven colonies were screened for the presence of the 3b hairpin construct using the intron specific primers AIB 214 / AIB 215 (Table 3.6). Among the eleven colonies screened by colony PCR using the intron specific forward and reverse primers, eight colonies showed good bands in PCR, one colony showed faint amplification and two colonies showed no amplification (Figure 4.20b). Plasmid was isolated from the fourth colony that showed good band in colony PCR and confirmed by PCR using the primer pairs AIB 190 (M13 specific reverse primer) and AIB 214 (intron specific forward primer); and a single primer AIB 1 (CMV coat protein specific forward primer). Specific products of approximately 850 bp and 900 bp respectively were obtained in PCR (Figure 4.20c). The plasmid on restriction digestion with single enzyme *Bam*H1, as well as *Sac*1 got linearised and, double restriction with both *Bam*H1 and *Sac*1 enzymes released the 1 kb hairpin construct from the vector (Figure 4.20d). The plasmid was further confirmed by sequencing.

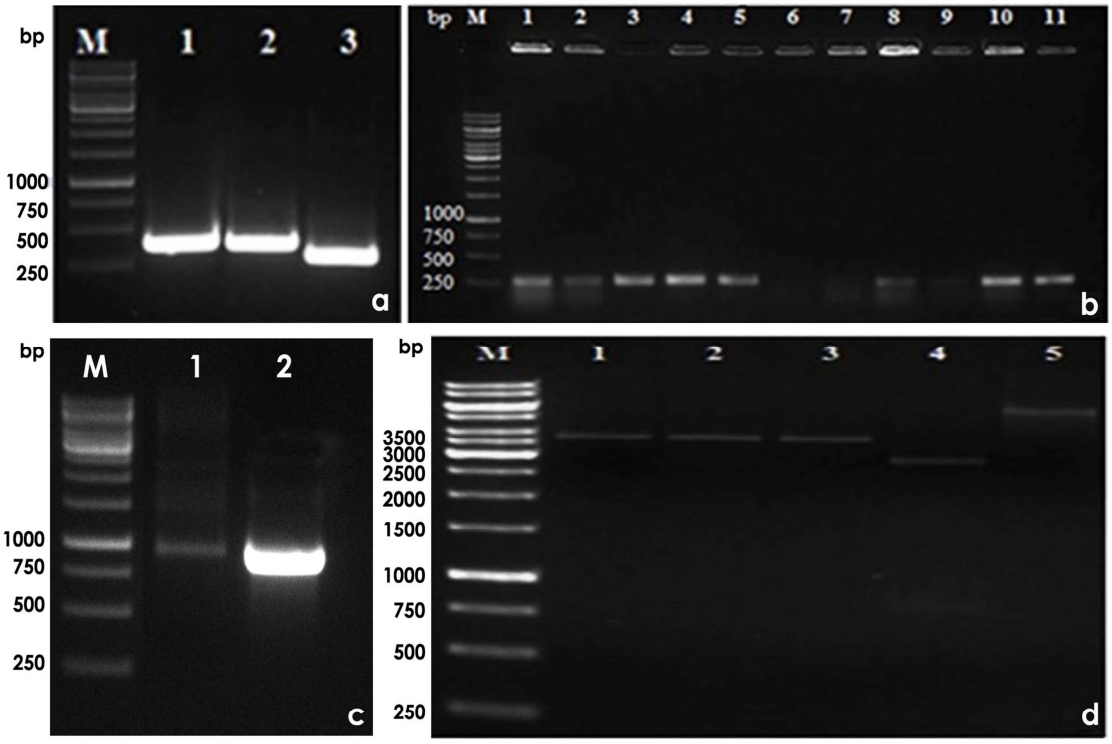

Figure 4.20 Confirmation of 3b hairpin construct in cloning vector pTZ57R/T in *E.coli*, (a) amplification of 3b sense construct using primers AIB 211 and AIB 212 (Lane 1: product size 350bp), antisense using primers AIB 212 / AIB 213 (Lane 2: product size 350bp) and intron using primers AIB 214 / AIB 215 (Lane 3: product size 240bp) from phytoene desaturase 6th intron from tomato, **(b)** colony PCR using intron primers AIB 214 / AIB 215 to screen the transformed colonies; Lane 1–11 represents different colonies, **(c)** PCR of plasmid isolated from positive colonies using AIB 190 / AIB 214 giving approx. 850 bp product (Lane 2) and, a single primer PCR using AIB1 giving 800 bp product (Lane 3), **(d)** Restriction analyses of hairpin construct where Lane1, 2 and 3 represents single restriction of plasmid using *Bam*H1, *Sac*1 and *Nsi*1 respectively. Lane 4: double restriction of plasmid using *Bam*H1 and *Sac*1 releasing the 1 kb hairpin construct and Lane 5: unrestricted plasmid; Lane M: 1 kb ladder. Details of primers are given in Table 3.6.

4.3.1.3 Cloning of the 3b hairpin construct in to the binary vector and mobilization to Agrobacterium

The recombinant pTZ57R/T vector harboring the 3b hairpin construct, isolated from the transformed *E. coli* DH5α cells was double restricted using the enzymes *Bam*H1 and *Sac*1 that released the 1kb hairpin construct from the recombinant vector (Figure 4.21a). The binary vector pBI121 was also double restricted with the same enzymes that released the GUS gene of 2 kb (Figure

4.21a). The hairpin construct purified from the double restricted pTZ57R/T vector was ligated with double restricted pBI121 vector, using T_4 DNA ligase. The ligated product was transformed into *E. coli* DH5α using the InstaTA cloning kit and colonies were screened by PCR using the intron specific primers AIB 267 / AIB 268 which gave a product of approximately 250 bp (Figure 4.21b), and the intron and promoter specific primers AIB 267 / AIB 108 respectively, that gave a specific product of approximately 800 bp size (Figure 4.21c). The recombinant plasmid was isolated from the selected colonies and confirmed by PCR using four set of primers (Details of primers are given in Table 3.10 and Table 3.6). On amplification with the intron specific primers AIB 214 / AIB 215, approximately 250 bp product was obtained (Figure 4.21d, Lane 1); on amplification with AIB 267 / AIB 108, approximately 800 bp product was obtained (Figure 4.21d, Lane 2), on amplification of the plasmid with the primers AIB 268 / AIB 269 (intron specific forward and nos terminator specific reverse), approximately 900 bp product was obtained (Figure 4.21d, Lane 3) and finally, on amplification with the intron specific primers AIB 267 / AIB 268 (primers without flanking restriction enzymes), approximately 250 bp product was obtained (Figure 4.21d, Lane 4). Double restriction of the plasmid with *Bam*H1 and *Sac*1 released the complete1 kb hairpin construct from the vector (Figure 4.21e, Lane 4).

Transformation of *Agrobacterium* strain EHA105 with the recombinant plasmid by triparental mating yielded colonies on 48 h of incubation. These were then streaked onto YEB agar media with the antibiotics kanamycin and rifampicin at a concentration of 50 µg/mL, and incubated for 24–36 h. Colonies were screened from this master plate using AIB 267 / AIB 268 and AIB 267 / AIB 108 (Table 3.10) which gave products of size approx. 250 bp and 800 bp respectively (same as shown in Figure 4.21b and c) PCR using the *Agrobacterium* specific primers, AIB 121 / AIB 122 and AIB 258 / AIB 259 gave products of size 948 bp and 898 bp respectively (Figure 4.21f and g). Plasmid from positive *Agrobacterium* colony was confirmed by PCR using the same set of primers used for confirmation of the 3b hairpin construct in pBI121 in *E. coli* and, similar results were obtained (Figure 4.21d).

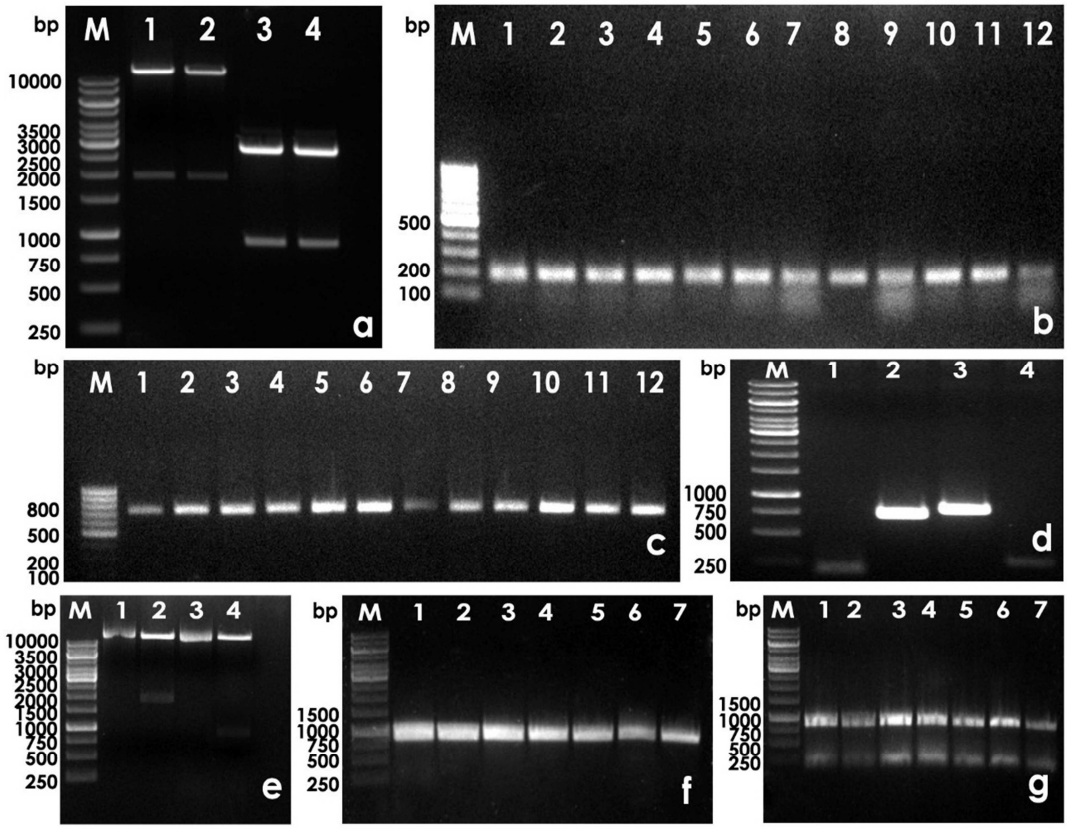

Figure 4.21 Confirmation of 3b hairpin construct in binary vector pBI121 in *E. coli* and *Agrobacterium,* (a) double restriction of pBI121 using the enzymes *Bam*H1 and *Sac*1 releasing the 2 kb GUS gene (Lanes 2 and 3) and, double restriction of pTZ57R/T vector with *Bam*H1 and *Sac*1 releasing the 1 kb hairpin construct, **(b)** colony PCR using intron primers AIB 267 / AIB 268 giving approx. 240 bp product, **(c)** colony PCR using AIB 267 / AIB 108 giving an 800 bp product; Lane 1–7 represents different colonies, **(d)** PCR confirmation of transformed plasmid; Lane 1: PCR using primers AIB 214 / AIB 215 giving approx. 240 bp product, Lane 2: PCR using primers AIB 267 / AIB 108 giving 800 bp product, Lane 3: PCR using primers AIB 268 / AIB 269 giving 900 bp product and Lane 4: PCR using the primers AIB 267 / AIB 268, **(e)** restriction analyses of hairpin construct; Lane1: unrestricted pBI121 plasmid, Lane 2: pBI121 double restricted with *Bam*H1 and *Sac*1 releasing the 2 kb GUS gene, Lane 3: transformed pBI121 unrestricted and Lane 4: transformed plasmid double restricted using *Bam*H1 and *Sac*1 releasing the 1 kb hairpin construct **(f)** and **(g)** PCR done for confirmation of 3b hairpin construct in *Agrobacterium* where **(f)** show the amplification of different colonies using the primers AIB 121 / AIB 122 giving 900 bp product and **(g)** shows the amplification of colonies using the primers AIB 258 / AIB 259 giving 898 bp product. Lane M: 1kb ladder, Lane 1–12: different colonies. Details of primers are given in Table 3.10 and Table 3.6.

4.3.2 Hairpin construct preparation using 2b gene

Initially, 2b gene was amplified from CMV infected black pepper leaves using gene specific primers, cloned and sequenced and the clones on colony PCR using the 2b gene specific primers AIB183 and AIB 184 gave product of approx. 420 bp (Figure 4.22a). The 2b gene comprised of 336 nucleotides (Figure 4.19) potentially coding for 111 amino acids (Figure 4.7). From this clone of the 2b gene, the sense and antisense constructs of the 2b gene were amplified with flanking restriction enzyme sites and overhangs. Phytoene desaturase, 6th intron of size 224 bp from *Solanum lycopersicum* was inserted between the 336 bp sense and antisense strands of 2b gene initially in pBSK vector. This was then mobilized into the binary vector pBI121 and finally transferred to *Agrobacterium tumefaciens* strain EHA105. The confirmation of the construct assembled at ICAR–Indian Institute of Spices research was confirmed by PCR, restriction digestion and sequencing.

4.3.2.1 Confirmation of 2b hairpin construct by PCR and restriction digestion

PCR of the plasmid pBI121 carrying the 2b hairpin construct in both *E. coli* and *Agrobacterium* using the intron primers AIB 214 / AIB 215 amplified the 250 bp intron (Figure 4.22b, Lane1), PCR performed using the primers AIB 267 / AIB 108 gave a product of approx. 800 bp (Figure 4.22b, Lane 2) and PCR of the plasmid using the primers AIB 268 / AIB 269 amplified a region of approx. 850 bp (Figure 4.22b, Lane 3). Double restriction of hairpin construct using the enzymes *Bam*H1 and *Sac*1 released the 1 kb hairpin construct (Figure 4.22c). This construct was named as pBI121CMV2bHP.

4.3.3 Sequencing of the recombinant plasmids in *E. coli* and *Agrobacterium*

Recombinant plasmid pBI121 having the 3b hairpin construct was isolated from transformed *E. coli and Agrobacterium* and confirmed by sequencing. Sequencing showed that the 3b hairpin construct is 922 bp with the 224 bp intron inserted between the 336 bp sense and antisense gene of 3b (coat protein). The intron was present in the antisense orientation. The restriction

enzymes *Bam*H1 and *Sac*1 flank the construct on both sides, the intron is flanked by the *Nsi1* restriction enzyme sites (Figure 4.23a). The construct was named as pBI121CMV3bHP. On sequencing of the recombinant plasmid with 2b hairpin construct, the total hairpin construct was 922 bp with the 336 bp sense and antisense construct of complete 2b gene sandwiching the 224 bp intron present in sense orientation (Figure 4.23b). The 2b hairpin construct is also flanked by the restriction enzyme sites *Bam*H1 and *Sac*1 similar to that in pBI121CMVCPHP but the intron is flanked by the *Hind*III restriction enzyme sites on both sides whereas in case of pBI121CMV3bHP the enzyme *Nsi*1 flanked the intron.

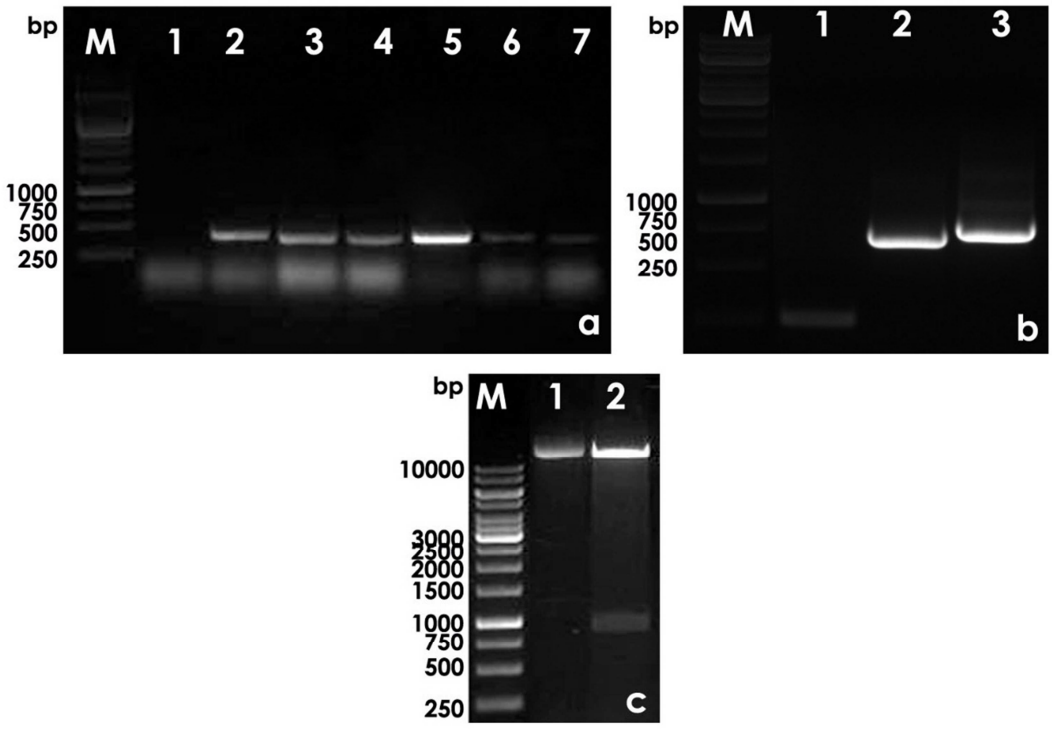

Figure 4.22 Amplification of 2b gene and confirmation of 2b hairpin construct in binary vector pBI121, (a) screening of the colonies transformed with the 2b gene using the 2b specific primers, AIB183 / AIB 184 giving approx. 420 bp product, **(b)** Confirmation of the 2b hairpin construct in the binary vector pBI121 in both *E. coli* and *Agrobacterium* using different primer sets; Lane 1: PCR of the plasmid using the intron primers AIB 214 / AIB 215 amplifying the 250 bp intron, Lane 2: PCR of the plasmid using the primers AIB 267 / AIB 108 giving a product of approx. 800 bp and Lane 3: PCR of the plasmid using the primers AIB 268 and AIB 269 amplifying approx. 850 bp product, (c) restriction analyses of hairpin construct; Lane1: unrestricted plasmid and Lane 2: double restriction of plasmid using *BamH*1 and *Sac*1 releasing the 1 kb hairpin construct. Details of primers used for confirmation are given in Table 3.6 and Table 3.10.

3b Hairpin construct

ggatccATGGACAAATCTGAATCAACCAGTGCTGGTCGTAACCGTCGGCGTCGTCCGCGTCGCGGTTCCCGCTCCGCCTCCTCCTCTGCGGAT
GCTACATTTAGAGTCCTGTCGCAACATCTTTCGCGACTCATCAAGACGTTAGCAGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGT
AGTGAGCGTTGTAAACCTGGATACACGTTCACCTCGATTACCCTGAAGCTACCAAAAATAGACCAAGGGTCTTATTATGGCAAAAGGTTGTTA
CTTCCTGATTCAGTCACTGAGTTCATAAGAAGCTTGTTTCGCGCATTCAAATTCAAGTTAATCatgcatGTGAGCTAATCACGAGTAAATTTC
TCCCTCTTGTAGTTATTTTGTTAAACTTCCCTAATAAGCTGTAAAGTTGATTAGAATTCTAAAAAAAAAAATCTGTAAAATTGATAATTCAATC
ACACCTATGGGACTTTACTAACCTTAAAAGAGCATAAAAGTTCATTACTTCTTCATTGGACCTTTTGTGTGCAGCTAAAATATTAAATTCTTT
GATATAATTTGCAGatgcatGATTAACTCGAATTTGAATGCGCGAAACAAGCTTCTTATCGAACTCAGTGACTGAATCAGGAAGTAACAACCT
TTTGCCATAATAAGACCCTTGGTCTATTTTTGGTAGCTTCAGGGTAATCGAGGTGAACGTGTATCCAGGTTTACAACGCTCACTACCCACAAA
GGTTGGGTGGTTAATAGTAGGACGACCAGCTGCTAACGTCTTGATGAGTCGCGAAAGATGTTGCGACAGGACTCTAAATGTAGCATCCGCACA
GGAGGAGGCGGAGCGGGAACCGCGACGCGGACTACCCCGACGGTTACGACCAGCACTGGTTGATTTAGATTAGTCCCTgagctc

2b Hairpin construct

ggatccATGGAATTGAACGAAGGCGCAATGACAAACGTCGAACTCCAGCTGGCTCGCATGATGGAGGTGAAGAGACAGAGACGAAGGTCTCCA
AGAAGAATCGACGGGGACGAGGTCACAAAAGTCCCAGCGAGAGGGCGCGTTCAAATCTCAGACTGTTCCGTTTTCTACCATTTTTTCAGATAG
ATGGTTCGGAACTGGTAGGGATGTACCACCGTGCGCGCGCGGTGGAGCTGTCCGAGTCTGAGGCCCCTTGTTTTCCGTTACCAGCGGAAGAAG
ACCATGATTTTGACGATACGGATTGGTTTGCTGGCAACGAATGGGCGGAAGGTGCGTTTTGAaagcttCTGCAAATTATATCAAAGAATTTAA
TATTTTAGCTGCACACAAAAGGTCCAATGAAGAAGTAATGAAGTTTTATGCTCTTTTAAGGTTAGTAAAGTCCCATAGGTGTGATTGACTTAT
CAATTTTACAGATTTTTTTTTTAGAATTCTAATCAACTTTACAGCTTATTAGGGAAGTTTAACAAAATAACTACAAGAGGGAGAAATTTACTC
GTGATTAGCTCACaagcttTTCAAAACGCACCTTCCGCCCATTCGTTGCCAGCAAACCAATCCGTATCGTCAAAATCATGGTCTTCTTCCGCT
GGTAACGGAAAACAAGGGGCCTCAGACTCGGACAGCTCCACCGCGCGCGCACGGTGGTACATCCCTACCAGTTCCGAACCATCTATCTGAAAA
AATGGTAGAAAACGGAACAGTCTGAGATTTGAACGCGCCCTCTCGCTGGGACTTTTGTGACCTCGTCCCCGTCGATTCTTCTTGTGAGACCTT
CGTCTCTGTCTCTTCACCTCCATCATGCGAGCCAGCTGGAGTTCGACGTTTGTCATTGCGCCTTCGTTCAATTCCATgagctc

Figure 4.23 Sequence of *Cucumber mosaic virus* 3b and 2b hairpin construct in in *E. coli* and *Agrobacterium.* The 224 bp intron is inserted between the 336 bp sense and antisense gene of 3b / 2b making the total size of the construct 922 bp including the restriction enzyme sites. ggatcc is *Bam*H1 site, aagctt is the *Hind*III site, gagctc is the *Sac*1 site and atgcat is the *Nsi*I site.

4.4 Production of transgenic lines of black pepper using the constructs developed

4.4.1 Somatic embryo production

The different stages of somatic embryo production of three varieties are shown in Figure 4.24. Germination of the seeds were seen in all the three varieties within two weeks (Figure 4.24a). Out of 1400 Panniyur–1 seeds placed 700 germinated and out of 1100 seeds each of Sreekara and IISR–Thevam, 550 and 770 seeds got germinated respectively. Thus germination percentage was highest in IISR–Thevam (70%) followed by Panniyur–1 and Sreekara (each 50%). Primary somatic embryos emerged from the germinated seedlings in 45–75 days in the same media in all three varieties (Figure 4.24b). Percentage of primary somatic embryo production was highest in Sreekara (60%) followed by

IISR–Thevam and Panniyur (each 50%). The primary somatic embryo clumps in different stages from free globular to torpedo shape were carefully detached and inoculated again on SH full strength medium free of hormones with lesser concentration of sucrose (1.0%) and gelled with 0.8% agar and incubated in complete darkness to promote secondary embyogenesis. Secondary embryos were seen proliferating from the brownish yellow tissue at the root pole of the primary ones in 45–60 days in all the varieties (Figure 4.24c). Cyclic secondary somatic embryos (embryogenic mass) were obtained from the variety Sreekara in the full strength SH medium free of hormones with 1% sucrose and 0.8% agar and is maintained by subculturing into fresh medium of same composition (SH medium with 1.0% sucrose) and cyclic somatic embryos of Panniyur–1 and IISR–Thevam were obtained by repeated subculturing in SH media with sucrose concentration of 0.75% and maintained by subculturing at an interval of 15 days (Figure 4.24d). These were transformed using *Agrobacterium* having the hairpin constructs pBICMV2bHP and pBICMV3bHP.

4.4.2 *Agrobacterium* mediated transformation and regeneration

The cyclic somatic embryos of IISR–Thevam and Sreekara were used for *Agrobacterium* mediated transformation with the EHA105 cells harboring the pBI121CMV2bHP construct and with the EHA105 cells harboring the construct pBI121CMV2bHP, in different experiments (Jiby and Bhat, 2011). A total of 25 transformation events were performed using each hairpin construct prepared and each variety. For each transformation experiment, approximately one gram cyclic somatic embryos were used. The embryogenic mass after co–cultivation produced some growing points within 15–30 days in the SH medium with 0.8% agar, 30% sucrose and selection agent kanamycin added at a concentration of 25 µg/mL, and the antibiotic cefotaxime added at a concentration of 100 µg/mL to control the growth of *Agrobacterium*. From approximately 1 g of the embryogenic mass used for transformation, four to six growing points were observed in both the varieties.

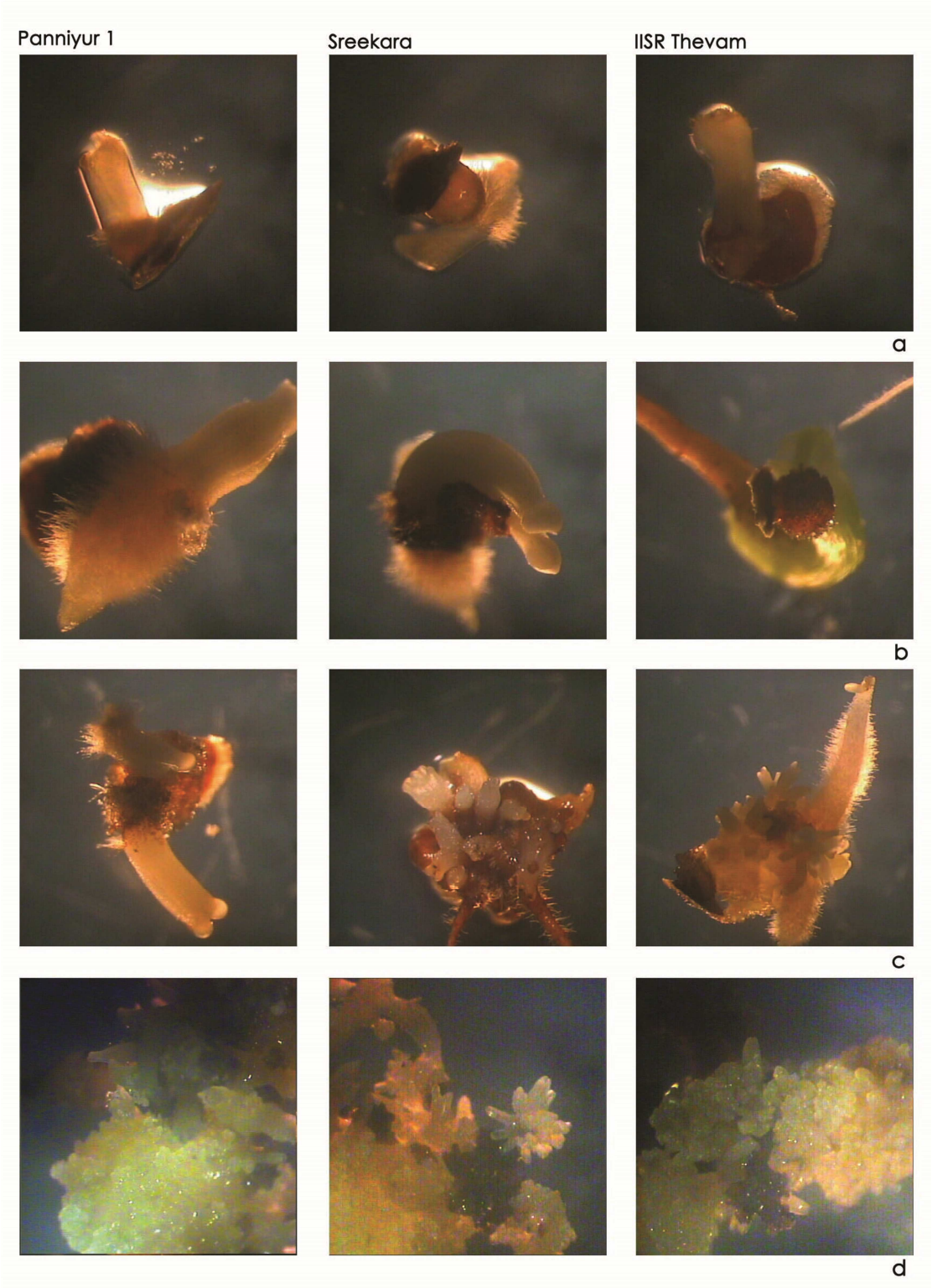

Figure 4.24 Different stages of somatic embryo production of three varieties of black pepper as seen under the stereomicroscope, (a) germinating seedlings, **(b)** primary embryos emerging from the germinated seedlings, **(c)** secondary embryos and, **(d)** cyclic somatic embryos.

These growing points were excised and incubated in the SH medium with 30% sucrose, with kanamycin (50 µg/mL) and 100 µg/mL cefotaxime, in dark for 15–30 days. The proliferated embryos obtained were transferred to SH liquid medium with 3% sucrose and cefotaxime (100 µg/mL) without kanamycin. The regenerated plantlets of the variety of IISR–Thevam and Sreekara of both the hairpin constructs were then maintained in Woody plant medium (WPM) for better root formation. A total of 26 plantlets of pBI121CMV2bHP transformants including both varieties were transferred to and maintained in WPM. Similarly 24 pBI121CMV3bHP transformants including both varieties were transferred to the WPM. The different stages of the *Agrobacterium* mediated transformation and regeneration process of IISR–Thevam and Sreekara are shown in Figure 4.25. A total of 100 control, non–transformed plantlets of variety IISR Thevam and Sreekara produced from 1 g cyclic somatic embryos of each variety were also maintained in the SH–30 solid medium without any hormones or antibiotics (Figure 4.26). These plantlets were taken as the non–transformed control for the screening of the transformants. Details of transformation events and the plantlets regenerated are given in Table 4.9.

Table 4.9 Details of transformation experiments carried out in somatic embryos of black pepper

Constructs	Variety	No: of transformation experiments performed	No: of growing points observed	No: of cotyledonary stage embryos produced in selection medium	No: of regenerated plantlets survived in liquid medium	No; of plantlets transferred to woody plant medium
pBI121CMV2bHP	IISR–Thevam	25	125	150	44	14
	Sreekara	25	100	110	30	12
pBI121CMV3bHP	IISR–Thevam	25	119	130	32	12
	Sreekara	25	80	90	25	12
Control plants	IISR – Thevam					50
	Sreekara					50

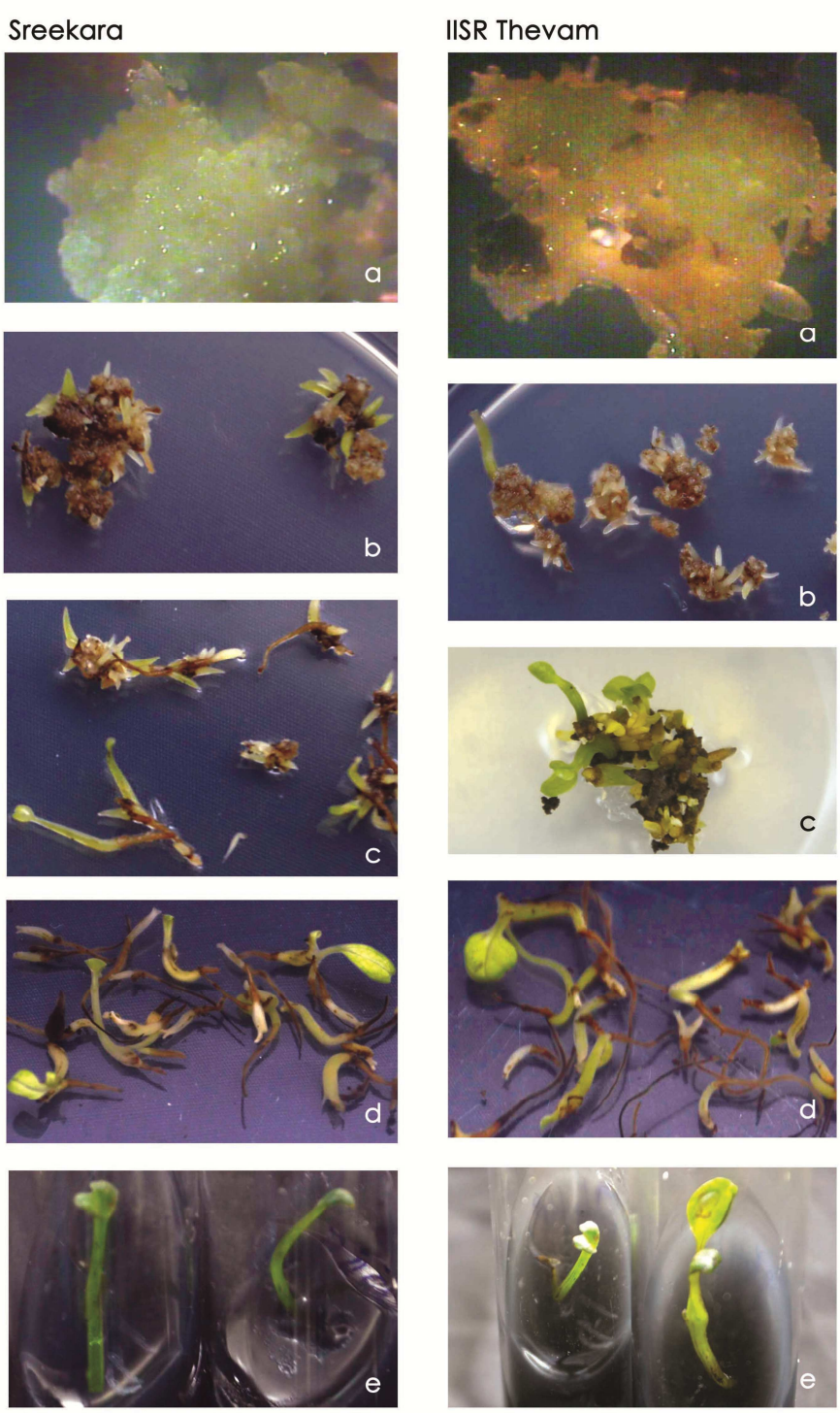

Figure 4.25 Transformation and regeneration of plantlets from embryogenic mass of Sreekara and IISR–Thevam transformed using the pBI121CMV2bHP construct, (a) co–cultured embryogenic mass in SH medium, **(b)** growing points in the selection medium, **(c)** plantlets emerging from the growing points, **(d)** plantlets in liquid medium and **(e)** plantlets in Woody plant medium.

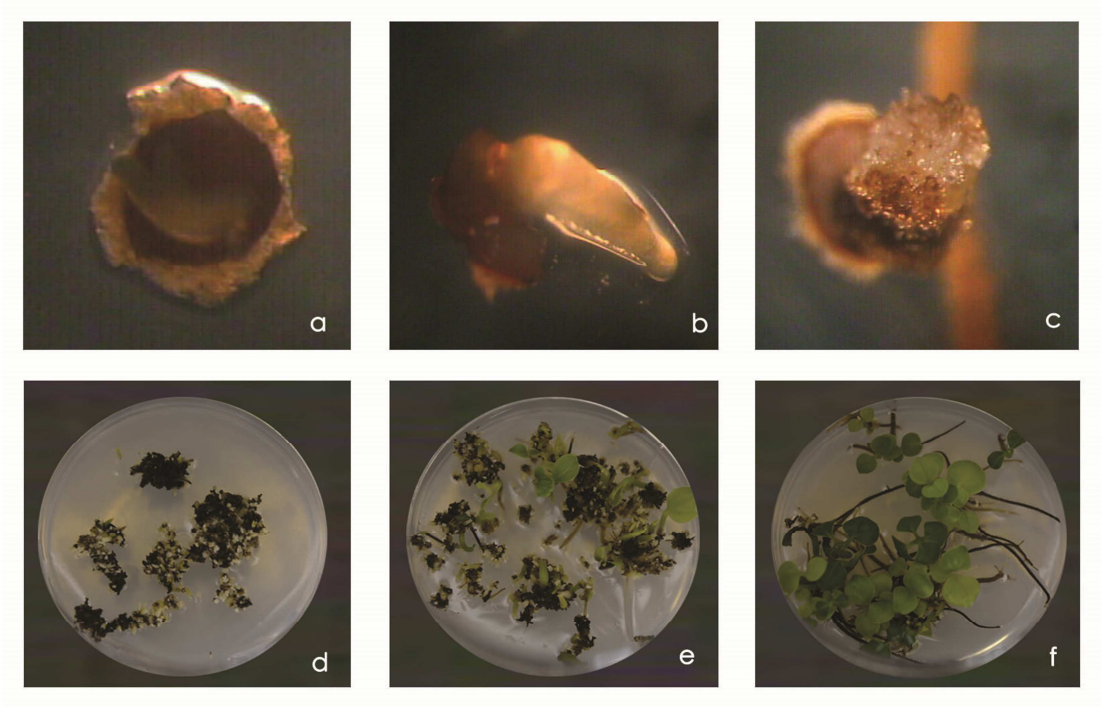

Figure 4.26 Different stages of somatic embryo production and regeneration of IISR–Thevam plantlets used as non–transformed control, (a) inoculated seed, **(b)** germinating embryo, **(c)** somatic embryo stage, **(d)** subcultured embryogenic mass, **(e)** plantlets emerging from the embryogenic mass and, **(f)** plantlets at two leaf stage with developed roots.

4.4.3 Screening of putative transformants

4.4.3.1 DNA PCR

Total genomic DNA was extracted from 50 mg of putative transformed plantlets from the selection media using the protocol of Hareesh and Bhat, (2008). PCR of the pBI121CMV2bHP transformants of IISR–Thevam and Sreekara plantlets were done using two sets of primers. One set was the AIB 108 and AIB 267 that amplified the region from the 3' end of the intron to the CaMV 35S promoter giving an amplicon of approximately 800 bp size. The other set was the AIB 268 / AIB 269 that amplified the region from 5' end of intron to the nos terminator region amplifying a region of approx. 750 bp size. All the ten plantlets of both varieties on PCR using the primers AIB 267 / AIB 108 gave expected amplicon of 750 bp (Figure 4.27a and b). However, only the Sreekara plants gave specific expected amplicons of approx. 750 bp using the primer sets AIB 268 / AIB 269 (Figure 4.27d). There was non–specific amplification with

double bands in the IISR–Thevam plantletss screened by these primers (Figure 4.27c)

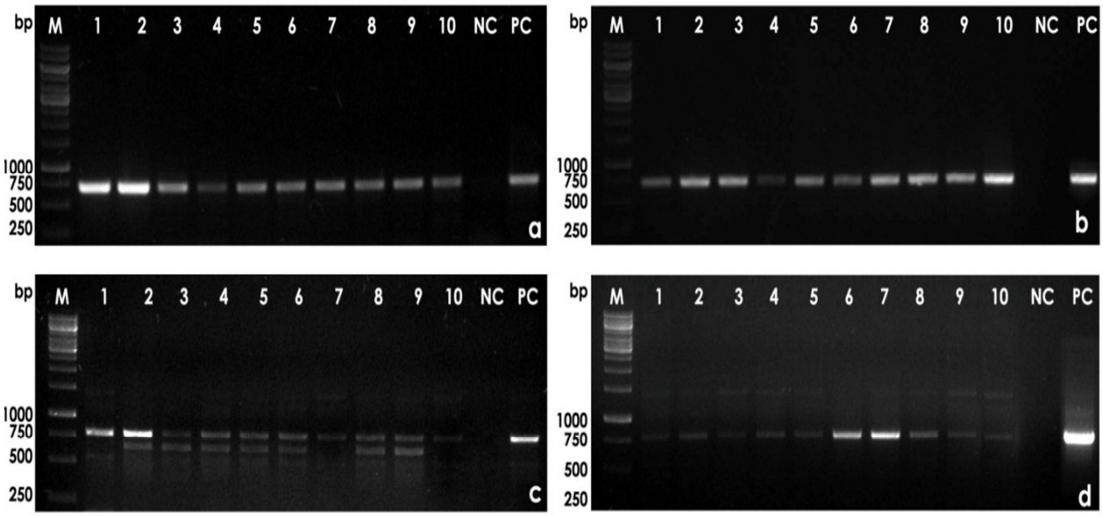

Figure 4.27 Screening of the pBI121CMV2bHP transformants by DNA PCR, (a) DNA PCR of IISR–Thevam transformed plantlets by the primers AIB 108 / AIB 267 giving approx. 750 bp product, **(b)** DNA PCR of Sreekara plantlets using the primers AIB 108 / AIB 267 giving approx. 750 bp product, **(c)** DNA PCR of the same IISR–Thevam plantlets using the primers AIB 268 / AIB 269 giving approx. 800 bp and, **(d)** DNA PCR of the Sreekara plantlets using the primers AIB 268 / AIB 269 giving an expected amplicon of approx. 800 bp. Lane M: 1 kb ladder, Lane 1–10: transformed plantlets, Lane NC: Non–transformed control and Lane PC: Plasmid pBI121CMV2bHP from transformed *E. coli*. Details of primers are given in Table 3.10 and the sketch of location of primers is shown in Figure 3.7.

Similarly the pBI121CMV3bHP putative transformants of both varieties were screened by PCR using two sets of primers. However, the AIB 268 / AIB 269 primers that gave non–specific amplification for pBI121CMV2bHP transformants of IISR–Thevam variety, was replaced by kanamycin specific primers (AIB 119 / AIB 120). Out of the ten plantlets of IISR–Thevam variety transformed with pBI121CMV3bHP, four plantlets gave strong bands and two plants showed faint amplification (Figure 4.28a). Amongst the ten plantlets of pBI121CMV3bHP transformants of Sreekara variety, six showed faint amplification (Figure 4.28b). When the kanamycin specific primers were used, though amplification was there in most of the plantlets in the two varieties of both the constructs, non–specific amplification in the non–transformed control was seen (Figure 4.28c and d).

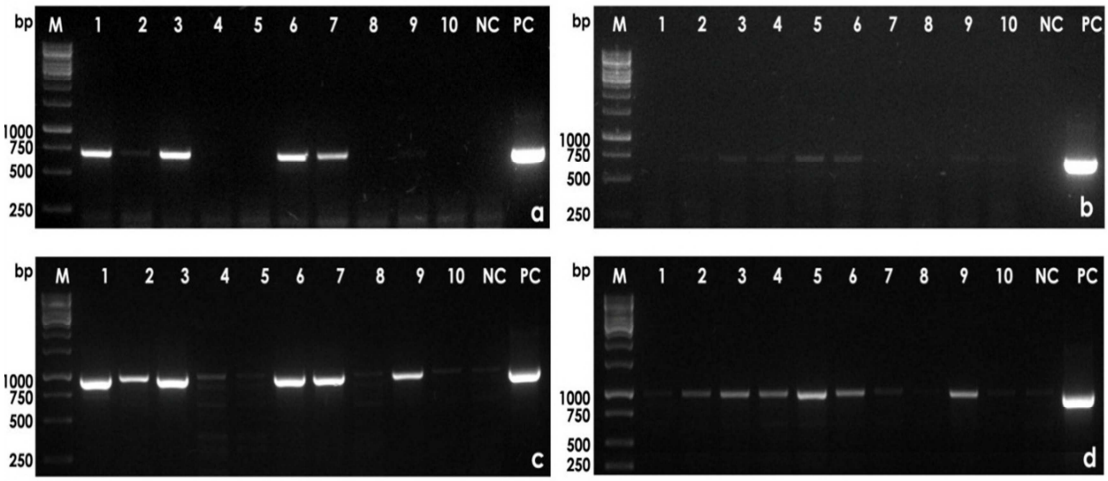

Figure 4.28 Screening of the pBI121CMV3bHP transformants by DNA PCR, (a) DNA PCR of IISR–Thevam transformed plantlets by the primers AIB 108/ AIB 267 giving approx. 750 bp product, **(b)** DNA PCR of Sreekara plantlets using the primers AIB 108 / AIB 267 giving approx. 750 bp product, **(c)** DNA PCR of the same IISR–Thevam plantlets using the primers AIB 119 / AIB 120 giving approx. 1000 bp and**, (d)** DNA PCR of the Sreekara plantlets using the primers AIB 119 / AIB 120 giving an expected amplicon of approx. 1000 bp. Details of primers are given in Table 3.10 and the sketch of location of primers is shown in Figure 3.7. Lane M: 1 kb ladder, Lane 1–10: transformed plantlets, Lane NC: Non–transformed control and Lane PC: Plasmid pBI121CMV2bHP from transformed *E. coli*. Details of primers are given in Table 3.10 and the sketch of location of primers is shown in Figure 3.7.

4.4.3.2 RT–PCR

Two sets of primers were used for RT–PCR of total RNA extracted from five plantlets of each variety transformed using pBI121CMV2bHP/ pBI121CMV3bHP constructs. The first set was the AIB 267/ AIB 108 and the second set was kanamycin specific primers, AIB 119/ AIB 120. None of the IISR–Thevam plantlets of both constructs got amplified in any of the RT–PCR experiments (Figure 4.29 and Figure 4.30; a and c). In case of Sreekara plantlets, one pBI121CMV2bHP transformant amplified with both the primer sets (Figure 4.29b and d) and three pBI121CMV3bHP plantlets amplified in RT–PCR using both sets of primers (Figure 4.30b and d). Unlike the non–specific amplification seen in DNA PCR with kanamycin specific primers, specific product of approx. 1 kb was obtained in RT–PCR using these primers.

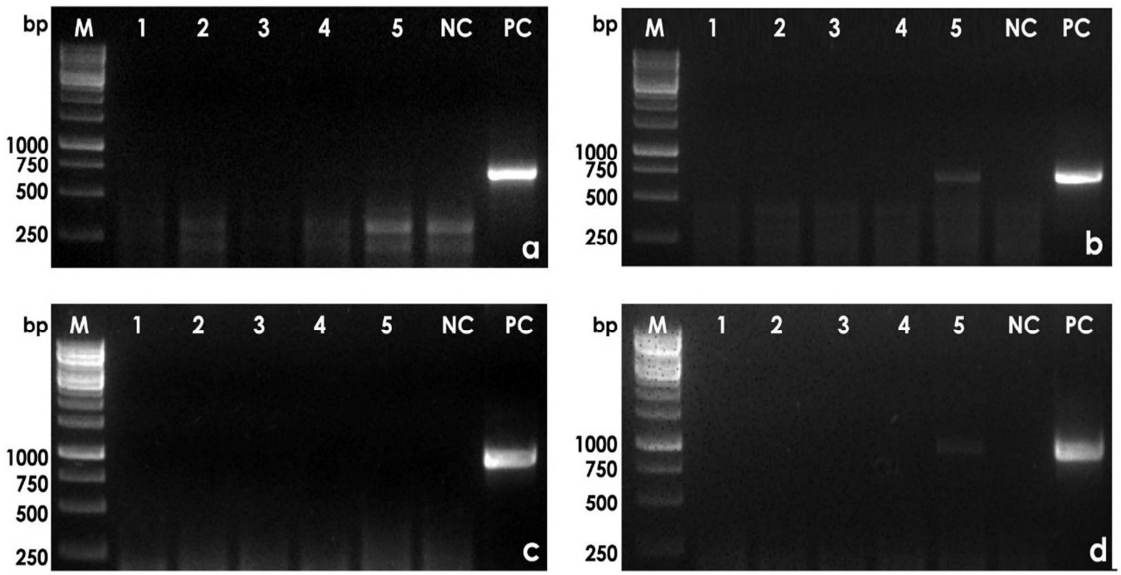

Figure 4.29 Screening of the pBI121CMV2bHP transformants by RT–PCR, (a) RT–PCR of IISR–Thevam transformed plantlets by the primers AIB 108 / AIB 267, **(b)** RT–PCR of Sreekara plantlets using the primers AIB 108 / AIB 267 giving approx. 750 bp product in the fifth lane alone, **(c)** RT–PCR of the same IISR–Thevam plantlets using the primers AIB 119 / AIB 120 giving approx. 800 bp amplicon in the PC and, **(d)** RT–PCR of the Sreekara plantlets using the primers AIB 119 / AIB 120 giving an expected amplicon of approx. 1000 bp. Lane M: 1 kb ladder, Lane 1–10: transformed plantlets, Lane NC: Non–transformed control and Lane PC: Plasmid pBI121CMV2bHP from transformed *E. coli* (for PCR control). Details of primers are given in Table 3.10 and the sketch of location of primers is shown in Figure 3.7.

4.5 Development of cleft grafting method for challenge inoculation of CMV in black pepper

4.5.1 Transmission efficiency of CMV through cleft grafting in black pepper

The stock plants for grafting were selected from the replicated cuttings of the mother plants screened by RT–PCR using the CMV CP specific primers AIB 1 and AIB 2 (Figure 4.1). On testing the stock plants for cleft grafting using these primers, 650 bp product was obtained in all the plants. Out of 30 grafts performed using healthy scion and infected stock, 22 grafts survived showing a success of 73%. In the case of self–grafts success was 76% (out of 30 self–grafts, 23 survived). Good graft union was observed in all the successful grafts and callus tissue was seen around the graft union in 4–6 weeks (Figure 4.31a).

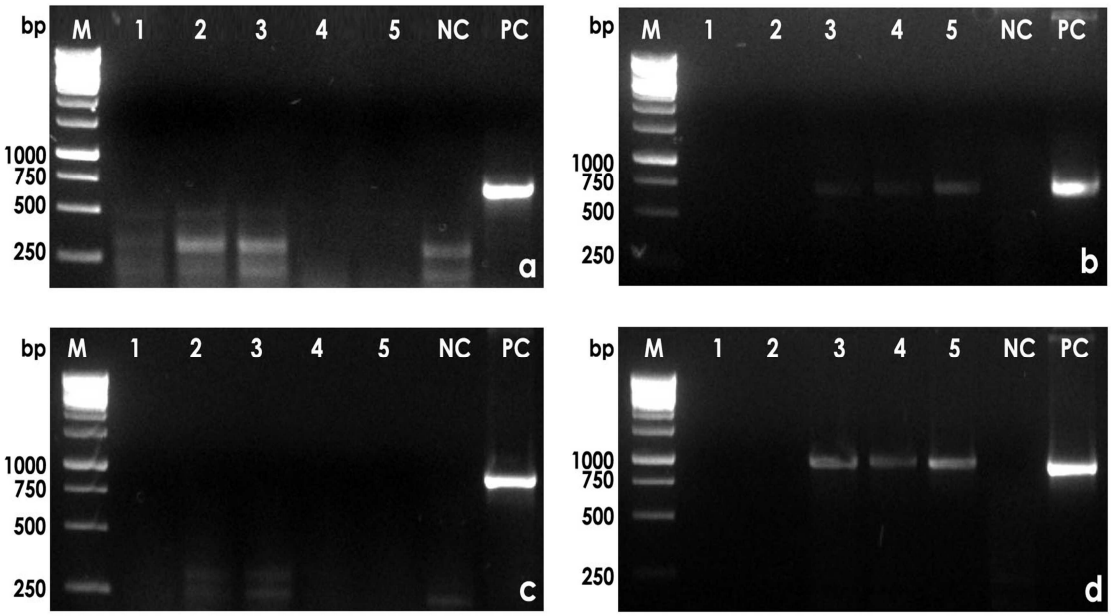

Figure 4.30 Screening of the pBI121CMV3bHP transformants by RT–PCR, (a) RT–PCR of IISR–Thevam transformed plantlets by the primers AIB 108 / AIB 267, **(b)** RT–PCR of Sreekara plantlets using the primers AIB 108 / AIB 267 giving approx. 750 bp product in the third, fourth and fifth lanes, **(c)** RT–PCR of the same IISR–Thevam plantlets using the primers AIB 119 / AIB 120 and, **(d)** RT–PCR of the Sreekara plantlets using the primers AIB 119 / AIB 120 giving an expected amplicon of approx. 1000 bp in Lanes 3, 4 and 5. Lane M: 1 kb ladder, Lane 1–10: transformed plantlets, Lane NC: Non–transformed control and Lane PC: Plasmid pBI121CMV2bHP from transformed *E. coli* (for PCR control). Details of primers are given in Table 3.10 and the sketch of location of primers is shown in Figure 3.7.

Among grafted plants with infected stock, two plants showed mild symptoms, two plants showed moderate symptoms while the remaining plants showed severe symptoms (Figure 4.31e). The symptoms appeared similar to that observed in the plants naturally infected with CMV. Though there was difference in the timing and progression of symptoms, all the plants with CMV infected stock developed mild to severe symptoms, 4–6 weeks after grafting (Figure 4.32).

Transmission of CMV to the scion plants was tested by RT–PCR and RT–qPCR in the total RNA isolated from young leaves that newly emerged from the scions of the grafted plants (Figure 4.31b and c). All the 22 control plants tested (with infected stock and healthy scion) were positive in RT–PCR using AIB 183

and AIB 184 (2b gene specific primers), amplifying approximately the 420 bp region of RNA2 and the primers AIB 243 / AIB 279 amplifying the 750 bp region of RNA1 of CMV (RT–PCR of twelve plants shown in Figure 4.33a and b) and the Ct value was found to be 10 to 17 in RT–qPCR (Figure 4.33c). Positive control (naturally infected plant) gave 420 and 750 bp with corresponding primers in the RT–PCR and the Ct value of the positive control in RT–qPCR was found to be 14. Negative control did not amplify in RT–PCR or RT–qPCR. Thus 100% transmission of CMV through cleft grafting in black pepper was noted.

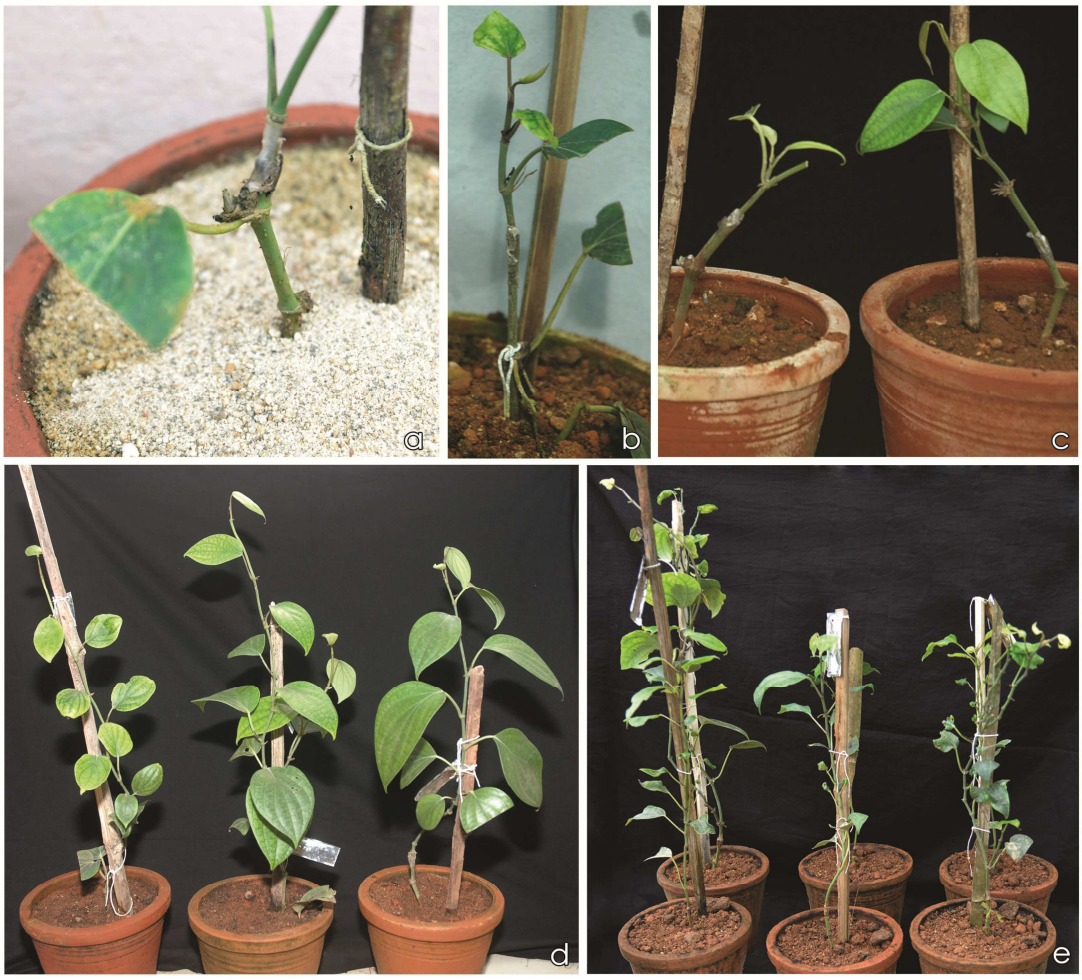

Figure 4.31 Different stages of cleft grafting in black pepper, (a) union of stock and scion, of a grafted plant, **(b)** and **(c)** grafted plants showing new leaves coming from the scion, **(d)** transgenic grafts with transgenic scion and *Cucumber mosaic virus* infected stock and, **(e)** non–transgenic control grafts with healthy scion and *Cucumber mosaic virus* infected stock.

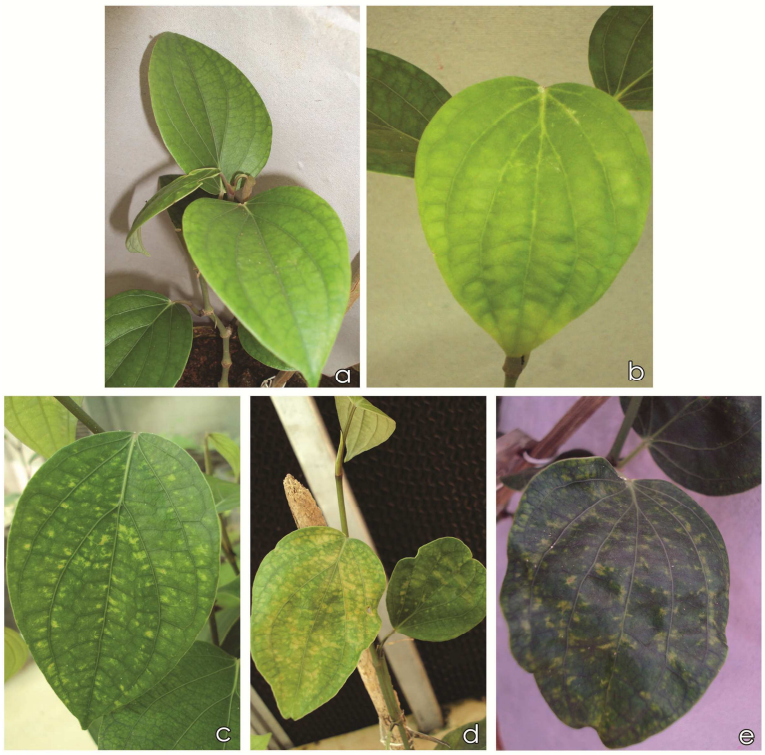

Figure 4.32 Different symptoms observed in the grafted (challenge inoculated) plants, (a) symptomless, **(b)** mild, **(c)** moderate, **(d)** severe and, **(e)** wild non–grafted.

4.5.2 Challenge inoculation of transgenic plants by cleft grafting and screening for presence of CMV

Transgenic black pepper plants carrying the CP of CMV in the sense orientation, available at ICAR–IISR, were grafted onto CMV infected stocks. Out of 24 transgenic grafts performed, 18 survived indicating 75% success of grafting. Among the transgenic grafted plants, two plants showed mild symptoms (T2 and T16); three showed moderate symptoms (T3, T7, T12) and the remaining did not show any symptoms of infection (Table 4.10). In the case of eight transgenic lines tested, except one transgenic line (CS121; replicates–T12 and T13) all others were found to be negative in RT–PCR using 2b specific primers (Table 3.6). The Ct values of tested positive plants in RT–qPCR were 12 and 14 (replicates of CS 121) and all other lines showed high Ct values indicating very low viral load (Table 4.10). Only one transgenic line (both replicates) gave specific product of 120 bp when the products of RT–qPCR were loaded on gel, while other transgenic lines did not yield any product (Figure 4.34). The summary of success of grafting and transmission of CMV through cleft grafting is given in Table 4.11.

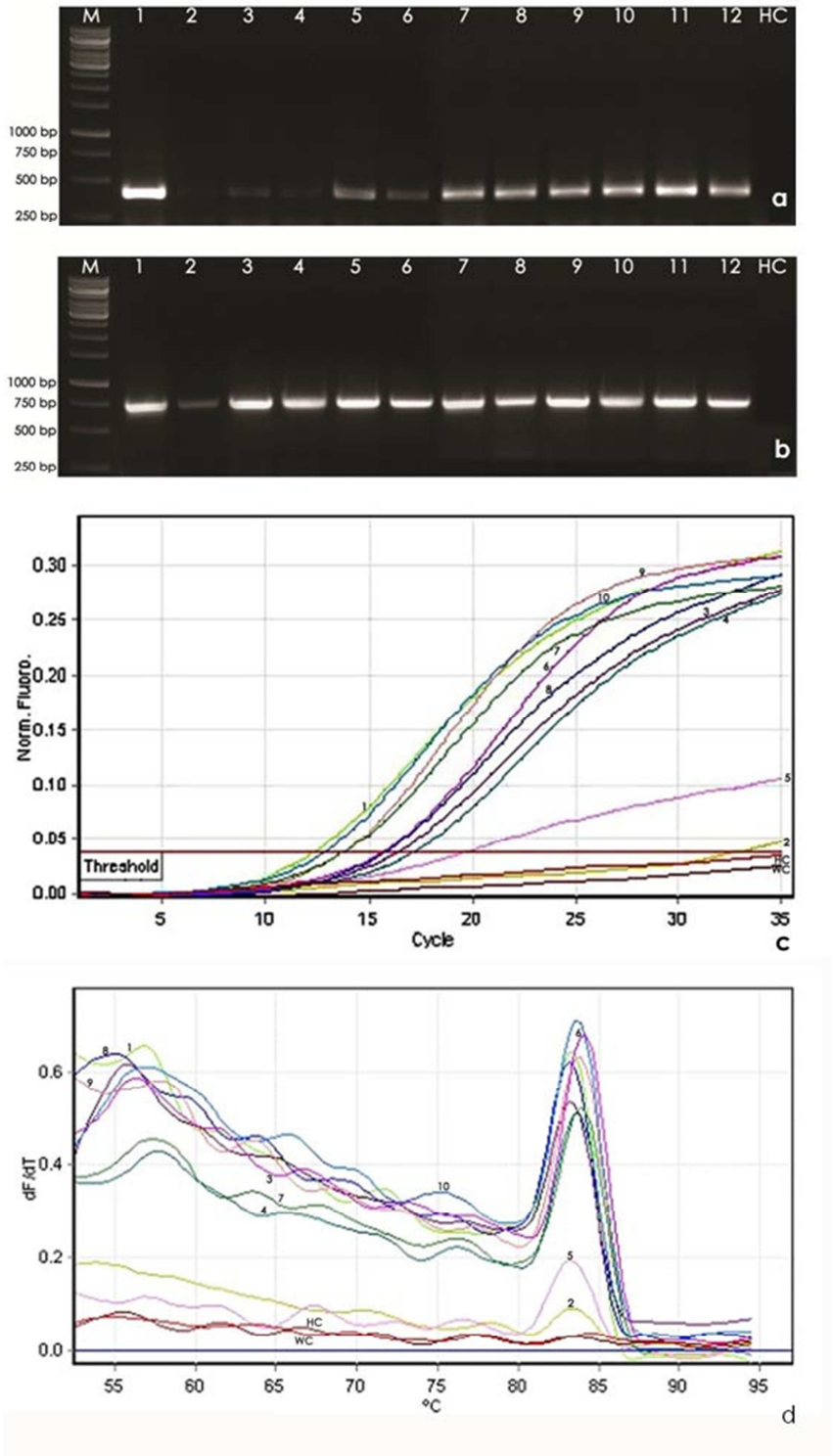

Figure 4.33 Testing of non–transgenic grafted (challenge inoculated) plants for presence of *Cucumber mosaic virus* by RT–PCR and RT–qPCR (a) RT–PCR using AIB 183 / AIB 184 amplifying the 420 bp region of RNA2 of *Cucumber mosaic virus* and, **(b)** RT–PCR using AIB 243 and AIB 279 amplifying the 750 bp region of RNA1 of *Cucumber mosaic virus* and, **(c)** annealing curve of the samples obtained in RT–qPCR. Lane M: 1 kb ladder, Lane 1–12: grafted plants and Lane 13: healthy control **(d)** melting curve of the samples showing specific peaks in RT–qPCR.

Table 4.10 Screening of the transgenic and non–transgenic grafted (challenge inoculated) black pepper plants for presence of *Cucumber mosaic virus*

Non–transgenic plants	Symptom	Results		Transgenic plants	Replicates	Symptoms	Results	
		RT–PCR	**RT–qPCR (cT value)**				**RT–PCR**	**RT–qPCR (cT value)**
NT1	Severe	+	12.38	CS110	T1	Nil	–	NA
NT2	Moderate	+	10.56		T2	Mild	–	32.33
NT3	Severe	+	15.14	CS116	T3	Moderate	–	NA
NT4	Severe	+	15.79	CS117	T5	Nil	–	30.19
NT5	Severe	+	16.42		T6	Nil	–	30.62
NT6	Severe	+	19.65		T7	Moderate	–	NA
NT7	Severe	+	12.86	CS119	T8	Nil	–	27.60
NT8	Severe	+	17.19	CS120	T9	Nil	–	30.04
NT9	Severe	+	13.77		T10	Nil	–	NA
NT10	Severe	+	11.71		T11	Nil	–	NA
NT11	Mild	+	20.20	CS121	T12	Moderate	+	14.87
NT14	Severe	+	23.06		T13	Nil	+	12.16
NT15	Moderate	+	16.95	CS143	T14	Nil	–	30.51
NT16	Severe	+	17.27		T15	Nil	–	NA
NT19	Severe	+	17.62		T16	Mild	–	29.82
NT20	Severe	+	13.84	CS144	T17	Nil	–	30.96
NT21	Severe	+	16.63		T19	Nil	–	32.06
NT23	Severe	+	16.38		T20	Nil	–	30.88
NT24	Severe	+	16.74	**T: Transgenic grafts; NT: Non–transgenic grafts**				
NT25	Severe	+	12.93	**(+) indicates positive; (–) indicates negative**				
NT26	Mild	+	32.79					
NT27	Severe	+	14.32					

Table 4.11 Success of grafting and transmission of *Cucumber mosaic virus* in transgenic and non–transgenic black pepper plants

Type of grafts	No: of grafts performed	No: of grafts established	Percentage of success	Symptoms			Testing for presence of CMV using CP primers	
				Mi/Ns	Mo	Se	RT–PCR	RT–qPCR
T	24	18	75	2/13	3	–	2 (+)	2 (+)
NT	30	22	73	2/0	2	18	18 (+)	18 (+)
SG	30	23	76	–	–	–	–	–

T : transgenic grafts
NT: non–transgenic grafts (CMV infected stock and healthy scion)
SG : Self grafts
Mi: mild; Ns: non symptomatic; Mo: moderate; Se: severe

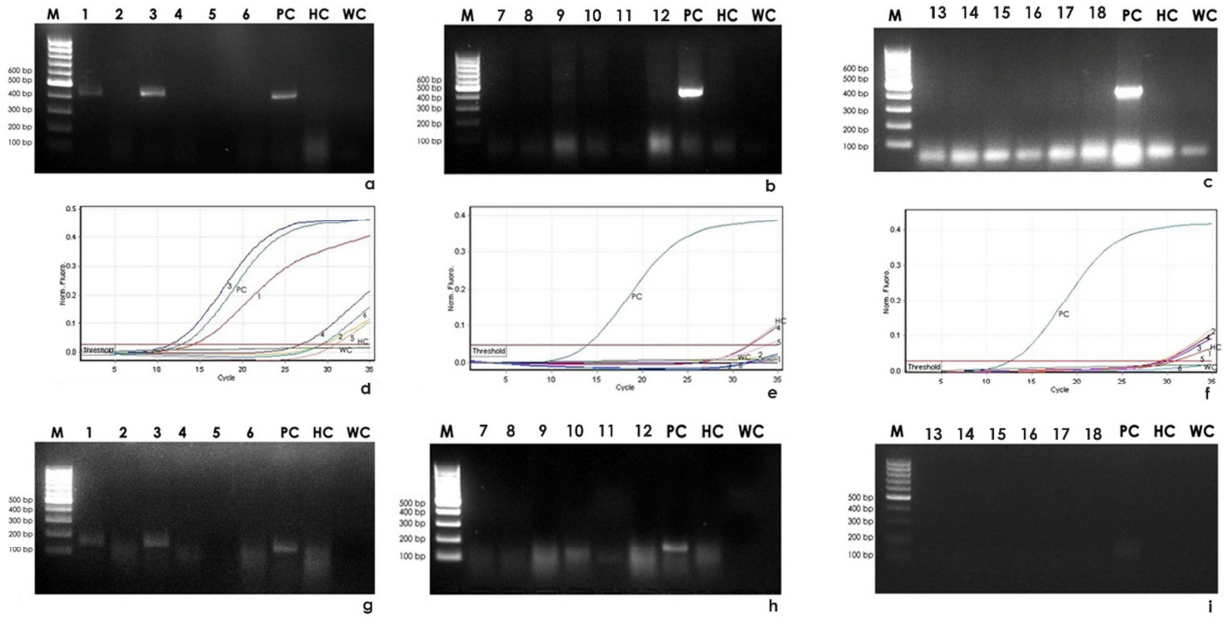

Figure 4.34 Testing of grafted (challenge inoculated) transgenic black pepper plants for presence of *Cucumber mosaic virus* by RT–PCR and RT–qPCR, (a), (b), (c) testing of grafted transgenic plants by RT–PCR using virus specific primers AIB183 / AIB184, amplifying the 420 bp region of RNA2 of CMV, **(d), (e), (f)** RT–qPCR using AIB 239 / AIB 240 and, **(g), (h), (i)** RT–qPCR products loaded in 1.5% gel. Lane M: 1 kb ladder, Lane 1–6: grafted transgenic plants (T12, T14, T13, T15, T16 and T17), Lane 7–12: grafted plants (T1, T2, T3, T5, T6 and T7), Lane 13–18: grafted transgenic plants (T8, T9, T10, T11, T18, T19 and T20) Lane PC– naturally infected plant, Lane HC–healthy control and WC–water control.

CONTENTS

DISCUSSION

Though many advances have been made in the management of plant diseases, there is 30–40% reduction in the yield of crops due to pests and pathogens of plants, threatening the food security globally (Flood, 2010). The spread of these disease causing agents to new territories as a consequence of global warming further worsens the scenario (Bebber *et al*., 2013). Among the different pathogens, viruses are one among the top five production constraints that cause diseases in almost all the crops grown for food and/or fiber (Hull, 2014). Recent reports suggest the presence of viruses, in plants presumed to be 'healthy' (Muthukumar *et al*., 2009). The crop losses caused due to the viral pathogens are highly significant especially in the developing countries that mainly sustain on agriculture, and where crop–free seasons cannot be seen (Fargette *et al*., 2006).

The highly evolving and adapting nature of the viruses, the spread of viruses and/or their vectors and the emergence of new viruses and virus diseases, makes the management strategies more difficult (Anderson *et al*., 2004; Malmstrom and Alexander, 2016). Viruses are obligate intracellular parasites that express their genetic material with the help of the plant translational machinery, and this closeness between the pathogen and plant makes the chemical methods of virus control more strenuous (Lindbo and Falk, 2017). Thus, there is an ever increasing urge for ingenious management strategies to meet the production demands and, to overcome the issues related to toxicity and resistance to pesticides.

Cucumber mosaic virus (CMV) with worldwide distribution is one among the top five threatening plant viruses, infecting more than 1200 species from 100 plant families (Scholthof *et al*., 2011). The virus causes stunting in black pepper, the most important spice crop, in India (Sarma *et al*., 2001), Brazil (Eiras *et al*., 2004) and Sri Lanka (de Silva *et al*., 2001) leading to the loss of crop yield and quality. As black pepper is mainly propagated vegetatively, spread of the virus occurs through infected stem cuttings and, insect vectors also spread the virus in a non–persistent manner. Due to its importance and the absence of resistant

genes in black pepper germplasm, engineering CMV resistance in black pepper is the best way to combat the infection and with the first successful demonstration of engineered resistance in tobacco (Abel *et al*., 1986), many virus resistant plants have been produced using similar strategies and some were even commercialized.

The discovery of RNA interference (RNAi) mediated resistance and hairpin vector constructs made the mechanism of genetic engineering more intelligible (Broderson and Voinnet, 2006). However, some proteins encoded by the viral gene sequences act as suppressors of RNAi (Kasschau and Carrington, 1998) and, it was understood that the difference in the transgene and the infecting viral RNA sequences, by more than 15–20% may lead to the failure of RNAi (Jones *et al*., 1998; Savenkov and Valkonen, 2001). Thus, a thorough study of the viral genomes and proper selection of viral transgene sequences are inevitable parts for the successful demonstration of RNAi mediated resistance mechanism in transgenic plants. Also, some crops are infected by both the subgroup I and subgroup II isolates of CMV; hence, the biggest task is to prepare a transgene construct that can provide resistance to many CMV isolates. The current investigation was pursued to analyze the molecular genetic structure of black pepper isolate of CMV and design a suitable transgene construct that can target more than one CMV isolate. Also, production of transgenic black pepper plants with proper transgene constructs was attempted. Lastly, development of a challenge inoculation method for CMV in black pepper was undertaken in this study.

5.1 Complete genome sequencing of black pepper isolate of CMV

Understanding genome of the virus is essential for devising better diagnostic and management strategies by selecting the most appropriate gene for developing pathogen derived resistance. So far, only the coat protein sequence of black pepper isolate of CMV was cloned and sequenced in India (Bhat *et al*., 2005c). This study characterized for the first time the complete genome sequence of black pepper isolate of CMV (BP) from India, and analyzed its phylogeny with other isolates reported from different parts of the world. The

complete genome sequence of BP isolate of CMV was found to be 8.615 kb (Table 4.1). In a previous study on complete genome sequencing, the total genome size of a subgroup IB CMV isolate infecting tomato in India was found to be 8.614 kb (Koundal *et al.* 2011). In another study, the total genome size of a subgroup II CMV isolate from India, infecting cucumber was found to be 8.623 kb (Kumari *et al*., 2013).

5.1.1 Multiple sequence alignment

Multiple sequence alignment of the different genes revealed a deletion of nine nucleotides in the putative methyltransferase domain of 1a gene of RNA1 (Figure 4.8) which is also reflected in the deduced amino acid sequences (Figure 4.9). This deletion was also observed in the CMV cucumber isolate from Malaysia (CLW2) and tomato isolate from China (HM3). The length of the 1a gene in these three isolates was 2973 bases whereas, in all other subgroup IB isolates considered in this study, it was 2982 bases. Interestingly, this deletion was not seen in the black pepper isolate from China. The length of the 1a gene of all the subgroup IA isolates were also found to be 2982 bases but, the length of the 1a gene in the subgroup II isolates varied from 2976–2982 bases. In addition to the role of 1a protein as a component of viral replicase (Palukaitis *et al*., 1992), it is known to possess elicitor functions for defense responses in plants (Kim *et al*., 1997; Diveki *et al*., 2004; Kang *et al*., 2012). Also, studies show that mutations in the 1a protein effect the resistance responses to CMV (Kang *et al*., 2012). On sap inoculation from black pepper to *Nicotiana tabacum* and *Chenopodium amaranticolor,* typical mosaic symptoms and chlorotic lesions were observed respectively in these host plants, which is an indication that the virus is replicating. However, whether this deletion has any functional significance is yet to be studied. No such anomalies were seen in the other genes and the length of the nucleotide and deduced amino acid sequences of all the genes were found to be similar to that of the subgroup IB isolates considered in this study.

5.1.2 Percent identity analyses

Percent identity analyses performed in the present study show that the BP isolate shared close identity with subgroup IB isolates (Table 4.2–4.5), with

the highest identity (of 95–98%) to the *Capsicum annuum* isolate from Karnataka (KO), India for all the RNAs, nucleotide and deduced amino acid sequences except for the complete RNA3, the 3a nucleotide and the deduced 3a protein. The RNA3, the 3a nucleotide and the 3a deduced amino acid sequence of the BP isolate shared highest identity (of 95–97%) to the CLW2 isolate. In the current study, analyses also show that the 3' UTR of the three RNAs were highly conserved (Figure 4.10). Similar observations were reported earlier (Roossinck, 2002; Koundal *et al*., 2011; Kumari *et al*., 2013).

The highest percent identity (95–98%) of the BP isolate towards the KO isolate was in compliance to the earlier studies where high level of conservation was reported among the CMV isolates infecting different hosts in a particular region (Bhadramurthy *et al*., 2009). High percent identity (89–98%) of the BP isolate towards CLW2 isolate showed that, these are genetically related, which might be due to the fact that CMV move between the countries or regions along with the infected materials, as reported earlier (Mazidah *et al*., 2012). However, the percent identity of the BP isolate to the black pepper isolate from China (WN1) was less (80–96%) compared to the percent of identity with the KO and CLW2 isolates. The less sequence identity between the two black pepper isolates and, more identity of the BP isolate to KO and CLW2 isolates suggests the adaptation of virus to new hosts and geographic locations, respectively. Earlier studies have reported high sequence identity among the Indian isolates of CMV (Madhubala *et al*., 2005; Srivastava and Raj, 2004). The percent identity of the BP isolate with the subgroup IA isolates was 68–96% and that with the subgroup II isolates was 51–82% (Table 4.2–4.5). Among the different genes, the 3b gene shared the highest sequence identity of 80–98% with the isolates of different subgroups and 2b gene shared the least sequence identity of 51–95%. Similar results of percent identity analyses were reported earlier (Koundal *et al*., 2011).

The percent identity analyses of the 3' UTRs show that the 3' UTR of the three RNAs of the BP isolate are highly conserved (91% identity), compared to the 5' UTR of the three RNAs (52–80%). The subdivision of the subgroup I into IA and IB is mainly done based on the 3' UTR nucleotide sequence of RNA3 of CMV (Roossinck *et al*., 1999). The high percent identity of the 3' and 5' UTR of the

three RNAs of BP isolate of CMV (of 85–97% and 82–98% respectively) to CMV subgroup IB isolates and lesser identity of the above regions of the BP isolate to subgroup IA isolates (of 86–92% and 81–92% respectively), proves that the present isolate belong to subgroup IB. Similarly, in the previous study, the ND isolate of CMV from India was classified as subgroup IB (Koundal *et al*., 2011). However, the Palampur isolate from India was classified as subgroup II based on the sequence analyses of coat protein and 3' UTR (Kumari *et al*., 2013).

5.1.3 Phylogenetic analyses

Phylogenetic analyses of all the genes of the BP isolate at the nucleotide and deduced amino acid level showed its close clustering towards subgroup IB isolates (Figure 4.11–4.16) which is in accordance with the previous study (Roossinck, 2002), where all the Asian isolates were placed in subgroup IB. This is in contrast to the study in which an Asian isolate of CMV from Palampur, India, was placed in subgroup II (Kumari *et al*., 2013), confirming the worldwide distribution of subgroup IA and II strains of CMV unlike the subgroup IB strains. In all the phylogenetic trees, at the nucleotide and deduced amino acid level, BP isolate of CMV was clustered with the KO isolate (Figure 4.11–4.16), except the 3a nucleotide and deduced 3a amino acid trees, where the BP isolate was clustered with the CLW2 isolate (Figure 4.15).

New isolates of viruses originate due to the adaptation to host plants and the environment. Specific adaptation of CMV to soybean plant was reported from Indonesia (Hong *et al*., 2003). In the present investigation, the closeness of the BP isolate to the KO isolate infecting *Capsicum annuum*, from Karnataka, India and the less sequence identity among the black pepper isolates (BP isolate and WN1 from China) suggest the strong role of geographic locations in the evolution of different isolates of the virus. Previously, based on coat protein sequence it was reported that the BP isolate belonged to subgroup I (Bhat *et al*., 2005c). Phylogenetic analysis together with percent identity analysis of the coding and the non–coding genes confirm the grouping of CMV–BP isolate (used in the study) in subgroup IB. Among the different genes of the BP isolate at nucleotide and deduced amino acid level, the 3b gene was the most conserved and 2b gene

was the most variable in percent identity and phylogenetic analysis, as reported by many earlier workers (Koundal *et al*., 2011; Roossinck, 2002).

5.1.4 Nucleotide diversity studies

The evolutionary constraints on each coding gene might be the probable reason for the apparent differences in their phylogenetic trees, the 3b tree being the most compact (Figure 4.16) and the 2b being the most variable (Figure 4.14). Thus, the evolutionary forces acting on the different genes were studied in the present investigation. Though all the genes of the current isolate showed haplotype diversity value nearly one, the highest nucleotide diversity was seen in 2b and 3b had the least (Table 4.6). The ratio of non–synonymous to synonymous substitution is consistent with the above data with 3b gene having the least but, 1a had the highest nearly 0.5 (Table 4.6), though the putative methyltransferase domain is highly conserved in 'Sindbis-like' supergroup of positive-strand RNA viruses (Mikhail *et al*., 1992). This is in contrary to a previous study, where the 1a gene had the lowest dN/dS (non–synonymous to synonymous substitution) ratio in spite of having the highest nucleotide diversity (Kim *et al*., 2014). Though all the genes of the BP isolate were undergoing negative selection, the value is greater for 1a gene indicating a possibility of 1a gene nearing a neutral evolution. This supports the study conducted previously where all the genes of CMV were undergoing negative selection and, 2b gene was reported to be undergoing weaker negative selection (Kim *et al*., 2014).

The high nature of conservation of RNA3 in the CMV isolates from different geographic locations as shown by the percent identity, phylogenetic and nucleotide diversity studies (in the present study) suggest its significance in viral RNA functions and interactions as reported earlier (Andreev *et al*., 2004), which might be due to the fact that coat protein interacts with itself or viral RNA but little with the host and, the interactions with the aphid vectors are also nonspecific and minimal (Roossinck, 2001). The role of 2b gene in host specificity might be the reason for its high variability shown in the current study.

This study thus, characterized for the first time, the complete genome sequence of black pepper isolate of CMV from India, and analyzed its phylogeny with other isolates reported worldwide. The isolate belongs to subgroup IB, has rare deletion in 1a gene and 3b gene is the most conserved among the subgroups.

5.2 Designing of siRNAs

RNAi is a natural phenomenon of RNA degradation activated by sequence specific RNAs. The mechanism is triggered by short interfering RNAs processed from the mRNAs arising from the transgenes. Length of the transgene used is very important for a successful RNAi. Though there are reports showing that sequences of 23–60 nt induces effective RNA silencing, transgenes with more than 100 nt is required for an effective RNAi and usually, 300–800 nt region is preferred for controlling plant viruses (Simon-Mateo and Garcia, 2011). In the current study, a computational online software 'dsCheck' was used to select a 400 bp region of the five genes of BP isolate suitable for dsRNA synthesis with minimum off–targets (Table 4.7). The regions with minimum off–targets were selected due to the fact that, the siRNAs synthesised from such regions will knock down only the target genes without affecting other gene functions (Jackson *et al*., 2003). This is the most important factor to be considered while designing siRNAs, as a single siRNA can target many mRNAs sharing sequence similarity (Semizarov *et al*., 2003). In a previous study based on these criteria, a 500 bp region, most suitable for dsRNA synthesis with minimal off–targets was selected, for different target genes of fruit borer (Asokan *et al*., 2012).

The silencing effect of the RNAi mechanism depends on many factors including the dsRNA concentration (Meyering–Vos and Muller, 2007; Shakesby *et al*., 2009), sequence of nucleotide selected (Araujo *et al*., 2006), and dsRNA fragment length (Mao *et al*., 2007; Saleh *et al*., 2006). Another crucial factor that limits the application of RNAi is the sequence polymorphism present in the target gene (Gordon and Waterhouse, 2007). Studies show that even a single mismatch between the siRNA and the target region of the transcript may hinder the efficiency of RNAi (Amarzguioui *et al*., 2003; Elbashir *et al*., 2002). This was supported by another study where the efficiency of designed siRNAs decreased

notably on inducing a nucleotide change between the siRNA and the mRNA target (Czauderna *et al*., 2003). Thus, the sequence variations in the 400 bp selected regions of all the genes of BP isolate of CMV were then compared with selected subgroup IB isolates available in the GenBank. Of all the genes of BP isolate of CMV, 3b gene had the lowest sequence polymorphism (of 17%) and 2b gene had the highest sequence variations (of 32 %) among the different isolates of the subgroup IB (Table 4.7). Previously in this study, nucleotide diversity analyses of the complete 1a, 2a, 2b, 3a and 3b genes of BP isolate of CMV, revealed similar pattern of sequence polymorphism with lowest nucleotide diversity seen in 3b gene (0.12255) and highest in the 2b gene (of 0.20663) (Table 4.6). The results were consistent with the earlier report where the level of gene conservation was highest in 3b gene and least in 2b gene (Roossinck, 2002). In some cases short siRNAs were found to be more effective compared to the long dsRNA region with high sequence polymorphism (Valentine *et al*., 2007). Hence, the 400 bp selected regions were further subjected to another online software system 'siDesign' to generate the siRNAs from these regions.

Some of the important aspects of designing siRNAs are the accessibility of the target mRNA to the siRNA. Any region in the mRNA that form secondary structures reduces the accessibility of the siRNAs thereby hindering the efficiency of RNAi (Shao *et al*., 2007; Tafer *et al*., 2008). The GC content of the siRNAs and the presence of palindromes, also affect the efficiency of RNAi by forming secondary structures that make the mRNA transcript inaccessible for the siRNA (Birmingham *et al*., 2007). The 'siDesign', generates all the possible siRNAs of 19 nucleotide length with their GC content and score. The siRNAs with good scores indicate that they are potential with low seed frequency, which is an indication of minimal off–targets.

On analyses of the selected regions of the five genes of BP isolate of CMV, many siRNAs were generated by this tool. However, the aim of the present study was to select the most conserved region among the different genes of black pepper isolate of CMV, most suitable for dsRNA synthesis and, generate the common siRNAs that can target the selected subgroup I isolates of CMV. Thus, four siRNAs were designed from the 3b gene with good score and minimum GC

content (Table 4.8) but no common siRNAs were designed from the other genes. All the four common siRNAs were generated from the 77–476 nucleotide region of the 3b gene from the 5' end and, these may help in the effective gene silencing by RNAi mechanism. Other than the 'siDESIGN' software, some of the other tools available for designing of potential siRNAs are the 'MysiRNA-Designer', 'Asi-Designer' and 'RNAxs' (Mysara *et al*., 2011).

5.3 Hairpin construct preparation

The RNAi has emerged as a powerful tool for engineering resistance in plants especially with the finding of hairpin transgene constructs. The basic structure of a hairpin transgene construct include an intron/spacer sandwiched between the sense and antisense strands of the transgene, inserted between a plant promoter and terminator. In the present investigation, the hairpin constructs of the 3b gene and the 2b gene were assembled in this way and, inserted in between the CaMV 35S promoter and nos terminator present in the binary vector, pBI121. Here, hairpin constructs were chosen due to the fact that, RNAi mediated resistance mechanism operated by using a single copy of the transgene in either sense or antisense orientation, as done in the starting stages of RNAi (Elmayan and Vaucheret, 1996: Waterhouse and Helliwell, 2003), resulted in unstable, delayed resistance with low efficiency (Duan *et al*., 2012). Later, it was known that multiple copies of the transgene enhanced the efficiency of RNAi to a greater extent (Swaney *et al*., 1995), especially when these were assembled in the form of a hairpin (Stam *et al*., 1998). Subsequently, the RNAi mediated by hairpin RNA was found to be highly efficient (Chuang and Meyerowitz, 2000; Smith *et al*., 2000). In both the 3b and 2b hairpin constructs, an intron (phytoene desaturase 6th intron from tomato) separated the corresponding sense and antisense strands of the 3b/2b genes (Figure 4.23). Though spacers can be used to separate the sense and antisense strands, as in the first generation hairpin vectors (Pooggin *et al*., 2003; Purushothama *et al*., 2015), here, in this study, intron was used because, an intron in the place of spacer augmented the RNAi mediated resistance as proved by many researchers (Smith *et al*., 2000; Wesley *et al*., 2001).

For both the 3b and 2b hairpin construct preparation, the binary vector pBI121 was used as the vector backbone. Among the different binary vectors available, pBI121 was the most commonly preferred, by more than 40% of the researchers working on transformation (Komori *et al*., 2007). This vector was derived from the pBIN series of vectors, and has kanamycin resistance gene that aid in the screening of transformants. In a previous transformation study, black pepper was transformed with the binary vector pBI121 *via Agrobacterium* mediated, and the transgenic plants were screened by the histochemical GUS staining (Jiby and Bhat, 2011). Another attractive feature of the pBI121 vector is that the transgene construct can be easily inserted into the vector by replacing the 2.0 kb GUS gene, by restriction of pBI121 with the enzymes *Bam*HI and *Sac*I, that make it possible to clone the desired gene using these restriction enzyme sites (Shade *et al*., 1994, Jones *et al*., 1998; Charity *et al*., 1999). Here, in the present study, both the 3b/2b hairpin constructs were inserted in the pBI121 vector by replacing the GUS gene using the restriction enzymes *Bam*HI and *Sac*I. Studies also report the use of pBI121 as the binary vector for transformation, by replacing or without replacing the GUS gene (Retheesh and Bhat, 2011; Jiby and Bhat, 2011). Though the large size (of 14758 bp) and the stringent nature of the plasmid pBI121, make the transformation work tedious, its advantageous considering the fact that high copy number plasmids may result in multiple integration in the genome, which may lead to silencing of the transgene. The large size of the plasmid also helps in avoiding the integration of sequences from the vector backbone into the plant genome (Ye *et al*., 2007).

5.3.1 Preparation of 3b hairpin construct

Here, in this study, the 3b gene (coat protein) from BP isolate of CMV was chosen for the hairpin vector construct preparation. This was because the 3b gene is the most conserved of all the CMV genes, among the subgroups, as proved by the sequence polymorphism study (accomplished in this investigation) of the complete coat protein gene as well as that of the 400 bp region selected by the 'dsCheck'. Also, the four common potential siRNAs designed from this region indicate that the hairpin construct can confer resistance to many CMV isolates from subgroup I. In a previous study, the hairpin construct of CMV CP on either

sides of the spacer of size 1445 bp was used for the transformation of tobacco and resistant lines showing the production of siRNAs were obtained (Kalantidis *et al*., 2002). The binary vector used was pART27 driven by the CaMV 35S promoter. In the same study another binary vector pATC940 was also used (Kalantidis *et al*., 2002).

In the present study, for the preparation of 3b hairpin construct, the region from 1–337 bp was chosen starting from the 5' end of 3b gene of CMV BP isolate as selected by the 'dsCheck'. However, the region from the start codon of the 3b protein was chosen to ensure that the selected gene can form a functional protein. Besides, the 'siDesign' computational tool showed that, four common and potential siRNAs will be generated from this region. Initially, the sense and antisense constructs of the selected region of 3b gene was amplified by PCR using primers flanked with the restriction enzymes, *Bam*HI and *Nsi*I for sense construct; *Sac*I and *Nsi*I for antisense construct. The intron was amplified using primers flanked with *Nsi*I. The restriction enzyme sites were followed by six base pair overhangs to facilitate the proper restriction of the PCR products. The PCR fragments were restricted with the corresponding restriction enzymes and, in a single step ligated with the cloning vector pTZ57R/T which was also double restricted with the enzymes, *BamH*I and *Sac*I. During ligation, the concentration of the intron was increased compared to that of the sense and antisense, so that the ratio of concentration of sense, intron, antisense and vector was 3:8:3:1. This was to ensure the proper ligation of the intron in between the sense and the antisense constructs.

The pTZ57R/T vector is a high copy number plasmid of size 2886 bp and ligation with the hairpin construct increased the size of the vector to approx. 3900 bp. The pTZ57R/T vector was preferred for initial cloning due to the small size and the relaxed nature of the plasmid. Further, the recombinant colonies can be easily detected by blue white screening. The ligated product was transformed into the *E. coli* DH5α cells and plated on LB agar plates with ampicillin, X–gal and IPTG and, colonies were screened by blue white screening. The recombinant nature of these cells were then confirmed by colony PCR, plasmid PCR, restriction digestion and sequencing (Figure 4.20).

After confirmation, the hairpin construct assembled in the cloning vector was restricted with *BamH*I and *Sac*I that released the hairpin fragment of approx. 1 kb size (Figure 4.21). The binary vector pBI121 was also restricted with the enzymes, *Bam*HI and *Sac*I, which released the GUS gene of 2 kb (Figure 4.21). The released hairpin fragment was ligated to the restricted binary vector and transformed into the *E. coli* DH5α cells. Here, the transformed *E. coli* DH5α cells were plated on LB agar medium with kanamycin, as the binary vector pBI121 had the kanamycin resistant gene instead of ampicillin resistant gene present in the cloning vector pTZ57R/T. Only the recombinant cells grew on the medium with kanamycin. The recombinant cells were confirmed for the presence of the hairpin construct (Figure 4.21) and was mobilized to the *Agrobacterium* strain EHA105 by triparental mating.

Among the different methods available for mobilization of a transgene construct from *E. coli* to *Agrobacterium*, triparental mating was the most preferred due to the higher transformation efficiency (Ditta *et al*., 1980). Mobilization of the gene construct into *Agrobacterium* can also be done by electroporation and freeze thaw method. In the transformation study of vanilla, the transgene construct was mobilized from *E. coli* to *Agrobacterium* using freeze thaw method (Retheesh and Bhat, 2011). However, the efficiency of transformation was very low when the freeze thaw method was used (Hofgen and Willmitzer, 1988) but comparatively higher when the electroporation method was used (Shen and Forde, 1989).

In the present study, three different strains were used for triparental mating; the DH5α cells carrying the recombinant binary plasmid, the *Agrobacterium* strain EHA105 and the helper strain pRK2014, for mobilizing the plasmid from *E. coli* to *Agrobacterium*. The *E. coli* cells had kanamycin resistance but not rifampicin resistance, the non–transformed *Agrobacterium* strain had rifampicin resistance but not kanamycin resistance and pRK2014 cells had ampicillin resistance but not kanamycin/rifampicin resistance, so only the *Agrobacterial* cells transformed with the recombinant plasmid grew on the minimal agar medium with rifampicin and kanamycin.

5.3.1.1 Confirmation of 3b hairpin construct

Confirmation of the recombinant clones of *Agrobacterium* was done by colony PCR and plasmid PCR using the same set of primers used for the confirmation of construct in *E. coli.* However, *Agrobacterium* specific primers were also used for confirmation, to ensure that *Agrobacterium* cells are selected and to avoid the chances of *E. coli* contamination with the *Agrobacterium* transformants. The colony PCR and the PCR of the plasmid from the transformed *Agrobacterium* cells gave specific products of expected band size (Figure 4.21). Upon restriction of the recombinant plasmid isolated from the transformed *Agrobacterium* cells, with the restriction enzymes *Bam*HI and *Sac*I, approx. 1 kb hairpin construct was released instead of the 2 kb GUS gene that was released from the pBI121 plasmid isolated from the non–transformed *Agrobacterial* cells. Sequencing of the 3b hairpin construct showed that the total construct was approx. 921 bp with the intron sandwiched between the sense and antisense constructs of 3b gene in the sense orientation (Figure 4.23a).

5.3.2 Preparation and confirmation of 2b hairpin construct

The low level of resistance and the absence of RNAi mediated silencing, often observed in plants transformed with CP hairpin construct, is attributed to the silencing suppressor function of 2b gene present in this *cucumovirus* (Morroni *et al*., 2008). Also, there are reports showing that the cucumoviruses and potyviruses have evolved mechanisms to suppress the kind of resistance developed by RNA interference made functional by the use of a single copy of the transgene (Brignetti *et al*., 1998; Voinnet *et al*., 1999). In a previous study, the CMV CP hairpin construct sandwiching an intron spacer from *Arabidopsis* actin2 was used for the transformation of *Nicotiana benthamiana* plants and 50% resistance was observed in the transgenic lines. However, the resistance was found to be 100% when the plants were transformed with inverted repeats of RNA2 (Chen *et al*., 2004). Hence, 2b gene was also chosen for hairpin vector construction in the present study, in order to have a better comparison of resistance in the plants transformed with these hairpin constructs. In an earlier study, the hairpin gene construct targeting the 2b gene of CMV was used for the

transformation of *Arabidopsis* and 64% resistance was observed in the transgenic plants (Qu *et al*., 2007).

In the present investigation, the 2b hairpin construct was assembled in a similar method adopted for 3b hairpin construct preparation. Unlike the 3b hairpin construct preparation, the 2b hairpin construct was initially assembled in the cloning vector pBlueScript SK(+) in between the *Bam*HI and *Sac*I restriction enzyme sites (Figure 3.9). Also, here the intron was flanked with the *Hind*III restriction enzyme sites instead of the *Nsi*I sites that flank the intron in the 3b hairpin construct. The construct was then cloned in binary vector pBI121 and mobilized to the *Agrobacterial* strain EHA105. The hairpin construct was confirmed by plasmid PCR, restriction digestion (Figure 4.22) and sequencing. The total construct was approx. 922 bp including the flanking restriction enzyme sites. In contrary to the 3b hairpin construct, the intron present in the 2bHP construct was in the antisense orientation (Figure 4.23).

Other than the 3b and the 2b genes, there are reports on the use of replicase hairpin construct for transformation of plants. In a previous study the hairpin construct of partial replicase gene of size 1138 bp was cloned in between the attL recombinant sites of a Gateway entry vector. This was then mobilized in to a binary vector pEKH2IN2 and transformed initially in to TOP10 competent cells and, kanamycin was used for the selection of recombinant cells. Similar to the confirmation of the 3b and 2b hairpin constructs done in this study, the replicase hairpin construct was confirmed by restriction with the enzyme *Eco*RI. Similar to the pBI121 used in this study, the pEKH2IN2 was also driven by the CaMV 35S but in addition to the kanamycin resistant gene, the vector had hygromycin phosphotransferase resistant gene. The *Agrobacterial* cells used were EHA105 in to which the hairpin construct assembled, was mobilized by triparental mating. This construct was then used for the *Agrobacterium* mediated transformation of precultured cotyledonary explants of tomato and high level of resistance to two different isolates of CMV (O and Y) were obtained. The resistance was correlated to the presence of siRNAs, detected before and after the challenge inoculation (Ntui *et al*., 2014).

In another study, the hairpin construct of 3' UTR was used for the *Agrobacterium* mediated transformation of tobacco. The hairpin construct was assembled by multiple cloning and ligation, first in an intermediate vector and then in the binary vector pCAMBIA–1300 driven by the CaMV 35S promoter. Finally, this was mobilized in to the *Agrobacterial* cells, EHA105 by electroporation. Delayed resistance was shown by the transgenic tobacco plants on challenge inoculation with the virus (Duan *et al*., 2008).

5.4 Production of transgenic lines of black pepper

5.4.1 Somatic embryo production

The most important step in the production of any transgenic plant is the initiation and validation of a suitable regeneration system. For the efficient regeneration of the transformants, suitable explants have to be identified, which is very crucial for successful transformation and regeneration. Many studies on transformation have focused on the use of somatic embryos as explants and have procured excellent results (Ashakiran *et al*., 2011; Vidal *et al*., 2010; Pavingerova *et al*., 2011). Recently, a protocol for transformation of cassava with high efficiency was developed using the somatic embryos as explants (Nyaboga *et al*., 2015). In case of black pepper, a transformation protocol using the somatic embryos induced from the micropylar portions of mature seeds have been successfully manifested (Nair and Dutta, 2006). In the present study, somatic embryos were developed from three varieties of black pepper, Panniyur–1, Sreekara and IISR-Thevam, using the protocol demonstrated by Nair and Dutta, (2006), with slight modification by Jiby and Bhat, (2011). All the three varieties of scooped out micropylar region, germinated within two weeks and the highest percentage of germination was seen in IISR–Thevam. Unlike germination, the highest percentage of somatic embryo production was shown by the variety Sreekara. Both the primary and secondary somatic embryos from all the three varieties emerged in 45–75 days in the SH solid medium without additional hormones. Cyclic secondary somatic embryos (embryogenic mass) were obtained from the variety Sreekara in the full strength SH medium free of hormones with 1% sucrose and 0.8% agar and was maintained by subculturing

into fresh medium of same composition (SH medium with 1.0% sucrose) and cyclic somatic embryos of Panniyur–1 and IISR–Thevam were obtained by repeated subculturing in SH media with sucrose concentration of 0.75% and maintained by subculturing at an interval of 15 days (Figure 4.24). In a previous study, the optimum concentration of sucrose for cyclisation of somatic embryos was found to be 0.7% for Panniyur–1 and 1% for both the Sreekara and IISR–Thevam varieties (Shina and Bhat, 2016). In the present study, the concentration of sucrose was further reduced from 1% to 0.75% for IISR–Thevam variety. This was to promote the formation of cyclic somatic embryos, and to prevent the regeneration of these embryos into plantlets, as the undifferentiated somatic embryos are better explants for transformation.

5.4.2 *Agrobacterium* mediated transformation

In the present study, transformation of somatic embryos of both IISR–Thevam and Sreekara varieties were carried out using the EHA105 isolates carrying the hairpin constructs pBI121CMV3b/pBI121CMV2b, following the protocol of Jiby and Bhat, (2011). For this, approx. 1g of the embryogenic mass was co–cultured with the recombinant EHA105 isolates and the transformed embryos were selected using increasing kanamycin concentration. After several rounds of selection, 50 putative transformants including both varieties transformed with each hairpin construct individually, were rooted in the Woody plant medium (Figure 4.25, Table 4.9). From the same 1g of non–transformed embryogenic mass, hundred plantlets were regenerated and were rooted in the SH full strength medium without any hormones (Figure 4.26, Table 4.9). Previously, *Agrobacterium* mediated transformation of somatic embryos of black pepper using the GUS construct resulted in the establishment of nine transformed black pepper plantlets that survived after the hardening process, from approx. 1g of embryogenic mass (Jiby and Bhat, 2011). In the same study, from approx. 1g of non–transformed somatic embryos, 200 plantlets were hardened. Thus, a significant reduction in the regeneration efficiency was noted in case of the transformed embryos compared to the non–transformed ones. The repeated use of the antibiotics in selection medium, added for controlling the *Agrobacterial* strain might be the main factor for the reduction of the number of

plantlets regenerated from the transformed somatic embryos. The infection caused by the *Agrobacterium* and the continuous use of antibiotics were reported as the prime reason for the reduction in the regeneration efficiency in the previous study (Jiby and Bhat, 2011) and in similar studies conducted earlier (Husnain *et al*., 1997; Krishna Raj *et al*., 1997; and Singh *et al*., 2002).

5.4.3 Screening of putative transformants by PCR and RT–PCR

Polymerase chain reaction (PCR) is the basic technique preferred for the screening of the transformants. This is a fast, cost–effective and equally sensitive technique used for the prefatory identification of the transgenes. Many recent reports on transgenics have initially screened the transformed plants by PCR. In the current study, transformed plantlets that survived in the selection media (SH solid medium with 50 µg/mL kanamycin and 100 µg/mL cefotaxime) were subjected to PCR and RT–PCR analysis using different sets of primers. For this 50 mg of the transformed plantlets from ten different transformation events of both the varieties transformed using both the constructs separately, were subjected to DNA extraction followed by PCR. Three sets of primers were mainly used for the screening procedure, AIB 267 / AIB 108 (promoter specific forward and intron specific reverse), AIB 268 / AIB 269 (intron specific forward and nos terminator specific reverse) and AIB 119 / AIB 120 (kanamycin specific forward and reverse primers). PCR analysis of the ten *in vitro* IISR–Thevam and Sreekara plantlets transformed using the pBI121CMV2bHP revealed the presence of the transgene in all the plantlets, tested using the AIB 267 / AIB 108 giving an expected band size of approx. 800 bp (Figure 4.27). Though all these plantlets amplified in PCR using the AIB 268 / AIB 269 primers, giving a product of expected band size of approx. 800 bp, a double band was also seen in the IISR–Thevam plantlets (Figure 4.27c). The PCR analysis of the plantlets of both the varieties transformed with the pBI121CMV3bHP constructs were done using the AIB 267 / AIB 108 and the kanamycin specific primers (AIB 119 and AIB 120). Out of the ten plantlets of IISR–Thevam screened by PCR, four plantlets gave strong bands and two plantlets showed the presence of faint bands, using the primers AIB 267 / AIB 108. Similarly, five plantlets of the variety Sreekara showed faint amplification in PCR using the same set of primers (Figure 4.28). No

amplification was seen in the negative control using these primers. Though these plantlets amplified in PCR using the kanamycin set of primers, there was faint amplification in the negative control also.

From the results of the PCR analysis of DNA, five plantlets of each variety transformed with each construct were selected and subjected to RT–PCR analysis using the AIB 267 / AIB 108 and AIB 119 / AIB 120 (kanamycin specific primers). Out of the five plantlets of each variety, IISR–Thevam and Sreekara, screened by RT–PCR using the AIB 267 / AIB 108 primers, only one Sreekara plantlet amplified with a faint band of expected size. The result was consistent when the kanamycin primers were used for the PCR (Figure 4.29b, d). In case of the plantlets of these varieties transformed with the pBI1213bHP construct, three plantlets of the Sreekara variety amplified using both these sets of primers (Figure 4.30b, d). No non–specific amplification was seen in any of these plantlets including the non–transformed control. In the previous study on transformation of black pepper, 85 % of transformed hardened plants showed specific amplification in PCR using the kanamycin and GUS specific primers (Jiby and Bhat, 2011).

Though the PCR and RT–PCR analyses in the current study indicate the presence of transgene in these plantlets, more confirmatory tests are needed, as the sensitivity and reliability of the PCR technique was not 100% infallible as shown by many researchers previously (Barik *et al*., 2005, Hiei *et al*., 1994). The *Agrobacterial* association with the transformed plantlets may result in the false signal in the PCR tests (Shekhawat *et al*., 2008). Hence, transgenicity of the plantlets can be confirmed only by advanced blotting techniques, like dot blot and Southern blot for which the plantlets need to be hardened. However, among the different sets of primers used in the current study, the primers AIB 267 / AIB 108 are specific, the other two sets gave non–specific amplification in DNA PCR. These primers along with the kanamycin specific primers can be used for RT–PCR as no non–specific amplification was seen in RT–PCR using the kanamycin specific primers (AIB 119 / AIB 120). As both the hairpin constructs have a similar structure the same set of primers can be used for the screening of the plantlets transformed using these constructs, by PCR.

5.5 Development of cleft grafting method for challenge inoculation of CMV in black pepper

A thorough understanding of the viral diseases and investigating resistance in the host, are the major challenges for a successful disease management programme. Evaluation for resistance for a particular virus involves the successful transmission of virus to the host plants. In case of black pepper, infectious clones of CMV are not available. Sap transmission from black pepper to *Chenopodium* and *Nicotiana* were found to be successful but not to black pepper, the major factors might be the woody nature of black pepper and a harder and hydrophobic leaf lamina. The presence of oxidants, phenols and other inhibitors and low viral concentrations also affect sap transmission (de Silva *et al*., 2002). Hence, cleft grafting as a means of transmission of CMV was attempted in black pepper in the present investigation.

A series of grafting experiments were performed in this study, using the CMV infected stock and healthy black pepper plants as scion (non–transgenic control). Also, self–grafts were done, where the scion excised from a particular stock plant was grafted on to the same stock at the excised position. From these experiments, the success of grafting was found to be 73% for non–transgenic controls and 76% for self–grafts. The transmission of CMV through cleft grafting was studied by testing the newly emerged leaves of the scion of the non–transgenic (infected stock and healthy scion) established grafts by RT–PCR and RT–qPCR. All the plants showed amplification in RT–PCR and RT–qPCR irrespective of the primer sets used (Figure 4.33). Thus, the transmission efficiency of CMV through cleft grafting in black pepper was found to be 100%. Another study reported 100% success in grafting and transmission of *Mungbean yellow mosaic virus* in mungbean (Akhtar and Haq, 2003). Transmission efficiency of *Cassava brown streak virus* to cassava by chip bud grafting was found to be 70–100%, in a previous study (Wagaba *et al*., 2013).

In the present study, the utility of the cleft grafting method was also tested by screening the transgenic lines of black pepper carrying the CMV coat protein sense construct, previously produced (Jiby, 2011). The scions from these transgenic lines were grafted onto CMV infected black pepper stock plants in

replicates and, presence of virus was studied in the newly emerged leaves by RT–PCR and RT–qPCR. The success of grafting in these plants was found to be 75%. On testing the newly emerged leaves of scion of the established grafts, RT–PCR showed the presence of the virus in only one transgenic line (both the replicates) among the eight transgenic lines tested (Figure 4.34a, b). The RT–qPCR results were consistent with the results of RT–PCR with only one transgenic line (both replicates) showing an annealing curve with a low cT value indicating a high viral load (Figure 4.34c).

Thus, an efficient and reliable method for transmission of CMV was developed in the present study that can be used for screening of black pepper germplasm both conventional and transgenically modified, for CMV resistance. The level of resistance in each genotype can be studied which is a necessity for any breeding programme. The method is advantageous, considering the fact that the chance for escaping infection is zero in case of grafting but not in aphid transmission due to the host vector preference mechanism. Inoculation of test plants can be done at the 4 leaf stage uniformly and can be easily handled under greenhouse conditions. Development of the disease within four to six weeks of inoculation facilitates the better study of interaction of the virus with the host plant.

Another advantage of graft transmission of viruses, is that the initial viral inoculum used for transmission of virus to a recipient plant will be high, compared to that of sap inoculation and aphid transmission, thereby ensuring the successful spreading of the virus. The only requirement for a successful virus transmission by grafting, is the connection of vascular system between the stock and the scion. Even, compatible or functional union between the stock and scion is not essential, a contact with a mechanical grafting device contaminated with the virus can transmit the disease (Goldschmidt, 2014).

In the present study, transmission of the virus was achieved by the grafting of a healthy or transgenic scion onto the virus infected stock plants and, movement of the virus from bottom of the plant to the top was studied by testing the newly emerged leaves from the scion, for the presence of virus. However, in

many transformation studies, infected scion is grafted onto a healthy rootstock, thereby studying the movement of the virus from the top to the bottom of the plant and newly emerged leaves from the rootstock were tested for the presence of virus. For example, a simple single–leaflet method of grafting has been developed for the transmission of *Tomato chlorosis virus* (ToCV) that was not sap transmissible (Lee *et al*., 2017). Though the virus can be transmitted by means of whitefly, the method was tedious involving complicated procedures. The single–leaflet grafting method consisted of grafting of a single leaflet from ToCV infected tomato onto healthy tomato seedling and 87.8% transmission of the virus from infected to the healthy seedling was noted (Lee *et al*., 2017). Similarly, in another study, 100% transmission of the *Tomato mosaic virus* (ToMV) from infected scion to healthy tomato was achieved by grafting (both cleft and leaf grafting methods) and by the sap inoculation of standard virus inoculum in phosphate buffer (Yadav and Yadav, 2017). Further, *Mungbean yellow mosaic virus* was also transmitted in a similar way from the infected scion to the healthy stock (Akhtar and Haq, 2003).

In many transgenic studies, transgenic rootstocks and infected scion was used, instead of infected stock and transgenic scion. The aim of such experiments was to study the transmission of resistant signals from the transgenic plant to the non-transgenic ones. In a study conducted previously, the non–transgenic tomato and tobacco plants were grafted onto the transgenic tobacco rootstocks which were silenced for two genes, essential for the multiplication of *Tobamovirus* and virus was introduced into the transgenic plants by inoculating the leaves of the scion. The results showed that the RNA silencing was transmitted from the stock to the scion (Ali *et al*., 2016). Thus, in the present study chances are there for the transmission of resistant signals from the transgenic scion to the CMV infected stock that may ultimately lead to the reduction of viral load in the stock plants.

SUMMARY AND CONCLUSIONS

6.1 Summary

Cucumber mosaic virus (CMV) ranks one among the top five predominant plant viruses and has emerged as a serious pathogen of black pepper during the past few decades in India and other black pepper producing countries across the world. The virus causes stunted disease in black pepper affecting the production and quality of the berries leading to the consequential drop in yield. The severity and high incidence of the disease make this the third major production constraint of black pepper. This *Cucumovirus* member, with a tripartite genome has the broadest host range of any known virus and is unmanageable by chemicals or traditional control methods. Among the different pathogen derived approaches used for engineering resistance against this most notorious virus, RNAi sounds to be highly promising. Hence, this study focused on developing transgenic black pepper plants using the hairpin construct of 3b and 2b genes of black pepper isolate of CMV. The study was accomplished through five prime objectives that included the complete genome sequencing of black pepper isolate of CMV, designing of common siRNAs targeting many subgroup IB strains of CMV, somatic embryo production from three different varieties of black pepper, *Agrobacterium* mediated transformation of the somatic embryos produced and testing of the regenerated putative transformants. Finally, a challenge inoculation method for CMV in black pepper was also developed to facilitate the screening of plants for resistance to this virus and its utility was tested in the transgenic plants carrying the CMV coat protein in the sense orientation, already available.

The complete genome sequencing of CMV from black pepper was accomplished to get a thorough understanding of the genomic structure of the present isolate under study. Further, percent identity analyses, phylogenetic relationships and nucleotide diversity studies were performed that helped in analyzing the level of conservation in each gene of the three RNAs, the relationship of the black pepper isolate with other subgroup I and II strains of

CMV and the evolutionary forces driving these genes. An important finding of the study was a rare nine nucleotide deletion in the putative methyltransferase domain of 1a gene of RNA1 which was present in two other strains of CMV among the hundred 1a gene sequences available in the GenBank. The corresponding deletion of three amino acids was also observed in the multiple sequence alignment of the deduced amino acids of the 1a protein. Interestingly, the deletion was not found in the black pepper strain of CMV from China and not even in the CMV isolate from long pepper, a closely related species of black pepper.

The percent identity analyses proved that all the genes of the current isolate shared highest sequence similarity to the subgroup IB strains at the nucleotide and amino acid level. Phylogenetic trees constructed, clearly marked the clustering of the black pepper isolate with the subgroup IB strains. These studies together classify the black pepper isolate of CMV as subgroup IB. Finally, the nucleotide analyses performed in this study showed that the 2b gene is the most diverse of all the five genes and 3b gene is the highly conserved among the different subgroups of CMV. The percent identity analyses also inferred similar observations. Regarding the evolutionary forces acting on these genes, all the genes were undergoing negative selection except for the 1a gene that was nearing neutral selection. As a whole, out of the three RNAs of the present isolate, RNA2 was highly tolerant to amino acid changes and RNA1 was the least tolerant. Whether the deletion of three amino acids in the deduced 1a protein, compared to that of the other strains, has any significance on the function is inconclusive and, may be further investigated in detail.

Once the complete gene sequences of different RNAs of the current isolate was ascertained, these were subjected to a computational tool 'dsCheck' to find out the most suitable region for dsRNA synthesis. Based on the earlier observations regarding the appropriate length of the transgene and the parameters of the software, a 400 bp region was selected from all the five genes (except the 336 bp 2b gene), as the most suitable regions for the hairpin construct preparation. The selected regions were subjected to sequence

polymorphism study and again the results were consistent with complete genome sequence data, confirming the 3b gene as the highly conserved and the 2b gene as the least conserved among the different genes. Further, the potential siRNAs that will be coded from the selected regions were designed *in silico* with the help of another computational tool 'siDesign' and though potential siRNAs were designed from all the five genes, with maximum specificity and minimum off–targets, four common siRNAs were generated from the 3b gene and none from other genes. The common siRNAs designed from the 3b gene will target all the subgroup IB strains taken in the current investigation.

Hairpin constructs were then assembled using the 3b gene, targeting the region from where the four common siRNAs were designed. Also, previous studies show that the 2b gene of the CMV functions as silencing suppressor with the ability to overcome post transcriptional gene silencing, hence the 2b gene of CMV was also chosen for the hairpin vector construction. Hairpin constructs of 3b and 2b genes were initially assembled in the cloning vectors pTZ57R/T and pBluescript SK+ respectively. This was achieved by PCR of sense, antisense and the phytoene desaturase 6th intron amplified from tomato, using primers flanked with proper restriction enzymes, followed by the restriction of the PCR products using the corresponding enzymes and one step ligation with the double restricted cloning vector. For the preparation of 3b hairpin construct, the restriction enzymes used were *Bam*HI, *Sac*I and *Nsi*1. For the 2b hairpin construct preparation, instead of the *Nsi*I site, *Hind*III restriction enzyme site was used. The cloning vector ligated with the hairpin construct was transformed in to *E. coli* DH5α cells. After the confirmation of the hairpin construct by colony PCR, plasmid PCR and restriction digestion, both the hairpin constructs were released from their respective cloning vectors by double restriction with *Bam*HI and *Sac*I, ligated to the double restricted binary vector and again transformed initially in to *E. coli DH5α* cells and later in to the *Agrobacterium* strain EHA105 by triparental mating. The construct was confirmed by PCR analyses and restriction digestion. The plasmids isolated from the recombinant *E. coli* and *Agrobacterium* cells on sequencing, revealed that the intron of size 224 bp was inserted in

between the sense and antisense constructs of 3b and 2b gene making the two hairpin constructs nearly 922 bp. These were designated as pBI121CMV3bHP and pBI121CMV2bHP respectively.

These constructs were used for the *Agrobacterium* mediated transformation of black pepper somatic embryos. For this, somatic embryos were produced from three varieties of black pepper, Panniyur–1, Sreekara and IISR–Thevam induced on the micropylar region of the mature berries. The secondary somatic embryos emerging from the primary embryos, and the cyclic somatic embryos were maintained by regular subculturing. The cyclic somatic embryos were used as explants for the transformation study. Nearly 25 transformation experiments were carried out using each construct and each of the two varieties, Sreekara and IISR–Thevam. As Panniyur–1 somatic embryos failed to regenerate, these were not taken for the transformation study. After several rounds of selection, a total of 26 plantlets of IISR–Thevam transformed using the hairpin constructs were transferred to the Woody plant medium. Similarly 24 plantlets of the variety Sreekara transformed with the hairpin constructs were transferred to the Woody plant medium.

From the transformed embryos in the selection medium, 50 mg of the well proliferated and regenerated embryos at the cotyledonary stage were screened for the presence of transgene by DNA PCR and RT–PCR using specific sets of primers. All the IISR–Thevam and Sreekara plantlets transformed using the pBI121CMV2bHP construct, showed specific amplification in PCR using one set of primers whereas the IISR–Thevam plantlets alone showed double bands in the PCR using a second set of primers. In case of the plantlets transformed using the pBI121CMV3bHP construct, six plantlets of IISR–Thevam and seven plantlets of Sreekara amplified in PCR using the first set of primers. When the kanamycin specific primers were used non–specific amplification was seen in both varieties transformed using both the constructs. In RT–PCR anlyses only the plantlets from the Sreekara variety amplified. Among the five Sreekara plantlets transformed using the pBI121CMV2bHP analyzed by RT–PCR, one plantlet gave specific product in RT–PCR. Three plantlets of the same variety transformed

using the pBI121CMV3bHP amplified in RT–PCR irrespective of the primer sets used.

Finally, a challenge inoculation method for CMV in black pepper was developed by cleft grafting. A series of cleft grafting experiments were performed that included self grafts, where scion excised from a particular plant was grafted on to the same stock and, non–transgenic controls where the scion excised from a healthy black pepper plant was grafted on to the CMV infected stock. The success of grafting in these experiments ranged from 73–75% and transmission of CMV from infected to healthy scion was found to be 100%, as tested by RT–PCR and RT–qPCR. Using this challenge inoculation method, eight transgenic lines of black pepper carrying the CMV coat protein sense construct were screened for resistance. Except one transgenic line, all others were negative for the virus in RT–PCR and RT–qPCR which is an indication of resistance in these plants.

6.2 Conclusions

The major achievements of the present investigation are:-

1. Complete genome of CMV from black pepper was sequenced and the sequences of different RNAs and the details were deposited in the NCBI GenBank.
2. The CMV isolate from black pepper was classified as subgroup IB based on the percent identity, phylogenetic analyses and nucleotide diversity studies.
3. A rare deletion of nine nucleotides was seen in the methyltransferase domain of 1a gene of RNA1 as revealed by the multiple sequence alignment of the 1a sequences.
4. The 1a gene was found to be nearing neutral selection and other genes were under negative selection pressure.
5. Among the different genes, 3b gene was highly conserved and 2b gene was the least conserved among the different subgroups.

6. The most suitable region for dsRNA synthesis was determined from the different genes of CMV isolate of black pepper.
7. Four potential and common siRNAs were designed from the 3b gene *in silico* that can target many subgroup IB strains of CMV.
8. Hairpin constructs of the 3b and 2b gene was prepared in suitable binary vectors and transformed in to *Agrobacterium* cells.
9. Cyclic somatic embryos were developed from three varieties of black pepper and maintained by regular subculturing.
10. *Agrobacterium* mediated transformation was carried out using cyclic somatic embryos of two varieties and regenerated plantlets were transferred to the Woody plant medium.
11. The transformed plantlets were screened for the presence of transgene by DNA PCR and RT–PCR.
12. Challenge inoculation method was developed for screening of transgenic plants of black pepper for CMV resistance based on cleft grafting.
13. Using this method transgenic black pepper plants carrying the CMV coat protein sense construct were screened.

6.3 Future line of work

1. Construction of infectious clones from black pepper isolate of CMV
2. Finding the significance of the deletion in 1a gene of RNA1 of CMV by constructing deletion mutants.
3. Production of large number of transgenic plants of different varieties of black pepper using the hairpin constructs prepared in the current study.
4. Challenge inoculation and screening for resistance to CMV in the transgenic plants by cleft grafting method developed in the present study.

5. Analysis of mechanism of resistance in the transgenic lines.

REFERENCES

Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986). Delay of disease development in transgenic plants that express the *Tobacco mosaic virus* coat protein gene. *Science* **232(4751)**: 738–43

Agarwal S, Kanwar K (2007). Comparison of genetic transformation in *Morus alba* L. *via* different regeneration systems. *Plant Cell Reports* **26(2)**: 177–85

Agrios GN (1978). Plant Pathology II edition, pp: 466–470

Ahmad N, Fazal H, Abbasi BH, Rashid M, Mahmood T, Fatima N (2010). Efficient regeneration and antioxidant potential in regenerated tissues of *Piper nigrum* L. *Plant Cell Tissue and Organ Culture* **102(1)**: 129–34

Akhtar KP, Haq A (2003). Standardization of a graft inoculation method for the screening of mungbean germplasm against *Mungbean yellow mosaic virus* (MYMV). *The Plant Pathology Journal* **19(5)**: 257–9

Ali ME, Waliullah S, Kobayashi K, Yaeno T, Yamaoka N, Nishiguchi M (2016). Transmission of RNA silencing signal through grafting confers virus resistance from transgenically silenced tobacco rootstocks to non–transgenic tomato and tobacco scions. *Journal of Plant Biochemistry and Biotechnology* **25(3)**: 245–52

Alsheikh M, Suso HP, Robson M, Battey N, Wetten A (2002). Appropriate choice of antibiotic and *Agrobacterium* strain improves transformation of antibiotic sensitive *Fragaria vesca* and F. v. semperflorens. *Plant Cell Reports* **20(12)**: 1173–80

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C (2005). Particle bombardment and the genetic enhancement of crops: myths and realities. *Molecular Breeding* **15(3)**: 305–27

Amarzguioui M, Holen T, Babaie E, Prydz H (2003). Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Research* **31(2)**: 589–95

Anandaraj M, Ramana KV, Sarma YR (1996). Sequential inoculation of *Phytophthora capsici*, *Radopholus similis* and *Meloidogyne incognita* in causing slow decline of black pepper. *Indian Phytopathology* **49(3)**: 297–9

Anandaraj M, Sarma YR (1995). Diseases of black pepper (*Piper nigrum* L.) and their management. *Journal of Spices and Aromatic Crops* **4(1)**: 17–23

Anderson JM, Palukaitis P, Zaitlin M (1992). A defective replicase gene induces resistance to *Cucumber mosaic virus* in transgenic tobacco plants. *Proceedings of the National Academy of Sciences* **89(18)**: 8759–63

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P (2004). Emerging infectious diseases of plants: pathogen pollution, climate

change and agrotechnology drivers. *Trends in Ecology and Evolution* **19(10)**: 535–44

Andreev IA, Kim SH, Kalinina NO, Rakitina DV, Fitzgerald AG, Palukaitis P, Taliansky ME (2004). Molecular interactions between a plant virus movement protein and RNA: force spectroscopy investigation. *Journal of Molecular Biology* **339(5)**: 1041–1047

Araujo RN, Santos A, Pinto FS, Gontijo NF, Lehane MJ, Pereira MH (2006). RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. *Insect Biochemistry and Molecular Biology* **36(9)**: 683–93

Arencibia AD, Carmona ERC, Tellez P, Chan MT, Yu SM, Trujillo LE, Oramas P (1998). An efficient protocol for sugarcane (*Saccharum* spp. L) transformation mediated by *Agrobacterium tumefaciens*. *Transgenic Res*earch **7**: 213–222

Arif M, Azhar U, Arshad M, Zafar Y, Mansoor S, Asad S (2012). Engineering broad-spectrum resistance against RNA viruses in potato. *Transgenic Research* **21(2)**: 303–11

Ashakiran K, Chidambareswaran M, Govindasamy V, Sivankalyani V, Girija S (2011). Somatic embryogenesis for *Agrobacterium* mediated transformation of tomato (*Solanum lycopersicum* L.). *International Journal of Biotechnological Applications* **3(2)**: 72–9

Asokan R, Nagesha SN, Manamohan M, Krishnakumar NK, Mahadevaswamy HM, Prakash MN, Chandra GS, Rebijith KB, Ellango R (2012). Common siRNAs for various target genes of the fruit borer, *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae). *Current Science* **25**: 1692–9

Asurmendi S, Berg RH, Smith TJ, Bendahmane M, Beachy RN (2007). Aggregation of *Tobacco mosaic virus* coat protein plays a role in coat protein functions and in coat protein mediated resistance. *Virology* **366(1)**: 98–106

Atal CK, Banga SS (1962). Phytochemical studies on stem of *P. longum*. *Indian Journal of Pharmacology* **24**: 105

Babu KN, Ravindran PN, Peter KV (1997). Protocols for Micropropogation of Spices and Aromatic Crops. Indian Institute of Spices Research, Calicut, Kerala, pp: 35

Barik DP, Mohapatra U, Chand PK (2005). Transgenic grasspea (*Lathyrus sativus* L.): factors influencing *Agrobacterium* mediated transformation and regeneration. *Plant Cell Reports* **24(9)**: 523–31

Baulcombe DC (2002). Viral suppression of systemic silencing. *Trends in Microbiology* **10**: 306–308

Baulcombe DC (2004). RNA silencing in plants. *Nature* **431**: 356–363

Baulcombe DC, Saunders GR, Bevan MW, Mayo MA, Harrison BD (1986). Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. *Nature* **321(6068)**: 446

Bebber DP, Ramotowski MA, Gurr SJ (2013). Crop pests and pathogens move polewards in a warming world. *Nature Climate Change* **3(11)**: 985

Beclin C, Berthome R, Palauqui JC, Tepfer M, Vaucheret H (1998). Infection of tobacco or *Arabidopsis* plants by *Cucumber mosaic virus* counteracts systemic post-transcriptional silencing of nonviral (trans) genes. *Virology* **252**: 313–7

Bendahmane M, Chen I, Asurmendi S, Bazzini AA, Szecsi J, Beachy RN (2007). Coat protein-mediated resistance to *Tobacco mosaic virus infection* of *Nicotiana tabacum* involves multiple modes of interference by coat protein. *Virology* **366(1)**: 107–16

Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409(6818)**: 363

Bevan M (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12(22)**: 8711–21

Bevan MW, Flavell RB, Chilton MD (1983). A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* **304(5922)**: 184

Bhadramurthy V, Bhat AI (2009). Biological and molecular characterization of *Bean common mosaic virus* associated with vanilla in India. *Indian Journal of Virology* **20(2)**: 70–77

Bhat AI, Devasahayam S, Preethi N, Hareesh PS, Tressa T (2005b). *Planococcus citri* (Risso) an additional mealy bug vector of *Badnavirus* infecting black pepper (*Piper nigrum* L.) in India. *Entomon* **30(1)**: 85–90

Bhat AI, Devasahayam S, Sarma YR, Pant RP (2003). Association of a *Badnavirus* transmitted by mealybug (*Ferrisia virgata*) with black pepper (*Piper nigrum* L.) in India. *Current. Science* **84**: 1547–1550

Bhat AI, Devasahayam S, Venugopal MN, Suseela Bhai R (2005a). Distribution and incidence of black pepper (*Piper nigrum* L.) in Karnataka and Kerala, India. *Journal of Plantation Crops* **33(1)**: 59–64

Bhat AI, Faisal TH, Madhubala R, Hareesh PS, Pant RP (2004). Purification, production of antiserum and development of enzyme linked immunosorbent assay based diagnosis for *Cucumber mosaic virus* infecting black pepper (*Piper nigrum* L.). *Journal of Spices and Aromatic Crops* **13**: 16–21

Bhat AI, Hareesh PS, Madhubala R (2005c). Sequencing of coat protein gene of an isolate of *Cucumber mosaic virus* infecting black pepper (*Piper nigrum* L.) in India. *Journal of Plant Biochemistry and Biotechnology* **14**: 37–40

Bhat AI, Madhubala R, Hareesh PS, Anandaraj M (2006). Detection and characterization of the phytoplasma associated with a phyllody disease of black pepper (*Piper nigrum* L.) in India. *Scientia Horticulturae* **107(2)**: 200–4

Bhat AI, Siljo A, Devasahayam S (2012). Occurrence of symptomless source of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L.) varieties and a wild *Piper* species. *Archives of Phytopathology and Plant Protection* **45**: 1000–1009

Bhat SR, Chandel KP, Malik SK (1995). Plant regeneration from various explants of cultivated *Piper* species. *Plant Cell Reports* **14(6)**: 398–402

Bidney D, Scelonge C, Martich J, Burrus M, Sims L, Huffman G (1992). Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. *Plant Molecular Biology* **18**: 301–313

Birmingham A, Anderson E, Sullivan K, Reynolds A, Boese Q, Leake D, Karpilow J, Khvorova A (2007). A protocol for designing siRNAs with high functionality and specificity. *Nature Protocols* **2(9)**: 2068

Blevins T, Rajeswaran R, Aregger M, Borah BK, Schepetilnikov M, Baerlocher L, Farinelli L, Meins Jr F, Hohn T, Pooggin MM (2011). Massive production of small RNAs from a non–coding region of *Cauliflower mosaic virus* in plant defense and viral counter defense. *Nucleic Acids Research* **39(12)**: 5003–14

Boff MIC, Sartoari DV, Bogo A (2006). Effects of extracts of *Piper nigrum* L. on the bean weevil, *Acanthoscelides obtectus* (Say). *Revista Brasileria de Armazenamentho* **31**: 17–22

Bonfim K, Faria JC, Nogueira EO, Mendes EA, Aragao FJ (2007). RNAi mediated resistance to *Bean golden mosaic virus* in genetically engineered common bean (*Phaseolus vulgaris*). *Molecular Plant Microbe Interactions* **20(6)**: 717–26

Borges F, Martienssen RA (2015). The expanding world of small RNAs in plants. *Nature Reviews Molecular Cell Biology* **16(12)**: 727

Bouhida M, Lockhart BE, Olszewski NE (1993). An analysis of the complete sequence of a *Sugarcane bacilliform virus* genome infectious to banana and rice. *Journal of General Virology* **74(1)**: 15–22

Brar GS, Cohen BA, Vick CL, Johnson GW (1994). Recovery of transgenic peanut (*Arachis hypogaea* L.) plants from elite cultivars utilizing ACCELL technology. *Plant Journal* **5**: 745–753

Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *The EMBO Journal* **17**: 6739–46

Broderson P, Voinnet O (2006). The diversity of RNA silencing pathways in plants. *Trends in Genetics* **22(5)**: 262–280

Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM (2009).Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome–wide reductions in cytosine methylation. *Journal of Virology* **83**: 5005–5013

Bull SE, Owiti JA, Niklaus M, Beeching JR, Gruissem W, Vanderschuren H (2009). *Agrobacterium* mediated transformation of friable embryogenic calli and regeneration of transgenic cassava. *Nature Protocols* **4(12)**: 1845

Canto T, Palukaitis P (1998). Transgenically expressed *Cucumber mosaic virus* RNA 1 simultaneously complements replication of *Cucumber mosaic virus* RNAs 2 and 3 and confers resistance to systemic infection. *Virology* **250(2)**: 325–36

Carr JP, Gal–On A, Palukaitis P, Zaitlin M (1994). Replicase–mediated resistance to *Cucumber mosaic virus* in transgenic plants involves suppression of both virus replication in the inoculated leaves and long–distance movement. *Virology* **199**: 439–447

Carr JP, Marsh LE, Lomonossoff GP, Sekiya ME, Zaitlin M (1992). Resistance to *Tobacco mosaic virus* induced by the 54–kDa gene sequence requires expression of the 54 kDa protein. *Molecular Plant Microbe Interactions* **5**: 397–404

Cerutti H (2003). RNA interference: traveling in the cell and gaining functions? *Trends in Genetics* **19**: 39–46

Chan MT, Chang HH, Ho SL, Tong WF, Yu SM (1993). *Agrobacterium* mediated production of transgenic rice plants expressing a chimeric α–amylase promoter / P–glucuronidase gene. *Plant Molecular Biology* **22**: 491–506

Charity JA, Anderson MA, Bittisnich DJ, Whitecross M, Higgins TJ (1999). Transgenic tobacco and peas expressing a proteinase inhibitor from *Nicotiana alata* have increased insect resistance. *Molecular Breeding* **5(4)**: 357–65

Chellappan P, Masona MV, Vanitharani R, Taylor NJ, Fauquet CM (2004). Broad spectrum resistance to ssDNA viruses associated with transgene–induced gene silencing in cassava. *Plant Molecular Biology* **56(4)**: 601–11

Chen S, Songkumarn P, Liu J, Wang GL (2009). A versatile zero background T–vector system for gene cloning and functional genomics. *Plant Physiology* **150(3)**: 1111–21

Chen YK, Lohuis D, Goldbach R, Prins M (2004). High frequency induction of RNA–mediated resistance against *Cucumber mosaic virus* using inverted repeat constructs. *Molecular Breeding* **14(3)**: 215–26

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology* **115(3)**: 971–80

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004). Factors influencing *Agrobacterium* mediated transformation of monocotyledonous species. *In Vitro Cellular & Developmental Biology–Plant*. **40(1)**: 31–45

Chowrira GM, Akella V, Lurquin PF (1995). Electroporation mediated gene transfer into intact nodal meristems *in planta*. *Molecular Biotechnology* **3(1)**: 17–23

Christou P, McCabe DE, Martinell BJ, Swain WF (1990). Soybean genetic engineering–commercial production of transgenic plants. *Trends in Biotechnology* **8**:145–51

Chuang CF, Meyerowitz EM (2000). Specific and heritable genetic interference by double–stranded RNA in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* **97(9)**: 4985–90

Chung BN, Yoon JY, Palukaitis P (2013). Engineered resistance in potato against *Potato leafroll virus*, *Potato virus A* and *Potato virus Y*. *Virus Genes* **47(1)**: 86–92

Cillo F, Finetti–Sialer MM, Papanice MA, Gallitelli D (2004). Analysis of mechanisms involved in the *Cucumber mosaic virus* satellite RNA–mediated transgenic resistance in tomato plants. *Molecular Plant Microbe Interactions* **17(1)**: 98–108

Cillo F, Palukaitis P (2014). Transgenic resistance. *Advances in Virus Research* **90**: 35–146

Cirilli M, Geuna F, Babini AR, Bozhkova V, Catalano L, Cavagna B, Dallot S, Decroocq V, Dondini L, Foschi S, Ilardi V (2016). Fighting Sharka in peach: current limitations and future perspectives. *Frontiers in Plant Science* **7**: 1290

Clough GH, Hamm PB (1995). Coat protein transgenic resistance to *Watermelon mosaic virus* and *Zucchini yellow mosaic virus* in squash and cantaloupe. *Plant Disease* **79(11)**: 1107–9

Cogoni C, Macino G (1999). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA–dependent RNA polymerase. *Nature* **399(6732)**: 166

Collmer CW, Howell SH (1992). Role of satellite RNA in the expression of symptoms caused by plant viruses. *Annual Review of Phytopathology* **30(1)**:419–42

Cuozzo M, O'Connell KM, Kaniewski W, Fang RX, Chua NH, Tumer NE (1988). Viral protection in transgenic tobacco plants expressing the *Cucumber mosaic virus* coat protein or its antisense RNA. *Nature Biotechnology* **6(5)**: 549

Czauderna F, Fechtner M, Dames S, Ayguen H, Klippel A, Pronk GJ, Giese K, Kaufmann J (2003). Structural variations and stabilizing modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Research* **31(11)**: 2705–16

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001). Comparative analysis of transgenic rice plants obtained by *Agrobacterium* mediated transformation and particle bombardment. *Molecular Breeding* **7(1)**: 25–33

Dalmay T, Horsefield R, Braunstein TH, Baulcombe DC (2001). SDE3 encodes an RNA helicase required for post–transcriptional gene silencing in *Arabidopsis*. *The EMBO Journal* **20(8)**: 2069–77

Danilova SA, Kusnetsov VV, Dolgikh YI (2009). A novel efficient method for maize genetic transformation: Usage of *Agrobacterial* monolayer. *Russian Journal of Plant Physiology* **56(2)**: 258–63

Darlington CD, Wylie AP (eds.) (1961). Chromosome atlas of flowering plants 2nd edn. George Unwin, London, pp: 184–185

Davino S, Panno S, Rangel EA, Davino M, Bellardi MG, Rubio L (2012). Population genetics of *Cucumber mosaic virus* infecting medicinal, aromatic and ornamental plants from northern Italy. *Archives of Virology* **157(4)**: 739–45

de Block M, Herrera–Estrella L, Van Montagu M, Schell J, Zambryski P (1984). Expression of foreign genes in regenerated plants and in their progeny. *The EMBO Journal* **3(8)**: 1681–9

de Framond AJ, Barton KA, Chilton MD (1983). Mini–Ti: a new vector strategy for plant genetic engineering. *Nature Biotechnology* **1(3)**: 262

de La Riva GA, Gonzalez–Cabrera J, Vazquez–Padron R, Ayra–Pardo C (1998). *Agrobacterium tumefaciens*: a natural tool for plant transformation. *Electronic Journal of Biotechnology* **1(3)**: 24–5

de Silva DPP, Jones P, Shaw MW (2001). Isolation, purification and identification of *Cucumber mosaic virus* in Sri Lanka on black pepper (*Piper nigrum* L.). *Sri Lankan Journal of Agricultural Sciences* **38**: 17–27

de Silva DPP, Jones P, Shaw MW (2002). Identification and transmission of *Piper yellow mottle virus* and *Cucumber mosaic virus* infecting black pepper (*Piper nigrum* L.) in Sri Lanka. *Plant Pathol*ogy **51**: 537–545

Deeshma KP, Bhat AI (2014). Further evidence of true seed transmission of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L.). *Journal of Plantation Crops* **42**: 289–293

Delbreil B, Guerche P, Jullien M (1993). *Agrobacterium* mediated transformation of *Asparagus officinalis* L. long–term embryogenic callus and regeneration of transgenic plants. *Plant Cell Reports* **12(3)**:129–32

Denli AM, Hannon GJ (2003). RNAi: an ever–growing puzzle. *Trends in Biochemical Sciences* **28(4)**:196–201

Dhekney SA, Li ZT, Dutt M, Gray DJ (2008). *Agrobacterium* mediated transformation of embryogenic cultures and plant regeneration in *Vitis rotundifolia* Michx. (muscadine grape). *Plant Cell Reports* **27(5)**: 865–72

Ding SW, Shi BJ, Li WX, Symons RH (1996). An interspecies hybrid RNA virus is significantly more virulent than either parental virus. Proceedings of the National Academy of Sciences of the United States of America **93**: 7470–7474

Ditta G, Stanfield S, Corbin D, Helinski DR (1980). Broad host range DNA cloning system for gram–negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proceedings of the National Academy of Sciences* **77(12)**: 7347–7351

Diveki Z, Salanki K, Balazs E (2004). The necrotic pathotype of the *Cucumber mosaic virus* (CMV) Ns strain is solely determined by amino acid 461 of the 1a protein. *Molecular Plant Microbe Interactions* **17(8)**: 837–45

Dong JJ, Teng WM, Buchholz WG, Hall TC (1996). *Agrobacterium* mediated transformation of Javanica rice. *Molecular Breeding* **2**: 267–276

Donson J, Kearney CM, Turpen TH, Khan IA, Kurath G, Turpen AM, Jones GE, Dawson WO, Lewandowski DJ (1993). Broad resistance to *Tobamoviruses* is mediated by a modified *Tobacco mosaic virus* replicase transgene. *Molecular Plant Microbe Interactions* **6(5)**: 635–42

Doolittle SP (1916). A new infectious mosaic disease of cucumber *Phytopathology* **6**: 145–147

Dorman HJ, Deans SG (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology* **88(2)**: 308–16

Dougherty WG, Parks TD (1995). Transgenes and gene suppression: telling us something new? *Current Opinion in Cell Biology* **7**: 399–405

Draper J, Scott R, Armitage P (1988). Plant genetic transformation and gene expression: a laboratory manual. Oxford: Blackwell Scientific Publications

Draper J, Davey MR, Freeman JP, Cocking EC, Cox BJ (1982). Ti plasmid homologous sequences present in tissues from *Agrobacterium* plasmid–transformed Petunia protoplasts. *Plant and Cell Physiology* **23(3)**: 451–8

Dronne S, Jullien F, Caissard JC, Faure O (1999). A simple and efficient method for *in vitro* shoot regeneration from leaves of lavandin (*Lavandula intermedia* Emeric ex Loiseleur). *Plant Cell Reports* **18(5)**: 429–33

Duan C, Wang C, Guo H (2008). Delayed resistance to *Cucumber mosaic virus* mediated by 3' UTR–derived hairpin RNA. *Chinese Science Bulletin* **53(21)**: 3301–10

Duan CG, Wang CH, Guo HS (2012). Application of RNA silencing to plant disease resistance. *Silence* **3(1)**: 5

Duarte MLR, Albuquerque FC (1991). Fusarium disease of black pepper in Brazil. In: Sarma YR, Premkumar T (eds) Black pepper diseases. National Research Centre for Spices, Calicut, pp: 39–54

Dubey VK, Chandrasekhar K, Srivastava A, Aminuddin, Singh VP, Dhar K, Arora PK (2015). Expression of coat protein gene of *Cucumber mosaic virus* (CMV subgroup IA) Gladiolus isolate in *Nicotiana tabacum*. *Journal of Plant Interactions* **10(1)**: 296–304

Duque AS, de Araujo SS, Dos Santos DM, Fevereiro MP (2004). Optimisation of a selection scheme using kanamycin to improve transformation of *Medicago truncatula* cv. Jemalong. *Plant Cell, Tissue and Organ Culture* **78(3)**: 277–80

Dutta I, Saha P, Das S (2008). Efficient *Agrobacterium* mediated genetic transformation of oilseed mustard [*Brassica juncea* (L.) Czern.] using leaf piece explants. *In Vitro Cellular Developmental Biology–Plant* **44**: 401–411

Edwardson JR, Christie RG (1991). The potyvirus group, Agricultural Experimental Station Monograph series No.16, vols. I-IV. University of Florida pp: 1244

Eiras M, Boari AJ, A Colariccio, Chaves AIR, Briones MRS, Figueira AR, Harakava R (2004). Characterization of isolates of the *Cucumovirus–Cucumber mosaic virus* present in Brazil. *Journal of Plant Pathology* **86**: 61–69

Elbashir SM, Harborth J, Weber K, Tuschl T (2002). Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26(2)**: 199–213

Elmayan T, Vaucheret H (1996). Expression of single copies of a strongly expressed 35S transgene can be silenced post transcriptionally. *The Plant Journal* **9(6)**: 787–97

Eng L, Jones P, Lockhart B, Martin RR (1993). Preliminary studies on the virus diseases of black pepper in Sarawak. In: Pepper Industry–Problems and Prospects, (Eds. Ibrahim MY, Bong CF) and Ipor IB), University Pertanian, Malayasia, pp: 149–161

English,JJ, Mueller E, Baulcombe DC (1996). Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**: 179–188

Fang X, Qi Y (2016). RNAi in plants: an Argonaute–centered view. *The Plant Cell* **28(2)**: 272–85

Fargette D, Konate G, Fauquet C, Muller E, Peterschmitt M, Thresh JM (2006). Molecular ecology and emergence of tropical plant viruses. *Annual Reviews in Phytopathology* **44**: 235–60

Finer JJ, McMullen MD (1990).Transformation of cotton (*Gossypium hirsutum* L.) *via* particle bombardment. *Plant Cell Reports* **8(10)**: 586–9

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998). Potent and specific genetic interference by double–stranded RNA in *Caenorhabditis elegans*. *Nature* **391(6669)**: 806

Firoozabady E, Heckert M, Gutterson N (2006). Transformation and regeneration of pineapple. *Plant Cell Tissue and Organ Culture* **84(1)**: 1

Fitch MM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990). Stable transformation of papaya *via* microprojectile bombardment. *Plant Cell Reports* **9(4)**: 189–94

Fitch MM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1992). Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of *Papaya ringspot virus*. *Nature Biotechnology* **10(11)**: 1466

Fitchet M (1990). Tissue culture propagation of black pepper (*Piper nigrum* L.). *Acta Horticulturae* **275**: 285–91

Flavell RB, Dart E, Fuchs RL, Fraley RT (1992). Selectable marker genes: safe for plants? *Nature Biotechnology* **10(2)**: 141

Flood J (2010). The importance of plant health to food security. *Food Security* **2(3)**: 215–31

Fraile A, Alonso–Prados JL, Aranda MA, Bernal JJ, Malpica JM, Garcia–Arenal F (1997). Genetic exchange by recombination or reassortment is infrequent in natural populations of a tripartite RNA plant virus. *Journal of Virology* **71**: 934–940

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR (1983). Expression of bacterial genes in plant cells. *Proceedings of the National Academy of Sciences* **80(15)**: 4803–7

Francki RIB, Mossop DW, Hatta T (1979). *Cucumber mosaic virus.* CMI/AAB Descriptions of plant viruses, No.213

Fromm M, Taylor LP, Walbot V (1985). Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proceedings of the National Academy of Sciences* **82(17)**: 5824–8

Fuchs M, Gonsalves D (1995). Resistance of transgenic hybrid squash ZW–20 expressing the coat protein genes of *Zucchini yellow mosaic virus* and *Watermelon mosaic virus* 2 to mixed infections by both *potyviruses*. *Nature Biotechnology* **13(12)**: 1466

Fuchs M, Gonsalves D (2007). Safety of virus resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. *Annual Review of Phytopathology* **45**: 173–202

Fuchs M, Klas FE, McFerson JR, Gonsalves D (1998). Transgenic melon and squash expressing coat protein genes of aphid–borne viruses do not assist the spread of an aphid non–transmissible strain of *Cucumber mosaic virus* in the field. *Transgenic Research* **7(6)**: 449–62

Fuentes A, Carlos N, Ruiz Y, Callard D, Sanchez Y, Ochagavia ME, Seguin J, Malpica–Lopez N, Hohn T, Lecca MR, Perez R (2016). Field trial and molecular characterization of RNAi–transgenic tomato plants that exhibit resistance to *Tomato yellow leaf curl geminivirus*. *Molecular Plant Microbe Interactions* **29(3)**: 197–209

Gaba V, Zelcer A, Gal–On A (2004). Cucurbit biotechnology–the importance of virus resistance. *In Vitro Cell Developmental Biology–Plant* **40**: 346–358

Gal–On A, Wolf D, Wang Y, Faure JE, Pilowsky M, Zelcer A (1998). Transgenic resistance to *Cucumber mosaic virus* in tomato: blocking of long–distance movement of the virus in lines harboring a defective viral replicase gene. *Phytopathology* **88(10)**: 1101–7

Gao M, Kawabe M, Tsukamoto T, Hanada H, Tao R (2010). Somatic embryogenesis and *Agrobacterium* mediated transformation of Japanese apricot (*Prunus mume*) using immature cotyledons. *Scientia Horticulturae* **124(3)**: 360–7

Garfinkel DJ, Simpson RB, Ream LW, White FF, Gordon MP, Nester EW (1981). Genetic analysis of crown gall: fine structure map of the T–DNA by site–directed mutagenesis. *Cell* **27(1)**: 143–53

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A (2003). ExPASy: the proteomics server for in–depth protein knowledge and analysis. *Nucleic Acids Research* **31(13)**: 3784–3788

Gaudin V, Vrain T, Jouanin L (1994). Bacterial genes modifying hormonal balances in plants. *Plant Physiology and Biochemistry* **32**: 11–29

Gelvin SB (2003). *Agrobacterium* mediated plant transformation: the biology behind the "gene–jockeying" tool. *Microbiology and Molecular Biology Reviews* **67(1)**: 16–37

Gelvin SB (2005). Agricultural biotechnology: gene exchange by design. *Nature* **433(7026)**: 583

Goldschmidt EE (2014). Plant grafting: new mechanisms, evolutionary implications. *Frontiers in Plant Science* **5**: 727

Golemboski DB, Lomonossoff GP, Zaitlin M (1990). Plants transformed with a *Tobacco mosaic virus* nonstructural gene sequence are resistant to the virus. *Proceedings of the National Academy of Sciences* **87(16)**: 6311–5

Gonsalves C, Xue B, Yepes M, Fuchs M, Ling K, Namba S, Chee P, Slightom JL, Gonsalves D (1994). Transferring *Cucumber mosaic virus*–white leaf strain coat protein gene into *Cucumis melo* L. and evaluating transgenic plants for protection against infections. *Journal of the American Society for Horticultural Science* **119(2)**: 345–55

Gonzalez AE, Schopke C, Taylor NJ, Beachy RN, Fauquet CM (1998). Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz.) through *Agrobacterium* mediated transformation of embryogenic suspension cultures. *Plant Cell Reports* **17(11)**: 827–31

Gorbalenya AE, Koonin EV, Donchenko AP, Blinov VM (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Research* **17**: 4713–30

Gordon KH, Waterhouse PM (2007). RNAi for insect proof plants. *Nature Biotechnol*ogy **25**: 1231–1232

Gordon–Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willetts NG, Rice TB (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *The Plant Cell* **2(7)**: 603–18

Govindarajan VS, Stahl WH (1977). Pepper–chemistry, technology, and quality evaluation. *Critical Reviews in Food Science and Nutrition* **9(2)**: 115–225

Guo HS, Garcia JA (1997). Delayed resistance to *Plum pox potyvirus* mediated by a mutated RNA replicase gene: involvement of a gene silencing mechanism. *Molecular Plant Microbe Interactions* **10**: 160–170

Hadi MZ, McMullen MD, Finer JJ (1996). Transformation of 12 different plasmids into soybean *via* particle bombardment. *Plant Cell Reports* **15(7)**: 500–5

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic acids symposium series* **41(41)**: 95–98. [London]: Information Retrieval Ltd., c1979–c2000

Hamilton AJ, Baulcombe DC (1999). A species of small antisense RNA in post transcriptional gene silencing in plants. *Science* **286(5441)**: 950–2

Hammond SM, Caudy AA, Hannon G.J (2001). Post–transcriptional gene silencing by double–stranded RNA. *Nature Reviews Genetics* **2**: 110–119

Han SN, Oh PR, Kim HS, Heo HY, Moon JC, Lee SK, Kim KH, Seo YW, Lee BM (2007). Effects of antibiotics on suppression of *Agrobacterium tumefaciens* and

plant regeneration from wheat embryo. *Journal of Crop Science and Biotechnology* **10**: 92–8

Hareesh PS, Bhat AI (2008). Detection and partial nucleotide sequence analysis of *Piper yellow mottle virus* infecting black pepper in India. *Indian Journal of Virology* **19(2)**: 160–7

Hareesh PS, Madhubala R, Bhat AI (2006). Characterization of *Cucumber mosaic virus* infecting Indian long pepper (*Piper longum* L.) and betel vine (*Piper betle* L.) in India. *Indian Journal of Biotechnology* **5**: 89–93

Hassani–Mehraban A, Brenkman AB, van den Broek NJ, Goldbach R, Kormelink R (2009). RNAi–mediated transgenic *tospovirus* resistance broken by intraspecies silencing suppressor protein complementation. *Molecular Plant Microbe Interactions* **22(10)**: 1250–7

He Y, Jones HD, Chen S, Chen XM, Wang DW, Li KX, Wang DS, Xia LQ (2010). *Agrobacterium* mediated transformation of durum wheat (*Triticum turgidum* L. var. durum cv Stewart) with improved efficiency. *Journal of Experimental Botany* **61(6)**: 1567–81

Hellens R, Mullineaux P, Klee H (2000). Technical focus: a guide to *Agrobacterium* binary Ti vectors. *Trends in Plant Science* **5(10)**: 446–51

Helliwell C, Waterhouse P (2003). Constructs and methods for high–throughput gene silencing in plants. *Methods* **30(4)**: 289–95

Hellwald KH, Palukaitis P (1994). Nucleotide sequence and infectivity of *Cucumber mosaic cucumovirus* (strain K) RNA2 involved in breakage of replicase mediated resistance in tobacco. *Journal of General Virology* **75(8)**: 2121–5

Hemenway C, Fang RX, Kaniewski WK, Chua NH, Tumer NE (1988). Analysis of the mechanism of protection in transgenic plants expressing the *Potato virus* X coat protein or its antisense RNA. *The EMBO Journal* **7(5)**: 1273–80

Hernalsteens JP, Van Vliet F, De Beuckeleer M, Depicker A, Engler G, Lemmers M, Holsters M, Van Montagu M, Schell J (1980). The *Agrobacterium tumefaciens* Ti plasmid as a host vector system for introducing foreign DNA in plant cells. *Nature* **287(5783)**: 654

Herrera–Estrella L1, Block MD, Messens E, Hernalsteens JP, Montagu MV, Schell J(1983). Chimeric genes as dominant selectable markers in plant cells. *The EMBO Journal* **2(6)**: 987–95

Hiei Y, Ohta S, Komari T, Kumashiro T (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T–DNA. *The Plant Journal* **6(2)**: 271–82

Hoekema A, Hirsch PR, Hooykaas PJ, Schilperoort RA (1983). A binary plant vector strategy based on separation of vir and T–region of the *Agrobacterium tumefaciens* Ti–plasmid. *Nature* **303(5913)**: 179

Hofgen R, Willmitzer L (1988). Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Research* **16(20)**: 9877

Hong JS, Masuta C, Nakano M, Abe J, Uyeda I (2003). Adaptation of *Cucumber mosaic virus* soybean strains (SSVs) to cultivated and wild soybeans. *Theoretical and Applied Genetics* **107(1)**: 49–53

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993). New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* **2(4)**: 208–18

Howard RA (1973). Notes on the Piperaceae of Lesser Antilles. *Journal of the Arnold Arboretum* **54**: 377–411

Hull R (2014). Matthew's Plant Virology. Academic Press USA. pp: 1056

Hu Q, Niu Y, Zhang K, Liu Y, Zhou X (2011). Virus–derived transgenes expressing hairpin RNA give immunity to *Tobacco mosaic virus* and *Cucumber mosaic virus*. *Virology Journal* **8(1)**: 41

Husnain T, Malik T, Riazuddin S, Gordon MP (1997). Studies on the expression of marker genes in chickpea. *Plant Cell Tissue and Organ Culture* **49(1)**: 7–16

Indurker, Misra SHS, Eapen S (2007). Genetic transformation of chickpea (*Cicer arietinum*) with insecticidal crystal protein gene using particle gun bombardment. *Plant Cell Reports* **26**: 755–763

Ingram HM, Power JB, Lowe KC, Davey MR (1999). Optimisation of procedures for microprojectile bombardment of microspore–derived embryos in wheat. *Plant Cell Tissue and Organ Culture* **57(3)**: 207–10

Ivic–Haymes SD, Smigocki AC (2005). Biolistic transformation of highly regenerative sugar beet (*Beta vulgaris* L.) leaves. *Plant Cell Reports* **23(10–11)**: 699–704

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology* **21(6)**: 635

Jacquemond M (2012). *Cucumber mosaic virus*. *Advances in Virus Research* **84**: 439–504

Jagger IC (1916). Experiments with the cucumber mosaic disease. *Phytopathology* **6**: 148

James C (2009). Global status of commercialized biotech. GM Crops 2008. ISAAA, Ithaca, NY

Jan FJ, Fagoaga C, Pang SZ, Gonsalves D (2000). A single chimeric transgene derived from two distinct viruses confers multi–virus resistance in transgenic plants through homology–dependent gene silencing. *Journal of General Virology* **81(8)**: 2103–9

Jardak–Jamoussi R, Winterhagen P, Bouamama B, Dubois C, Mliki A, Wetzel T, Ghorbel A, Reustle GM (2009). Development and evaluation of a *Grapevine fanleaf virus* inverted repeat construct for genetic transformation of grapevine. *Plant Cell Tissue and Organ Culture* **97(2)**: 187–96

Jefferson RA (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* **5(4)**: 387–405

Jefferson RA, Kavanagh TA, Bevan MW (1987). GUS fusions: beta–glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6(13)**: 3901–7

Ji LH, Ding SW (2001). The suppressor of transgene RNA silencing encoded by *Cucumber mosaic virus* interferes with salicylic acid–mediated virus resistance. *Molecular Plant Microbe Interactions* **14(6)**: 715–24

Jiby MV (2011). Agrobacterium mediated transformation of black pepper using sequences from *Cucumber mosaic virus* and *Piper yellow mottle virus*. *Ph.D. Thesis,* Mangalore University, Karnataka, India

Jiby MV, Bhat AI (2011). An efficient *Agrobacterium* mediated transformation protocol for black pepper (*Piper nigrum* L.) using embryogenic mass as explant. *Journal of Crop Science and Biotechnology* **14(4)**: 247–54

Jones AL, Johansen IE, Bean SJ, Bach I, Maula AJ (1998). Specificity of the resistance to pea seed-borne mosaic potyvirus in transgenic peas expressing the viral replicase (NIb) gene. *Journal of General Virology* **79**: 3129–3137

Jones NB, Van Staden J (1995). Plantlet production from somatic embryos of *Pinus patula*. *Journal of Plant Physiology* **145(4)**: 519–25

Jones NB, van Staden J (2001). Improved somatic embryo production from embryogenic tissue of *Pinus patula*. *In Vitro* Cellular and Developmental Biology–Plant **37(5)**: 543–9

Johnson SR, Strom S, Grillo K (2007). Quantification of the impacts on US agriculture of biotechnology derived crops planted in 2006. National Center for Food and Agriculture Policy. http://www.ncfap.org

Joseph B, Joseph D, Philip VJ (1996). Plant regeneration from somatic embryos in black pepper. *Plant Cell Tissue and Organ Culture* **47(1)**: 87–90

Kalantidis K, Psaradakis S, Tabler M, Tsagris M (2002). The occurrence of *Cucumber mosaic virus* specific short RNAs in transgenic tobacco expressing virus derived double stranded RNA is indicative of resistance to the virus. *Molecular Plant Microbe Interactions* **15(8)**: 826–33

Kalbande BB, Patil AS (2016). Plant tissue culture independent *Agrobacterium tumefaciens* mediated *In planta* transformation strategy for upland cotton

(*Gossypium hirsutum*). *Journal of Genetic Engineering and Biotechnology* **14(1)**: 9–18

Kang BC, Yeam I, Jahn MM (2005). Genetics of plant virus resistance. *Annual Review of Phytopathology* **43**: 581–621

Kang WH, Seo JK, Chung BN, Kim KH, Kang BC (2012). Helicase domain encoded by *Cucumber mosaic virus* RNA1 determines systemic infection of Cmr1 in pepper. *PLoS One* **7(8)**: e43136

Kasschau KD, Carrington JC (1998). A counterdefensive strategy of plant viruses: suppression of post transcriptional gene silencing. *Cell* **95(4)**: 461–70

Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC (2003). P1/HC–Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. *Developmental Cell* **4(2)**: 205–17

Kenel F, Eady C, Brinch S (2010). Efficient *Agrobacterium tumefaciens* mediated transformation and regeneration of garlic (*Allium sativum*) immature leaf tissue. *Plant Cell Reports* **29(3)**: 223–30

Kerschen A, Napoli CA, Jorgensen RA, Muller AE (2004). Effectiveness of RNA interference in transgenic plants. *FEBS Letters* **566(1–3)**: 223–8

Kester DE, Davies FT Jr, Geneve RL (2002). Hartmann and Kester's plant propagation: principles and practices. 7th ed. 411–460

Ketting RF, Haverkamp TH, van Luenen HG, Plasterk RH (1999). Mut–7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99(2)**: 133–41

Khawale RN, Singh SK, Garg G, Baranwal VK, Ajirlo SA (2006). *Agrobacterium* mediated genetic transformation of Nagpur mandarin (*Citrus reticulata* Blanco). *Current Science* **25**: 1700–5

Kim KM, Seo JK, Kwak HR, Kim JS, Kim KH, Cha BJ, Choi HS (2014). Molecular genetic analysis of *Cucumber mosaic virus* populations infecting pepper suggests unique patterns of evolution in Korea. *Phytopathology* **104**: 993–1000

Kim SJ, Lee SJ, Kim BD, Paek KH (1997). Satellite–RNA mediated resistance to *Cucumber mosaic virus* in transgenic plants of hot pepper (*Capsicum annuum* cv. Golden Tower). *Plant Cell Reports* **16(12)**: 825–30

Kjemtrup S, Sampson KS, Peele CG, Nguyen LV, Conkling MA, Thompson WF, Robertson D (1998). Gene silencing from plant DNA carried by a geminivirus. *The Plant Journal* **14(1)**: 91–100

Klas FE, Fuchs M, Gonsalves D (2006). Comparative spatial spread overtime of *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV) in

fields of transgenic squash expressing the coat protein genes of ZYMV and WMV, and in fields of nontransgenic squash. *Transgenic Research* **15(5)**: 527–41

Komari T, Kubo T (1999). Methods of genetic transformation: *Agrobacterium tumefaciens*. *Molecular Improvement of Cereal Crops*, Springer, Dordrecht 43–82

Komori T, Imayama T, Kato N, Ishida Y, Ueki J, Komari T (2007) . Current status of binary vectors and superbinary vectors. *Plant Physiology* **145(4)**: 1155–60

Kothari SL, Joshi A, Kachhwaha S, Ochoa Alejo N (2010). Chilli peppers–a review on tissue culture and transgenesis. *Biotechnology Advances* **28(1)**: 35–48

Kotsuka K, Tada Y (2008). Genetic transformation of golden pothos (*Epipremnum aureum*) mediated by *Agrobacterium tumefaciens*. *Plant Cell Tissue and Organ Culture* **95(3)**: 305–11

Koundal V, Haq QM, Praveen S (2011). Characterization, genetic diversity, and evolutionary link of *Cucumber mosaic virus* strain New Delhi from India. *Biochemical Genetics* **49(1–2)**: 25–38

Krens FA, Molendijk L, Wullems GJ, Schilperoort RA (1982). *In vitro* transformation of plant protoplasts with Ti–plasmid DNA. *Nature* **296(5852)**: 72

Krishna Raj S, Bi YM, Saxena PK (1997). Somatic embryogenesis and *Agrobacterium* mediated transformation system for scented geraniums (*Pelargonium* sp.Frensham'). *Planta* **201(4)**: 434–40

Kueh TK, Sim SL (1992). Occurrence and management of wrinkled–leaf disease of black pepper. In P.Wahid, D.Sitepu, S.Deciyanto and U.Superman (eds.), Proc. The International Workshop on Black pepper diseases, Bander, Lampung Indonesia, Institute for spice and medicinal crops, Bogor, Indonesia, pp: 227–233

Kumar S, Singhal V, Roshan R, Sharma A, Rembhotkar GW, Ghosh B (2007). Piperine inhibits TNFα induced adhesion of neutrophils to endothelial monolayer through suppression of NF–k and IkB kinase activation. *European Journal of Pharmacology* **575**: 177–186

Kumari R, Bhardwaj P, Singh L, Zaidi AA, Hallan V (2013). Biological and molecular characterization of *Cucumber mosaic virus* subgroup II isolate causing severe mosaic in cucumber. *Indian Journal of Virology* **24(1)**: 27–34

Kung YJ, Yu TA, Huang CH, Wang HC, Wang SL, Yeh SD (2010). Generation of hermaphrodite transgenic papaya lines with virus resistance *via* transformation of somatic embryos derived from adventitious roots of in vitro shoots. *Transgenic Research* **19(4)**: 621–35

Kusano M, Tohyama K, Bae CH, Riu KZ, Lee HY (2003). Plant regeneration and transformation of Kentucky Bluegrass (*Poa pratensis* L.) *via* the plant tissue culture. *Korean Journal of Plant Biotechnology* **30**: 115–121

Lapidot M, Gafny R, Ding B, Wolf S, Lucas WJ, Beachy RN (1993). A dysfunctional movement protein of *Tobacco mosaic virus* that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *The Plant Journal* **4(6)**: 959–70

Lazo GR, Stein PA, Ludwig RA (1991). A DNA transformation–competent *Arabidopsis* genomic library in *Agrobacterium*. *Nature Biotechnology* **9(10)**: 963

Leclercq J, Lardet L, Martin F, Chapuset T, Oliver G, Montoro P (2010). The green fluorescent protein as an efficient selection marker for *Agrobacterium tumefaciens* mediated transformation in *Hevea brasiliensis* (Mull. Arg). *Plant Cell Reports* **29(5)**: 513–22

Lee H, Kim MK, Choi HS, Kang JH, Ju HJ, Seo JK (2017). Efficient transmission and propagation of *Tomato chlorosis virus* by simple single–leaflet grafting. *The Plant Pathology Journal* 33(3): 345

Lee YH, Jung M, Shin SH, Lee JH, Choi SH, Her NH, Lee JH, Ryu KH, Paek KY, Harn CH (2009). Transgenic peppers that are highly tolerant to a new *Cucumber mosaic virus* pathotype. *Plant Cell Reports* **28(2)**: 223–32

Lee YH, Kim HS, Kim JY, Jung M, Park YS, Lee JS, Choi SH, Her NH, Lee JH, Hyung NI, Lee CH (2004). A new selection method for pepper transformation: callus–mediated shoot formation. *Plant Cell Reports* **23(1–2)**: 50–8

Leelavathi S, Sunnichan VG, Kumria R, Vijaykanth GP, Bhatnagar RK, Reddy VS (2004). A simple and rapid *Agrobacterium* mediated transformation protocol for cotton (*Gossypium hirsutum* L.): embryogenic calli as a source to generate large numbers of transgenic plants. *Plant Cell Reports* **22(7)**: 465–70

Leibman D, Wolf D, Saharan V, Zelcer A, Arazi T, Yoel S, Gaba V, Gal–On A (2011). A high level of transgenic viral small RNA is associated with broad *potyvirus* resistance in cucurbits. *Molecular Plant Microbe Interactions* **24(10)**: 1220–38

Li DD, Shi W, Deng XX (2003). Factors influencing *Agrobacterium* mediated embryogenic callus transformation of Valencia sweet orange (*Citrus sinensis*) containing the pTA29–barnase gene. *Tree Physiology* **23(17)**: 1209–15

Li GS, Yang AF, Zhang JR, Bi YR, Shan L (2000). Transformation of maize calluses and regeneration of herbicide transgenic plant. *Chinese Science Bulletin* **20**: 2181–2184

Li HQ, Sautter C, Potrykus I, Puonti–Kaerlas J (1996). Genetic transformation of cassava (*Mamhot esculenta* Crantz). *Nature Biotechnology* **14**: 736–740

Li HW, Lucy AP, Guo HS, Li WX, Ji LH, Wong SM, Ding SW (1999). Strong host resistance targeted against a viral suppressor of the plant gene defence silencing mechanism. *The EMBO Journal* **18**: 2683–91

Li MR, Li HQ, Wu GJ (2006). Study on factors influencing *Agrobacterium* mediated transformation of *Jatropha cuicas*. *Journal of Molecular Cell Biology* **39**: 83–87

Li ZN, Fang F, Liu GF, Bao MZ (2007). Stable *Agrobacterium* mediated genetic transformation of London plane tree (*Platanus acerifolia* Willd.). *Plant Cell Reports* **26(5)**: 641–50

Librado P, Rozas J (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25(11)**: 1451–1452

Lin HX, Rubio L, Smythe AB, Falk BW (2004). Molecular population genetics of *Cucumber mosaic virus* in California: evidence for founder effects and reassortment. *Journal of Virology* **78**: 6666–6675

Lin SS, Wu HW, Jan FJ, Hou RF, Yeh SD (2007). Modifications of the helper component–protease of *Zucchini yellow mosaic virus* for generation of attenuated mutants for cross protection against severe infection. *Phytopathology* **97**: 287–296

Lindbo JA, Falk BW (2017). The Impact of "Coat Protein–Mediated Virus Resistance" in Applied Plant Pathology and Basic Research. *Phytopathology* **107(6)**: 624–34

Lindbo JA, Silva–Rosales L, Proebsting WM, Dougherty WG (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *The Plant Cell* **5(12)**: 1749–1759

Linnaeus C (1753). Species Plantarum, London

Liu G, Bao M (2003). Adventitious shoot regeneration from in vitro cultured leaves of London plane tree (*Platanus acerifolia* Willd.). *Plant Cell Reports* **21(7)**: 640–4

Liu Z, Scorza R, Hily JM, Scott SW, James D (2007). Engineering resistance to multiple Prunus fruit viruses through expression of chimeric hairpins. *Journal of the American Society for Horticultural Science* **132(3)**: 407–14

Lius S, Manshardt RM, Fitch MM, Slightom JL, Sanford JC, Gonsalves D (1997). Pathogen–derived resistance provides papaya with effective protection against *Papaya ringspot virus*. *Molecular Breeding* **3(3)**: 161–8

Lockhart BEL (1990). Evidence for a double stranded circular genome in a second group of plant viruses. *Phytopathology* **80**: 127–131

Lockhart BEL, Kiratiya–Angul K, Jones P, Eng L, de Silva DPP, Olszewski NE, Lockhart N, Deema N, Sangalang J (1997). Identification of *Piper yellow mottle virus*, a mealy bug–transmitted badnavirus infecting *Piper* spp. in Southeast Asia. *European Journal of Plant Pathology* **103**: 303–11

Lopez M, Humara JM, Rodriguez R, Ordas RJ (2000). Factors involved in *Agrobacterium tumefaciens* mediated gene transfer into *Pinus nigra* Arn. ssp. salzmannii (Dunal) Franco. *Euphytica* **114(3)**: 195

Lupina T, Cripps H (1987). UV spectrophotometric determination of piperine in pepper preparations: collaborative study. *Journal Association of Official Analytical Chemists* **70(1)**: 112–3

Madhubala R, Bhadramurthy V, Bhat AI, Hareesh PS, Retheesh ST, Bhai RS (2005). Occurrence of *Cucumber mosaic virus* on vanilla (*Vanilla planifolia* Andrews) in India. *Journal of Biosciences* **30(3)**: 339–50

Madhusudhanan K, Rahiman BA (2000). The effect of activated charcoal supplemented media to browning of *in vitro* cultures of *Piper* species. *Biologia Plantarum* **43(2)**: 297–9

Maju TT, Soniya EV (2012). *In vitro* regeneration system for multiplication and transformation in *Piper nigrum* L. *International Journal of Medicinal and Aromatic Plants* **2(1)**: 178–84

Malmstrom CM, Alexander HM (2016). Effects of crop viruses on wild plants. *Current Opinion in Virology* **19**: 30–6

Malyshenko SI, Kondakova OA, Nazarova JV, Kaplan IB, Taliansky ME, Atabekov JG (1993). Reduction of *Tobacco mosaic virus* accumulation in transgenic plants producing non–functional viral transport proteins. *Journal of General Virology* **74(6)**: 1149–56

Manamohan M, Chandra GS, Asokan R, Deepa H, Prakash MN, Kumar NK (2013). One step DNA fragment assembly for expressing intron containing hairpin RNA in plants for gene silencing. *Analytical Biochemistry* **433(2)**: 189–91

Mao YB, Cai WJ, Wang JW, Hong GJ, Tao XY, Wang LJ, Huang YP, Chen XY (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology* **25(11)**: 1307

Marano MR, Baulcombe D (1998). Pathogen–derived resistance targeted against the negative–strand RNA of *Tobacco mosaic virus*: RNA strand–specific gene silencing? *The Plant Journal* **13(4)**: 537–46

Mariashibu TS, Subramanyam K, Arun M, Mayavan S, Rajesh M, Theboral J, Manickavasagam M, Ganapathi A (2013). Vacuum infiltration enhances the *Agrobacterium*–mediated genetic transformation in Indian soybean cultivars. *Acta Physiologiae Plantarum* **35(1)**: 41–54

Martinez F, Lafforgue G, Morelli MJ, Gonzalez–Candelas F, Chua NH, Daros JA, Elena SF (2012). Ultradeep sequencing analysis of population dynamics of virus escape mutants in RNAi–mediated resistant plants. *Molecular Biology and Evolution* **29(11)**: 3297–307

Masuta C, Ueda S, Suzuki M, Uyeda I (1998). Evolution of a quadripartite hybrid virus by interspecific exchange and recombination between replicase components of two related tripartite RNA viruses. *Proceedings of the National Academy of Sciences* **95(18)**: 10487–92

Mathew PJ, Mathew PM, Kumar V (2001). Graph clustering of *Piper nigrum* L. (black pepper). *Euphytica* **118(3)**: 257–264

Mathew PM (1958). Studies on Piperaceae. *Journal of Indian Botanical Society* **37**: 155–171

Mathew VH, Rao RS (1984). *In vitro* responses of black pepper (*Piper nigrum* L.). *Current Science* **53**: 183–186

Mathias RJ, Boyd LA (1986). Cefotaxime stimulates callus growth, embryogenesis and regeneration in hexaploid bread wheat (*Triticum aestivum* L. em. thell). *Plant Science* **46(3)**: 217–23

Mayavan S, Subramanyam K, Arun M, Rajesh M, Dev GK, Sivanandhan G, Jaganath B, Manickavasagam M, Selvaraj N, Ganapathi A(2013). *Agrobacterium tumefaciens* mediated *In planta* seed transformation strategy in sugarcane. *Plant Cell Reports* **32(10)**: 1557–74

Mazidah M, Yusoff K, Habibuddin H, Tan YH, Lau WH (2012). Characterization of *Cucumber mosaic virus* (CMV) causing mosaic symptom on *Catharanthus roseus* (L.) G. Don in Malaysia. *Pertanika Journal of Tropical Agricultural Science* **35(1)**: 41–53

McCabe DE, Swain WF, Martinell BJ, Christou P (1988). Stable transformation of soybean (*Glycine max*) by particle acceleration. *Nature Biotechnology* **6(8)**: 923

McCullen CA, Binns AN (2006). *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. *Annual Reviews of Cell and Developmental Biology* **22**: 101–27

McGarvey PB, Montasser MS, Kaper JM (1994). Transgenic tomato plants expressing satellite RNA are tolerant to some strains of *Cucumber mosaic virus*. *Journal of the American Society for Horticultural Science* **119(3)**: 642–7

Meyer HJ, Van Staden J, Allen S (1992). The use of antibiotics to control systemic bacteria in *in vitro* cultures of *Piper nigrum* cv Kuching. *South African Journal of Botany* **58(6)**: 500–4

Meyering–Vos M, Muller A (2007). RNA interference suggests sulfakinins as satiety effectors in the cricket *Gryllus bimaculatus*. *Journal of Insect Physiology* **53(8)**: 840–8

Meyers B, Zaltsman A, Lacroix B, Kozlovsky SV, Krichevsky A (2010). Nuclear and plastid genetic engineering of plants: comparison of opportunities and challenges. *Biotechnology Advances* **28(6)**: 747–56

Mikhail NR, Eugene VK and Alexander EG (1992). Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis–like' supergroup of positive–strand RNA viruses. *Journal of General Virology* **73**: 2129–2134

Mitter N, Worrall EA, Robinson KE, Li P, Jain RG, Taochy C, Fletcher SJ, Carroll BJ, Lu GM, Xu ZP (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nature Plants* **3(2)**: 16207

Mochizuki T, Ohki ST (2011). Single amino acid substitutions at residue 129 in the coat protein of *Cucumber mosaic virus* affect symptom expression and thylakoid structure. *Archives of Virology* **156(5)**: 881–6

Mohanty D, Chandra A, Tandon R (2016). Germline transformation for crop improvement. In: Rajpal V, Rao S, Raina S (eds) *Molecular Breeding for Sustainable Crop Improvement*. Sustainable Development and Biodiversity, Springer, Cham **11**: 343–395

Montes C, Castro A, Barba P, Rubio J, Sanchez E, Carvajal D, Aguirre C, Tapia E, Dell P, Decroocq V, Prieto H (2014). Differential RNAi responses of *Nicotiana benthamiana* individuals transformed with a hairpin–inducing construct during *Plum pox virus* challenge. *Virus Genes* **49(2)**: 325–38

Moran R, Garcıa R, Lopez A, Zaldua Z, Mena J, Garcıa M, Armas R, Somonte D, Rodrıguez J, Gomez M, Pimentel E (1998). Transgenic sweet potato plants carrying the *δ*–endotoxin gene from *Bacillus thuringiensis* var. tenebrionis. *Plant Science* **139(2)**: 175–84

Morroni M, Thompson JR, Tepfer M (2008). Twenty years of transgenic plants resistant to *Cucumber mosaic virus*. *Molecular Plant Microbe Interactions* **21**: 675–684

Mossop DW, Francki RIB (1977). Association of RNA 3 with aphid transmission of *Cucumber mosaic virus. Virology* **81**: 177–181

Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K (2000). *Arabidopsis* SGS2 and SGS3 genes are required for post transcriptional gene silencing and natural virus resistance. *Cell* **101(5)**: 533–42

Mundembe R, Matibiri A, Sithole–Niang I (2009). Transgenic plants expressing the coat protein gene of *Cowpea aphid–borne mosaic potyvirus* predominantly convey the delayed symptom development phenotype. *African Journal of Biotechnology* **8(12)**: 2686–2690

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–497

Muthukumar V, Melcher U, Pierce M, Wiley GB, Roe BA, Palmer MW, Thapa V, Ali A, Ding T (2009). Non–cultivated plants of the Tallgrass Prairie Preserve of

northeastern Oklahoma frequently contain virus-like sequences in particulate fractions. *Virus Research* **141(2)**: 169–73

Mysara M, Garibaldi JM, ElHefnawi M (2011). MysiRNA-designer: a workflow for efficient siRNA design. *PLoS One* **6(10)**: e25642

Orczyk AN, Orczyk W (2000). Study of the factors influencing *Agrobacterium* mediated transformation of pea (*Pisum sativum* L.). *Molecular Breeding* **6(2)**: 185–94

Nair KP (2011). Agronomy and economy of black pepper and cardamom: The 'King' and 'Queen' of Spices. Elsevier

Nair RR, Dutta GS (2003). Somatic embryogenesis and plant regeneration in black pepper (*Piper nigrum* L.). Direct somatic embryogenesis from tissues of germinating seeds and ontogeny of somatic embryos. *The Journal of Horticultural Science and Biotechnology* **78(3)**: 416–21

Nair RR, Dutta GS (2006). High frequency plant regeneration through cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.). *Plant Cell Reports* **24(12)**: 699–707

Nair RR, Sasikumar B, Ravindran PN (1993). Polyploidy in a cultivar of black pepper (*Piper nigrum* L.) and its open pollinated progeny. *Cytologia* **58**: 27–31

Nakano M, Hoshino Y, Mii M (1994). Regeneration of transgenic plants of grapevine (*Vitis vinifera* L.) *via Agrobacterium rhizogenes* mediated transformation of embryogenic calli. *Journal of Experimental Botany* **45(5)**: 649–56

Nandakumar R, Babu S, Kalpana K, Raguchander T, Balasubramanian P, Samiyappan R (2007). *Agrobacterium* mediated transformation of indica rice with chitinase gene for enhanced sheath blight resistance. *Biologia Plantarum* **51(1)**: 142–8

Napoli C, Lemieux C, Jorgensen R (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co–suppression of homologous genes in trans. *The Plant Cell* **2(4)**: 279–89

National Academics of Sciences and Medicine (2016). Genetically engineered crops: Experiences and prospects. The National Academic Press, Washington DC

Nazeem PA, Augustin M, Rathy K, Sreekumar PK, Rekha CR, Shaju KV, Peter KV, Girija D, Kesavachandran R (2004). A viable protocol for large scale *in vitro* multiplication of black pepper (*P. nigrum* L.). *Journal of Plantation Crops* **32**: 163–8

Nazeem PA, Joseph L, Geetha CK, Nair GS (1992). *In vitro* cloning of black pepper (*Piper nigrum* L.). *Journal of Plantation Crops* **20**: 257–259

Nester EW, Gordon MP, Amasino RM, Yanofsky MF (1984). Crown gall: a molecular and physiological analysis. *Annual Review of Plant Physiology* **35(1)**: 387–413

Niazian M, Noori SA, Galuszka P, Tohidfar M, Mortazavian SM (2017). Genetic stability of regenerated plants *via* indirect somatic embryogenesis and indirect shoot regeneration of *Carum copticum* L. *Industrial Crops and Products* **97**: 330–7

Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH (2006). Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nature Biotechnology* **24(11)**: 1420

Noris E, Lucioli A, Tavazza R, Caciagli P, Accotto GP, Tavazza M (2004). *Tomato yellow leaf curl Sardinia virus* can overcome transgene–mediated RNA silencing of two essential viral genes. *Journal of General Virology* **85(6)**: 1745–9

Nouri S, Arevalo R, Falk BW, Groves RL (2014). Genetic structure and molecular variability of *Cucumber mosaic virus* isolates in the United States. *PLoS One* **9(5)**: 1–12

Ntui VO, Kynet K, Khan RS, Ohara M, Goto Y, Watanabe M, Fukami M, Nakamura I, Mii M (2014). Transgenic tobacco lines expressing defective *Cucumber mosaic virus* replicase–derived dsRNA are resistant to CMV–O and CMV–Y. *Molecular Biotechnology* **56(1)**: 50–63

Nyaboga EN, Njiru JM, Tripathi L (2015). Factors influencing somatic embryogenesis, regeneration, and *Agrobacterium* mediated transformation of cassava (*Manihot esculenta* Crantz.) cultivar TME14. *Frontiers in Plant Science* **6**: 411

Orczyk AN, Orczyk W, Przetakiewicz A (2000). *Agrobacterium* mediated transformation of cereals from technique development to its application. *Acta Physiologiae plantarum* **22(1)**: 77–88

Osbourn JK, Sarkar S, Wilson TM (1990). Complementation of coat protein–defective *Tobacco mosaic virus* (TMV) mutants in transgenic tobacco plants expressing TMV coat protein. *Virology* **179(2)**: 921–5

Otani M, Mii M, Handa T, Kamada H, Shimada T (1993). Transformation of sweet potato [*Ipomoea batatas* (L.) Lam.] plants by *Agrobacterium rhizogenes. Plant Science* **94**: 151–159

Oz MT, Eyidogan F, Yucel M, Oktem HA (2009). Optimized selection and regeneration conditions for *Agrobacterium* mediated transformation of chickpea cotyledonary nodes. *Pakistan Journal of Botany* **41(4)**: 2043–54

Ozawa K (2009). Establishment of a high efficiency *Agrobacterium* mediated transformation system of rice (*Oryza sativa* L.). *Plant Science* **176(4)**: 522–7

Pailey PV, Rama Devi L, Nair VG, Menon MR, Nair MRGK (1981). Malformation of leaves in black pepper. *Journal of Plantation Crops* **9**: 61–62

Palukaitis P, Roossinck MJ, Dietzgen RG, Francki RIB (1992). *Cucumber mosaic virus. Advances in Virus Research* **41**: 281–348

Pang SZ, Jan FJ, Carney K, Stout J, Tricoli DM, Quemada HD, Gonsalves D (1996). Post transcriptional transgene silencing and consequent *tospovirus* resistance in transgenic lettuce are affected by transgene dosage and plant development. *Plant Journal* **9**: 899–909

Pang SZ, Jan FJ, Gonsalves D (1997). Nontarget DNA sequences reduce the transgene length necessary for RNA mediated tospovirus resistance in transgenic plants. *Proceedings of National Academic Science USA* **94**: 8261–8266

Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jha A, Tyagi OD, Prasad AK, Wengel J, Olsen CE, Boll PM (1997). Phytochemistry of the genus *Piper*. *Phytochemistry* **46**: 597–673

Parrott WA, All JN, Adang MJ, Bailey MA, Boerma HR, Stewart CN Jr. (1994). Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. Kurstaki insecticidal gene. *In Vitro Cell Developmental Biology* **30**: 144–149

Parveez GK, Masri MM, Zainal A, Yunus AM, Fadilah HH, Rasid O, Cheah SC (2000). Transgenic oil palm: production and projection. *Biochemical Society Transactions* **28(6)**: 969–72

Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn T, Hohn B, Potrykus I (1984). Direct gene transfer to plants. *The EMBO Journal* **3(12)**: 2717–22

Pathi KM, Tula S, Tuteja N (2013). High frequency regeneration *via* direct somatic embryogenesis and efficient *Agrobacterium* mediated genetic transformation of tobacco. *Plant Signaling and Behavior* **8(6)**: e24354

Patil BL, Ogwok E, Wagaba H, Mohammed IU, Yadav JS, Bagewadi B, Taylor NJ, Kreuze JF, Maruthi MN, Alicai T, Fauquet CM (2011). RNAi mediated resistance to diverse isolates belonging to two virus species involved in Cassava brown streak disease. *Molecular Plant Pathology* **12(1)**: 31–41

Pavingerova D, Briza J, Niedermeierova H, Vlasak J (2011). Stable *Agrobacterium* mediated transformation of Norway spruce embryogenic tissues using somatic embryo explants. *Journal of Forest Science* **57(7)**: 277–80

Peden KW, Symons RH (1973). *Cucumber mosaic virus* contains a functionally divided genome. *Virology* **53(2)**: 487–92

Peltola H, Soderlund H, Ukkonen E (1984). SEQAID: A DNA sequence assembling program based on a mathematical model. *Nucleic Acids Research* **12**: 307–321

Petri C, Wang H, Alburquerque N, Faize M, Burgos L (2008). *Agrobacterium* mediated transformation of apricot (*Prunus armeniaca* L.) leaf explants. *Plant Cell Reports* **27(8)**: 1317–24

Philip VJ, Dominic J, Triggs GS, Dickinson NM (1992). Micropropagation of black pepper (*Piper nigrum* L.) through shoot tip cultures. *Plant Cell Reports* **12**: 41–44

Polito VS, McGranahan G, Pinney K, Leslie C (1989). Origin of somatic embryos from repetitively embryogenic cultures of walnut (*Juglans regia* L.) Implications for *Agrobacterium* mediated transformation. *Plant Cell Reports* **8(4)**: 219–21

Pooggin M, Shivaprasad PV, Veluthambi K, Hohn T (2003). RNAi targeting of DNA virus in plants. *Nature Biotechnology* **21(2)**: 131

Pooggin MM (2017). RNAi–mediated resistance to viruses: a critical assessment of methodologies. *Current Opinion in Virology* **26**: 28–35

Potrykus I (1991). Gene transfer to plants: assessment of published approaches and results. *Annual Review of Plant Biology* **42(1)**: 205–25

Prakasam V, Subbaraja KT, Bhakthavatsalu CM (1990). Mosaic disease–a new record in black pepper in Lower Palneys. *Indian Cocoa Arecanut and Spices Journal* **13**: 104–105

Pratap D, Kumar S, Raj SK, Sharma AK (2011). *Agrobacterium* mediated transformation of eggplant (*Solanum melongena* L.) using cotyledon explants and coat protein gene of *Cucumber mosaic virus*. *Indian Journal of Biotechnology* **10**: 19–24

Pratap D, Raj SK, Kumar S, Snehi SK, Gautam KK, Sharma AK (2012). Coat protein–mediated transgenic resistance in tomato against a IB subgroup *Cucumber mosaic virus* strain. *Phytoparasitica* **40(4)**: 375–82

Praveen S, Kushwaha CM, Mishra AK, Singh V, Jain RK, Varma A (2005). Engineering tomato for resistance to tomato leaf curl disease using viral rep gene sequences. *Plant cell Tissue and Organ Culture* **83(3)**: 311–8

Prins M, de Haan P, Luyten R, van Veller M, van Grinsven M, Goldbach R (1995). Broad resistance to *tospoviruses* in transgenic tobacco plants expressing three tospoviral nucleoprotein gene sequences. *Molecular Plant Microbe Interactions* **8**: 85–91

Prins M, Kikkert M, Ismayadi C, Graauw WD, Haan PD, Goldbach R (1997). Characterization of RNA–mediated resistance to *Tomato spotted wilt virus* in transgenic tobacco plants expressing NSM gene sequences. *Plant Molecular Biology* **33**: 235–243

Prins M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M (2008). Strategies for antiviral resistance in transgenic plants. *Molecular Plant Pathology* **9(1)**: 73–83

Purseglove JW, Brown EG, Green CL, Robbins SRJ (1981). *Spices.* Vol. 1. Longman

Purushothama ACR, Brosseau C, Giguere T, Sano T, Moffett P, Perreault JP (2015). Small RNA derived from the virulence modulating region of the potato spindle tuber viroid silences callose synthase genes of tomato plants. *The Plant Cell* **27(8)**: 2178–94

Qiu Y, Zhang Y, Wang C, Lei R, Wu Y, Li X, Zhu S (2018). *Cucumber mosaic virus* coat protein induces the development of chlorotic symptoms through interacting with the chloroplast ferredoxin I protein. *Scientific Reports* **8(1)**: 1205

Qu F, Ye X, Hou G, Sato S, Clemente TE, Morris TJ (2005). RDR6 has a broad–spectrum but temperature–dependent antiviral defense role in *Nicotiana benthamiana*. *Journal of Virology* **79**: 15209–15217

Qu J, Ye J, Fang R (2007). Artificial micro RNA mediated virus resistance in plants. *Journal of Virology* **81(12)**: 6690–9

Qu R, Bhattacharya M, Laco GS, de Kochko A, Subba Rao BL, Kaniewska MB, Elmer JS, Rochester DE, Smith CE, Beachy RN (1991). Characterization of the genome of *Rice tungro bacilliform virus*: Comparison with *Commelina yellow mottle virus* and Caulimoviruses. *Virology* **185**: 354–364

Rajeswaran R, Golyaev V, Seguin J, Zvereva AS, Farinelli L, Pooggin MM (2014). Interactions of *Rice tungro bacilliform pararetrovirus* and its protein P4 with plant RNA–silencing machinery. *Molecular Plant Microbe Interactions* **27(12)**: 1370–8

Raj Mohan K (1985). Standardization of tissue culture techniques in important horticultural crops. PhD Thesis submitted to Kerala Agricultural University

Rao AL, Francki RI (1982). Distribution of determinants for symptom production and host range on the three RNA components of *Cucumber mosaic virus*. *Journal of General Virology* **61(2)**: 197–205

Rao AQ, Bakhsh A, Kiani S, Shahzad K, Shahid AA, Husnain T, Riazuddin S (2009). The myth of plant transformation. *Biotechnology Advances* **27(6)**: 753–63

Ravindran PN (2000). Black pepper (*Piper nigrum* L.). Hardwood academic publishers, The Netherlands

Ravindran PN, Nair MK (1984). Pepper varieties. *Indian Cocoa Arecanut and Spices Journal* **7(3)**: 67–69

Reddy DV, Sudarshana MR, Fuchs M, Rao NC, Thottappilly G (2009). Genetically engineered virus–resistant plants in developing countries: current status and future prospects. *Advances in Virus Research* **75**: 185–220

Register JC III, Beach RN (1988). Resistance to *Tobacco mosaic virus* in transgenic plants results from interference with an early event in infection. *Virology* **166**: 524–532

Rema J, John CZ, Mini PM (1995). *In vitro* plant regeneration of economically important *Piper* species. (*P. nigrum* L., *P. betle* L., *P. longum* L., and *P. chaba* Hunt). *Proceedings of Seventh Kerala Science Congress*, Palakkad, pp: 321–324

Remila KG, Neelakandan N (1994). Morphological abnormalities due to phyllody disease of black pepper (*Piper nigrum* L.). *Phytomorphology* **44**: 111–114

Retheesh ST, Bhat AI (2011). Genetic transformation and regeneration of transgenic plants from protocorm–like bodies of vanilla (*Vanilla planifolia* Andrews) using *Agrobacterium tumefaciens*. *Journal of Plant Biochemistry and Biotechnology* **20(2)**: 262

Rheede HV (1688). *Hortus Indicus Malabaricus*, Amsterdam: Joannis van Someren. 1678–1692

Ribeiro SG, Lohuis H, Goldbach R, Prins M (2007). *Tomato chlorotic mottle virus* is a target of RNA silencing but the presence of specific short interfering RNAs does not guarantee resistance in transgenic plants. *Journal of Virology* **81(4)**: 1563–73

Rodriguez–Negrete EA, Carrillo–Tripp J, Rivera–Bustamante RF (2009). RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *Journal of Virology* **83(3)**: 1332–40

Roossinck MJ (2001). *Cucumber mosaic virus*, a model for RNA virus evolution. *Molecular Plant Pathology* **2(2)**: 59–63

Roossinck MJ (2002). Evolutionary history of *Cucumber mosaic virus* deduced by phylogenetic analyses. *Journal of Virology* **76(7)**: 3382–7

Roossinck MJ, Sleat D, Palukaitis P (1992). Satellite RNAs of plant viruses: structures and biological effects. *Microbiological Reviews* **56(2)**: 265–79

Roossinck MJ, Zhang L, Hellwald KH (1999). Rearrangements in the 5′ nontranslated region and phylogenetic analyses of *Cucumber mosaic virus* RNA 3 indicate radial evolution of three subgroups. *Journal of Virology* **73(8)**: 6752–8

Rubino L, Lupo R, Russo M (1993). Resistance to *Cymbidium ringspot virus* infection in transgenic plants expressing full–length viral replicase gene. *Molecular Plant Microbe Interactions* **6**: 729–34

Sailaja M, Tarakeswari M, Sujatha M (2008). Stable genetic transformation of castor (*Ricinus communis* L.) *via* particle gun–mediated gene transfer using embryo axes from mature seeds. *Plant Cell Reports* **27(9)**: 1509

Salaj T, Moravcikova J, Vookova B, Salaj J (2009). *Agrobacterium* mediated transformation of embryogenic tissues of hybrid firs (*Abies* spp.) and regeneration of transgenic emblings. *Biotechnology Letters* **31(5)**: 647–52

Saleh MC, van Rij RP, Hekele A, Gillis A, Foley E, O'Farrell PH, Andino R (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nature Cell Biology* **8(8)**: 793

Sanford JC (2000). The development of the biolistic process. *In Vitro Cellular and Developmental Biology–Plant* **36(5)**: 303–8

Sanford JC, Johnston SA (1985). The concept of parasite–derived resistance deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology* **113(2)**: 395–405

Sanford JC, Klein TM, Wolf ED, Allen N (1987). Delivery of substances into cells and tissues using a particle bombardment process. *Particulate Science and Technology* **5(1)**: 27–37

Santra M, Santra DK, Rao VS, Taware SP, Tamhankar SA (2005). Inheritance of β–carotene concentration in durum wheat (*Triticum turgidum* L.ssp.durum). *Eucalypta* **144**: 215–221

Sarma YR, Anandaraj M, Devasahayam S (1992). Diseases of unknown etiology of black pepper (*Piper nigrum* L.). In Proceedings of Internet Workshop on Black pepper diseases (eds) P.Wahid, D. sitepu, S Deciyanto and U.Superman, Institute for spice and medicinal crops, Bogor, Indonesia, pp: 133–143

Sarma YR, Anandaraj M (1997). Phytophthora foot rot of black pepper. In Agnihotri VP, Sarbhoy AK and Singh DV (eds.), Management of threatening diseases of national importance, Malhotra Publishing House, India, pp: 237–248

Sarma YR, Kiranmai G, Sreenivasulu P, Anandaraj M, Hema M, Venkataramana M, Murthy AK, Reddy DVR (2001). Partial characterization and identification of a virus associated with stunt disease of black pepper (*Piper nigrum* L.) in South India. *Current Science* **80**: 459–462

Sarma YR, Solomon JJ, Ramachandran N, Anandaraj M (1988). Phyllody disease of black pepper (*Piper nigrum* L.). *Journal of Plantation Crops* **16**: 69–72

Sarria R, Torres E, Angel F, Chavarriaga P, Roca WM (2000). Transgenic plants of cassava (*Manihot esculenta*) with resistance to Basta obtained by *Agrobacterium*–mediated transformation. *Plant Cell Reports* **19(4)**: 339–44

Sasikumar B, Veluthambi K (1994). Kanamaycin sensitivity of cultured tissues of *Piper nigrum* L. *Journal of Spices and Aromatic Crops* **3**: 158–60

Sasikumar B, Veluthambi K (1996). Strain and vector specificity in *Agrobacterium* black pepper interaction. *Journal of Plantation Crops* **24**: 597–602

Savary S, Ficke A, Aubertot JN, Hollier C (2012). Crop losses due to diseases and their implications for global food production losses and food security. *Food Security* **4**: 519–537

Savenkov EI, Valkonen JP (2001). Coat protein gene mediated resistance to *Potato virus* A in transgenic plants is suppressed following infection with another *potyvirus*. *Journal of General Virology* **82(9)**: 2275–8

Schenk RU, Hildebrandt AC (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* **50**: 199–204

Scholthof KB, Adkins S, Czosnek H, Palukaitis P, Jacquot E, Hohn T, Hohn B, Saunders K, Candresse T, Ahlquist P, Hemenway C (2011). Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology* **12(9)**: 938–54

Schwind N, Zwiebel M, Itaya A, Ding B, Wang MB, Krczal G, Wassenegger M (2009). RNAi–mediated resistance to Potato spindle tuber viroid in transgenic tomato expressing a viroid hairpin RNA construct. *Molecular Plant Pathology* **10(4)**: 459–69

Scorza R, Hily JM, Callahan A, Malinowski T, Cambra M, Capote N, Zagrai I, Damsteegt V, Briard P, Ravelonandro M (2007). Deregulation of plum pox resistant transgenic plum 'honeysweet'. *Acta Horticulturae* **738**: 669–673

Scott IM, Jensen HR, Philogene BJR, Arnason JT (2008). A review of *Piper* spp. (Piperaceae)–Phytochemistry, insecticidal activity and mode of action. *Phytochemistry Reviews* **7**: 65–75

Semizarov D, Frost L, Sarthy A, Kroeger P, Halbert DN, Fesik SW (2003). Specificity of short interfering RNA determined through gene expression signatures. *Proceedings of the National Academy of Sciences* **100(11)**: 6347–52

Seppanen P, Puska R, Honkanen J, Tyulkina LG, Fedorkin O, Morozov SY Atabekov JG (1997). Movement protein–derived resistance to triple gene block–containing plant viruses. Journal *of General Virology* **78**: 1241–1246

Shade RE, Schroeder HE, Pueyo JJ, Tabe LM, Murdock LL, Higgins TJ, Chrispeels MJ (1994). Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles. *Bio/technology* **12(8)**: 793

Shakesby A, Wallace IS, Isaacs HV, Pritchard J, Roberts DM, Douglas AE (2009). A water–specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology* **39(1)**: 1

Shao Y, Chan CY, Maliyekkel A, Lawrence CE, Roninson IB, Ding Y (2007). Effect of target secondary structure on RNAi efficiency. *RNA* **13**: 1631–1640

Sharma N, Rathore M, Singh NK, Gothalwal R, Ahmed Z, Kumar N (2009). Studies on *in vitro* regeneration and kanamycin sensitivity in cucumber (*Cucumis sativus* L.) cv. Poinsett. *Indian Journal of Crop Science* **4(1–2)**: 55–9

Shashidhara S, Lokesh MS, Lingaraju S, Palakshappa MG (2009). Integrated disease management of foot rot of black pepper caused by *Phytophthora capsici* L. *Karnataka Journal of Agricultural Sciences* **22**: 444–447

Shekhawat UK, Ganapathi TR, Srinivas L, Bapat VA, Rathore TS (2008). *Agrobacterium*–mediated genetic transformation of embryogenic cell suspension cultures of *Santalum album* L. *Plant Cell Tissue and Organ Culture* **92(3)**: 261–71

Shen WJ, Forde BG (1989). Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Research* **17(20)**: 8385

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989). Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* **338(6212)**: 274

Shina Sasi, AI Bhat (2016). Optimization of cyclic somatic embryogenesis and assessing genetic fidelity in six varieties of black pepper (*Piper nigrum* L). *Journal of Medicinal Plants Studies* **4(4)**: 109–115

Shintaku MH, Zhang L, Palukaitis P (1992). A single amino acid substitution in the coat protein of *Cucumber mosaic virus* induces chlorosis in tobacco. *Plant Cell* **4**: 751–757

Sievers Fabian, Andreas Wilm, David Dineen, Toby J Gibson, Kevin Karplus, Weizhong Li, Rodrigo Lopez (2011). Fast scalable generation of high–quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* **7(1)**: 539

Sijen T, Wellink J, Hendriks J, Verver J, van Kammen A (1995). Replication of *Cowpea mosaic virus* RNA1 or RNA2 is specifically blocked in transgenic *Nicotiana benthamiana* plants expressing the full–length replicase or movement protein genes. *Molecular Plant Microbe Interactions* **8**: 340–348

Siju S, Madhubala R, Bhat AI (2007). Sodium sulphite enhances RNA isolation and sensitivity of *Cucumber mosaic virus* detection by RT–PCR in black pepper. *Journal of Virological Methods* **141(1)**: 107–10

Sim SL, Jafar R, Powel J, Davey MR (1998). Development of an *Agrobacterium* mediated transformation system for black pepper (*Piper nigrum* L.). *Acta Horticulturae* **461**: 349–353

Simon–Mateo C, Garcia JA (2006). MicroRNA–guided processing impairs *Plum pox virus* replication, but the virus readily evolves to escape this silencing mechanism. *Journal of Virology* **80**: 2429–2436

Simon–Mateo C, Garcia JA (2011). Antiviral strategies in plants based on RNA silencing. *Biochimica et Biophysica Acta – Gene Regulatory Mechanisms* **1809(11–12)**: 722–31

Singareddy V, Sheri VR, Muddanuru T, Tatineni R, Jain RK, Sankaraneni CR, Kodeboyina VS, Mulpuri S (2018). Genetic engineering of sunflower (*Helianthus annuus* L.) for resistance to necrosis disease through deployment of the *Tobacco streak virus* coat protein gene. *Plant Cell Tissue and Organ Culture* **135(2)**: 263–77

Singh ND, Sahoo L, Sonia, Jaireal PK (2002). *In vitro* shoot organogenesis and plant regeneration from cotyledonary node and leaf explants of pigeon pea (*Cajanus cajan* L. Millsp). *Physiology and Molecular Biology of Plants* **8**: 113–140

Singh Z, Jones RAC, Jones M (1995). Identification of *Cucumber mosaic virus* subgroup I isolates from banana plants affected by infectious chlorosis disease using RT–PCR. *Plant Disease* **79**: 713–716

Sitepu D, Kasim R (1991). Black pepper diseases in Indonesia and their control strategy, In: Disease of black pepper proceedings of the International pepper community workshop on joint research for the control of black pepper diseases, Goa, India (eds Sarma YR, Premkumar T), National research centre for spices, Calicut, India pp: 13–28

Sivaraman K, Kandiannan K, Peter KV, Thankamani CK (1999). Agronomy of black pepper (*Piper nigrum* L.). *Annual Review of Journal of Spices and Aromatic Crops* **8**: 1–18

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000). Gene expression: total silencing by intron–spliced hairpin RNAs. *Nature* **407(6802)**: 319–320

Smith RH, Hood EE (1995). *Agrobacterium tumefaciens* transformation of monocotyledons. *Crop Science* **35(2)**: 301–9

Smyth DR (1999). Gene silencing: plants and viruses fight it out. *Current Biology* **9(3)**: R79

Soards AJ, Murphy AM, Palukaitis P, Carr JP (2002). Virulence and differential local and systemic spread of *Cucumber mosaic virus* in tobacco are affected by the CMV 2b protein. *Molecular Plant Microbe Interactions* **15(7)**: 647–53

Srinivasan K (2007). Black pepper and its pungent principle–piperine: a review of diverse physiological effects. *Critical Reviews in Food Science and Nutrition* **47(8)**: 735–748

Srivastava A, Raj SK (2004). High molecular similarity among Indian isolates of *Cucumber mosaic virus* suggests a common origin. *Current Science* **25**: 1126–31

Srivastava A, Raj SK (2008). Coat protein–mediated resistance against an Indian isolate of the *Cucumber mosaic virus* subgroup IB in *Nicotiana benthamiana*. *Journal of Biosciences* **33(2)**: 249

Stam M, Viterbo A, Mol JN, Kooter JM (1998). Position–dependent methylation and transcriptional silencing of transgenes in inverted T–DNA repeats: Implications for post transcriptional silencing of homologous host genes in plants. *Molecular Cell Biology* **18**: 6165–6177

Suzuki M, Kuwata S, Masuta C, Takanami Y (1995). Point mutations in the coat protein of *Cucumber mosaic virus* affect symptom expression and virion accumulation in tobacco**.** *Journal of General Virology* **76**: 1791–1799

Svabova L, Smykal P, Griga M, Ondrej V (2005). *Agrobacterium*–mediated transformation of *Pisum sativum in vitro* and *in vivo*. *Biologia Plantarum* **49(3)**: 361–70

Swaney S, Powers H, Goodwin J, Rosales LS, Dougherty WG (1995). RNA mediated resistance with nonstructural genes from the *Tobacco etch virus* genome. MOI. *Plant Microbe Interactions* **8**: 1004–1011

Tafer H, Ameres SL, Obernosterer G, Gebeshuber CA, Schroeder R, Martinez J, Hofacker IL (2008). The impact of target site accessibility on the design of effective siRNAs. *Nature Biotechnology* **26(5)**: 578

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30(12)**: 2725–9

Tang H, Ren Z, Krczal G (2000). An evaluation of antibiotics for the elimination of *Agrobacterium tumefaciens* from walnut somatic embryos and for the effects on the proliferation of somatic embryos and regeneration of transgenic plants. *Plant Cell Reports* **19(9)**: 881–7

Taskin KM, Ercan AG, Turgut K (1999). *Agrobacterium tumefaciens* mediated transformation of sesame (*Sesamum indicum* L.). *Turkish Journal of Botany* **23(5)**: 291–6

Tenllado F, Garcia–Luque I, Serra MT, Diaz–Ruiz JR (1995). *Nicotiana benthamiana* plants transformed with the 54–kDa region of the *Pepper mild mottle tobamovirus* replicase gene exhibit two types of resistance responses against viral infection *Virology–New York* **211**:170

Tenllado F, Garcia–Luque I, Serra MT, Diaz–Ruiz JR (1996). Resistance *to Pepper mild mottle tobamovirus* conferred by the 54–kDa gene sequence in transgenic plants does not require expression of the wild–type 54–kDa protein. *Virology* **219(1)**: 330–5

Tennant P, Fermin G, Fitch MM, Manshardt RM, Slightom JL, Gonsalves D (2001). *Papaya ringspot virus* resistance of transgenic Rainbow and SunUp is affected by

gene dosage, plant development, and coat protein homology. *European Journal of Plant Pathology* **107(6):** 645–653

Thomas CL, Jones L, Baulcombe DC, Maule AJ (2001). Size constraints for targeting post–transcriptional gene silencing and for RNA–directed methylation in *Nicotiana benthamiana* using a *Potato virus* X vector. *The Plant Journal* **25(4)**: 417–25

Thomas PE, Lawson EC, Zalewski JC, Reed GL, Kaniewski WK (2000). Extreme resistance to *Potato leafroll virus* in potato cv. Russet Burbank mediated by the viral replicase gene. *Virus Research* **71(1–2)**: 49–62

Thompson JD, Gibson T J, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25(24)**: 4876–4882

Thompson JR, Langenhan JL, Fuchs M, Perry KL (2015). Genotyping of *Cucumber mosaic virus* isolates in western New York State during epidemic years: characterization of an emergent plant virus population. *Virus Research* **210**: 169–77

Tijsterman M, Ketting RF, Plasterk RH (2002). The genetics of RNA silencing. *Annual Review of Genetics* **36(1)**: 489–519

Torreblanca R, Cerezo S, Palomo–Rios E, Mercado JA, Pliego–Alfaro F (2010). Development of a high throughput system for genetic transformation of olive (*Olea europaea* L.) plants. *Plant Cell Tissue and Organ Culture* **103(1)**: 61–9

Torreblanca R, Palomo Rios Elena, Cerezo, Sergio Mercado Jose, Pliego–Alfaro, Fernando (2009). *Agrobacterium*–mediated transformation of olive (*Olea europaea* L.) embryogenic cultures. *Acta Horticulturae* **839**: 387–391

Tousch D, Jacquemond M, Tepfer M (1994). Replication of *Cucumber mosaic virus* satellite RNA from negative–sense transcripts produced either in vitro or in transgenic plants. *Journal of General Virology* **75(5)**: 1009–14

Tricoli DM, Carney K J, Russell PF, McMaster JR, Groff DW, Hadden KC, Himmel PT, Hubbard JP, Boeshore ML, Quemada HD (1995). Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to *Cucumber mosaic virus*, *Watermelon mosaic virus* 2, and *Zucchini yellow mosaic virus*. *Bio/Technology* **13**: 1458–1465

Tripathi AK, Jain DC, Kumar S (1996). Secondary metabolites and their biological and medical activities of *Piper* species plants. *Journal of Medicinal and Aromatic Plants* **18**: 302–321

Tripathi S, Suzuki J, Gonsalves D (2007). Development of genetically engineered resistant papaya for *Papaya ringspot virus* in a timely manner. In Plant–Pathogen Interactions. Humana Press pp. 197–240

Tzfira T, Citovsky V (2002). Partners–in–infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. *Trends in Cell Biology* **12(3)**: 121–9

Umit A, Ilhan Kadir, Akgun KO (2009). Antifungal activity of aqueous extracts of spices against bean rust (*Uromyces appendiculatus*). *Allelopathy Journal* **24**: 0973–1046

Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer JJ (1993). Development of the particle inflow gun. *Plant Cell Tissue and Organ Culture* **33(3)**: 237–46

Valentine TA, Randall E, Wypijewski K, Chapman S, Jones J, Oparka KJ (2007). Delivery of macromolecules to plant parasitic nematodes using a *Tobacco rattle virus* vector. *Plant Biotechnology Journal* **5**: 827–834

Van de Velde W, Mergeay J, Holsters M, Goormachtig S (2003). *Agrobacterium rhizogenes*–mediated transformation of *Sesbania rostrata*. *Plant Science* **165(6)**: 1281–8

van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2**: 291–299

Vanderschuren H, Stupak M, Futterer J, Gruissem W, Zhang P (2007). Engineering resistance to geminiviruses–review and perspectives. *Plant Biotechnology Journal* **5(2)**: 207–20

Vasil IK (2005). The story of transgenic cereals: the challenge, the debate, and the solution–a historical perspective. *In Vitro Cellular and Developmental Biology–Plant* **41(5)**: 577–83

Vasil IK, Vasil V (1999). Transformation of wheat *via* particle bombardment. In Plant Cell Culture Protocols. Humana Press pp: 349–358

Vassilakos N, Bem F, Tzima A, Barker H, Reavy B, Karanastasi E, Robinson DJ (2008). Resistance of transgenic tobacco plants incorporating the putative 57 kDa polymerase read through gene of *Tobacco rattle virus* against rub–inoculated and nematode transmitted virus. *Transgenic Research* **17(5)**: 929–41

Vidal JR, Kikkert JR, Wallace PG, Reisch BI (2003). High–efficiency biolistic co–transformation and regeneration of' Chardonnay (*Vitis vinifera* L.) containing npt–II and antimicrobial peptide genes. *Plant Cell Reports* **22(4)**: 252–60

Vidal N, Mallon R, Valladares S, Meijomin AM, Vieitez AM (2010). Regeneration of transgenic plants by *Agrobacterium*–mediated transformation of somatic embryos of juvenile and mature *Quercus robur*. *Plant Cell Reports* **29(12)**: 1411–22

Voinnet O, Pinto YM, Baulcombe DC (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proceedings of the National Academy of Sciences* **96(24)**: 14147–52

Wagaba H, Beyene G, Trembley C, Alicai T, Fauquet CM, Taylor NJ (2013). Efficient transmission of cassava brown streak disease viral pathogens by chip bud grafting. *BMC Research Notes* **6(1)**: 516

Wagiran (2003). Development of biolistic transformation of Impatiens balsamina L. Master of philosophy Thesis, University of Reading, UK

Wahid P, Sitepu D, Deciyanto S, Ujang Superman D (1992). In proceedings of the international workshop on black pepper diseases (eds. Sitepu and Ujang–Superman), Agency for agricultural research and development, Research institute for spices and medicinal crops. Bogor, Indonesia

Wang PG, Zoubenko O, Tumer NE (1998). Reduced toxicity and broad spectrum resistance to viral and fungal infection in transgenic plants expressing pokeweed antiviral protein II. *Plant Molecular Biology* **38(6)**: 957–964

Wang R, Du Z, Bai Z, Liang Z (2017). The interaction between endogenous 30S ribosomal subunit protein S11 and *Cucumber mosaic virus* LS2b protein affects viral replication, infection and gene silencing suppressor activity. *PloS One* **12(8)**: e0182459

Wang Y, Tzfira T, Gaba V, Citovsky V, Palukaitis P, Gal–On A (2004). Functional analysis of the *Cucumber mosaic virus* 2b protein: pathogenicity and nuclear localization. *Journal of General Virology* **85**: 3135–47

Waterhouse PM, Graham MW, Wang MB (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proceedings of the National Academy of Sciences* **95(23)**: 13959–64

Waterhouse PM, Helliwell CA (2003). Exploring plant genomes by RNA induced gene silencing. *Nature Reviews Genetics* **4(1)**: 29

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP (2001). Construct design for efficient, effective and high–throughput gene silencing in plants. *The Plant Journal* **27(6)**: 581–90

Wintermantel WM, Zaitlin M (2000). Transgene translatability increases effectiveness of replicase–mediated resistance to *Cucumber mosaic virus*. *Journal of General Virology* **81(3)**: 587–95

Wu G, Kaper JM (1995). Competition of viral and satellite RNAs of *Cucumber mosaic virus* for replication *in vitro* by viral RNA–dependent RNA polymerase. *Research in Virology* **146(1)**: 61–7

Wu X, Beachy RN, Michael T, Wilson A, Shaw JG (1990). Inhibition of uncoating of *Tobacco mosaic virus* particles in protoplasts from transgenic tobacco plants that express the viral coat protein gene. *Virology* **179(2)**: 893–5

Xia GM, Li ZY, He CX, Chen HM, Richard B (1999). Transgenic plant regeneration from wheat (*Triticum aestivum* L) mediated by *Agrobacterium tumefaciens*. *Acta Physiologica Sinica* **25**: 22–28

Xiao YH, Yin MH, Hou L, Pei Y (2006). Direct amplification of intron containing hairpin RNA construct from genomic DNA. *Biotechniques* **41(5)**: 548–52

Xing Y, Ji Q, Yang Q, Luo Y, Li Q, Wang X (2008). Studies on *Agrobacterium* mediated genetic transformation of embryogenic suspension cultures of sweet potato. *African Journal of Biotechnology* **7(5)**: 534–540

Xu G, Sui N, Tang Y, Xie K, Lai Y, Liu Y (2010). One–step, zero–background ligation–independent cloning intron–containing hairpin RNA constructs for RNAi in plants. *New Phytologist* **187(1)**: 240–50

Yadav K, Yadav P (2017). The effect of different mechanism of transmission on transavaibility of *Tomato Mosaic Virus*. *Journal of Pharmacognosy and Phytochemistry* **6(6)**: 448–50

Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notsuka K, Hibi T, Hayashi T, Matsuta N (2000). Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. *Plant Cell Reports* **19(7)**: 639–46

Yan P, Shen W, Gao X, Duan J, Zhou P (2009). Rapid one–step construction of hairpin RNA. *Biochemical and Biophysical Research Communications* **383(4)**: 464–8

Yan P, Shen W, Gao X, Li X, Zhou P, Duan J (2012). High–throughput construction of intron–containing hairpin RNA vectors for RNAi in plants. *PLoS One* **7(5)**: e38186

Yang Y, Bao M, Liu G (2010). Factors affecting *Agrobacterium* mediated genetic transformation of embryogenic callus of *Parthenocissus tricuspidata* Planch. *Plant Cell Tissue and Organ Culture* **102(3)**: 373–80

Yao W, Ruan M, Qin L, Yang C, Chen R, Chen B, Zhang M (2017). Field performance of transgenic sugarcane lines resistant to *Sugarcane mosaic virus*. *Frontiers in Plant Science* **8**:104

Ye X, Gilbertson A, Peterson MW, inventors (2007). Vectors and methods for improved plant transformation efficiency. US Patent Application No. US2007/0074314 A1

Yoshikawa N, Saitou Y, Kitajima A, Chida T, Sasaki N, Isogai M (2006). Interference of long–distance movement of *Grapevine berry inner necrosis virus*

in transgenic plants expressing a defective movement protein of *Apple chlorotic leaf spot virus*. *Phytopathology* **96(4)**: 378–85

Zambryski P, Joos H, Genetello C, Leemans J, van Montagu M, Schell J (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *The EMBO Journal* **2(12)**: 2143–50

Zhai H, Liu QC (2003). Studies on genetic transformatiom of embryogenic suspension cultures of sweet potato. *Agricultural Sciences in China* **2**: 791–7

Zhang BH, Liu F, Liu ZH, Wang HM, Yao CB (2001). Effects of kanamycin on tissue culture and somatic embryogenesis in cotton. *Plant Growth Regulation* **33**: 137–149

Zhang K, Liu J, Zhang Y, Yang Z, Gao C (2015). Biolistic genetic transformation of a wide range of Chinese elite wheat (*Triticum aestivum* L.) varieties. *Journal of Genetics and Genomics* **42(1)**: 39–42

Zhang P, Potrykus I, Puonti Kaerlas J (2000). Efficient production of transgenic cassava using negative and positive selection. *Transgenic Research* **9**: 405–415

Zhang X, Yuan YR, Pei Y, Lin SS, Tuschl T, Patel DJ, Chua NH (2006). *Cucumber mosaic virus*–encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes and Development* **20(23)**: 3255–68

Zhang X, Zhao H, Gao S, Wang WC, Katiyar–Agarwal S, Huang HD, Raikhel N, Jin H (2011). *Arabidopsis* Argonaute 2 regulates innate immunity via miRNA393 mediated silencing of a Golgi–localized SNARE gene, MEMB12. *Molecular Cell* **42(3)**: 356–66

Zhao D, Song GQ (2014). Rootstock–to–scion transfer of transgene–derived small interfering RNA s and their effect on virus resistance in nontransgenic sweet cherry. *Plant Biotechnology Journal* **12(9)**: 1319–28

Zhao ZY, Cai T, Tagliani L, Miller M, Wang N, Pang H, Rudert M, Schroeder S, Hondred D, Seltzel J, Pierce D (2000). *Agrobacterium* mediated sorghum transformation. *Plant Molecular Biology* **44**: 789–798

Ziebel H, Murphy AM, Groen SC, Tungadi T, Westwood JH, Lewsey MG, Moulin M, Kleczkowski A, Smith AG, Stevens M, Powell G, Carr JP (2011). *Cucumber mosaic virus* and its 2b RNA silencing suppressor modify plant aphid interactions in tobacco. *Science Reporter* **1**: 187

Zou C, Zhang Z, Wu S, Osterman JC (1998). Molecular cloning and characterization of a rabbit eIF2C protein. *Gene* **211**: 187–194

Zvereva AS, Pooggin MM (2012). Silencing and innate immunity in plant defense against viral and non–viral pathogens. *Viruses* **4(11)**: 2578–97

INTERNET RESOURCES

AICRPS – http://www.aicrps.res.in
dsCheck – http://dsCheck.RNAi.jp/
FAOSTAT, 2018 – http://www.fao.org/faostat
International Pepper Community (IPC), 2017 – www.ipcnet.org
National Centre for Biotechnology Information – www.Ncbi.nlm.nih.gov
National Center for Food and Agriculture Policy– http://www.ncfap.org
OligoCalc – http://www.northwestern.edu/biotools/ oligocalc.html
ORF finder – www.ncbi.nlm.nih.gov/projects/gorf
siDesign – http://www.dharmacon.com
Spices Board India 2018 – www.indianspices.com

ANNEXURE I

CULTURE MEDIA, ANTIBIOTIC STOCKS AND REAGENTS

CULTURE MEDIA

1.1 Luria Bertani (LB) HiVeg Broth, Miller

Constituent	Weight (g)
HiVeg hydrolysate	10
Yeast extract	5
Sodium chloride	10
pH adjusted to 7.5, volume made up to 1 litre, and sterilized by autoclaving	

1.2 LB HiVeg Agar, Miller

Constituent	Weight (g)
Bacto tryptone	5
Bacto yeast extract	1
Sodium chloride	10
Agar	15
pH adjusted to 7.5, volume made up to 1 litre, agar added and sterilized by autoclaving	

1.3 YEB Broth

Constituent	Weight (g)
Peptone	5
Yeast extract	1
Beef extract	5
Sucrose	5
Magnesium sulphate heptahydrate	0.24
Volume made up to 1 litre, pH adjusted to 7.2 and sterilized by autoclaving	

1.4 YEB Agar

Constituent	Weight (g)
Peptone	5
Yeast extract	1
Beef extract	5
Sucrose	5
Magnesium sulphate heptahydrate	0.24
Agar	15
pH adjusted to 7.2, volume made up to 1 litre, agar added and sterilized by autoclaving	

1.5 Liquid M9 Minimal Medium

Constituents	Volume (mL)
5X M9 salts	200
20% glucose solution	20
Volume made up to 1 litre and sterilized by autoclaving	

1.6 M9 Minimal Medium (Agar)

Constituents	Volume (mL)
5X M9 salts	200
20% glucose solution	20
Agar	15g
Volume made up to 1 litre, agar added and sterilized by autoclaving	

1.7 5X Minimal Salts composition

Constituents	Weight (g)
$Na_2HPO_4.7H_2O$	64
KH_2PO_4	15
NaCl	2.5
NH_4Cl	5.0
Volume made up to 1 litre	

1.8 SH Medium

Stock	Constituents	Weight (g)	Stock Vol (mL)	Vol/L (mL)
A	KNO_3	25	500	50
	$MgSO_4.7H_2O$	4.00		
	$NH_4H_2PO_4$	3.00		
	$CaCl_2.2H_2O$	2.00		
B	$MnSO_4.2H_2O$	1.00	500	5
	H_3BO_3	0.50		
	$ZnSO_4.7H_2O$	0.10		
	KI	0.10		
	$CuSO_4.5H_2O$	0.02		
	Na_2MoO_4	0.01		
	$CoCl_2$	0.01		
C	$FeSO_4.7H_2O$	1.50	500	5
	Na_2EDTA	2.00		
D	Inositol	25	250	10
E	Thiamine HCl	0.50	500	10
	Nicotinic Acid	0.05		
	Pyridoxine HCl	0.05		
	Sucrose	10–30 g/L		
	Agar	8 g/L		
pH adjusted to 5.5–5.8, volume made up to 1 litre, agar added (for solid medium) and sterilized by autoclaving				

1.9 Woody plant Medium

Stock	Constituents	Weight (g)	Stock Vol (mL)	Vol/L (mL)
A	NH_4NO_3	10.000	250	10
	$Ca(NO_3)_2.4H_2O$	13.900		
B	K_2SO_4	24.750	500	20
C	$CaCl_2$	1.812	250	10
D	KH_2PO_4	4.250	250	10
	H_3BO_3	0.155		
	Na_2MoO_4	0.00625		
E	$MgSO_4.7H_2O$	9.250	250	10
	$MnSO_4.H_2O$	0.5575		
	$ZnSO_4.7H_2O$	0.215		
	$CuSO_4.5H_2O$	0.00625		
F	$FeSO_4.7H_2O$	0.695	250	10
	Na_2EDTA	0.9325		
G	Thiamine HCl		250	10
	Nicotinic	0.025		
	Acid	0.0125		
	Pyridoxine	0.0125		
	Hcl	0.050		
	Glycine			
H	Myo Inositol	2.500	250	10
	Sucrose	30 g/L		
	Agar	8 g/L		
	Charcoal	2 g/L		
Volume made up to 1 litre, pH adjusted to 5.5–5.8, agar added (for solid medium) and sterilized by autoclaving				

ANTIBIOTIC STOCKS FOR TISSUE CULTURE

1.9 Cefotaxime (50 mg/mL)

Cefotaxime (250 mg) dissolved in 5 mL HPLC grade water and filter sterilized.

2.0 Kanamycin (50 mg/mL)

Kanamycin (250 mg) dissolved in 5 mL HPLC grade water and filter sterilized.

2.1 Rifampicin (50 mg/mL)

Rifampicin (250 mg) dissolved in 5 mL DMSO and filter sterilized.

REAGENTS FOR CLONING AND TRANSFORMATION

2.2 Ampicillin stock solution (50 mg/mL)

Ampicillin sodium salt (100 mg) dissolved in 2 mL of deionized water and filter sterilized.

2.3 X–Gal stock solution (20 mg/mL)

X–Gal (5–bromo–3–indolyl–β–D–galactopyranoside) (40 mg) dissolved in 2 mL N, N–dimethylformamide. Stored at –20°C in the dark. Alternatively, X–Gal solution, commercially available was used

2.4 IPTG stock solution (0.200 mg/mL)

IPTG (isopropyl–β–D–thiogalactopyranoside) (0.400 mg) dissolved in 2 mL deionized water. Filter sterilized, aliquoted and stored at 4°C. Alternatively, IPTG solution, commercially available was used

2.5 LB–ampicillin X–Gal/IPTG plates

LB agar (100 mL) was prepared and autoclaved. Medium was allowed to cool to 55°C, 100 µL of ampicillin stock solution (50 mg/mL), 200 µL of X–Gal stock solution (20 mg/mL), and 20 µL of IPTG solution (200 mg/mL) was added, mixed gently and poured to the plates. the plates were placed in laminar air flow for 30 min for solidifying of agar.

ANNEXURE II

STOCK SOLUTIONS/ REAGENTS/ BUFFERS

REAGENTS FOR DNA ISOLATION

2.1 DNA Extraction Buffer

Constituents	Volume (mL)
1 M Tris–HCl (pH 8)	5.0
0.5 M EDTA (pH 8)	0.4
5.0 M NaCl	14.0
CTAB	2%
Sterilized by autoclaving. Added 1% PVP and 500 µL of β–mercapto ethanol before use	

2.2 Phenol: chloroform: isoamyl alcohol (25:24:1)

Tris saturated phenol (25 mL), chloroform (24 mL) and isoamyl alcohol (1 mL) was mixed.

2.3 10% CTAB

CTAB (10 g) dissolved in 100 mL of water. Sterilized by autoclaving.

2.4 Chloroform: isoamyl alcohol

Chloroform (24 mL) and isoamyl alcohol (1 mL) mixed.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

2.5 Ethidium bromide (EtBr) (10 mg/mL)

Constituent	Weight or Volume
EtBr	100 mg
Distilled water	10 ml
Components were mixed and stirred on a magnetic stirrer for 1 h to ensure complete solubility. This was then transferred to an amber colored bottle and stored.	

2.6 50x Tris Acetic acid EDTA buffer (TAE)

Constituent	Weight or Volume
Tris base	242 g
Glacial acetic acid	7.1 ml
0.5 mM EDTA (pH 8.0)	100 ml
Volume was made up to 1000 mL with sterile double distilled water	

2.7 1x TAE

Constituent	Volume (mL)
50x TAE	10
Distilled water	490

ANNEXURE III

CHEMICALS, INSTRUMENTS AND COMPANIES

CHEMICALS	
1 kb DNA ladder (250 bp – 10 kb)	Thermo Scientific, Massachusetts, USA
6X loading dye	Thermo Scientific, Massachusetts, USA
*Bam*H1	Thermo Scientific, Massachusetts, USA
E. coli DH5α	Thermo Scientific, Massachusetts, USA
*Nsi*1	Thermo Scientific, Massachusetts, USA
pBR322 (50 ng/µL)	Thermo Scientific, Massachusetts, USA
pTZ57R/T	Thermo Scientific, Massachusetts, USA
Revert aid RT	Thermo scientific, Massachusetts, USA
RNasin	Thermo Scientific, Massachusetts, USA
*Sac*1	Thermo Scientific, Massachusetts, USA
QuantiFast™ SYBR Green PCR Master Mix	Qiagen, Hilden, Germany
Taq DNA polymerase	Genei, Merck Biosciences, USA
GeneJET gel extraction kit	Thermo Scientific, Massachusetts, USA
GeneJET Plasmid Miniprep Kit	Thermo Scientific, Massachusetts, USA
InsTAclone PCR cloning kit	Thermo Scientific, Massachusetts, USA
SERVICES	
Primers	Integrated DNA Technologies, Coralville, Iowa, USA.
Sequencing	Eurofins, Bangalore, India
INSTRUMENTS	
Gel documentation system (GeneSys)	Syngene, Synoptics Group, Cambridge, UK
Biophotometer plus	Eppendorf, Hamburg, Germany
Thermocycler	Mastercycler Gradient, Eppendorf, Hamburg, Germany

PUBLICATIONS

Research publications in peer reviewed journals

Revathy KA, Bhat AI (2019). Designing of siRNAs for various target genes of *Cucumber mosaic virus* subgroup IB. *Indian Journal of Biotechnology* 18:119–125. NAAS Rating : 6.29

Revathy KA, Bhat AI (2017). Complete genome sequencing of *Cucumber mosaic virus* from black pepper revealed rare deletion in the methyltranferase domain of 1a gene. *VirusDisease* 28: 309–314. NAAS Rating: 5.90

Research Publications in symposiums/seminars

- **Revathy KA, Bhat AI**, Graft inoculation of *cucumber mosaic virus* and screening for resistance in transgenic black pepper. In abstracts of 30th Kerala Science Congress organized by KSCSTE, CWRDM and Govt. Brennen College, Thalassery on 28–30 Jan 2018.
- **Revathy KA, Manamohan M, Bhat AI**, Designing of siRNAs for various target genes of *Cucumber mosaic virus* subgroup IB. In abstracts of National Seminar in Genome Informatics in Cancer and Agri Bioinformatics organized by KAU and Mercy College, Palakkad on 5th January 2017.
- **Revathy KA, Bhat AI**, Complete genome sequencing of *Cucumber mosaic virus* from black pepper revealed rare deletion in the methyltransferase domain of 1a gene. In abstracts of 6th International Conference 'Plant, Pathogens and People; Challenges in Plant, Pathology to Benefit Humankind' organized by Indian Phytopathological Society, New Delhi held on February 23–27, 2016.
- **Revathy KA, Bhat AI**, RNAi vector construction using 2b gene from black pepper isolate of *Cucumber mosaic virus.* In abstracts of National Symposium on Understanding host-pathogen interaction through science of omics held by Indian Phytopathological Society on March 16–17, 2015.
- **Revathy KA, Shina Sasi, Bhat AI**, Characterization of *Cucumber mosaic virus* infecting black pepper based on RNA2 and RNA3 sequences. In abstracts of PlacrosymXXI ; International Symposium on Plantation Crops, held at Kozhikode, Kerala on 10–12 December 2014.

NCBI Sequence submission

Revathy KA, Bhat AI (2016). Complete genome sequencing of Cucumber mosaic virus from black pepper revealed rare deletion in methyltransferase domain of 1a gene. Data accessible at NCBI GenBank database, KU947029, KU947030, KU947031

Indian Journal of Biotechnology
Vol. 18, April 2019, pp 119-125

Designing of siRNAs for various target genes of *Cucumber mosaic virus* subgroup IB

Revathy K A[1,2] and Bhat A I[1*]

[1]Division of Crop Protection, ICAR–Indian Institute of Spices Research, Marikunnu, Kozhikode 673012, Kerala, India
[2]University of Calicut, Malappuram, 673635, Kerala, India

Received 13 July 2018; revised 10 March 2019; accepted 18 March 2019

Cucumber mosaic virus (CMV) is a major production constraint in black pepper causing stunted disease. Resistant varieties are unavailable and control measures are not effective till now. Recently, RNA interference (RNAi) is the most promising strategy for combating virus infection in plants but the effectiveness depends on sequence specificity between the transgene and the targeting virus. This study was undertaken to design the most suitable region for double stranded RNA synthesis with maximum specificity and minimized off–targets for all the five genes of CMV from black pepper. A 400 bp off–target minimized region identified from each of the five genes was subjected to sequence polymorphism study with selected CMV subgroup IB strains and common 'siRNAs' were designed *in silico*. As 3b gene had the least variations (of 17%) with four common and potential siRNAs designed from this region *in silico*, a hairpin construct was assembled using this region in *Agrobacterium* that can be used for developing black pepper resistant to selected CMV subgroup IB strains.

Key words: *Cucumber mosaic virus*, black pepper, RNA interference, siRNA designing, hairpin construct

Introduction

Black pepper is known as the king of spices, one of the precious spice in the world and has played a major role in India's international trade. From an economic point of view, pepper yield is often low and variable. Viral disease known as stunted disease contribute to low yields and reduced fruit quality. Diseased plants show mottling and mosaic on leaves. Stunting of whole plant is seen under severe cases, spike length is greatly reduced and spikes are poorly filled causing yield reduction. Association of *Cucumber mosaic virus* (CMV) with the disease is reported from India, China, Sri Lanka and Brazil[1]. CMV spreads non–persistently through aphids and infected stem cuttings. Besides black pepper, more than 1200 crops from 100 plant families are affected by this virus[2].

CMV is a plus sense RNA virus (Genus: *Cucumovirus*, Family: Bromoviridae) with three RNAs enclosed in separate particles. RNA1 codes for a single gene 1a whereas RNA2 and RNA3 code for two genes each. The genes 1a and 2a have role in replication[3], 2b gene acts as the silencing suppressor[4], 3a gene codes for the movement protein and 3b gene, the coat protein important in transmission of the virus by aphids[5]. CMV strains are classified into subgroup I and II, subgroup I is further subdivided into IA and IB based on serology, symptomology and hybridization studies[6]. Complete genome sequencing of black pepper isolate of CMV revealed that the isolate belongs to subgroup IB.

Chemicals to control viruses are lacking and resistant varieties are unavailable. Thus, developing pathogen derived resistance (PDR) is the best management strategy, for which selection of suitable transgene is important. Recent studies show that transgenes in the form of hairpin structures where sense and antisense strands of the same sequence separated by an intron, will trigger RNA silencing and confer systemic resistance against viral genomes[7]. The mechanism known as RNA interference (RNAi) has been successfully used for conferring resistance to pathogens in crops and also for improving the quality traits in crops. However, the effectiveness of the mechanism depends on the sequence similarity between the transgene and the viral genome[8]. Thus, sequence polymorphism in the target gene limits the application of RNAi to a great extent. Hence, this study was undertaken to identify the most appropriate region among five genes of the current isolate for

*Author for correspondence:
Tel: +91–495–2731410
aib65@yahoo.co.in

VirusDis.
DOI 10.1007/s13337-017-0386-4

ORIGINAL ARTICLE

Complete genome sequencing of cucumber mosaic virus from black pepper revealed rare deletion in the methyltransferase domain of 1a gene

K. A. Revathy[1,2] · A. I. Bhat[1]

Received: 1 April 2017 / Accepted: 10 June 2017

Abstract The complete genome of cucumber mosaic virus (CMV) from black pepper was sequenced and compared with CMV isolates from subgroups I and II reported worldwide. Percent identity and phylogenetic analyses clearly indicated that the CMV isolate from black pepper (BP) belongs to subgroup IB. Sequence analyses also showed the presence of a rare deletion of nine nucleotides in the putative methyltransferase domain of 1a gene which was observed only in two more isolates of CMV among one hundred 1a gene sequences of CMV for which sequence information is available in the database. Interestingly this deletion is not present in the black pepper isolate of CMV from China (WN1) and from Indian long pepper that is closely related to black pepper. Percent identity analyses showed that the 3′untranslated region (UTR) of the three RNAs of the BP isolate were conserved with 91% identity whereas the 5′UTR of three RNAs showed 52–80% identity. The level of gene conservation among the subgroups was highest in coat protein and lowest in 2b. The values of nucleotide diversity studies were further consistent with the above data. The ratio of non-synonymous to the synonymous substitution of the five genes of three RNAs was in the order 1a > 2a > 2b > 3a > 3b and less than one for all the genes, indicating purifying selection. These clearly reflect that the protein encoded by RNA1 is highly tolerant to amino acid changes followed by that of RNA2 and, RNA3 is the least tolerant correlating to its functional importance.

Keywords Cucumber mosaic virus · Black pepper · Complete genome · Phylogenetic analysis · Nucleotide diversity

Introduction

Black pepper (*Piper nigrum* L.) (Piperaceae) is one of the economically important spice crops of India mainly grown in the states of Kerala and Karnataka. Known as the King of spices, it originated in the tropical evergreen forests of Western Ghats of India [23]. Though, India has the largest area under black pepper cultivation, its productivity is often low due to biotic and abiotic stresses. Cucumber mosaic virus (CMV) (genus: *Cucumovirus*, family *Bromoviridae*) is one of the major production constraint of black pepper in India. Along with piper yellow mottle virus, it causes stunted disease in black pepper which is the third major disease of this spice crop. In a survey conducted in 2004 in India, high incidence and severity of the disease was reported from black pepper plantations located especially at high altitudes such as Idukki and Wayanad districts of Kerala where the mean incidence ranged from 29 to 45% respectively and, Kodagu and Hassan districts of Karnataka where the mean incidence was 14.9 to 5.2% [4]. Besides black pepper CMV infects other related species such as Indian long pepper and betel vine [12]. Association of CMV with the disease has also been reported from Brazil [10] and Sri Lanka [7, 8]. Typical symptoms include stunting of plant, mottling and mosaic on leaves, reduction of spike length and poor filling of spikes leading to yield

Electronic supplementary material The online version of this article (doi:10.1007/s13337-017-0386-4) contains supplementary material, which is available to authorized users.

✉ A. I. Bhat
aib65@yahoo.co.in

[1] Division of Crop Protection, ICAR-Indian Institute of Spices Research, Marikunnu, Kozhikode, Kerala 673012, India

[2] University of Calicut, Malappuram, Kerala 673635, India

Published online: 17 June 2017

The doctoral thesis is submitted to University of Calicut, Malappuram, Kerala, India. Research described in this thesis was performed at Division of Crop Protection, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India.

Layout and design of the thesis : **Revathy K. A.**

Cover Design : **Sudhakaran A.**