CLONING AND EXPRESSION OF COAT PROTEIN GENE OF *SWEET POTATO LEAF CURL VIRUS* (SPLCV)

by

SRUTHY G.S.

(2013 - 09 - 107)

THESIS

Submitted in partial fulfilment of the

requirements for the degree of

B. Sc.-M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

B. Sc.-M. Sc. (INTEGRATED) BIOTECHNOLOGY

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695 522

KERALA, INDIA

2018

DECLARATION

I hereby declare that the thesis entitled "**CLONING AND EXPRESSION OF COAT PROTEIN GENE OF *SWEET POTATO LEAF CURL VIRUS* (SPLCV)**" is a bonafied record of research done by me and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship, or other similar title, of any other society or university.

Place: Vellayani

Date: **28-11-2018**

SRUTHY G.S.

(2013-09-107)

स्वच्छ भारत
एक कदम स्वच्छता की ओर

भा.कृ.अनु.प- केंद्रीय कन्द फसल अनुसंधान संस्थान

(भारतीय कृषि अनुसंधान परिषद, कृषि और किसान कल्याण मंत्रालय, भारत सरकार)
श्रीकार्यम, तिरुवनन्तपुरम-695 017, केरल, भारत

3

ICAR- CENTRAL TUBER CROPS RESEARCH INSTITUTE

(Indian Council of Agriculture Research, Ministry of Agriculture and Farmers Welfare, Govt. of India)
Sreekariyam, Thiruvananthapuram-695 017, Kerala, India

ISO 9001:2008

Dr. T.MAKESHKUMAR
Principal Scientist (Plant Pathology)
Division of Crop protection

e-mail: makeshkumar.t@icar.gov.in;
makeshctcri@gmail.com
Mobile: 9447158546

CERTIFICATE

Certified that this thesis entitled **"Cloning and expression of coat protein gene of sweet potato leaf curl virus (SPLCV)"**is a record of research work done by **Ms. Sruthy,G.S. (2013-09-107)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Sreekariyam
Date: 28/11/2018

Dr. T. Makeshkumar
(Major Advisor, Advisory committee)
Principal Scientist
Division of Crop Protection
ICAR-Central Tuber Crops Research Institute,
Sreekarivam

Phone : +91-471-2598551 to 2598554
Director (Per) : +91-471-2598431
(Res) : +91-471-2597211
Sr. Admn. Officer : +91-471-2598193
Fax : +91-471-2590063
E-mail : director.ctcri@icar.gov.in
ctcritvm@gmail.com
Web : http://www.ctcri.org

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Sruthy G.S. (2013-09-107) a candidate for the degree of B.Sc. - M.Sc. (Integrated) Biotechnology, agree that the thesis entitled "**Cloning and expression of Coat protein gene of *Sweet potato leaf curl virus* (SPLCV)**" may be submitted by Ms. Sruthy G.S. in partial fulfillment of the requirement for the degree.

28/11/18

Dr. T Makeshkumar

(Chairman, Advisory Committee)

Principal Scientist

Division of Crop Protection

ICAR-Central Tuber Crops Research Institute, Sreekariyam

Dr. Swapna Alex

(Member, Advisory Committee)

Professor and Head

Department of Plant Biotechnology

College of Agriculture, Vellayani

28/11/18

Dr. M. L. Jeeva

(Member, Advisory Committee)

Principal Scientist

Division of Crop Protection

ICAR-Central Tuber Crops Research Institute, Sreekariyam

28/11/18

Dr. K. Umamaheswaran

(Member, Advisory Committee)

Associate Director (RARS (SR)) & Professor

Department of Plant Pathology

College of Agriculture, Vellayani

28/11/18

Dr. R. Selvarajan

(External examiner)

Principal Scientist

(Plant pathology- virology)

ICAR-National Rsearch Centre for Banana, Thiruchirapalli,Tamilnadu

ACKNOWLEDGEMENT

Firstly, I would like to express my sincere gratitude to my advisor Dr. T. Makeshkumar for the continuous support of my study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my study. It was such a different learning experience for me to be his student.

Besides my advisor, I would like to thank the rest of advisory committee members, ***Dr. Swapna Alex, Dr. M.L. Jeeva*** *and* ***Dr. K. Umamaheswaran****, for their insightful comments and encouragement, scholarly wisdom they offered me. I also wish to express my sincere thanks to* ***Dr. Archana Mukherjee*** *Director, ICAR- CTCRI,* ***Dr. Anilkumar,*** *Dean College of Agriculture, Vellayani ,and* ***Dr. C.A. Jayaprakas****,Head of the division of plant protection for giving me the opportunity to be part of the CTCRI family for the while.*

My acknowledgement would be lacking if I don't mention my gratitude to my dear teacher ***Dr. Soni K.B.*** *and also* ***Dr. S.S. Veena,*** *Principal Scientist, ICAR-CTCRI, Thiruvanathapuram, for her constant help, encouragement and valuable suggestions during the research work and preparation of the thesis.*

A few lines are too short to make a complete account of my deep sincere thanks to ***Jayakrishnan chettan*** *and* ***Dr. T. R. Resmi*** *for there timley help and guidance.*

Special thanks go to the Transgenic Lab team, for everything that they taught me about lab work and for forgiving my mistakes and shortcomings. This thesis would not have been possible without their help and support. ***Dr.Anjali, Dr. Asha S, Adarsh chettan Dr. Karthikeyan S, Mr.Arutselvan , Dr. Sangeetha, Merlin chechi, Sumayya chechi, Iype chettan, Divya chechi*** *and my dear friend* ***Vishnunarayanan,*** *who was always there for me with a smile whenever I needed help or inspiration. They all have taught me various lessons of life and also made my lab memories wonderful.*

I owe a big thanks to all my dearest friends ***Shahana, Anji, Dathy , Bimal, saban, Athul, Achu, Bhagya, Pakkaran and Adithya*** *and all my classmates for their invaluable care, constant*

support and motivation. To my senior ***Aarathy*** *for helping and advising me throughout my work. All Heads and Staffs of ICAR-CTCRI, Sreekariyam and College of Agriculture, Vellayani for their uninterrupted help in providing necessary facilities and for extension of laboratory facilities for conducting the research work. I thank them for the kind cooperation extended at all stages.*

Last but not the least, I would like to thank my family: my dear parents and chettai for their love, blessing, prayers and encouragement. Thanks go without saying to the God almighty for everything and giving me strength to overcome my own setbacks. I am very much indebited to him for the blessings showered on me with which I was able to complete this research work successfully and in time.

Sruthy G.S.

DEDICATED TO MY DEAR

PARENTS AND FAMILY

CONTENTS

LIST OF TABLES

10

LIST OF FIGURES

12

13

LIST OF APPENDICES

14

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Micro litter
µM	Micro molar
3'	Three prime
5'	Five prime
A	Adenine
BLAST	Basic Local Arrangement Search Tool
bp	Base pair
C	Cytosine
cm	Centi metre
CP	Coat protein
CTAB	Cetyl trimethyl ammonium bromide
CTCRI	Central Tuber crops Research Institute
DNA	Deoxynucleic acid
E.coli	Estereshia coli
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
F	Forward primer
FAOSTAT	Food and Agriculture Organization Statistical database
g	Gram
G	Guanine
h	Hour

15

ha-	Hectare
IgG	Immunoglobulin G
Kb	Kilo base pair
kDa	Kilo Dalton
kg	Kilo gram
L	Litter
m	Meter
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Milimolar
NCM	Nitro cellulose membrane
nm	Nanomolar
°C	Degree Celsius
ORF	Open reading frame
PCR	Polymerase chain reaction
pM	Picomolar
R	Riverse primer
Rpm	Rotation per minute
RTC	Root and tuber crops
Sec	Seconds
SPCSV	Sweet potato chlorotic stunt virus
SPFMV	Sweet potato feathery mottle virus

16

SPLCV	Sweet potato leaf curl virus
SPLV	Sweet potato latent virus
SPMMV	Sweet potato mild mottle virus
SPVD	Sweet potato viral diseases
TBS	Tris buffered saline
T	Thymine
U	Unit
V	Volt

INTRODUCTION

18

1. INTRODUCTION

The cassava, sweet potato, yams and aroids serve as necessary part of agriculture, offer staple food for millions of people in diverse regions of the world. In many parts of the developing countries this has been widely used as a staple food for carbohydrate intake besides being used as animal feed and also in industries.

Among the diverse root and tuber crops, sweet potato *(Ipomoea batatas)* is the most important root crop after potato and cassava, ranks seventh in global food crop production. Sweet potato is a perennial dicotyledonous herbaceous crop that belongs to the family of *Convolvulaceae*. This crop can be consider as food security crop because of its wider adaptability, versatility, nutritional value, resistance to production stresses and high yield per unit area (Kays, 2005).

Sweet potatoes are grown broadly in many countries with tropical or subtropical climatic zones (Loebenstein *et al.*, 2003). Even though sweet potato originated in Central/ South America, about 80% of sweet potato production is concentrated in China and Sub Saharan Africa. In India, sweet potato is cultivated in about two lakh hectares of land yielding ca 1 million tonnes (FAOSTAT, 2015) and in Kerala, 312 ha of land are under sweet potato production yielding 488 tonnes (FIB, 2013).

Sweet potato is considered as a crop with greater potential due to its nutritional qualities such as carbohydrate content, richness in beta carotene, vitamin C and B6. This can also be considered as an industrial crop used in animal food supplement.

Sweet potatoes are propagated vegetatively through vine cuttings and are prone to many systemic infections. Usage of these planting materials may reduce the rate of sweet potato yields (Clark and Hoy, 2006). The

19

production of sweet potato gradually declines due to the diseases caused by fungi, bacteria, nematodes, viruses, and mycoplasma (Clark and Moyer, 1988). The combination of several biotic and abiotic factors, including mutations, viruses and other pathogens will also led to decline in yield and quality (Clark *et al.,* 2002; 2003).

Yield reduction in sweet potato is mainly caused by weevil followed by viruses (Ndunguru and Kapinga, 2007). More than 30 viruses have been known to cause infections in sweet potato and viruses in the nine families such as, *Bromoviridae, Bunyaviridae, Caulimoviridae, Closteroviridae, Comoviridae, Flexiviridae, Geminiviridae, Luteoviridae* , and *Potyviridae,* are considered as major threats to the crop. Half of these viruses were described as DNA viruses belonging to *Geminiviridae* and *Caulimoviridae* (Clark *et al.,* 2006).

The large family *Geminiviridae* composed of gemini virueses with circular, single-stranded DNA (ssDNA) genomes packaged within geminate particles. The *Geminiviridae*, classified into nine genera such as *Becurtovirus, Begomovirus, Eragrovirus, Mastrevirus, Curtovirus, Topocuvirus, Grablovirus, Capulavirus* and *Turncurtovirus* (Varsani *et al.,* 2017) on the basis of its genomic organization and biological properties.

Begomovirues are among the most widespread and damaging plant viruses in the world. Begomoviruses are transmitted by the whiteflies (*Bemisia tabaci)*. These viruses have single or bipartite genome and mainly cause infection in dicotyledonous plants (Leke *et al.,* 2014). A lot of begomovirus species (*Ipomoea yellow vein virus* (IYVV), *Sweet potato leaf curl Georgia virus* (SPLGV), *Sweet potato leaf curl virus* (SPLCV), *Sweet potato feathery mottle virus* (SPFMV), and *Sweet potato chlorotic stunt virus* (SPCSV)) have been reported to infect *Ipomoea* species (Lotrakul *et al.,* 2002; Lozano *et al.,* 2009).

20

Sweet potato leaf curl virus (SPLCV) and related sweepo viruses have been found in many countries, including Brazil, China, India, Italy, Kenya, Korea, Peru, Spain and Uganda (Banks *et al.,* 1999; Fuentes and Salazar, 2003; Briddon *et al.*, 2006; Kwak *et al.,* 2006; Luan *et al.,* 2006; Miano *et al.,* 2006; Lozano *et al.,* 2009; Paprotka *et al.,* 2010; Albuquerque *et al.,* 2011; Wasswa *et al.,* 2011).

Substantial yield loss in some sweet potato cultivars due to sweepo viruses reported by Fauquet and Stanley, 2003; Clark and Hoy, 2006).Young sweet potato plants infected with SPLCV exhibit symptoms such as upward curling of the leaves and vein swelling however, only a few symptoms remain in mature plants and most plants become symptomless. Even with the lack of symptoms, it can cause up to a 30% decrease in yield (Clark and Hoy, 2006).

Sweet potato leaf curl virus, an important limitation for sweet potato production. Diagnosis, identification, and characterization are essential for developing an appropriate management strategy. Serological detection of SPLCV from the extract is currently unavailable due to the shortage of an antiserum exact for SPLCV. The purification of virus for antiserum production has not been successful yet. Coat protein gene has been utilized for developing antiserum in different viruses (Abouzid *et al., 2002*). The way of producing antiserum using coat protein by cloning and expressing the gene is very much useful when the purification of virus is difficult.

The present study highlights the cloning and expression of coat protein gene of SPLCV for the production of antiserum, allows the development of relevant and inexpensive method for the detection of SPLCV infections.

REVIEW OF LITERATURE

22

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Root crops are plants that build up starchy roots, tubers, stems, bulbs or corms that act as food stores for the plant. They give a considerable part of world's food supply and are also a vital source of animal feeds. On a worldwide basis, approximately 55 percent of roots and tuber production is consumed as food; the residue is used as planting material, as animal feed or in the production of starch, distilled spirits, alcohol and a range of other minor products. These crops usually have low labor supplies and can be stored in the ground for periods (AATF, 2015).

Roots and tuber crops play an essential part in the human diet. Root and Tuber crops (RTCs), including cassava, sweet potato, yams, potato, and other root crops are essential to the agriculture and food security of more than 100 countries and on the whole component of the diet for 2.2 billion people (NHB, 2011).

2.2 SWEET POTATO *(Ipomoea batatas (L.) Lam.)*

Sweet potato is cultivated mostly in tropical and subtropical lowland agro-ecologies, although it is well adapted to other zones and can be grown over widely diverse environments. The sweet potato is a perennial, even though it is grown as an annual.

The sweet potato, a member of the *Convolvulaceae* or morning glory family, is grown for its enlarged storage roots; sometimes the tender leaves and shoots are consumed as a green leafy vegetable. Sweet potato is a hexaploid plant with 90 chromosomes. Most species of the genus *Ipomoea* have 30 chromosomes.The details of systematic classification of sweet potato is described in Table: 1

23

2.2.1 Origin and distribution

Sweet potato is the seventh largest food crop. The origin of sweet potato is Central America, but at present it is broadly distributed in many tropical and subtropical countries in diverse climacteric regions. Sweet potato can be grown all around the year and it can with stand adverse climatic conditions; thus it is considered as an "insurance crop."

The crop is primarily vital in Southeast Asia, Oceania, and Latin America regions and China claims about 90% of total world production. It was rapidly introduced to Europe, and later spread to Africa, perhaps from Spain or directly from tropical America. The Portuguese also carried sweet potatoes to India and Southeast Asia (Hall and Phatak, 1993; Martin and Jones, 1986).

2.2.3 Nutritional value

Sweet potatoes are considered as a typical food security crop. The crop can be harvested steadily. They are rich in dietary fibre, minerals, vitamins, and bioactive compounds such as phenolic acids and anthocyanins. Due to its unique features and nutritional value National Aeronautics and Space Administration (NASA) preferred sweet potatoes as a complete crop to be included into the menus for space missions (Benjamin, 2007).

Orange flesh sweet potatoes are rich in carotenoids, and they can improve vitamin A status, in developing countries. Sweet potato storage roots are good source of carbohydrates, considerable quantities of vitamin C, and moderate quantities of thiamin, riboflavin and niacin. Orange-fleshed cultivars contain high quantities of pro-vitamin A (Faber *et al.*, 2002).

These micronutrient disorders are known to impact negatively on the growth and development of children and also to reduce resistance to disease in both children and adults. Micronutrient deficiencies, specifically vitamin A, iron

24

and zinc, are public health problems in South Africa (SAVACG, 1996) and elsewhere. The nutrient content of 100 g of raw sweet potato given in the Table: 2.

2.2.4 Global production

Sweet potato ranks fifth in developing countries and is the sixth most important food crop after rice, wheat, potatoes, maize and cassava. Over 105 million tones are produces globally in each year. Approximately 95% of the global production centered in developing countries (CIP, 2017).

In India, sweet potato is cultivated in about two lakh hectares of land yielding ca 1 million tonnes (FAOSTAT, 2015) and in Kerala, 312 ha of land are under sweet potato production yielding 488 tonnes (FIB, 2013). Sweet potato is largely cultivated as rainfed crop in eastern India particularly the states of Odissa, West Bengal, Uttar Pradesh, Bihar and Jharkhand accounting for 77% of area and 82% of production of India (Edison *et al.,* 2009).

2.3 PRODUCTION CONSTRAINTS

Sweet potato production is affected by several pathogens such as fungi, bacteria, nematodes, viruses, and mycoplasma (Clark and Hoy, 2006). Among these viruses are the major threats to the production (Njeru *et al.,* 2008). In many parts of the world the reason for yield reduction is usage of virus infected tubers and vine cuttings for propagation. Around 80-90% crop losses have been recorded in areas affected by viral complexes (Mukasa *et al.,* 2004). In India incidence of *Sweet potato leaf curl* virus was reported by (Makeshkumar *et al.,* 2007).

2.4 VIRAL DISEASES IN SWEET POTATO

Sweet potato is cultivated through vegetative propagation using storage roots, shoot tips and stem cuttings. The high occurrence of viruses in sweet potato is the consequence of using infected stem cuttings as planting materials repeatedly and presence of insect vectors (aphids and whiteflies). These

viruses have a well-organized mechanism for their persistence and distribution in planting material.

Viral diseases frequently a reason for decrease in the yield and superiority of storage roots (Clark and Moyer 1988; Loebenstein *et al.*, 2003). Over 30 viruses from various genera including *Begomovirus, Carlavirus, Cavemovirus, Crinivirus, Cucumovirus, Enamovirus, Ipomovirus, Nepoviurus, Potyvirus, Solendovirus* and *Tospovirus* cause infections in sweet potato.

2.5 VIRUS INFECTING SWEET POTATO

In India occurrence of *Sweet potato leaf curl virus, Sweet potato feathery mottle virus, Sweet potato chlorotic stunt virus*, *Sweet potato mild mottle virus* and *Sweet potato latent virus* have been reported (Kumar *et al.,* 1991). Worldwide in total, more than 30 viruses cause diseases in sweet potato from these 23 have been assigned a formal taxonomic position by the ICTV.

Over 20 different viruses belonging to at least nine families infect sweet potato worldwide (Clark *et al.,* 2012; Valverde *et al.,* 2007). Several of these viruses occur in East Africa, including *Sweet potato feathery motte virus* (SPFMV), (genus Potyvirus), *Sweet potato mild mottle virus* (SPMMV, genus Ipomovirus). Others includes those in the family Closteroviridae which, includes *Sweet potato chlorotic streak virus* (SPCSV,genus Crinivirus), *Sweet potato chlorotic fleck virus* (SPCFV, genus Carlavirus) and *Sweet potato leaf curl virus* (SPLCV, genus begomovirus)

(Wasswa *et al.,* 2011). SPLCV is transmitted by *Bemisia tabaci* or through vegetative propagation. The virus also infects species of the *Convolvulaceae* which acts as alternative hosts after insect transmission (Valverde *et al.,* 2007). SPLCV occurs worldwide (Briddon *et al.,* 2006; Lotrakul *et al.,* 1998). The viruses infecting sweet potato are given in Table: 3.

2.6 GEMINIVIRIDAE

The family Geminiviridae represents a variety of plant viruses, and cause extensive crop losses throughout the world (Hanley-Bowdoin *et al.,* 1999; Briddon and Stanley, 2006). Members of this group cause severe diseases in wide range of species, and are accountable for a significant amount of crop damage worldwide. Most of these viruses play an intense agricultural importance (Rybicki *et al.,* 2000).

Based on genome organization, nucleotide sequence similarities, and biological properties, the *Geminiviridae* has been classified into nine genera such as *Becurtovirus, Begomovirus, Eragrovirus, Mastrevirus, Curtovirus, Topocuvirus, Grablovirus, Capulavirus* and *Turncurtovirus* (Varsani *et al.,* 2017) Figure: 1. The genome can either be single component nucleotides between 2500-3100. In some of the begomoviruses exhibit two similar sized components each between 2600 and 2800 nucleotides.

2.7 BEGOMOVIRUS

Begomoviruses are transmitted by whitefly (*Bemisia tabaci* Genn.) and enclose either monopartite (DNA-A) or bipartite (DNA-A and DNA-B) genome.

The genomes of begomoviruses native to the New World (NW) are mostly bipartite, whereas most Old World (OW) begomoviruses are monopartite and associated with additional ssDNA molecules; alphasatellites and betasatellites (Paprotka *et al.,* 2010). In the bipartite viruses, the genes required for viral replication and encapsidation are encoded by DNA-A and those required for movement, occur on DNA-B. This circular single-stranded DNA of approximately 1.3–1.4 kb with some sequence homology with genomic components of nanoviruses, encodes a replication-associated protein (Saunders *et al.,* 2002; Mansoor *et al.,* 2003).

There are a number of begomoviruses that have or require only one genomic component for infection. These monopartite begomoviruses include

Ageratum yellow vein virus (AYVV) (Tan *et al.,* 1995), *Cotton leaf curl virus* (CLCuV) (Briddon *et al.,* 2006), *Tomato yellow leaf curl virus* (TYLCV) (Navot *et al.,* 1991), and *Ipomoea yellow vein virus* (IYVV) (Banks *et al.,* 1999). Their genomic components have been suggested to be equivalent to DNA-A of bipartite begomoviruses (Tan *et al.,* 1995; Zhou *et al.,* 1998). The genome organization of begomovirus depicted in Figure: 2.

2.8 *SWEET POTATO LEAF CURL VIRUS*

Sweet potato leaf curl virus, belonging to the genus *Begomovirus* (SPLCV) (Onuki *et al.*, 2000). The incidence of SPLCV was first reported by Shinkai (1979) and Liao *et al.* (1979) from Japan and Taiwan. The virus has also been reported from USA, Brazil, Mexico, China, Korea, Puerto Rico (Lotrakul *et al.* 2002), and Kenya (Miamo *et al.*, 2006) and Peru (Fuentes and Salazar 2003).

The virus is transmitted by *B. tabaci* biotype B in a persistent manner and by grafting, recently seed trasmision of white fly infestation was reported by Kim *seeing that et al.,* 2015. Several *Ipomoea* species were susceptible to SPLCV. SPLCV can cause up to 30% reductions in yield. Osaki and Inouye, 1991 reported that the cellular alteration in the nucleoplasm, seeing that fibrillar rings and crystalline arrays of virus-like particles in infected sweet potato plants. Whole genome sequence of SPLCV (AF104036, 2,828nts) has been determined by Lotrakul and Valverde (1999). Its genomic DNA and organization is similar to that of monopartite begomoviruses. Co infection of SPLCV and SPFMV induce severe leaf distortion, general chlorosis and stunting (Kokkinos and Clark, 2004). The list of Sweet potato leaf curl virus strains reported are detailed in Table: 4.

2.8.1 Genome organisation

They have small, circular, single-strand DNA genomes consist of monopartite genome encapsidated in twinned icosahedral particles (Stanly *et al.,* 2005). The viral DNA has two open reading frames (ORFs - V1 and V2 in the virion sense and four ORFs (C1, C2, C3 and C4) in the complementary sense, separated by an intergenic region (IR). The AV1 gene encodes the viral coat

protein (CP) essential for viral transmission by *B. tabaci*. The viral properties such as its accumulation, symptom development and virus movements are encoded by AV2 gene (Padidam *et al.,* 1996). The replication-associated protein (Rep or C1), the transcriptional activator protein (TrAP or C2), are encoded by AC1 gene which controls viral gene expression, the replication-enhancer protein (REN or C3), required for viral DNA replication and C4 gene is a suppressor of posttranscriptional gene silencing (PTGS) (Noueiry *et al.,* 2000). The genome organization of *Sweet potato leaf curl virus* depicted in Figure: 3.

2.8.2 Symptomatology and host range

The *Sweet potato leaf curl virus* infected leaves shows inward leaf curling, vein swelling, darkening of tuber skin, and grooving of tubers in some sweet potato cultivars (Pardina *et al.*, 2012). Most of the symptoms disappear from leaves during aging process. Host range study revealed that SPLCV infection is restricted to plants in the genus *Ipomoea* within the family *Convolvulaceae* (Ling *et al.,* 2011).

SPLCV is transmitted gradually by the whitefly *B. tabaci* (Lotrakul *et al.,* 1998) (Figure: 4) and through vegetative propagation of sweet potato, using storage roots, shoot tips and stem cuttings. Also transmission through seeds was reported (Kim *et al.,* 2015). These propagation methods, in turn, lead to accumulation of viruses over subsequent propagations. Recently Zang *et al.,* (2014) reported that artificial infection methods for sweepo virus using agro inoculation is a highly efficient means of studying infectivity in sweet potato. Sweet potato leaf curl disease symptoms are detailed in Figure: 5.

2.9 VIRAL DETECTION METHODS

A number of diagnostics methods have been developed for the detection of sweet potato viruses which rely upon the biological, serological, and nucleic acid properties of these viruses (Valverde *et al.,* 2007; 2008). Detection and identification of these viruses are difficult because of low concentration and uneven distribution within the plant (Esbenshade and Moyer, 1982). Along with

these, presence of latex and phenolic compounds in sweet potato tissues (Abad and Moyer, 1992), occurrence of mixed infections, and multiple viral strains (Valverde *et al.,* 2008) augments the situation.

2.9.1 Serological based methods

Enzyme-linked immunosorbent assay (ELISA) and serologically specific electron microscopy (SSEM) (Cadena-Hinojosa and Campbell, 1981; Hoyer *et al.,* 1996; Fuentes *et al.,* 1996; Souto *et al.,* 2003; Gutierrez *et al.,* 2003; Untiveros *et al.,* 2007) is the most common serological methods used for the detection of several viruses infecting sweet potato plants.

Several diagnostic methods have been developed for the detection of SPLCV, based on symptoms on indicator hosts which rely upon the extensive host range study conducted by Ling *et al.* (2008) on SPLCV. Serological assays are currently unavailable for the detection of SPLCV due to difficulties in obtaining purified SPLCV antigen for antisera production.

Proper diagnosis and identification are essential for the development of appropriate management programs (Valverde *et al.,* 2008) for plant viruses. Unless fungal or bacterial infection, viral diseases cannot be controlled in a simple way. Control of viral diseases relies upon establishment, development, and dispersal of viruses in plants. Eradication of sources of infection and elimination of alternative hosts as well as vectors are the widely used methods to get rid of viral diseases (Clark and Moyer, 1988). Use of virus-free planting material, followed by the use of resistant cultivars is also widely accepted methods as a control strategy (Clark and Moyer, 1988; Loebenstein *et al.,* 2003; Valverde *et al.,* 2007; 2008).

2.9.2 Nucleic acid based methods

The development of reverse transcription (RT) and polymerase chain reaction (PCR) techniques has allowed virologists to compare these sweet potato viruses at the molecular level. Information obtained from comparative study of

biological and biochemical properties along with phylogenetic relationships with other plant viruses also can help to clarify the ambiguity of the taxonomy of sweet potato viruses. In addition to the conventional use of diagnostic host range and indicator plants, these molecular and serological techniques are useful for detection of viruses in sweet potato plants leading to the improvement of more practical and more efficient virus indexing programs than those used in the past.

2.9.3 Polymerase chain reaction

Virus detection using Polymerase chain reaction (PCR) methods were first published in the early 1990s (Vunsh *et al.,* 1990) and theoretically accessible the user extreme levels of specificity and sensitivity utilizing gel electrophoresis for interpretation of results. Advances in the field of molecular biology, nucleic acid based methods such as reverse transcription (RT) and the Polymerase Chain Reaction (PCR) began to be used in plant virus detection (Hsu *et al.,* 2005). Several degenerative primers have been designed to recognize the conserved regions of viral genomes of many species or the whole virus genus or family (Chen *et al.,* 2001; Posthuma *et al.,* 2002).

PCR is the well suited technique for the detection and identification of geminiviruses because they replicate via a double stranded, circular DNA intermediate and the replicative form which can serve as a template for PCR amplification (Stanley, 1991). Rojas *et al* ., (1993) designed degenerative primers coding conserved regions in DNA-A and DNA-B which serve as general primers for amplifying fragments of Gemini viruses. Wyatt and Brown (1996) used AV494/AC1048 degenerative primers targeting the middle or core region of coat protein. The primer SPG1/SPG2 has been used to identify several isolates of SPLCV in sweet potato plants due to its high sensitivity and its highly conserved annealing regions on open reading frames AC2 and AC1. Lotrakul *et al.,* 1998; Li *et al.,* 2004, used the same primers in their study to detect gemini virus infestation on sweet potato.

31

2.9.4 Rolling circle amplification

The perceptive of gemini viruses is transformed by one of the most recent technique known as, Rolling circle amplification (RCA). Very minute quantity of DNA sample can be utilized for diagnosis of all viruses. The bacteriophage Φ29 DNA polymerase used to amplify single or double stranded circular DNA template exponentially (Dean *et al.,* 2001; Inoue-Nagata *et al.,* 2004). This polymerase enzyme exhibit high fidelity DNA replication by the proof reading activity (Estaban *et al.,* 1993; Johne *et al.,* 2009).

The restriction enzymes were used to digest the RCA amplified products and can be used for direct DNA sequencing (Inoue-Nagata *et al.,* 2004). This technique consider all over the world for the virus detection due to the benefits of low cost, and smooth handling (Kushwaha *et al.,* 2010).

Haible *et al.,* 2006 used this method to construct tandemly repeated concatemers of whole genomes and these genome fragments were used for the generation of infectious clones in (*Ageratum yellow vein virus* (AYVV) ,*Tomato leaf curl virus* (TLCV)) and (*Squash leaf curl virus* (SqLCV)). RCA technique is proven to be an excellent tool for the classification of viral DNAs in infected plant materials (Fuji *et al.,* 2004) Figure: 6.

2.10 GENE EXPRESSION STUDIES

Serological methods are widely used in the detection of viral infection (Portsmann and Kiessing, 1992). Currently, serological methods, such as enzyme-linked immunosorbent assay (ELISA), to detect *Sweet potato leaf curl virus* (SPLCV) are not available due to the difficulties in obtaining purified virions that can be used as antigen for antiserum production. It is possible that either SPLCV virus particles occur in low concentration in plant tissues or viral particles are not stable after standard purification procedures (Gutiérrez *et al.,* 2015).

32

Escherichia coli (E.coli) is considered as a main host organism to produce high quantities protein of interest. Currently recombinant DNA technology along with its ease to manipulate and its rapid growing rate in a less expensive media used in the expression of proteins (antigen) for antibody production. More than a few approaches were used for the expression of antigens. One of the aspects is to express proteins in *E.coli* as to clone genes of interest into an expression vector coding for an amino terminus (Ashoub *et al.,* 2009).

2.10.1 Importance of coat protein gene expression in bacterial system

Cloning and expressing viral coat protein (CP) genes in bacteria can prevail over the difficulties in obtaining purified plant virus preparations for antiserum production (Meng *et al.,* 2003). In 2002 Abouzid *et al.,* prepared polyclonal antiserum specific for CP gene of four begomovirus- *Bean golden mosaic virus* (BGMV), *Tomato yellow leaf curl virus* (TYLCV), *Cabbage leaf curl virus* (CLCV), and *Tomato mottle virus* (ToMV) expressed in *E. coli*

33

1: Systematic classification

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnolyiophyta
Class	Magnoliospsida
Subclass	Asteridae
Order	Solanales
Family	*Convolvulaceae*
Genus	*Ipomoea*
Species	*batatas*

Table 2: Nutritional value of sweet potato

Characteristics	Equivalent weight
Starch (% fresh weight, FW)	18-28
Starch grain	2-40
Amylose (% starch)	8-32
Total sugars (% FW)	1.5-5.0
Proteins (% FW)	1.0-3.0
Fibers (% FW)	1.0
Vitamin A (microgram/ 100mg/FW)	900
Vitamin C(mg/100mg/FW)	35
Minerals (%FW)	1.0
Anti- nutritional compounds	Trypsin inhibitors

Table 3: List of viruses reported to infect sweet potato

Sl.No.	Virus name	Abb.	Family/genus	Transmission	Reference
1	*Sweet potato feathery mottle virus*	SPFMV	*Potyviridae (Potyvirus)*	Aphid	Moyer and Kennedy,1978; Skai *et al.*,1997
2	*Sweet potato virus G*	SPVG	*Potyviridae (Potyvirus)*	Aphid	Colinet *et al.*, 1994
3	*Sweet potato latent virus*	SPLV	*Potyviridae (Potyvirus)*	Aphid	Colinet *et al.*,1997
4	*Sweet potato leaf curl virus*	SPLCV	*Geminiviridae (Begomovirus)*	Whiteflies	Lotrakul and valverde 1999
5	*Sweet potato mild specking virus*	SPMSV	*Potyviridae (Potyvirus)*	Aphids	Alvarez *et al.*, 1997
6	*Sweet potato mild mottle virus*	SPMMV	*Potyviridae (Potyvirus)*	Aphids	Colinet *et al.*,1997
7	*Sweet potato chlorotic stunt virus*	SPCSV	*Caulimoviridae (Crinivirus)*	Aphids	Funetes and Salazar 1992

8	*Sweet potato collusive virus*	SPCV	*Caulimovirus*	*	Paproka *et al.*,2009
9	*Sweet potato virus 2*	SPV2	*Potyviridae*	Aphids	Lozano *et al.*, 2009
10	*Sweet potato virus C*	SPVC	*Potyviridae*	Aphids	Luan *et al.*, 2007
11	*Sweet potato symptomless virus*	SPSMV 1	*Gemniviridae*	*	Lozano *et al.*, 2009
12	*Sweet potato chlorotic fleck virus*	SPCFV	*Betaflexiviridae*	*	Lozano *et al.*, 2000
13	*Sweet potato vein clearing virus*	SPVCV	*Caulimoviridae*	*	Cohen *et al.*, 1997
14	*Sweet potato leaf speckling virus*	SPLSV	*Luteoviridae*	*	Cuellar *et al.*, 2011
15	*Sweet potato leaf curl Georgia virus*	SPLCGV	*Gemini viridae*	White flies	Lottrakl *et al.*,2003

16	*Sweet potatoC6 virus*	SPC6V	*Betaflexiviridae*	*	Lobenstein *et al.*,2009
17	*Sweet potato pakakuy virus*	SPPV	*Caulimoviridae*	*	Kreuze *et al.*,2009
18	*Sweet potato golden vein associated virus*	SPGVaV	*Begomovirus*	White flies	Kil *et al.*,2014

*- Not reported.

Table 4: List of *Sweet potato leaf curl virus* strains reported. 37

Sl. No.	GenBank accession No.	Species/Strain name	Country	Reference
1	EF456742	*Sweet potato leaf curl canary virus (SPLCCaV)*	Spain	Lozano *et al.*, 2009
2	KX611145	*Sweet potato leaf curl Georgia virus (SPLCGV)--[AR-MDZ]*	Argentina	Martino *et al.*, 2017
3	AJ132548	*Sweet potato leaf curl virus-[Sicily]*	Italy- Sicily	Briddon *et al.*, 2005
4	AJ586885	*Ipomoea yellow vein virus (IYVV)*	Italy	Banks *et al.*, 1999
5	FJ560719	*SPLCV Korean isolate (SPLCV-KR)*	Korea	Kim *et al.*,2015
6	DQ512731	*Sweet potato leaf curl virus (SPLCV-CN)*	China	Luan *et al.*, 2006
7	HQ333142.1	*Sweet potato leaf curl virus (SPLCV-US)*	United sates	Lotrakul *et al.*,1998
8	KM050768.1	*Sweet potato leaf curl virus*	India	Makeshkumar *et al.*, 2014

38

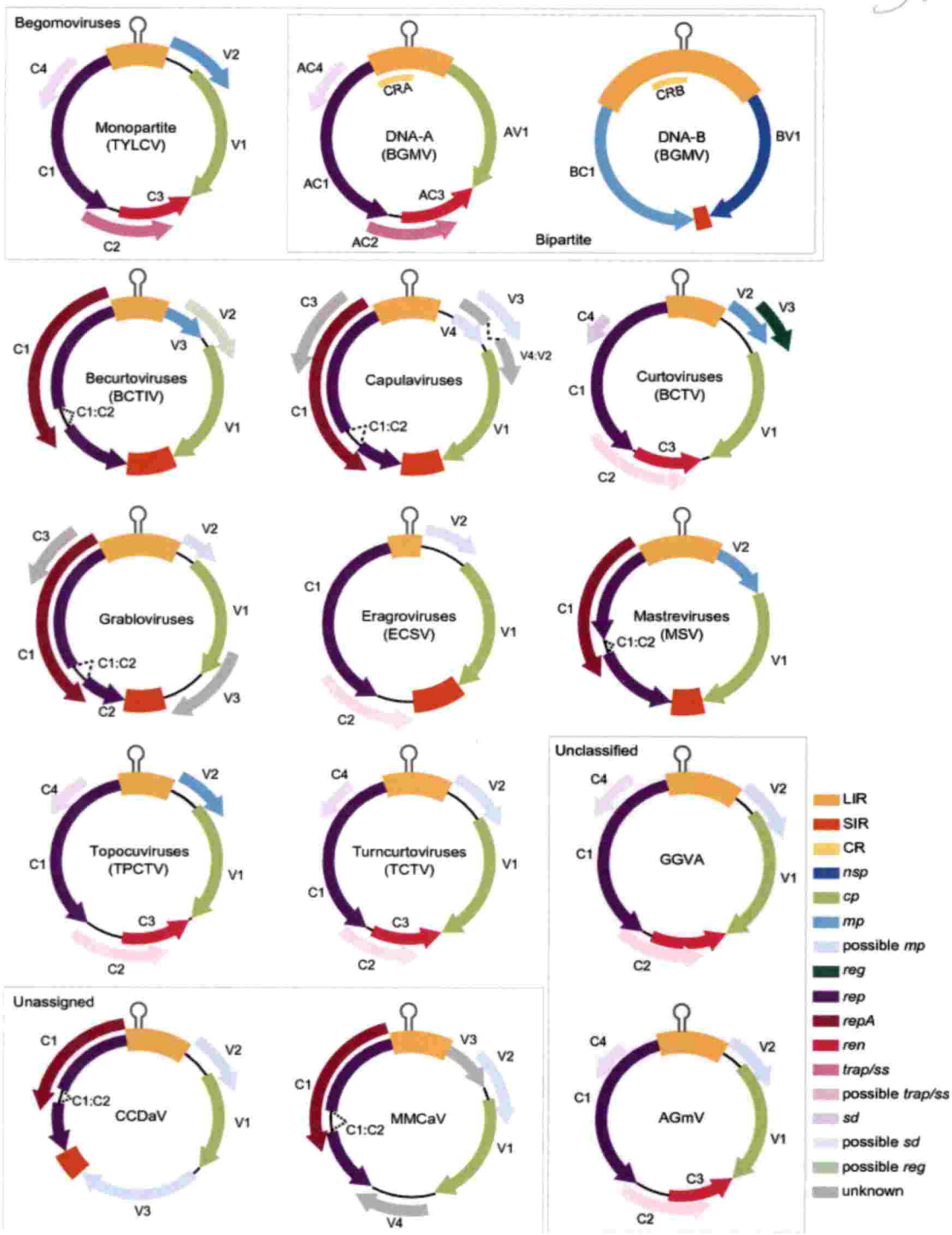


Figure 1: Gemini virus classification, Varsani *et al.*, 2017.

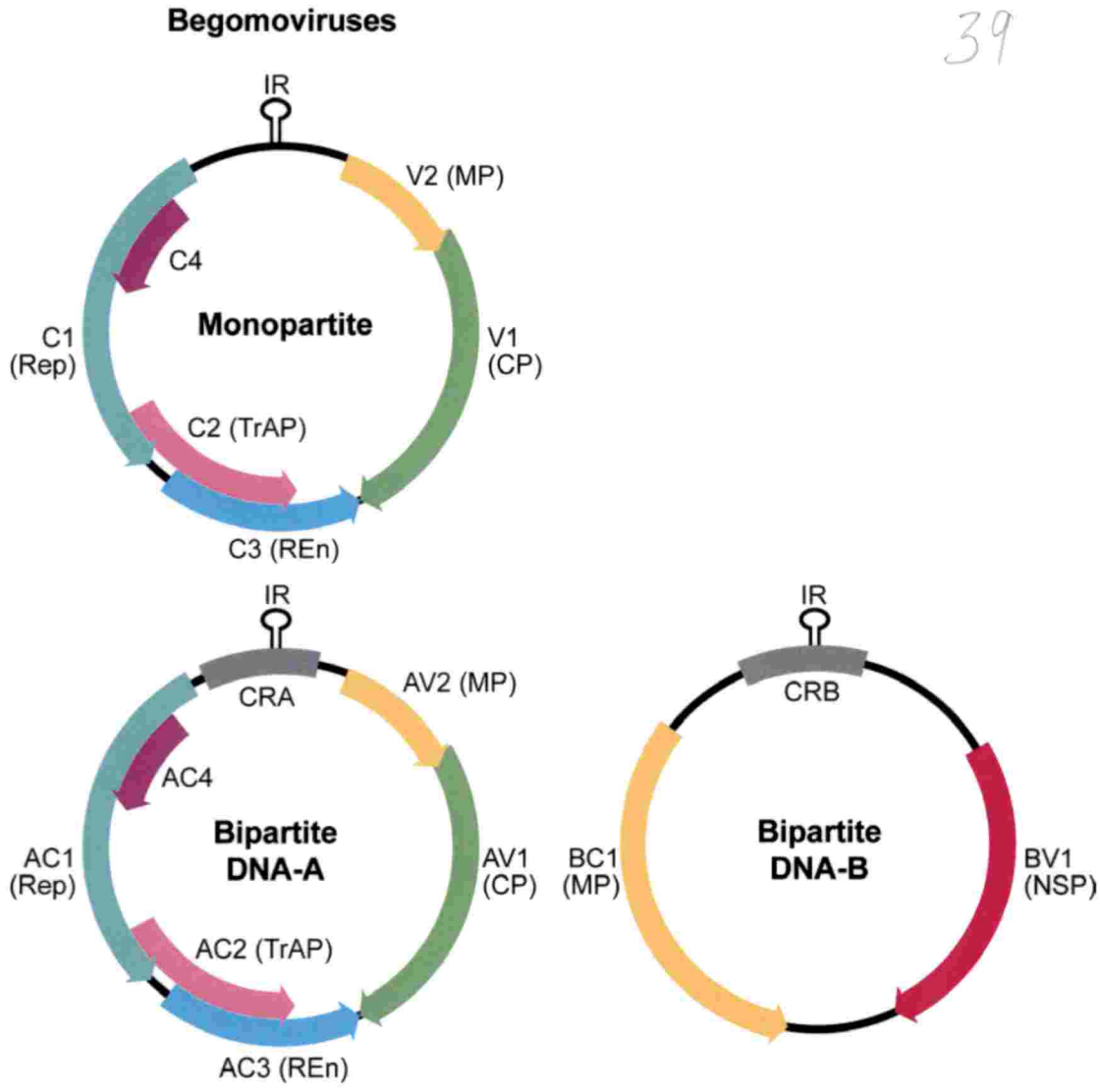


Figure 2: Genome organization of Begomoviruses.

40

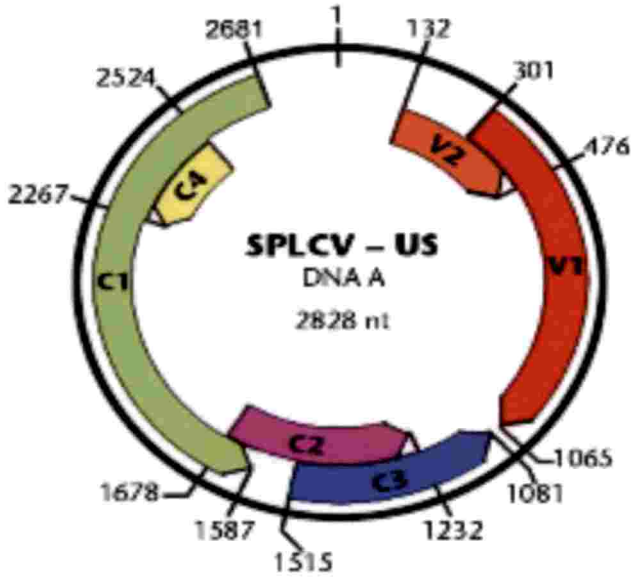


Figure 3: Typical genome organization of *Sweet potato leaf curl virus.*

Figure 4: White fly (*B. tabaci*) infestation on sweet potato leaves.

41

Cupping of the leaves
(*Ipomoea batatas*)

Yellow vein symptoms
(Ipomoea setosa)

Leaf blistering
(Ipomoea nil)

Extreme vein clearing
(*Ipomoea tricolor*)

Leaf distortion
(*Ipomoea batatas*)

Severe upward leaf curl
and mosaic symptoms
(*Ipomoea batatas*)

5

Figure 5: Symptoms of SPLCV in various *Ipomoea* species.

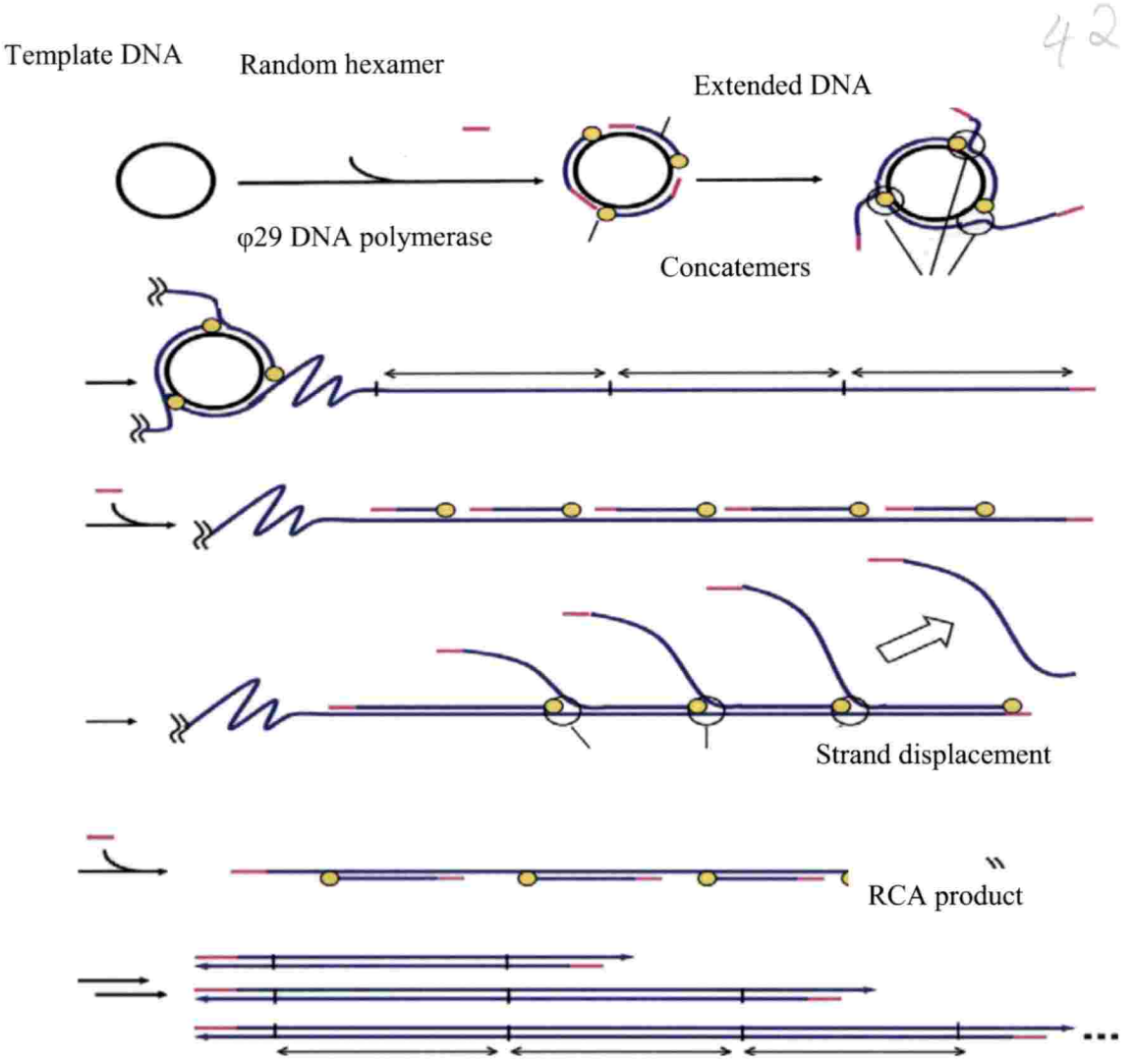


Figure 6: Schematic representation of Rolling Circle Amplification, Fujii *et al.*, 2006

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Cloning and expression of coat protein gene of *sweet potato leaf curl virus*" was carried out at the division of crop protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvanathapuram during 2017-2018. Details pertaining to the experimental material and procedures used in the study are elaborated in this chapter.

3.1 SAMPLE COLLECTION

Sweet potato samples belonging to various accessions were collected randomly from the germplasm collection maintained at ICAR-CTCRI, Thiruvananthapuram and Regional Centre-CTCRI, Bhubaneswar. Forty samples were collected from the field and from that 10 samples exhibiting leaf curl disease symptoms and suspected of virus infection was collected. The samples were photographed and symptoms were recorded and scored. The geographical origin of selected accessions is represented in the table 6.

3.2 NUCLEIC ACID EXTRACTION

For carrying out polymerase chain reaction (PCR) based detection of the viruses infecting sweet potato, DNA isolation is a prerequisite. PCR was performed on DNA isolated from infected leaf samples using different virus specific primers.

3.2.1 Standardization of DNA isolation protocol

The extraction of good quality DNA from sweet potato was difficult due to the presence of high amounts of polyphenols and mucilage.

3.2.2 DNA Isolation

For genomic DNA isolation, some modifications were done on CTAB method of DNA extraction (Doyle and Doyle, 1990).β- Mercaptoethanol and PVP was added to the final concentration of 2% (v/v). The buffer was pre- heated to 60°C in water bath .The samples (100 mg) were chilled and pulverized to the fine

45

powder by liquid nitrogen using a sterile motar and pestle and transferred in to a sterile 2ml centrifuge tubes containing 1ml of freshly prepared warm extraction buffer (Appendix I). The content was homogenized by gentile inversion and then incubated at 60°C in water bath for 30min with intermediate shaking. Then it was centrifuged at 12,000 rpm at 10minutes at room temperature. The supernatant was transferred to another sterile microfuge tubes with a clean sterile pipette tips. To this 10µL RNase was added and incubated at 37°C for 1 hour. The homogenate was extracted with an equal volume of 24:1(v/v) chloroform/isoamyl alcohol and mixed well by inversion for 5-10min and centrifuged at 15,000 rpm for 10 min at room temperature. To the aqueous phase, 0.8 volume of chilled isopropanol was added and mixed by inversion. The mixture was incubated at -20°C for at least 1hour or overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 15,000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was washed in 0.5ml of 70% ethanol twice, each time centrifuging at 12,000 g for 5min at room temperature and discarding the supernatant. The pellet was air dried 30-40 min and dissolved in 50µL of sterile distilled water. The extracted DNA was then stored at -20°C.

3.3 ANALYSIS OF THE EXTRACTED DNA

3.3.1 Agarose gel electrophoresis

The integrity and quality of the extracted DNA was checked in agarose gel electrophoresis. An agarose gel of 0.8 % was prepared in 1X TAE buffer (Appendix II) and ethidium bromide (EtBr) was added, 0.5µl/l. An aliquot of the DNA sample (2µl) mixed with the loading dye was loaded in each of the wells of the gel. The gel was run at 5 V/cm for 30 min. The gel was then visualized under UV light and the image was documented using Azure gel documentation system. The quantity of DNA was also measured using spectrophotometer (Denovix DS 11+ spectrophotometer).

46

3.4 MOLECULAR DETECTION OF VIRUSES USING POLYMERASE CHAIN REACTION

In order to detect the presence of *Sweet potato leaf curl virus* infected samples through PCR, different sets of primers were used and their details were provided in the Table 5 and Table 6. The primers were synthesized from Integrated DNA Technologies (IDT), USA. The synthesized primers (100 pM) were diluted to a final concentration of 10 pM with sterile water to obtain the working solution. The components of the mixture were optimized as listed below:

Water	12.2 µl
10X Taq buffer	2.5 µl
200M dNTP	1.0 µl
10Pm Forward primer	1.0 µl
10Pm Reverse primer	1.0 µl
Template DNA	2.0 µl
Taq polymerase	0.3 µl
Total volume	20 µl

Amplification was performed in a BioRad C100 Touch Thermocycler (Germany) with 38 cycles of initial denaturation 94°C for 2 min 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min, followed by a final extension cycle of 72°C for 10 min. PCR products were separated by electrophoresis on 1.2% (w/v) agarose gel and stained with ethidium bromide.

3.5 WHOLE GENOME AMPLIFICATION USING ROLLING CIRCLE AMPLIFICATION (RCA)

Rolling Circle Amplification (RCA) was carried out with the total DNA isolated from the selected samples for whole genome amplification. This amplification needs only little amount of DNA (10-20 ng) as template. The reaction mixture composed of 2µl of Φ 29 DNA polymerase buffer (10X), 2µl of exo-resistant

47

random hexamer primers (500μM) and 2μl of dNTPs (10mM). Template DNA was added to this mixture, mixed well and denatured for 3 min at 94°C and cooled down to room temperature. After cooling, 4 μl of Pyrophosphatase (0.1 U/ μl) and 0.7 μl of Φ 29 DNA polymerase (10 U/ μl) were added and incubated for 18-20 hrs at 30°C followed by heat inactivation at 65°C for 10 min. The products of RCA were analysed in 0.8% gel.

3.5.1 Restriction analysis of RCA products

RCA products were subjected to restriction digestion with *Bam*HIfor obtaining 2.7 kb fragments which represents full length genomic DNA respectively. Similarly, PBS2KS+ vector DNA vector was also restricted with *Bam*HI for ligating the restricted RCA products.

The components of the restriction mixture were optimized as listed below:

Cutsmart buffer (10X)	1.0 μl
*Bam*HI HF	0.5 μl
Template DNA /PBS2KS+ vector DNA	2.0 μl
Water	6.5 μl
Total volume	10 μl

Restriction digestion was carried out at 37°C for 25 min followed by incubation at 65°C for 10 min for enzyme inactivation. The restricted products along with PCR Marker (1 kb plus) from 'Thermo Scientific' were separated on agarose gel (1%). The gel was viewed under gel documentation system.

3.6 CLONING AND TRANSFORMATION

3.6.1 Cloning of RCA restricted products

The eluted DNA A fragment was cloned into PBS2KS+ vector DNA and transformed into *Escherichia coli* DH5α using manual method as described by Sambrook *et al.,* (2000). The recombinant clones obtained were analyzed by colony PCR method.

48

The cloning and transformation protocol was performed in three consecutive days.

Day 1: *E. coli* DH5α cells revived in Luria agar medium were used for the transformation procedure. The ligation mix was prepared with the components as listed below.

Vector PBS2KS+ vector DNA (restricted with *BamHI* HF)	2.0 µl
10x ligation buffer	1.5 µl
RCA product (restricted with *BamH1* HF)	8.0 µl
T4 DNA ligase	2.0 µl
Total volume	15.0 µl

After vortexing, the ligation mix was centrifuged for a few sec and incubated overnight at 4°C.

Day 2:

Preparation of competent cells

To fresh 50 ml LB broth (Appendix VI), 500µl overnight grown *E. coli* DH5α cells were inoculated and incubated at 37 °C with shaking at 200 rpm for 90 min. The cells were pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. Supernatant was discarded and the pellet was washed with 10 ml 100mM $MgCl_2$, mixed well and centrifuged at 10,000 rpm for 10 min at 4°C. The cell was then dispersed with 10 ml of 100 mM $CaCl_2$ and kept in ice for 1hr. After1hr, centrifuged at 10,000 rpm for 10 min. To the pellet, 1 ml of 100 mM $CaCl_2$ was added, mixed well and again kept in ice for 1 hr. The cells will now become competent for transformation.

Transformation

A volume of 50 µl of competent cells were added to each tube containing ligation mix and kept on ice for 1 hr. The mixture was heat shocked at 42°C for 2 min and immediately the tubes were quenched into ice. Then, 1ml of the LB medium was added to each tube and incubated at 37°C with shaking at 200 rpm for 1 hr. The cells were then concentrated by centrifuging at 7000 rpm for 1 min. Finally the cell suspensions were spread on LA ampicillin /X gal/ IPTG agar plates. The plates were incubated overnight at 37°C and observed for the growth of colonies next day.

Day 3:

Analysis of recombinant colonies

Analysis of the positive colonies containing the insert was confirmed through colony PCR using coat protein specific primers M13F/R, SPG1/SPG2, SPG3/SPG4, PW285 1/2.

3.6.2 Plasmid isolation of transformed white colonies

Colonies which showed positive in colony PCR were selected for plasmid isolation for carrying out the downstream applications like further confirmation for the presence of inserts through restriction and sequencing.

Plasmid isolation was done using the manual method formulated as follows.

A single colony (Transformed white colony) was inoculated on 2 ml LB broth with appropriate antibiotic [Here, ampicillin (50 µg/ml)] and incubated overnight with shaking of 150 rpm at 37°C. Next day, bacterial cells were pelletized by centrifuging at 10,000 rpm for 10 min at room temperature. After discarding the supernatant, the pellet was resuspended in 100 µl Buffer P1 (Appendix VI) and vortexed. To this,10 µl activated RNase (10 mg/ml) was added and incubated for 5 min at 37°C. To this, 200 µl buffer P2 (Appendix VI) was added and gently mixed by inverting 6 to 7 times and incubated on ice for 5 min. To this, 150 µl of buffer

50

P3 (Appendix VI) was added, mix gently by inverting 6 to 7 times and incubated on ice for 5 min. The mixture was centrifuged at 15,000 rpm for 30 min. The supernatant was collected and again centrifuged at 15,000 rpm for 20 min. To the supernatant was collected, 0.8 volume of isopropanol was added and incubated on -20°C for 1 hr. After incubation, the mixture was centrifuged at 15,000 rpm for 15 min and the supernatant was discarded. The pellet was washed with 70% ethanol by centrifuging for 15,000 rpm for 15 min. After discarding the supernatant, the pellet was air-dried, resuspended in sterile distilled water and stored at -20 °C.

3.6.3 Confirmation of recombinant clones using restriction analysis

Restriction digestion using particular enzymes was performed for the confirmation of insert in the particular white colony.

The components of the restriction mixture were optimized as listed below:

Cutsmart buffer (10X)	1.0 µl
BamHI	0.5 µl
Water	6.5 µl
Plasmid DNA	2.0 µl
Total volume	10 µl

3.6.4 Sequence analysis

The cloned SPLCV whole genome in PBS2KS+ vector was sequenced at Scigenome Pvt. Ltd. The sequence were analysed and blasted using the Basic Local Alignment Search Tool (BLASTn) in the NCBI website.

3.7 CLONING OF SPLCV COAT PROTEIN GENE

Using the full length clone of SPLCV sequence information, specific primers were designed to amplify the full coat protein gene of SPLCV. The full length clone DNA as tempelate, and SPLCV CP gene specific primers (SPLCV F -5'GCA CAT ATG TAT GAC AGG GCG AAT TCC CGT 3'; SPLCV R -5' AAC CAT GGT TAT TGT GTG AAT C3') were used to amplify the full-length coat protein

51

(CP) gene (a *NdeI* restriction site on SPLCV F and a *KpnI* restriction site on SPLCV R are underlined).

3.7.1 PCR Amplification of coat protein gene

The components of the mixture were optimized as listed below:

Water	12.2 µl
10X Taq buffer	2.5 µl
200M dNTP	1.0 µl
10Pm Forward primer	1.0 µl
10Pm Reverse primer	1.0 µl
Template DNA	2.0 µl
Taq polymerase	0.3 µl
Total volume	20 µl

Amplification was performed in a BioRad C100 Touch Thermocycler (Germany) with 38 cycles of initial denaturation 94°C for 2 min 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min, followed by a final extension cycle of 72°C for 10 min. PCR products were separated by electrophoresis on 1.2% (w/v) agarose gel and stained with ethidium bromide. Amplified prodcuts corresponding to the expected size were excised from agarose gels and further purified by gel elution method.

3.7.2 Gel elution of amplified whole CP fragments

Extraction of the whole CP regions of SPLCV was carried out with QIAEX-II gel extraction kit (QIAGEN, Germany). The PCR products were resolved on agarose gel (1%) and the amplicon was excised from the gel using a clean sharp scalpel. The gel slice was placed into a pre-weighed 2 ml tube and its weight was recorded. Then, thrice the volume gel solubilising buffer was added to the gel slice and it was incubated at 50°C for 10 min occasionally inverting it every 3 min until the gel slice dissolved completely. Then the sample was centrifuged at 13,000 rpm for 30 sec and the supernatant was discarded. The pellet was washed with 500 µl

52

QX1 buffer, vortexed and centrifuged for 30 sec at 13,000 rpm and removed the supernatant completely with a pipette for removing residual agarose contaminants. The pellet was washed twice with 500 µl PE buffer, vortexed and centrifuged for 30 sec at 13,000 rpm and removed the supernatant completely with a pipette for removing residual salt contaminants. The pellet is air-dried for 10-15 min or until it becomes transparent pellet. Sterile distilled water (20 µl) was added to the pellet, resuspended by vortexing and incubated for 5 min at room temperature. The sample was centrifuged for 30 sec and the supernatant containing purified DNA was transferred to a fresh tube and stored at -20°C.

3.7.3 Cloning and transformation

The eluted DNA fragment was cloned into PTZ5R/T vector DNA (Appendix IX) and transformed into *E. coli* DH5α using manual method as described by Sambrook *et al.,* (2000). The recombinant clones obtained were analyzed by colony PCR method.

The cloning and transformation of full coat protein gene was performed as per the protocol described in Section 3.6 with the ligated product mentioned here: The ligation mix was prepared with the components as listed below.

Vector PTZ57R/T vector DNA	1 µl
10x ligation buffer	1 µl
Coat protein insert	2 µl
T4 DNA ligase	1 µl
Nuclease free water	5 µl
Total volume	10 µl

After vortexing, the ligation mix was centrifuged for a few sec and incubated overnight at 4°C. This ligated product was used for transformation.

53

3.7.4 Analysis of recombinant colonies

Analysis of the positive colonies containing the insert was confirmed through colony PCR using coat protein specific primers.

Plasmid DNA isolation of transformed white colonies

Colonies which showed positive in colony PCR were selected for plasmid isolation for carrying out the downstream applications like further confirmation for the presence of inserts through restriction and sequencing. Plasmid DNA isolation was done as described earlier (section 3.6.2).

3.7.5 Confirmation of recombinant clones using restriction analysis

While analyzing all the enzymes, *SacI* and *HindIII* give the SPLCV–CP protein (Inframe). Restriction digestion using *SacI* and *HindIII* was performed for the confirmation of insert. The components of the restriction mixture were optimized as listed below:

Cutsmart buffer	1 µl
Plasmid	2 µl
SacI and *HindIII*	1µl
Nuclease free water	5 µl
Total volume	10 µl

3.7.6 Sequence analysis

The cloned CP gene in PTZ57R/T vector was sequenced at Scigenome Pvt. Ltd. and the sequence was analysed and blasted using the Basic Local Alignment Search Tool (BLASTn) in the NCBI website.

54

3.7.7Sub cloning in to expression vectors

The SPLCV coat protein gene was excised from the PTZ57R/T vector by restriction enzymes (*SacI* and *HindIII*) and subcloned into the expression vector pET28a+ (New England Biolabs, Beverly, MA).

The ligation mix was prepared with the components as listed below,

pET28a+ vector (*SacI* and *HindIII* digested)/pMAL-p5x,/pQE-31	1 µl
Ligation buffer	1 µl
T4 DNA ligase	2 µl
Coat protein gene (*SacI* and *HindIII* digested)	1 µl
Nuclease-free water	5 µl
Total volume	10 µl

The ligation solutions were mixed and incubated overnight at 16˚C.

Transformation

A volume of 50 µl of competent cells were added to each tube containing ligation mix and kept on ice for 1 hr. The mixture was heat shocked at 42°C for 2 min and immediately the tubes were quenched into ice. Then, 1ml of the LB medium was added to each tube and incubated at 37°C with shaking at 200 rpm for 1 hr. The cells were then concentrated by centrifuging at 7000 rpm for 1 min. Finally the cell suspensions were spread on LA ampicillin agar plates (Appendix VII). The plates were incubated overnight at 37°C and observed for the growth of colonies next day. Plasmid DNA isolation was done as described earlier (section 3.6.2).

3.7.8 Analysis of recombinant colonies

Analysis of the positive colonies containing the insert was confirmed through Restriction digestion with *Sac*I and *HindIII.* The components of the restriction

mixture were optimized as listed below:

55

Cutsmart buffer	1 µl
Plasmid	2 µl
*Sac*I and *Hin*dIII	1 µl
Nuclease free water	5 µl
Total volume	10 µl

3.7.9 Sequence analysis:

The cloned CP gene in pET28a+vector is sequenced at Scigenome Pvt. Ltd. and sequence were analysed and blasted using the Basic Local Alignment Search Tool (BLASTn) in the NCBI website. Sequence were transated using Expasy search tool and aligned using MUSCLE software with available sequences from NCBI website.

3.8. STANDARDISATION OF OPTIMAL EXPRESSION CONDITION FOR SPLCV CP

The single colony carrying the SPLCV-CP insert was grown in 10 mL of LB medium supplemented with ampicillin, and incubated at 37°C, 200 rpm for overnight in an incubator shaker and this culture is used as starter culture for expression. Fifty mL of fresh LB medium with Ampicillin was then inoculated with 1 % of the starter culture, and then incubated at 37°C, 200 rpm until the OD_{600} reaches 0.5. After obtaining the desired optical density, the protein expression was induced by the addition of lmM IPTG and incubated at 37°C, 200 rpm for 3-4 h. Simultaneously a non induced culture lacking IPTG was used as the control. 5mL culture was drawn at different incubation time intervals (1, 2, 3 and 4 h), pellatised and the level of protein expression was examined. Moreover different IPTG concentrations (ranging from 0.5 mM to 2mM) and different temperature conditions (ranging from 22°C to 37°C) were used to standardize the optimal expression. The obtained culture was then centrifuged at 8,000 rpm for 5 min and then resuspended in TE buffer (10 mM Tris HCL, pH 8.0, containing 10

56

mM EDTA). The filtrates were then denatured and run on a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

3.8.1 SDS- PAGE gel preparation

Stacking and Resolving gel composition for 12% SDS-Polyacrylamide gel preparation were described below:

Resolving Gel (1mm plate) 12%

Water	1.76 ml
30% Acrylamide/Bisacrylamide solution	2.14 ml
1.5M Tris pH	1.4 ml
10% SDS	54 ml
10% APS (freshly prepared)	54 ml
TEMED	5.4 µl
Stacking gel (1mm) 12%	
Water	1.82 ml
30% Acrylamide/Bisacrylamide solution	454 µl
1M Tris pH	334 µl
10% SDS	27 µl
10% APS (Freshly prepared)	27 µl
TEMED	2.7 µl

3.8.2 Visualization of proteins in the gels

To visualize the fixed protein, the gel was paced in 40% distilled water, 10% acetic acid,and 50% methanol solution containing 0.25% Coomassie Brilliant Blue R-250 and incubated at over night at room temperature on a rocking shaker. Transfered the gel in to a mixture of 67.5% distilled water, 7.5% actetic acid, and 25% methanol and placed on a rocking shaker until the excess dye has been removed. The proteins in the gel appeared deep blue bands.

3.8.3 SDS-PAGE gel analysis

Destaining was done and the bands were observed, Composition of de staining solution described earlier 3.8.2.

3.8.3 Dot Immuno Blot Assay (DIBA)

The Nitro cellulose membrane (NCM) containing the transferred protein was then placed in a petridish and 25 mL of 5% blocking solution was added. The membrane was incubated for I h at room temperature with gentle osclilation (20 rpm). After incubation, the blocking solution was discarded and the membrane was washed in IX TBST (pH 7.5) (Appendix VIII) thrice for 5 min. Then commercially available anti-ACMV polyclonal antibody (DSMZ, Germany) diluted in TBST-SDM to 1:1000 was added and the membrane was incubated at 4 °C overnight. After incubation the primary antibody was discarded and the membrane was washed twice in IX TBST with constant agitation for 5 min each. The membrane was then incubated with alkaline phosphatase linked anti mouse lgG (secondary conjugate antibody) diluted to l: 10,000 in TBST– SDM and incubated for 1 h at room temperature. The conjugate antibody solution was discarded and the membrane was washed twice in IX TBST for 5min each. After the final washing the membrane was placed in a clean petridish and the substrate BCIP/INBT solution was added and incubated at room temperature for 10 min in dark. The membrane was analysed for purple colour development. After the colour development, reaction was stopped by washing the membrane thrice in distilled water for 10 min. The membrane was dried on a blotting paper and photographed.

58

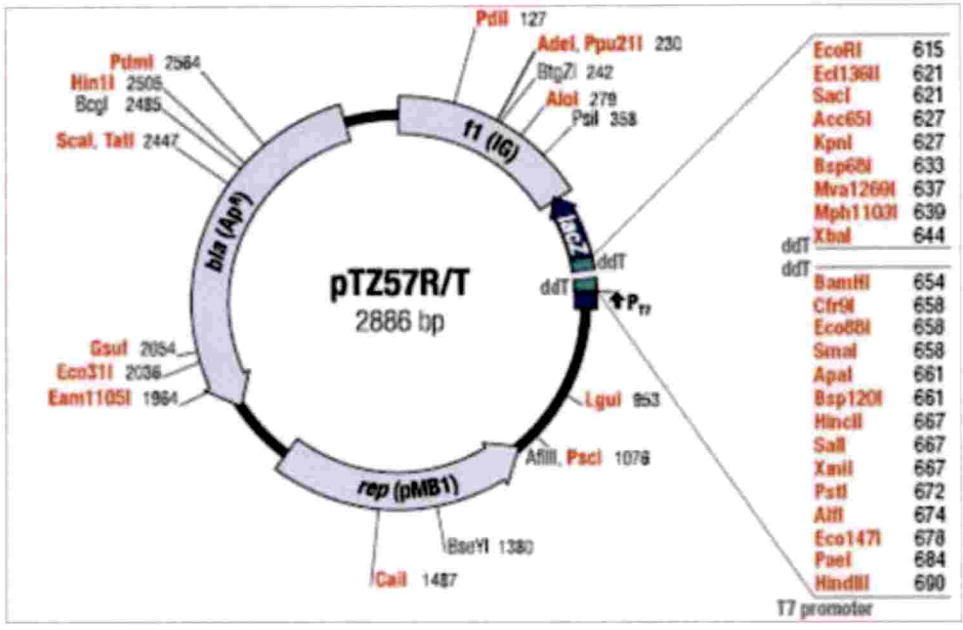
Table 5: Details of primers used for diagnosis of *Sweet potato leaf curl virus* from collected samples.

SPG1	5'CCC CKG TGC GWR AAT CCA T3'	AC4/AC1	900 bp
SPG2	5'ATC CVA AYW TYC AGG GAG CTA A3'	AC1/AC4	
SPG 3	5' ACT TCG AGA CAG CTA TCG TGC C3'	AV1/AV2	1100 bp
SPG 4	5'AGC ATG GAT TCA CGC ACA GG3'	AC2/AC3	
PW 285 1	5' TAA TTC GAA CTG CAG TTC CGT ATT TCA GTT3'	AC1	500 bp
PW 285 2	5'GCT AGA GGA GGC CTG CAG ACT GCT AAC GAC3'	AC1	

59

Table 6: Details of primers used for whole coat protein gene amplification.

Sl. No	Primer name	Sequence	Amplified region	Amplicon size
1	SPLCV F	5'TAT GAC AGG GCG AAT TCC CGT3'3'	Whole CP	800 bp
2	SPLCV R	5' AAC CAT GGT TAT TGT GTG AAT C3'		
3	SPLCV CP R	5' GGC TGT ATG GGA CCT CCT GAA3'	Partial CP	475 bp
4	SPLCV CP F (Partial	5'CCC TAC ACT GGG AAT GCT GT3'		



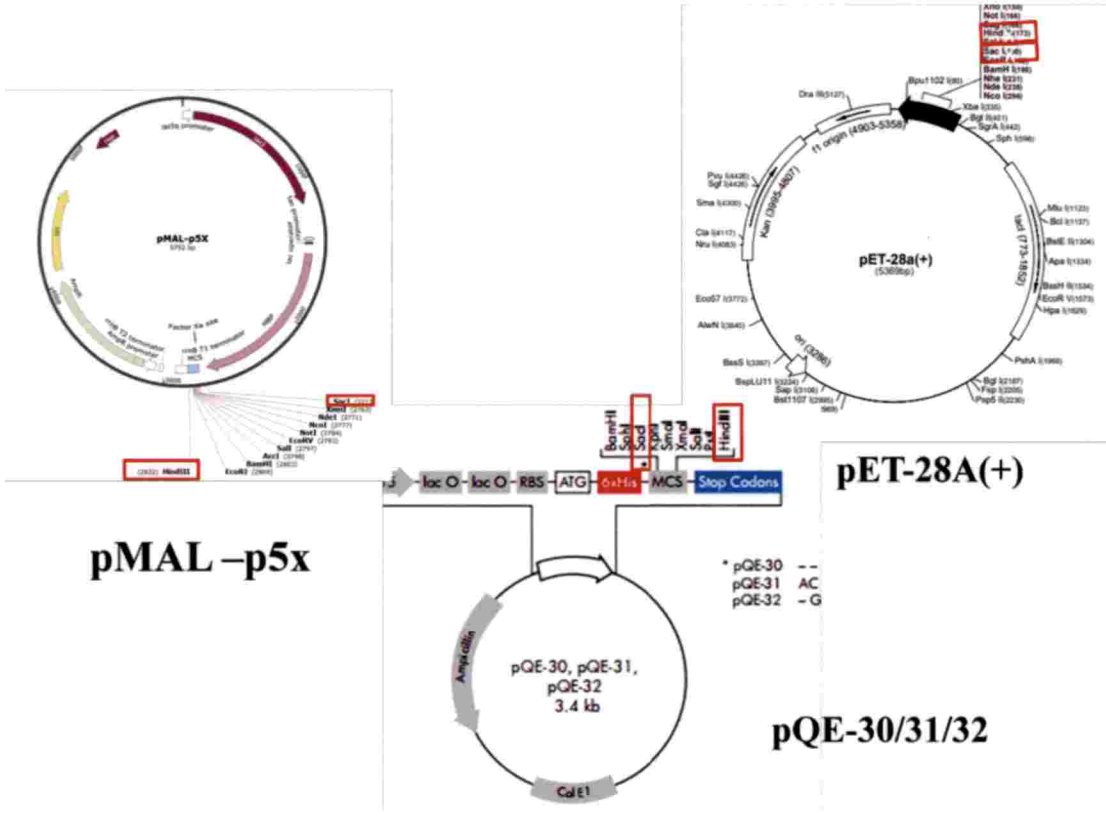


Figure 7: Vector map of vectors used in the study.

RESULTS

62

4. RESULTS

The results of the study entitled "Cloning and expression of coat protein gene of Sweet potato leaf curl virus" carried out at the Division of Crop protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2018-2019 are presented in this chapter.

4.1 SAMPLE COLLECTION

Sweet potato samples belonging to various accessions were collected randomly from the germplasm collection maintained at ICAR-CTCRI, Thiruvananthapuram and Regional Centre Bhubaneshwar. Most of the samples collected were symptomatic with inward leaf curling, upward curling on leaf edges vein swelling, interveinal chlorosis, chlorotic spots, blistering on entire leaf surface and generalized yellowing. Asymptomatic leaf samples also showed prominant infection sometimes which seemed to be mild chlorosis. 35 samples were collected and labeled by their accession numbers and symptomatology was recorded by visual observation. The sample details and symptoms observed are represented in the Table no 6 and representative samples are shown in Figure 8. The representative sample set was used for further test and analysis.

4.2 NUCLEIC ACID EXTRACTION

4.2.1 Isolation of DNA

DNA isolation from representative 30 samples was carried out using modified CTAB method. The extracted DNA was run on agarose gel (1%) and visualized under UV to observe the bands. The spectrometric readings and the gel images of isolated DNA were as shown in Table: 8 and Figure: 9.

4.3 MOLECULAR DETECTION OF VIRUSES

Polymerase chain reaction was employed as a part of molecular detection and diagnosis. Virus specific primes were used for the detection of *Sweet potato*

63

leaf curl virus. Primers were synthesized from Integrated DNA Technologies (IDT), USA.

4.3.1 PCR for detection of SPLCV

Detection of SPLCV was carried out using virus specific primers (Table no 4) providing corresponding PCR conditions. A non template control was used as negative control. Band representing the amplicon size 470 bp for SPLCV partial CPprimers were observed for SPLCV positive samples in agarose gel (1%) electrophoresis (Figure 10). No amplification was observed in negative control indicating that there is no non specific primer binding.

4.4 ROLLING CIRCLE AMPLIFICATION OF SPLCV GENOME

Three samples (S731, S1336 and Sree Nandhini) showing SPLCV infection were selected for whole genome amplification using rolling circle amplification (RCA) technique. The amplified products were obtained as a shear of concatamers while running in 1% agarose gel(Figure 11).While analyzing the genome of available SPLCV isolates from the NCBI, it was found that a unique *Bam* H1 site is present. *Bam* H1 is used to restrict the RCA products and 2.7kb fragment (Figure 12) obtained. The fragments were further confirmed by M13F/R, and gene specific primers SPG1/2, SPG3/4 and PW285 1/2.

pBS 2IKS+ vector is also digested with *Bam* H1 and inserts were purified by chloroform extraction method and checked in 1% agarose gel. The extracted fragments were cloned in pBS2IKS+ and transformed in to DH5α cells. The recombinant positive clones were confirmed by blue white screening in LB Ampicillin Xgal/ IPTG (Figure 13). Around 15-20 colonies were obtained in each plate. Grid plating were maintatin the transformed white colonies. The white colonies were selected for plasmid isolation and restriction analysis. Plasmid DNA was isolated from 10 colonies which show the shift. Out of the 15 plasmids, 10 plasmids were showed positive and selected for restriction digestion. The two positive samples were sequenced at Agrigenome DNA sequencing facility,

64

Cochin with their primers. The sequencing results were obtained as electrophorogram.

4.6 SEQUENCE ANALYSIS

Positive plasmids were sequenced at Agrigenome, Cochin for DNA sequencing, with M13R primers gave the partial sequence from 362 to 1128, SPG4 primers gave the sequence from 971 to 2185, SPG4 gave the sequence from 1487 to 2345 and PW285.2 gave the sequence from 2199 to 2345.All these sequences were aligned manually and analysed. The sequencing results were obtained as 90% similarity with Sweet potato leaf curl Greece isolates.NCBI BLAST results of inserted sequence were shown in Figure: 17. Phylogenetic tree was constructed with similar sequences using online NCBI blast analysis software. The tree constructed showed similarity with SPLCV –Chinese isolate Figure: 18

The sequence obtained is given below (1897 nt):

GGATCCATTGACGTTTGCTCTGGCTTTTACCATGTATGATAATGAGCCCACTA
CTGCTAAGATCCGTATGGATCTTAGAGATAGATTGCAGGTCTTGAAGAAATTC
TCTGTTACTGTTTCTGGTGGGCCTTATAGCCACAAGGAGCAGGCGTTAGTTAG
GAGGTTTTTTAACAGTTTGTATAATCATGTTACTTATAATCATAAGGAGGAAG
CTAATTATGAGAATCATTTAGAGAATGCGTTAATGTTGTATTCTGCTAGCAGT
CATGCTAGCAATCCTGTGTATCAAACCCTGCGTTGCAGGGCTTATTTTTATGA
TTCGCACAATAATTAATAAAGAAGTACTTTTATATCATCTTTGCAATCTATTAC
ATCGACTTCTTCTACCCACAAGATTTCTTGTGGTAAATGTCTAATTACATACAC
TAAATTATATAATGAAAAATAACCTAAACTAGCTAGACTATTACAAATTCGCC
ATTTAAGGCGATTGGAAATACCAGTCCAATTGGGAATAACACCAGTCAGACG
CGTTGTTATGATCCGGAATTGGAGGAATATCTTCTGGAATCCAATTTCCCTCC
TGTTCCGGCGGTTGACTTGAAGCTGCAACTTGATGACCAATGGATCCCCCGGG
CTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGC
CCGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTA
ATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA
CAACATACGAGCCGGGAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTG
AGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAA
CCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGTGGTGATGATGCTGACA
GCCGCAGCCCTTCCACACTATCCTAGTCCTCGTCTCAGGAACTTTCCTCTTGGC
CTTCTTCGCCTCTGTGTGCAGTGGCTCCTGAATGGGACACTTCCTCTTGATTCC
AGAAGGGAGATTGGACATCGCAGAATATAGCGTTTGCTGTTGCCCAATTCTTG
AGTGCTCCTTGCTCTGGTTTGTCCAACCAGAGTTTAAATGAGGAACCCTCTCC
TGGATTGCATAGGAAGATAGTGGGAATTCCGCCTTTAATTTGAACTGGCTTTC

65

CGTATTTACAGTTGGATTGCCAGTCCTTCTGGGCCCCCATGAACTCTTTAAAG
TGCTTTAGGTATTGGGGGTTGACGTCATCAATGACGTTATACCAAGCACTATT
GCTATACACCTTTGGGCTTAGGTCCAAATGTCCACACAGATAATTATGTGGGC
CTAAAGATCTAGCCCAAACTGTTTTACCTATTCTACTTGGGCCTTCTATAACTA
TACTAATGGGCCTATCTGGCCGCGCAGCGGCATCCATGACATTATCAGCGGCC
CAATCGCTGATAATGTCAGGTACATTATTGAAAGAAGAAGAGGAAAAAAGTG
AAGAATATACAGAAGGAGGAGGAGAAAAAATCCTATCTAAATTATTATTAAT
ATTATGGAATTGAAGAACAAAGTCTTTTGGACACAATTCCTTTATTATCTGTA
ACGCTTCAGATTTACACCCAGTATTTAATGCCTGGGCGTAAGCATCGTTAGCC
GTTTGCTGACCTCCTCTAGCACTTCTTCCGTCGACCTGGAAGACTCCACTGTCA
ACAAAATCTTGGTCCTTTTCAATGTAGCCTTGACATCGGAAGAGGACTTTGCT
GATTGAATTGGGTGTATGTGTAGGGACGGGCCTGGGTGGAG

4.4 PCR AMPLIFICATION OF WHOLE COAT PROTEIN GENE

Amplification of coat protein (CP) gene was carried out using specific primers which amplifies the whole CP region of SPLCV. A non template control was used as the negative control. Band representing the amplicon size 754bp for SPLCV CP primers were observed in agarose gel (1%) electrophoresis (Figure: 19). No amplification was observed in negative control indicating that there is no non specific primer binding. PCR conditions were described in 3.7.1.

4.7 CLONING OF COAT PROTEIN GENE IN TA CLONING VECTOR

The SPLCV whole CP insert was eluted from the gel and cloned in TA vector PTZ57R/T. The cloning ligation mixtures proportions were described in 3. 7.2.

4.7.1 Cloning and transformation

The amplicons representing 754 bp for whole CP was eluted and the amplified products were cloned using InsTA PCR Cloning Kit (Fermentas, USA) for further sequence analysis and observing the suitable restriction site for identifying the ORF of CP protein. Transformation was carried out in *E.coli*DH5α cells. Recombinant clones were confirmed by blue white screening in LA Ampicillin Xgal/IPTG plates (Figure 20). Grid Plates were prepared to maintain recombinant white colonies. These recombinant colonies were checked for presence of insert using colony PCR with SPLCV partial CP primers. Out of 25

colonies 18 colonies showed positive which were selected for plasmid isolation. The insertion of gene of interest was confirmed by the colony PCR described (3.6.2) and restriction digestion of plasmid with *SacI* and *HindIII.* The positive clones were sending for sequencing in Agrigenome.

4.7.2 Sequence analysis

The positive plasmid clone was sent to Agrigenome for DNA sequencing, with the M13F primers. The sequencing results were obtained as electrophoregram. From the sequencing results, it was found that clone was shown 98% similarity with the CTCRI-TVM isolate. These sequences were used for further expression studies. NCBI BLAST results of inserted sequence were shown in Figure: 24.The sequence obtained is given below (958 nts):

>SP-
T9CTAGATTGCACATATGTATGACAGGGCGAATTCCCGTTTCGCGGAGA
TTTCATCCCTATGGGGGGAGACCGGTAAGACGGAGGCTAAACTTCGAG
ACAGCTATCGTGCCTTATACTGGGAATGCTGTCCCAATTGCTGCCCGAA
GCTATGTCCCGGTTTCAAGAAGCGTCCGGATGAAGAGAAAGAGGGGT
GACCGTATCCCGAAGGGTTGTGTGGGTCCCTGTAAGGTCCAGGATTAT
GAGTTCACGATGGACGTTCCCCACACGGGAACGTTTGTCTGTGTCTCG
GATTTTACAAGGGGAACTGGGCTTCTTCATCGCCTGGGTAAGCGTGTG
TGTGTTAAGTCAATGAGTATAGATGGGAAGGTCTGGATGGATGATAAT
GTGGCCAGGAGAGATCACACCAATATCATCACGTATTGGTTGATCCGT
GACAGAAGGCCCAATAAGGATCCGCTGAACTTTGGGCAGATATTCACC
ATGTACGATAATGAGCCCACTACTGCTAAGATCCGAATGGATCTGAGG
GATAGAATGCAGGTCCTAAAGAAATTCTCTATTACAGTTTCAGGAGGT
CCATACAGCCACAAGGAGCAGGCTTTGGTTAGAAAGTTTTTTAAGGGT
TTGTATAACCATGTAACTTACAATCACAAGGAAGAAGCTAAGTACGAG
AATCACTTAGAAAATGCGCTTATGCTGTATAGTGCTAGTAGTCATGTTA
GTAATACTGTGTATCAGACCCTGCGTTGCAGGGCTTATTTTTATGATTC
ACACAATAACCATGGTTAATCGGATCCCGGGCCCGTCGACTGCAGAGG
CCTGCATGCAAGCTTTCCCTATAGTGAGTCGTATTAGAGCTTGTCGTAA

67

TCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC
CACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGG

4.8 SUB CLONING OF SPLCV CP WHOLE GENOME IN TO EXPRESSION VECTORS

Whole genome of SPLCV CP was eluted from the gel and sub cloned into expression vector pET-28a+ DNA and transformed into *E.coli* expression BL-21 DE3 cells. The recombinant clones were confirmed by LA-Kanamycin plates for pET28a+, LA-Ampicillin plates for pMALp5x and pQE30/31/32. Presence of insert in recombinant colonies was confirmed by colony PCR using SPLCV CP primers (Figure: 26) and restriction digestion analysis (Figure: 28). From the LA-Kanamycin plate, Out of 25 colonies 18 colonies are selected for plasmid isolation. From the LA- Ampicillin plates for pMALp5x and pQE30/31/32, out of 15 colonies 3 and 4colonies selected for plasmid isolation. SPLCV-CP gene insert concentration in pQE30/31/32 and pMALp5x is very less; details were mentioned in Table 9. So further expression studies pET28a+ expression vector was selected.

4.9 EXPRESSION EXPERIMENTS

Among the various IPTG concentrations and different time and temperature of incubation, for optimizing the expression of the CP gene, it was found that the protein expression was optimum when expressed at 37°C for 3 h with an IPTG concentration of 1 mM. The expressed protein showed a molecular weight of appropriate size of 32 kDa, when analysed on 12% SDS-PAGE (Figure29).

4.10 SEQUENCE ANALYSIS

Nucleotide sequences of SPLCV-CP were translated in to protein sequences using ExPASy (Figure: 30) and aligned with available sequences in NCBI BLAST using MUSCLE-Multiple Sequence Alignment tool (Figure: 31).

68

4.11 CONFIRMATION OF EXPRESSED PROTEIN THROUGH SEROLOGICAL ASSAY

Expressed proteins were confirmed by Dot immune blot assay (DIBA). Two SPLCV-CP protein positive samples were obtained (Figure 32).

69

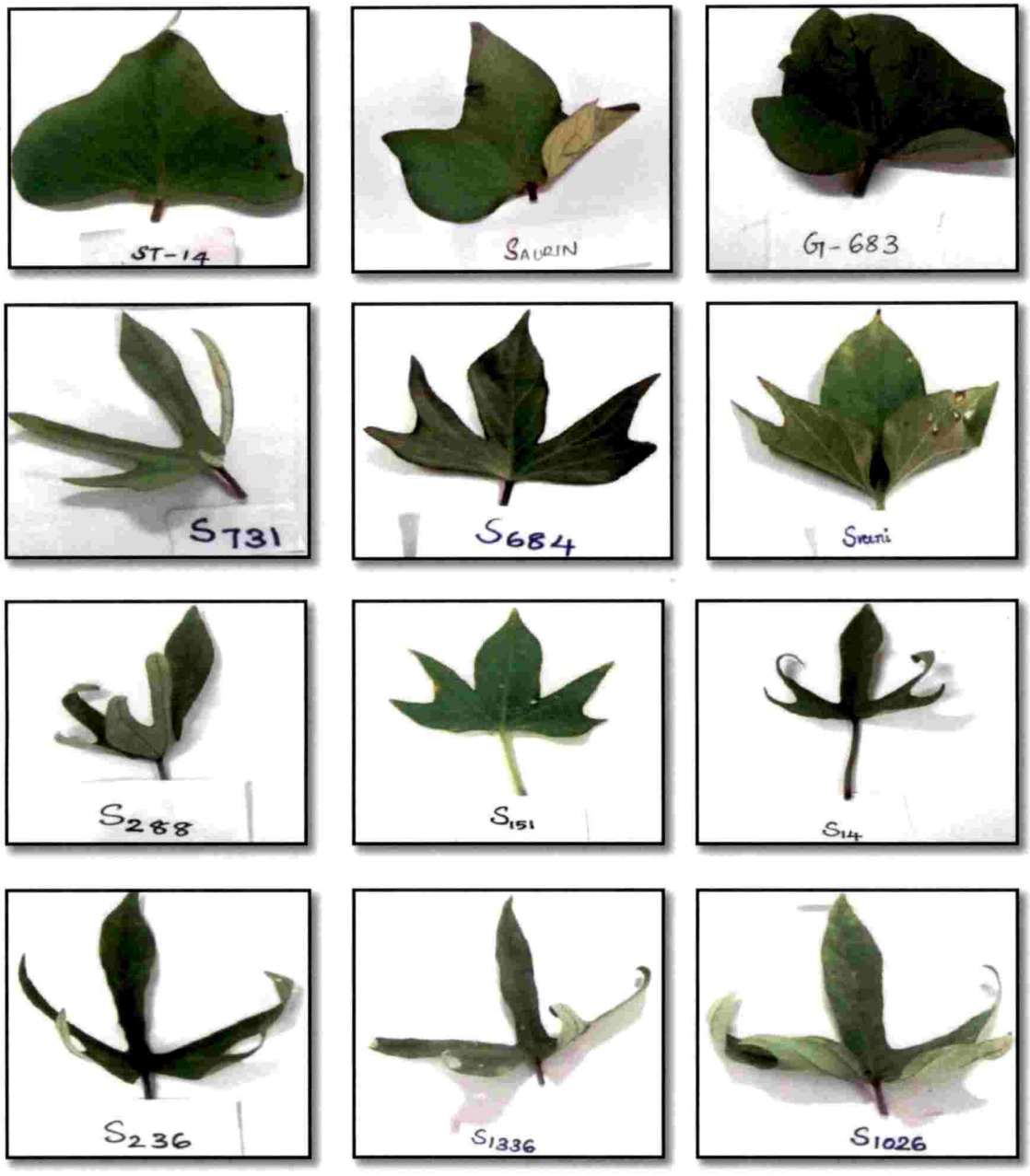


Figure 8: Representative sample set (symptom details in Table 6)

70

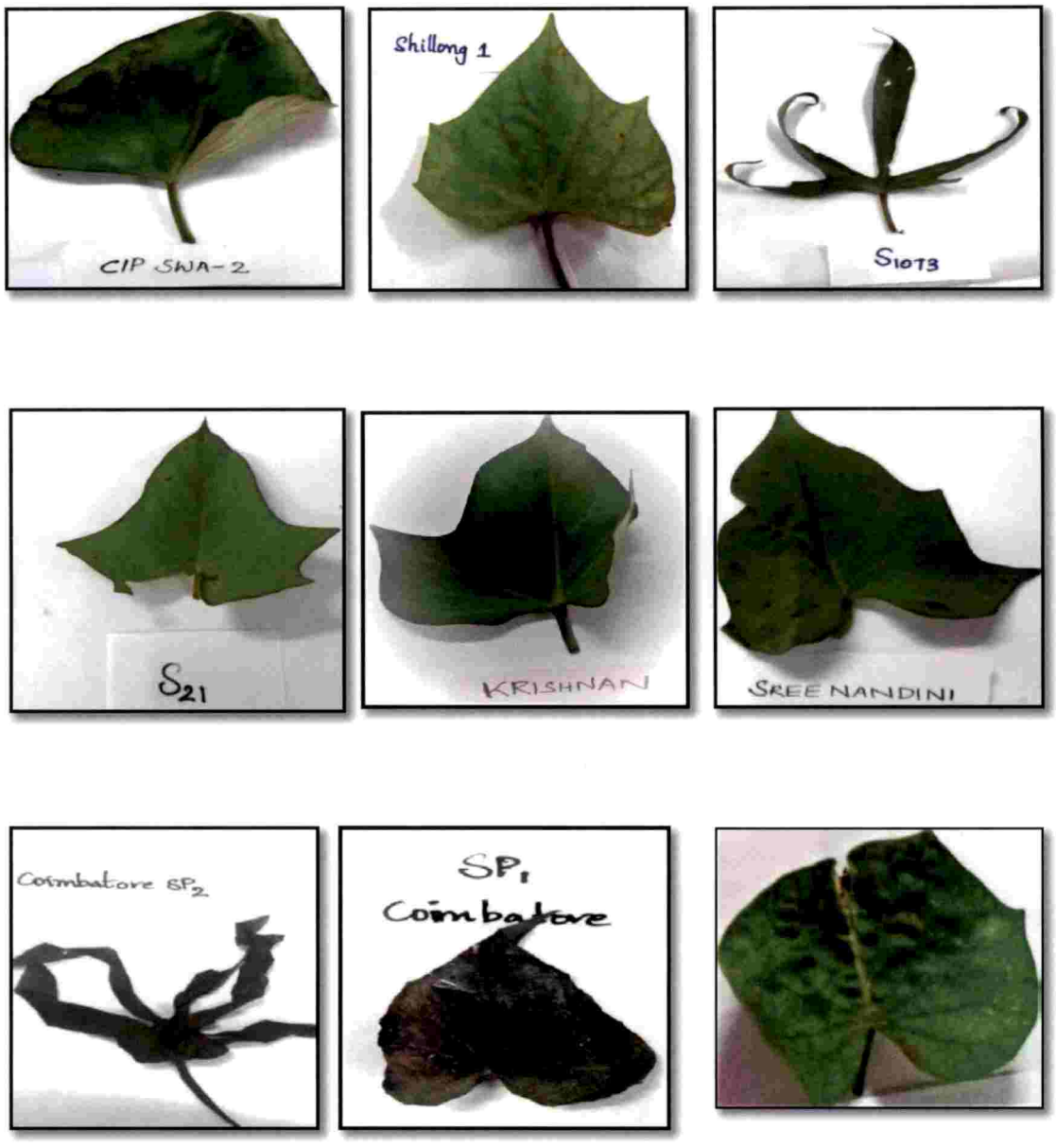


Figure 8: Representative sample set (continued)

Figure 8: Representative sample set (continued)

Table 7: Representative sample set, location and symptoms observed

Sl. No	Sample ID	Place of collection	Symptoms
1	S151	CTCRI	Generalized yellowing
2	S14	CTCRI	Upward curling
3	S286	CTCRI	Downward curling, Blistering
4	S288	CTCRI	Chlorotic spots
5	S1026	CTCRI	Severe upward curling
6	S1336	CTCRI	Upward curling
7	S236	CTCRI	Upward curling, Leaf narrowing
8	S1335	CTCRI	Severe upward curling, Leaf blistering
9	S684	CTCRI	Upward curling, Chlorosis
10	S1073	CTCRI	Upward curling
11	S730	CTCRI	Chlorosis
12	Shillong	CTCRI	Yellowing
13	Sreeni	CTCRI	Yellowing, Upward curling
14	Nongpon	CTCRI	Vein clearing
15	Saurin	CTCRI	Chlorotic spots, Curling
16	S286	CTCRI	Severe Upward curling
17	S288	CTCRI	Upward curling
18	S1026	CTCRI	Vein clearing and generalized yellowing
19	Krishnan	CTCRI	Chlorotic spots, Upward curling
20	Sree Nandhini	CTCRI	Chlorotic spots
21	ST-14	CTCRI	Upward curling

Table 7 (Continued): Representative sample set, location and symptoms observed

73

22	CIP	CTCRI	Mild upward curling
23	Gautham	CTCRI	Chlorotic spots
24	G-683	CTCRI	Leaf blisters
25	G-497	CTCRI	Mild upward curling
26	SP3	CTCRI	Leaf puckering
27	SP1	CTCRI	Leaf blistering, Upward curling
28	SP2	CTCRI	Leaf puckering
29	S21	CTCRI	Leaf blisters
30	S101	CTCRI	Yellowing, Mild chlorotic spots

Table 8: Quantification of DNA of representative set isolated using Spectrometric readings.

74

Sl. No	Sample ID	Amt. of DNA (µg/ml)	Purity(A_{260}/A_{280})
1	S151	173.86	2.08
2	S14	179.34	1.80
3	S125	240.45	1.89
4	S236	222.04	1.93
5	S258	1027.44	2.00
6	S286	986.71	1.67
7	S288	306.30	2.01
8	S1026	182.36	2.03
9	S1336	254.12	2.03
10	S236	258.85	2.09
11	S731	308.51	2.09
12	S481	213.08	2.0
13	S1335	172.40	2.05
14	S684	208.80	2.06
15	S1332	119.70	1.86
16	S1073	100.50	1.83
17	S730	105.45	1.90
18	Shillong	95.45	1.88
19	Sreni	99.50	1.84
20	Nongpon	141.41	1.96
21	Saurin	185.90	2.10
22	Krishnan	332.34	2.13
23	Sree Nandhini	500.71	2.29
24	ST-14	240.47	2.23

Table 8 (Continued): Quantification of DNA of representative set isolated using Spectrometric readings

25	CIP	571.00	2.10
26	Gautham	429.45	2.17
27	SP3	146.64	2.2
28	SP1	156.72	2.4
29	SP2	100.12	2.15
30	S11	450.71	1.56
31	S21	398.34	2.05
32	S101	245.67	1.84
33	S231	238.93	1.65
34	G-497	345.76	2.12

Table 9: Details of vectors selected for the study

Vectors used	Method of selection	Size of the vector(kb)
pET28a+	Kanamycin	5.4
pQE30/31/32	Ampicillin	3.5
pMALp5x	Ampicillin	5.7

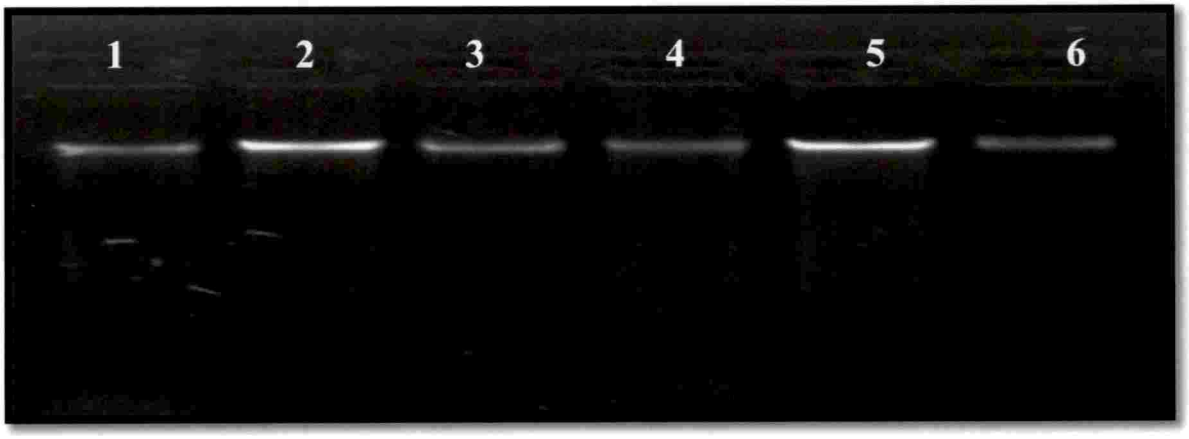
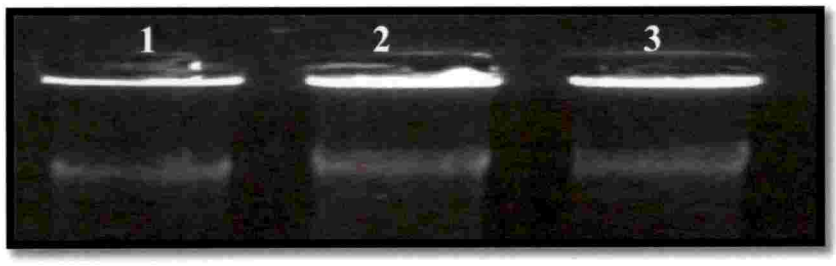


Figure 9: Gel profile of DNA isolated by CTAB method (2%).

Lane 1- CIP, Lane 2-SreeNandhini, Lane 3- Saurin, Lane 4- Krishnan, Lane 5- G683, Lane 6- ST14.

Figure 10: Agarose gel electrophoresis of sweet potato samples positive for SPLCV CP partial primers.

Lane 1- 1kb ladder(DNA ladder Thermo Scientific), Lane 2- S1026, Lane 3- S1336, Lane 4- S236, Lane 5- S731, Lane 6- S481, Lane 16- positive sample.



77

Figure 11: Gel image showing RCA product

Lane 1- Sree Nandhini, Lane 2- S731, Lane 3- S1336.

Figure 12: Gel image showing Restriction digestion of RCA product with *BamH*I.

Lane 1- Restricted PBS2KS+, Lane 2- Sree Nandhini , Lane 3- S731, Lane 4- S1336.

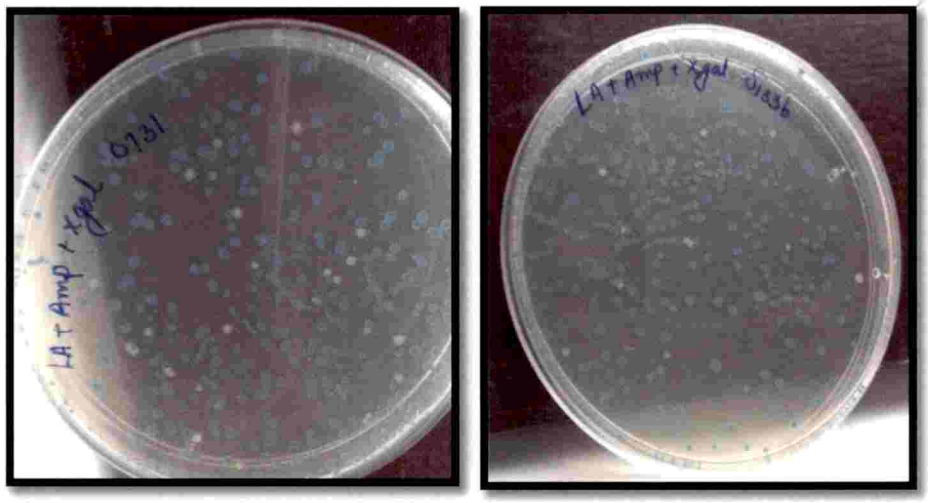


Figure 13: Blue – white colony screening in LA-AX1plates.

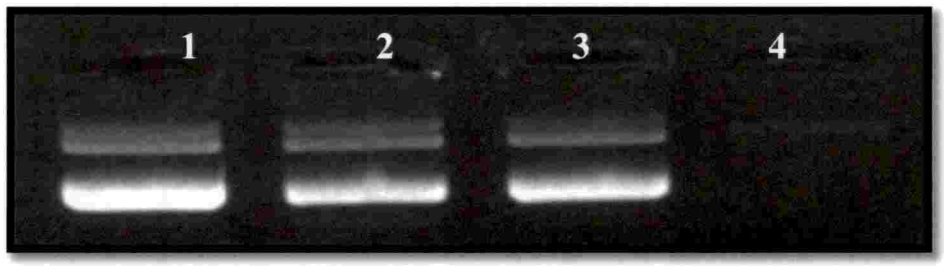


Figure 14: Gel image showingplasmid DNA isolated from transformed white colonies.

Lane 1- S1336(2), Lane 2- S 1336(4), Lane 3- S731(1), Lane 4-

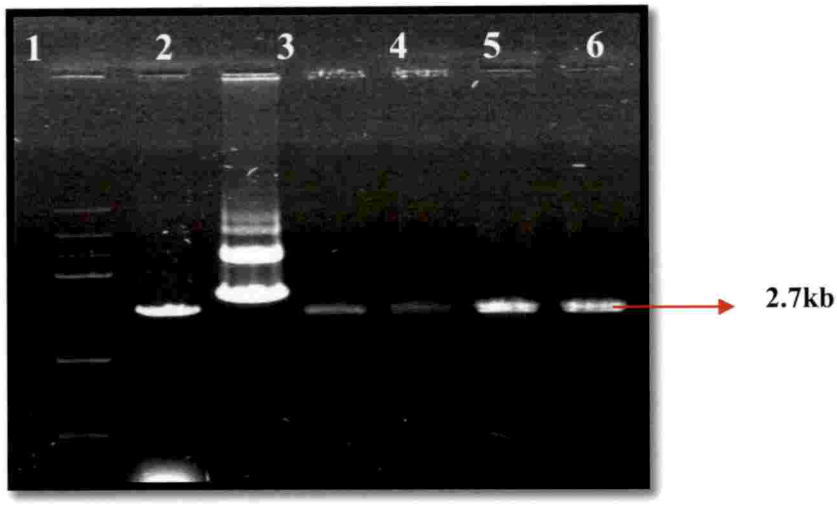


Figure 15: Gel image (0.6%) showing restriction analysis of recombinant clones with *BamHI*.

Lane 1- 1 Kb ladder (DNA ladder Thermo Scientific , Lane 2- PBS2KS+ /*BamHI* , Lane 3- PBS2KS+ uncut, Lane 4- S731 (1), Lane 5- S731(4), Lane 6- S1336(2), Lane 7- S1336(4).

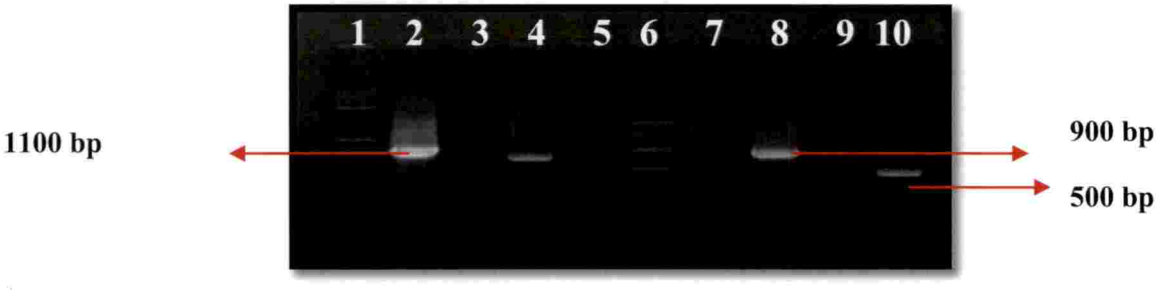


Figure 16: Agarose gel electrophoresis of RCA clone positive for G3/G4, M13F/G4, G3/M13R, G1/G2 and PW285.1/2.

Lane 1- 1Kb ladder (DNA ladder Thermo Scientific), Lane 2-G3/G4, Lane 3- N, Lane 4-M13f/G4, Lane 5-N, Lane 6- G3/M13R, Lane 7- N, Lane 8- G1/G2, Lane 9-N, Lane10-

80

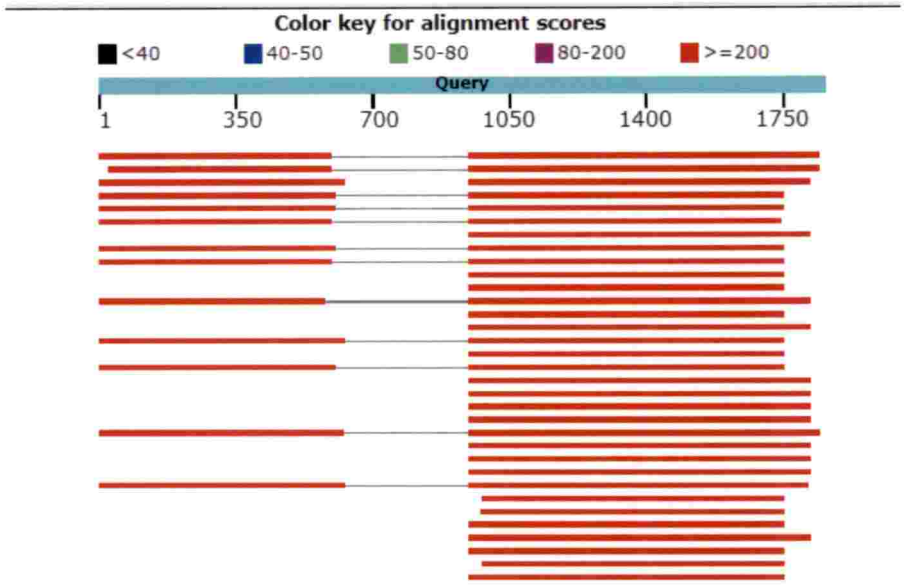


Figure 17: Blast analysis of the SPLCV genome.

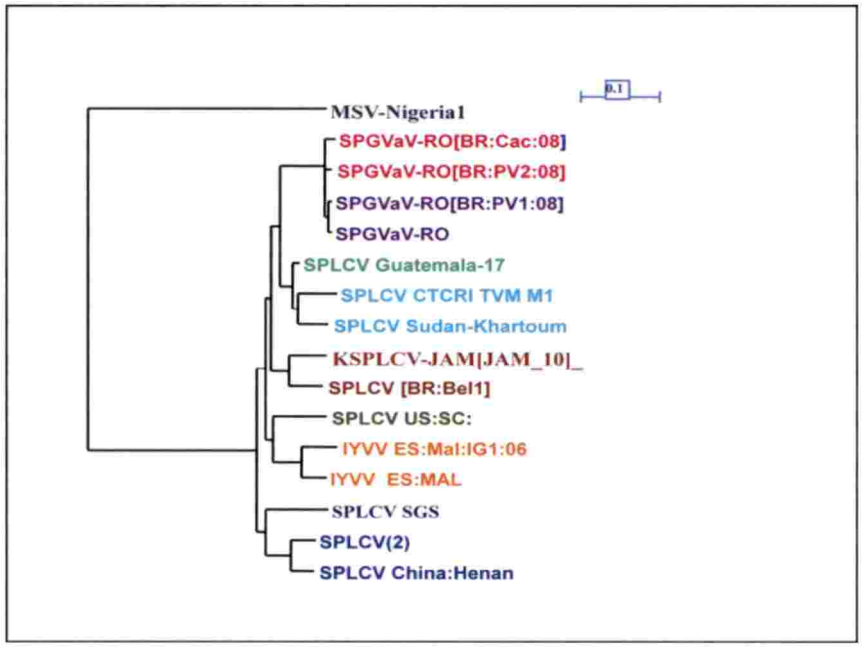


Figure 18: Phylogenetic tree construction of SPLCV sequence.

81

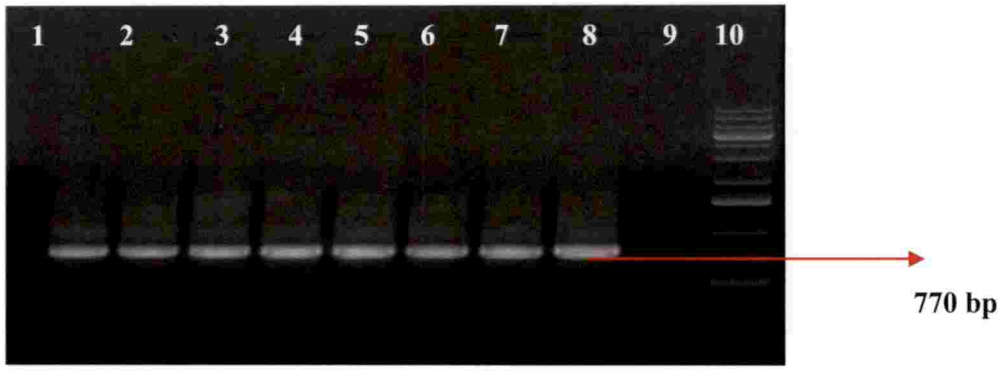


Figure 19: Gel image showing primer standardization of SPLCV whole coat protein gene.

Lane 1-Tm-60°C, Lane 2-Tm-59.7°C, Lane 3-Tm-59.1°C, Lane 4-Tm-58.1°C, Lane 5-Tm-57°C, Lane 6-Tm-56.2°C, Lane 7-Tm-55.5°C, Lane 8-Tm-55°C, Lane 9-Negative control, Lane 10-1 kb

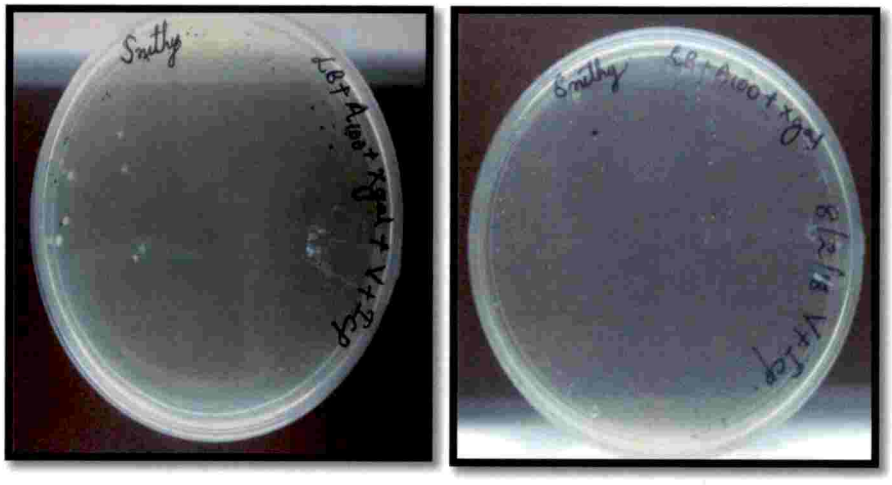


Figure 20: Blue – white colony screening in LA-AX1plates.

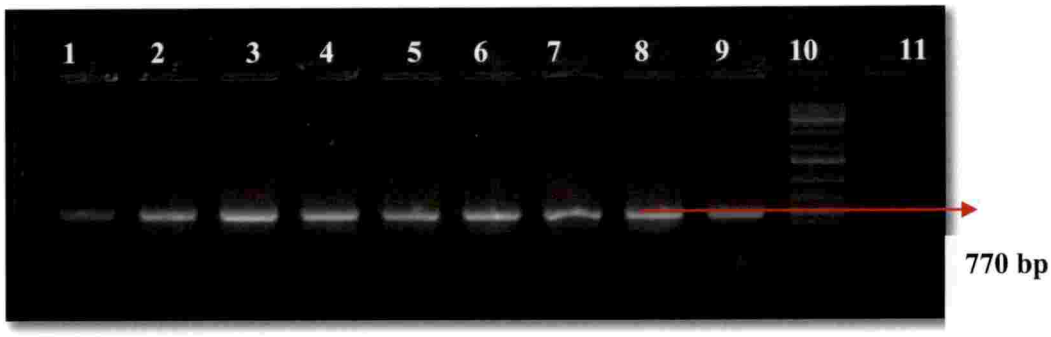


Figure 21: Colony PCR to confirm SPLCV-CP inserts.

Lanes 1 to 9 represent the colony PCR amplification of SPLCV- CP in *E.coli cells*, Lane 10- represent 1 kb plus (DNA ladder Thermo Scientific), and Lane 11- represents negative control.

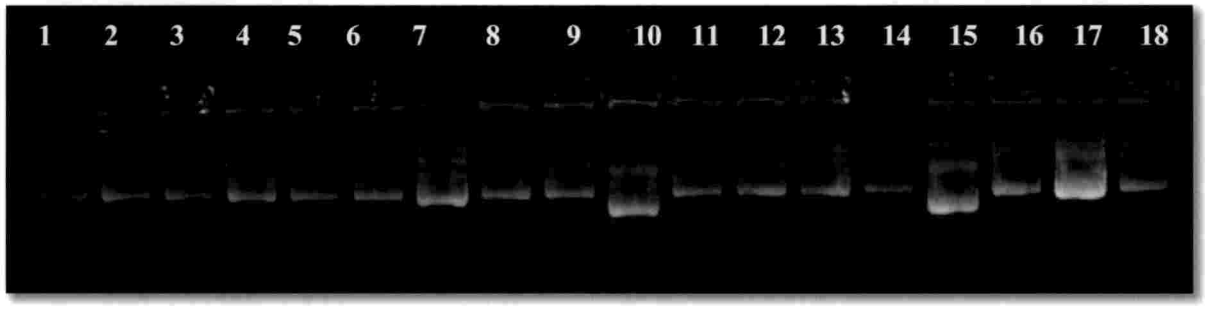


Figure 22: Plasmid isolated from recombinant clones.

Lanes 1 to 18 represent the plasmid isolated from positive *E.coli*

83

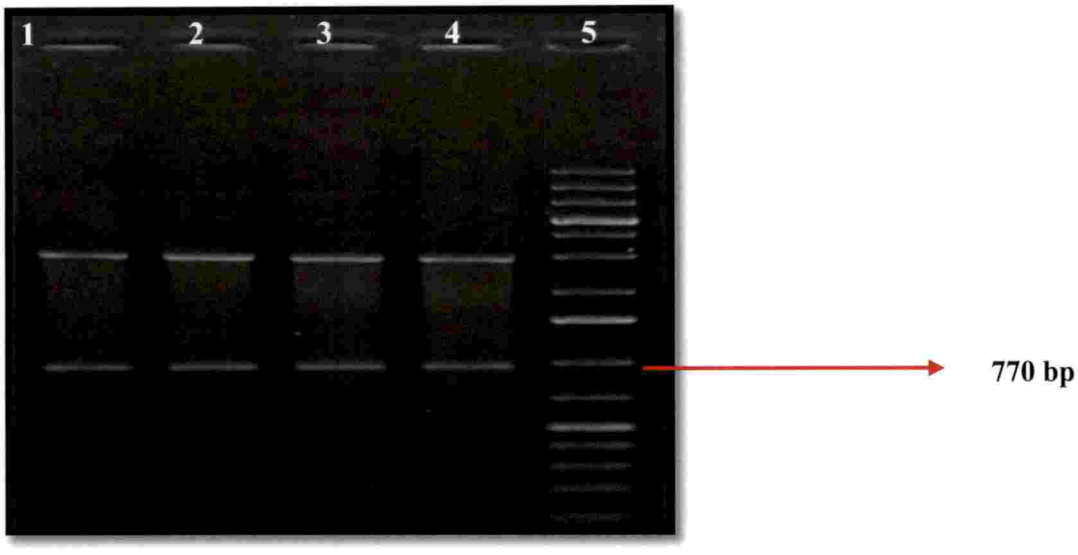


Figure 23: Restriction analysis with *SacI* and *HindIII*.

Lane-1to 4-SPLCV-CP gene insert, Lane 5- 1 kb plus (DNA ladder Thermo Scientific).

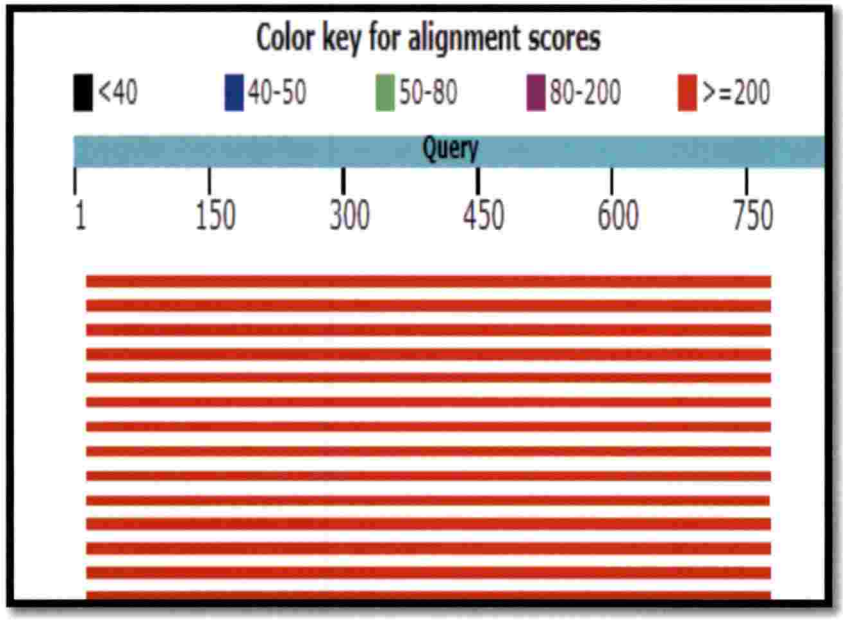


Figure 24: BLAST analysis of the SPLCV CP gene

84

Figure 25: Transformation in BL21 DE3 cells

Plate A-pET28a+, Method of selection- Kanamycin resistance
Plate B- pQE 31, Method of selection- Ampicillin resistance.
Plate C- pMALp5X, Method of selection- Ampicillin resistance.

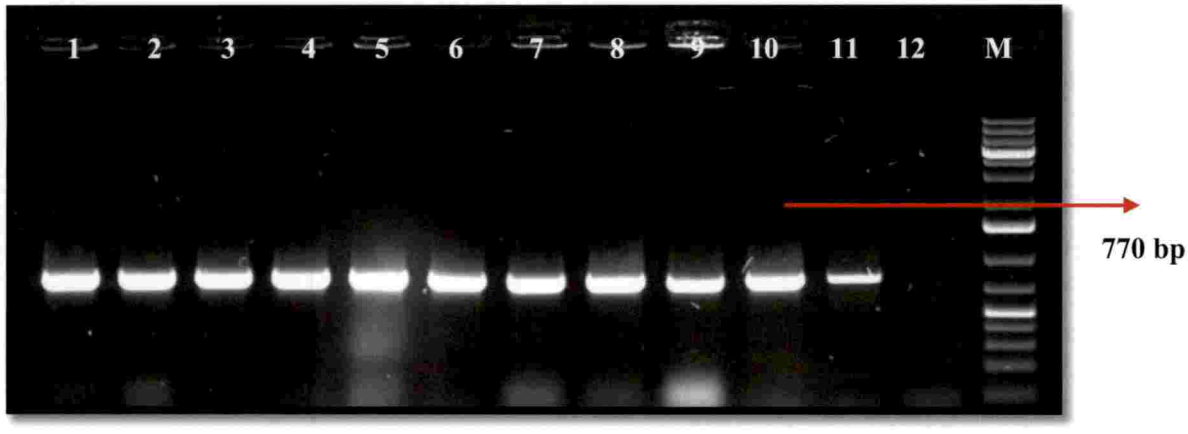


Figure 26: Colony PCR to confirm SPLCV-CP gene amplification.

Lane 1 to 11- SPLCV-CP positive clones, Lane 12- Negative control, Lane M- 1kb plus DNA ladder.

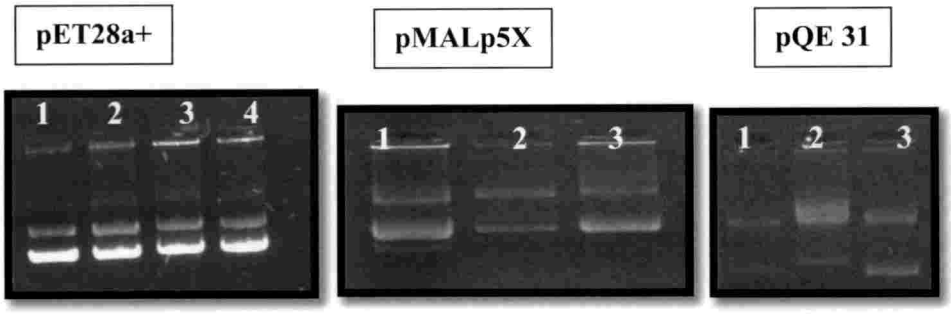


Figure 27: Plasmid isolation from transformed colonies

Lane 1to 4 plasmid isolated from pET28a+, Lane 1to 3 plasmid isolated from pMALp5X, Lane 1to 3 plasmid isolated from pQE31

pQE 31

Lane 1- 1kb ladder, Lane-2 uncut plasmid, Lane 3,7and 9 SPLCV-CP gene insert.

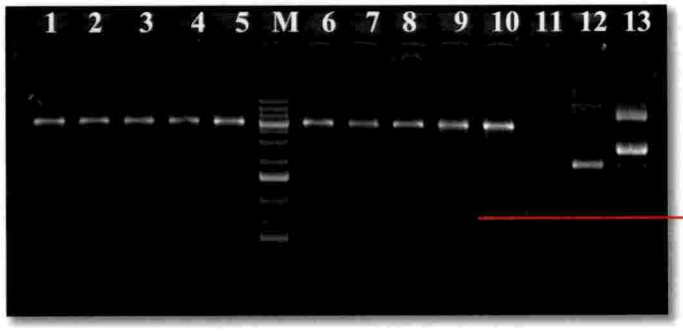
770 bp

pMALp5X

Lane 1- Ikb ladder, Lane 4 and 6 SPLCV- CP insert.

770 bp

pET28a+

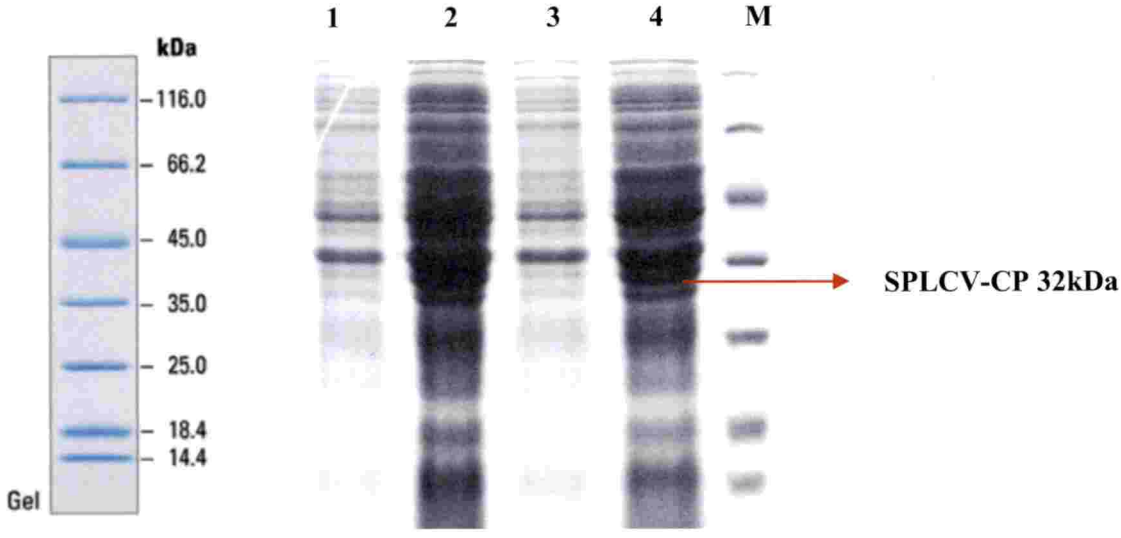


Lane M- Ikb ladder, Lane 1 and 10 SPLCV- CP insert, Lane 12 and 13 Uncut plasmid.

770 bp

Figure 28: Confirmation of Recombinant clones with *Sac*I and *Hind*III Double digestion.

87



Lane 1- Uninduced sample, Lane 2- Induced protein sample 1, Lane 3- Uninduced sample 2, Lane 4- Induced protein sample 2, Lane M- Pre stained protein marker.

Figure 29: SDS-PAGE analysis of the SPLCV CP gene

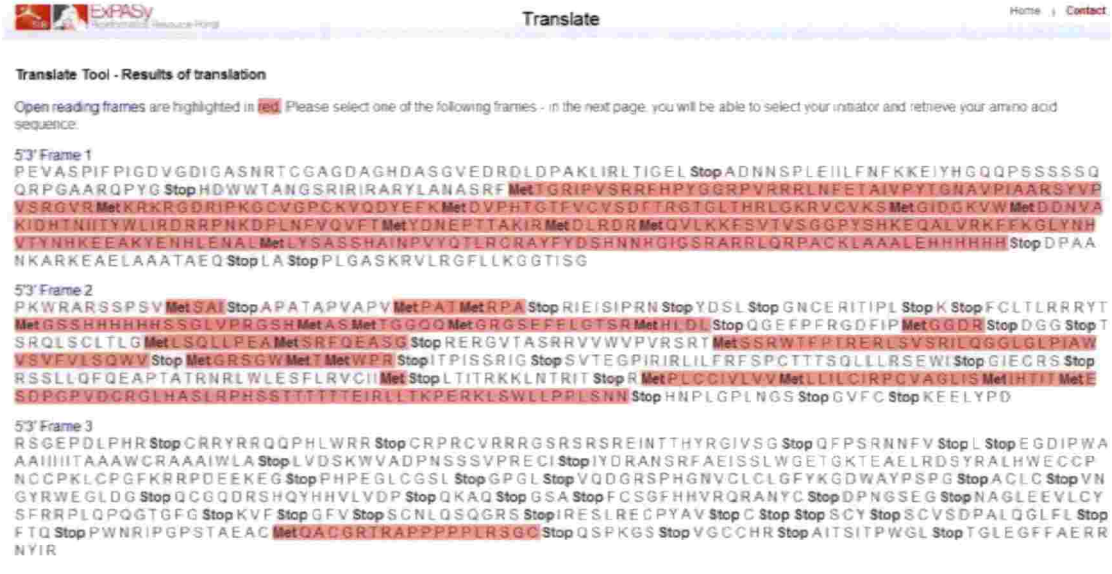
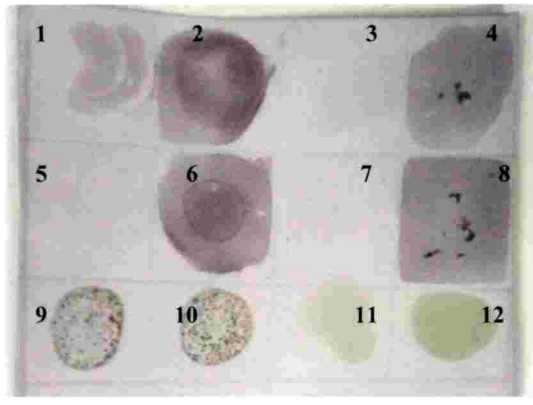


Figure 30: Protein sequence-In frame analysis using ExPASy tool.

88

```
CLUSTAL multiple sequence alignment by MUSCLE (3.8)

CTCRI           MTGRIPVSRRFHPYGGRPVRRRLNFETAIVPYTGNAVPIAARSYVPVSRGVRMKRKRGDR
SGS             MTGRIPVSRRFHPYGGRPVRRRLNFETAIVPYTGNAVPIAARSYVPVSRSVRMKRKRGDR
                ************************************************.**********

CTCRI           IPKGCVGPCKVQDYEFKMDVPHTGIFVCVSDFTRGTGLTHRLGKRVCVKSMGIDGKVWMD
SGS             IPKGCVGPCKVQDYEFTMDVPHTGIFVCVSDFTRGTGLLHRLGKRVCVKSMSIDGKVWMD
                ****************.********************** ************.********

CTCRI           DNVAKIDHTNIIIYWLIRDRRPNKDPLNFVQVFTMYDNEPTTAKIRMDLRDRMQVLKKFS
SGS             DNVAKRDHTNIIIYWLIRDRRPNKDPSNFGQVFTMYDNEPTTAKIRMDLRDRMQVLKKFS
                ***** ******************* ** ******************************

CTCRI           VTVSGGPYSHKEQALVRKFFKGLYNHVTYNHKEEAKYENHLENALMLYSASSHAINPVYQ
SGS             ITVSGGPYSHKEQALVRKFFKGLYNHVTYNHKEEAKYENHLENALMLYSASSHVSNTVYQ
                :**************************************************. *.***

CTCRI           TLRCRAYFYDSHNN--------------------------
SGS             TLRCRAYFYDSHNNQIGSRARRLQRPACKLAAALEHHHHHH
                **************
```

Figure 31: Multiple sequence alignment of SPLCV-CP Using MUSCLE tool.



Lanes 1 and 5- SPLCV-CP protein uninduced sample I, Lanes 2 and 6- SPLCV protein induced sample I, Lanes 3 and 7- SPLCV-CP protein uninduced sample II, Lanes 4 and 8- SPLCV-CP protein induced sample II, Lanes 9 and 10- SPLCV infected leaf sample (positive control), Lanes 11 and 12- Negative control.

Figure 32: DIBA results showing the expressed SPLCV CP

DISCUSSION

90

5. DISCUSSION

Sweet potato (*Ipomoea batatas (L.) Lam.*) is the one of the food security crop in the family of *Convolvulaceae.* Sweet potato is widely accepted because of its wider adaptability, high temperatures, tolerance, easy to propagate and maintain, and yields well in adverse conditions. Sweet potato production is limited by several pathogens such as bacteria, viruses, fungi, nematodes and mycoplasma.

Viruses are the major constraints for the sweet potato production. *Sweet potato leaf curl virus (SPLCV),* a whitefly-transmitted *geminivirus* is one of the important among these and was first reported in Japan and Taiwan (Osaki and Inouye, 1991). Infection of *Sweet potato leaf curl virus* shows symptoms such as inward leaf curling, vein swelling, darkening of tuber skin, and grooving of tubers in some sweet potato cultivars (Pardina *et al.*, 2012).

The infection causes 40-60% reduction in the tuber yield (Clark and Hoy, 2006) which is of the major worldwide concerns. Since the crop being of socio-economic importance, strategies for the development of viral resistant crop needs to be developed.

Different diagnostic methods have been developed for SPLCV detection. Molecular hybridization using a SPLCV specific probe (coat protein gene) has been used to detect SPLCV from sweet potato field collected samples (Valverde *et al.,* 2004a; 2004b). PCR, using specific and degenerate primers and real-time PCR assays have been successfully used to detect SPLCV from indicator hosts and sweet potato plants infected with this virus (Lotrakul and Valverde, 1999; Li *et al.,* 2004; Kokkinos and Clark, 2006; Valverde *et al.,* 2008). Due to the difficulties in obtaining purified SPLCV virions that can be used as antigens for antisera production, serological assays are not currently available.

In our study *Sweet potato leaf curl virus* (SPLCV) was detected in leaf samples collected from ICAR- CTCRI germplasm and Regional center of CTCRI Bhuvaneswar. Thirty five samples were collected and genomic DNA isolated. DNA isolation from sweet potato leaves is difficult because of the presence poly

phenol contents. Due to the unavailability of SPLCV specific antiserum, PCR based detection is carried out for initial screening of samples. SPLCV CP partial primers were used to detect viral infection. Which gave 470 bp size of fragment was obtained. Ten samples were shown SPLCV positive bands in PCR with SPLCV CP partial primers. Rolling Circle Amplification (RCA) was carried out to amplify whole genome sequence of *Sweet potato leaf curl virus* (SPLCV). Paptroka *et al.,* (2010) reported Rolling Circle Replication in SPLCV. They used pBluescript SKII+ vector for cloning and *BamHI, PstI* and *SstI* restriction enzymes. They reported several sequence variants of sweet potato begomoviruses *Sweet potato leaf curl virus* isolates (FJ969832), and *Sweet potato leaf curl virus* (FJ969834). In the present study PBS2KS+ vector and unique *BamHI* restriction site is selected for cloning. Sequencing of S1336 sample shows 90% similarity with *Sweet potato leaf curl virus* Greece isolate (KF697069.1).

In this study *BamHI* site is the unique restriction site in the SPLCV genome and it is in the AV1 region of the SPLCV genome. So new primers were designed for the whole coat protein gene amplification. For the inframe and correct orientation cloning, restriction enzymes *NdeI* and *KpnI* in the upstream and downstream primers were incorperated, which facilitated the successful cloning of the SPLCV CP in pET 28a+. Insertion of several restriction enzymes for the inframe cloning has also been reported by other researchers (Bragard *et al.,* 2000; Kadkhodayan *et al.,* 2000; Liu *et al.,* 2001; Petrzik *et al.,* 2001).

Apart from the pET vector series, several other vector systems have also been used for the efficient cloning including pGEX (Bicka *et al.,* 2001; Jacob and Usha, 2002; Petrzik *et al.,* 2001), pTBG(H) ,pVEX (Saini and Vrati, 2003), and pMAL-c2E (Gutierrez *et al* ., 2015). Among the bacterial strains, *E. coli* BL21 cells are the most preferred one for the *in vitro* protein expression (Bragard *et al.,* 2000; Petrzik *et al.,* 2001; Jacob and Usha, 2002; Saini and Vrati, 2003). Gutierrez *et al.,* (2015) used *E. coli JM 109* competent cells for SPLCV-CP gene expression. They incorporate the SPLCV coat protein gene ,downstream from the mal E gene of E. coli, which encoded a maltose-binding protein (MBP), resulting in a

92

cytoplasmic expression of a fusion protein (MBP * CP). The fusion protein was isolated by affinity chromatography using an amylose-resin column, and the target passenger protein of 32 kDa was cleaved from the MBP by enterokinase, and separated from MBP by 10% (w/v) SDS-PAGE.

Among the varying standardization factors for the optimum protein expression, the expression level of transformed BL21 cells appeared to be the optimum at the optical density of 0.5 cultures with 1 mM IPTG concentration when incubated at 37° C for 3 h.

The protein produced during expression was in the insoluble form. Raikhy *et al.,* (2007) reported that the incubation at 37°C would result in the protein to be in the insoluble form, while at 28°C the protein will be present in the soluble form. It was stated that when large sized proteins are expressed in *E. coli* or when small sized proteins were over expressed then insoluble protein complexes, difficult to hydrolyse (Koschorreck *et al.,* 2005). But in this study we have achieved high level of protein expression at 37°C. Analysis of the use of various IPTG concentrations in protein expression systems were reported, viz., 1 mM (Bragard *et al.,* 2000; Raikhy *et al.,* 2007; Thomas and Baneyx, 1996; Kadkhodayan *et al.,* 2000; Jacob and Usha, 2002), 0.1 mM concentration (Petrzik *et al.,* 2001).

Our studies showed that an optimum IPTG concentration for the SPLCV CP expression was 1 mM. Moreover incubation for 3 h was sufficient for optimum protein expression of SPLCV-CP. However incubation at 16°C for overnight for other potyviral CP are also reported (Kadkhodayan *et al.,* 2000).

The present study helped to detect the *Sweet potato leaf curl* virus by serological assays, which allows the development of relevant and inexpensive method for the detection of SPLCV infections.

SUMMARY

94

6. SUMMARY

The study entitled "Cloning and expression of coat protein gene of *sweet potato leaf curl virus*" was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2017-2018. The objective of the study was to clone and express coat protein gene of *Sweet potato leaf curl virus*. The important findings of the above studies are summarized in this chapter.

Sweet potato samples having virus infection symptoms were collected from the repository of ICAR-CTCRI, Trivandrum and field samples of Bhuvaneswar. These samples had common symptoms such as vein clearing, upward and downward curling, generalized yellowness, vein swelling and blisters formation. The total DNA was isolated from these infected leaf samples. The PCR based detection method was adopted for disease diagnosis. SPLCV partial coat protein primers used for the initial screening of sweet potato samples.

Rolling Circular Amplification was carried out to obtain Whole genome sequence of *Sweet potato leaf curl virus*. The S1336 sample selected for whole genome amplification were cloned in PBS2KS+ and confirmed by polymerase chain reaction with SPG4/G3, M13F/G4, G3/M13R,SPG1/G2 and PW285.1/2. Restriction digestion with *BamHI* was also performed to confirm the clones. Sequencing was done with PW285.2, SPG1, SPG4 and M13 primers. Sequencing of S1336 sample showed 90% similarity with *Sweet potato leaf curl virus* Greece isolate (KF697069.1). For whole genome amplification *BamH1* unique restriction site was used but there is an internal BamH1 site near to the AV1 region of the SPLCV-CP gene so new SPLCV whole coat protein primers were designed for the expression studies. S1336 sample was selected for expression studies, which showed 98% similarity with the CTCRI isolate (KM050768.1). The coat protein gene of the SPLCV isolate was expressed in heterologous expression systems pET 28a+ vector, and the subsequent protein was used for the production of SPLCV specific antiserum. The CP gene was cloned in pET 28a+ vector and transformed

95

into BL21 cells. The BL21 clone containing the inframe CP gene was subjected to protein expression by IPTG induction. The protein expression was optimum at 37°C for 3 h with an IPTG concentration of 1 mM. The expressed protein showed a molecular weight of appropriate size of 32 kDa, when analysed on 12% SDS-PAGE. Expressed protein confirmed by Dot Immuno Blot Assay (DIBA).

REFERENCES

7. REFERENCES

AATF (2015).Report. Afrian Agricultural Technology Foundation, Africa.

Abad, J. A. and Moyer, J. W. 1992. Detection and distribution of Sweet potato feathery mottle virus in sweet potato by invitro transcribed RNA probes (riboprobes), membrane immunobinding assay, and direct blotting. *Phytopathol.*82:300-305.

Abouzid, A. M., Freitas-Astúa, J. Purcifull, D. E., Polston, J. E., Beckham, K. A., Crawford, W. E., Petersen, M. A., Peyser, B. Patte, C. and Hiebert, E. 2002. Serological studies using polyclonal antisera prepared against the viral coat protein of four begomoviruses expressed in Escherichia coli. *Plant Dis.* 86: 1109–1443.

Albuquerque, L. C., Inoue-Nagata, A. K. and Pinheiro, B. 2011. A novel monopartite begomovirus infecting sweet potato in Brazil. *Arch. Virol.* 156: 129–148.

Ashoub, A. and Mervat M. M .2008. Sequence and comparison analysis of coat protein gene and 3` non coding region of *sweet potato feathery mottle virus* (SPFMV) isolated from Egypt. *J. Genet. Cytol.* 37: 17-24.

Banks, G., Bedford, I., Beitia, F., Rodriguez, C. E., and Markham, P. 1999. A novel geminivirus of Ipomoea indica (Convolvulacae) from southern Spain. *Plant Dis.* 83: 486–486.http://dx.doi.org/10.1094/PDIS.1999.83.5.486B

Benjamin, A. 2007. Sweet Potato: A Review of Its Past, Present, and Future Role in Human Nutrition. *Advances in Food and Nutrition Research,* 52, 1-59. http://dx.doi.org/10.1016/S1043-4526(06)52001-7

Bradgard, C. Dinecan, G. H., and Wesley, S.V. 2000. Virus like particles assembles in plants and bacteria expressing the coat protein gene of Indian peanut clump *J. Gen virol.*J8: 267-272.

Briddon, R. Bull, S. and Bedford, I. 2006. Occurrence of *Sweet potato leaf curl virus* in Sicily. *Plant Pathol.* 55: 286–286.

Briddon, R.W. and Stanley, J. 2006. Subviral agents associated with plant single-stranded DNA viruses. *Virol.* 344: 198-210.

Cadena-Hinojosa, M.A. & Campbell, RN. 1981. Serological detection of feathery mottle virus strains in sweet potatoes and *Ipomoea incarnata. Plant Dis.* 65: 412-414.

Chen, M.J. 1972. Electron microscopic studies of plant pathogenic mycoplasma found in Taiwan.*Proceedings of the national council.* Republic of china, 5:61-77.

Chen, X.Y., Chen, F.X., Yuan, Z.N., and Zhuang, B.H. 2001. Effect of virus-elimination on some physiological indices in sweet potato. *J.Fujian Agri. Uni.*30:449-453.

CIP (2017). Annual Report. International Potato Centre, Lima, Peru.

Clark, C. A. and Moyer, J. W. 1988. Compendium of Sweet Potato Diseases, The American Phyto pathological Society. St. Paul, Minnesota, USA.

99

Clark, C. A. and Hoy, M. W. 2006. Effects of common viruses on yield and quality of Beauregard sweet potato in Louisiana. *Plant Dis.* 90: 83-88.

Clark, C. A. and Valverde, R. A. 2001. Viruses and sweet potato cultivar decline in Louisiana, USA. Pp. 62-69. In: International Workshop on Sweetpotato Cultivar Decline Study, 2000. Eds: Nakazawa, Y. and Ishiguru, K. Miyakonojo, Japan.

Clark, C. A., Valverde, R. A., Fuentes, S. Salazar, L. F., and Moyer, J. W. 2012. Research for improved management of sweetpotato pests and diseases: Cultivar decline. *Acta Hort.* 583: 103-112.

Cohen, J., Loebenstein, G. Milgram, M., Pearlsman, M. and Leshman, O. 1997.Ipomoea crinkle leaf curl caused by a whitefly transmitted gemini like virus. *Annal. Appl. Biol.* 131: 245-282.

Colinet, D., Kummert, J., and Lepoivre, P.1994. The complte nucleotide sequences of coat protein cistron and the 3' non- coding region of a newly identified potyvirus infecting sweet potato, as compaired to those of *Sweet potato feathery mottle virus. Arch. Virol.*139:327- 336.

Colinet, D., Nguyen, M., Kummert, J., Lepovre, P. and Xia, F. Z. 1998. Differenciation among poty virus infecting Sweet potato based on genus- and virus specific reverse transcription polymerase chain reaction. *Plant Dis.*82: 223-229.

Dean, F.B., Nelson, J.R., Giesler, T.L., and Lasken, R.S.2001. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Res.* 11, 1095–1099.

Edison, S. Hegde, V. Makesh Kumar, T. Srinivas, T. Suja, G. and Padmaja, G. 2009. Sweet potato in the Indian sub continent. In: The Sweet Potato. Lobenstein, G. and Thottappilly, G. (Eds.). *Springer Science Publishers*, pp.391-414.

Esbanshade, P. R. and Moyer, J. W. 1982. Indexing for *Sweet potato feathery mottle virus* in sweet potato using enzyme linked immune sorbent assay. *Plant.Dis*.66:911-913.

Esterhuizen, L. L., Heerden, S. W. and Rey, M. E. 2012. Genetic Identification of Two Sweet- Potato-Infecting Begomoviruses in South Africa. *Arch. Virol.* 157: 2241-2245.

Faber, M., Venter, S. L., and Benadé, A. J. S. 2002. Increased vitamin A intake in children ,South African community. *Public Health Nutrition*, 5, 11–16.

FAOSTAT [Food and Agriculture Organization Statistical Database], 2015[Online].

Fauquet, C. M. and Stanley, J. 2003. Geminivirus classification and nomenclature; progress and problems. *Ann. Appl. Biol.* 142:165-189.

FIB. 2013. *Farm Guide* Farm Information Bureau, Govt. of Kerala, Thiruvananthapuram, pp.81-94.

101

Fuentes, S. and Salazar, L. 2003: First report of *Sweet potato leaf curl virus* in Peru. *Plant Dis.* 87: 98–98.

Fuentes, S., Mayo, M.A., Jolly, C.A., Nakano, M., Querci, M., and Salazar, L.F. 1996. A novel luteovirus from *sweet potato, sweet potato leaf speckling virus*. *Ann. of Appl. Biol.* 128: 491-504.

Gutierrez, D. L., Fuentes, S. and Salazar, L. F. 2003. Sweet Potato Virus Disease (SPVD): Distribution, Incidence and Effete on Sweet Potato Yield in Peru. *Plants Dis.* 87:297-302.

Gutierrez, M., Watanabe, M., and Todd, A. J. 2015 Expression of gastrin-releasing peptide by excitatory interneurons. *Molecular Pain*, 10: 79.

Haible, D., Kober, S. and Jeske, H. 2006. Rolling circle amplification revolutionize diagnosis and genomics of geminiviruses. *J. Virol. Meth.*135:9-16.

Hall, M. R. and Phatak, S. C. 1993. Sweet potato *ipomoea batatas(L)Lam.*(pp 693-708). Genetic improvement of vegetable crops- Pergamon press, Oxford,833pp.

Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S., and Robertson, D. 1999. Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. Critical Reviews in Plant Sciences 18: 71-106.

Harrison, B. D. 1985. Advances in geminivirus research. *Phyto. Pathol.* 23: 55–82.

102

Hsu, Y. C., Yeh, T. J., and Chang Y.C. 2005. A new combination of RT-PCR and reverse dot blot hybridization for rapid detection and identification of potyviruses.*J.viorol,methods*. 128:54-60.

Inoue-Nagata, A. K., Albuquerque, L.C., Rocha, W. B., and Nagata, T. 2004. A simple method for cloning the complete begomovirus genome using the bacteriophage phi29 DNA polymerase. *J. Virol Methods* 116: 209–211.

Jacob, T. and Usha, R. 2001. 3Terminal sequence analysis of the RNA genome of the Indian isolate of Cardamom mosaic irus: a new member of genus Maclurairus of Potyiridae. *Virus Genes* 23, 81–88.

Kadkhodayan, S. Knapp, M. S., Schmidt, J. J. and Fabes, S. E. 2000. Cloning, expression and one step purification of the minimal essential domain of the light chain of Botulinum Neurotoxin Type A. *Prot. Exp. Purif.* 19: 125-130.

Kays, S. J. 2005. Sweet potato production worldwide: assessment, trends and the future. *Acta Hortica*.670:19-25.

Kim, J. Kil, E. J., Kim, S. Seo, H. Byun, H. S. and Park, J. 2015. Seed transmission of Sweet potato leaf curl virus in sweet potato *(Ipomoea batatas)*. *Plant Path.* 64 (6):1284-291

Kokkinos, C. D. and Clark, C. A. 2004. Real Time PCR assays for detection and quantification of sweet potato viruses. *Plant Dis*. 90:783- 788.

Koschorreck, M. Fischer, M. Barth, S. and Pleiss, J. 2005. How to find soluble proteins: a comprehensive analysis of alpha/beta hydrolases for recombinant expression in *E. coli.* BMC Genomics 6: 49-52.

Kushwaha, S.2011.Impact of supplementation of Drumstick (moringa oleifera) and Amarnath (Amarathus tricolor) Punjab Agricultural University, Ludhiana. India.

Kumar, C. A., Mandal, B. B., and Sreevasthava, M. 1991. Occurrence of SPFMV in germplasm of *Ipomoea batatas .Current Science* 60: 345-348.

Kwak, H. R., Kim, M. K.and Chung M. N. 2006. Virus disease incidences of sweet potatoes in Korea. *J. Plant Pathol.* 22: 239–47.

Leke, W.N., Mignouna, D.B., Brown, J. K., and Kvarnheden, A.2014. Begomovirus disease complex: emerging threat to vegetable production systems of West and Central Africa. *Agric Food Sec.* 4:1-14.

Li, R. Salih, S. and Hurtt, S. 2004. Detection of Geminiviruses in Sweet potato by Polymerase Chain Reaction. *Plant Dis.* 88: 1347-1351. http://dx.doi.org/10.1094/PDIS.2004.88.12.1347

Liano, C. H., Chein, I. C., Chin, R. J. and Han Y.H.1979. A study of sweet potato diseases in Taiwan. *J.Agric.Res.China 28*: 124-128.

Ling, K. S., Harrison, H. F., Simmons, A. M., Zhang, S. C., and Jackson, D.M 2011. Experimental host range and natural reservoir of sweet potato leaf curl virus in the United States. *Crop Protec.*30(8):1055-1062.

104

Ling, K. S., Jackson, D. M., Harrison, H. F. and Simmons, A. M. 2008. Field evaluation on yield effects of U.S.A. heirloom sweet potato cultivars infected by *Sweet potato leaf curl virus. Crop Prot.* 29:757-65

Loebenstein, G. Fuentas, S. Cohen, J. and Salazar, L.F. 2003. Sweet potato In: Lobenstein G, Thottapally G (Eds) virus and virus like diseases of major crops in developing counteris, luwer academic publishers Dordrecht, The Netherlands pp-223-264.

Lotrakul, P. Valverde, R. A., Clark, C. A., Sim, J. and De La Torre, R. 1998. Detection of a Geminivirus Infecting Sweet Potato in the United States. *Plant Dis.* 82: 1253-1257. http://dx.doi.org/10.1094/PDIS.1998.82.11.1253

Lotrakul, P. Valverde, R.A., Clark, C.A. and Fauquet, C. M. 2002. Properties of a Begomovirus isolated from sweet potato infected with *Sweet potato leaf curl virus.* Revista Mexicana de Fitopatologia 21: 128-136.

Lozano, G. Nunez, A. Valverde, R. and Castillo, J. 2004. Begomoviruses infecting sweet potato in Spain (poster). In: Proceedings, 2nd European Whitefly Symposium. October 5-9, Cavtat, Croatia

Lozano, G. Trenado, H. P., Valverde, R. A., Castillo, J. 2009. Novel begomovirus species of recombinant nature in sweet potato (*Ipomoea batatas*) and *Ipomoea indica:* taxonomic and phylogenetic implications. *Journal of Gen. Virol.* 90: 2550–6245.

105

Luan, Y.S., Zhang, J. and An, L. J. 2006. First report of Sweet potato leaf curl virus in China. *Plant Dis.* 90: 11-18

Makeshkumar, T. Prakashkrishnan, B. S., Hedge V. and Edison S. 2007. Sweet potato leaf curl disease – a new emerging virus problem of sweet potato in India. In:ICRISAT(Ed) 10th Plant Virus Epidemiology Symposium on controlling Epidemics of emerging and established Plant virus Disease-15-19 october 2007, Hyderabad, India,pp 68-69.

Mansoor, S. Briddon, R. Zafar, Y. and Stanley, J. 2003. Geminivirus disease complexes: an emerging threat. Trends *Plant Sci.* 8: 128–134. http://dx.doi.org/10.1016/S1360-1385(03)00007-4

Martin, F. W. and Jones, A. 1986. Breeding sweet potatoes. *Plant breeding Reviews* 4, 313-345.

Meng, B., Credi, R., Petrovic, N., Tomazic, I. and Gonsalves, D. 2003. Antiserumto Recombinant Virus Coat Protein Detects Rupestris stem pitting associated virus in Grapevines. *Plant Dis.* 87, 515-522.

Miano, D. W., LaBonte, D. R. and Clark, C. A. 2008. Identification of molecular rmarkers associated with sweet potato resistance to sweet potato virus disease in Kenya. *Euphytica* 160, 15–24.http://dx.doi.org/10.1007/s10681-007-9495-2

106

Miano, D.W., LaBonte, D.R., Clark, C.A., Valverde, R.A., Hoy, M.W., Hurtt, S., and Li, R. 2006. First report of a begomovirus infecting sweetpotato in Kenya. *Plant Dis.* 90: 18-20.

Mukasa, S. B., Rubaihayo, P. R. and Valkonen, J. P. T. 2004. Incidence of Viruses and Virus-like Diseases in Sweet Potato in Uganda. *Plant Dis.* 87:336-340.

Mukasa, S. B., Rubaihayo, P. R. and Valkonen. 2006. Intractions between co-infections in sweet potato plants, *plant pathol.* 53: 458-467.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. *Tomato yellow leaf curl virus:* A whitefly-transmitted geminivirus with a single genomic component. *Virol.*185: 151-161.

NHB [National Horticulture Board], 2011[Online]. Available: http://nhb.org.in [12 August 2011].

Njeru, R. Mburu, M. Cheramgoi, E. Gibson, R. Kiburi, Z. Obudho, E. and Yobera, D. 2004. Studies on the physiological effects of viruses on sweet potato yield in Kenya. *Ann. Appl. Biol.* 145(1): 71-76.

Njeru, M. C., Bagabe, D. Nkezabahizi, D. Kayiranga, J. Kajuga, L. Butare and Ndirigue, J. 2008. Viruses infecting sweet potato in Rwanda: occurrence and distribution, *Annals of Appl. Biol.* 153(2): 215-221.

Noueiry, A. O., Lucas, W. J. and Gilbertson, R. L.2000. Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. *Cell* 76:925–932.

Onuki, M. Honda, Y. and Hanada, K. 2000. Geminate Particle Morphology of Sweet Potato Leaf Curl Virus in Partially Purified Preparation and Its Serological Relationship to Two Begomoviruses by Western Blotting. J. *Gen. Plant Pathol.* 66: 182-184. http://dx.doi.org/10.1007/PL00012942

Osaki, T. and Inouye, T. 1991. Transmission characteristics and cytopathology of a whitefly-transmitted virus isolated from sweet potato leaf curl disease. *Agric. Biol.* 43: 11-19.

Padidam, M. Beachy, R. N. and Fauquet, C. M. 2000. The role of AV2 ("Precoat") and coat protein in viral replication and movement in tomato leaf curl geminivirus. *Virol.* 1996, 224: 390–404.

Padidam, M., Sawyer, S. and Fauquet, C. M. 2012. Possible emergence of new geminiviruses by frequent recombination. *Virol.* 265, 218–225.

Paprotka, T. Boiteux, L. S., Fonseca, M. E., Resende, R. O., Jeske, H. Faria, J. C. and Ribeiro, S. G. 2010. Genomic diversity of sweet potato geminiviruses in a Brazilian germplasm bank. *Virus Res.* 149: 224–233.

Paprotka, T. Boiteux, L.S., Fonseca, N. Resende, R. O., Heske, H. Faria, J. C. 2012. Genomic diversity of sweet potato geminiviruses in a Brazilian germplasm bank. *Virus Res.* 149(2): 224-33.

Pardina, P. R., ,Luque, A. Nome, C. Colomba, F. L., Delgado, S. F. and DiFeo, L. 2012. First report of sweet potato leaf curl virus infecting sweet potato in Argentina. *Australian plant Disease Notes*.7 :157-160.

Pardina, P. R., Luque, A. Nome, C. Colmba, E. L., Delgado, S. F. and Di Feo, L. 2012. First report of *Sweet potato leaf curl virus* infecting sweet potato in Argentina. Australian *Plant Dis.* 7:157-60

Petrzik, K. Mraz, I. Kubelkova, D. 2001. Preparation of recombinant coat protein of Prunus necrotic ringspot virus. *Acta Virol* 45:61–63.

Porstmann, T. and Kiessig, S. T. 1992. Enzyme immunoassays techniques. An overview. *J. Immunological Methods*, 150: 5-21.

Posthuma, K. I., Adams, A. N., Hong, Y.and Kirby ,M.J. 2002. Detection of Strawbery crinkle virus in plants and aphids by RT-PCR using conserved L gene sequences.*J. Plant Path.*51:230-245.

Raikhy, G. Hallan, V. Kulshrestha, Sharma, M. L., Ram, R. and Zaidi A. A. 2007. Molecular characterisation of a Carnation etched ring virus isolate from India. *Acta Virologica* 47: 105-111.

Rojas, M. R., Gilbertson, R. L., Russell, D. R., and Maxwell, D. P. 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. *Plant Dis.* 77:340-347.

Rojas, M. R., Hagen, C. Lucas, W. J. and Gilbertson, R. L. 2005.Exploiting chinks in the plant's armor: Evolution and emergence of geminiviruses. *Annu. Rev. Phyto pathol.* 43:361–394.

Rybicki, E.P. 2000. A phylogenetic and evolutionary justification for three genera of Geminiviridae. *Arch. Virol.* 139:49-77.

Saini, M.and Vrati, S. 2003. A Japanese encephalitis virus peptide present on Jhoson grass mosaic virus-like particles induces virus neutralizing antibodies and protects mice against lethal challenge. *j virol* 77:3487-3494.

Salazar, L.F. and Fuentes, S. 2001. Current knowledge on major virus diseases of sweet potatoes. In: International Workshop on Sweetpotato Cultivar Decline Study, Pp. 14-19.

Saunders, K. Bedford, and I. Stanley, J. 2002. Adaptation from whitefly to leafhopper transmission of an autonomously replicating nanovirus-like DNA component associated with ageratum yellow vein disease. *J. Gen. Virol.* 83: 907–913

SAVACG (1996).Report. The South African Vitamine A Consultation Group, Africa.

Shinkai, A., Maejima, I., Iwasaki, M., Sakamoto, S., Muramine, S., Ide, Y. and Yamakawa, T. 1979. In Report on Investigation of Sweet Potato Leaf Curl Symp tom. pp. 1-38, Agriculture, Forestry and Fisheries Research Council, Tokyo (in Japanese).

Souto, E.R., Sim, J., Chen, J., Valverde, R.A., and Clark, C.A. 2003. Properties of strains of *Sweet potato feathery mottle virus* and two newly recognized potyviruses infecting sweetpotato in the United States. *Plant Dis.* 87: 1226-1232

Stanley, J. Bisaro, D. M., Briddon, R. W., Brown, J. K., Fauquet, C. M., Harrison, B. D., Rybicki, E. P. and Stenger, D. C.2005. Family Geminiviridae. In

110

Virus Taxonomy Classification and Nomenclature of Viruses Eighth Report of the International Committee on the Taxonomy of Viruses. *Elsevier Academic Press*; 301–326.

Stanley, J. Frischmuth, T. and Ellwood, S. 1990. Defective viral DNA ameliorates symptomsof geminivirus infection in transgenic plants. *Proc. Natl. Acad. Sci.* U.S.A.87: 6291–6295.

Tan, P. H. N., Wong, S. M., Wu, M., Bedford, I. D., Saunders, K., and Stanley, J. 1995. Genome organization of *Ageratum yellow vein virus*, a monopartite whitefly- transmitted geminivirus isolated from a common weed. *J. Gen. Virol.* 76:2915-2922.

Thomas, J. G. and Baneyx, F. 1996. Protein misfolding and inclusion body formation in recombinant cells over expressing heat -shock proteins. J. *Biol. Chem.* 271: 11141-11147.

Untiveros, M. Fuentes, S. and Salazar, F. L. 2007. Synergistic Interaction of *Sweet Potato Chlorotic Stunt Virus* (Crinivirus) with Carla-, Cucumo-, Ipomo-, and Potyviruses Infecting Sweet Potato. *Plant Dis.* 91:669- 676.

Valverde, R. A., Lozano, G. Navas-Castillo, J., Ramos, A. and Valdes, F. 2004. First report of Sweet potato chlorotic stunt virus and Sweet potato feathery mottle virus infecting sweet potato in Spain. *Plant Dis.* 88: 428-458

Valverde, R. A., Clark, C. A. and Valkonen, J. P. 2007. Viruses and Virus Disease Complexes of Sweet Potato. *Plant Viruses,* 1, 116-126.

Valverde, R. A., Kokkinos, C. D and Clark, C. A. 2008. White transmission of sweet potato viruses, *virus res.* 100,123-128.

Valverde, R. A., Kokkinos, C. D. and Clark, C. A. 2004. Sweet potato leaf curl virus: detection by molecular hybridization. *Phyto pathol.* 94: 100-105

Varsani, A. Shepherd, D.N., Rybicki, E.P., and Martin, D.P. 2017 A highly divergent South African geminivirus species illuminates the ancient evolutionary history of this family. *Virol J* 6:36.

Vunsh, R., Rosner, A., and Sten, A.1990. The use of polymerase chain rection (PCR) for the detection of *Bean yellow mosaic virus* in gldiolous. *Ann.Appl.Biol.*117:561-569.

Wassawa, P. Otto, B. Maruthi, M. N., Mukasa, S.B., Monger, W. and Gibson, R. W. 2011. First identification of a sweet potato begomovirus in Uganda; characterization, detection, and distribution .*Plant pathol.* 60: 1030-1039.

Wyatt, S. D., and Brown, J. K. 1996. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathol.* 86:1288-1293.

Zhang, Y.Q. and Guo, H. C. 2014. Research progress on the tip meristem culture of sweetpotato. Chinese *Agri. Sci. Bull.* 21 (3), 74–76.

Zhou, X., Liu, Y., Robinson, D.J., and Harrison, B.D. 1998. Four DNA-A variants among Pakistani isolates of cotton leaf curl virus and their affinities to DNA-A of geminivirus isolates from okra. *J. Gen. Virol.* 79:915-923.

APPENDICES

113

APPENDIX I

Reagents required for plant total DNA isolation

1. CTAB DNA Extraction Buffer

Tris HCl (pH 8.0)	: 100 mM
EDTA	: 20 mM
NaCl	: 1.4 M
CTAB	: 2 %
PVP	: 2 % (w/v)
β-mercaptoethanol	: 0.2 % (v/v)

(Freshly added prior to DNA extraction) Autoclave and store at room temperature)

2. **Chloroform-Isoamyl alcohol (24: 1)**

Chloroform	: 24 ml
Isoamyl alcohol	: 1 ml

3. **TE Buffer**

Tris HCl (pH 8.0)	: 10 mM
EDTA	: 1 mM

APPENDIX II

Reagents required for Agarose gel electrophoresis

TAE Buffer (50X)

114

Tris base	: 242g
Glacial acetic acid	: 57.1 ml
0.5 M EDTA (pH 8.0)	: 100 ml

APPENDIX III

Luria Agar Medium

Luria Agar Medium 35 g of LA (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

APPENDIX IV

Luria Broth Medium

20 g of LB (HiMedia) in 1000 ml distilled water. Autoclave and store at room temperature

APPENDIX V

Preparation of LA Ampicillin /X gal /IPTG Plates

1. Ampicillin stock (50 mg/ml) Dissolve 2.5 g Ampicillin in 50 ml deionized water. Filter sterilizes and store at 20°C in the dark.

2. X gal stock (20 mg/ml) Dissolve 0.2 g X gal in 10 ml N, N Dimethylformamide. Store at -20°C in the dark.

3. IPTG stock (100 mM) Dissolve 1.7 g IPTG in 50 ml deionized water. Filter sterilizes and store at 4°C in the dark. Before pouring the plates, allow the LA

115

medium (1L) to cool to 55°C, then add 1ml ampicillin stock (making the final concentration to 50µg/ml). Mix gently and pour the plates. Dry the plates opened at room temperature under UV light for 30 min. Add 40µl X gal stock solution to the plate and spread evenly using a sterile spatula. Allow it to diffuse completely into the medium for about 10 min. Then add 40µl IPTG stock solution, spread evenly using a sterile spatula. Pre-warm the LA ampicillin /X gal /IPTG Plates at 37oC for 20 min before use.

APPENDIX VI

Reagents required for plasmid isolation

1. Buffer P1

Tris (pH 8.0)	: 50 mM
EDTA (pH 8.0)	: 10 mM

Autoclave and store at 4°C

2. Buffer P2 (freshly prepared)

NaOH	: 200mM
SDS	: 1 %

Autoclave and store at room temperature

4. Buffer P3

Potassium acetate : 3 M

116

Adjust the pH to 5.5 with glacial acetic acid. Do not autoclave. Use autoclaved distilled water. Store at 4°C

APPENDIX VII

Reagents required for SDS PAGE

1.1X running buffer (TGB Tris Glycine buffer) 1L

Tris base 3g (25mM)

Glycine 14.4g (190mM)

SDS-1g(0.1%)

pH adjusted to 8.3, make up to 1L using distilled water (Do not autoclave)

2. 30% acrylamide/ bisacrylamide solution (50 ml)

Acrylamide 14.6g

Bis acrylamide 0.4g

Dissolve in dd H_2O and stored at 4°C.

3. **1.5 M Tris pH 8.8 (50 ml)**

Tris 9.08g

4. **1M Tris pH 6.8 (50 ml)**

Tris 6.07g

APPENDIX VIII

Reagents required for DIBA

1. **Tris Buffered Saline (TBS)**

Tris	:	0.02M
NaCl	:	0.5 M
Distilled Water	:	800 ml

Adjust the pH to 7.5 with 1 M HCl and make up to 1L

117

2. Blocking solution (TBS-SDM)

TBS + 5% spray dried milk

3. Substrate buffer

Tris	:	0.1 M
NaCl	:	0.1 M
$MgCl_2$	:	5 mM
Distilled Water	:	800 ml

Adjust the pH to 9.5 with 1 M HCl and make up to 1L

4. **Substrate solution**

Solution A

Dissolve 75 mg of NBT in 1ml N, N Dimethylformamide

Solution B

Dissolve 50 mg of BCIP in 1 ml N, N Dimethylformamide

Add NBT and BCIP to the substrate buffer just before use to give a final concentration of 0.33mg/ml and 0.175 mg/ml respectively

ABSTRACT

CLONING AND EXPRESSION OF COAT PROTEIN GENE OF *SWEET POTATO LEAF CURL VIRUS* (SPLCV)

by

SRUTHY G.S.

(2013 - 09 - 107)

Abstract of the Thesis

Submitted in partial fulfilment of the

requirements for the degree of

B. Sc.-M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

B. Sc.-M. Sc. (INTEGRATED) BIOTECHNOLOGY

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695 522

KERALA, INDIA

2018

120

9. ABSTRACT

The study entitled "Cloning and expression of coat protein gene of *sweet potato leaf curl virus* was carried out at the division of crop protection, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2017-2018. The objective of the study was to clone and express the coat protein gene of *sweet potato leaf curl virus.*

Sweet potato samples with Sweet potato leaf curl symptoms were collected from the germplasm repository of ICAR-CTCRI Thiruvananthapuram and field samples from regional station Bhuvaneshwar. Initial screening of *Sweet potato leaf curl virus* was done with the specific partial coat protein primers which give the bands with the amplicon size of 470bp. Out of 35 samples 10 samples were shows the SPLCV positive infection. Whole genome amplification of *Sweet potato leaf curl virus was* done by rolling circle amplification method. PBS2KS+ vector is used in the cloning purpose. Recombinant colonies were confirmed by restriction analysis with *BamHI.* Sequencing was done by SPLCV specific primers PW285.2, SPG1, SPG4, M13 R and T3. Sequences shows maximum similarity (90%) with *Sweet potato leaf curl Greece virus* isolates (KF697069.1).

For the whole coat protein gene amplification new primers were designed by adding *NdeI* and *KpnI* restriction digestion sites. Standardization of primer was done and kept melting temperature at 55.9°C for further PCR amplification. Whole coat protein gene amplification was done which give the bands at 776 bp. Eluted PCR products were cloned in PTZ57R/T vector and transformed in to DH5α cells. Plasmids were isolated from recombinant colonies and confirmation was done with restriction digestion with *SacI* and *HindIII.* Sequence analysis was done for further studies which show 98% similarity with Sweet potato leaf curl virus CTCRI isolate.

The recombinant PTZ57R/T vector is restricted with *SacI* and *HindIII* is eluted and cloned into expression vectors such as pMALp5X, pET28a+ and pQE

expression vectors. SPLCV-CP insert concentration was high in pET28a+, hence further expression studies pET28a+ was selected. The CP gene was cloned in pET 28a+ vector and transformed into BL21 cells. The BL21 clone containing the inframe CP gene was subjected to protein expression by IPTG induction. The protein expression was optimum at 37°C for 3 h with an IPTG concentration of 1 mM. The expressed protein showed a molecular weight of appropriate size of 32 kDa, when analysed on 12% SDS-PAGE. Expressed protein confirmed by Dot Immuno Blot Assay (DIBA). This expressed protein can be used for the production of antiserum. These aids in the serological detection of Sweet potato leaf curl *virus* infection.