Agrobacterium Mediated Transformation of Tomato Leaf Curl Virus (ToLCV) Coat Protein (CP) gene into Tomato

A Project Report

Submitted by

K. Bhuvana Kamath
C. Divya
&
S. Gauthaman

in partial fulfillment for the award of the degree of

Bachelor of Technology
in
Industrial Biotechnology

Under the guidance of
Dr. S. Sadasivam

DEPARTMENT OF BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY
COIMBATORE – 641 006

ANNA UNIVERSITY: CHENNAI 600 025

April 2006

ANNA UNIVERSITY: CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report entitled "***Agrobacterium* Mediated Transformation of Tomato Leaf Curl Virus (ToLCV) Coat Protein (CP) gene into Tomato**" is the bonafide work of

Ms. K. Bhuvana Kamath	-	Register No. 71202204007
Ms. C. Divya	-	Register No. 71202204008
Mr. S. Gauthaman	-	Register No. 71202204009

Who carried out the project work under my supervision.

SIGNATURE

Dr.S.Sadasivam

HEAD OF THE DEPARTMENT

Department of Biotechnology
Kumaraguru College Of Technology
Chinnavedampatti
Coimbatore-641006.

SIGNATURE

Dr.S.Sadasivam
Prof and HOD
SUPERVISOR

Department of Biotechnology
Kumaraguru College Of Technology
Chinnavedampatti
Coimbatore-641006.

CERTIFICATE OF EVALUATION

College : KUMARAGURU COLLEGE OF TECHNOLOGY

Branch : Industrial Biotechnology

Semester : Eighth Semester

Sl.No.	Name of the Students	Title of the Project	Name of the Supervisor with Designation
01	K. Bhuvana Kamath	**"*Agrobacterium* Mediated Transformation of Tomato Leaf Curl Virus (ToLCV) Coat Protein (CP) gene into Tomato"**	Dr. S. Sadasivam HOD.
02	C. Divya		
03	S. Gauthaman		

The Report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Industrial Biotechnology of Anna University were evaluated and confirmed to be report of the work done by the above students and then evaluated.

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This is to certify that **Ms.C.Divya,** student of B.Tech (Industrial Biotechnology), Kumaraguru College Of Technology, Coimbatore, has successfully completed a Research Project entitled **"*Agrobacterium* Mediated Transformation of Tomato Leaf Curl Virus (To LCV) Coat Protein (CP) gene into Tomato"** from 16th January 2006 to 28th March 2006 in our Research and Development Laboratory.

We wish her all the best for her future.

For **Indo-American Hybrid Seeds (India) Pvt. Ltd.,**

Ganesh S. Hegde
General Manager – HRD & Admin.

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General Manager – HRD & Admin.

G V Jagadish
Dr.G.V.Jagadish 29/03/2006
Associative Director-QA.

ACKNOWLEDGEMENT

We thank the lord Almighty for the multitudes of His tender mercies and abounding grace that has made it possible for us to complete this project.

We are extremely grateful to Dr. Manmohan Attavar, CMD, IAHS for providing us an opportunity to do this project at IAHS pvt ltd.

It gives us immense pleasure in expressing our deep sense of gratitude to Dr.S.Sadasivam HOD, KCT

We express our hearty gratitude to Dr.G.V Jagdish for his valuable guidance and encouragement throughout our training period.

We are thankful to Dr.Devaraja for his constant and valuable guidance throughout our project.

We express our sincere thanks to Smt.S.K Indira and also the other lab staffs Ms.C.M.Mamatha, Mr.K.Ajith Kumar and Mr.Anthonyswamy for demonstrating the experiments.

Last but not the least, we express our respect to our beloved parents for their blistering and solicitous blessings and we thank our friends for their best wishes and encouragement.

ABSTRACT

Tomato is the world's largest vegetable crop and is known as a protective supplementary food. **Tomato leaf curl virus (ToLCV)** is a Geminivirus that has caused a severe epidemic leading to complete crop failure. The objective of this project is to confer pathogen-derived resistance to tomato plants against ToLCV via coat protein (CP) mediated protection. Healthy tomato plants were maintained under green house conditions. Healthy whiteflies *Bemisia tabaci* were made to feed on ToLCV infected tomato plants. These viruliferous flies were allowed to feed on the young tomato plantlets to produce infection. The coat protein gene of ToLCV was isolated by PCR from genomic DNA of infected tomato leaves and cloned into *E.coli* DH5α strain carrying pUC19 plasmid. This was followed by sub-cloning of the CP gene into pGPTV a binary vector. Tri-parental mating was carried out between *E.coli* carrying pGPTV-CP and Agrobacterium using helper *E.coli* strain. The transconjugants were screened using PCR and the *Agrobacterium* clones carrying the CP gene were selected. Tomato seedlings were raised from embryos and leaves from these seedlings were used for leaf disk preparation. The selected clones of *Agrobacterium* were co-cultivated with tomato leaf disks and allowed to develop into callus.

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ABBREVIATIONS

AAP - Acquisition Access Period

ACMV – African Cauliflower Mosaic Virus

Amp - Ampicillin

AMV - Alfalfa Mosaic Virus

BAP - Benzyl Amino Purine

BCTV - Beet Curly Top Virus

BGMV - Bean Golden Mosaic Virus

bp – base pair

CP - Coat Protein

CTAB - Cethyltrimetyl Ammonium Bromide

ds – Double stranded

E.coli – Escherichia coli

EDTA – Ethylenediaminetetraacetate

IAA - Indole Acetic Acid

IAP - Inoculation Access Period

IPTG – Isopropyl - β - D- Thiogalactopyronoside

KRS - kanamycin,rifampicin and streptomysisn

LB - Leuria Bertini

Mr – Molecular weight

MS - Murashige and Skoog

MSV - Maize Streak Virus

PCR - Polymerase Chain Reaction

PDR – Pathogen Derived Resisitance

pGPTV - plasmid Glucuronidase Plant Transformation Vector

PR - Pathogenesis Related

pUC – plasmid of University of California

PVX - Potato X Potevirus

RCR - rolling circle DNA replication

RT media - Regeneration for Tomato

SA - Salicylic acid

ss – single stranded

TAE – Tris acetate

TE - Tris EDTA

TGMV - Tomato Golden Mosaic Virus

TMV - Tobacco Mosaic Virus

ToLCBV -Tomato leaf curl Bangalore virus

ToLCNdV -Tomato leaf curl NewDelhi virus

ToLCV - Tomato leaf curl virus

ToLCVD - Tomato Leaf Curl Virus Disease

ToMV - Tomato Mosaic Virus

TPM – Tri Parental Mating

TRV - Tobacco Rattle Tobra Virus

TSWV - Tomato Spotted Wilt Virus

TYLCV - Tomato yellow Leaf Curl Virus

TYLCV-Th – Tomato Yellow Leaf Curl Virus Thailand Isolate

UV - Ultra Violet light

X-Gal – 5–Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside

INTRODUCTION

CHAPTER 1

1. INTRODUCTION

1.1 An introduction to crop plants:

Man is directly dependent upon plants for his survival because plants are the prime source of food, fiber and drugs. Vegetables play an extremely important role in providing nutrition to all sections of the society in India.

Tomato (*Lycopersicon esculentum*) is the world's largest vegetable crop and known as protective food because of its special nutritive value. Tomato is protective supplementary food. It is short duration crop and gives high yield. Tomato is used in preserved products like ketch-up, sauce, chutney, soup, paste, puree etc. It is a rich source of vitamins (A & C) and organic acid, essential amino acids and dietary fibers. It also contains minerals like iron, phosphorus. Tomato contains Lycopene and β-carotene pigments. The estimated area and production of tomato for India are about 3, 50,000 hectares and 53, 00,000 tons respectively. The average productivity of tomato in our country is merely 158 q/ha while its productivity in USA is 588 q/ha, in Greece 498 q/ha, in Italy 466 q/ha and 465 q/ha in Spain (Mehrota.R.S 1995). The maximum production and productivity have been shown by Uttar Pradesh (UP) followed by Karnataka, Punjab, West Bengal and Assam. The advantages of growing tomato are:

- Short duration vegetable crop.
- The branches of indeterminate plants keep growing and producing fruits until frost kills the plant.
- Tomato is well fitted in different cropping systems of cereals, grains, pulses and oilseeds.

1.2 Plant diseases:

Stakan and Harrar (1957) defined plant disease as, "a physiological disorder or structural abnormality that is harmful to the plant or to any of its part or products that reduces the economic value". Diseases caused by virus, viriod, mycoplasma and spiroplasma are among the major constraints for achieving maximum production on these group of crops. On an average, in India, there is a loss of about 10% in crop yields due to plant diseases (Mehrotra.R.S, 1995).

The scientific investigation of plant viral diseases did not begin until the late 19th century. The earliest known written record describing a viral disease is a poem in Japanese written by the Empress Koken in 752 A.D. and translated by T.Inouye as follows,

"In this village
It looks as if frosting continuously
For, the plant I saw
In the field of summer
The color of the leaves were yellowing"

The plant, identified as *Eupatorium lindleyanum*, has been found to be susceptible to the tobacco leaf curl Geminivirus, which causes a yellowing disease (Osaki *et al*; 1985).

1.3 Plant viruses:

All known plant viruses are small, most having 4 to 10 genes in their genome. Most plant viruses have positive sense RNA, although some have negative sense, ambisense or ds-RNA and a few small DNA viruses. In most viruses, the genetic information is encapsulated by a protein coat. All geometric viruses are either rods or spheres. Due to small amounts of genetic information, viral replication depends on plant

In general, plant virus life cycle has 2 phases:

1. Replication within plant cells and subsequent spread of the infection throughout the plant.
2. The virion phase where the virus is transmitted from one plant to another. This usually involves vectors like insect, fungus, nematode etc. (William.O.Dawson and Mark.E.Hilf; 1992).

Plant DNA viruses can be classified into double stranded (ds) caulimoviruses and the single stranded (ss) geminiviruses. The latter have DNA genomes of 2.5-3.0 Kb and unique geminate (twinned) capsid morphology, hence the name geminiviruses. These icosahedra are joined together at a site where one morphological subunit is missing from each. They infect many important crop plants including monocots and dicots. Some Geminiviruses are transmitted by whiteflies and others by leafhoppers in a persistent manner, but the virus do not replicate in their vectors and are not seed transmitted. Few of them may be transmitted by mechanical inoculation. Each member in this group, appear to have a relatively narrow host range.

1.4 Tomato leaf curl virus (ToLCV):

ToLCV belongs to subgroup III of geminiviruses which includes viruses that infect dicots and are transmitted by whitefly both monopartite and bipartite genomes have been reported

The symptoms caused by the virus are as follows (Dr. Singh S.J, 1993)

1. Severe stunting of plants with downward rolling and wrinkling of leaves.
2. Newly emerging leaves exhibit slight yellow coloration and later show curling symptoms.

4. Nodes and internodes are significantly reduced in size.
5. Infected plants look pale and produce more lateral branches giving bushy appearance.
6. Fruits from infected plants are small and deformed.

Studies conducted at IIHR (Indian Institute of Horticulture Research), showed that the disease progresses from February to June when dry and hot season with low humidity prevails. The incidence may even reach up to 100% during these months. This type of weather conditions favor whitefly multiplication and disease spread. This disease is transmitted by a single whitefly species (*Bemisia tabaci*). The whitefly population and ToLCV incidence remain comparatively less during winter and rainy seasons. The virus perpetuates on many weed hosts like *Acanthospermum hispidium*, *Blainvella rhomboids* etc. These hosts act as potential source of inoculums of ToLCV.

1.5 Control measures:

The various measures taken to control viral diseases are

1) The breeding of resistant or immune cultivates by classic genetic procedures
2) Control of vectors
3) Use of antiviral agents and chemicals
4) Production of transgenic plants containing viral genes that confer resistance to the virus.

1.5.1 Genetic protection:

It is the most important means of protecting crops from developing systemic disease. It requires knowledge about the genes that provide resistance to virus disease and such involves "cultivar resistance" rather than "species resistance". The major problems faced by this form variation and the costs of breeding program weighed against the possible gains in crop yield are high. Also, the process being a long term one has reduced the practical application of such a control measures.

1.5.2 Control of vectors:

This can be achieved in various ways by isolation, by breaking the cycle of the vector, virus and host plant and by creating artificial barriers to exclude the vector. Screens or cages may be used to ward off flying insects. But these methods are ineffective and not advisable to gain complete protection.

1.5.3 Antiviral agents and chemicals:

Antiviral agents are mainly inhibitors that are obtained from plant extracts, in and from insects. Some inhibitors also include antimetabolites. Earlier certain systemic fungicides were used to suppress symptoms of virus infection without any effect on the amount of virus produced in the leaves. Salicylic acid (SA) induces pathogenesis related (PR) proteins in plants and confers resistance to pathogens. However, there has been no successful control on a commercial scale by application of antiviral agents and chemicals.

1.5.4 Transgenic plants:

It is now possible to introduce almost any foreign gene into a plant and obtain expression of that gene. This makes possible to transfer genes for resistance or immunity to a particular virus across species, genes and family boundaries. The presence of one virus in the plant can interfere with infection by another virus or strain, resulting in cross protection. Transgenic expression of viral coat protein genes, non coat viral genes, satellite RNAs, Ribozymes, PR proteins and virus specific antibodies in a variety of crop plants have been carried out to provide a useful degree of protection against the virus concerned.

Polston and Ernest Hiebert (University of Florida, plant pathologist) snipped a piece off the gene that regulates replication in the tomato yellow leaf curl virus and spliced that genetic information into tomato plants. Their hope was that the transferred gene would give the tomato's immune system the ability to recognize the virus and shut it down before it damages the plant.

The extent of protection provided by such transgenic plants is correlated with the degree of expression of coat protein gene.

This approach of developing viral resistance plants is a more specific and efficient method when compared to the rest. Thus transgenic plants expressing viral coat protein genes offer the best approach. Also the tremendous loss in the tomato crop due to ToLCV has stressed the need to develop ToLCV resistant transgenic tomato plant. Thus, this piece of research work will deal with the isolation, cloning and transformation of the ToLCV coat protein mediated by *Agrobacterium tumefaciens* into tomato.

REVIEW OF LITERATURE

CHAPTER 2

2. REVIEW OF LITERATURE

2.1 Plant diseases:

Plant diseases have a tremendous impact on human affairs. Failure in food crops due diseases result in food shortage, famine and drastically affects the economy of the country. Amongst the prokaryotes, the economically important pathogenic organisms are members of the phytoplasma group and some from a small number of bacterial genera. Majority of the plant pathogens are fungi. Most of them are saprophytes and almost 8000 species cause plant diseases (Slater.A.*et al.*, 2003). Such diseases will have a major effect on the yield and commercial value. For e.g. in 1946-47, the wheat rust epidemic was responsible for food shortage as well as seeds in Madhya Pradesh (Padmanabhan, 1973). The bacterial blight of rice was reported in 1951 in Bombay (Srinivasan *et al*, 1959) and due to the introduction of a susceptible rice variety, the disease became pandemic within two years. Apart from these nematodes and parasites also pose a major threat to crop plants. For eg: lesion nematode and the potato cyst of potatoes, dagger and root-knot nematode of apples and sting and stubby root nematode of tomato plants. Pesticides and insecticides have been the main control measures used to reduce the incidence of bacterial and fungal diseases. One successful biotechnological approach to pest resistance is that of Bt cotton. Transgenic cotton, potato and maize plants were produced that carried in them the endotoxin gene isolated from *Bacillus thuringiensis.* These transgenic plants were resistant against insect pests like cotton bollworm, Colorado beetle and European corn borer respectively (Slater.A.*et al.*, 2003).

2.2 Viral diseases:

A plant is susceptible to a particular virus if it possesses all the components necessary for viral replication and systemic infection. Dawson and Hilf (1992) have listed the virus-host interaction types in order of decreasing susceptibility. Viral diseases are among the major constraint for achieving maximum production of food crops. This is because environmental and physical pressures lead to high levels of recombination between viruses (Howarth J.A *et al.*, 1989) (Timmermans *et al.*1994), making it a mammoth task to control such diseases. In May 1999, an epidemic of severe tomato leaf curl virus disease (ToLCVD) caused complete crop failure in Kolar district of Karnataka (Banks G.K *et al.*, 2001). Dasgupta.I and colleagues in 2003 have reported the percentage of yield loss due to some important viral diseases of crops in India. Four out of the ten diseases mentioned were the mosaic and the leaf curl diseases that were caused by the Begomovirus group. The cotton leaf curl virus caused yield losses of 68-71% and the tomato leaf curl virus causing the main loss of 100% (data collected during epidemic years).

Using plant genetic engineering techniques, transgenic potato and tobacco plants have been developed that are resistant to potato virus X and tobacco mosaic virus. These transgenic plants produced viral coat protein and they showed considerable resistance to infection by their respective viruses (Maarten .J.C. *et al.*, 1994).

2.3 Geminiviruses:

Geminiviruses are a group of plant DNA viruses that pose a major threat to economically important crops like tomato, tobacco, sugarcane, wheat, maize, potato, bean etc. Among these the tomato crops have been susceptible to the ToLCV leading to complete crop failure in

0.7-0.8 * $10^{6)}$ and one type of coat protein polypeptide (Mr- 27-34 * 10^{13}) (Francki.R.I.B *et al*,1993). The genomes of Geminiviruses are organized in 3 ways: bipartite, monopartite and atypical based on the number of individual DNA molecules it contains. Subgroup I are called bipartite Geminiviruses which have a genome comprised of two components, designated DNA A and DNA B. each component is encapsidated in a separate geminate particle with totally six ORF's (DNA A-4, DNA B-2) e.g.:- ACMV. Subgroup II includes viruses that have monopartite genome containing 4 ORF's e.g. - MSV and WDV. Several Geminiviruses have properties intermediate between the above two subgroups. The genomes of BCTV, ToLCV and TYLCV resemble both monopartite and bipartite viruses and are called 'Atypical Geminiviruses' (Timmermas *et al.*, 1994).

The number of ORF's differs between the monopartite and bipartite viruses. But all geminiviruses share a common feature which is the intergenic region containing typical transcription initiation and termination sequences which control viral replication process (Timmermas *et al.*, 1994). The differences between monocot type infecting and dicot type infecting DNAs have been closely examined by Howarth A.J *et al.*, (1989). First is that monocot-types have two intergenic regions where as dicots have only one. Second is the presence of a 80 nucleotides complementary strand DNA covalently bound to the 5' end of ribonucleotides which act as primers of complementary strands synthesis in the monocot type viruses and is absent in the dicot-type.

2.3.1 Classification:

Based on geographical distribution those infecting dicotyledonous plants are classified as "old world" and "new world gemininiviruses". Bean Golden Mosaic Virus (BGMV) and Tomato Golden Mosaic Virus

Curl Virus (TYLCV) and Tomato Leaf Curl Virus (ToLCV) belong to the old world Geminiviruses (Howarath J.A *et al.*, 1989).

Based on vector and host range they are divided into three subgroups. Subgroup I includes those viruses that infect monocotyledonous plants and transmitted by leafhoppers e.g.:- Maize Streak Virus (MSV). Second subgroup are leafhopper transmitted viruses infecting dicotyledonous plants e.g.:- Beet Curly Top Virus (BCTV). The third subgroup comprises of whitefly transmitted viruses infecting dicotyledonous plants (Dean E.Rochester *et al.*, 1994).

Phylogenic analysis of Geminiviruses was carried out based on amino acid sequences of 16 geminiviruses replication – associated proteins and 15 coat proteins. Trees based on coat proteins add two main branches which positively correlated with vector specificities for either monocots or dicots (Howarth A.J *et al.*, 1989). A composite phylogenetic tree based on amino acid sequences of coat protein and replication associated proteins showed that the monopartite monocot infecting geminiviruses are ancestral to bipartite class. This transition from monopartite to bipartite is known to have occurred in 3 steps: Infection of dicots, Acquisition of whitefly, transmission and acquisition of a bipartite genome. Intermediates in this transition may have resembled viruses of the atypical class such as ToLCV and TYLCV (Timmermans *et al.*, 1994).

2.3.2 Replication:

Geminivirus replication seems to be confined to the cell nucleus. In infected plants, viral particles accumulate almost exclusively in the nuclei. In the dicot-specific geminivirus infection results in major cytopathological changes in the nucleus (Timmermans *et al.*,1994). Evidence suggests that replication occurs via the rolling circle model is known to be involved in replication. Any deletions or alterations in its structure can interfere with replication. It contains a palindromic sequence within this region is a very highly conserved 9-base sequence that is closely related to the bacteriophage ϕX174Seq that is known to initiate rolling circle DNA replication (RCR) (Bruce A.Voyles,2002).

This 9bp sequence encodes the C1 protein which initiates the RCR. This protein nicks the *ori* site in the virus- sense DNA and remains covalently linked to the 5' side of the nick. The 3'end is extended by DNA polymerase. When replication proceeds to complete the circle and the *ori* site is regenerated on the progeny strand, C1 protein cleaves this site and transferred to the progeny strand to initiate a new round of replication. Thus, this protein not only initiates but also terminates RCR. It also facilitates the unwinding of the dsDNA acting in conjugation with *rep* helicase (Eugene *et al.*, 1992).

2.4 ToLCV:

The ToLCV genome falls under the atypical category and is known to have both monopartite and bipartite genomes. One isolate called Tomato leaf curl Bangalore virus (ToLCBV) has a monopartite genome where as the Tomato leaf curl NewDelhi virus (ToLCNdV) has a bipartite genome with both the components required for symptom development and systemic movement (Orawan *et al.*, 2002). The DNA within the virions is positive strands. The ORF's occur in both positive and negative strands and appear to have numerous overlapping reading frames (Bruce A.Voyles, 2002).

The complete genome of Thailand isolate of TYLCV-Th is of the bipartite type. The A - DNA had six ORF's and B – DNA has two ORF's. Sequence comparisons showed that TYLCV–Th maintained the greatest degree of similarity with the ToLCV isolated in Australia (Dean E. Rochester *et al.*, 1994).

The complete genome of the Bangalore isolate of ToLCV is shown in **Fig 2.1**. It is a single stranded circular DNA of 2759 bp (Accession no: AF165098) (Muniyappa *et al.*, 2000). It clearly shows 6 ORF's encoding 6 proteins needed for replication and encapsidation. The absence of genes for movement shows that this virus depends on nutrient movement within phloem to move through various parts of the plant.

Fig 2.1: Genome map of ToLCV- Ban4 (Ref: www.ncbi.nih.in)

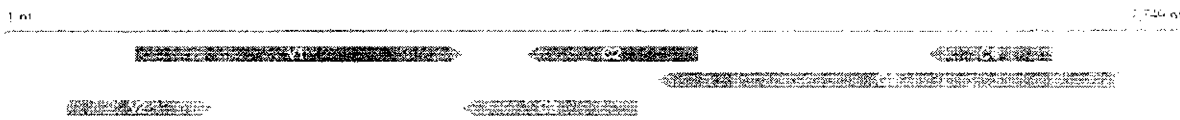


Table 2.1: Genome of ToLCV- Ban4. (Ref: www.ncbi.nih.in)

Product Name	**Start**	**End**	**Strand**	**Length**	**Gi**	**GeneID**	**Locus**
V2 protein	141	488	?	115	21165977	944494	V2
V1 (coat protein)	301	1071	?	256	21165976	944497	V1
C3 protein	1074	1478	-	134	21165980	944495	C3
C2 protein	1219	1623	-	134	21165979	944493	C2
C1 (replication-associated protein)	1526	2608	-	360	21165978	944498	C1
C4 protein	2158	2451	-	97	21165981	944496	C4

2.4.1 Vector and hosts:

White flies (*Aleyrodidae*) are known to transmit about 70 disease agents mainly of tropical and subtropical plants. The ToLCVD is known to be transmitted by a single whitefly species *Bemisia tabaci* (*Gennadius*). Only the first instar of the larva is mobile and it does not move far. Adults are winged and many generations may be produced in a year. The nymphs of *B.tabaci* are phloem feeders (Mathews R.E.F.1992). Whiteflies require 30 minutes to acquire and transmit the virus. They become viruliferous in 6 hour after acquisition feeding. The acquisition threshold for whiteflies was 31minutes and resulted in 3% transmission. A minimum feeding period of 32 minutes was required by a viruliferous whitefly to cause infection on tomato test plant, this gave 4% transmission. Starving the vector for 1 hour pre-acquisition or one hour pre – inoculation result in higher level of transmission of ToLCV. Extended feeding period beyond 1 hour resulted in reduced transmission level. Whiteflies could also acquire the virus from the cotyledonary leaves of infected tomato plant with resultant of 20% transmission. After an acquisition access of 24 hour to a ToLCV source, male and female whiteflies retain their infectivity for 5 and 53 days respectively (Singh S.J, 1993).

Nymphs also can acquire and transmit the virus. Percentage parasitism of *B.tabaci* nymphs on tomato, weed species and cotton respectively was 0.7%, 2 to 6% and 19%. The highest mean number of nymphs per plant (125) was recorded on cotton grown in the glass house (Ramappa H.K *et al.*, 1998).

Transmission of circulative viruses involves the passage of viral particles from the gut into the hemocoel, then into the salivary gland and ultimately through saliva into another plant. The vector specificity of transport from the hemocoel into the salivary gland. Such virus-vector specificity is determined both by the virus and by the insect (Timmermans *et al.*, 1994).

ToLCV – Tomato plant interaction falls under type I interaction, in which the virulent virus not only replicates and systematically invades the plant but also induces symptoms and reduce yield (Dawson and Hilf., 1992).Ramappa H.K and colleagues (1998) have studied the contribution of tomato and alternative host plants to ToLCV inoculum pressure in different areas of South India. ToLCV was identified in 13 weed species commonly found in Karnataka like *Parthinium hysterophorous L., Solarium nigeum L., Ageratumconyzoides L., and Datura stramonium L.*, ToLCV isolated from these weeds were transmitted successfully to tomato by *B. tabaci*. This showed that the weeds act as a reservoir of ToLCV.

2.4.2 Losses due to ToLCVD:

Experiments conducted at Indian Institute of Horticulture Research (IIHR), Bangalore has revealed that loss in yield of tomato is as high as 92.5%. However, the loss in yield varied with the age of the plant at the time of infection. When plants get infected within 20days after transplanting, they remain stunted and produce very few fruits and the loss in yield reach up to 92.5%. However if plants are infected at 35 and 50days after transplanting, the yield loss was 74.1% and 28.9% respectively. This indicates that earlier the infection, higher the yield loss and vice versa. (Sastry and Singh, 1973). In Karnataka alone, 100% ToLCV infection was observed in tomato plants during the summer months, causing yield losses ranging from 27-90% (Devaraja; *et. al.* 2005). Thus the tremendous losses of the tomato crop have stressed the need to develop viral resistant tomato hybrids.

2.5 Role of coat protein:

From the ToLCV genome structure shown previously it is clear that the coat protein is encoded by the V1 gene containing 771bp. The nucleotide sequence of the coat protein gene of ToLCV Bangalore isolate is as follows:

Accession no: Z48182 (Hong.Y.G *et al.*, 1995).

ORIGIN

1 atgtccaagc gtccggcaga tatgatcatt tccgggcccg tctcgaagta tcgccgactt
61 ctgagctcaa tcagcccata cagcaagcgt gctgctgtcc gcattgtccg cggcacaaag
121 ggaaaggaat gggccaacag gcccatgaat cggaagccca tgttttacag gatgttcaga
181 ggtcctgatg ttcctagagg ctgtgagggt ccatgtaagg tccaatcctt tgagtcaaga
241 cacgatataa ttcatatagg gaaggtcatg tgtattagtg atgtcactcg cggtacgggg
301 ttaacacata gagttggtaa gcggttttgt gtcaagtcag tatacgtttt gggtaaaata
361 tggatggatg agaatatcaa gactaagaat cacacgaata gtgttatgtt ttttcttgtt
421 cgtgaccgtc gccctgttga taagccacag gactttggag atgtgttcaa tatgtttgac
481 aacgagccta gcactgcaac tgtgaagaat atgcatagag atcgttatca ggtgttgagg
541 aagtggcatg caactgtcac tggtggacag tacgcttcaa aggaacaggc attagtgaag
601 gagtttgtta aggttaataa ttacgttgtt tataaccagc aagaggctgg gaaatatgag
661 aatcattctg agaatgcgtt gatgttgtat atggcatgta ctcatgcctc taatcctgtg
721 tatgctacgc ttaagattcg tatttacttc tacgattctg taaccaattg a

This is the most abundant protein found in infected tissues. The coat protein serves many important functions for the virus. Firstly, it encapsulates the ss DNA genome and protects it. Secondly, the coat protein has been suggested to regulate the transition from RF synthesis to viral ss DNA production such that the absence of coat protein early in infection would result in continuing RF and its presence later in the infection cycle would lead to encapsidation. Thirdly, for symptom and disease development coat protein plays a major role. The virus has to move through the plant to cause infection.

The movement can be viewed as two processes a) long-distance spread through the vascular system, and b) local spread (i.e. movement of viral DNA between cells). Since geminivirus replicate in the nucleus, another transport mechanism may be needed to facilitate movement in and out of the nucleus.

In bipartite geminiviruses, replication and encapsidation functions are encoded by DNA-A and systemic spread functions are encoded by DNA-B. The proteins encoded by DNA-B, BL1 (~ 33 kDa) and BR1 (~ 27 kDa) are both necessary for systemic infection (Lucas.J.W.*et al.*, 1994). Mutation of the coat protein of ACMV (bipartite) has little effect on the long distance spread, but symptom attenuation and delay was observed. Explanation for this observation requires a model based on the postulate that some level of interaction between the coat protein and BV1 movement protein is required. This interaction may be synergistic and may involve overlapping functions such as binding to viral DNA. Also BV1 and the coat protein share significant homology (Timmermans et al., 1994). Monopartite viruses are phloem-limited and depend largely on the nutrient flow for spread. The requirement of coat protein in this process suggests that virions mediate the movement.

2.6 Coat protein mediated protection:

Transformation of plants with portions of viral genomes frequently gives rise to lines of plants that are resistant to the virus from which the sequence was derived. These include segments from of viral genomes encoding capsid or coat proteins, sequence encoding proteins that are or may be subunits of viral replicase, sequences incapable of encoding proteins, entire genomes of defective interfering viruses and derived from the concept of cross protection which showed that the presence of one virus in a plant can interfere with infection by another virus or strain.

Sanford and Jonhson (1985) coined the term "parasite-derived resistance" or "pathogen-derived resistance" (PDR) which referred to the expression of viral gene product at either an inappropriate time, in inappropriate amounts, or in an inappropriate form during the infection cycle, thereby perturbing the ability of the pathogen to sustain an infection. Currently PDR has been divided into two major forms of protection a) coat-protein mediated protection and b) replicase-related proteins mediated protection.

The mechanism of coat-protein mediated resistance has been explained by Lomonossoff (1995) based on two models. In model one, a sub-cellular component acts as a receptor or uncoating site for invading virions and is responsible for initiating the uncoating process. In transgenic plants, accumulating coat protein, coat protein would bind to the receptor preventing the association of virions with the receptor and thus rendering them unable to initiate infection. In model two, the initiation of uncoating is triggered by a change in physiological conditions upon entry into the cell and initiation of uncoating is a reversible, dynamic process. In cytoplasm that contains transgene derived coat protein, the equilibrium between uncoating and recoating of the end of the virion would be shifted in favor of recoating. Both models predict that plants accumulating higher levels of coat protein would exhibit a greater degree of resistance.

The coat-protein strategy of disease resistance has the following advantages (Timmermans *et al.*, 1994):

- The strategy can generally be extended to other plant species and resistance against other viruses.
- In this case broad-spectrum resistance is expected and it mat not be necessary to isolate the CP gene of the pathogenic virus if the CP gene of a related virus or strain is available.

2.7 Field testing:

Many transgenic plants expressing coat protein genes for Tobacco Mosaic Virus (TMV), Alfalfa Mosaic Virus (AMV), Tobacco Rattle Tobra Virus (TRV) and Potato X Potevirus (PVX) have been developed. Field experiments with tomatoes suggest that transgenic experiment of coat protein were partially resistant to TMV and to strains L, 2, 2^2 of Tomato Mosaic Virus (ToMV). Less than 5% of transgenic plants showed systemic disease symptoms (Mathews R.E.F, 1991). Genetically engineered potatoes were produced that were resistant to potato virus X. They found that some transgenic lines of potatoes carrying the potato virus X coat protein gene had a reduced yield, whereas other lines had the same yield, but produced many elongated potatoes. Such elongated potatoes may not appeal to the consumers. (Maarten.J.C. *et al.*, 1994). When transgenic plants expressing CP of TMV were grown at higher temperatures, drastic reductions in coat protein accumulation were observed and resistance was overcome (Fitchen *et al.*, 1993).

CHAPTER 3

3. OBJECTIVES

This project has been formulated with the following objectives: -

1) To maintain Tomato leaf Curl Virus (ToLCV) culture on tomato plants.
2) To isolate coat protein (CP) gene of ToLCV.
3) To clone the CP gene into pUC19 vector.
4) To Subclone the CP gene into pGPTV binary vector.
5) To do *Agrobacterium* mediated transformation of CP gene into tomato plants.

MATERIALS & METHODS

CHAPTER 4

4. MATERIALS AND METHODS

4.1 Maintenance of virus culture

4.1.1 Raising of healthy tomato seedlings

Procedure:

1) Seeds of hybrid tomato Rashmi were sown into the nursery tray containing sterilized nursery media.
2) These trays were drenched with a solution of fungicide (Bavistin 0.1%) to suppress soil borne fungal pathogens and the seeds were allowed to germinate under green house conditions.
3) The seedlings were carefully transplanted to polybags containing farm yard manure, sand and red earth in equal ratio.
4) The seedlings were allowed to grow for 12 days; these healthy seedlings were used for inoculation experiments.

4.1.2 Raising of healthy whiteflies

Procedure:

1) Healthy whiteflies were raised and maintained on cotton plants.
2) The cotton plants were replaced with new plants at regular intervals to ensure healthy whitefly population.
3) Plants along with the flies were maintained within rooms covered with nets to prevent the entry of other insects and the escape of whiteflies.

4.1.3 Inoculation of tomato seedlings

Procedure:

1) A modified plastic pet jar is taken. Its bottom is removed and covered with muslin cloth. A hole is created in the side of the container and on its lid, which are closed with cotton. This is called acquisition bottle.
2) The lid of the container is removed and the healthy whiteflies were collected

3) The pet jar is then closed quickly.
4) A small portion of infected tomato plant is cut and inserted into the jar such that leaves are inside the jar and stalk project out through the hole in the lid.
5) Any gaps in the holes are filled with cotton.
6) The lower most portion of the stalk is scrapped off and a piece of cotton is tied around it with rubber band.
7) The stalk with the cotton is soaked into a dish of water.
8) This entire setup is enclosed in a netted cage which is in turn covered with the news paper.
9) This is to provide the dark environment which will allow the fly to feed on the infected leaves.
10) The time required for the fly to acquire virus varies from 30 minutes to days. This period is called AAP (Acquisition Access Period).
11) Then these infected white flies are collected using the same method and made to feed on raised seedlings for overnight. This period is called IAP (Inoculation Access Period).
12) These newly infected healthy plants were transferred to green house conditions and monitored for symptoms.

4.2 Isolation of ToLCV coat protein gene

Equipments: Microfuge (Beckman 22R microcentrifuge), UV-spectrometer (DU 720 General purpose UV/Vis spectrophotometer), PCR (Beckmans)

Materials: All chemicals used were of analytical grade.

4.2.1 Isolation of genomic DNA (Porebski *et al.*, 1997)

Reagent: Extraction Buffer: - (100ml) [10mM Tris-HCl (v/v), 1.4 M NaCl (v/v), 20mM EDTA (w/v), 2 % CTAB (w/v), 0.4 %, 2-

Bangalore), Sevag (24:1::chloform:isoamylalcohol), Isopropanaol, 5M NaCl, Absolute ethanol, 70 % ethanol (ice cold), 3M-sodium acetate, Tris – EDTA.

Procedure:

1) 150 mg of healthy and infected tomato leaf tisssue was crushed in 500 μl of extraction buffer (preheated at 50°C) in separate eppendorf tubes.
2) The samples were incubated at 50°C for 15 minutes in a waterbath.
3) 500 μl of chloroform: isoamylalcohol (24:1) was added.
4) The tubes were kept on shaker for 20 minutes.
5) The contents were centrifuged at 12000 rpm for 12 minutes at room temperature.
6) The supernatant was transferred to a fresh eppendorf tube and DNA was precipitated by adding equal volume of isopropanol (500 μl) and 0.5 volume of NaCl.
7) Tubes were incubated at -20°C for 3 hours.
8) The DNA was recovered by centrifugation at 12000 rpm for 12 minutes at 4°C.
9) The pellet was washed with 1.0 ml of absolute ethanol.
10) The pellet was resuspended in 500 μl of TE buffer and 10 μl of Rnase A was added and incubated at 37°C for 30 minutes.
11) Equal volume of phenol: chloroform was added and centrifuged at 12000 rpm for 12 minutes at room temperature.
12) The above step was repeated twice.
13) Equal volume of chloroform:isoamylalcohol (24:1) was added and centrifuged at 12000 rpm for 12 minutes at room temperature.
14) 0.1volume of 3M-sodium acetate & 2 volume of absolute alcohol was

15) Incubate at -20°C overnight.

16) DNA was recovered by centrifugation at 12000 rpm for 2 minutes at 4°C and the pellet was air-dried and washed with 1.0 ml of 70% ethanol.

17) The pellet was resuspended in 50 µl of TE buffer.

18) 20 µl of the sample was run on 0.8% agarose gel and observed under UV-Transilluminator.

4.2.2 Agarose gel electrophoresis (Sambrook *et al.*, 1989)

Reagent : 1X TAE buffer,0.04 M Tris – acetate, 0.001 M EDTA), Gel loading buffer 0.25% Bromophenolblue, 0.25% Xylene cyanol FF, 30%Glycerol in water (store at 4°C),Agarose Gel (0.8%) [Agarose (0.8g), TAE buffer (1X)- 100ml, Ethidium Bromide- 8µl].

Procedure:

Step 1: Setting up the electrophoresis apparatus.

1) The edges of a clean dry, gel tray were sealed with cellophane tape so as to form a mould. The combs were positioned 0.5 – 1.0 mm above the bottom of the tray so that the complete wells were formed.

2) Sufficient electrophoresis buffer (1X TAE) was prepared to fill the electrophoresis tank and to prepare gel.

3) 0.8% agarose gel was prepared by melting 0.4 g of agarose in 50 ml of 1X TAE buffer by heating.

4) Cooled to 60°C and ethidiumbromide (stock solution of 10 mg/ml in water) was added to get a final concentration of 0.5 µg/ml.

5) The gel was poured in gel casting unit with combs.

6) After the gel was completely set (30-45 minutes at room temperature), the combs and tapes were carefully removed and the gel was placed in the electrophoresis tank.

7) Sufficient electrophoresis buffer was poured in the electrophoresis tank to cover the gel.

Step 2: Loading of samples

1) 12 μl of DNA samples were taken in separate eppendorf tubes and 2 μl of gel loading buffer was added to each tube and mixed well.

Gel buffer serves for 3 purposes:

a) increases the density of the sample, ensuring that the DNA drops evenly into the well.

b) adds color to sample, thereby simplifying the loading process.

c) contains dyes that in an electric field move towards the anode at predictable rates.

2) The samples were loaded into appropriate wells using micropipette.

3) The lid of the gel tank was closed and electrical leads were attached so that the DNA will migrate towards the anode. A voltage of 1 – 5 V/cm was applied. The gel was run until the bromophenolblue and xylenecyanol FF migrated to the appropriate distance through gel.

4) After electrophoresis for 1.5 hours, the electric current was turned off and the leads were removed. The lid of the tank was opened and the gel was observed under UV Transilluminator.

4.2.3 Polymerase Chain Reaction (Sambrook *et al.*, 1989)

Reagent : 10X buffer [(final concentration 1X), 500M KCl, 10mM Tris-HCl, 15mM MgCl2, 0.1 % gelatin pH 8.3,], 25mM $MgCl_2$, Template, dNTP's (mixture of dATP,dCTP,dGTP,dTTP), Primers (Primer1 PINDc 1--5' TTAA<u>AAGCTT</u>GAGCGCGTCATGTG 3') and Primer2 PINDv2 (5' GAGT<u>AAGCTT</u>ATTTGTTTTTGTGGT 3') concentration 0.3mM) (Genei Bangalore) , Taq DNA polymerase 2.5μl/μl, Double distilled water (to makeup the volume).

Procedure (Table 4.1):

- Mixture of dNTP's (dATP, dGTP, dCTP and dTTP) final concentration of 0.225 mM
- Primers (NTP I and NTP II) final concentration of 0.3 μM
- Taq DNA polymerase
- Denaturation temperature - 94°C for 5 minutes
- Annealing temperature - 55°C for 1 minutes
- Extension temperature - 72°C for 1 minutes

4.2.4 Gel elution (Sambrook *et al.* 1989)

Reagent: Electrophoresed Agarose gel with amplified gene, Chloroform:isoamylalcohol, Equilibriated phenol, 3M sodiumacetate mixture, 70%ethanol.

Procedure:

1) After electrophoresis, the bands were located under ultraviolet fluorescence. An incision was made in the gel ahead of the desired band (towards positive electrode). A rectangular piece containing the band was cut from the gel and was put into an eppendorf tube.
2) The eppendorf tubes were kept in boiling water bath for 10 minutes to melt the agarose.
3) 400 μl of 2.5 M ammonium acetate was added and mixed well.
4) The tube was centrifuged at 12000 rpm at 28°C for 6 minutes.
5) Supernatant was transferred to a fresh eppendorf tube.
6) 2.5 volumes of distilled ethanol was added and incubated for overnight at 4°C.
7) The tubes were centrifuged at 12000 rpm for 12 minutes at 4°C.

9) Tubes were centrifuged at 12000 rpm for 12 minutes at 4°C.

10) The pellet was air dried for 15 minutes.

11) Pellet was dissolved in 50 µl of 1X TE buffer and stored at 4°C.

12) The concentration of the DNA was estimated using spectrophotometer.

4.2.5 Estimation of DNA

Reagent: Eluted DNA sample, ethidiumbromide.

Procedure:

1) Standard solution of known DNA concentration was prepared in the range of 0, 1, 5, 15, 30 and 60 ng/µl.
2) On a piece of parafilm, 2 µl of 5 µg/ml solution of ethidiumbromide was arranged and a standard curve was prepared by mixing 2 µl of standard DNA solution each with an individual 2ul of ethidiumbromide 0 , 1, 5, 15, 30 and 60 ng/µl standards were used.
3) The dilutions of DNA fragments preparation was done in TE buffer and 2 µl of ethidiumbromide on a parafilm.
4) The parafilm was then placed on a UV Transilluminator and visualized.
5) The fluorescence of the DNA fragment preparation was compared with standard curve in order to estimate its concentration.

4.3 Cloning and transformation of CP gene

4.3.1 Restriction digestion (Sambrook *et al.*, 1989)

Reagents: Plasmid preparation of pUC19 and λ-DNA, Restriction enzyme buffer, *Ecor I* and *XbaI* enzymes (Genei Bangalore), Autoclaved double distilled water, TAE buffer (1X)

Procedure (Table 4.2):

1) 10 µl of pUC19 was taken in a sterile microfuge tubes and mixed with sufficient double distilled water to give the required final volume (20 µl).
2) 2 µl of 10X restriction buffer was added and mixed by tapping the tubes.
3) 1 µl of restriction enzyme *Ecor I* and *Hind III* was added to the tubes and mixed well by tapping.
4) The tubes were spun for few seconds; both the tubes were incubated at 37°C waterbath for 3 hours.
5) The restriction was stopped by adding 1 µl of 0.5 M EDTA (pH 8.0).
6) The DNA samples were analyzed directly on 0.8% agarose gel by adding 3.3 µl of gel loading dye to each tube containing the reaction mixture.
7) The tubes were spun briefly and the mixture was loaded into slot cast on 0.8% agarose gel containing ethidiumbromide (0.5 µg/ml).
8) The gel was visualized using UV Transilluminator.

4.3.2 Ligation reaction (Sambrook *et al.*, 1989)

Reagents: Plasmid DNA, Insert DNA, Bacteriophage T4 DNA ligase (Genei Bangalore), 10X ligase buffer, Double distilled water.

Procedure(Table 4.3):

1) Two clean eppendorf tubes were taken.
2) In one eppendorf tube 3.3 µl of insert DNA (CP) was taken and to it 3.3 µl of vector DNA (pUC19) was added to give a molar concentration of 1:2
3) To the other eppendorf tube 6.6 µl of insert DNA (CP) was taken and to it 3.3 µl of vector DNA (pUC19) was added to give a molar

4) 2.5 μl of 10X ligase buffer and 1.0μl of ligase enzyme was added to each tube.

5) Double distilled water was added to make up the volume to 25 μl

6) The tubes containing the reaction mixture was incubated at -20°C for overnight.

7) The ligation mixture was used to transform the competent *E.coli* cells to check for successful ligation reaction.

4.3.3 Competent cells preparation and transformation (Sambrook *et al.*, 1989)

Reagent: Overnight culture of DH5α, 0.1M calcium chloride, Ampicillin (100mg/ml), pUC19 (Genei Bangalore), Samples (ligated).

Procedure:

1) Single colony of DH5α was picked from a freshly grown culture and inoculated into a 5 ml LB broth and incubated at 37°C for overnight.

2) The culture was scaled up by adding 0.5 – 0.8ml of overnight culture into 25 ml of LB broth and incubated for 3 hours at 37°C in shaker.

3) The cells were aseptically transferred to centrifuge tubes. The culture was cooled to 0°C by storing the tubes on ice for 10 minutes.

4) The media was decanted from cell pellet and tubes were kept inverted for 1 minute to allow the last traces of media to drain away.

5) The pellet was resuspended in 5ml of ice cold calcium chloride (100 mM) and stored on ice for 10-15 minutes. The tubes were intermittently mixed for the pellet to completely dissolve in calcium chloride.

6) The cells were recovered by centrifugation at 6000 rpm for 6 minutes at 4°C.

7) The supernatant was decanted from the cell pellet and tubes were kept in inverted position for 1 minute to allow the last traces of supernatant to drain away.
8) Pellet was resuspended in 1.5 ml of 10 mM calcium chloride and used for transformation.

4.3.4 α - Complementation

Reagents: Ligated mixture of pUC19/CP competent cells (DH5α) (Genei Bangalore) LB agar plate with ampicillin (100ng/ml), X –gal 20mg/ml , IPTG .

Procedure:

LAXI plate preparation

1) 40 μl of IPTG and 40 μl of Xgal were mixed thoroughly in an eppendorf tube.
2) This was then spread uniformly on LB plate (Amp) and allowed to dry.
3) The colonies, which were formed by the ligated samples, were taken and were inoculated on the LAXI plate and incubated at 37°C for overnight.

Steps:

1) 150 μl of competent cells were taken in an eppendorf tubes and 10 μl of ligated sample was added and the tubes were kept on ice bath for 20 minutes.
2) The tubes were transferred to water bath at 42°C for 2 minutes and Immediately transferred onto ice to chill for 2 minutes.
3) 800 μl of LB broth was added and incubated at 37°C for 1hour, this allows the bacteria to recover and express the antibiotic resistance marker encoded by plasmid.

5) The pellet was dissolved in 150 μl of fresh LB, and then plated on LB plates containing Xgal and IPTG.

4.3.5 Isolation of plasmid DNA (Pal Malinga, 1995)

Reagents: Solution I (50mM glucose; 25mM Tris (pH 8) and 10mM EDTA), Solution II (0.2N NaOH, 1% SDS), Solution III (5M potassium acetate- 60ml, glacial acetic acid- 11.5ml, distilled water – 28.5ml),
3M sodium acetate (Dissolve 408.1g of sodium acetate in 800ml of water. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1L with water. Dispense into aliquots and sterilize by autoclaving), LB media, STE solution (0.1M NaCl, 10mM Tris – Cl (pH 8), 1mM EDTA (pH 8) store at 4°C), TE buffer (Tris EDTA)(10mM Tris chloride (pH 8), 1mM EDTA (pH 8)), TAE buffer 1X (0.04 Tris acetate, 0.001M EDTA), Gel loading buffer (0.25% Bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water), Agarose gel (Agarose 0.8g, TAE buffer (1X)-100ml), ethidiumbromide, Isopropanol, Rnase A, Phenol:Chloroform; Isoamylalcohol (25:24:1), Chloroform:Isoamylalcohol (24:1), Absolute alcohol, 70% ethanol.

Procedure:

1) Transformed DH5α strain was inoculated into 50 ml of LB with 50 μl of ampicillin (50 μg/ml) and incubated at 28°C overnight on a shaker.
2) The culture was centrifuged at 12000rpm for 12 minutes at 4°C to pellet down the cells and the supernatant was discarded.
3) The pellet was resuspended in 3ml of solution I and incubated for 10 minutes at room temperature.
4) 6.0 ml of freshly prepared solution II was added and incubated for 10 minutes at room temperature.
5) 0.9 ml of alkaline phenol was added and mixed by vortexing.

6) Immediately 450 µl of 0.3 M sodium acetate (pH 4.8) was added and mixed by inverting and incubated at -20°C for 20 minutes.
7) Centrifuged at 12000 rpm for 12 minutes at 28°C and supernatant was collected.
8) 8 µl of RNase was added to the supernatant and incubated at 37°C for 30 minutes in water bath.
9) 800µl of phenol: chloroform: isoamylalcohol mixture was added and centrifuged at 12000 rpm for 12 minutes at 28°C this extraction step was repeated twice.
10) The aqueous layer was transferred to fresh tube and 800 µl of chloroform: isoamylalcohol was added, centrifuged at 12000 rpm for 12 minutes at 28°C.
11) To the supernatant 1.0 ml of absolute alcohol was added and kept at -20°C for 3-4 hours to precipitate DNA.
12) The tubes were centrifuged at 12000 rpm for 12 minutes at 4°C and the pellet was washed in 1.0 ml of ice cold 70% ethanol.
13) The pellet was dried briefly in speed vac for 15 minutes and resuspended in 50 µl of TE buffer.
14) The presence of plasmid was confirmed by running on 0.8% agarose gel.

4.4 Subcloning of CP gene into pGPTV

After plasmid isolation restriction digestion was carried out simultaneously for eluted samples of pUC19 and pGPTV using Ecor I and Hind III by same procedure under (4.3.1)

Following restriction the restricted sample were eluted from the Agarose gel according to the procedure (4.2.4) and ligated (both pUC19 and

After ligation competent cell preparation was carried out with the ligated pGPTV and pUC19 samples using the same procedure given under (4.3.3).

4.5 Plant tissue culture

Equipments: Sterile forceps, Scalpel, blotting paper (whatman), sterile brown sheets, tissue culture bottles, cling wrap, laminar air flow, temperature controlled growth room.

4.5.1 Surface sterilization of tomato seeds (M.S.Punia, 1998)

Reagents: Tomato seeds (Hybrid Rashmi), Tween 20 detergent, 70% ethanol, 0.12% mercuric chloride solution, Autoclaved distilled water.

Procedure:

1) Wash the tomato seeds in water containing few drops of tween 20 solution for 15 minutes with intermittent shaking under sterile conditions and decant the water.
2) Continue washing the seeds with sterile water until lather is completely removed.
3) Swab the laminar air flow cabinet with 70% methanol, within the cabinet rinse the seeds with autoclaved double distilled water for three times
4) Seeds were treated with 70% ethanol for 1 minute.
5) The seeds were treated with 0.12% mercuric chloride solution for 10 minutes.
6) The sterilant was decanted and washed with autoclaved double distilled water for three times for 1 minute successively.
7) The seeds were soaked in sterile water overnight to soften the seed coat.

4.5.2 Isolation of embryo from tomato seeds

Reagnts: Tomato seeds (Hybrid Rashmi), Sterile MS medium, Autoclaved double distilled water, 70% ethanol.

Procedure:

1) The tomato seeds soaked for overnight were placed on a sterile brown sheet.
2) Using a sterile scalpel, an incision was made on the sharper end of tomato seed.
3) The embryos were inoculated onto sterile Murashige & Skoog's (MS) medium.
4) The culture bottles were incubated at 25°C in growth room with a photoperiod of 16 hours light and 8 hours dark period.

4.5.3 Prepration of explants (M.S. Punia, 1998)

Procedure:

1) Leaves from 4 week old *in-vitro* grown healthy tomato plants were taken and cut on the margins using a sterile scalpel to get leaf discs of about 2cm x 2cm.
2) The cotyledons were wounded at both the ends by giving a gentle cut; the hypocotyls were cut into small pieces.
3) Leaf discs were placed in a regeneration medium with adaxial portion downward (most responsive portion to nutrients and the last tissue of the leaf to cease growing and dividing) and incubated under condition of light and temperature in growth room for two days.

4.5.4 Tri – parental mating (Pal Malinga, 1995)

Reagents: Rifampicin (10μg/ml), streptomycin (100 μg/ml), AB agar, YEP agar, kanamycin (100 μg/ml), 0.9% NaCl, *Agrobacterium tumefaciens* strain LBA 4404, *E.coli* pRK 2013, *E.coli* with pGPTV –

Procedure:

1) Streak *Agrobacterium tumefaciens* strain LBA 4404 on AB agar plate with rifampicin (10 µg/ml) and streptomycin (100 µg/ml) incubated at 28°C for overnight to obtain isolated colonies.
2) Streak *E.coli* harboring pRK 2013 on LB plates with kanamycin (100 µg/ml) and pGPTV – CP on LB plate with kanamycin (100 µg/ml) and incubated at 37°C overnight to get isolated colonies.
3) Mix isolated colony each of *E.coli* with pGPTV – CP and Agrobacterium strain LBA 4404 on a YEP plate without any antibiotics and incubate at 28°C overnight.
4) Take loop full of this mixed culture and dilute with 1ml of 0.9% of NaCl; make serial dilutions of this stock solution till 10^{-6} dilution.
5) Take the culture from 10^{0}, 10^{-2}, 10^{-4} and 10^{-6} dilutions on AB medium containing kanamycin (100 µg/ml), rifampicin (10 µg/ml) and streptomycin (100 µg/ml) incubate at 28°C for three days and observed for isolated colonies.

4.5.5 Co - cultivation of tomato leaf disc with *Agrobacterium tumefaciens* (M.S. Punia, 1998)

Procedure:

1) The isolated colonies from *Agrobacterium* (from 10^{-6} AB – KRS plate of TPM) having recombinant plasmid were inoculated in LB broth containing kanamycin (50 µg/ml) and were incubated overnight at 25°C.
2) The pre incubated tomato leaf discs were put in a sterile bottle containing *Agrobacterium tumefaciens* culture. The culture had to be diluted with LB broth such that the broth contains $5x10^{8}$ cells/ml (usually 1:20 dilutions for an overnight culture) and swirled gently for

3) The leaf discs were taken out and the excess of culture was blotted on a filter paper.

4) The infected leaf discs were transferred onto regenerating medium and incubated for two days in dark.

5) After 2 days the leaf discs were washed with double distilled water for 3 – 4 times and the leaf discs were blotted on sterile filter paper.

6) Placed on the selection media and incubated in growth rack for obtaining regenerates.

Table 4.1: Polymerase Chain Reaction (PCR)

	DNA (μl)	**Taq DNA polymerase (μl)**	**Primer I (μl)**	**Primer II (μl)**	**Buffer (μl)**	**dNTPs (μl)**	**DD Wate (μl)**
Healthy	10	1.0	1.0	1.0	2.5	1.5	8.0
Infected 1	10	1.0	1.0	1.0	2.5	1.5	8.0
λ DNA	10	1.0	1.0	1.0	2.5	1.5	8.0

Note: DD – Double Distilled

Table 4.2: Restriction Digestion

DNA sample	DNA (µl)	10X buffer (µl)	EcorI (µl)	Hind III (µl)	DD water (µl)
pUC 19	10.0	2.0	1.0	1.0	6.0
CP	10.0	2.0	1.0	1.0	6.0

Note: DD – Double Distilled

Table 4.3: Ligation Reaction

Molar Ratio	Vector DNA(µl) (pUC19)	Insert DNA CP (µl)	10X Buffer (µl)	Ligase Enzyme (µl)	DD Water (µl)
1:2	3.3	3.3	2.5	1.0	14.83
1:4	3.3	6.6	2.5	1.0	11.57

Note: DD – Double Distilled

RESULTS AND DISCUSSION

CHAPTER 5

5. RESULTS AND DISCUSSION

5.1. Maintenance of virus culture

The hybrid tomato seeds of the variety Rashmi were selected for this study. This is because earlier studies conducted by the University of Agricultural Sciences, Bangalore, India, revealed that this hybrid was susceptible to ToLCV-[Ban4] and to TYLCV (Israel) (Maruthi.M.N.*et al.*, 2003). Healthy tomato seedlings were grown in one week time, which were in the cotyledon (2leaf) stage (Fig:5.1a). After transplanting them to polybags, healthy tomato plants were obtained after 12days (Fig: 5.1b) and this was used for inoculation experiment. The healthy whiteflies were allowed to feed on ToLCV infected plants for 1day as acquisition access period (AAP) (Fig: 5.2a). The healthy whiteflies were maintained on cotton plants since the highest mean number of nymphs per plant (125) was recorded on cotton (Ramappa H.K *et al.*, 1998).These viruliferous whiteflies were allowed to feed on healthy tomato plants for 1day as inoculation access period (IAP) (Fig:5.2b). Flies were shown to become viruliferous within 6 hours and a minimum feeding period of 32 minutes resulted in 4% transmission (Singh S.J, 1993). Hence to obtained higher transmission efficiencies the IAP was extended to 1 day. Symptoms like curling and twisting of leaves were seen on the inoculated plants. After 1 week 40% infectionwas obtained. In 3 weeks time 100% infection was observed. Thus the extension of IAP showed positive results. Young, newly emerging leaf tissue was collected from the plants showing severe infection and subjected to genomic DNA isolation. The virus multiplication is known to be maximum in young leaves and hence it is a good source for the isolation of viral coat protein

5.2 Isolation of ToLCV coat protein gene:

Genomic DNA was isolated from the infected leaves and its presence was confirmed by agarose gel electrophoresis. A good concentration of genomic DNA was obtained which is clear from the thick bands seen in the gel (Fig:5.3). The eluted genomic DNA was subjected to PCR amplification using primers specific for ToLCV coat protein gene. The primers used were PINDc 1 (5' TTAAAAGCTTGAGCGCGTCATGTG 3') and PINDv2 (5' GAGTAAGCTTATTTGTTTTTGTGGT 3') (Orawan. C *et al* 2002). The CP gene was amplified and a band was noticed when subjected to agarose gel electrophoresis (Fig: 5.4). Its molecular weight was found to be 700 bp. This was in good correlation with the molecular weight of CP as reported by Hong.Y (1995). The CP gene was eluted from the gel and its concentration was estimated spectrophotometrically and found to be 995 ng/µl. This concentration was indeed very high and hence this was further used as the source of CP gene for the cloning experiments.

5.3 Cloning and transformation of CP gene:

pUC 19 was the vector selected to carry out the cloning experiment. They lack the *rop* gene which is normally located close to the origin of DNA replication and is involved in the control of copy number. As a result, these plasmids replicate to a much higher copy number than do other plasmids that carry a pMB1 (or CoLE1) origin (Sambrook *et al.*, 1989). The plasmid pUC19 and CP gene were subjected to restriction digestion using enzymes. *Hind III and EcoR I*, (Fig: 5.5).

Lane 1 – digested pUC19 vector

Lane 2 – undigested pUC – CP

Lane 3 – digested pUC – CP

Ligation was carried out in order to insert the CP gene into pUC19 vector. Upon digestion of the ligated sample insert CP was released. Thus 2 bands were obtained one corresponding to the linear pUC19 and other corresponding to the insert CP (Fig: 5.5, lane 3). The ligated samples were subjected to transformation using DH5α competent cells (Fig: 5.6a). The transformation experiment was confirmed by α - complementation since the pUC19 vector contains the amino terminal fragment of the LacZ and the recombinants were identified by histochemical screening (Sambrook *et al* 1989).

The recombinant clones were seen on the LAXI plate as white colonies (Fig: 5.6b). These colonies were selected and cultured in LB Amp broth, so that it can be subjected to plasmid isolation.

5.4. Sub – cloning of CP gene into pGPTV

Cloning into pUC19 alone is not sufficient since to carrying out *Agrobacterium* transformation a binary vector is necessary and pUC19 is not a binary vector (Slater.A.*et al*., 2003). Hence subcloning into pGPTV, a binary vector was necessary.

The vector (pGPTV) and the insert DNA (CP) were successfully ligated. This was confirmed by carrying out restriction analysis of the ligated samples (Fig: 5.7a).

Lane 1	- pGPTV- CP undigested
Lane 2, 4, 5	- pGPTV- CP digested
Lane 3	- Marker (λDNA) digested

The clones carrying pGPTV were selected and transformed into *E.coli* cells. The efficiency of competent cells was good and this resulted in the production of clones. Upon screening the transformants, the presence of recombinant (pGPTV-CP) was confirmed (Fig: 5.7b). Only those clones carrying the insert and that are resistant to antibiotic kanamycin can grow on the selection medium. These clones were used for tri-parental mating.

5.5. Plant tissue culture and *Agrobacterium* mediated transformation:

Tomato seeds were surface sterilized and the embryos were isolated (Fig: 5.8a i). After 1 week, the embryos developed into platelets (Fig: 5.8aii). Leaf disc preparation was carried out with 4-week-old plantlets.

Leaf disc were prepared and incubated on MS medium for 2 days. Meanwhile, tri-parental mating was carried out to facilitate the conjugal transfer of pGPTV – CP into *Agrobacterium* strain LBA 4404 through helper plasmid (pRK 2013). The transconjugants were grown on KRS selection medium (Fig: 5.8b). Only those *Agrobacterium* cells carrying all the three plasmids will form colonies. The transconjugants were screened by PCR technique and it was confirmed that CP was inserted into *Agrobacterium* (Fig: 5.9).

Lane 3, 5, 8, 11	- Unamplified plasmid DNA
Lane 2, 4, 6, 9, 10, 13	- Amplified plasmid DNA showing CP gene
Lane 7	- Marker DNA

The transformed *Agrobacterium* was co-cultivated with the previously prepared leaf discs (Fig: 5.10). Following the transformation the leaf discs were transferred onto selection medium. Purpose of selection media is to kill the *Agrobacterium* and induce leaf discs to initiate callus growth. Leaf explants of all the ToLCV resistant tomato lines except LA 1777 produced callus on MS supplemented with IAA and Kinetin concentrations ranging from 2-4 mg/l (Devaraja *et al.*, 2004).

Thus *Agrobacterium* carrying the gene of interest was obtained and this was used to carry out transformation of leaf explants. Due to want of time, the presence of the CP gene in the callus cells could not be confirmed and transgenic plants could not be produced from the callus. Hence further research is necessary to study the extent of transformation and expression of viral coat protein in tomato plants. Also the assessment of disease resistance in these transgenic plants and their progeny is necessary.

FIG - 5.1

Healthy Tomato Seedlings

a) Seedlings in Nursery Tray

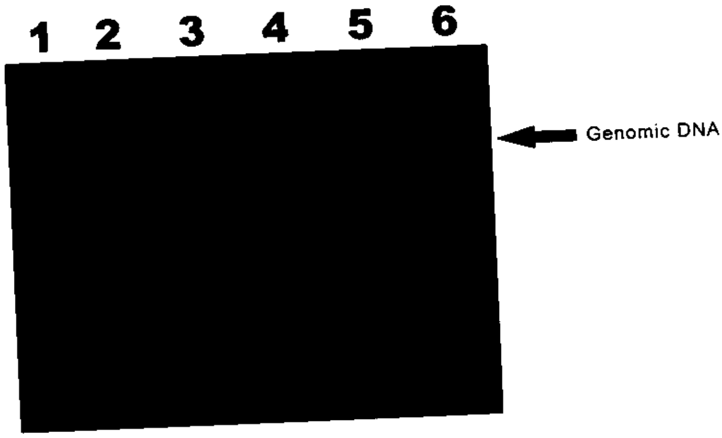
b) Seedlings in Polybags (12 days old)

FIG - 5.2

a) Acquisition of virus by white fly(vector)

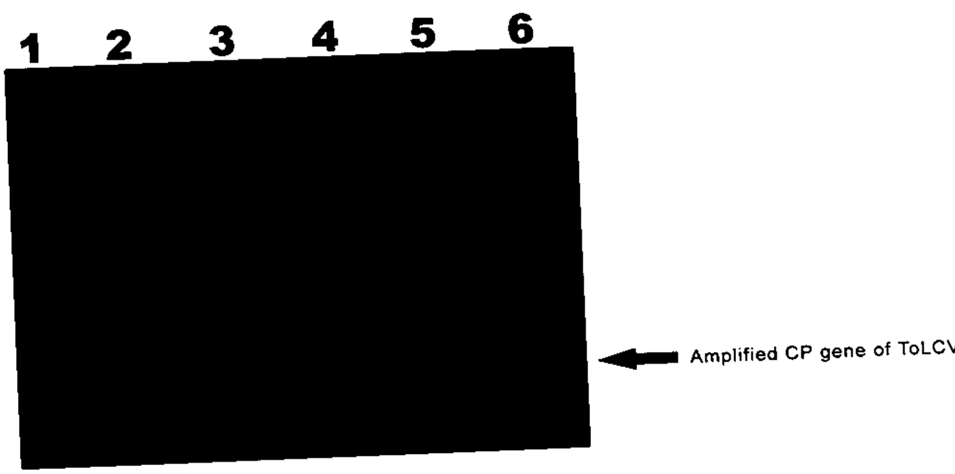
b) Inoculation of healthy seedlings with viruliferous fli

FIG - 5.3
Isolation of Genomic DNA



Lanes (1 to 6) : Genomic DNA from infected tomato leaves

FIG - 5.4
Polymerase chain reaction (PCR)



Lanes (2 to 6) : Amplified coat protein (CP) gene of Tomato leaf curl virus (ToLCV)

FIG - 5.5

Restriction Digestion

1 2 3 4 5 6

Lane 1 : pUC 19 digested (vector)

Lane 2 : pUC - CP undigested

Lane 3 : pUC - CP digested

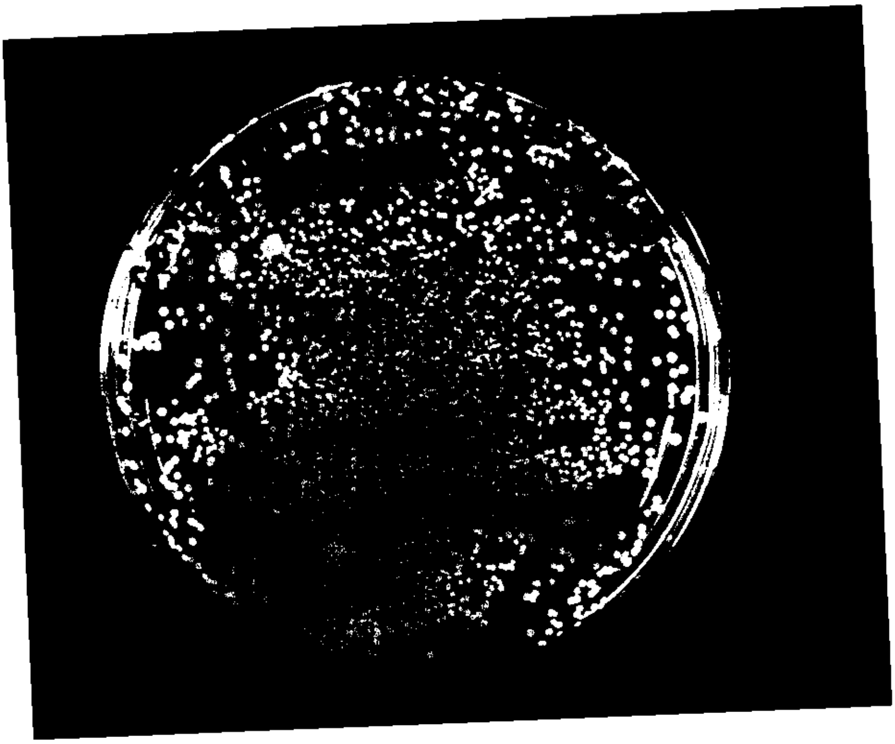
Lane 4 : Marker (λDNA) digested

Lane 5 : pGPTV undigested

FIG - 5.6

Cloning of pUC - CP into *E.coli* DH5α

a) Competent cells on LB plate (control)



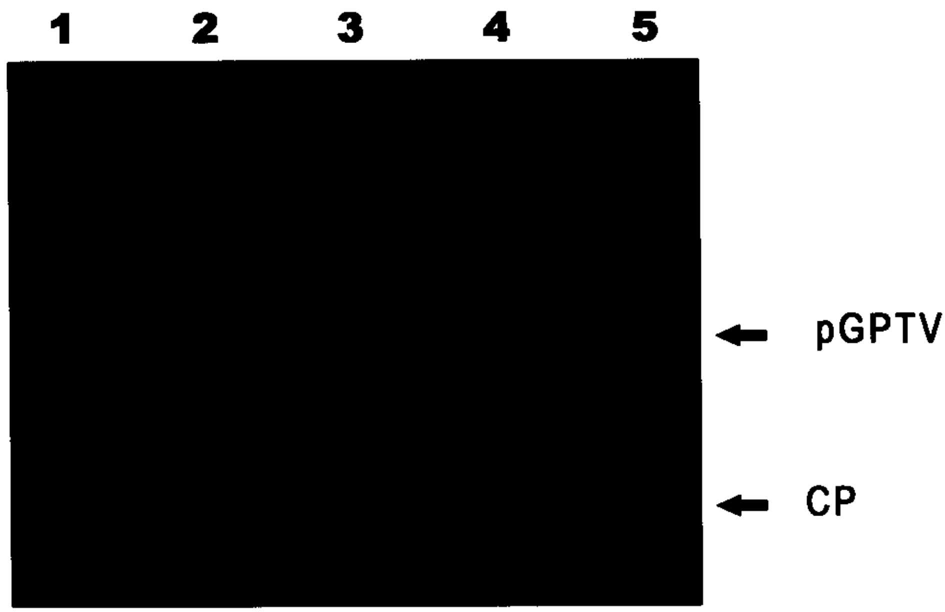
b)α- Complementation

B - Blue colo
W - White col
(Transformed

FIG - 5.7

Sub cloning of CP gene into pGPTV

a) Confirmation of ligation of CP gene into pGPTV



Lane 1 : pGPTV - CP undigested

Lane 2 : pGPTV - CP digested

Lane 3 : Marker (λDNA) digested

Lane 4 & 5 : pGPTV - CP digested

b) Screening of Transformants

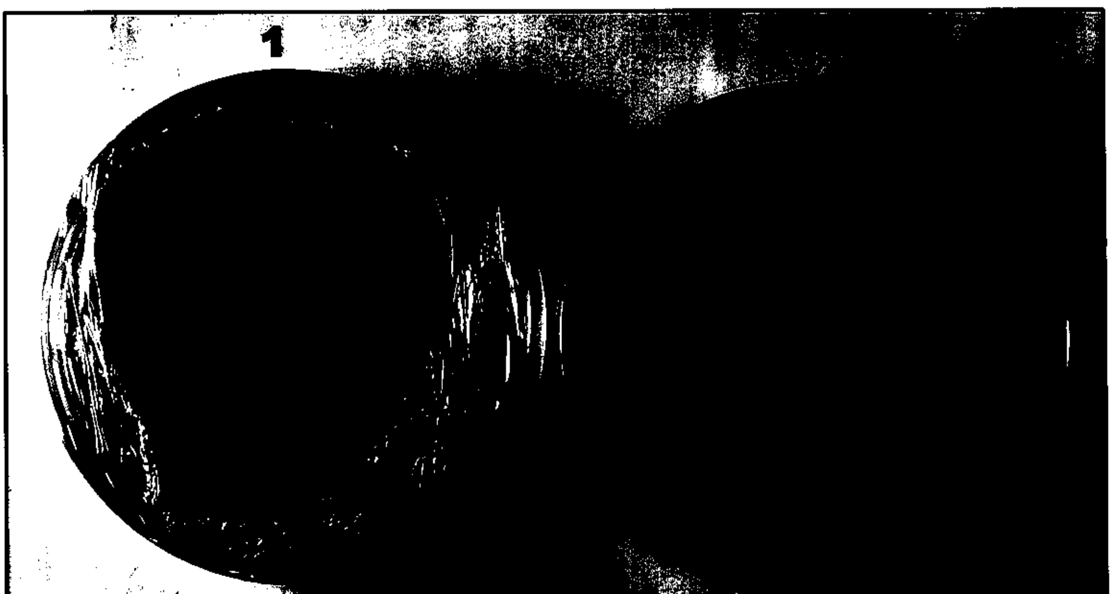
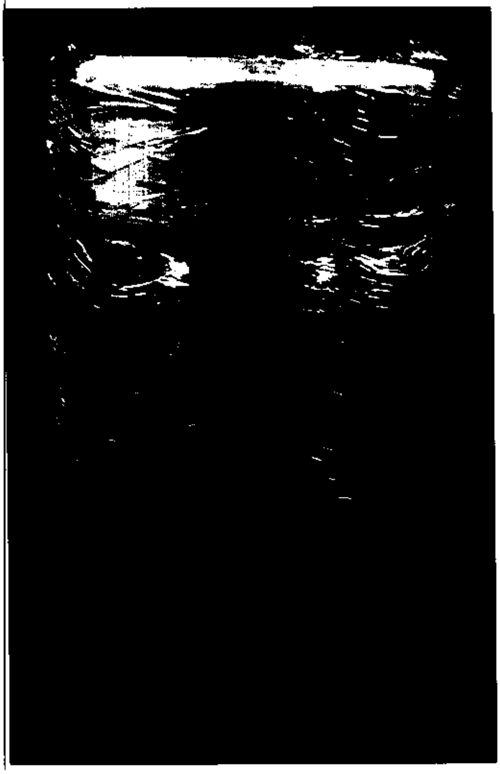
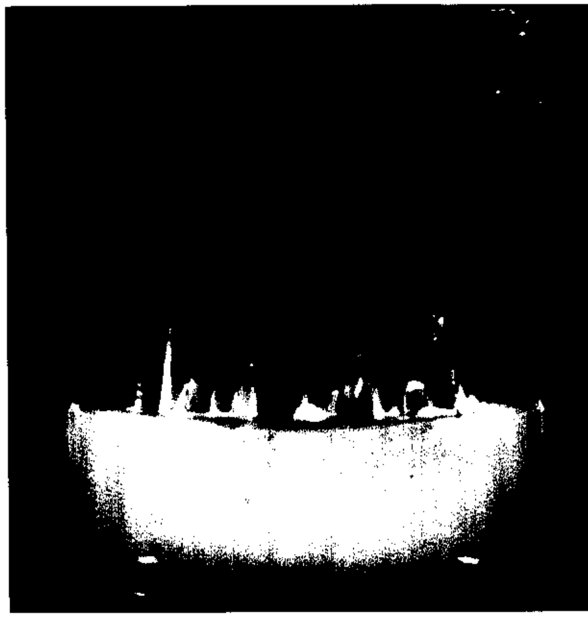


FIG - 5.8

a) Isolation of Embryo from tomato seeds



(i)



(ii) After 1 week

b) Triparental mating

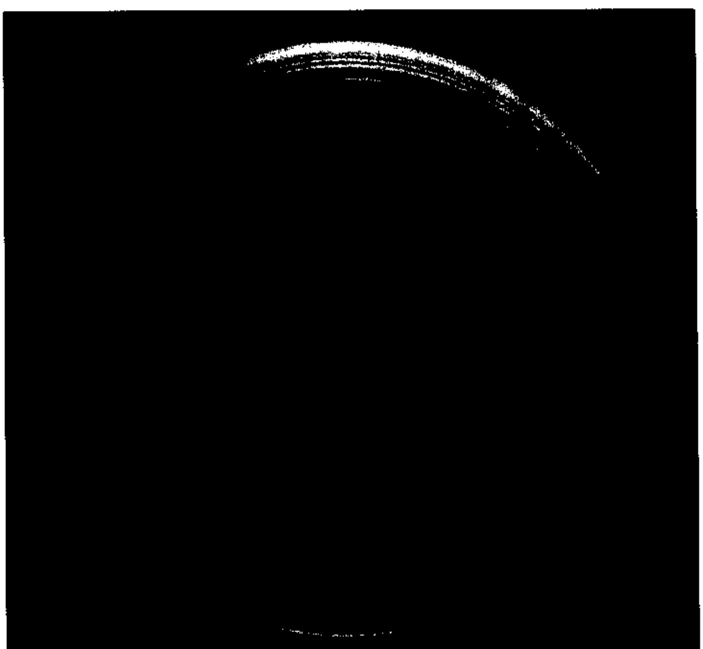
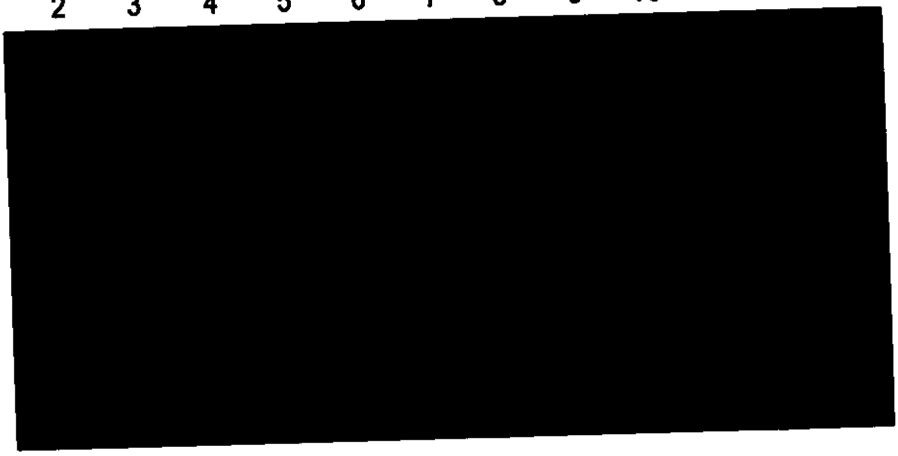


FIG - 5.9

Agarose gel showing PCR products



Lanes 3,5,8,11&12 : Unamplified Plasmid DNA samples

Lanes 2,4,6,9,10&13 : Amplified Plasmid DNA samples

Lane 7 : Marker (λDNA)

FIG - 5.10

Co - cultivation of Tomato leaf disc with *Agrobacterium tumefaciens*

CONCLUSIONS

CHAPTER 6

6. CONCLUSION

Viral disease cause serious losses world wide in horticulture and agriculture crops. Conventional breeding programs to develop resistance are effective, but also protracted and expensive and the results can be circumvented by virus variation. Transgenic plants carrying nucleotide sequences derived from plant viruses can exhibit increased resistance to viral disease. The ability to produce virus-resistant plants is clearly of agronomic significance. Alternative approaches to protect plants from viral infections are those of plant transformation and regeneration. Coat protein- mediated resistance is more widely applied than other approaches, perhaps in part because it was the first described, but also because it appears to provide a broader type of resistance. Alternative strategies and the combination of several strategies may provide even more broad protection as well as high levels of resistance. Some of the strategies that can be implemented in future research are:

1. The use of mutant viruses in conjugation with coat protein – mediated resistance, and the combination of coat protein – mediated resistance with other types of engineered resistance.
2. Various types of CP gene constructs can be used for transformation and the degree of resistance can be evaluated. Some types of constructs are:
    - Use of either sense or antisense transcripts of the CP gene.
    - A CP gene constructs lacking a start codon.
    - Use of antisense CP transcripts or sense transcripts containing a frameshift mutation.

3. To eliminate any possibility of coat protein accumulation in tissues where it is not accessible for the infecting virions, the use of different tissue specific promoters in the CP gene constructs is necessary.

This will in turn lead to the development of crop plants that in the field have heightened levels of resistance to viruses that are both mechanically transmitted and vector borne. A number of field trials are currently taking place to assess the usefulness of the resistance obtained with PDR. To date, these trials have been mainly carried out with plants transgenic for, and expressing, viral coat proteins and the results have been encouraging. For e.g. Fruit yields observed for transgenic tomato plants resistant to tomato aspermy virus were 50% higher than those for non transgenic control plants. With the world's population continuing to rise, the major target of modern agriculture is to be a sustainable increase in yield that keeps pace with the increasing number of mouths to feed and this can be possible only through modern plant biotechnology techniques.

APPENDIX

APPENDIX

REAGENTS

STE :

0.1 M NaCl

10 mM Tris-Cl (pH 8.0)

1.0 mM EDTA (pH 8.0)

(store at 4°C)

Solution I:

50 mg Glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

(store at 4°C)

Solution II:

0.2 N NaOH

1% SDS

Solution III:

5.0 M Potassiumacetate	–	60 ml
Glacial acetic acid	–	11.5 ml
Double Distilled Water	–	28.5 ml

Phenol : Chloroform

Mix equal amount of phenol and chloroform. Equilibriate the mixture by extracting several times with 0.1 M Tris ((pH 7.6). Store the equlibriated mixture under an equal volume of 0.01 M Tris-Cl (pH 7.6) at 4°C in dark

Extraction Buffer:

100 mM Tris-HCl

1.4 M NaCl

20 mM EDTA

2% CTAB (Cethyltrimetyl ammonium bromide)

0.4% 2 – mercaptoethanol

1% PVP

AB Medium:

AB salts(500 ml)

NH_4Cl - 0.5 g

$CaCl_2$ - 0.075 g

$MgS0_4.7H_20$ – 0.15 g

$FeS0_4.7H_20$ – 1.25 g

KCl - 0.075 g

AB buffer (500 ml) (pH 7.0)

K_2HP0_4 – 1.5 g

NaH_2P0_4 – 0.5 g

AB Agar Medium (100 ml)

20 ml of AB salts + 20 ml of AB buffer

0.5% D- Glucose, 1.5% Agar (pH5.5)

YEP Medium (pH7.0)

Peptone – 10 g

Yeast Extract – 10 g

NaCl – 5.0 g

Agar – 15 g

Leuria Bertini(LB) broth (pH 7.0):

Tryptone – 10 g

Yeast extract – 10g

NaCl – 5.0 g

Gel Loading Buffer (6X):

0.25% Bromophenolblue

0.25% Xylenecyanol FF

30% Glycerol in water

TE buffer (pH 8.0):

100 mM Tris-Cl (pH 8.0)

1.0 mM EDTA (pH 8.0)

TAE buffer (50X):

Tris – 242 g

Glacial acetic acid – 57.1 ml

0.5 M EDTA – 100 ml (pH 8.0)

MS Media (Murashige & Skoog) (pH 5.8):

Elements	mg/L
I. Macroelements	
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170

II. Microelements

$MnSO_4.4H_2O$	223
KI	0.83
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$Na_2Mo_4.2H_2O$	0.25
$CoCl_2.6H_20$	0.025
$FeSO4.7H_2O$	27.8
Sodium – EDTA	37.8

III. Aminoacids

Pyridine HCl	0.5 ml
Nicotinic Acid	0.5 ml
Thiamine HCl	0.3 ml
Glycine	2.0 ml

IV. Vitamins

Myo-Inositol	100
Sucrose	30000
Agar	8000

RT Media:

MS Media + BAP 2.5 mg/L (Benzyl Amino Purine) + IAA 0.1 mg/L (Indole Acetic Acid)

REFERENCES

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- Alan J Howart and George J Vandemark (1989), Phylogeny of Geminiviruses, J. Gen. Virol **70**, 2717-2727.
- Banks . G.K, Colvin. J, Chowdareddy.R.V, Maruthi M.N, Muniyappa V, Venkatesh M, Kiran kumar M, Padmaja A.S and Beitia F.J., (2001), First report of the *Bemisia tabacci* V biotype in India and an associated tomato leaf curl virus disease epidemic, Plant dis. Res.**85**:231.
- Bruce A. Voyles (2002), The biology of viruses, 2nd edition, McGraw Hill Publishers.p:191-193.
- Crick, F.M.C., and Watson,J.D.(1956). Structure of small viruses. Nature (London)**15**:473-475.
- Czosnek,H., Ber.R,Navot.N and Zamir.D (1988), Detection of tomato yellow leaf curl virus in lysates of plants and insects by hybridization with a viral DNA probe. Plant disease, **72**: 949-951.
- De Fazio, G, Kudamatsu, M., and Vicente, M.(1980). Virazole pretreatments for the prevention of TSWV systemic infection in tobacco plants *Nicotiana tabacum*,L.'White burley'.Fitopatol.Bras.**5**, 343-394.
- Dean E. Rochester, Jacqeline J. DePaulo, Claude M. Fauquet and Roger N. Beachy(1994), Complete nucleotide sequence of the geminivirus tomato yellow leaf curl virus, Thailand isolate. J.Gen. Virol.**75**, 477-485.
- Devaraja, Prandnya Gangatirkar, Sunitha S.N, Kirthi Narayanaswamy, Anjali Karande, Muniyappa V and Savithri H.S,(2004), Production of monoclonal antibodies to tomato leaf curl Bangalore virus, Ann.appl.Biol **144:**333-338.
- Devaraja, (1998), standardization of vegetative and tissue culture techniques for multiplication and maintenance of tomato leaf curl virus resistant tomato lines, Mysore J.Agric.Sci.,**38**(2):175-178.
- Eugene V. Koonin and Tatyana V. Ilyina (1992), Geminivirus replication proteins are related to prokaryotic plasmid rolling circle DNA replication initiator proteins. J.Gen. Virol.**73**, 2763-2766.
- Foina L.hughes, Edward P.Rybicki and Barbara vonWechmar M,(1992),Genome typing of southern African subgroup I geminiviruses, Journal of General Virology,**73**:1031-1040.

- Francki R.I.B, Robert G. Milne and Hatta.T.,(1993), Geminivirus group in Atlas of Plant viruses, Vol I, CBS publishers and distributors Chap3, p33-44.
- George P. Lomonosoff, (1995), Pathogen derived resistence to plant viruses, Annu. Rev. Phytopathol.**33**:323-343.
- Herman B.Scholthof and Karen-Berth G.Scholthof,(1996), Plant virus gene vectors for transient expression of foreign proteins in plants, Annu. Rev of Phytopathol, **34**:299-323.
- Holt. J, Colvin. J and Muniyappa V, (1999), Identifying control strategies for tomato leaf curl virus disease using an epidemiological model, Journal of Applied Ecology, **36**:625-633.
- Hong,Y.G. and Harrison,B.D, (1995) Nucleotide sequences from tomato leaf curl viruses from different countries: evidence for three geographically separate branches in evolution of the coat protein of whitefly-transmitted geminiviruses, J. Gen. Virol. **76** (PT 8), 2043-2049.
- John H. Fitchen and Roger N. Beachy(1993), Genetically engineered protection against viruses in transgenic plants, Annu. Rev. Micro Biol. **47**:739-763.
- Maarten J. Chrispeels and David E. Sadava, (1994), Plants, Genes and Agriculture, Jones and Bartlett Publishers,Edition I, Chp (13,15).
- Manjula C, Nagaraju and Muniyappa V,(2003), Evaluation of different oil formulation of Bio- Agent, *Beauveria bassiana* against different life stages of *Bemisia tabaci*, the vector of tomato leaf curl virus, Pl.Dis.Res.**18**(1):25-28.
- Marja C.P. Timmermans, Premdas O and Joachim Messing (1994), Geminiviruses and their uses as extrachromasomal replicons. Annu.Rev.Plant Physiol. Plant Mol. Biol. **45**:79-112.
- Mathews, R.E.F, Fundamentals of plant virology (1992), Academic press, Newzealand, p 168.
- Mehrota.R.S (1995), Plant Pathology, Tata - McGraw Hill Publishers 12^{th} Edition, Chapter – 1, 25 p:1, 655
- Muniyappa,V., Venkatesh,H.M., Ramappa,H.K., Kulkarni,R.S.,Zeidan,M., Tarba,C.Y., Ghanim,M. and Czosnek,H.(2000), Tomato leaf curl virus from Bangalore (ToLCV-Ban4): sequence comparison with Indian ToLCV isolates, detection in plants and insects, and vector relationships, Arch. Virol. 145 (8), 1583-1598.
- Orawan Chatchawankanphanich and Douglas P.Maxwell,(2002) Tomato leaf curl Karnataka virus from Bangalore, India, Appears

- Osaki, T., Yamada, M., and Inouye, T.(1985), Whitefly transmitted viruses from three plant species, Ann. Phyto-pathol.Soc.Jpn. **51**,82-83.
- Padmanaban,S.Y. (1973), The great Bengal Famine, Ann.Rev.Phytopath., **11**:11-26
- Pal Malinga(1995), Methods in Plant Molecular Biology, A Laboratory course Manual, Cold Spring Harbour-Laboratory Press.
- Porebski, S.L., Bailey, G., Baun, B.R.(1997), Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components, Plant Mol Biol.Rep, **15**:8-15.
- Punia, M.S.(1998), Plant Biotechnology and Molecular biology – A Laboratory manual, Scientific Publishers(India).
- Ramappa H. K, Muniyappa V and Colvin J,(1998), The contribution of tomato and alternative host plants to tomato leaf curl virus inoculum pressure in different areas of South India, Ann. Appl.Biol,**133**:187-198.
- Raskin.I, (1992). Role of Salicylic acid in plants, Annu. Rev. Plant Physiol. Plant Mol.Biol. **43**: 439-463.
- Sambrook, J., Fritsch, E.F., and Maniatis , T. (1989), Molecular cloning Laboratory manual, II edition, Cold Spring Harbor Laboratory Press.
- Sastry,K.S.M and Singh,S.J (1973), Assessment of losses in tomato by tomato leaf curl virus. Indian Journal of Mycology and plant pathology, **3**:50-54.
- Singh,S.J, (1993) Viral and mycoplasmal diseases of vegetable crop, Kalyani Publishers/Lyall bk depot. p110-115.
- Slater.A, Nigel Scott and Mark Fowler (2003), Binary Vectors for plant transformation in Plant Biotechnology, Oxford University Press, Chapter 4. p93.
- Srinivasan, M.C., M.J.Thirumalachar and M.K.Patel (1959), "Bacterial blight of rice", Curr.Sci. **28**:469-470.
- Stakman, E.C. and J.G.Harrar (1957), Principles of Plant Pathology., Ronald Press, NewYork, p581.
- William J.Lucas and Robert L. Gilbertson (1994), Annu. Rev. Phytopathol.**32**:387-411.
- William O Dawson and Mark. E. Hilf, (1992). Host range determinants of plant viruses, Annu.Rev, Plant Physiol. Plant Mol. Biol., **43**: 527-555.