MOLECULAR CHARACTERIZATION OF THE COAT PROTEIN OF A POTYVIRUS INFECTING SUGARCANE

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

TOMAL K. DATTAROY

PLANT TISSUE CULTURE DIVISION

NATIONAL CHEMICAL LABORATORY

PUNE 411008

INDIA

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **`Molecular Characterization of the Coat Protein of a Potyvirus Infecting Sugarcane'** submitted by Mr. Tomal K. Dattaroy was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Material obtained from other sources has been duly acknowledged in the thesis.

Dr. S.K. Rawal

(Research Guide)

to my parents
and
to the spirit of the research fellow...

...for one day in thy court is worth a thousand
Psalms 84:100

ACKNOWLEDGEMENT

I take this opportunity to pay my tributes to late Prof. John Barnabas, who was a source of inspiration.

I would like to express my deep sense of gratitude to Dr. S.K. Rawal, my Research Supervisor, for suggesting this problem, discussing the difficulties encountered, offering advice as to how to circumvent them, and most importantly, giving me the freedom to perform the experiments in my own way. On a personal level, I would like to thank him for his support in times of difficulty.

I would like to thank Dr. R.A. Mashelkar and Dr.A.F. Mascarenhas for permitting me to use the laboratory facilities at NCL. I would like to thank Dr. B.M. Khan for his critical evaluation of my progress.

Thanks are due to Dr. P. Sreenivasulu of S.V. University, Tirupati, for providing the antiserum to SCMV-N, to Dr. P.N. Harer of College of Agriculture, Pune, for seeds of sorghum, cv. M-35-1, and to Dr. K.N. Ganesh of Organic Chemistry (Synthesis) Division, NCL, for providing oligonucleotide primers and thermal cycling facilities. I take this opportunity to thank the participants of the Mahabaleshwar Symposium on Biology of Plant Viruses, 1993, and the anonymous netters of the Internet Molecular Biology Methods and Reagents Newsgroup, for their suggestions with respect to the different experiments performed.

I am deeply indebted to my colleagues, Sunita Mahajan and D. Vijay Kumar, for their support and encouragement. I am grateful to Dr. K. Rajmohan for his advice while on a visit to this lab during the formative part of this work. I also take this opportunity to thank my friends Rajendra Rane, Sanjeev Satyal, R. Ravi Kumar, M. Narasimha, A. Gopalakrishna and Vasant Jadhav for their suggestions and incidental help at different times during the course of the experiments described in this thesis. I thank my labmates Jawahar Mehra, Dr. D.K. Kulkarni, Mrs. Ulka Potdar, Gyanendra Tripathi and Adinpunya Mitra for coming together during moments of fun as well as of trouble, and thus making the lab a better place to work in. Thanks are due to all members of the scientific and supporting staff of the Plant Tissue Culture and Biochemical Sciences Divisions, NCL Library and NIV Library.

I am grateful to Dr. Amitabha Sarkar, Organic Chemistry (Synthesis) Division, NCL, for his timely help during the preparation of this thesis. I would also like to thank Sanjoy, Rasidul, Surojit, Mohan, Swati, Parag, Tapan, Saumen and Sourav for their help at all times during the preparation.

I am grateful to Dr. Paul Ratnasamy, Director, NCL, for permitting me to submit my findings in the form of a thesis. I would like to acknowledge the University Grants Commission, New Delhi, for a research fellowship.

Tomal K Dattaroy

Tomal K. Dattaroy

ABBREVIATIONS

BSA	Bovine serum albumin
BYMV	Bean yellow mosaic virus
bp	Base pairs
cv.	Cultivar
CYVV	Clover yellow vein virus
DMSO	Dimethyl sulphoxide
dpi	Days post inoculation
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid disodium salt
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HIV-I	Human immunodeficiency virus I
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-galactoside
JGMV	Johnsongrass mosaic virus
kb	Kilobases
kbp	Kilobase pairs
kDa	Kilodaltons
5'm^7GpppG	5' 7-methylguanosine
MDMV	Maize dwarf mosaic virus
MOPS	3-(*N*-morpholino)propanesulphonic acid
PeaMV	Pea mosaic virus
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PepMoV	Pepper mottle virus
PMSF	Phenyl methyl sulphonyl fluoride
PPV	Plum pox virus
PPV-NAT	Plum pox virus non-aphid transmissible
PSbMV	Pea seedborne mosaic virus
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis

SrMV	Sorghum mosaic virus
TAPS	3-tris(hydroxymethyl)methyl aminopropane sulphonic acid
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TEV	Tobacco etch virus
TMV	Tobacco mosaic virus
TuMV	Turnip mosaic virus
TVMV	Tobacco vein mottling virus
WMV-2	Watermelon mosaic virus 2
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
ZYMV	Zucchini yellow mosaic virus

BUFFERS, MEDIA AND REAGENTS

Luria-Bertani (LB) Medium

1% Bacto-tryptone

0.5% Yeast extract

0.5% NaCl

pH 7.0

LB-Agar

2% Bacto-agar in LB

LB-Agar-amp^{50}tet^{15}-IPTG-X-Gal

LB-Agar

50 μg/ml ampicillin

15 μg/ml tetracycline

80 μg/ml IPTG

16 μg/ml X-Gal

Top Agarose

1% Bacto-tryptone

0.8% NaCl

0.6% Agarose

TEG

50mM Glucose

25mM Tris-HCl, pH 8.0

10mM EDTA, pH 8.0

1X TAE

0.04(M) Tris-acetate

0.001(M) EDTA, pH 8.0

1X TBE

0.09(M) Tris-borate

0.001(M) EDTA, pH 8.0

20X SSC

3(M) NaCl

0.3(M) Sodium citrate, pH 7.0

20X SSPE

3.6(M) NaCl

0.2(M) Sodium phosphate, pH 7.7

20mM EDTA

TABLE OF CONTENTS

SUMMARY

Potyviruses form the largest group of viruses that infect plants and cause extensive damage to agricultural, pasture, horticultural and ornamental crops. Most potyviruses are transmitted by aphids, while there are some which are transmitted by fungi, mites or whiteflies. The virus particles are flexuous rods, 680-900 nm long and 11-15 nm wide.Each particle is made up of 2,000 copies of a single protein species of 30-37 kDa and one molecule of single stranded RNA of messenger polarity about 10,000 nucleotides long. The genome has single open reading frame encoding a large polyprotein precursor. This polyprotein precursor is cleaved autocatalytically by proteases encoded in the ORF into functional proteins.

The sugarcane mosaic virus is a definitive member of the potyvirus group and causes mosaic diseases in sugarcane, maize, sorghum and other poaceous plants. The sugarcane mosaic disease has caused extensive damage to the sugarcane crop in the United States and Australia. A large number of SCMV strains have been reported from different parts of the world and are differentiated on the basis of host reactions, serological, biochemical and molecular tests. Sequence data of the 3' terminal region, including the coat protein gene, of the SCMV-SC, the strain that infects sugarcane in Australia is available. The coat protein gene is 939 bases long and the protein has 313 amino acids. The 3' non-translated region has 235 nucleotides followed by a poly (A) tail.

Most of the available biochemical and molecular data on all potyviruses, including SCMV, are available from Australia, United States and Europe. There is very little data on these viruses from the Indian sub-continent. The disease symptoms have been seen on the sugarcane crop in Maharashtra, India, and the physiological changes in the plant that subsequently affects productivity studied. A preliminary molecular study of the pathogen is necessary to decide upon a plan for the control of the disease. With the advances in recombinant DNA technology, there are several methods for control of viral diseases in plants, especially the coat protein-mediated protection. Recombinant potyviral proteins are also being exploited as novel antigen presentation systems. The work presented in this thesis is a study that would serve as the basis for further studies for exploring the

possibility of protection against the disease and also, applying it in the field of vaccine research, as novel antigen presentation systems.

Infected leaves of sugarcane were collected from the field. Viruses were purified by several steps of differential centrifugation in borate buffer. The purified particles were tested for viability by inoculating them on inbred sorghum cv. M-35-1 seedlings under quarantined conditions. The viruses were separated by SDS-PAGE and blotted onto nitrocellulose membranes. A rabbit polyclonal antiserum to SCMV-N was used as a primary antibody and alkaline phosphatase conjugated goat anti-rabbit IgG antibody was used for the secondary reaction. A band at ~24 kDa gave strong cross reaction.

RNA was isolated from the particles using guanidium salts and urea. RNA was separated on 1% agarose gels under denaturing conditions. A high molecular weight band was seen above 9.5 kb. The characteristic eukaryotic ribosomal RNA bands were absent and therefore it was assumed that the preparation was free of any host RNA contamination. The size of the RNA as observed in the gel was in agreement with the size known in literature.

cDNA was synthesized and an aliquot of the reaction mixture was radiolabeled. The radiolabeled reaction was electrophoresed and autoradiographed. The 2nd strand synthesis showed a smear from ~7 kb down to ~500 bp. *Eco*RI adaptors were ligated to the cDNA which was then ligated to λgt11 *Eco*RI arms and was then packaged in an *in vitro* packaging system. The packaged phage was titrated on *E. coli* Y1090 cells in the presence of IPTG and X-Gal and a titre of 1.7×10^5 recombinant pfu/ml was obtained. The entire packaging mixture was amplified to a titre of 1.1×10^{14} recombinant pfu/ml. Primary and secondary screenings were performed using the rabbit polyclonal antiserum to SCMV-N and an alkaline phosphatase conjugated goat anti-rabbit IgG. Fifty three immunopositive plaques were picked from a primary screening of 2.2×10^4 pfu. Phage plugs were eluted and 100 pfu plated for the secondary screening which gave nearly 100% immunopositive plaques. These plaques were plugged out and DNA from liquid lysates prepared. The DNA upon digestion with *Eco*RI failed to release the insert. It was assumed that the *Eco*RI site had got modified in the vector.

Oligonucleotide primers were designed on the basis of the sequence data of SCMV-SC and covered the flanking ends of the capsid protein. These were:

VCPP1 - 5' d(TCCACCAAGCTGGAACAGTC) 3' (sense primer)

VCPP2 - 5' d(GACTAGTGGTGCTGCTGCAC) 3' (antisense primer).

Polymerase chain reaction of DNA from λgt11 library and immunopositive plaques after decapsidation by heating were carried out. The cycling conditions were as follows: denaturation at 94°C for 1 min 20 sec, annealing at 37°C for 2 min 20 sec and extension at 72°C for 3 min. A single band of ~600 bp was always observed even after increasing the annealing temperature to 52°C. Reverse-transcriptase PCR was carried out using the viral RNA as template and the same results were obtained.

The capsid protein gene of most potyviruses is about 900-950 bases long. The PCR primers were chosen from the ends flanking the capsid protein sequence of SCMV-SC. The length of the gene between the two ends from where the primer sequences were chosen is 939 bases. However, PCR reactions of both the recombinant λgt11 DNA as well as cDNA from viral RNA of SCMV prepared in our laboratory have consistently amplified ~600 bp band. When pUC18 digested with *Alu*I was used as a marker, this band corresponded to the 679 bp band.

The PCR amplified DNA from this band was eluted with Gene Clean and the ~600 bp fragment was used as a probe for hybridization with viral RNA. Strong signals were seen in the region above 9.5 kb, thereby indicating that this PCR amplified fragment had a viral origin. The PCR amplified fragment was ligated into pMOS*Blue* T-vector, a vector especially designed for cloning of PCR products. Plasmid DNA was transformed into *E.coli* MOS*Blue* cells and recombinants were screened using IPTG and X-Gal. Plasmid DNA from white colonies was isolated and digested with *Eco*RI and *Hin*dIII to release a fragment similar in size to the PCR product. This insert was subcloned into pGEM3Z to enable bi-directional sequencing with SP6 and T7 primers using Sequenase™ Version 2.0. Sequence data is compared with the existing potyviral capsid protein gene sequences. This information will be critical for the planning of the strategy to control the infection.

The thesis embodied herein has been divided into three chapters. **Chapter 1** is the **Introduction**. This chapter reviews the current status of research in the area of potyviruses and sugarcane mosaic virus, and examines the scope of the current research. **Chapter 2** is entitled **Experimental Procedures**, where the methodologies of all the experiments performed have been described. **Chapter 3** is the chapter on **Results and Discussion**, where all the findings of the experiments performed are presented, discussed and conclusions drawn upon.

What is your aim in philosophy? To show the fly the way out of the fly-bottle...

Ludwig Wittgenstein

Philosophical Investigations

CHAPTER 1

INTRODUCTION

Viruses are as ancient as life itself. Viruses leave no fossils, but their rapid replication, large populations, high mutation rates, extensive genetic recombinations, gene duplications and ancient origin contribute to their variability and allow the possibility of following evolutionary change. Plant viruses are the master explorers of evolutionary space and a part of the rich tapestry of life on earth (1).

Plant viruses differ greatly in their survival strategies (2). The occurrence and course of epidemics of plant virus disease are the result of interplay of many factors and processes. These impose a variety of selection pressures on the viruses, and are conversely affected by modifications in their genomes. Virus isolates are selected for their ability to survive in different and fluctuating environmental conditions, especially temperature. In some cases, where host species have been exposed to infection continuously for many years, there is evidence of co-evolution of virus and host to a situation in which the viruses survive in but do not greatly damage their plant hosts (2).

1.1 Potyviruses

Potyviruses form the largest group of viruses that cause significant losses in agricultural, pasture, horticultural and ornamental crops (3, 4). The potyvirus group is named after its type member, Potato Virus Y (PVY) and is the largest of the 34 known plant virus groups and families (4). It encompasses 180 definitive members constituting 30% of all known plant viruses (3, 4).

1.1.1 The physical characteristics of potyviruses

The morphological characteristics of the virions are reported to be flexuous and rod-shaped. The dimensions of a potyvirus particle range from 680-900 nm in length and 11-15 nm in width (3, 4). Each virion is made up of 2,000 units of a single structural protein surrounding one molecule of single-stranded RNA that is, on an average, 10,000 nucleotides long and of messenger polarity. Definitive potyviruses are transmitted in a non-persistent manner by many aphid species, while some potyviruses have fungus, mite or whitefly vectors (3). All potyviruses examined so far are reported to induce characteristic pinwheel or scroll-shaped inclusion bodies in the cytoplasm of infected cells (5).

1.1.2 The genome

The genome has a protein (VPg) covalently attached to its 5' end and a single open reading frame (ORF) coding for a large polyprotein precursor and a poly (A) tail at the 3' end. This ORF is flanked by two non-coding regions (NCR) at the 5' and the 3' ends (4, 6-8).

The 3' non-coding region

The 3' non-coding region of different potyviruses has been described as heterogeneous in size, sequence and predicted secondary structure. The length varies from 147 to 499 nucleotides (9). Distinct potyviruses show a low sequence identity in the 3' NCR (up to 30 to 53%) as compared to that between strains of individual viruses (10). This region consists of pairs of direct repeats and is capable of forming secondary structures (11). Features seen common to all of them are the presence of AU-rich segments and that each of them can be predicted to fold into stable secondary structures (12). However, their predicted secondary structures appear to vary from virus to virus and their biological significance in potyviruses remains unclear (12, 13). In case of papaya ringspot virus (PRSV) strains W and P, no obvious common features of secondary structure were observed although extensive secondary structures were predicted with a wide range of free energies (14).

The 5' non-coding region

The length of the potyviral 5' NCR ranges between 144 to 205 nucleotides (3, 4). They are rich in A and have few G (4). Alignment of the 5' NCR of a few viruses like plum pox virus (PPV), tobacco etch virus (TEV), tobacco vein mottling virus (TVMV) and PVY have revealed a marked difference in sequence except for some conserved nucleotide blocks but this is not accompanied by the conservation of their predicted secondary structures (12). There is a highly conserved region of 12 nucleotides, TCAACACAACAT, which is termed as the *Potybox*, starting at position 13 in PVY and TVMV, and at position 14 in PPV and TEV (3). The conservation of these sequences suggests that they could play an important role in the viral life cycle, for example in the processes of encapsidation, translation or replication (3).

The PVY strain N (PVY^N) RNA genome has a 5' NCR of 185 bases (15). There are two other reading frames that are frequently punctuated by termination codons and are therefore not likely to code for proteins of significant size. The first AUG codon at position 185 is likely to be the initiator codon (15) since it is in a context (UCAAUGGC) similar to the consensus sequence for translation initiation in plants (AACAAUGGC) (16). Strains of PVY have a pentanucleotide motif (UUUCA) that is repeated three to five times in this region (17). This pentanucleotide block appears only once in the same region of some other potyviruses, except in case of TVMV, where it occurs thrice (17). This pentanucleotide conserved motif is not found at the 5' end of the complementary strand, which is in agreement with other conserved sequence elements and could have a role in the PVY life cycle (17).

1.1.3 Translation of the potyviral RNA

Translational initiation of most eukaryotic mRNAs occurs by a scanning mechanism (18). RNAs of picornaviruses, which are said to be the supergroup of potyviruses because of several similarities, have long 5' NCRs harbouring numerous AUG codons and lack 5'm^7GpppG caps. These viruses inhibit translation of host cell mRNA. However, translation of viral RNA is initiated at an internal ribosome entry site (IRES), resembling the internal initiation mechanism used in prokaryotes (18). Although the potyviral genomic RNA functions as mRNA, very little is known about its translation process. The RNA lacks a 5'm^7GpppG cap leading to the speculation that the mechanism of translation initiation differs from that of cellular mRNA (19). It has been seen that cap-independent enhancement of translation is associated with the 5' NCR in TEV (19). In PVY, the 5' NCR enabled the initiation of translation even when located in an internal position, thereby suggesting that the full length of 5' NCR could correspond to the IRES (20). This mechanism may not be a universal phenomenon in potyviruses, because the translation of the PPV RNA was suggested to proceed through a conventional leaky scanning mechanism (21). In the PPV, a functional ORF starts at position 36, but translation is initiated at position 147. The PPV 5' NCR was not able to drive *in vitro* an efficient internal entry of the ribosomes (21).

1.1.4 Proteolytic processing of the potyviral polyprotein precursor

The polyprotein ranges from 340 kDa to 368 kDa in molecular weight for different potyviruses (4). Linking proteins in the form of self-processing polyproteins is a strategy adopted in the replication of many viruses, notably the positive-stranded RNA viruses (22). In the picornaviridae, a single long open reading frame encodes a polyprotein, although full-length translation products are not normally observed due to extremely rapid primary intra-molecular (*cis*) cleavages mediated by virus-encoded proteases (22). The precursor polyprotein is cleaved autocatalytically by proteases coded by the viral genome within the open reading frame (8). The order of these products in the polyprotein (4) from the N-terminus is:

(i) P1

(ii) Helper component-protease (HC-Pro)

(iii) P3

(iv) $6K_1$, a 6 kDa protein

(v) Cylindrical inclusion protein (CI)

(vi) $6K_2$, a 6 kDa protein

(vii) Small nuclear inclusion protein (NIa) which includes VPg at the N-terminus

(viii) Large nuclear inclusion protein (NIb) which has RNA-dependent RNA polymerase activity

(ix) Coat protein (CP).

CP and VPg are the only gene products that have been detected in virus particles. HC-Pro, CI, NIa and NIb have been isolated from infected plants and characterized (3). Both NIa and NIb accumulate in the nucleus (8), although proteolysis and replication, the functions assigned to these proteins, are performed in the cytoplasm.

The requirement for three proteases is surprising because only a single proteolytic enzyme can carry out proteolysis in a polyprotein producing virus as in the como- and nepoviruses (23). Two selective advantages may have contributed to the presence of multiple proteases. First, increasing the number of proteases may have provided an additional means to regulate proteolysis through differential activities of the enzymes. Second, the differential proteases may have adapted to provide additional non-proteolytic viral functions. Several examples are now available to support the latter idea. The picornavirus 3C protease exhibits RNA binding activity that may be necessary for initiation of positive strand RNA synthesis (24). Additionally, the core domain of the Sindbis Virus CP is a serine-type protease, illustrating that a protease can provide both enzymatic and structural functions (25).

The NIa protease

The virus-encoded protease, NIa, is said to be the evolutionary homologue of the picornavirus 3C protease (26). It processes co- and post-translationally the larger viral precursor (27). The remaining proteolytic activities that are required for completion of the processing pathway reside in the HC-Pro and P1 protein (28). NIa is responsible for cleavages in the C-terminal two-thirds of the polyprotein whereas HC-Pro and P1 autocatalytically cleave at their respective C-termini.

NIa protease cleavage sites have been defined by sequences of seven amino acids which have been characterized experimentally (27). These are defined by the heptapeptide consensus sequence, EXXYXQ^S/G (Glu-Xaa-Xaa-Tyr-Xaa-Gln^Ser/Gly). The cleavage occurs between the sixth and the seventh dipeptide in the heptapeptide and the dipeptides are glutamine-serine or glutamine-glycine.

The cleavages occur at the following sites:

(i) Between C-terminus of P3 and N-terminus of $6K_1$

(ii) Between C-terminus of $6K_1$ and N-terminus of CI

(iii) Between C-terminus of CI and N-terminus of $6K_2$

(iv) Between C-terminus of $6K_2$ and N-terminus of NIa

(v) Between C-terminus of NIa and N-terminus of NIb

(vi) Between C-terminus of NIb and N-terminus of CP.

However, in case of pea seedborne mosaic virus (PSbMV) and turnip mosaic virus (TuMV), the cleavage at the $6K_2$-NIa junction is characterized by the presence of glutamic acid instead of glutamine (29, 30). In johnsongrass mosaic virus (JGMV), all the NIa cleavage sites except that at the NIb-CP junction, contained glutamic acid instead of glutamine (31).

NIa protease also appears to catalyze post-translational proteolysis of an internal cleavage site (32). The sequence of this internal cleavage site differs from the others but it cleaves after glutamic acid in several viruses. This site is located between the VPg and the protease domains of the NIa protein. This cleavage was proposed to be related to some step of viral RNA replication (4). However, experiments with TEV have shown full-length forms of NIa linked to the RNA (33), thereby suggesting that this cleavage is not strictly required for VPg activity during RNA replication.

The HC-Pro protease

The proteolytic activity of the HC-Pro is characterized by a conserved hexapeptide cleavage site at its C-terminus (4), YXVG^GX (Tyr-Xaa-Val-Gly^Gly-Xaa). Cleavage occurs at the glycine-glycine dipeptide (between the 4^{th} and the 5^{th} peptide) in the hexapeptide.

HC-Pro is essential for transmission of potyviruses by insect vectors. The helper component (HC) consists of a polypeptide of around 55 kDa (34) that is responsible for the binding of the virus particles to surfaces in the maxillary stylets (35). It has a role in the retention of the virus on the cuticular lining of the food canal and foregut of aphids (36). Transmission electron microscopy and immunogold labeling studies have shown that virions were retained at these sites only when aphids were fed on virions along with HC, a combination that is essential for transmission (36).

The P1 protease

The protein encoded by the 5' terminal region of the potyviral genome is known as the P1 protein (3). It is the most variable of those that have been sequenced (37) and the least conserved region in the entire polyprotein (23). The N-terminal half of P1 is hypervariable both in length and in sequence (23). It shows the greatest variation in molecular mass in over 30 potyviruses which have been studied by *in vitro* translations with a size range from 32 to 64 kDa (37). The C-terminus of P1 has been identified as a serine-type protease responsible for the autocatalytic cleavage between P1 and HC-Pro at a tyrosine-serine dipeptide (23, 37). In TEV, the C-terminal half of 147 amino acid residues consists of the complete functional protease, with a catalytic triad of histidine 214, aspartic acid 223 and serine 256 , whereas the N-terminal half of 157 amino acids is completely dispensable for proteolytic activity (23). P1 cleaves preferentially by an autoproteolytic mechanism, and its activity *in vitro* requires a cellular factor present in extracts from plant (wheat germ) but not animal (rabbit reticulocyte) cells (23). In TVMV, P1 was shown to exhibit a non-specific RNA binding activity (38) with a high net positive charge. This charge is far higher than any of the other potyvirus encoded protein. A high net positive charge is indicative of a nucleic acid binding protein. P1 had a very high affinity for RNA but its role has not been ascertained (38).

1.1.5 The multiplication of potyviruses

Multiplication of RNA viruses within the host cell involves four fundamental steps that overlap chronologically (39). These are, (1) decapsidation, which liberates the nucleic acid and makes it available for other processes; (2) translation, during which the viral RNA serves as messenger RNA, producing the structural and non-structural proteins coded by the viral RNA; (3) replication of the viral genome, yielding progeny RNA molecules; and (4) encapsidation of these progeny RNA strands.

Potyviral RNA-dependent RNA polymerase

On the basis of sequence similarities with RNA-dependent RNA polymerases of other positive strand RNA viruses, NIb was proposed to be the putative RNA polymerase (40). NIb genes share a relatively high degree of homology in potyviruses (41). There are 5 consensus motifs and these are:

(i) WNGSLKAELR

(ii) KVCVDDFNN

(iii) YCD(N)ADGSFDSSL

(iv) GNNSGQPSTVVDNT(S)LMVXXA

(v) GDD

The last two motifs are characteristic of strongly conserved RNA-dependent RNA polymerases in all animal, plant and bacterial positive strand RNA viruses (42, 43). GDD (glycine-aspartic acid-aspartic acid) is proposed to be the active site involved in RNA chain elongation or substrate binding (42). The first three motifs are invariable in all potyviruses and are probably indispensable for the activity of the polymerase (43).

Possible roles of other proteins in replication

Several potyviral proteins may be involved directly in the replication of viral RNA. The CI is an RNA helicase with ATPase activity and is said to facilitate unwinding of a double-stranded RNA duplex with 3' overhangs in the 3'-5' direction (44-46). The 6 kDa protein ($6K_2$) is an essential membrane-associated factor which probably anchors the VPg at the 5' end of the RNA to the membrane during replication (47). This has been postulated on the basis of the picornavirus 3A protein which is immediately upstream of the 3B protein (VPg in picornaviruses) and fulfils such a function (48). Four proteins are clustered in the order CI / $6K_2$ / NIa / NIb within the polyprotein and are processed by the NIa protease (27). Both NIa and NIb accumulate predominantly in the nucleus, although potyviral replication probably occurs in a membrane-associated complex in the cytoplasm (47). How these replication-associated proteins interact in the cytoplasm remains to be determined (49).

1.1.6 The coat protein

Coat protein (CP) monomers are reported to range in size from 30 to 45 kDa (8). Heterogeneity in apparent CP size is a common feature of potyviruses owing to degradation

during purification and/or storage (8). These degraded forms move faster on SDS-polyacrylamide gels as specific bands (50, 51). From biochemical and genetic analysis, however, it is clear that each potyvirus contains only a single type of capsid protein monomer (52).

Coat protein is the most characterized gene product of potyviruses. CP sequences of 42 strains of 20 distinct potyviruses have been determined using protein and / or gene sequencing techniques (3, 9). N-termini of CP of distinct potyviruses vary considerably in length and sequence whereas the C-terminal two-thirds of the protein are highly similar, as revealed by sequence comparisons and biochemical analysis (9, 53). The significant properties of the N-terminus (54-59) are:

(i) It is the only large region in the entire CP that is unique to a potyvirus.

(ii) It is immunodominant.

(iii) It contains virus specific epitopes.

Mild proteolysis by trypsin of purified potyvirus particles revealed that N- and C-terminal regions of the CP are exposed on the particle surface (56). The surface-exposed N-terminal region can vary in length from 30 to 95 amino acids, depending on the virus, whereas the length of surface-exposed C-terminus is only 18 to 20 amino acids in different potyviruses (9, 56). Removal of N-and C-termini by proteolysis leaves a fully assembled virus particle composed of coat protein cores consisting of 215 to 227 amino acids (3). These core particles appear indistinguishable from untreated native particles by electron microscopy and are still infectious, suggesting that N- and C-termini are not required in particle assembly or for infectivity during mechanical inoculation (56, 60).

1.1.7 The movement of potyviruses

Within the plant - from cell to cell and from one tissue to another

Virus movement represents an important component in determining pathogenicity and virulence. Movement of plant viruses throughout their hosts appears quite remarkable

because they must cross the plant cell wall to move from one cell to another after multiplication (61). The general pathway for entry used by animal viruses is either surface fusion or receptor-mediated endocytosis. In plants, the cell wall acts as a barrier to the extra-cellular release and subsequent uptake of viral particles to any of these pathways. Plant viruses, therefore, have evolved a distinct mechanism for cell-to-cell movement (61).

Introduction of the virus particle into the host cell occurs as a result of mechanical or biological damage to the integrity of the cell wall and plasma membrane (61). Progressive virus infection occurs as a result of their ability to spread into adjacent cells. These possibly circumvent the cell wall by exploiting the plasmodesmata (62), the plant functional analogue of gap junctions in animals, which provide cytoplasmic continuity between adjacent cells (61). The channels are typically constricted and are probably modified to facilitate the entry of virus particles which are relatively larger in size. Most plant viruses are presumed to encode at least one protein that helps in movement from cell-to-cell. The coat protein, along with the movement proteins, is required for cell-to-cell movement. The movement proteins modify the functions of the plasmodesmata probably by re-arrangements of their components in the cytoplasmic sleeve and subtle regulatory changes that modulate the dimensions (61).

Potyviral analogues of movement proteins?

Despite extensive study of potyviruses, the requirements for cell-to-cell movement and long-distance transport are poorly understood (63). A potyviral protein possessing a dedicated movement function is yet to be identified. P1 protein of TVMV has been proposed to be involved in the cell-to-cell spread of the infection (40) on the basis of its sequence similarity to the 30 kDa movement protein of TMV (4, 64). The RNA binding properties of P1 protein of TVMV (38) give reason for speculating its function to be similar to that proposed for the TMV 30 kDa protein where it converts the molecule into an easily transportable form (64).

The role of the coat protein in potyviral transport

The involvement of CP in potyvirus movement was revealed by the analysis of TEV (65). In addition to virion assembly, CP plays significant role in cell-to-cell movement and long distance transport. Two possibilities are envisaged. Firstly, cell-to-cell movement may

require the assembly and translocation of virions between host cells. Alternatively, a non-virion ribonucleoprotein may be the transported form (65), analogous to that proposed for several other viruses that do not require virus assembly prior to movement (61). Potyviruses differ in that CP may participate in the function of the ribonucleoprotein complex or may interact with the host or other viral factors to potentiate transport in a manner comparable to a movement protein (65).

Mutations in the N-terminus did not affect virion assembly, but partially inhibited cell-to-cell movement, indicating that CP has other roles to play in translocation, besides assembly (65). These mutations inhibited systemic translocation through the vascular tissues which was rescued by complementation with the transgenic wild-type CP, thereby confirming the role of CP in long-distance translocation (65). Mutations in the CP core domain debilitate the interactions necessary for encapsidation and possibly affect protein-protein or protein-RNA interactions involved in the formation of a non-virion transport complex. RNA sequences involved in assembly of virions are yet to be identified for their role in movement (65). Factors interacting with CP for virus movement are yet to be identified. CP is probably the only viral protein necessary in potyviruses. However, a movement function may be performed by one or more structural proteins that are involved in other essential functions during replication and infection (63).

From one plant to another

A major difference between plant virus systems and their vertebrate counterparts is that vertebrate viruses spread mostly without a vector, or are spread by arthropod vectors that they infect; whereas most plant viruses rely on transmission by vectors that in most instances are not themselves infected. Vertebrate viruses, but not plant viruses, are selected for their ability to overcome antibody-mediated defence reactions (2). Almost all potyviruses rely for their survival and spread mainly on transmission by aphids. These can be acquired and inoculated by aphids within a few minutes and are usually not retained by their vector insects for more than an hour or two. Vector specificity is not highly developed and most individual potyviruses can be spread by several species of aphid (2).

The roles of the coat protein and the helper component - an insight into vector-pathogen interactions

Successful transmission of potyviruses by their aphid vectors depends upon the interaction of two viral encoded proteins, CP and HC (66). Enzymatic removal the N-terminal domain of the CP results in the loss of aphid transmissibility (67). A DAG (aspartic acid-alanine-glycine) conserved motif in the N-terminal region is said to play a role in aphid transmissibility (2). Deletions of DAG motif abolished aphid transmission in TVMV (68). Changes in the second or third amino acid in the DAG motif is a consistent feature of non-transmissible potyvirus isolates. Amino acids downstream of this motif were also postulated to have an effect on aphid transmissibility (68). This motif was suggested to be expanded to DAGX, although the fourth amino acid is not highly conserved (69).

It has been suggested that the helper component (HC) functions either by acting as a bridge binding to the DAG motif in the CP and to retention sites in the aphid mouthparts or by indirectly mediating an interaction between DAG and the aphid (66). The HC of non-transmissible isolate of TEV was found to be functional in the transmission of another isolate, implying that it does not recognize the CP of the former. HC of PVY can assist the transmission of non-transmissible isolate of TEV (70); adaptation of CP to its own HC is lost. It has been proposed that more than one site in HC may be functionally related to aphid transmissibility (71). Cleavage at DAGX sites by a trypsin-like enzyme might release the particles from their retention sites in the aphid feeding apparatus so that they could be egested and act as inoculum during subsequent exploratory probes by the aphids (2). Such cleavages are not known to destroy infectivity (72).

When potyviruses are subcultured several times without the use of aphid vectors, they tend to lose their aphid transmissibility (73), presumably because aphid non-transmissible variants outgrow their transmissible progenitors. In some cases the key change is in the virus particle protein, but in others, it is in the helper component (74). Both these proteins must, therefore, be conserved and retain their mutual adaptation if they are to function in transmission and the virus is to maintain its biological fitness (2).

The properties of the genome organization in potyviruses can be summarized in the following table (Table 1.1):

TABLE 1.1

GENOME ORGANIZATION OF POTYVIRUSES

Virus	Host	Genome	ORF	5' NCR	3' NCR	Polyprotein	Ref.
TEV	Tobacco	9496 nt	9264 nt	144nt	186 nt	3054 aa, 346 kDa	75
TVMV	Tobacco	9471 nt	9015 nt	206 nt	251 nt	3005 aa, 340 kDa	76
PPV-NAT	Plum, Peach, Apricot	9741 nt	9375 nt	146 nt	220 nt	3125 aa, 353.8 kDa	77
PPV	*Prunus* genus	9786 nt	9531 nt	146 nt	219 nt	3140 aa, 355 kDa	78
PVY^N	Potato, Tobacco	9704 nt	9189 nt	185 nt	331 nt	3063 aa	15
PVY^H	Potato	9703 nt	9183 nt	189 nt	330 nt	3061 aa	17
PSbMV	Pea seed	9924 nt	9618 nt	143 nt	163 nt	3206 aa, 364 kDa	30
PRSV	Papaya	10326 nt	10032 nt	85 nt	209 nt	3863 aa, 381 kDa	79
TuMV	Crucifers, oilseed rape, *Arabidopsis*	9830 nt	9489 nt	129 nt	209 nt	3863 aa, 358 kDa	29
PepMoV	Pepper	9640 nt	9204 nt	167 nt	265 nt	3068 aa	80
JGMV	Poaceous crops, Johnsongrass	9766 nt	9156 nt	135 nt	475 nt	3052 aa	31

Note: nt - nucleotides, aa - amino acids

1.2 Sugarcane Mosaic Virus

Sugarcane mosaic, in combination with soilborne diseases, principally *Pythium* root rot and red rot, caused a near collapse of the Louisiana sugarcane industry in the 1920s (81, 82). The sugarcane mosaic virus (SCMV) is a definitive member of the potyvirus group (83). The virus causes mosaic diseases in sugarcane, maize, sorghum and other poaceous crops (84, 85). In the early 1980s, an SCMV epidemic seriously affected sugarcane yields in the Isis cane growing area of Queensland, Australia, and combined with severe drought, resulted in losses upto 40% in some susceptible cultivars (86).

1.2.1 The physiological effect of SCMV infection

SCMV infection affects chlorophyll content, carboxylating enzymes and normal mineral metabolism (87). Existence of photorespiration in the infected leaves of sugarcane and a subsequent deviated carbon pathway, resulting in reduced rate of carbon assimilation, has been reported (88). A deranged carbon flow due to infection affecting productivity was also reported (88). Also reported was the low level of synthesis of malate, which is a key intermediate and an increased level of glycollate synthesis (88). The presence of phospho*enol*pyruvate carboxylase in infected leaves was shown to be low (88).

1.2.2 SCMV isolates worldwide and their taxonomic status

A large number of SCMV strains have been reported from different parts of the world and these were differentiated on the basis of host reactions and serological tests. A great deal of confusion exists concerning the status and the relationships of the virus isolates included as strains of SCMV (84). Strains originating in sugarcane were designated as SCMV, whereas those that originated in maize were designated as MDMV (59). Until recently, these strains were believed to belong to the same virus, since the majority induced similar symptoms in sugarcane, maize and sorghum, and they appear to be related serologically. Recent immunological studies have shown that antibodies directed to the surface exposed N-terminus of potyviral CP are virus specific whereas those directed at the conserved core region also recognize other distinct potyviruses (56).

In Australia, four strains of SCMV from 23 naturally infected species of Poaceae on the basis of natural and experimental host ranges, symptoms in certain differential plants and antigenic properties were identified. These were JG, SC, BC and Sabi (89) and the molecular weights of their coat proteins were 33.7, 34.2, 39.1 and 40.3 kDa, respectively (90). In the United States, there are 13 strains of SCMV designated as SCMV-A, -B, -C, -D, -E, -F, -G, -H, -I, -J, -K, -L and -M (59). SCMV and MDMV strains from Australia and the United States have been grouped as four distinct potyviruses on the basis of reactivities of cross-absorbed virus-specific antibodies and these are JGMV, MDMV, SCMV and SrMV (59). HPLC peptide-profiling of tryptic digests of CP of these viruses further confirmed their classification into four distinct groups (91). These four viruses are now categorized under the SCMV sub-group of potyviruses (59).

A probe from the 3' NCR from SCMV-SC was specific only for SCMV strains and did not hybridize to RNA from other potyviruses (92). Comparisons of the nucleotide sequences of potyvirus genomes revealed that some parts of the genome, but not 3' NCR, had local regions of high sequence identity that could lead to cross-hybridization between distinct potyviruses. Nucleic acid hybridization with the 3' NCR of the potyvirus genome as the probe was shown to be a relatively simple means of distinguishing between distinct potyviruses and their strains (92).

The sequence of the 3'-terminal 1343 nucleotides of the SC strain of SCMV (SCMV-SC) was compared with the 1376 nucleotides at the 3'-terminus of MDMV-B (93). The SCMV-SC sequence includes an ORF which codes for the viral CP of 313 amino acids followed by a 3' NCR of 235 nucleotides and a poly (A) tail. The MDMV-B sequence codes for the capsid protein of 328 amino acids and has a 3' NCR of 236 nucleotides. The CP of SCMV-SC has 92% homology with that MDMV-B except for the region between amino acid residues 27 and 70 of SCMV-SC. This region of SCMV-SC is smaller (44 residues) than the equivalent region in MDMV-B (59 residues) and has only 22% identity with the MDMV-B sequence. Possible mechanisms for generating such sequence diversity include deletion, frameshift mutation, gene duplication and recombination. But examination of the nucleic acid sequence data reveals that the sequence diversity is not the result of a simple deletion or a frameshift mutation. Although there is evidence of some gene duplication in this region in both SCMV-SC and MDMV-B, the duplicated sequence in SCMV-SC is different from that

duplicated in MDMV-B and there is little sequence identity between the common regions. A possible explanation for the sequence diversity is recombination but searches of protein and nucleic acid databases with both divergent sequences failed to produce any significant identities with other potyviral CP or genomic sequences. The origin of this diversity is unknown, but sequence analysis of the corresponding regions of other isolates assigned by serology to the SCMV group will establish whether one of the two sequence types predominates (93).

1.2.3 The incidence of the disease in India

Of the more than 100 diseases that affect sugarcane in India, mosaic is the most widely distributed and upto 8 strains have been reported (94, 95). SCMV is known to be transmitted either through setts or from infected plants to healthy ones by insects (96). SCMV is difficult to eliminate by heat treatment from the stock used for the propagation of sugarcane. Plant tissue culture techniques are applied for the generation of virus-free plant material by culturing the apical meristems (97). Sugarcane plants raised through tissue culture from excised shoot apices were successfully transferred to soil where they were grown to maturity. All the parent plants had characteristic symptoms, whereas over 90% of the cultured plants were free from mosaic infection. Second generation plants grown from seed setts from tissue cultured plants also showed no symptoms. However, the sugarcane cultivar CO 740, which was developed as virus-free (96) was found to be showing mosaic infection in the subsequent generations (88). The possibility of the existence of photorespiration in the infected leaves leading to a deviated carbon pathway and a reduced rate of carbon assimilation was proposed. Productivity was affected because of the deranged carbon flow (88). Virus incidence caused an economic loss by at least 15% in India (98). Although potyviruses are known to infect poaceous crops in India (95), there is no molecular data available on the different strains.

1.3 Aim and scope of the present work

Results of Ghorpade and Joshi (88) evidently direct towards the conclusion that the virus-free sugarcane plantlets raised through apical meristem culture (96) were not virus-resistant. It was necessary to explore for methods by which virus resistant cultivars could be generated and the loss in productivity minimized.

1.3.1 Viruses in tropical agriculture and their control

Viruses are especially troublesome for tropical and sub-tropical agriculture in the developing world where diagnostic and preventive technologies are few (99). Vectors proliferate because of local farming practices and the prohibitive costs of chemical control. Once infected, there are few economically or biologically viable ways to cure a susceptible crop plant of a virus. Preventing the virus from reaching the host can involve physical, chemical and biological methods (100). Use of healthy planting stock and virus-free seeds, control of vectors with pesticides, traps and screens, and breeding for virus resistance are the various options (99, 100).

Among the complications of virus infections are:

(i) High mutation rates creating resistance-breaking isolates more rapidly than conventional breeding programmes can introduce new resistance genes,

(ii) Reservoirs of virus intolerant (symptomless) wild species,

(iii) Mixed infections with unrelated viruses, and,

(iv) Persistence in vectors which are economically and environmentally impossible to treat (99).

Crop losses owing to a virus infection can be high, given favourable climatic conditions for its vector, the proximity of plants in a large monoculture and the lack of a suitable host resistance gene (99).

Resistance in the host plant

Host resistance is the main means of control of plant virus diseases (100). Resistance is mainly controlled at a single genetic locus, although more complex systems are known. Dominant resistance alleles are strongly associated with virus localising mechanisms and normally involving local lesions. Incompletely dominant and recessive alleles allow the spread of the virus, but inhibit multiplication or symptom development. Fully recessive may be associated with complete immunity. Few resistance genes have proved exceptionally durable. Acquisition of virulence can be associated with loss of general pathogenic fitness, but in some cases this can be restored by further selection of the virus in resistant hosts. A virus may contain several virulence determinants and may develop a stable gene-for-gene relationship with a host having several resistance genes. it may be possible to design robust, oligogenic resistance systems which will be difficult for the virus to overcome (100).

Host genes for potyvirus resitance

A few genes conferring resistance to potyvirus infection have been identified. *Ry* gene in potato confers resistance to PVY infection (99). *Rsv* is a gene in soybean that confers resistance to soybean mosaic virus (SMV) infection and has a simple mode of inheritance (101). Crossing of susceptible and resistant soybean cultivars was carried out and the progeny characterized for chromosomal location of *Rsv* using RFLPs and microsatellites or single sequence repeats (SSRs) as genetic markers. A single dominant gene, *Hss*, conferring a temperature-insensitive lethal systemic necrotic reaction to SMV in common bean, has been identified (102). *Hss* is not a resistance gene against SMV except when present with additional genes, but rather a gene conferring true hypersensitivity to the virus to the extent of being lethal. The dominant inhibitor gene, *I*, confers resistance to bean common mosaic virus (BCMV) infection in common bean (103) and is linked to *Hss* (102). *I* gene has also been characterized and an RAPD marker identified (103).

Breeding for potyvirus resistance

Cultivars resistant to potyvirus infection have been identified in the germplasm of different crops (104, 105). Several wild relatives and interspecific hybrids of sugarcane showed resistance to SCMV-H (104). Breeding programmes with the objective of being able

to incorporate mosaic resistance of this diverse germplasm into new cultivars were established. It is widely believed that new developments in sugarcane cultivar improvement can be expected from the use of unexplored germplasm. However, in a complex polyploid crop species such as sugarcane, back-crossing to introduce specific genes is very difficult as major genetic reassortments occur during meiosis (86).

1.3.2 The concept of cross-protection

The practice of using mild, symptomless or attenuated strains of viruses to protect field crops against closely related strains and reduce yield losses is referred to as cross-protection (106). Successful cross-protection is judged by the ability of the first virus to suppress or delay disease symptoms caused by the super-infecting virus. There are several hypotheses that have been proposed for the possible mechanism of cross-protection. These include blocking of the replication of the second virus by the depletion of an essential component of the host by the former, encapsidation of the nucleic acid of the infecting strain by the inducing strain, and blocking of the uncoating of the infecting strain by the protecting strain (107). Mechanisms responsible for this phenomenon have not been elucidated. There are disadvantages, though. First, the protecting strain could mutate to a highly virulent form. Second, it could act synergistically with a non-related virus resulting in a disease condition that is more severe than that caused by either virus alone. Third, it might be a severe pathogen for a different crop. Fourth, the protecting strain itself could cause a small but significant loss in yield (107).

1.3.3 Pathogen-derived resistance

The concept of non-conventional protection was developed in 1980s (108). The basis of this concept is that if one understands the molecular interactions involved in virus-functioning, then mechanisms can be invented for interfering with those interactions. The concept proposed that a host expressing particular parasite sequences may circumvent disease. This was termed as pathogen-derived resistance (PDR). The pioneering work in this area was done in 1986 when a transgenic tobacco plant expressing the TMV CP gene showed suppressed or delayed symptoms when inoculated with TMV (107). Since then, CP-mediated resistance against virus infection has been employed in several plants against a variety of viruses (109). The question of how CP confers resistance remains unanswered. Furthermore,

the nature of virus infection and disease development makes it likely that resistance is effected at several stages (109).

Another method of PDR is replicase-mediated resistance (110). It is considered that expression of replicase-derived sequences in transgenic plants at the protein level interferes with the normal functioning and / or assembly of viral replicase enzyme complexes. This could lead to a general, marked inhibition of viral replication in the cells of these transgenic plants.

Attenuation of symptoms of helper viruses by satellite RNAs (111) and reduction of symptoms by defective-interfering RNAs (99, 111) are some of the virus-targeted resistance strategies. Transgenic plants expressing antisense RNAs to the CP gene and 3' NCR were shown to be resistant to infection (111, 112). The 2'-5' oligoadenylate (2-5A) pathway is a part of the mammalian antiviral response system induced by interferons and this was reconstructed in plants to achieve multiple virus infection (113). The key enzyme, 2-5A synthetase polymerizes ATP to 2-5A which activates a specific ribonuclease that degrades viral RNA. Transgenic plants were constructed to express a mammalian 2-5A synthetase cDNA (113) and a broad-spectrum of resistance was observed.

1.3.4 Pathogen-derived resistance in potyviruses

Coat protein mediated resistance

In potyviruses, mild strain cross-protection has been attempted at the field level to protect courgette plants against severe strains of zucchini yellow mosaic virus (ZYMV) (114). One of the earliest examples of the breadth of protection conferred by potyviral CP genes is that of transgenic tobacco plants expressing the SMV CP gene (115). SMV is not pathogenic for tobacco, but these plants showed resistance to infection by two serologically unrelated potyviruses, PVY and TEV. This evidence for a broad spectrum of resistance led to the possibility of plants being protected against many potyviruses by using a limited number of different CP genes. Following this, tobacco plants were engineered to express the CP genes of PRSV (116), WMV 2 and ZYMV (117). In case of the former, the plants were resistant to detrimental effects caused by the heterologous potyviruses TEV, PepMoV and PVY (116). In case of the latter two, plants were resistant to six other potyviruses which were BYMV, PVY,

PeaMV, CYVV, PepMoV and TEV (117). It was also observed that the plants expressing the WMV 2 CP gene showed better protection against these viruses than those expressing the ZYMV gene (117). Transgenic plants expressing a CP gene,therefore, will show at least a noticeable level of protection against symptom development when challenged by other potyviruses. It was also observed that plant lines accumulating the most CP were not the most resistant (115). There was no correlation between the accumulation of potyviral CP and the level of resistance (118).

Transgenic plants expressing mutated TEV CPs (truncated at amino- or carboxy-terminals, or both) were more effective in CP-mediated resistance to TEV than transgenic plants that expressed the full-length TEV CP (119). Truncated CPs are in some way dysfunctional and are more effective at disrupting the normal virus-host relationship than the full-length CP. The speculation is that these molecules are dysfunctional in the process of systemic transport, and, as a consequence of being incorporated into the virions, generated defective virus or ribonucleoprotein complexes. The reported broad spectrum resistance (115) may also involve this phenomenon where CPs in a heterologous system may be dysfunctional resulting in limited systemic movement of the virus. Transgenic plants expressing antisense CP RNA or sense CP RNA with a frameshift mutation interfered with the replication of RNA of the infecting potyvirus (119).

Resistance using non-structural genes

Plants expressing the TVMV NIa gene were highly resistant to TVMV but susceptible to TEV and PVY (120). It is possible that since the VPg from the recombinant NIa is present outside its usual context (as a part of a polyprotein consisting of several replication-associated polypeptides), it might interfere with the processes involving the viral-encoded VPg. This could include events at the early stages of RNA replication or in the steps involved in the packaging of progeny RNA. Alternatively, the presence of the protease might alter the processing of the polyprotein during translation (120). Those plants expressing the CI gene were susceptible to all the three viruses (120). It has been suggested that all replication-associated protein genes cannot be used to engineer resistance to potyviruses.

Tobacco plants expressing the NIb (RNA-dependent RNA polymerase or replicase) gene were found to be resistant to PVY^{O} infection, but plants that expressed the same

sequence with a deletion of the widely conserved replicase GDD motif (42) were susceptible (121). This had a narrower spectrum of resistance than coat-protein mediated protection.

Resistance through untranslatable RNAs

A construct harbouring the 5' NCR of TEV ligated to a mutated, untranslatable TEV CP gene was introduced in tobacco (122). Some plants were highly resistant and some susceptible, with a few of the susceptible lines recovering after initial symptom development. One possibility is that the untranslatable transcript may associate with the minus-sense replicative intermediate to arrest replication (123). Another speculation is that an inducible, cytoplasmic-based, cellular activity degrades specific RNA sequences (122). In the recovery phenotype, this RNA degradation system is activated only after virus infection, and also by the additive levels of transgene and viral RNAs present. In highly resistant lines, the activity may be fully induced by the transgene transcript (122). Homozygous, transgenic plants expressing the PVY CP mRNA, but rendered untranslatable by the introduction of a stop codon immediately after the start codon, showed virus-specific resistance (124).

1.3.5 Current status of research in pathogen-derived resistance to potyviruses in sugarcane

The CP of SCMV-SC was transiently expressed in sugarcane protoplasts (86). Considerable research has been undertaken, especially in Australia, to develop an efficient, meristematic tissue transformation and regeneration system for the introduction of genes of agronomic importance, such as the CP gene of SCMV into elite, SCMV-susceptible sugarcane cultivars (86, 125).

Scope in the current scenario

An efficient meristematic tissue regeneration system for sugarcane exists in our laboratory. To proceed towards a transgenic approach for protection of the sugarcane plant from SCMV infection, it is necessary to study the pathogen in detail first. No molecular data on the potyviruses infecting poaceous crops in India are currently available. The findings of the work being presented will help identify the pathogen by scientific diagnostic methods as

well as accurately determine their taxonomic status with respect to the different potyviral strains found worldwide.

1.3.6 Exploring newer vistas for the present work

Apart from the agronomical importance of the current work, there are other biotechnological applications relevant to medicine and industry. Viruses have been engineered to express antigenic epitopes for vaccine development. A poliovirus antigen chimaera containing an epitope from a transmembrane glycoprotein of human immunodeficiency virus type 1 (HIV gp41) was constructed (126). Antiserum to this chimaera was shown to be specific for HIV-1 gp41 in peptide-binding assays by Western Blotting. The results established the potential of using poliovirus for the presentation of foreign antigens and suggested that the chimaeras could offer an approach for the development of an HIV vaccine. Empty capsids of the human pathogenic parvovirus B19 can be produced in a baculovirus system (127). Intact heterologous proteins can be incorporated and presented on the capsid surface to develop vaccines as well carry out studies in cell targeting and gene therapy. Hybrid virus-like particles carrying repetitive copies of the polypeptides or proteins exposed on the surface may provide efficient means of antigen presentation of the immune system (126-129). Sub-unit vaccines derived from fermentation technology is most likely to be the most expedient and simple means of delivering a licensable recombinant product (128).

Multiple copies of the JGMV CP synthesized in *E. coli* can readily form potyvirus-like particles (PVLPs) (130). Potyvirus particles treated with trypsin to remove the surface-exposed N- and C-termini can be dissociated and reassociated into PVLPs. All necessary information required for polymerization of the CP is located within the core region. Synthesis in *E. coli* of native CP of JGMV and hybrid protein molecules containing foreign antigens resulted in the intracellular formation of PVLPs which were immunogenic (129). Antisera were shown to be specific for the antigenic epitopes. The foreign antigens used were (a) an octapeptide epitope from the malarial parasite, *Plasmodium falciparum*, and (b) a 26 kDa protein, Sj26-Glutathione-S-transferase from *Schistosoma japonicum*. Also used was the decapeptide luteinizing hormone releasing hormone.

Foot and mouth disease virus epitopes were successfully fused to the surface of cowpea mosaic virus that infected cowpea and was highly immunogenic (131). Similarly, chimaeric tobacco mosaic virus (TMV) particles expressing malarial epitopes on the surface were found to be infective in tobacco and highly immunogenic for the epitope they carried (132). These candidate sub-unit vaccines can be safely produced in high volumes without the expense of maintaining sterile liquid cultures (132). Such an approach using potyviruses as carriers is yet to be carried out.

The results of the present work will guide us to whether the potyviruses infecting sugarcane in India can be exploited as novel antigen presentation systems. Given the current scenario of healthcare and the risks involved in using live, attenuated vaccines, the requirement of novel, recombinant, heterologous antigen presentation systems needs to be looked into.

The study of biology is partly an exercise in natural aesthetics. We derive as much of our pleasure as biologists from the continuing realization of how economical, elegant and intelligent are the accidents of evolution that have been maintained by selection. A virologist is among the luckiest of biologists because he can see into his chosen pet down to the details of all its molecules. The virologist sees how an extreme parasite functions using just the most fundamental aspects of biological behaviour.

David Baltimore
Nobel Lecture, 1976

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1 Virus Particles

2.1.1 Symptomatology

Leaves of sugarcane (*Saccharum officinarum*, L., cv. CO 740 and cv. CO 8014) were harvested from the fields of College of Agriculture, Pune, India. Leaves that showed yellow streaks were collected. The intensity of yellow streaks increased with the age of the leaves and that of the plant. Older leaves looked chlorotic.

2.1.2 Isolation of virus particles from infected leaves

The method described by Gough and Shukla (90) was modified and used to isolate virus particles from infected leaves of sugarcane. The leaves were thoroughly washed in sterilized deionized water, the midrib removed and chopped into 1 cm^2 pieces. The leaf pieces were cooled at 4°C overnight. Twenty-five gm of chopped leaf material was homogenized in 125 ml of viral homogenization buffer (VHB).

VHB composition:

0.5 (M) sodium borate (boric acid-NaOH), pH 8.0

0.15% thioglycollic acid

10 mM EDTA, pH 8.0

1 mM PMSF

The sap was clarified by centrifugation at 8,000 X *g* at 4°C for 10 min with 1/3rd volumes each of chloroform and carbon tetrachloride. Triton X-100 was added to the recovered aqueous phase to a final concentration of 5%. The mixture was stirred at 4°C overnight. Stirring was continued till the detergent got uniformly mixed and the sap obtained was clear. This was then centrifuged at 8,000 X *g* for 10 min at 4°C. The supernatant was centrifuged at 100,000 X *g* for 90 min at 4°C in a Sorvall OTD Combi ultracentrifuge. The pellet obtained was resuspended in VHB by stirring at 4°C overnight.

The resuspended pellet was centrifuged at 8,000 X *g* for 10 min at 4°C. The supernatant was subjected to another round of ultracentrifugation at 100,000 X *g* for 90 min

at 4°C. The pellet was resuspended in viral resuspension buffer (VRB) (1 ml for 50 gm of initial leaf material).

VRB composition

50 mM sodium borate buffer (boric acid-NaOH), pH 8.0

1 mM PMSF

2.1.3 Protein estimation

Bradford's method of protein estimation (133) was used to determine the yield of viral particles. This method estimates the amount of protein in a solution by the principle of dye-binding.

Solutions

1. BSA

BSA at a concentration of 0.5 mg/ml was used as a standard protein solution. The expected absorbance of this solution at 280 nm is 0.33.

2. Bradford's colour reagent

The composition of 100 ml of Bradford's colour reagent was as follows:

Coommassie Brilliant Blue G-250	10 mg
95% Ethanol	5 ml
H_3PO_4	9.66 ml

The volume was made up to 100 ml with deionized water and filtered through Whatman 3MM filter paper.

Assay

BSA (0.5 mg/ml) was diluted in 50 mM Tris-HCl, pH 7.8. The amounts of protein taken were 0, 2, 4, 6, 8 and 10 μg. 1 ml of Bradford's Colour Reagent was added to 100 μl of diluted protein and vortexed. The dye was allowed to bind for 2 min and absorbance read at 595 nm. The absorbance value obtained for the reference (blank) was subtracted from the other observed values which were then plotted against the concentration of protein and the linear regression was calculated. The prepared viral suspension was diluted in 50 mM Tris-HCl, pH 7.8 and the estimation carried out as above.

The following precautions were necessary:

(i) The Bradford's colour reagent was prepared fresh for every protein estimation. Protein standard assays were carried out at every estimation.

(ii) While carrying out the protein standard assays, only 2-3 samples were taken at a time, the dye-binding allowed to go on and then the next 2-3 samples were taken. This was done since it was observed that with increasing time the amount of dye bound to the protein increased and thereby caused a hyperbolic increase in the absorbance values of the latter samples. Time allowed for binding of the dye was kept constant this way.

2.1.4 Electrophoretic separation of proteins on polyacrylamide gels

Analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide sub-units that minimize aggregation. SDS, a strongly anionic detergent, and 2-mercaptoethanol, a reducing agent, along with heat, are used to dissociate the proteins (134, 135). The denatured polypeptides bind SDS and become negatively charged. The amount of SDS bound is almost proportional to the molecular weight of the polypeptide and is independent of its sequence. By using markers of known molecular weight, it is therefore possible to estimate the molecular weight of a polypeptide chain.

SDS-polyacrylamide gel electrophoresis is carried out with a discontinuous buffer system. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone (stack) on the surface of the resolving gel. Discontinuous buffer systems can concentrate all of the complexes in the sample into a very small volume and thereby increase the resolution. All components of the system contain 0.1% SDS. The sample and the stacking gel contain Tris-HCl, pH 6.8, the upper and the lower buffer reservoirs contain Tris-glycine, pH 8.3, and the resolving gel contains Tris-HCl, pH 8.8.

Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross-linked by a bi-functional agent such as *N,N'*-methylene-*bis*-acrylamide. The effective range of separation depends on the concentration of acrylamide used to cast the gel and on the degree of cross-linking.

The glass plates were assembled with 1 mm Teflon spacers sandwiched between them at the edges. The spacers were coated with sealing grease to prevent leakages and the assembly was held together using clamps.

Composition of 12.5 % SDS-polyacrlamide gels

	Resolving Gel		Stacking Gel
	Mini Gels 5 cm X 9 cm	Large Gels 13.5 cm X 14 cm	
30 % acrylamide	4.16 ml	10.4 ml	1.67 ml
1 % *N,N'*-methylene-*bis*-acrylamide	1.00 ml	2.5 ml	1.3 ml
1 (M) Tris-HCl, pH 8.8 (pH 6.8 for stacking gels)	1.675 ml	4.188 ml	1.25 ml
Distilled water	2.16 ml	5.4 ml	5.6 ml
10 % SDS	100 µl	250 µl	100 µl
degassed mixture under vacuum and added:			
TEMED	7.5 µl	18.75 µl	5 µl
10 % Ammonium persulphate	100 µl	250 µl	75 µl

Ammonium persulphate solution was always prepared fresh just before use.

The resolving gel mixture was poured into the gap between the two glass plates allowing sufficient gap for the stacking gel. The gel was overlaid with water saturated n-butanol to prevent oxygen from diffusing into the gel and inhibiting polymerization. Once polymerization was complete, the n-butanol was drained off and the stacking gel was poured. A Teflon comb was immediately inserted to mould the wells.

Gel loading buffer

The gel loading buffer had the following composition:

125 mM Tris-HCl, pH 6.8

700 mM 2-mercaptoethanol

2% SDS

20% glycerol

0.0025% bromophenol blue

Samples were mixed with an equal volume of gel loading buffer and heated in a boiling water bath for 5 min before loading.

Gel electrophoresis buffer

The gel was electrophoresed in a buffer which had the following composition:

25 mM Tris

250 mM glycine

0.1% SDS.

The pH of the solution was 8.3.

Electrophoresis was carried out at a constant current of 20 mA in gel electrophoresis buffer till the bromophenol blue dyefront reached the bottom of the gel. After completion of electrophoresis, gels were either stained with silver salts or blotted onto nitrocellulose membranes for immunological detection.

2.1.5 Staining of proteins separated on polyacrylamide gels with silver salts

Staining of proteins separated on SDS-polyacrylamide gels is usually achieved with the dye, Coommassie Brilliant Blue R250. However, the stain is of limited sensitivity. The low yields of viral particles required a method that would be sensitive, reproducible and easy to handle. Ultra-sensitive staining of trace amounts of proteins can be achieved by a selective reduction of ionic silver to its metallic form (136, 137).

Silver is used as light-sensitive agent in photography. It was also used earlier to visualize cellular structures. Adaptation of staining methods using silver salts to the detection of proteins in polyacrylamide matrices resulted in an approximately 100-fold increase in sensitivity over Coommassie Blue (136). In this method, a polyacrylamide gel containing separated proteins is soaked in silver nitrate ($AgNO_3$) solution. This is followed by the use of most common photographic developers to obtain a negative image. Exposure of this image to light may cause a photoreversal unless the remaining silver salts are removed by rinsing the gel in a photographic fixer followed by extensive washes in water (136).

The method of Blum *et al* (137) was modified.

Solutions:

1. Fixative: 50 % methanol, 12 % acetic acid.
2. 0.1 % sodium thiosulphate ($Na_2S_2O_3$)
3. Stain: 0.2 % $AgNO_3$, 0.0703% formaldehyde (HCHO)
4. Developer: 6% Na_2CO_3, 0.04% $Na_2S_2O_3$, 0.00925% HCHO

Following electrophoresis, the gel was stored in the fixative for a minimum of 1 hour but the duration of fixation could be extended for a sufficiently long period of time. The gel was given 3 washes with 50% ethanol of 20 min duration each. This was followed by one wash in 0.1% $Na_2S_2O_3$ for 2 min. It was rinsed with distilled water and then soaked in the stain in dark for 20 min, followed by extensive washing with distilled water. The gel was transferred to the developer which was rapidly discarded once the molecular weight marker bands appeared. It was followed by another extensive rinse with distilled water. The gel was stored in the fixative.

$Na_2S_2O_3$ prevents the formation of insoluble silver salts in the developer which may precipitate on the gel surface and darken the background (6). Na_2CO_3 makes the developing solution alkaline facilitating the oxidation of HCHO. HCHO serves as a silver reducing agent and is converted to formic acid during development (136).

The following precautions were taken. All solutions were freshly prepared in deionized water prior to use. Care was taken while handling the gel to prevent the formation of artifacts by fingerprints. The duration of washing and staining in the different steps of the protocol was exactly followed. The developing was allowed to proceed till the required number of molecular weight marker bands just began appearing and the solution was promptly discarded to prevent the appearance of non-specific artifacts in the lanes.

2.1.6 Immunological detection of virus-specific protein immobilized on solid supports

Western blotting (138) is the method by which electrophoretically fractionated proteins are transferred from a gel to a solid support and probed with reagents that are specific for particular sequences of amino acids. Usually, the probes are antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the solid support. This method is extremely useful for the identification and quantitation of specific proteins in complex mixtures. Electrophoretic separation of proteins is almost always carried out under denaturing conditions and therefore, any problems of solubilization, aggregation and co-precipitation of the target protein with adventitious proteins are eliminated (135).

Biological material

Primary antibody

Rabbit polyclonal antiserum to N strain of SCMV (a kind gift of Dr. P. Sreenivasulu, S.V. University, Tirupati, India).

Secondary Antibody

Goat anti-rabbit IgG conjugated with alkaline phosphatase (Promega, USA).

Solutions

Towbin buffer

39 mM Glycine

48 mM Tris

0.37% SDS

20% Methanol.

The solution had a pH between 7.8 to 8.4.

Tris-buffered saline (TBS)

100 mM Tris-HCl, pH 8.0

150 mM NaCl

TBST

0.05% Tween-20 (detergent) in TBS

Blocking solution

1% BSA in TBST

Alkaline phosphatase buffer (AP buffer)

100 mM Tris-HCl, pH 9.5

100 mM NaCl

5 mM $MgCl_2$

NBT

50 mg/ml nitro blue tetrazolium in 70% dimethylformamide

BCIP

50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 70% dimethylformamide

After electrophoresis, the gel was equilibrated in Towbin buffer for 30 min to 1 hour with gentle shaking. The cassette was loaded onto the electroblotting unit (Hoeffer Scientific, USA) along with the nitrocellulose membrane. The gel was towards the negative electrode and the membrane towards the positive electrode. Transfer was carried out in Towbin buffer overnight at a current of 0.6 to 0.8 amps.

The nitrocellulose membrane was equilibrated in TBS for 30 min followed by floating on TBST until evenly wet. It was submerged and rinsed briefly in the same buffer twice. Protein transfer was authenticated by staining with Ponceau S, a dye that binds to all proteins immobilized on nitrocellulose (135). This stain was rapidly rinsed out for the subsequent tests by washing extensively with deionized water. Non-specific protein binding sites were saturated by incubating the membrane in the blocking solution for 30 min. The blocking solution was replaced by an appropriate dilution of the SCMV antiserum (1:800 in TBST) and incubation was carried out for 30 min. The membrane was washed thrice with TBST to remove unbound antibodies. Each wash was carried out for 15 min.

The membrane was transferred to TBST containing appropriate dilution (1:8000 in TBST) of the secondary antibody. The incubation was allowed to go on for 30 min for binding of the secondary antibody to rabbit IgG. The membrane was again washed thrice with TBST to remove unbound antibodies. Each wash was carried out for 15 min.

The membrane was blotted dry and transferred to colour development solution (10 ml AP Buffer, 66 µl NBT, 33 µl BCIP). Once reactive areas turned purple (within a maximum of 15 min), colour development was stopped and the membrane was rinsed thoroughly in deionized water. The membrane was either kept moist and photographed or air-dried and stored for record.

2.1.7 Infectivity tests of viral preparations

Any pathological isolate has to be checked for its integrity and viability before performing any biochemical studies. The viral particles that were isolated from the field infected leaves had been checked for their serological relationship to SCMV. However, it was necessary to check the viability of the isolated and purified viral particles.

Indicator hosts are used for infectivity assays. These are plants that show disease symptoms when physically inoculated with the viral preparations under quarantined conditions. Sorghum cv. M-35-1 (a kind gift of Dr. P.N. Harer, College of Agriculture, Pune, India) was used as the host for these experiments.

A metal chamber fitted with a fluorescent lamp was used to simulate quarantined conditions. This chamber was kept in a culture room with temperature maintained at about 25°C. The fluorescent lamp was appropriately switched on manually to provide a 12 hour light-12 hour dark photoperiod.

Soil was sterilized and sorghum seeds sown. Plants were watered every third day. Flag leaves of 10-day old seedlings were inoculated at the base with the viral suspension in borate buffer (VRB) by gentle rubbing with the pipette tip. Symptoms were observed every day.

2.2 The Nucleic Acid

2.2.1 Elimination of ribonucleases from the experimental system

To obtain good preparations of viral RNA it is necessary to minimize the activity of ribonucleases liberated during cell lysis (135, 139). Improper care results in RNA preparations contaminated with RNases from outside sources. RNases are very stable at very high temperatures necessitating extreme precautions.

The following precautions were taken to minimize RNase contamination:

Glassware : All glassware were treated with 0.1% diethyl pyrocarbonate (DEPC) overnight and then autoclaved after draining off the solution. This was followed by baking at 200°C for 4 hours.

Plasticware : All plasticware were siliconized to render their surfaces hydrophobic. This was followed by treatment with 0.1% DEPC overnight and subsequent autoclaving.

Solutions : All solutions were prepared in deionized water that contained 0.1% DEPC and subsequently autoclaved. It is imperative to post-treatment denature the DEPC by autoclaving since it modifies the purine residues in RNA and $-NH_2$ moeities in amino compounds such as Tris. All solutions were stored in DEPC treated, autoclaved, baked containers.

Some plasticware such as electrophoresis units were found to be reacting to DEPC. These were treated with 3% hydrogen peroxide overnight and subsequently rinsed extensively with DEPC treated, autoclaved deionized water (135).

Disposable surgical gloves were worn while preparing solutions and performing experiments to prevent contamination from fingertips, which happen to be a major source of ribonucleases.

2.2.2 Isolation of potyviral RNA

In addition to all the above precautions, chaotropic agents such as guanidium salts and reducing agents such as 2-mercaptoethanol which inactivate RNases (140, 141) were used for the isolation of RNA. The method of Frenkel *et al* (10) was followed.

Solution A:

6 (M) guanidium-HCl

0.2 (M) sodium acetate, pH 5.2

10 mM 2-mercaptoethanol

Solution B:

6 (M) guanidium-HCl

0.2 (M) sodium acetate, pH 5.2

10 mM EDTA, pH 8.0

Solution C:

7 (M) urea

100 mM Tris-HCl, pH 7.5

0.1 mM EDTA, pH 8.0

0.1% SDS

Other Solutions and Reagents:

3 (M) sodium acetate, pH 5.2

Distilled phenol containing 0.1% 8-hydroxyquinoline and saturated with

100 mM Tris-HCl, pH 8.0

Chloroform : isoamyl alcohol in the ratio 24:1.

The viral suspension was mixed with 10 volumes of solution A and 10 volumes of chilled absolute ethanol. The mixture was kept at -70°C for 2 hours and then centrifuged at 16,000 X *g* for 15 min at 4°C. The pellet was dissolved in 5 volumes of solution B and 5 volumes of chilled absolute ethanol, and again kept at -70°C for 2 hours. The mixture was centrifuged at 16,000 X *g* for 15 min at 4°C.

The pellet was dissolved in 2.5 volumes of solution C. An equal volume of phenol was added and the suspension centrifuged at 16,000 X *g* for 15 min at 20°C. An equal volume of phenol : chloroform : isoamyl alcohol in the ratio 25:24:1 was added to the recovered aqueous phase and the suspension recentrifuged under the same conditions. The aqueous phase obtained here was rendered free of any traces of phenol by centrifuging with an equal volume of chloroform : isoamyl alcohol.

RNA was precipitated from the aqueous phase with 0.1 volumes of 3 (M) sodium acetate, pH 5.2 and 2 volumes of absolute ethanol at -70°C overnight. The RNA precipitate was washed extensively with 70% ethanol to remove any traces of salts, vacuum-dried and dissolved in deionized water for subsequent studies. However, for storage, the pellet was kept in 70% ethanol at -70°C.

2.2.3 Separation of RNA in denaturing agarose gels

RNA molecules do not have hydrodynamically equivalent conformations in aqueous solutions, and therefore, several methods were developed in which molecular weights of RNA could be determined by gel electrophoresis under denaturing conditions (142). Formaldehyde (HCHO) was the first denaturant used for the electrophoretic analysis of RNA under denaturing conditions (143). Denaturation of certain GC-rich helical regions of some RNAs without appreciable degradation required the presence of formamide (142).

Reagents

Reagent A (gel electrophoresis buffer-10X stock)

0.5 (M) MOPS, pH 7.0

0.01 (M) EDTA, pH 7.5.

This solution was kept in dark at 4°C.

Reagent B

294 µl reagent A + 706 µl distilled water

Reagent C

89 µl HCHO (37%) + 706 µl formamide

Reagent D (gel loading buffer)

322 µl reagent B

5 mg xylene cyanol

5 mg bromophenol blue

400 mg sucrose

178 µl HCHO (37%)

500 µl formamide

Reagent E

0.5 mg/ml ethidium bromide

Gel preparation

1% agarose in 1X MOPS/EDTA

0.5 g agarose

5 ml Reagent A

36 ml distilled water

The above was heated to mix the agarose and cooled to 60°C. 9 ml of 37% HCHO was added making the final concentration of HCHO to 2.2 (M). This was mixed well and poured into a gel-casting mould.

Sample preparation

The RNA sample was dried under vacuum and was dissolved sequentially in the following manner:

2.2 µl Reagent B

4.8 µl Reagent C

1.0 µl Reagent E

The above was heated to 70°C for 10 min and quenched on ice. Reagent E (ethidium bromide) was added for better staining of low amounts of RNA without any significant background (144). 1.5 µl of Reagent D was added, mixed well and the sample loaded on the gel.

Electrophoresis

Electrophoresis was carried out in 1X MOPS/EDTA. Pre-electrophoresis was done at 60 V for 30 min. After loading the samples, electrophoresis was carried out at 60 V for 1 hour and then at 100 V for 1-2 hours. Halfway through the process, the electrophoresis buffer was remixed thoroughly and poured back into the electrophoresis unit. Any ionic imbalance at the electrodes was thereby taken care of. The gel was photographed over an ultra-violet transilluminator fitted with a Polaroid camera. Whenever necessary, the gel was prepared for blotting onto nitrocellulose membranes.

2.3 The Library

cDNA synthesis and cloning involves a complex series of enzymatic steps for the copying of mRNA into double-stranded cDNA, and subsequently preparing the termini for vector ligation (145). Many approaches have been used to generate cDNA libraries, and these have tried to preserve as much of the original sequence as possible to improve cloning efficiency and to facilitate screening and subsequent analysis (146).

2.3.1 cDNA synthesis

Potyviral RNA is of messenger polarity. The RNA isolated from the viral particles was taken as such and cDNA synthesized. Riboclone™ cDNA Synthesis System (Promega, USA) was used as per the manufacturer's instructions (145, 147).

First-strand synthesis

A 1:10 dilution of avian myeloblastosis virus reverse transcriptase (AMV-RT) was made in a dilution buffer of 10 mM potassium phosphate, pH 7.4, 2 mM DTT, 10% glycerol and 0.2% Triton X-100. An oligo(dT)-*Xba*I primer-adaptor was used to drive the first strand synthesis. The sequence of the primer-adaptor molecule was:

5' GTCGACTCTAGA$(dT)_{15}$ 3'.

0.5 µg primer per µg of mRNA was recommended. It was made up to 8 µl with distilled water. The solution was heated to 70°C for 5 min, cooled slowly to room temperature and centrifuged briefly.

The first-strand synthesis reaction was assembled at room temperature. The reaction conditions were:

50 mM Tris-HCl, pH 8.3

75 mM KCl

10 mM $MgCl_2$

0.5 mM spermidine

10 mM DTT

4 mM sodium pyrophosphate

1 mM each of dATP, dCTP, dGTP, dTTP

1 unit/μl RNasin ribonuclease inhibitor

0.5 μg primer/μg RNA

10 units AMV-RT/μg RNA

For the tracer reaction, 4 μl of the total reaction mixture was taken and 4 μCi of α-^{32}P-dATP was added. Both reactions were carried out at 14°C for 1 hour. The labeled reaction was stopped by adding 1 μl 0.2 (M) EDTA. The volume was made up to 100 μl with distilled water and stored at -20°C.

Second-strand synthesis

The volume of the second-strand synthesis reaction was made up in such a way that the final volume was 5 times the first-strand reaction. The final reaction conditions were:

50 mM Tris-HCl, pH 7.6

100 mM KCl

5 mM $MgCl_2$

0.1 mM NAD

10 mM ammonium sulphate

50 μg/ml BSA

0.2 mM each of dATP, dCTP, dGTP, dTTP

0.1 mM spermidine

0.8 mM sodium pyrophosphate

5 mM DTT

8 units/ml RNase H

230 units/ml *E. coli* DNA polymerase I

10 units/ml *E. coli* DNA ligase

For the tracer reaction, 10 µl of the reaction was taken and 4 µCi of α-^{32}P-dATP was added. Both the reactions were carried out at 14°C for 4 hours. The tracer reaction was stopped by adding 9 µl of 0.5 (M) EDTA and the volume was made up to 100 µl with distilled water. This was stored at -20°C.

The second strand reaction was heated at 70°C for 10 min, briefly centrifuged and placed on ice. T4 DNA polymerase at 2 units per µg of input RNA was added and the reaction carried out at 37°C for 10 min. The reaction was stopped with 10 µl of 0.2 (M) EDTA.

cDNA was extracted by adding an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and centrifuging briefly. The aqueous phase was collected and cDNA precipitated with 0.5 volumes of 7.5 (M) ammonium acetate and 2.5 volumes of chilled ethanol at -20°C for 1 hour. The precipitate was washed with chilled ethanol, dried and dissolved in 50 µl TE.

Both the labeled tracer reactions were also extracted with phenol : chloroform : isoamyl alcohol and precipitated with ammonium acetate and chilled ethanol. The precipitates were dissolved in 10 µl TE each.

Analysis of cDNA synthesis products by denaturing gel electrophoresis

The products of the cDNA synthesis reaction was analyzed on a 1.4% alkaline agarose gel cast in 50 mM NaCl, 1 mM EDTA. Electrophoresis was carried out in 30 mM NaOH, 1 mM EDTA (148, 149). The sample buffer contained 0.0125% bromophenol blue, 10 mM NaOH and 10% glycerol. λ DNA digested with *Hin*dIII was end-filled with Klenow fragment of *E. coli* DNA polymerase I, α-^{32}P-dATP, dCTP and dGTP (145). The reaction was carried out in the same buffer that is used for *Hin*dIII restriction reactions at room temperature for 10 min and was stopped with the addition of 0.1 volumes of 0.2 (M) EDTA. This was the molecular weight marker used to analyze the size of the cDNA synthesis products.

A pre-electrophoresis run was carried out at 20 V for 30 min. The labeled samples were loaded on the gel and electrophoresis carried out for 12 hours at 12 V. The gel was dried under vacuum and exposed on an X-ray film (Kodak) with intensifying screen at -70°C.

2.3.2 Ligation of *Eco*RI adaptors to cDNA

Phosphorylation of the 5'-OH end of the shorter oligomer of the Eco*RI adaptor*

Synthetic oligonucleotides have a 5'-OH group which has to be phosphorylated by the addition of the terminal γ-phosphate of ATP by T4 polynucleotide kinase (135). The shorter oligomer of the adaptors had a sequence:

5' GCGGCCGCACCTGCAATG 3'

The reaction conditions were:

3 μg single-stranded DNA (adaptor molecule)

1 mM ATP

7 mM Tris-HCl, pH 7.6

1 mM $MgCl_2$

0.5 mM DTT

0.8 units/μl T4 polynucleotide kinase.

Incubation was carried out for 15 min at 37°C, after which all the reaction components (except the DNA) were doubled to maintain identical conditions and incubation was continued at 37°C for 45 min.

The phosphorylated DNA was extracted with phenol : chloroform : isoamyl alcohol and precipitated with 0.1 volumes of 3(M) sodium acetate, pH 5.2 and 3 volumes of absolute ethanol at -70°C. The precipitate was washed with 70% ethanol and dried.

Annealing of Eco*RI adaptors*

The longer oligomer of *Eco*RI adaptors had the sequence:

5' AATTCATTGCAGGTGCGGCCGC 3'

This was added to the dried phosphorylated shorter oligomer. the volume was made up to 8 μl with distilled water. The mixture was heated to 70°C for 5 min and gradually cooled to room

temperature for efficient annealing of the adaptors. The annealed adaptors were centrifuged and stored at -20°C.

Ligation of annealed **Eco*RI* adaptors to cDNA**

The annealed *Eco*RI adaptors were ligated to the synthesized cDNA (147).

The reaction components were:

6 μg of annealed adaptors

appropriate volume of synthesized cDNA

30 mM Tris-HCl, pH 7.8

10 mM $MgCl_2$

10 mM DTT

1 mM ATP

0.1 mg/ml acetylated BSA

0.25 units/μl T4 DNA ligase

The reaction was carried out at 14°C for 16 hours. Ligase was heat-inactivated at 70°C for 10 min and the reaction was cooled on ice.

Phosphorylation of 5'-OH end of adaptor-ligated cDNA

The longer oligomer of the annealed adaptor had a 5'-OH group. This was phosphorylated under the same conditions with T4 polynucleotide kinase (147). After incubation at 37°C for 30 min, the adaptor-ligated, phosphorylated cDNA was extracted from the mixture with phenol : chloroform : isoamyl alcohol and precipitated with 0.1 volumes of 3 (M) sodium acetate, pH 5.2 and 3 volumes of absolute ethanol at -70°C for 2 hours. The precipitate was washed with 70% ethanol, dried and dissolved in distilled water and was stored at -20°C.

2.3.3 Ligation of cDNA to λgt11 vector and packaging of phage DNA

λgt11 vector is commonly used for immunological screening (135). DNA fragments up to 7.2 kb are cloned into the unique *Eco*RI site located in *lacZ*. The presence of a suppressor protein is required in the host for propagation and screening. In a non-suppressing host, amber mutations will prevent cell lysis and allow intra-cellular accumulation of the desired fusion protein. λgt11 can be grown in a recA$^-$ host. The temperature-sensitive repressor allows control of bacteriophage replication and production of fusion proteins (150).

1 μg of vector DNA (λgt11-*Eco*RI arms) was ligated to the insert DNA. Ligation conditions were as described earlier except that 0.5 units of T4 DNA ligase were used per μl of reaction volume and the reaction was carried out at room temperature for 3 hours. The ligated vector-insert DNA was packaged into Packagene™ (Promega, USA) λ DNA packaging system (147). Packaging was carried out at 22°C, for 2 hours. The packaged phage was diluted in phage buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$) and a drop of chloroform added to it. This was mixed by gentle inversion and the chloroform was allowed to settle down. The packaged phage was stored at 4°C.

Titration of packaged phage

Titration was carried out in *E. coli* Y1090 which was grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) supplemented with 0.2% maltose and 10 mM $MgSO_4$. Y1090 cells were maintained on LB $Amp^{50}Tet^{25}$. The packaged phage was diluted in phage buffer and 100 μl of each dilution was adsorbed onto 100 μl of overnight grown Y1090 cells at 37°C for 30 min. The following were added to molten top agarose:

350 μg/ml X-gal

140 μg/ml IPTG

10 mM $MgSO_4$.

3 ml of molten top agarose was added to each phage dilution, mixed well and spread on LB agar plates. These plates were incubated at 37°C overnight and were observed for the appearance of blue and white plaques.

Amplification of the λgt11 cDNA library

The entire packaged mixture was adsorbed onto *E. coli* Y1090 cells for 20 min at 37°C and this was then mixed with molten top agarose supplemented with 10 mM $MgSO_4$. The mixture was poured on LB agar plates and incubated at 42°C for 12 hours. Plates were overlaid with 15 ml of SM buffer (0.1% gelatin in phage buffer) and shaken at 4°C overnight (135).

The SM buffer was harvested and centrifuged at 7,000 X *g* for 30 min at 4°C. The supernatant was stored in small aliquots with either chloroform at 4°C or 7% dimethyl sulphoxide (DMSO) at -70°C. The amplified phage was titrated again after appropriate dilutions in phage buffer for the appearance of blue and white plaques.

2.3.4 Immunological screening of λgt11 cDNA library for SCMV-CP clones

Appropriate dilutions of the amplified phage in phage buffer were made and adsorbed onto *E. coli* Y1090 cells and mixed with molten top agarose supplemented with 10 mM $MgSO_4$ (135, 147, 151). The mixture was poured on LB agar plates and incubated at 42°C for 3 hours 30 min. A nitrocellulose disc was impregnated with 10 mM IPTG by soaking the disc in the solution for 10 min and gently blotting it dry. This disc was overlaid on the plate. This was incubated for another 4 hours at 37°C. Positions were marked asymmetrically using a sterile needle. The filter was removed and rinsed in TBST. The petri dish was stored at 4°C for later analysis.

Non-specific protein binding sites were saturated by incubating the membrane in the blocking solution (1% BSA in TBST) for 30 min. The blocking solution was replaced by an appropriate dilution of the SCMV antiserum (1:800 in TBST) and incubation was carried out for 45 min. The membrane was washed with TBST thrice to remove unbound antibodies. Each wash was carried out for 15 min.

The membrane was transferred to TBST containing appropriate dilution (1:8000 in TBST) of the secondary antibody. The incubation was allowed to go on for 45 in for binding of the secondary antibody to rabbit IgG. The membrane was again washed thrice with TBST to remove unbound antibodies. Each wash was carried out for 15 min.

The membrane was blotted dry and transferred to colour development solution (10 ml AP Buffer, 66 µl NBT, 33 µl BCIP). Once reactive areas turned purple (within a maximum of 15 min), colour development was stopped and the membrane rinsed thoroughly in deionized water. The membrane was either kept moist and photographed or air-dried and stored for record.

The positive signals on the membrane were traced onto a transparency sheet which was aligned with the master plate. Positive plaques were plugged out and transferred to 1 ml of SM buffer with 30 µl of chloroform. The phage was allowed to elute into the SM buffer at 4°C overnight. The eluent from each plug was centrifuged and stored either in chloroform at 4°C or in 7% DMSO at -70°C.

The eluents were diluted again in phage buffer, re-plated and screened for immunopositive plaques for the second time. The positive plaques from the secondary screening were plugged out, phage particles eluted and amplified for further experiments.

2.3.5 Isolation and analysis of recombinant λ DNA

The plaque eluent was diluted in phage buffer and adsorbed onto *E. coli* Y1090 cells at 37°C for 45 min (135). This was added to 10 ml of LB supplemented with 10 mM $MgSO_4$ and pre-warmed at 37°C. This was incubated at 37°C for 6 hours. 100 µl of chloroform was added and shaken for 5 min at 37°C to lyse all unlysed cells.

Cellular debris were centrifuged down. Cellular nucleic acids in the supernatant were degraded with 1µg/ml RNase A and 1 µg/ml DNase I at 37°C for 30 min. An equal volume of 20 mM Tris-HCl, pH 7.4, 2.1 (M) NaCl, 10 mM $MgSO_4$, 20% polyethylene glycol 8,000 was added to the solution. The mixture was incubated at 0°C (ice-water) for 1 hour and then centrifuged at 10,000 X *g* for 20 min at 4oC. The pellet was resuspended in SM buffer (5% of the original lysate) and DNA was extracted with buffered phenol. The λ DNA was

precipitated with an equal volume of isopropanol at -70°C for 20 min. The precipitate was dissolved in distilled water.

The recombinant λgt11 DNA was digested with the restriction enzyme *Eco*RI to release the insert (135). Digestion was carried out at 37°C for 3 hours. The reaction products were analysed by electrophoresis in 0.8% agarose gels in 1X TBE, followed by staining with 0.5 μg/ml ethidium bromide and observed over an ultra-violet transilluminator.

2.4 Amplification by Polymerase Chain Reaction

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of a known sequence (135). Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a thermostable DNA polymerase. These oligonucleotides typically have different sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified. The template is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then allowed to cool down to a temperature that facilitates the annealing of the oligonucleotide primers to their target sequences, after which the annealed primers are extended with the DNA polymerase. The cycle of denaturation, annealing and DNA synthesis is repeated many times. The products of one round of amplification serve as templates for the next. Each successive cycle, therefore, essentially doubles the amount of the desired DNA product. The major product of this exponential reaction is a segment of double-stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the two primers (135).

2.4.1 Screening of λgt11 library by PCR

cDNA inserts from individual bacteriophage clones can be enzymatically amplified using PCR (152) and this technique can be directly adapted to obtain specific target cDNA directly from a λgt11 library (153, 154). PCR can be effectively used to screen cDNA libraries for the presence of specific clones.

Primers for the PCR were designed (155) on the basis of the sequence data of the coat protein gene of SCMV-SC, a strain of SCMV infecting sugarcane in Australia (93). The 5' upstream primer covered the start of the CP coding region. It was designated as VCPP1 and its sequence was:

5' d(TCCACCAA***GCT***GGAACAGTC) 3'

The codon GCT codes for alanine which is the N-terminal amino acid of the coat protein. The 3' downstream primer covered the stop signal of the CP coding region. It was designated as VCPP2 and its sequence was:

5' d(GA***CTA***GTGGTGCTGCTGCAC) 3'

CTA is the anticodon for the stop signal UAG in the SCMV-SC reading frame.

The λgt11 library was aliquoted into appropriate dilutions in distilled water and the phage was disrupted to release the DNA by heating at 70°C for 5 min. The disrupted phage was immediately chilled on ice. The components of the reaction were:

10 mM TAPS, pH 8.8

50 mM KCl

1.5 mM $MgCl_2$

0.01% gelatin

100 pmol each primer

200 μM each dNTP

3 units *Taq* DNA polymerase.

Before adding the enzyme, the reaction mixture was heated to 94°C to inactivate any possible inherent proteases in the library which could act on the *Taq* DNA polymerase.

The reaction mixture was overlaid with mineral oil and thermal cycling for 30 cycles was commenced. The cycling conditions were:

(i) denaturation at 94°C for 1 min 20 sec,

(ii) annealing at 37°C for 2 min 20 sec,

(iii) extension at 72°C for 3 min.

A final extension step at 72°C for 7 min was added. The reaction products were analyzed by electrophoresis on a 1% agarose gel in 1X TBE.

2.4.2 Analysis of immunopositive λgt11 plaques by PCR

It was reasoned that the same principle underlying the screening of λgt11 libraries by PCR (153, 154) could be applied to go for a tertiary screening of the immunopositive plaques obtained in the secondary screening. The immunopositive plaque eluent was diluted in distilled water and heated to decapsidate the phage and release the DNA. PCR was carried out in the same manner as the λgt11 library, except that this time 2 sets of reactions were carried out with different annealing temperatures.

In the first set, the annealing temperature was 37°C, as in the reactions for the λgt11 library. In the second set, the annealing temperature was raised to 52°C and the reaction was repeated because the degree of specificity of PCR is in part a function of the temperature at which the primers are annealed.

2.4.3 Amplification of viral RNA by reverse transcriptase-PCR

PCR methodology has been adapted to generate cDNA fragments for specific sequences and amplify them (156). In this method, the first strand synthesized by reverse transcriptase using mRNA as a template serves as a template for the second strand synthesis. The two strands then undergo the routine exponential amplification reaction.

First strand synthesis by reverse transcriptase

The viral RNA was heated to 94°C for 5 min and snap-chilled on ice to denature all possible secondary structures. The first-strand reaction was driven by 0.5 μg oligo(dT)-*Xba*I primer-adaptor (Riboclone™, Promega, USA). This was added to the RNA, heated to 70°C

for 5 min and slowly cooled to room temperature to allow annealing to occur. AMV-RT was diluted 10-fold and the RT reaction was carried out in the PCR buffer.

The reaction components were:

Viral RNA

10 mM TAPS, pH 8.8

50 mM KCl

1.5 mM $MgCl_2$

0.01% gelatin

1 unit/μl RNasin ribonuclease inhibitor

10 units AMV-RT

0.5 μg oligo (dT)-*Xba*I primer

This mixture was kept at room temperature for 10 min. The reaction was carried out at 42°C for 3 hours and was stopped by heating at 94°C for 5 min to denature the RNA-cDNA hybrid and to inactivate the enzyme. The mixture was snap-chilled on ice.

PCR of the first strand reaction product

The RT reaction was diluted 5-fold and the PCR primers were added to it. The final reaction components were:

10 mM TAPS, pH 8.8

50 mM KCl

1.5 mM $MgCl_2$

0.01% gelatin

100 pmol of each primer (VCPP1, VCPP2)

3 units Taq DNA polymerase.

The thermal cycling conditions were the same. Annealing of the primers to the template was carried out at 52°C. The amplified fragments were separated by electrophoresis on a 1% agarose gel in 1X TBE.

2.4.4 Elution of PCR-amplified fragments from agarose gels

The RT-PCR products were separated by electrophoresis on a preparative 1% agarose gel in 1X TAE. The desired fragment was sliced out after observation on a UV transilluminator. The principle of binding of DNA to glass (157) was used to purify the DNA fragment from the agarose matrix.

Reagents

(i) Sodium iodide solution

6 (M) sodium iodide

0.12(M) sodium sulphite

(ii) Silica suspension

Silicon dioxide powder in TE buffer in the proportion 1:5 (w/v)

(iii) Washing solution

10 mM Tris-HCl, pH 7.4

0.5 mM EDTA

50 mM NaCl

50% ethanol

2.5-3 volumes of chilled sodium iodide solution was added to the agarose piece and incubated at 55°C for about 20 min to dissolve the agarose completely. The suspension was kept on ice and 1-2 µl of silica suspension per µg of expected DNA was added to it. The suspension was kept on ice for 30 min with intermittent mixing by tapping. The suspension was centrifuged and the supernatant discarded. The silica-DNA complex was washed with

500 µl of washing solution thrice. The DNA was eluted out of the complex by incubating it at 55°C for 5 min in distilled water. The mixture was centrifuged and the supernatant collected. This step was repeated to extract the maximum amount of DNA from the complex. The purity of the eluted product was checked by gel electrophoresis. The eluted product was re-amplified under identical conditions to confirm the specificity of the primers for this fragment of amplified DNA.

2.4.5 Northern hybridization of viral RNA to RT-PCR product

Viral RNA was electrophoresed under denaturing conditions on a 1% agarose gel in 1X MOPS/EDTA with HCHO and formamide (135). The gel was washed in several changes of distilled water to remove HCHO. The gel was soaked in 0.05 (N) NaOH for 20 min and then rinsed with water. The gel was soaked in 20X SSC for 20 min (135). The RNA was transferred from the gel to the nitrocellulose membrane by capillary transfer (135). Blotting was carried out using 20X SSC as the medium of transfer through a Whatman 3MM filter paper wick. The membrane was saturated in 20X SSC and placed on the gel. A stack of filter paper towels were placed on the gel and was compressed using a weight. The transfer was allowed to go on for 24 hours.

Positions of the wells were marked on the nitrocellulose membrane. It was peeled off from the gel. A UV torch was used to observe the efficiency of transfer on both the gel as well as the membrane. The membrane was saturated with 6X SSC for 5 min and air-dried for 30 min. The nucleic acid was immobilized on the membrane by vacuum-baking at 80°C for 2 hours.

The membrane was equilibrated in the hybridization buffer at 42°C for 1 hour.

Hybridization buffer

50% formamide

0.5% BSA

1% SDS

5X SSPE

Random primer labeling of DNA

The eluted RT-PCR product was labeled (147) with α-^{32}P-dCTP using the Prime-a-Gene™ System (Promega, USA) which is based on the method of random priming labeling of DNA (158, 159). A mixture of random hexanucleotides is used to prime DNA synthesis *in vitro* from any linear double-stranded template DNA (17). It is possible to generate probes of extremely high specific activity. The DNA was denatured by boiling for 2 min and snap-chilled on ice. The components of the priming reaction were:

Denatured DNA

50 mM Tris-HCl, pH 8.0

5 mM $MgCl_2$

2 mM DTT

200 μM HEPES, pH 6.6

5.2 A_{260} units/ml random hexadeoxyribonucletides

333.33 mM each of dATP, dGTP, dTTP

50 μCi α-^{32}PdCTP

20 μg acetylated BSA

5 Units Klenow fragment of *E.coli* DNA polymerase I.

The priming reaction was carried out at room temperature for 1 hour and was stopped by boiling for 2 min. It was snap-chilled on ice and EDTA was added to a final concentration of 20 μM. The labeled probe was added directly to the hybridization buffer in which the membrane was being equilibrated. Hybridization was carried out at 42°C for 16 hours.

The membrane was washed (135) in the following sequence:

(i) Two washes in 2X SSPE, 0.1% SDS for 10 min each at room temperature.

(ii) One wash in 0.1X SSPE, 0.1% SDS for 10 min at room temperature.

(iii) Two washes in 0.1X SSPE, 0.1% SDS for 30 min each at 42°C.

The membrane was checked for radioactive counts with a Geiger-Muller counter and wrapped in Saran Wrap.It was exposed to an X-ray film (XAR; Kodak, USA) with intensifying screen at -70°C.

2.4.6 Cloning of PCR amplified product in plasmid vectors

*Cloning in pMOS*Blue *T-vector*

The template-independent activity of thermostable DNA polymerases preferentially adds a single adenosine residue to the 3' end of *in vitro* amplified double-stranded DNA (160). Such PCR products can then be inserted into compatible thymidine-tailed vectors (161, 162). The pMOS*Blue* T-vector (Amersham, UK) has been specifically constructed for this application. A thymidine residue was added to *Eco*RV site in the multiple cloning region.

The eluted RT-PCR product was ligated into the pMOS*Blue* T-vector. The reaction components were:

RT-PCR product

50 ng vector DNA

30 mM Tris-HCl, pH 7.8

10 mM $MgCl_2$

5 mM DTT

0.5 mM ATP

2-3 Weiss units T4 DNA ligase.

The ligation reaction was carried out at 16°C overnight.

Transformation of ligated plasmid DNA into MOS*Blue *cells

Competent MOS*Blue* cells (Amersham, UK) were thawn and mixed well with an appropriate aliquot of the ligation reaction. The tubes were left on ice for 30 min. A heat shock at 42°C for exactly 40 sec was given after which the tubes were placed on ice for 2 min. 80 µl of LB medium was added and the cells were allowed to recover at 37°C for 1 hour.

The cells were then plated on LB-agar amp^{50}tet^{15} -IPTG-X-Gal plates and incubated at 37°C overnight.

Screening for recombinants

Positive colonies were picked after screening by blue-white selection (disruption of *lacZ* gene). Each isolated colony was grown in 10 ml LB-amp^{50}tet^{15} overnight. Plasmid DNA was isolated by the alkaline lysis method (135). Cells were pelleted down by centrifugation and resuspended in TEG buffer. This was chilled on ice. 2 volumes of 0.2 (M) NaOH, 1% SDS was added and mixed well to allow the lysis of cells to occur. 1.5 volumes of 3 (M) potassium acetate, pH 4.8 was added and mixed well. The mixture was centrifuged at 16,000 X *g* for 15 min at 4°C and the supernatant carefully collected. Plasmid DNA was precipitated out slowly with an equal volume of isopropanol at 4°C for 4-6 hours. The precipitate was washed extensively with 70% ethanol, dried and dissolved. The DNA was digested with *Eco*RI and *Hin*dIII, two unique sites flanking the site of insertion in the multiple cloning site of the vector, at 37°C for 3 hours. pMOS*Blue* T-vector plasmids that contained the RT-PCR product were designated as the pMTV series.

The colonies were also screened for recombinants directly by PCR. A colony was picked and diluted in distilled water. The solution was boiled for 5 min to rupture the cells and inactivate the DNases. The mixture was centrifuged and an aliquot of the supernatant was taken for PCR. The reaction was carried out in an identical manner as for the λgt11 library, except that the annealing was carried out at 52°C. The products of the restriction digestion and colony-PCR were analyzed by electrophoresis on a 1% agarose gel in 1X TBE.

Cloning in pGEM3Z

The insert released from the pMOS*Blue* T-vector after digestion with *Eco*RI and *Hin*dIII was cloned into pGEM3Z (Promega, USA) which had been linearized by digestion with the two enzymes to generate appropriate cohesive ends(147). Ligation was carried out and the product was transformed into fresh, competent *E.coli* JM109 cells (135). The cells were plated on LB-agar amp^{100} plates with IPTG and X-gal. Positive colonies were screened by blue-white selection. Cells from individual colonies were grown in 10 ml LB-amp^{100}, plasmid DNA prepared (135) and digested with *Eco*RI and *Hin*dIII to release the insert. The

reaction product was analyzed by electrophoresis on a 1% agarose gel in 1X TBE. pGEM3Z plasmids that contained the *Eco*RI-*Hin*dIII fragment excised from the pMTV series were designated as the pZTV series.

2.5 Sequencing of Amplified DNA

The enzymatic method of DNA sequencing (163) generates separate populations of radiolabeled oligonucleotides that begin from a fixed point and terminate randomly at a fixed residue. Every base in the DNA has an equal chance of being the variable terminus. Each population consists of a mixture of oligonucleotides whose lengths are determined by the location of a particular base along the length of the original DNA. These populations of oligonucleotides are then resolved by electrophoresis under conditions that can discriminate between individual DNAs that differ in length by as little as one nucleotide. When these are loaded into adjacent lanes of a sequencing gel, the order of nucleotides can be read directly from an autoradiographic image of the gel (135).

In this method, a specific primer is used for extension by the DNA polymerase. Base-specific chain termination occurs as a result of the presence of dideoxynucleoside triphosphates (ddNTPs). 2',3'-ddNTPs differ from conventional dNTPs in that they lack a hydroxyl (-OH) residue at the 3' position of the deoxyribose. They can be incorporated by DNA polymerases into a growing DNA chain through their 5' triphosphate groups. However, the absence of a 3'-OH residue prevents the formation of a phosphodiester bond with the succeeding dNTP. Further extension of the DNA chain is therefore impossible. Thus, when a small amount of one ddNTP is included with the conventional dNTPs in a reaction mixture for DNA synthesis, there is a competition between extension of the chain and infrequent, but specific, termination. The products of the reaction are a series of oligonucleotide chains whose lengths are determined by the distance between the terminus of the primer used to initiate DNA synthesis and the sites of premature termination. By using four different ddNTPs in four separate enzymatic reactions, populations of oligonucleotides are generated that terminate at positions occupied by every G, A, T or C in the template strand.

2.5.1 Purification of plasmid DNA

Purified, RNA-free, supercoiled plasmid DNA must be used for sequencing. Plasmid DNA isolated from large scale (50 ml or 100 ml) cultures was treated with RNase A at a final concentration of 200 ng/μl at 37°C for 2 hours (135). DNA was extracted using phenol : chloroform : isoamyl alcohol and precipitated from the aqueous phase with 0.1 volumes of 3 (M) sodium acetate, pH 5.2 and 2 volumes of absolute ethanol at -20°C overnight. The precipitate was washed extensively with 70% ethanol, dried and dissolved in deionized water.

An equal volume of 13% PEG 8,000, 1.6 (M) NaCl was added on ice to the dissolved DNA to selectively precipitate out the supercoiled plasmid (135). The mixture was immediately centrifuged at 10,000 X *g* for 10 min at 4°C and the precipitate vacuum-dried and redissolved in deionized water. DNA was re-extracted with phenol : chloroform : isoamyl alcohol to remove any traces of PEG 8,000 from it. It was precipitated again with sodium acetate and ethanol. The precipitate was washed extensively with 70% ethanol to remove salts and any minute traces of PEG 8,000, residues of which could interfere with the sequencing reactions. Purity of the product was checked on an agarose gel.

2.5.2 Sequencing reaction using Sequenase™ Version 2.0

The methods described in the product instruction manual (US Biochemical, USA) was followed (164).

Denaturation of plasmid DNA

Plasmid DNA was denatured in the presence of 200 mM NaOH, 200 μM EDTA, at 37°C for 30 min. This was neutralized with 0.1 volumes of 3 (M) sodium acetate, pH 5.2 and the DNA was precipitated with 2-4 volumes of absolute ethanol at -70°C for 15 min. The precipitate was dried after washing extensively with 70% ethanol.

Annealing of primers to DNA

The pMTV series were plasmids derived from pMOS*Blue* T-vector which has T7 and U19 promoters. The pZTV series were derived from pGEM3Z vector which has SP6 and T7 promoters. Sequencing reactions were driven with only the T7 primer in case of the pMTV

plasmids as template whereas in case of the pZTV plasmids, both SP6 and T7 primers were used to drive the reaction.

The dried, supercoiled plasmid DNA was dissolved in deionized water and the volume was made up to 10 µl in 1X Sequenase buffer. The components of the solution were:

Primer : template in a 1:1 molar stoichiometry

40 mM Tris-HCl, pH 7.5

20 mM $MgCl_2$

50 mM NaCl.

A small excess of template and primer could possibly give better results. Therefore, the concentrations of both the primer as well as the template was adjusted accordingly. Annealing was carried out at 37°C for 30 min after which the annealed template-primer complex was brought to room temperature. It was imperative to use this complex within four hours.

Labeling reaction

The 5X Labeling Mix was diluted 2.5 fold. The final concentrations of the components were 2.5 µM each of dGTP, dCTP and dTTP. Reading of sequences close to the primer necessitated the dilution of the labeling mix 10 fold.

Sequenase™ Version 2.0 (US Biochemical, USA) is a genetic variant of the bacteriophage T7 DNA polymerase created by *in vitro* manipulation where the 3'-5' exonuclease activity of the wild-type enzyme is completely removed (165). The enzyme was diluted 8 fold in ice-cold 10 mM Tris-HCl, pH 7.5, 5 mM DTT and 0.5 mg/ml BSA. 3.25 units of Sequenase™ Version 2.0 was used per reaction. Pyrophosphatase was added (0.00125 units) to inhibit the slow, sequence-dependent reversal of the DNA polymerase reaction by pyrophosphorolysis.

The following reaction mixture was assembled on ice:

Annealed template : primer	10 µl
0.1 (M) DTT	1µl
Diluted labeling mix	2µl
α-^{35}S-dATP	0.5 µl (5 µCi)
Diluted Sequenase™ Version 2.0	1 µl

The total volume of the mixture was 15.5 µl. For reactions that required reading of sequences close to the primer, 1 µl of Mn^{2+} buffer (0.15 (M) sodium isocitrate, 0.1 (M) $MnCl_2$) was added. The labeling reaction was carried out for 2-3 min at 20°C and the extension-termination rapidly carried out.

Extension -termination of sequencing reactions

Four tubes labeled G, A, T and C were kept ready. 2.5 µl of the termination mix for each nucleotide was transferred and pre-warmed at 37°C. Each termination mix consisted of:

80 µM each of dGTP, dATP, dTTP and dCTP

8 µM of the respective ddNTP (ddGTP, ddATP, ddTTP or ddCTP)

50 mM NaCl.

Extension-termination was carried out rapidly by transferring 3.5 µl of the labeling reaction to the appropriate termination mix and incubating at 37°C for 5 min. The reaction was stopped by the addition of 4 µl of stop solution that contained 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. Samples labelled with ^{35}S was stored up to one week at -20°C till electrophoresis was carried out.

2.5.3 Denaturing polyacrylamide gel elctrophoresis of sequencing reaction products

Under optimal conditions, 300 or more bases can be read starting at the bottom of a gel. Factors affecting the resolution of a sequence include the quality of reagents used, the polymerization, the temperature of the gel during electrophoresis and proper drying of the gel after electrophoresis.

Casting the gel

The gel was cast sandwiched between a water-jacketed thermostatic plate and a notched plate on a gel platform using 0.4 mm spacers. The notched plate was made hydrophilic by coating with Bind-Silane (Pharmacia LKB, Sweden) to enable the binding of the polyacrylamide gel to the glass. The thermostatic plate was coated with Repel-Silane (Pharmacia LKB, Sweden) to make the surface hydrophobic.

100 ml of the polyacrylamide solution contained:

1X TBE

6% Acrylamide

0.3% *N,N'*-methylene-*bis*-acrylamide

7 (M) Urea

75 µl TEMED

700 µl 10% ammonium persulphate.

Ammonium persulphate solution was always prepared fresh.

Electrophoresis

The gel sandwich was fixed on to the Macrophor System (Pharmacia LKB, Sweden). A shark-tooth comb was placed and the wells were thoroughly flushed with 1X TBE. Pre-electrophoresis in 1X TBE at 2,000 Volts, 55°C for 45 min was carried out. Samples were denatured by heating at 80°C for 5 min and snap-chilled. 3.5 µl of each sample was loaded on one of the four adjacent wells marked G, A, T and C. Electrophoresis was carried out for a fixed period, depending on the region of the sequence to be read. Usually this was for either

1.5 hours or 2 hours. Subsequent loads of samples were made at fixed intervals of time after preparing them in the way described above. This ensured reading of different regions of the same sequence in the different loads and finally overlapping them after comparison.

Post-electrophoresis procedures

The gel, along with the glass plate, was washed in 15% methanol, 10% acetic acid for 1-2 hours for fixation and for the removal of urea. The gel was dried at 80°C for a minimum of 1-4 hours. Radioactive signals were checked using a hand-held Geiger-Muller counter. A sheet of X-ray film (XAR) (Kodak, USA) was placed, under safelight conditions, directly over the dried gel and sandwiched between two glass plates. This was wrapped in a black cloth and kept in dark. The time of exposure depended on the number of radioactive counts per second read by the Geiger-Muller counter after which the X-ray film was developed. The sequence was read and analyzed using the DNASIS software (Hitachi, Japan).

The White Queen laughed with delight, and stroked Alice's cheek. Then she began:

"` First the fish must be caught'
That is easy: a baby, I think could have caught it.
`Next, the fish must be bought'
That is easy: a penny, I think would have bought it.

"`Now cook me the fish!'
That is easy, and will not take more than a minute.
`Let it be in a dish!'
That is easy, because it already is in it.

"`Bring it here! Let me sup!'
It is easy to set such a dish on the table.
`Take the dish-cover up!'
Ah, that *is so hard that I fear I'm unable.*

"For it holds it like glue-
Holds the lid to the dish, while it lies in the middle:
Which is easiest to do,
Un-dish-cover the fish, or dish cover the riddle?"

Lewis Carroll
Through the Looking Glass and What Alice Found There

CHAPTER 3

RESULTS AND DISCUSSION

3.1 The Virus Particles

3.1.1 Symptoms seen on the infected leaves

Leaves of sugarcane (*Saccharum officinarum,* L., cv. CO 740 and cv. CO 8014) were harvested from the fields of College of Agriculture, Pune. Entire fields of the crop showed the characteristic yellow streaks observed during infection with sugarcane mosaic virus. The intensity of the mosaic symptoms was observed to increase with the age of the leaves as well as that of the plant. While the older leaves looked completely chlorotic (Fig. 1), the young emerging leaves were essentially uninfected.

3.1.2 The viral protein

The virus from the infected leaves of sugarcane was isolated as intact capsid. The virus yield was estimated by assaying the coat protein after several rounds of differential centrifugation (90) and using Bradford's method (133). The protein values varied from 75 μg to 135 μg of protein per gram of infected leaf material. Tender and greener leaves closer to the meristematic sheath gave lower yields. Yields from leaves from young, three-month old plants were also low. The yield obtained was 75.88 μg per gram of infected leaf material. Older leaves gave an average yield of 134.64 μg per gram of infected leaf material. In general, older leaves gave higher yields of coat protein and in turn, the virus particles than younger ones. The estimated protein yields were used to determine the amounts of coat protein to be used for RNA isolation from intact and virulent virus particles.

Silver-stained SDS-PAGE profiles of the coat protein from purified virus particles solubilized and denatured in presence of SDS showed several bands (Fig. 2). Molecular weight markers in the range 14 kDa to 66 kDa were loaded along with the protein preparations. Two intense bands were seen in the high molecular weight regions with apparent molecular mass of around 45 kDa and 50 kDa, while one such band was seen with an apparent molecular mass of about 24 kDa. Very faint bands are seen in the other regions. The possibility of host cell proteins contaminating the preparation was ruled out because the virus particles were sedimented and purified by repeated differential centrifugation.

A rabbit polyclonal antiserum to the N strain of SCMV was used to probe the denatured viral coat protein preparation fractionated by SDS-PAGE and followed by transfer and immobilization on nitrocellulose membranes. Immunoblot profiles (Fig. 3) showed a very intense band with the apparent molecular mass of about 24 kDa. A high molecular weight band of an apparent molecular mass of 45 kDa was also seen. This band was less intense. Faint low molecular weight bands were also observed.

Heterogeneity in apparent coat protein size is a common feature of potyviruses owing to degradation during purification and / or storage (8). These degraded forms move faster on SDS-polyacrylamide gels as specific bands (50, 51). From biochemical and genetic analysis, however, it is clear that each potyvirus contains only a single type of coat protein monomer (52). Coat protein monomers have been shown to range in size from 30 to 45 kDa (8). The CPs of four Australian strains of SCMV, JG, SC, BC and Sabi are reported to have molecular masses of 33.7, 34.2, 39.1 and 40.3 kDa (90). In the present study, the most intense signal obtained in the immunoblot analysis was in the range of about 24 kDa. Faint low molecular weight bands could possibly be the degraded forms that move faster on SDS-polyacrylamide gels (50, 51).

The serological relation of the viral preparation to the N strain of SCMV has been ascertained. The rabbit antiserum was raised against SCMV-N and its reactivity to the sap of infected sorghum lines by ELISA tested (P. Sreenivasulu and T. Satyanarayana, S.V. University, Tirupati; personal communication, 1993). The positive signal on the immunoblot confirms the presence of coat protein in the virus preparation. It also confirms that the formation of mosaic symptoms on infected sugarcane leaves is by a potyvirus. The serological relationship to SCMV-N gives reason to believe that the mosaic in our sugarcane fields is caused by SCMV.

3.1.3 Infectivity assay of the viral preparation

Any pathological isolate has to be checked for its viability and infectivity prior to performing any biochemical studies. The viral particles, isolated and purified from the field infected sugarcane leaves by differential centrifugation, were checked for their serological relationship to SCMV. However, it was necessary to check the viability and integrity of these viral particles, and also the symptoms caused.

Indicator hosts are used for infectivity assays. These are plants that show disease symptoms when physically inoculated with the viral preparations under quarantined conditions. In the present study, ten-day old seedlings of sorghum cv. M-35-1 were used as indicator hosts. The flag leaf of 50 seedlings was inoculated with SCMV preparation from infected sugarcane leaves, corresponding to about 300 ng of coat protein. The inoculated leaf developed chlorotic lesions by the third day. By the sixth day, the symptoms were also manifested on the first leaf which also curled up and was severely mottled (Fig. 4). By day nine, all the seedlings died due to progression of the disease and acute chlorosis. The infectivity test was repeated four times using 50 seedlings for each assay.

Both the serological results as well as the viability tests of the viral particles (from host reaction studies) gave us the scope to pursue further studies. Analysis of the purified and denatured protein on SDS-PAGE and immunoblot profiles substantiated the authenticity of SCMV coat protein. Coupled with these biochemical tests, the viability tests carried out using the purified viral preparations gave reason to believe that the virus particles had possibly retained their integrity and infectivity during the isolation and purification process.

Figure 1: Infected leaves of field grown sugarcane (*Saccharum officinarum,* L., cv. CO 740) displaying mosaic symptoms.

Figure 1

Figure 2: Silver stained SDS-PAGE profile.
Left to right: Lane 1- purified viral protein; Lane 2- Molecular weight marker (sizes from top to bottom are 66, 45, 36, 29, 24, 20.1 and 14.2 kDa)

Figure 3 Immunoblot of purified viral protein separated by SDS-PAGE against SCMV-N antiserum.

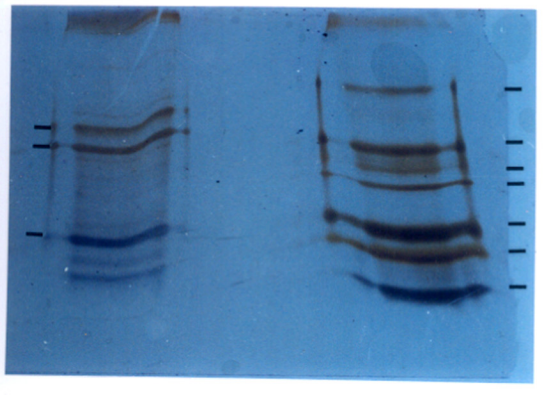

Figure 2

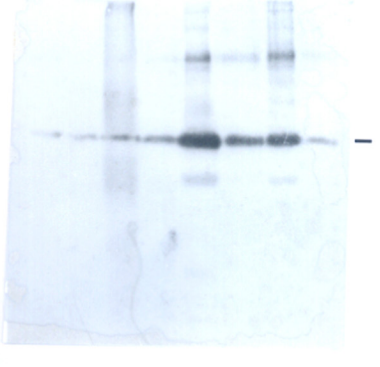

Figure 3

Figure 4 Infectivity assay on sorghum (cv. M-35-1) under quarantined conditions to test viability of purified viral preparation

(a, b) 13 day old uninfected seedlings (3 dpi)

(c, d) 13 day old infected seedlings (3 dpi) -site of infection and mosaic symptoms observed.

(e, f) 16 day old infected seedlings (6 dpi) - chronic infection and mottling of seedlings observed.

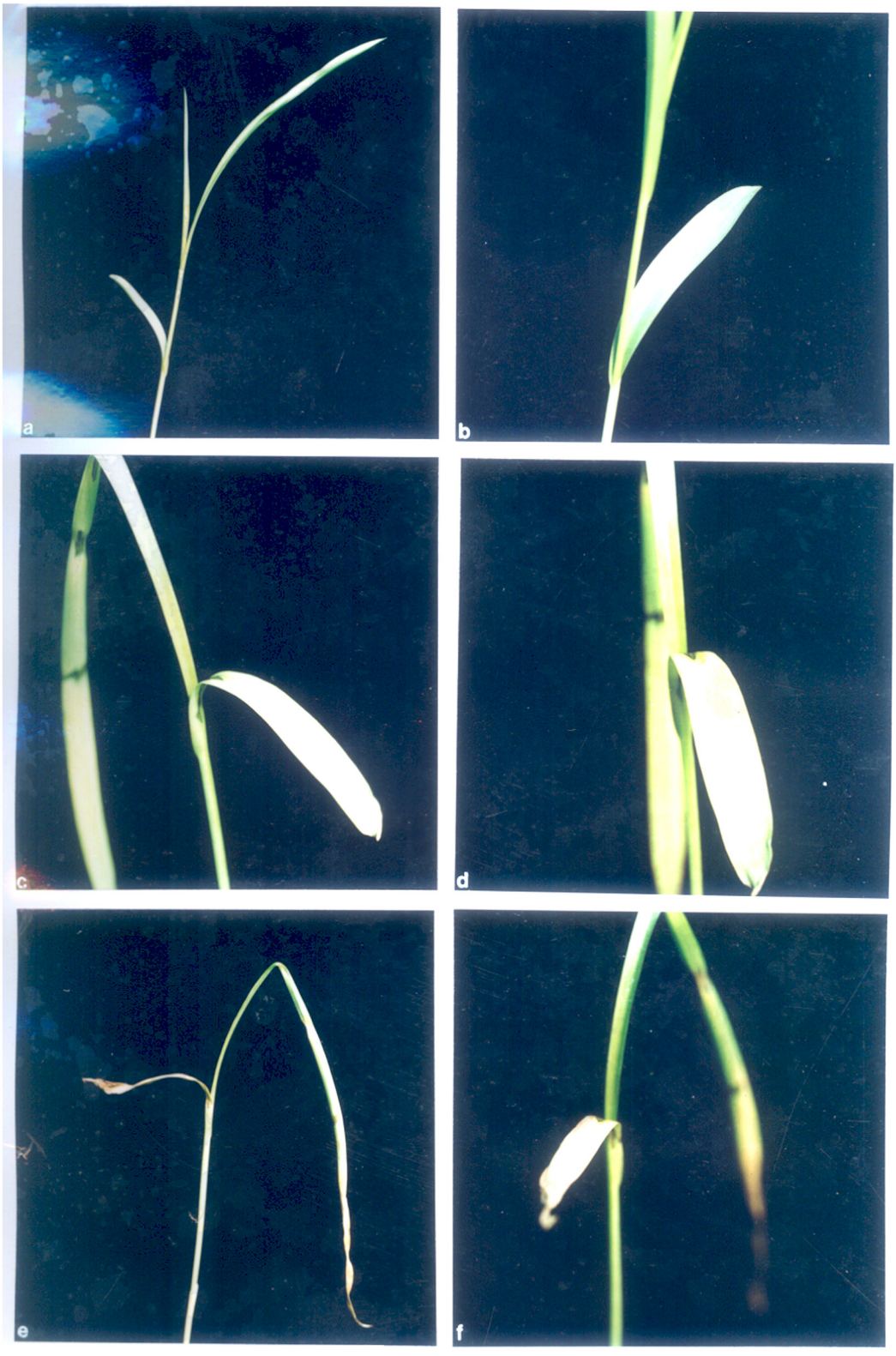

Figure 4

3.2 The Nucleic Acid

3.2.1 Viral RNA as seen in denaturing gels

Denaturing formaldehyde-formamide gels run in MOPS/EDTA showed fluorescence of RNA prepared from viral particles purified and isolated from infected sugarcane leaves (as described in Chapter 2, Experimental Procedures), and also tested for infectivity. BRL RNA Ladder (Gibco-BRL, USA), of sizes 0.24, 1.35, 2.37, 4.4, 7.46 and 9.49 kb, was loaded along the side of the sample. Fluorescence was observed as a smear in a region of ~ 12 to 0.3 kb (Fig. 5), although the bulk of the fluorescence was in the region of ~ 4.0 to 0.5 kb.

Potyviral genomic RNA is reported to be between 9.5 kb and 12 kb (15, 17, 29-31, 75-80). Eukaryotic RNA preparations show characteristic ribosomal RNA bands that were not seen in the present viral RNA preparation. Hence, the RNA preparation from the purified virus particles from infected sugarcane was assumed to be pure and free of contaminants. A smear was observed on the denaturing gel that could be possible because of ribonuclease contamination or as a consequence of shear.

3.2.2 cDNA synthesis

Since, potyviral RNA is of messenger polarity together with a poly (A) tail, the RNA isolated from the viral particles can be used as the template for cDNA synthesis. In the present study, Riboclone™ cDNA Synthesis System (Promega, USA) was used as per the manufacturer's instructions (145, 147) to synthesize cDNA from the RNA prepared from the virus particles isolated from infected sugarcane leaves.

Autoradiographs of the labeled second strand of the cDNA synthesized showed a smear from the region of 6.5 kb downwards (Fig. 6). λ DNA digested with *Hin*dIII and end-filled with α-^{32}P dATP in presence of Klenow fragment of *E. coli* DNA polymerase I was used as the molecular weight size marker. The SCMV RNA has a 3' untranslated region together with a poly (A) tail and just upstream of this region is the virus coat protein gene (93). Since the cDNA synthesis in the present work was driven using an oligo (dT) primer, the mixed population of double-stranded cDNAs would thus contain the CP coding region.

3.2.3 The library

λgt11 is an expression vector commonly used for immunological screening of cDNA libraries (Fig. 7) (135). DNA fragments upto 7.2 kb can be cloned into the unique *Eco*RI site located in *lacZ*. In the present study, the second strand cDNA inserts synthesized using the viral RNA as the template were ligated to the arms of λgt11 at the *Eco*RI sites and packaged. Titration was carried out on plates that contained IPTG and X-Gal. Packaged λgt11 phages gave a titre of 1.7 X 10^5 recombinant pfu/ml. The entire packaging mixture was amplified on LB-plates by infecting *E. coli* Y1090 cells. The amplified library was harvested and the titre obtained was 1.1 X 10^{14} recombinant pfu/ml. The ratio of clear to blue plaques was 2.2:1. Typical recombinant efficiencies of 5-9 pfu/μg of vector arms are achieved when using a positive control DNA (147).

Approximately, 22,000 pfu were plated for the primary screening. Fifty-three immunopositive plaques were picked up (Fig. 8a), phages eluted out of the plaque plugs and secondary screening carried out. One hundred pfu were plated for each secondary screening and 100% immunopositive plaques obtained (Fig. 8b).

λgt11 recombinant DNA was isolated and digested with the restriction endonuclease *Eco*RI to release the insert. The insert, however, could not be released from the DNA isolated from any of the immunopositive plaques (Fig. 9).

*Eco*RI sites flanking the inserts of λgt11 clones are reported to get lost due to modification, probably as a result of mutation during the process of insert ligation. This has been seen in case of clones with cDNA inserts of zucchini yellow mosaic virus (ZYMV), a potyvirus (166).

3.2.4 Amplification of potyviral sequences by PCR

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of a known sequence (135). Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a thermostable DNA polymerase. These oligonucleotides typically have different sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified. The major product of

this exponential reaction is a segment of double-stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers . The length of this segment of DNA is defined by the distance between the two primers (135).

Polymerase chain reaction offers several advantages compared to more traditional methods of diagnosis (167). As in serology, a wide range of selectivities is possible. Depending on the choice of primers, the method facilitates the detection of a specific pathogen or many members of a group of related pathogens. This may be considered as a valuable extension of the serological methods used in the identification of potyviruses (168).

λ-PCR

Releasing of inserts from recombinant λ DNA by polymerase chain reaction has been carried out using λgt11 specific sequencing primers (152). PCR methodology has been used to screen λgt11 libraries for specific clones using primers specific for the gene of interest (153, 154). In the present instance, the latter strategy was adopted to screen for the SCMV CP gene in the total λgt11 library raised from viral RNA purified from infected sugarcane leaves as well as the from immunopositive clones.

Primers for the PCR were designed (155) on the basis of available the sequence data of the coat protein gene of SCMV-SC (93). The 5' upstream primer covered the start of the CP coding region. It was designated as VCPP1 and its sequence was:

5' d(TCCACCAA***GCT***GGAACAGTC) 3'

The codon GCT codes for alanine which is the N-terminal amino acid of the coat protein. The 3' downstream primer covered the stop signal of the CP coding region. It was designated as VCPP2 and the sequence read:

5' d(GA***CTA***GTGGTGCTGCTGCAC) 3'

CTA is the anticodon for the stop signal UAG in the SCMV-SC reading frame.

The total crude lysate from the total λgt11 library was decapsidated by heat lysis to give the DNA template. At a primer annealing temperature of 37°C to the template DNA, the presence of several amplified bands was observed (Fig. 10, lanes 2 to 4). The approximate

sizes of the major amplified bands were 100 bp (also observed in the zero template control and attributed to amplification due to primer-dimer formation), 400 bp, 600 bp, 800 bp, 2 kbp and 3.5 kbp, in ascending order. The fact that so many bands were amplified may be attributed to the following reasons. First, the low temperature of annealing may lead to the non-specific annealing of primers to the template molecule. Second, the presence of host cell (*E. coli*) DNA in the phage lysate may provide additional templates for non-specific annealing of primers and subsequent amplification. The first report where PCR methodology had been applied for screening a total λgt11 library for a particular gene used an annealing temperature of 37°C (153).

The possibility for SCMV CP specific amplification by PCR using the immunopositive plaques from the secondary screening of the λgt11 library was considered. The immunopositive λgt11 clones constituted a part of the total λgt11 library. Therefore, it should also show the results observed above with the use of the same set of primers under identical conditions. The primers when annealed at 37°C to the template DNA from immunopositive λgt11 plaques showed amplification of only two bands. One band was larger than 564 bp and the other smaller (λ DNA digested with *Hin*dIII molecular weight marker) (Fig. 10, 11). The amplification of only two bands template DNA from immunopositive λgt11 confirmed the speculation that the observed high molecular weight bands were due to the presence of *E. coli* DNA in the phage pool. These bands were conspicuous by their absence when DNA from immunopositive plaques was used as the template (Fig. 11).

However, the possibility of non-specific annealing of the primers to the vector DNA at 37°C still remained. A predicted annealing temperature on the basis of the T_m and the GC content of the primers was calculated using the DNASIS software (Hitachi, Japan) and this was determined to be 52°C. Amplification of template DNA from SCMV CP immunopositive plaques at this annealing temperature gave the same results as observed earlier at an annealing temperature of 37°C (Fig. 12). This suggested that the amplified products were not spurious and had indeed a potyviral origin.

RT-PCR

PCR methodology has been adapted to generate cDNA fragments for specific sequences and to amplify them (156). In this method, the first strand synthesized by reverse

transcriptase using mRNA as the template serves as the template for the second strand synthesis. The two strands then undergo the routine exponential amplification reaction.

The same set of primers as were used for λ-PCR were also used for reverse transcriptase-PCR. Purified viral RNA from intact viruses was used as the template for first-strand cDNA synthesis and PCR. The annealing temperature was maintained at 52°C. Here again, amplification of two bands of the sizes as observed in λ-PCR were seen (Fig. 13a). The larger of the two bands, ~600 bp in size (estimated with the use of pUC18 digested with *Alu*I as the molecular weight size marker) was eluted out and used as a template for further amplification under identical conditions (Fig. 13b). The small band that was nearly ~400 bp in size was disregarded in all further studies, since the ~600 bp band came closest to the reported potyviral coat protein size.

A possibility that vector λ DNA could get amplified in the λ-PCR was speculated. This possibility was, however, eliminated since the amplified products in both the λ-PCR of template DNA from SCMV-CP immunopositive plaques and RT-PCR were of similar molecular weight. Also, the higher molecular bands observed in the PCR of the total library were absent in the PCR products of immunopositive plaques (Fig. 10), eliminating the possibility of any such non-specific amplification.

3.2.5 Northern hybridization of the amplified potyviral product with viral RNA

The relationship of the amplified product with RNA prepared from the purified viral preparations had to be established. RNA from viruses isolated from infected sugarcane leaves was electrophoresed under denaturing conditions on a 1% agarose gel in 1X MOPS/EDTA with HCHO and formamide (135). The RNA was transferred by capillary action onto a nitrocellulose membrane in 20X SSC using a Whatman 3MM filter paper as a wick.

The amplified product of ~600 bp was eluted out of an agarose gel, precipitated and used as a probe for hybridization with viral RNA. The amplified DNA was labeled with α-^{32}P dCTP by the method of random priming (147). Labeled DNA was hybridized at 42°C to the viral RNA immobilized on the nitrocellulose membrane. Intense signals were seen in the regions above 9.5 kb upto ~12 kb (Fig. 14). No low molecular weight fluorescent smear was seen in the gel. Consequently, no low molecular weight hybridization signals were seen

either. No hybridization signals were seen with total RNA from tissue-cultured sugarcane leaves used as the uninfected control. Leaves from tissue-cultured sugarcane plant were used since it was not possible to get unambiguously uninfected leaves from field-grown plants.

Potyviral genomic RNA is reported to be between 9.5 kb and 12 kb (15, 17, 29-31, 75-80). Eukaryotic RNA preparations show characteristic ribosomal RNA bands and these were not seen in the viral RNA preparation. Therefore, the RNA preparation from the viral particles was assumed to be pure and free of contaminants. The genome of the sugarcane-infecting potyvirus reported in this study can thus be assigned a size of ~9.5 to 12 kb.

From the above results, it is concluded that the ~600 bp amplified fragment both from λ-PCR as well as the RT-PCR is specific for the potyviral RNA from the infected leaves of sugarcane. The speculation that λ DNA could get amplified in the λ-PCR was once again eliminated.

The fact that the amplified ~600 bp product came from the λgt 11 clones that gave immunopositive reaction to antibodies raised against the coat protein of the N strain of SCMV gives credence to the assumption that the cDNA insert from these clones was indeed the SCMV coat protein gene. This was substantiated by the amplification of the same ~600 bp DNA fragment when RT-PCR was performed using the viral RNA as the template.

Figure 5 Denaturing formaldehyde agarose gel electrophoresis of RNA in 1X MOPS-EDTA
Left to right
Lane 1- RNA isolated from purified viral protein,
Lane 2- Molecular weight marker, RNA ladder (BRL, USA). Sizes from top to bottom are 9.49, 7.46, 4.4, 2.37, 1.35 and 0.24 kb

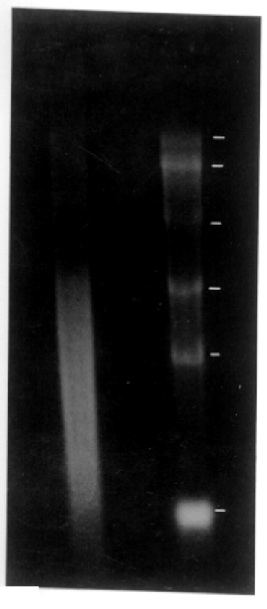

Figure 5

Figure 6 Autoradiogram of first and second strand cDNA synthesis tracer reactions(labeled with α^{32}P-dATP) electrophoresed on 1.4% alkaline agarose gel
Left to right
Lane 1- First strand reaction
Lane 2- Second strand reaction (synthesized smear begins between 9.4 and 4.3 kbp and goes down to 564 bp)
Lane 3-Molecular weight marker (λ DNA digested with *Hin*dIII, end filled with Klenow fragment of *E. coli* DNA polymerase I and α^{32}P-dATP). Sizes from top to bottom are, 23.1, 9.49, 6.55, 4.3 , 2.3, 2.0. 0.564 and 0.125 kbp.

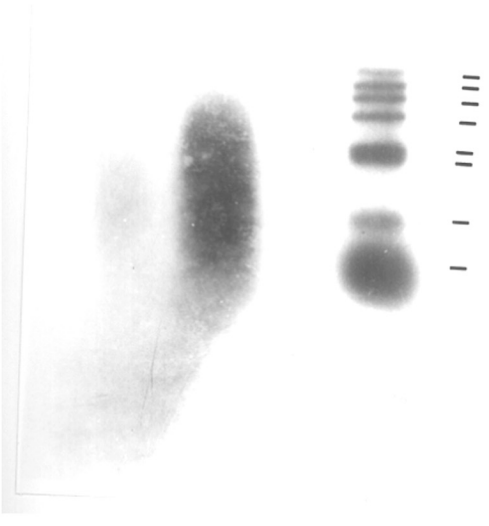

Figure 6

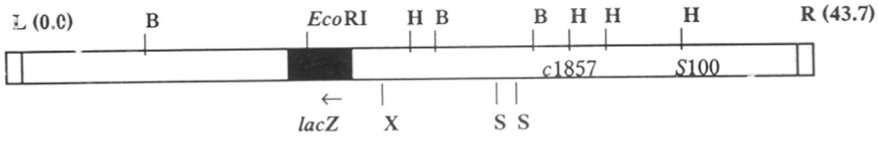

Figure 7 Structural map of the λgt11 vector.
L- left end (0.0 bp), R- right end (43.7 kbp). The site of insertion is at *Eco*RI (19.6 kbp),upstream from the β-galactosidase translation termination codon. Restriction sites are, B- *Bam*HI, H- *Hin*dIII, S- *Sal*I, X- *Xba*I. The direction of transcription of the *lacZ* gene is indicated by the arrow. *c*1857 is the temperature-sensitive *c*I repressor and *S*100, the amber mutation.

Figure 8 Immunoscreening of λgt11-SCMV cDNA library with rabbit polyclonal antiserum to SCMV-N

(a) Primary screening

(b) Secondary screening

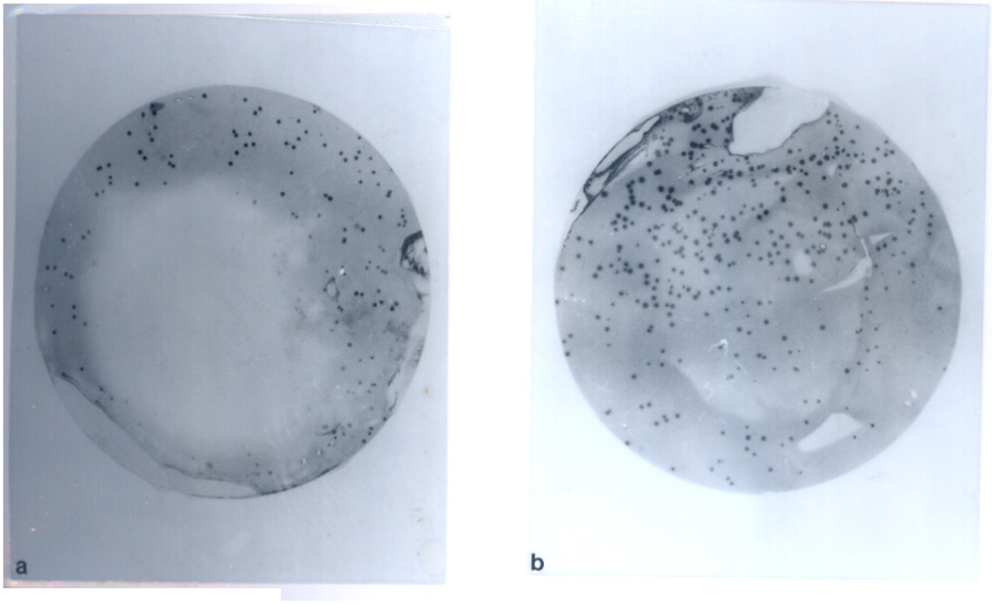

Figure 8

Figure 9 λgt11-SCMV immunopositive plaque DNA digested with *Eco*RI

(a) Left to right-

Lanes 1 and 2- λgt11-SCMV 1 and 2 DNA digested with *Eco*RI, Lanes 3 and 4- λgt11-SCMV 1 and 2 undigested, Lane 5- λ DNA digested with *Hin*dIII

(b) Left to right

Lane 1- λ DNA digested with *Hin*dIII, Lane 2- λgt11-SCMV 3 DNA digested with *Eco*RI, Lane 3 -λgt11-SCMV 3 DNA undigested.

Note the lack of released insert in the gel.

Figure 10 Screening of λgt11-SCMV library by polymerase chain reaction (Annealing at 37°C).

Left to right

Lane 1- zero template control, Lanes 2-4- λgt11-SCMV library in aliquots of 4, 8 and 16 μl, Lane 5- Molecular weight marker (λ DNA digested with *Eco*RI and *Hin*dIII), Lane 6-7- λgt11-SCMV 1 in aliquots of 8 and 16 μl, Lane 8-9- λgt11-SCMV 2 in aliquots of 8 and 16 μl.

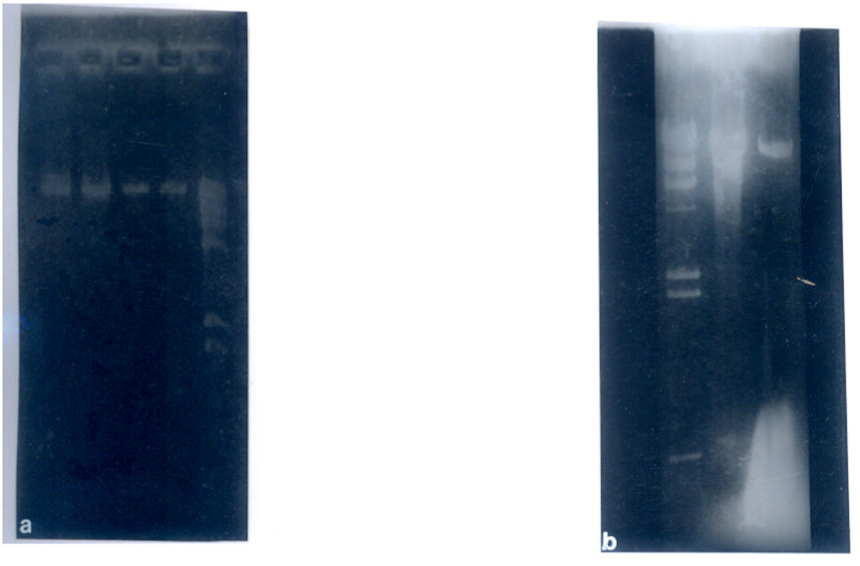

Figure 9

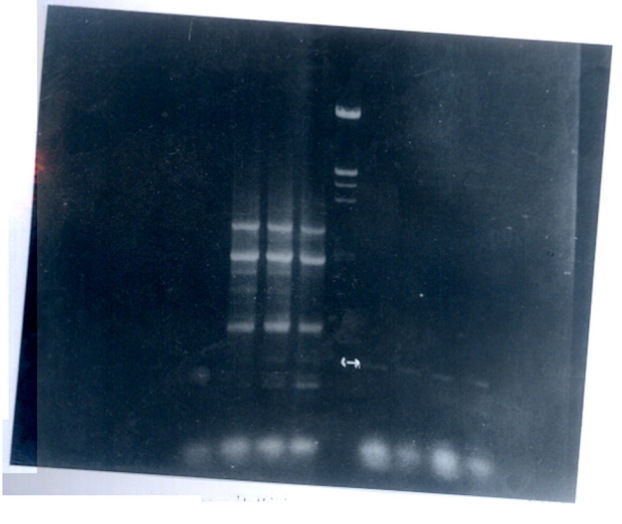

Figure 10

Figure 11 Preparative gel electrophoresis of amplified products of screening of λgt11-SCMV library by polymerase chain reaction.
Left to right
Lanes 1 and 3- λgt11-SCMV 1 and λgt11-SCMV 2, respectively, Lane 2- λ DNA digested with *Eco*RI and *Hin*dIII. Sizes from top to bottom are, 21.3, 5.1, 4.9, 4.3, 3.5, 2.0, 1.9, 1.6, 1.4, 0.95, 0.83, 0.564 and 0.125 kbp.

Figure 12 Screening of λgt11-SCMV immunopositive plaques by polymerase chain reaction (Annealing at 52°C).
Left to right
Lanes 1-4- λgt11-SCMV 1, 5-8- λgt11-SCMV 2, Lanes 9-11- λgt11-SCMV 3 (Each in an aliquot of 4μl), 12- zero template control, 13- λ DNA digested with *Hin*dIII. Sizes from top to bottom are, 23.1, 9.49, 6.55, 4.3 , 2.3, 2.0. 0.564 and 0.125 kbp.

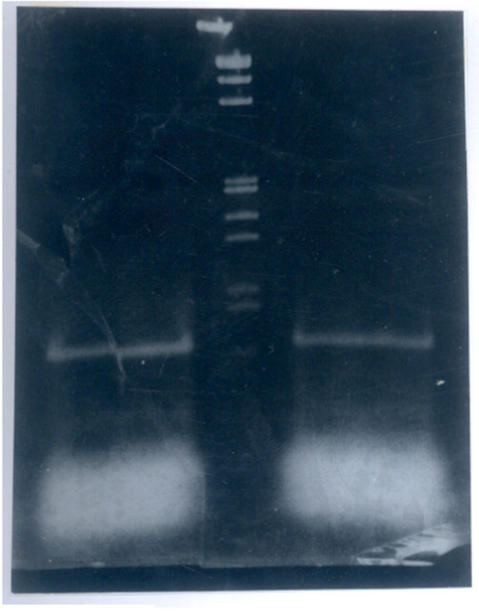

Figure 11

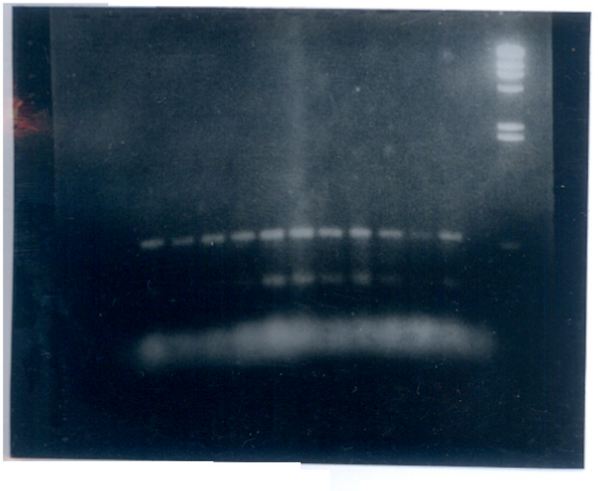

Figure 12

Figure 13 Reverse transcriptase-polymerase chain reaction

(a) Left to right

Lane 1- λ DNA digested with *Hin*dIII, Lane 2- zero RNA control, Lane 3-4 RT-PCR product (0.5 and 1 μg template RNA, respectively), Lane 5- Infected sugarcane leaf total RNA, Lane 6- Tissue cultured sugarcane leaf total RNA (control)

(b) RT-PCR ampified product re-amplified by PCR

Left to right

Lane 1- pUC18 DNA digested with *Alu*I (size of the highest band is 679 bp), Lane 2- zero template control, Lane 3- reamplified RT-PCR product.

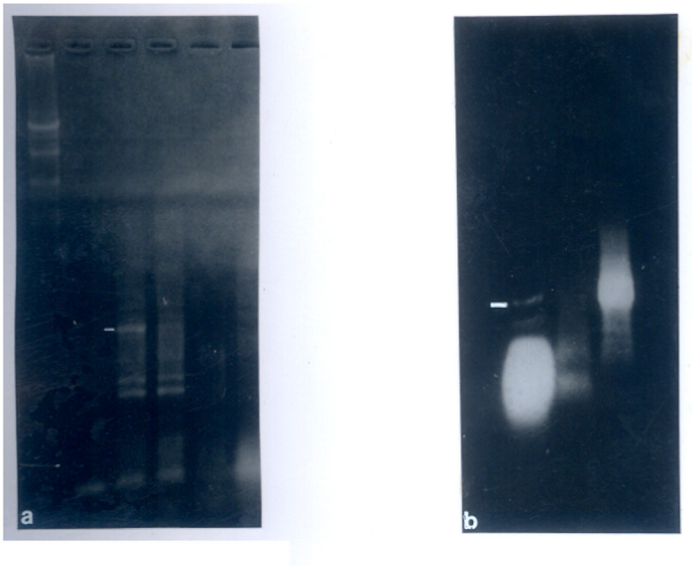

Figure 13

Figure 14 Northern hybridization of viral RNA to RT-PCR product

(a) Denaturing formaldehyde agarose gel electrophoresis in 1X MOPS-EDTA of RNA

Left to right

Lane 1- Molecular weight marker, RNA ladder (BRL, USA). Sizes from top to bottom are 9.49, 7.46, 4.4, 2.37, 1.35 and 0.24 kb, Lane 2-RNA isolated from purified viral protein, Lane 3- Tissue cultured sugarcane leaf total RNA (control).

(b) Hybridization of radioactively labeled RT-PCR product to RNA. Hybridization signals are seen only in the region above 9.49 kb and not in the control lane.

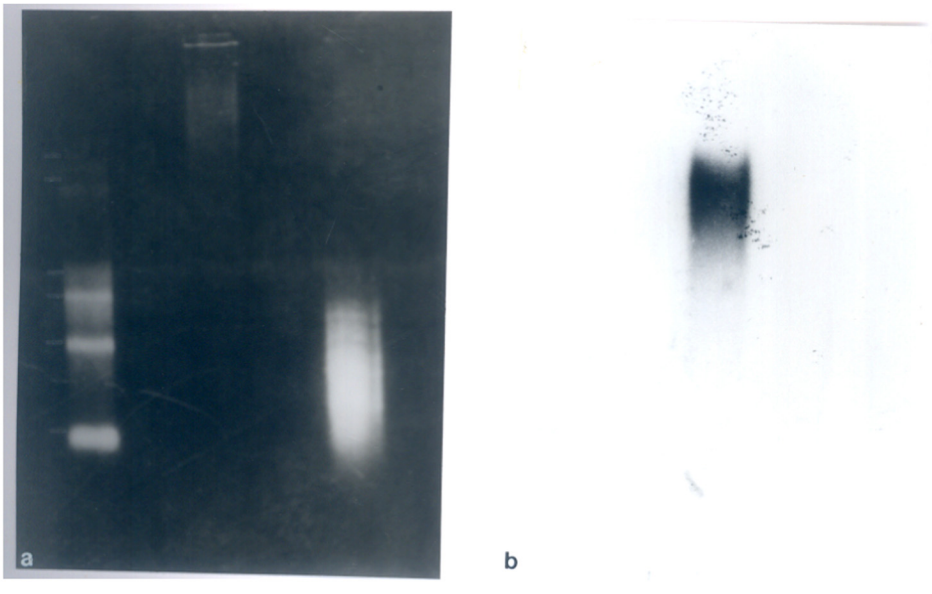

Figure 14

3.3 Cloning and sequencing of the amplified potyviral product

3.3.1 Cloning in plasmid vectors: pMOS*Blue* T and pGEM3Z

It was necessary to determine the nucleotide sequence of this amplified product. For ease in sequencing, the eluted DNA of ~600 bp was ligated into pMOS*Blue* T-vector (Amersham, UK), a vector specific for cloning PCR amplified fragments. The template-independent activity of thermostable DNA polymerases preferentially adds a single adenosine residue to the 3' end of *in vitro* amplified double-stranded DNA (160). Such PCR products can then be inserted into compatible thymidine-tailed vectors (161, 162). The multiple cloning site of pMOS*Blue* T-vector has an *Eco*RV site with T-overhangs that enables the ligation of PCR products with 3' A-overhangs (Fig. 15).

The clones in the pMOS*Blue* T-vector were designated as the pMTV series. The clones were selected by blue-white selection on ampicillin and tetracycline. Plasmid DNA isolated from 10 ml *E. coli* MOS*Blue* cultures was digested with *Eco*RI and *Hin*dIII. The insert released from this digestion corresponded to the PCR-amplified band of ~603 bp (ϕX174 DNA digested with *Hae*III molecular weight marker) (Fig. 16a). The positive white colonies were also screened for recombinants directly by PCR. A colony was picked and diluted in 50 µl distilled water. The solution was boiled for 5 min to rupture the cells and inactivate the DNases. The mixture was centrifuged and an aliquot of the supernatant was taken for PCR. The reaction was carried out in an identical manner as for the total λgt11 library with annealing at 52°C. Similar results as with the λgt11-PCR were obtained. The colony-PCR showed several high molecular weight amplified products and thus confirmed the earlier speculation that the observed high molecular weight bands in the PCR of the total λgt11 library were due to the presence of *E. coli* DNA.

pMTV23, a positive clone with the putative SCMV-CP gene, was digested with *Hin*dIII, *Eco*RI, *Pst*I, *Sac*I, *Sph*I, *Kpn*I, *Eco*RV, *Sma*I, *Xba*I, *Bam*HI and *Sca*I singly (Fig.16b, 16c). All these sites are located in the multiple cloning site of the pMOS*Blue* T-vector. Digestion with these restriction endonucleases linearized the plasmid in each case and no low molecular weight bands were seen. These results gave reason to conclude that the putative SCMV coat protein gene lacked sites for the above restriction endonucleases.

pMOS*Blue* T-vector has sites for T7 and U19 sequencing primers. Since only the T7 sequencing primer was available, the *Eco*RI-*Hin*dIII insert from pMTV23 was cloned in pGEM3Z (Promega, USA). Cloning in pGEM3Z enabled the use of T7 and SP6 sequencing primers, both of which were readily available. The pGEM3Z vector (Fig. 17) was double-digested with *Eco*RI and *Hin*dIII thereby generating staggered ends and deleting the multiple cloning site. The *Eco*RI-*Hin*dIII insert from pMTV23 was ligated into the staggered sites of pGEM3Z and the ligated plasmid transformed into fresh, competent *E. coli* JM109 cells. The clones were selected by blue-white selection on ampicillin and were designated as the pZTV series. Plasmid DNA from these clones was isolated and digested with *Eco*RI and *Hin*dIII. The released insert had the same size of ~603 bp as the RT-PCR product (Fig. 18).

3.3.2 Sequencing of the amplified potyviral product

pZTV231 was a clone that contained the *Eco*RI-*Hin*dIII insert from pMTV23. pMTV23 was sequenced using the T7 primer whereas pZTV231 was sequenced using both SP6 and T7 primers (Fig.19).The extent of readable sequence from the T7 primer was 354 bases and that from the SP6 primer was 248 bases. Homology searches in the EMBL database were carried out. Homology search in the EMBL database showed that the 248 bp sequence from the SP6 primer had a 56.5% identity in a 232 bp overlap with the *Streptococcus pneumoniae* bacteriophage CP-1 collar protein (169-171). The 354 bp sequence from the T7 primer had a 58.7% identity in a 107 bp overlap with the *Saccharomyces cerevisiae* chromosome III complete sequence (172).

All the three sequences were compared for homology with the SCMV-SC CP coding region (93) using the DNASIS software (Hitachi, Japan). All of these showed homologies ranging from 49% to 55% for the different regions. The coat protein nucleotide sequence of potyvirus PVY^{O} (173, 174) has sequence idendities ranging from 62% to 65% for different regions with the SCMV-SC CP coding region, whereas it had a sequence identity of 84% with the Chilean strain of PVY (173, 175). Comparisons of the sequences of pZTV231 from both the primers with the sequences of the CP genes of the two PVY strains showed homologies ranging from 33% to 42% for the different regions.

The low sequence homology of pZTV231 clone with known potyviral sequences gives reason to believe that the potyvirus isolated from the infected leaves of sugarcane found

in the fields of India is possibly different from those reported elsewhere. So far, no molecular data is available on potyviruses found in India. It is, therefore, deduced that the virus isolated from the fields is indeed different from those reported in literature from other geographical locations.

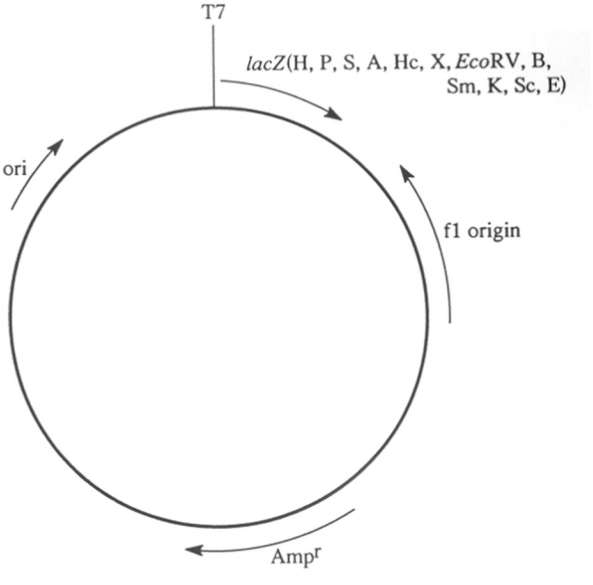

Figure 15 pMOS*Blue* T-vector map (2887 bp).
The site of insertion is at *Eco*RV (located 95 bases downstream of the start site) in the *lacZ* gene. The sites in the multiple cloning site are; H- *Hin*dIII, P- *Pst*I, S- *Sal*I, A- *Acc*I, Hc- *Hin*cII, X- *Xba*I, B- *Bam*HI, Sm- *Sma*I, K- *Kpn*I, Sc- *Sac*I, E- *Eco*RI.

Figure 16 Cloning of potyviral amplified product in pMOS*Blue* T-vector

(a) Left to right

Lane 1- λ DNA digested with *Hin*dIII, Lanes 2-3, pMTV22 and pMTV23, respectively,digested with *Eco*RI and *Hin*dIII, Lane 4- pMTV23 undigested, Lane 6-7- Colony-PCR of pMTV22 and pMTV23, respectively, Lane 7- zero template control.

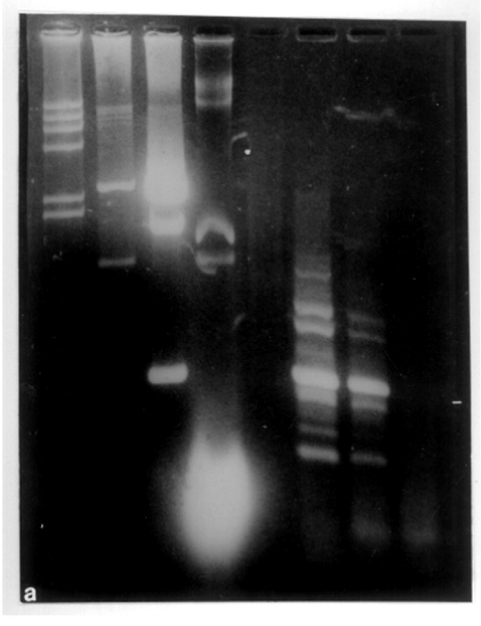

Figure 16

Figure 16

(b) Left to right
Lane 1- pUC18 DNA digested with *Alu*I, Lane 2-pMTV23 digested with *Eco*RI and *Hin*dIII, Lane 3- ϕX174 DNA digested with *Hae*III (sizes from top to bottom are 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp), Lanes 4-8- pMTV23 digested with *Hin*dIII, *Eco*RI, *Pst*I, *Sac*I, *Sph*I, respectively, Lane 9- pMTV23 undigested, Lane 10-λ DNA digested with *Hin*dIII.

(c) Left to right
Lane 1-pMTV23 digested with *Eco*RI and *Hin*dIII, Lane 3- pUC18 DNA digested with *Alu*I, Lane 4- pMTV23 undigested, Lane 6-ϕX174 DNA digested with *Hae*III, Lanes 7-13- pMTV23 digested with *Kpn*I, *Eco*RV, *Sma*I, *Xba*I, *Bam*HI, *Sca*I and *Sac*I, respectively.

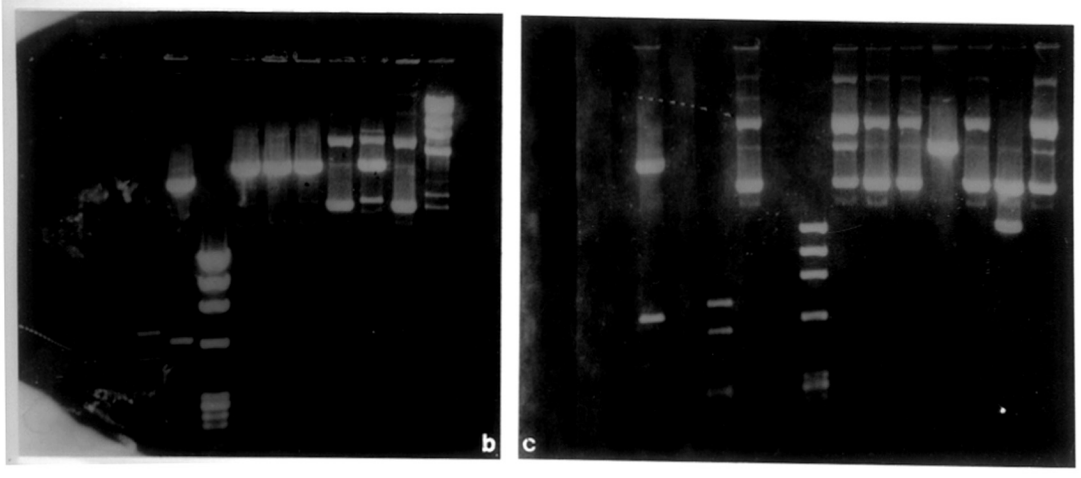

Figure 16

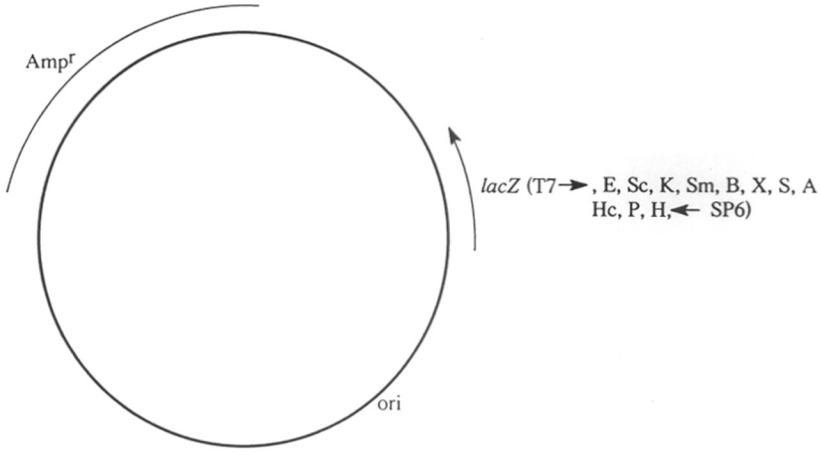

Figure 17 pGEM3Z vector map (2743 bp).
The sites in the multiple cloning site in *lacZ* are: T7→- T7 transcription start site (1), E- *Eco*RI (5), Sc- *Sac*I (15), K- *Kpn*I (21), Sm- *Sma*I (23), B- *Bam*HI (26), X- *Xba*I (32), S- *Sal*I (38), A- *Acc*I (39), Hc- *Hin*cII (40), P- *Pst*I (48), H- *Hin*dIII (56), ←SP6- SP6 transcription start site (69). Figures in brackets are the locations of the respective sites.

Figure 18 Cloning of *Eco*RI-*Hin*dIII fragment of pMTV23 in pGEM3Z
Left to right
Lane 2- pZTV231 undigested, Lane 3- pZTV231 *Eco*RI-*Hin*dIII digest, Lane 4- pZTV232 undigested, Lane 5- pZTV232 *Eco*RI-*Hin*dIII digest, Lane 6- pZTV233 undigested, Lane 7- pZTV233 *Eco*RI-*Hin*dIII digest, Lane 8- ϕX174 DNA digested with *Hae*III.

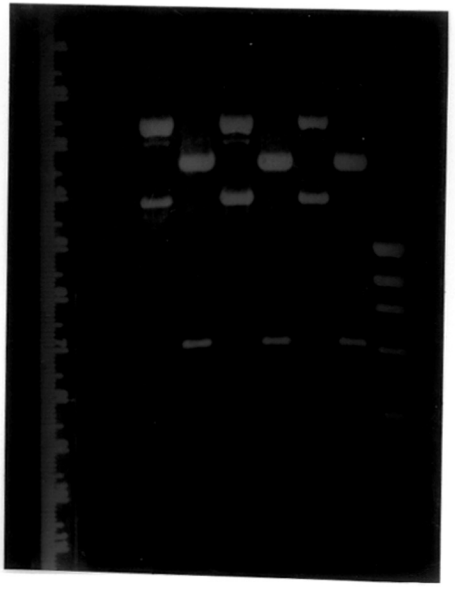

Figure 18

Figure 19 Autoradiogram of sequencing reactions carried out with pZTV231 as template
Left to right-(each load in the order G, A, T, C) reaction with SP6 primer first and second loads; reaction with T7 primer first and second loads

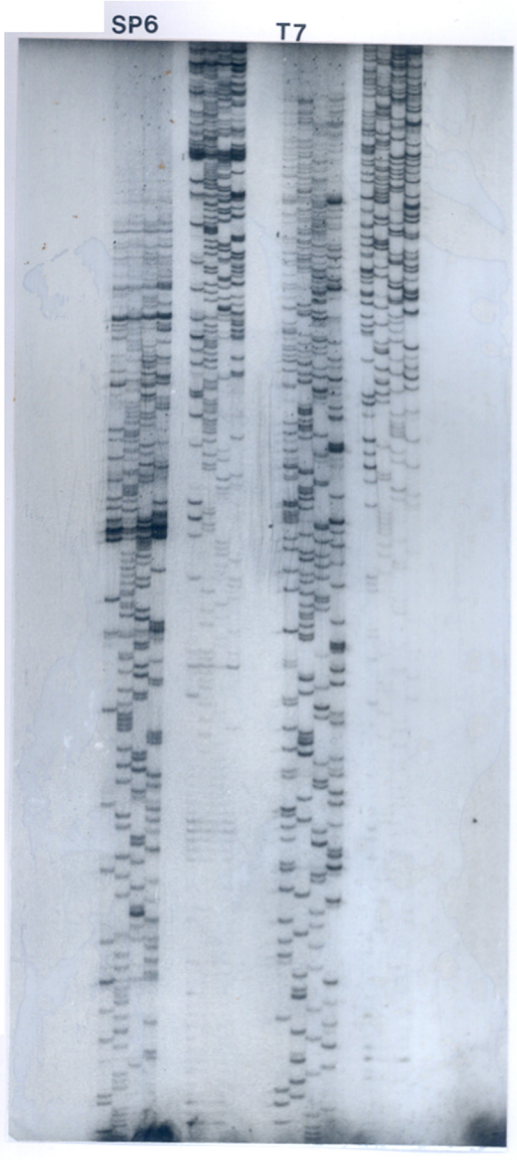

Figure 19

GCGGCCGCGC TTGGACCAGA TGTGGAATTT GTTCTATTAG CAACTGATGG
TCTTTGGGAT TACATAAAAA GCTCTGAAGC TGTAGCTTTT GTCGAGATCA
ACTACGTCAA CATGGTGATG TCCAGTTGGC CTGTGAGGCA CTTCGTCAGA
AACCTCTGGA TCAACGGCCC AAGACAATAT TAGCATAGTC ATAGCTGACT
TCGGGAGGAC AAAT

Figure 20 (a) Sequence of 214 bases of pMTV23 from 5' to 3' end using T7 sequencing primer

TTATACATAG AAACAATTCA TATCACGTTC TTTGAAGAAG TTTCTCAATA
TGACAAAAGA AAAGGAAATC CAGACAAGAA AGCACACGAT CTTCATCAGT
ACTTCACACG TTCACTATTA CCCAAAAGAT TACCTATCAA TTCACCCTAT
AAGAATATAC ATATATTTGC GGCTGCCAGC AGAAAATGCT AACTCAGTAA
TCAGAAAGTA ATATACACAT TGAATGACTA GTTCTACTGT AATACAGG

Figure 20 (b) Sequence of 248 bases of pZTV231 from 5' to 3' end using the SP6 sequencing primer

TTGGACCAGA TGTGGAATTT GTTCTATTAG CAACTGATGG TCTTTCGGGA
TTACATAAAA AGCTCTGAAG CTGTAGCTTT TCGTCGAGAT CAACTACCGT
CAACATGGTG ATGTCCAGTT CGGCCTGTCG AGGCACTTGG CGTCAGAAAG
CTCTGGATCA ACGGTCCCAA GACAATATTA GCATAGTCAT AGCTGACTTC
GGGGAGGACA AATTGGAAGG TGTTCCCCGA TGAAAGACCA AACATGTTCT
TGGAACTAAG TCAAGCAGTA GTGACTGTAG GGGTTGTTAA TAGGAATTGT
TCGATACAGT AGAACTAGTC ATTCAAGGAT ATACTTCTAT ACTAGTACAT
TTCC

Figure 20 (c) Sequence of 354 bases of pZTV231 from 5' to 3' end (reverse direction) using the T7 sequencing primer

3.4 Conclusions

The sugarcane mosaic virus is a definitive member of the potyvirus group and causes mosaic diseases in sugarcane, maize, sorghum and other poaceous plants. The sugarcane mosaic disease has caused extensive damage to the sugarcane crop in the United States, Australia and India. A large number of SCMV strains have been reported from different parts of the world and are differentiated on the basis of host reactions, serological, biochemical and molecular tests.

Most of the available biochemical and molecular data on all potyviruses, including SCMV, are available from Australia, United States and Europe. There is very little data on these viruses from the Indian sub-continent. The disease symptoms have been seen on the sugarcane crop in Maharashtra, India, and the physiological changes in the plant that subsequently affect productivity studied (88). A molecular study of the pathogen is necessary to decide its taxonomic position and to devise plans for the control of the disease.

The following evidences gave reason to conclude that the leaves of sugarcane in the fields were indeed infected by a potyvirus. Analysis of the purified and denatured viral coat protein on SDS-PAGE and immunoblot profiles substantiated the authenticity of a 24 kDa SCMV coat protein. However, it was found to be smaller in size than that reported from Australia and the United States. The serological relationship of the purified virus particles with the N strain of SCMV was ascertained. Coupled with these biochemical tests, the viability tests carried using the purified viral preparations proved that the virus particles retained their integrity during the isolation and purification process. The ability of the purified preparation to infect other poaceous species i.e., sorghum, was also amply demonstrated. The molecular mass of the coat protein of SCMV-SC, an Australian strain, is 34.2 kDa (90), and the length of its coat protein gene is 939 bases (93). The size of the coat protein reported here is ~24 kDa and the length of the PCR-amplified product using primers specific for the flanking sites of the coat protein gene is ~600 bp. Although, the coat protein was smaller than those reported in literature, the epitopes for antigenicity to antibodies raised against the coat protein of SCMV-N were retained.

Potyviruses have a single RNA molecule between 9.5 kb to 12 kb in length (15, 17, 29-31, 75-80). The reasons for concluding that the nucleic acid had a potyviral origin are:

(i) The fluorescence in the gel started at a region higher than 9.49 kb and went up to 12 kb. The characteristic eukaryotic ribosomal RNA bands were absent.

(ii) The amplification of the nucleic acid template occurred when primers specific for SCMV-SC CP were used for the polymerase chain reaction.

(iii) The amplified product hybridized with the viral RNA in the region above 9.49 kb and did not show any hybridization signals in the lower molecular weight regions as well as to the eukaryotic host RNA from the uninfected tissue cultured sugarcane leaf.

(iv) Based on the results that the eukaryotic ribosomal RNA bands were absent and the amplified product did not hybridize with the control leaf RNA, the possibility of the eukaryotic origin of the RNA used in all the experiments is ruled out.

While the serological and molecular data pointed towards the existence of a potyvirus related to SCMV infecting sugarcane, the partial sequence data of the PCR-amplified putative coat protein gene did not conclusively locate its taxonomic status. Potyviral sequences are reported to have low sequence identities among unrelated strains. The present investigation is the first report on molecular data on a potyvirus found in India that infects sugarcane leaves with manifestation of mosaic symptoms. The low sequence identity with reported potyviral strains may be because of the geographical location of the origin of the virus. Isolation and characterization of full-length genes using the PCR-amplified product as a probe will accurately point towards the taxonomic location of this Indian isolate of SCMV and lead to development of strategies for the protection of the crop from infection.

BIBLIOGRAPHY

1. **Keese, P., Gibbs, B.** (1993) *Curr. Opin. Genet. Develop.*, **3**, 873-877.
2. **Harrison, B.D., Robinson, D.J.** (1988) *Phil. Trans. Royal Soc. Ser. B*, **321**, 447-462.
3. **Shukla, D.D., Frenkel, M.J., Ward, C.W.** (1991) *Can. J. Plant Pathol.*, **13**, 178-191.
4. **Riechmann, J.L., Lain, S., Garcia, J.A.** (1992) *J. Gen. Virol.*, **73**, 1-16.
5. **Edwardson, J.R.** (1974) *Florida Agricultural Experiment Station Monograph*, Series 4, 225 pp.
6. **Hari, V., Siegel, A., Rozek, C., Timberlake, W.E.** (1979) *Virology*, **92**, 568-571.
7. **Hari, V.** (1981) *Virology*, **112**, 391-399.
8. **Dougherty, W.G., Carrington, J.C.** (1988) *Annu. Rev. Phytopathol.*, **26**, 123-143.
9. **Ward, C.W., Shukla, D.D.** (1991) *Intervirology*, **32**, 269-296.
10. **Frenkel, M.J., Ward, C.W., Shukla, D.D.** (1989) *J. Gen. Virol.*, **70**, 2775-2783.
11. **Dougherty, W.G., Allison, R.F., Parks, T.D., Johnston, R.E., Feild, M.J., Armstrong, F.B.** (1985) *Virology*, **146**, 282-291.
12. **Turpen, T.** (1989) *J. Gen. Virol.*, **70**, 1951-1960.
13. **Hay, J.M., Fellowes, A.P., Timmerman, G.M.** (1989) *Arch. Virol.*, **107**, 111-122.
14. **Quemada, H., L'Hostis, B., Gonsalves, D., Reardon, I.M., Heinrikson, R., Hiebert, E.L., Sieu, L.C., Slightom, J.L.** (1990) *J. Gen. Virol.*, **71**, 203-210.
15. **Robaglia, C., Durand-Tardif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S., Casse-Delbart, F.** (1989) *J. Gen. Virol.*, **70**, 935-947.
16. **Lutcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F., Scheele, G.A.** (1987) *EMBO J.*, **6**, 43-46.
17. **Thole, V., Dalmay, T., Burgyan, J., Balazs, E.** (1993) *Gene*, **125**, 149-156.
18. **Oh, S.-K., Sarnow, P.** (1993) *Curr. Opin. Genet. Develop.*, **3**, 295-300.
19. **Carrington, J.C., Freed, D.D.** (1990) *J. Virol.*, **64**, 1590-1597.

20. **Levis, C., Astier-Manifacier, S.** (1993) *Virus Genes*, **7**, 367-379.
21. **Riechmann, J.L., Lain, S., Garcia, J.A.** (1991) *Virology*, **185**, 544-552.
22. **Ryan, M.D., Drew, J.** (1994) *EMBO J.*, **13**, 928-933.
23. **Verchot, J., Carrington, J.C.** (1995) *J. Virol.*, **69**, 1582-1590.
24. **Audino, R., Rieckhof, G.E., Achacoso, P.L., Baltimore, D.** (1993) *EMBO J.*, **12**, 3587-3598.
25. **Choi, H.-K., Tong, L., Minor, W., Dumas, P., Boege, U., Rossmann, M.G., Wengler, G.** (1991) *Nature*, **354**, 37-43.
26. **Carrington, J.C., Dougherty, W.G.** (1987) *Virology*, **160**, 355-362.
27. **Carrington, J.C., Dougherty, W.G.** (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3391-3395.
28. **Carrington, J.C., Cary, S.M., Parks, T.D., Dougherty, W.G.** (1989) *EMBO J.*, **8**, 365-370.
29. **Nicolas, O., Laliberte, J.-F.** (1992) *J. Gen. Virol.*, **73**, 2785-2793.
30. **Johansen, E., Rasmussen, O.F., Heide, M., Borkhardt, B.** (1991) *J. Gen. Virol.*, **72**, 2625-2632.
31. **Gough, K.H., Shukla, D.D.** (1993) *Intervirology*, **36**, 181-192.
32. **Dougherty, W.G., Parks, T.D.** (1991) *Virology*, **183**, 449-456.
33. **Carrington, J.C., Haldeman, R., Dolja, V.V., Restrepo-Hartwig, M.A.** (1993) *J. Virol.*, **67**, 6995-7000.
34. **Thornbury, D.W., Pirone, T.P.** (1983) *Virology*, **125**, 487-490.
35. **Berger, P.H., Pirone, T.P.** (1986) *Virology*, **153**, 256-261.
36. **Ammar, E.D., Jarlfors, U., Pirone, T.P.** (1994) *Phytopathology*, **84**, 1054-1060.
37. **Wisler, G.C., Purcifull, D.E., Hiebert, E.** (1995) *J. Gen. Virol.*, **76**, 37-45.
38. **Brantley, J.D., Hunt, A.G.** (1993) *J. Gen. Virol.*, **74**, 1157-1162.
39. **David, C., Gargouri-Bouzid, R., Haenni, A.-L.** (1992) *Prog. Nucl. Acids Res. Mol. Biol.*, **42**, 157-227.
40. **Domier, L.L. Shaw, J.G., Rhoads, R.E.** (1987) Virology, **158**, 20-27.
41. **Liu, J., Peng, X., Mang, K.** (1995) *Sci. China (Ser. B)*, **38**, 160-168.
42. **Kamer, G., Argos, P.** (1984) *Nucl. Acids Res.*, **12**, 7269-7282.

43. **Poch, O., Sauvagey, L., Delarue, M., Tordo, N.** (1989) *EMBO J.*, **8**, 3867-3874.

44. **Lain, S., Riechmann, J.L., Garcia, J.A.** (1990) *Nucl. Acids Res.*, **18**, 7003-7006.

45. **Lain, S., Martin, M.T., Riechmann, J.L., Garcia, J.A.** (1991) *J. Virol.*, **65**, 1-6.

46. **Eagles, R.M., Balmori-Melian, E., Beck, D.L., Gardner, R.C., Forster, R.L.S.** (1994) *Eur. J. Biochem.*, 224, 677-684.

47. **Restrepo-Hartwig, M.A., Carrington, J.C.** (1994) *J. Virol.*, **68**, 2388-2397.

48. **Semler, B.L., Anderson, C.W., Hanecak, R., Dorner, L.F., Wimmer, E.** (1982) *Cell*, **28**, 405-412.

49. **Li, X.H., Carrington, J.C.** (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 457-461.

50. **Huttings, H., Mosch, W.H.M.** (1974) *Neth. J. Plant. Pathol.*, **80**, 19-27.

51. **Hiebert, E., McDonald, J.G.** (1976) *Virology*, **70**, 144-150.

52. **Allison, R.F., Dougherty, W.G., Parks, T.D., Johnston, R.E., Kelly, M.E., Armstrong, F.B.** (1985) *Virology*, **147**, 309-316.

53. **Shukla, D.D., Ward, C.W.** (1988) *J. Gen. Virol.*, **69**, 2703.2710.

54. **Shukla, D.D., Ward, C.W.** (1989) *Adv. Vir. Res.*, **36**, 273-314.

55. **Shukla, D.D., Ward, C.W.** (1989) *Arch. Virol.*, **106**, 171-200.

56. **Shukla, D.D., Strike, P.M., Tracy, S.L., Gough, K.H., Ward, C.W.** (1988) *J.Gen. Virol.*, **69**, 1497-1508.

57. **Shukla, D.D., Tribbick, G., Mason, T.J., Hewish, D.R., Geysen, H.M., Ward, C.W.** (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8192-8196.

58. **Shukla, D.D., Jilka, J., Tosic, M., Ford, R.E.** (1989) *J. Gen. Virol.*, **70**, 13-23.

59. **Shukla, D.D., Tosic, M., Jilka, J. Ford, R.E., Toler, R.W., Langham, M.A.C.** (1989) *Phytopathology*, **79**, 223-229.

60. **Jagadish, M.N., Ward, C.W., Gough, K.H., Tulloch, P.A., Whittaker, L.A., Shukla, D.D.** (1991) *J. Gen. Virol.*, **72**, 1543-1550.

61. **Deom, C.M., Lapidot, M., Beachy, R.N.** (1992) *Cell*, **69**, 221-224

62. **Mushegian, A.R., Koonin, E.V.** (1993) *Arch. Virol.*, **133**, 239-257.

63. **Dolja, V.V., Haldeman-Cahill, R., Montgomery, A.E., Vandenbosch, K.A., Carrington, J.C.** (1995) *Virology*, **206**, 1007-1016.

64. **Citovsky, V., Knorr, D., Schuster, G., Zambryski, P.** (1990) *Cell*, **60**, 637-647.

65. **Dolja, V.V., Haldeman, R., Robertson, N.L., Dougherty, W.G., Carrington, J.C.** (1994) *EMBO J.*, 13, 1482-1491.

66. **Pirone, T.P.** (1991) *Sem Virol.*, 2, 81-87.

67. **Salomon, R.** (1989) *Res. Virol.*, **140**, 453-455.

68. **Atreya, P.L., Atreya, C.D., Pirone, T.P.** (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7887-7891.

69. **Atreya, P.L., Lopez-Moya, J.J., Chu, M., Atreya, C.D., Pirone, T.P.** (1995) *J. Gen. Virol.*, **76**, 265-270.

70. **Pirone, T.P., Thornbury, D.W.** (1983) *Phytopathology*, 73, 872-875.

71. **Huet, H., Gal-On, A., Meir, E., Lecoq, H., Raccah, B.** (1994) *J. Gen. Virol.*, 75, 1407-1414.

72. **Hiebert, E., Tremaine, J.H., Roland, W.P.** (1984) *Phytopathology*, **74**, 411-416.

73. **Simons, J.N.** (1976) *Phytopathology*, **66**, 652-654.

74. **Granier, F., Durand-Tardif, M., Casse-Delbart, F., Lecoq, H., Robaglia, C.** (1993) *J. Gen. Virol.*, **74**, 2737-2742.

75. **Allison, R.F., Johnston, R.E., Dougherty, W.G.** (1986) *Virology*, **154**, 9-20.

76. **Domier, L.L, Franklin, K.M., Shahabuddin, M.,Hellman, G.M., Overmeyer, J.M., Hiremath, S.T., Siaw, M.F.E., Lomonosoff, G.P., Shaw, J.G., Rhoads, R.E.** (1986) *Nucl. Acids Res.*, **14**, 5417-5430.

77. **Maiss, E., Timpe, U., Brisske, A., Jelkmann, W., Casper, R., Himmler, G., Mattanovich, D., Katinger, H.W.D.** (1989) *J.Gen. Virol.*, **70**, 513-524.

78. **Lain, S., Riechmann, J.L., Garcia, J.A.** (1989) *Vir. Res.*, 13, 157-172.

79. **Yeh , S.-D., Jan, F.-J., Chiang, C.-H., Doong, T.-J., Chen, M.-C., Chung, P.-H., Ban, H.-J.** (1992) *J. Gen. Virol.*, 72, 2531-2541.

80. **Vance, V.B., Moore, D., Turpen, T.H., Bracker, A., Hollowell, V.C.** (1992) *Virology*, **191**, 19-30.

81. **Abbott, E.** (1960) *Sugar Bull.*, **39**, 23-27.

82. **Grisham, M.P.** (1994) *Plant Dis.*, **78**, 729-732.

83. **Matthews, R..E.F.** (1982) *Intervirology*, **17**, 1-99.

84. **Pirone, T.P.** (1972) *CMI/AAB Descriptions of Plant Viruses* **No.88.**

85. **Teakle, D.S., Shukla, D.D., Ford, R.E.** (1989) *CMI/AAB Descriptions of Plant Viruses* No.342.

86. **Smith, G.R., Ford, R., Frenkel, M.J., Shukla, D.D., Dale, J.L.** (1992) *Arch. Virol.*, 125, 15-23.

87. **Ghorpade, L.N., Joshi, G.V.** (1981) *Ind. J. Plant Physiol.*, 24, 1-7.

88. **Ghorpade, L.N., Joshi. G.V.** (1980) *Ind. J. Exp. Biol.*, 18, 1202-1203.

89. **Teakle, D.S., Grylls, N.E.** (1973) *Aust. J. Agric. Res.*, 24, 465-477.

90. **Gough, K.H., Shukla, D.D.** (1981) *Virology*, 111, 455-462.

91. **McKern, N.M., Shukla, D.D., Toler, R.W., Jensen, S.G., Tosic, M., Ford, R.E., Leon, O., Ward, C.W.** (1991) *Phytopathology*, 81, 1025-1029.

92. **Frenkel, M.J., Jilka, J.M., Shukla, D.D., Ward, C.W.** (1992) *J. Virol. Meth.*, 36, 51-62.

93. **Frenkel, M.J., Jilka, J.M., McKern, N.M., Strike, P.M., Clark, Jr., J.M., Shukla, D.D., Ward, C.W.** (1991) *J. Gen. Virol.*, 72, 237-242.

94. **Mali, V.R., Garud, T.B.** (1978) *FAO Plant Prot. Bull.*, 26, 28.

95. **Kondaiah, E., Nayudu, M.V.** (1984) *Curr. Sci.*, 53, 273-275.

96. **Hendre, R.R., Mascarenhas, A.F., Nadgir, A.L., Pathak, M., Jagannathan, V.** (1975) *Ind. Phytopathol.*, 28, 175-178.

97. **Hollings, M.** (1965) *Annu. Rev. Phytopathol.*, 3, 367-396.

98. **Ghorpade, L.N., Joshi, G.V.** (1979) *Studies on Productivity in Sugarcane var. CO 740 Infected by Mosaic Virus.* (29th Annual Convention of the Deccan Sugar Technologists' Association, Pune, India).

99. **Wilson, T.M.A., Davies, J.W.** (1994) *Outlook Agric.*, 23, 33-39.

100. **Fraser, R.S.S.** (1992) *Euphytica*, 63, 175-185.

101. **Yu, Y.G., Maroof, M.A.S., Buss, G.R., Maughan, P.J., Tolin, S.A.** (1994) *Phytopathology*, 84, 60-64.

102. **Kyle, M.M., Providenti, R.** (1993) *Theor. Appl. Genet.*, 86, 189-196.

103. **Haley, S.D., Afanador, L., Kelly, J.D.** (1994) *Phytopathology*, 84, 157-160.

104. **Grisham, M.P., Burner, D.M., Legendre, B.L.** (1992) *Plant Dis.*, 76, 360-362.

105. **Gillaspie, Jr., A.G., Wright, J.M.** (1993) *Plant Dis.*, 77, 352-354.

106. **Sequeira, L.** (1984) *Trends Biotechnol.*, **2**, 25-29.

107. **Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T., Beachy, R.N.** (1986) *Science*, **232**, 738-743.

108. **Sanford, J.C., Johnston, C.A.** (1985) *J. Theor. Biol.*, **113**, 395-405.

109. **Beachy, R.N., Loesch-Fries, S., Tumer, N.E.** (1990) *Annu. Rev. Phytopathol.*, **28**, 451-474.

110. **Carr, J.P., Zaitlin, M.** (1993) *Sem. Virol.*, 4, 339-347.

111. **Wilson, T.M.A.** (1993) *Proc. Natl. Acad. Sci. USA*, 90, 3134-3141.

112. **Powell, P.A., Stark, D.M., Sanders, P.R., Beachy, R.N.** (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6949-6952.

113. **Truve, E., Kelve, M., Aaspollu, A., Kuuksalu, A., Seppanen, P., Saarma, M.** (1994) *Arch. Virol.*, **Suppl. 9**, 41-50.

114. **Walkey, D.G.A., Lecoq, H., Collier, R., Dobson, S.** (1992) *Plant Pathol.*, **41**, 762-771.

115. **Stark, D.M., Beachy, R.N.** (1989) *Bio/Technology*, **7**, 1257-1262.

116. **Ling, K., Namba, S., Gonsalves, C., Slightom, J.L., Gonsalves, D.** (1991) *Bio/Technology*, **9**, 752-758.

117. **Namba, S., Ling, K., Gonsalves, C., Slightom, J.L., Gonsalves, D.** (1992) *Phytopathology*, **82**, 940-946.

118. **Lindbo, J.A., Silva-Rosales, L., Dougherty, W.G.** (1993) *Sem. Virol.*, 4, 369-379.

119. **Lindbo, J.A., Dougherty, W.G.** (1992) *Molec. Plant-Microbe Interact.*, 5, 144-153.

120. **Maiti, I.B., Murphy, J.F., Shaw, J.G., Hunt, A.G.** (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 6110-6114.

121. **Audy, P., Palukaitis, P., Slack, S.A., Zaitlin, M.** (1994) *Molec. Plant-Microbe Interact.*, **7**, 15-22.

122. **Dougherty, W.G., Lindbo, J.A., Smith, H.A., Parks, T.D., Swaney, S., Proebsting, W.M.** (1994) *Molec. Plant-Microbe Interact.*, 7, 544-552.

123. **Lindbo, J.A., Dougherty, W.G.** (1992) *Virology*, **189**, 725-733.

124. **Smith, H.A., Swanney, S.L., Parks, T.D., Wernsman, E.A., Dougherty, W.G.** (1994) *Plant Cell*, **6**, 1441-1453.

125. **Gambley, R.L, Bryant, J.D., Masel, N.P., Smith, G.R.** (1994) *Aust. J. Plant Physiol.*, **21**, 603-612.

126. **Evans, D.J., McKeating, J., Meredith, J.M., Burke, K.L., Katrak, K., John, A., Ferguson, M., Minor, P.D., Weiss, R.A., Almond, J.W.** (1989) *Nature*, **339**, 385-388.

127. **Miyamura, K., Kajigaya, S., Momoeda, M., Smith-Gill, S.J., Young, N.S.** (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 8507-8511.

128. **Kaslow, D.C., Bathurst, I.C., Barr, P.J.** (1992)*Trends Biotechnol.*, **10**, 388-391.

129. **Jagadish, M.N., Hamilton, R.C., Fernandez, C.S., Schoofs, P., Davern, K.M., Kalnins, H., Ward, C.W., Nisbet, I.T.** (1993) *Bio/Technology*, **11**, 1166-1170.

130. **Jagadish, M.N., Huang, D., Ward, C.W.** (1993) *J. Gen. Virol.*, 74, 893-896.

131. **Usha, R., Rohll, J.B., Spall, V.E., Shanks, M., Manle, A.J., Johnson, J.E., Lomonosoff, G.P.** (1993) *Virology*, **197**, 366-374.

132. **Turpen, T.H., Reinl, S.J., Charoenvit, Y., Hoffman, S.L., Fallarme, V., Grill, L.H.** (1995) *Bio/Technology*, 13, 53-57.

133. **Bradford, M.M.** (1976) *Anal. Biochem.*, 72, 248-254.

134. **Laemmli, U.K.** (1970) *Nature*, **227**, 680-685.

135. **Sambrook, J., Fritsch, E.F., Maniatis, T.** (1989) *Molecular Cloning - A Laboratory Manual* **(2nd Edition)** (Cold Spring Harbor; CSH Laboratory Press).

136. **Merril, C.R., Goldman, D., Van Keuren, M.L.** (1983) *Methods Enzymol.*, **96**, 230-239.

137. **Blum, H., Beier, H., Gross, H.J.** (1987) *Electrophoresis*, 8, 93-99.

138. **Towbin, H., Staehelin, T., Gordon, J.** (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.

139. **Blumberg, D.D.** (1987) *Methods Enzymol.*, 152, 20-24.

140. **Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J.** (1979) *Biochemistry*, 18, 5294-5299.

141. **MacDonald, R.J., Swift, G.H., Przybyla, A.E., Chirgwin, J.M.** (1987) *Methods Enzymol.*, 152, 219-227.

142. **Lehrach, H., Diamond, D., Wozney, J.M., Boedtker, H.** (1977) *Biochemistry*, **16**,

4743-4751.

143. **Boedtker, H.** (1971) *Biochim. Biophys. Acta*, **240**, 448-453.

144. **Gong, Z.** (1992) *BioTechniques*, 12, 74-75.

145. **Riboclone™ cDNA Synthesis System Technical Manual** (Promega Corporation, USA).

146. **Kimmel, A.R., Berger, S.L.** (1987) *Methods Enzymol.*, **152**, 307-316.

147. **Promega Protocols and Applications Guide** (1991) **2nd Edition**, (Promega Corporation, USA).

148. **Ogden, R., Adams, D.A.** (1987) *Methods Enzymol.*, **152**, 61-87.

149. **McDonnell, M.W., Simon, M.N., Studier, F.W.** (1977) *J. Mol Biol.*, **110**, 119-146.

150. **Young, R.A., Davis, R.W.** (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1194-1198.

151. **Huynh, T.V., Young, R.A., Davis, R.W.** (1985) in *DNA Cloning: A Practical Approach, Vol. 1* **(D.M. Glover, Ed.)** (Oxford; IRL Press).

152. **Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, R., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A.** (1988) *Science*, **239**, 487-491.

153. **Friedman, K.D., Rosen, N.L., Newman, P.J., Montgomery, R.R.** (1988) *Nucl. Acids Res.*, **16**, 8718.

154. **Friedman, K.J., Rosen, N.L., Newman, P.J., Montgomery, R.R.** (1990) in *PCR Protocols* **(M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, Eds.)** (San Diego; Academic Press).

155. **Arnheim, N., Erlich, H.A.** (1992) *Annu. Rev. Biochem.*, **61**, 131-156.

156. **Kawasaki, E.S.** (1990) in *PCR Protocols* **(M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, Eds.)** (San Diego; Academic Press).

157. **Vogelstein, B., Gillespie, D.** (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 615-619.

158. **Feinberg, A.P., Vogelstein, B.** (1983) *Anal. Biochem.*, **132**, 6-13.

159. **Feinberg, A.P., Vogelstein, B.** (1984) *Anal. Biochem.*, **137**, 266-267.

160. **Clark, J.M.** (1988) *Nucl. Acids Res.*, **16**, 9677-9686.

161. **Marchuk, D.** (1990) *Nucl. Acids Res.*, **19**, 1154.

162. **Holton, T.A., Graham, M.W.** (1990) *Nucl. Acids Res.*, **19**, 1156.

163. **Sanger, F., Nicklen, Coulson, A.R.** (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.

164. **Sequenase™ Version 2.0 DNA Sequencing Kit Product No. 70770** *Step-by-step Protocols* **8th Edition** (US Biochemical Corporation, USA).

165. **Tabor, S., Richardson, C.C.** (1989) *J. Biol. Chem.*, **264**, 6447-6458.

166. **Quemada, H., Sieu, L.C., Siemieniak, D.R., Gonsalves, D., Slightom, J.L.** (1990) *J. Gen. Virol.*, **71**, 1451-1460.

167. **Henson, J.M., French, R.** (1993) *Annu. Rev. Phytopathol.*, **31**, 81-109.

168. **Langeveld, S.A., Dore, J.-M., Memelink, J., Derks, A.F.L.M., van der Vlugt, C.I.M., Asjes, C.J., Bol, J.M.** (1991) *J. Gen. Virol.*, **72**, 1531-1541.

169. **Escarmi, S.C., Gomez, A., Garcia, E., Ronda, C., Lopez, R., Salas, M.** (1994) *Virology*, **133**, 166-171.

170. **Martin, A.C., Lopez, R., Garcia, P.** (1995) *Virology*, **211**, 21-32.

171. **Genbank Accession Number Z47794.**

172. **Genbank Accession Number X59720.**

173. **Sudarsono, W.S.L., Xiong, Z., Hellman, G.M., Wernsman, E.A., Weissinger, A.K., Lommel, S.A.** (1993) *Arch. Virol.*, **132**, 161-170.

174. **Genbank Accession Number X68226.**

175. **Genbank Accession Number X68221.**