

**A MUTAGENIC STUDY OF THE STRUCTURE AND  
ROLE OF TYMOVIRUS COAT PROTEINS**

This thesis contains no material which has been previously submitted for an award of a degree at this, or any other, University and is the original work of the author, except where acknowledged.

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of The Australian National University

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### Statement

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ATP	Adenosine 5' triphosphate
Bp	Bird's pair
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CITP	Cytidine 5' triphosphate
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleoside 5' triphosphate
dNTP	dideoxynucleoside 5' triphosphate
EDTA	Ethylenediamine tetraacetic acid
GTP	Guanosine 5' triphosphate
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ip	Isotonic pair
Ka or P	Kilobase
MES	Morpholinopropane sulphonic acid
MOPS	Morpholinopropane sulphonic acid
NBT	2,2,6,6-tetramethylpiperidine chloride
OD	Optical density
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDE	Sodium dodecyl sulphate
SDW	Sevic distilled water
UTP	Uridine 5' triphosphate

## ABBREVIATIONS

ATP	Adenosine 5' triphosphate
Bp	Base Pair
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CTP	Cytidine 5' triphosphate
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide 5' triphosphate
ddNTP	dideoxynucleotide 5' triphosphate
EDTA	Ethylenediamine tetraacetic acid
GTP	Guanosine 5' triphosphate
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Kb	Kilo base pair
Kd or D	Kilodalton
MES	Morpholinoethane sulphonic acid
MOPS	Morpholinopropane sulphonic acid
NBT	p-nitro blue tetrazolium chloride
OD	Optical density
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RT-PCR	Reverse transcribed-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
UTP	Uridine 5' triphosphate

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## ABSTRACT

The roles of viral coat proteins in infection of plant hosts have been determined for some viruses and include both cell-to-cell and long distance transport in the plant, transmission of the virus by insects and other vectors, and pathogenesis. This project examined the role of the turnip yellow mosaic virus - Blue Lake isolate (TYMV-BL) coat protein on infection by the virus and was approached in two ways. Firstly, the coat protein genes of six naturally occurring symptom variants of TYMV (the variants elicited severe, intermediate or mild symptoms) were isolated from their native host in Australia and sequenced to see if there were any differences which might explain the variation in symptoms. The mild variants were of most interest in investigating which amino acids were important in the attenuation of symptoms produced in infected plants. The nucleotide and amino acid sequences of the six variants were compared with that of TYMV-BL and differences were found in all variants. However, the only differences which seemed to correlate with symptoms were residues 42 and 49; glycine in the mild variants, alanine in TYMV-BL and the severe variants.

The second approach to investigating the role of the TYMV coat protein involved constructing recombinants of the coat protein. This was done by replacing parts of the turnip yellow mosaic virus coat protein with the corresponding part of the belladonna mottle virus (BMV) coat protein. Belladonna mottle virus is another tymovirus but its host range is different from that of TYMV. Ten recombinants were made and they produced different symptoms depending on which region of the protein was altered but none of the recombinants had a host range that differed from that of TYMV. Epitope analysis of the coat protein with antibodies raised against one of the systemically infecting N terminal recombinants showed that the

N terminus of the coat protein was immunodominant. The fact that the coat protein could tolerate changes to the N terminal region which stimulate a specific immune response while retaining the ability to systemically infect host plants suggested that it could be used for the presentation of foreign epitopes. This possibility was investigated by inserting a malaria antigen in place of the N terminus of the coat protein. The resulting mutant virus was able to systemically infect Chinese cabbage plants.

Studies of the recombinants also provided information on the structure of the TYMV coat protein. Replacement with parts of the BMV coat protein changed the isoelectric point of some of the systemically infecting recombinants including those with changes at the N terminus and in one of the putative  $\alpha$  helices. When the electrophoretic mobility of particles of infectious recombinants was analysed, only those of the recombinant with the changed  $\alpha$  helix had an altered mobility when compared with TYMV-BL. The other recombinants, including those with changed N termini, had mobilities identical to TYMV-BL even though their isoelectric points had been altered. This suggested that the region corresponding to the changed  $\alpha$  helix was exposed on the surface of the virus particle but that the N terminus and other regions were buried within the particle.

## CHAPTER ONE

### INTRODUCTION

#### 1.1 THE TYMOVIRUSES

The tymoviruses, of which turnip yellow mosaic virus (TYMV) is the type member, are a group of about 24 plant viruses having isometric particles approximately 30 nm in diameter. TYMV was first described almost 50 years ago (Markham and Smith, 1946). Since then it has become one of the most extensively studied plant viruses with isometric virions (Matthews, 1991).

Tymoviruses infect dicotyledonous plants with individual viruses having a narrow host range limited to either one plant family or to a few species of related families (Guy *et al.*, 1984). Tymoviruses have been isolated from plants in different countries on all continents with each virus usually restricted geographically (Hirth and Givord, 1988). There are some exceptions such as PoiMV which is found wherever poinsettias grow, and TYMV, which is found in Europe and Australia. Tymoviruses are easily transmitted by mechanical means and in the field TYMV is spread by flea beetles-*Phyllotreta* sp. (Markham and Smith, 1949). Plants infected with tymoviruses show bright yellow and green mosaic mottling (Markham and Smith, 1946; 1949). At a cytological level, infection by TYMV affects chloroplasts causing them to clump. Small vesicles are formed at the periphery of the chloroplasts as a result of invaginations of the chloroplast membranes (Chalcroft and Matthews, 1967; Lafleche and Bove, 1969). Several experiments have shown that replication of the virus is associated with chloroplasts in infected cells. Chloroplasts isolated from infected Chinese cabbage were able to catalyse synthesis of + strand RNA (Lafleche *et*

*al.*, 1972; Mouches *et al.*, 1974), and experiments where label was incorporated into newly synthesised RNA showed that an enzyme-template complex was associated with the chloroplast outer membrane (Laflèche *et al.*, 1972; Garnier *et al.*, 1980). Viral coat protein was detected in the cytoplasm overlying peripheral vesicles and, using this information along with the presence of the replicase, it was suggested that the peripheral vesicles were the sites of virus assembly (Matthews, 1991; Hatta and Matthews, 1976).

Tymoviruses were found to produce two types of particle which can be separated by centrifugation in density gradients: the top (or empty) component which comprises empty protein shells only, and the bottom (or full) component with protein shells containing the nucleic acid (Markham *et al.*, 1948). It was shown that only the nucleoprotein was infectious, demonstrating for the first time that the viral nucleic acid was responsible for infection (Markham *et al.*, 1948, Markham and Smith, 1949). The presence of empty capsids confirmed the importance of protein-protein interactions in stabilising the virus particles and in the architecture of the virions (Markham *et al.*, 1948). The nucleoproteins contain the genomic RNA of  $M_r$   $2.3 \times 10^6$  (Haselkorn, 1962) with 36% of their weight comprising RNA (Matthews, 1960). TYMV particles were found to contain a smaller subgenomic RNA of apparent  $M_r$   $0.3 \times 10^6$  which encodes the coat protein (Klein *et al.*, 1976, Pleij *et al.*, 1976, Ricard *et al.*, 1977) and this was also found in the bottom component. In other tymoviruses such as eggplant mosaic virus (EMV), wild cucumber mosaic virus (WCuMV) and okra mosaic virus (OkMV) the subgenomic RNA was found in the empty protein shells of the top component. Host tRNA molecules were also found in the top component of these viruses (Szybiak *et al.*, 1978) and in the top components of cocoa yellow mosaic virus (CoYMV) and Kennedyya yellow mosaic virus (KYMV) (Blok *et al.*, 1987). The tymoviruses belladonna mottle virus

(BMV), dulcamara mottle virus (DMV), erysimum latent virus (ELV), EMV, ononis yellow mosaic virus (OYMV) and WCuMV were found to contain host tRNA molecules in both the top and bottom particles (Blok *et al.*, 1987).

Tymovirus genomes have a high cytosine (32-42%) and a low guanosine (15-17%) content (Hirth and Givord, 1988), and a preference for cytosine in the third position of amino acid codons (Guilley and Briand, 1978). Both the genomic and subgenomic RNAs of tymoviruses are capped at the 5' termini with m<sup>7</sup>GpppGp (Klein *et al.*, 1976) and both have a 3' valine accepting tRNA-like structure (Pinck *et al.*, 1970). The 5' cap is thought to play a role in ribosome binding and stabilisation of the RNA for higher translation efficiency (Furuichi *et al.*, 1977; Shimotohno *et al.*, 1977). It is thought that the 3' tRNA-like structure plays some role in replication or regulation of translation (Giegé *et al.*, 1993). However, the 3' end of ELV does not have a tRNA-like structure (van Belkum *et al.*, 1987; Srifah *et al.*, 1992) so a definitive role for this structure in the life cycle of tymoviruses remains to be elucidated.

The structure of TYMV particles was investigated using X-ray diffraction techniques (Klug *et al.*, 1957; Klug and Finch, 1960) and by examination in the electron microscope (Huxley and Zubay, 1960; Nixon and Gibbs, 1960). These studies showed that the protein shell of the virus was made up of 180 identical subunits of the coat protein arranged in 12 groups of 5 (pentamers) and 20 groups of 6 (hexamers) with T=3 lattice symmetry. Studies on the protein monomer showed that it consisted of 189 amino acids with a total molecular weight of ~20,000 (Harris and Hindley, 1961; 1965).

The genomes of 7 tymoviruses and the coat protein genes of several others have been sequenced to date. The viruses whose complete genomes have been sequenced are:

- |      |  |
|------|--|
| TYMV | - type strain (Morch <i>et al.</i> , 1988)             |
|      | - Club Lake isolate (Keese <i>et al.</i> , 1989)       |
|      | - Blue Lake isolate (A. Meek, unpublished)             |
| EMV  | - Trinidad isolate (Osorio-Keese <i>et al.</i> , 1989) |
| OYMV | -Tintagel isolate (Ding <i>et al.</i> , 1989)          |
| KYMV | - Jervis Bay isolate (Ding <i>et al.</i> , 1990)       |
| ELV  | (Srifah <i>et al.</i> , 1992)                          |

The genome organisation of all these tymoviruses is similar with three open reading frames (ORFs) encoding the overlapping protein, replicase protein and coat protein. The sizes of the ORFs and encoded proteins vary between the different tymoviruses. The overlapping proteins range in size from  $M_r$  48.5K to 82K, the replicase proteins from  $M_r$  194K to 210K and the coat proteins from  $M_r$  19.6K to 21.5K (Srifah *et al.*, 1992).

TYMV genomic RNA was translated into two high molecular weight proteins, as well as the coat protein, in a rabbit reticulocyte lysate (Bénicourt *et al.*, 1978). These high molecular weight proteins of 195K and 150K were thought to be initiated at the same site on the genomic RNA (Bénicourt and Haenni, 1978) and both shared some amino acids. These authors also showed that the coat protein subgenomic RNA was preferentially translated compared with the genomic RNA. This led to a greater production of coat protein at later stages in the infection process. The 195K protein was shown to undergo post-translational cleavage to generate proteins of 120K and 78K which corresponded to the N and C termini respectively (Morch *et al.*, 1982). When the genomic sequence of TYMV was determined, it was shown to encode proteins of 206K, 69K and the 20K coat protein (Morch *et al.*, 1988). The 69K protein was translated starting from the 5' most AUG in the RNA

and overlapped the 206K protein. The 206K protein contained the amino acid sequences of the putative nucleotide binding and polymerase sites. Alignment of the sequences of various plant and animal RNA viruses had shown the existence of motifs characteristic of these sites (Kamer and Argos, 1984; Goldbach, 1987; Goldbach and Wellink, 1988; Argos, 1988; Gorbalenya *et al.*, 1989a; Gorbalenya *et al.*, 1989b). Previous studies had shown that the 206K (195K) protein was cleaved to yield two smaller proteins (Morch *et al.*, 1982). The nucleotide binding region mapped within the N terminal 120K protein which had been shown to be part of the TYMV replicase (Mouches *et al.*, 1984). The polymerase site mapped within the C terminal 78K protein. Later studies proved that the 206K protein was cleaved to yield proteins of ~150K and ~78K (Morch *et al.*, 1989). Mutants of TYMV were made to investigate the cleavage of the 206K protein to yield the smaller 150K and 70K proteins (Bransom *et al.*, 1991). A protease domain was identified in ORF 206 and mapped to amino acids 555-1051. Remote upstream sequences were also involved in the cleavage. The protease domain necessary for the proteolytic processing of the 206K protein was more precisely mapped to residues 731-885 of the 150K coding region of the 206K protein with cysteine<sup>783</sup> and histidine<sup>869</sup> being the probable active sites of the enzyme (Bransom and Dreher, 1994). Tymovirus-like proteinases were shown to constitute a distinct group within the viral papain-like enzymes (Rozanov *et al.*, 1995). When the strategies of expression of the genomes of 4 other tymoviruses OYMV, EMV, BMV and physalis mottle virus [PhMV]) were compared with TYMV they were found to be similar (Kadaré *et al.*, 1992).

An investigation of the roles of the 206K and overlapping 69K proteins was done by mutating the initiation codons of both proteins (Weiland and Dreher, 1989). Mutants with a changed initiation codon at the start of ORF 206 did not replicate in protoplasts supporting earlier



suggestions that this protein was involved in replication and both the 150K and 70K proteins were shown to be essential for RNA replication (Weiland and Dreher, 1993; Dreher and Weiland, 1994). Mutants of the initiation codon of ORF 69 replicated but only poorly and studies on the product of ORF 69 showed that clones with mutations in this protein replicated in protoplasts but did not spread to establish a systemic infection in plants (Bozarth *et al.*, 1992). This, and the fact that the protein shares some characteristics with other movement proteins such as expression early in infection and having a highly basic nature, led the authors to suggest that the 69K protein may be a movement protein. An investigation of the coat protein by Dreher's group showed that mutants which produced truncated or no coat protein were able to replicate in protoplasts but the amount of (+)-sense genomic RNA and subgenomic RNAs that accumulated were less than in wild-type infections. When inoculated onto Chinese cabbage or turnip plants, the mutants produced local lesions in the inoculated leaves but did not spread systemically. The coat protein was therefore not required for cell-to-cell movement of the virus but was necessary for long-distance movement in whole plants (Bransom *et al.*, 1995).

## 1.2 ROLES OF VIRUS COAT PROTEINS

### 1.2.1 THE ROLES OF VIRAL COAT PROTEINS IN MOVEMENT

#### 1. Viruses that require their coat proteins for cell-to-cell and long-distance movement:

##### Potexviruses

Mutants of the coat protein of potato virus X were constructed and analysed (Chapman *et al.*, 1992). Those mutants with in-frame deletions in the 5' end of the gene were able to replicate in protoplasts and systemically

infected plants although the morphology of the virions was abnormal. This result indicated that the N terminus of the coat protein was not required to be intact for virion formation. Viruses with a frame-shift mutation in the 5' end of the gene or with deletions in the central part of the coat protein gene did not accumulate even in the inoculated leaf. This suggested that even cell-to-cell spread of the virus required coat protein production and encapsidation of the viral RNA (Chapman *et al.*, 1992).

The function of the coat protein in the infection process of another potyvirus, papaya mosaic virus was examined by constructing mutants with insertions of 2 amino acids and premature termination codons near the N and C termini. A mutant in which most of the coat protein was deleted, as well as one which had a frame-shift introduced at the C terminus were also produced. No symptoms were detected on the local lesion host when inoculated with any of the mutants and none of the mutants replicated in a systemic host or produced any systemic symptoms. However, one of the mutants in which an alanine residue was deleted near the C terminus produced lesions in the local lesion host which led the authors to suggest that the complete coat protein was not necessarily required for infectivity. These lesions took up to 10 days longer to appear than those produced by infection with the wild-type transcript which indicated that the coat protein was necessary for efficient cell-to-cell movement of the virus and also for long-distance movement as the mutant did not systemically infect (Sit and AbouHaidar, 1993).

### **Potyviruses**

The role of the tobacco etch virus (TEV) coat protein was examined by constructing 4 coat protein mutants; 2 with single amino acid substitutions, 1 double amino acid substitution and 1 deletion of part of the N terminus (Dolja *et al.*, 1994). The single or double point mutations were made in

order to change conserved arginine and asparagine residues which were suggested to interact via a salt bridge within a predicted  $\alpha$ -helical core. The deletion mutation produced a protein lacking the N terminal sequence which had been shown to be exposed on the virion surface. The mutations had no effect on the ability of the viruses to replicate in protoplasts, but they did not move from cell-to-cell in inoculated leaves. The mutants with the single and double amino acid substitutions were restricted to single initially infected cells whereas the mutation with the N terminal deletion showed slow cell-to-cell movement in the inoculated leaves, and did not move systemically through the plant. When the mutants were inoculated onto plants containing a TEV coat protein transgene, the viral transgene was able to rescue the cell-to-cell and systemic movement defects of all the mutants. The mutant with the deletion at the N terminus of the coat protein was able to form virions with a normal appearance which showed that this part of the coat protein was not essential in maintaining virion structure. These results suggested that the functions of the TEV coat protein in virion assembly, cell-to-cell and long-distance movement were distinct and separate (Dolja *et al.*, 1994).

Further mutants of the TEV coat protein were made which involved changing a highly conserved serine residue to tryptophan in the core domain and deleting the C terminal domain which had been shown to be exposed on the virion surface. Both mutants replicated as well as parental virus in protoplasts. In whole plants the serine to tryptophan mutant was restricted to individual initially infected cells whereas the C terminal deletion mutant was able to move from cell-to-cell but not systemically. This latter mutant was also able to form virions and was therefore assembly competent which indicated that the C terminus was dispensable for virion formation. The cell-to-cell and long-distance movement defects of the serine to tryptophan mutant were rescued in plants expressing the TEV coat protein gene but the long-distance movement defect of the C terminal

deletion was not rescued. These results again showed that the TEV coat protein was involved in cell-to-cell and long-distance transport of the virus and that these two functions required distinct regions of the coat protein (Dolja *et al.*, 1995).

In contrast to results above with tobacco etch virus, a tobacco vein mottling virus (TVMV) mutant with an insertion of 4 amino acids close to the N terminus of the coat protein behaved like the parental virus in the amount of protein and RNA produced in protoplasts. In inoculated plants the mutant developed symptoms identical to those of native viral RNA or wild-type transcript. Mutants of TEV had shown that the N terminus of the coat protein was not essential for virion assembly (Dolja *et al.*, 1994). The results with TVMV suggested that the N terminus of the coat protein in this virus was dispensable for replication, cell-to-cell and long-distance movement (Klein *et al.*, 1994).

### Comoviruses

When coat protein deletion mutants of cowpea mosaic virus were constructed, the mutants were able to replicate in protoplasts. When the mutants were inoculated onto whole plants no symptoms were seen on inoculated or systemic leaves and no virus specific proteins were detected. These results indicated that the coat protein was required for cell-to-cell and long-distance movement of cowpea mosaic virus (Wellink and van Kammen, 1989).

### Carmoviruses

A mutant of turnip crinkle virus (TCV) in which the coat protein was deleted indicated that the protein was necessary for systemic movement of the virus. The mutant produced local lesions in the inoculated leaves of *Chenopodium amaranticolor* but these were smaller than those of a wild-

type virus infection. When *Brassica campestris* plants (a systemic host of TCV) were inoculated with mutant transcript, viral RNA could only be detected in the inoculated but not the systemic leaves. The requirement of the coat protein for intercellular movement of the virus appeared to be host dependent as no mutant viral RNA was detected in the inoculated leaves of *Nicotiana benthamiana* (another systemic host of TCV). The decreased ability of the mutant to move from cell-to-cell indicated that the coat protein was a host-dependent determinant of cell-to-cell movement (Hacker *et al.*, 1992).

A further mutant of the coat protein of turnip crinkle virus was constructed with the putative calcium binding amino acids aspartic acid<sub>155</sub> and aspartic acid<sub>157</sub> both changed to asparagine. The mutant was able to replicate and accumulated coat protein to wild-type levels in protoplasts. In whole plants the mutant was detected in inoculated leaves although at much lower levels than the wild-type RNA, and it was not detected in upper leaves of inoculated plants. The coat protein was therefore required for systemic spread and efficient cell-to-cell movement of the virus (Laakso and Heaton, 1993).

### **Cucumoviruses**

Coat protein mutants of cucumber mosaic virus showed that it was required for both cell-to-cell and long-distance movement of the virus (Suzuki *et al.*, 1991). When mutants with coat protein deletions were inoculated into protoplasts RNA replication was detected but at a very low level. No coat protein was detected in protoplasts stained with a fluorescent antibody. When inoculated onto whole plants no local lesions or systemic symptoms were produced. These results suggested that the coat protein was necessary for efficient replication in protoplasts and for cell-to-cell and long-distance movement in plants (Suzuki *et al.*, 1991).

Further work on coat protein deletion and frame-shift mutants of cucumber mosaic virus showed that coat protein mutants replicated in protoplasts but that accumulation of RNA later in the life cycle was affected. In whole plants coat protein mutants did not accumulate RNA in inoculated or systemic leaves which supported earlier work suggesting that the coat protein was necessary for cell-to-cell and long-distance movement of the virus (Boccard and Baulcombe, 1993).

### **Alfalfa mosaic virus**

The coat protein of alfalfa mosaic virus had been shown to be involved in genome activation, asymmetric plus-strand RNA accumulation and cell-to-cell spread of the virus. Mutational analysis of the alfalfa mosaic virus coat protein showed that these functions in the life-cycle of the virus could be mutated separately which indicated that they were associated with different domains of the protein. The coat protein was required for all three functions; mutants that produced no coat protein or a coat protein with an N terminal deletion of 20 amino acids were defective in all three functions. The N terminal amino acids 6-38 were involved in genome activation while the insertion of non-viral amino acids at position 85 abolished cell-to-cell spread of the virus. A mutant with a C terminal deletion of 21 amino acids was defective in plus strand RNA accumulation (Van der Vossen *et al.*, 1994).

## **2. Viruses that require their coat proteins for long-distance movement:**

### **Tobamoviruses**

Mutants were created by means of insertions and deletions in the tobacco mosaic virus (TMV) coat protein gene (Dawson *et al.*, 1988). These mutants prevented assembly of the virus into virions but the virus replicated and viral RNA was able to move from cell-to-cell. Systemic

infection by the mutant viruses was not as efficient as infection by wild-type virus. In addition, the symptoms produced by the mutant viruses were different from those of wild-type virus, the amount of coat protein accumulated by the mutants varied and the mutant coat proteins were smaller than the wild-type protein (Dawson *et al.*, 1988).

Further mutants of the tobacco mosaic virus coat protein were constructed and results suggested that the ability of the coat protein to assemble into virions was necessary for long distance movement of the virus (Saito *et al.*, 1990). A frame-shift mutation in the coat protein of tobacco mosaic virus which resulted in the production of a truncated coat protein (34 amino acids instead of 158 amino acids) prevented the systemic spread of the virus but did not affect replication or cell-to-cell movement of the virus. The mutant was complemented in transgenic plants containing the TMV coat protein gene and was able to move systemically in these plants (Holt and Beachy, 1991). Chimeras of two tobamoviruses were made by inserting the coat protein of odontoglossum ringspot virus (ORSV) in place of the TMV coat protein. These viruses differ in their ability to infect *Nicotiana tabacum*: ORSV is confined to the inoculated leaves whereas TMV spreads systemically. The TMV chimera expressing the ORSV coat protein replicated and moved from cell-to-cell as efficiently as wild-type TMV. However it was deficient in long distance movement and unable to systemically infect *N. tabacum* plants. (Hilf and Dawson, 1993).

### **Sobemoviruses**

The coat protein of southern bean mosaic virus (SBMV) was shown to be necessary for systemic movement of the virus through its ability to assemble into virions. Mutants that were unable to form particles were able to move from cell-to-cell but not systemically (Fuentes and Hamilton, 1993). Studies on another sobemovirus, rice yellow mottle virus (RYMV), using a frame-shift and a deletion mutant of the coat protein showed that the coat

protein mutants replicated in protoplasts as well as the wild-type virus although in whole plants viral RNA was only detected in inoculated leaves. The amount of viral RNA which accumulated in the mutant inoculated leaves was less compared to leaves inoculated with wild-type transcript or viral RNA. This suggested that while the coat protein was not absolutely necessary for cell-to-cell movement, movement was more efficient when the coat protein was present. Rice plants inoculated with transcripts of the mutants did not develop symptoms or accumulate virus particles four weeks after inoculation which demonstrated the role of the coat protein in systemic movement. (Brugidou *et al.*, 1995).

### **Bromoviruses**

The role of the brome mosaic virus coat protein in packaging of the viral RNA and systemic spread of the virus was examined by constructing mutants at the N terminus of the coat protein (Sacher and Ahlquist, 1989). In a mutant with the N terminal 25 amino acids of its coat protein deleted, coat protein accumulation was only moderately affected but RNA packaging in protoplasts was abolished. In plants inoculated with this mutant, no virions, viral RNA or symptoms were detected. Another mutant, with the N terminal 7 amino acids of the coat protein deleted, was able to package viral RNA in protoplasts and to produce systemic infection in whole plants. These results show the importance of the brome mosaic virus coat protein in systemic infection by the virus although the first 7 amino acids were dispensable for infection of plants (Sacher and Ahlquist, 1989).

Further mutants of the brome mosaic virus coat protein confirmed these results. N terminal mutants which lacked the first 7 amino acids and had internal point mutations in the coat protein were found to differentially affect virus translocation in different host plants. This suggested that both the coat protein and host factors were involved in virus spread. Larger deletions in the coat protein or a frame-shift which resulted



in decreased production of the coat protein prevented systemic spread of the virus. The coat protein was thus not required for cell-to-cell spread but was necessary for systemic infection by the virus (Flasinski *et al.*, 1995). Deletions in the coat protein gene of another bromovirus, cowpea chlorotic mottle virus (CCMV) did not prevent replication of the virus in protoplasts. A small amount of viral RNA was recovered from mutant-inoculated leaves but none was isolated from systemic leaves. Systemic infection of cowpea plants by the mutant did not occur which showed that the coat protein was required for long-distance movement (Allison *et al.*, 1990).

### Monopartite geminiviruses

Coat protein mutants of the monopartite geminivirus, beet curly top virus, (BCTV) were made which demonstrated that it was essential for spread of the virus in contrast to the bipartite geminivirus tomato golden mosaic virus (TGMV) (see section 3 below)(Briddon *et al.*, 1989). A frame-shift mutation in the coat protein prevented the virus infecting plants although the mutant virus was able to replicate in protoplasts. However the amount of single stranded DNA produced in protoplasts was less than that in the protoplasts inoculated with transcript from the parental clone. The authors concluded that the coat protein stabilised the ssDNA by encapsidation and also played a role in the switch from dsDNA to ssDNA synthesis. The results of these experiments showed that an intact coat protein was essential for spread of the virus and hence for the production of symptoms (Briddon *et al.*, 1989). Mutational analysis of the coat protein of another monopartite geminivirus, maize streak virus, showed that the coat protein was not required for replication but was essential for systemic spread of the virus. Viral DNA was detected only in the inoculated and not the systemic leaves of mutant-inoculated maize plants. The coat protein was also necessary for the appearance of classic disease symptoms on infected plants (Lazarowitz *et al.*, 1989).

### Furoviruses

The coat protein of the furovirus beet necrotic yellow vein virus was shown to be necessary for long-distance movement of the virus (Quillet *et al.*, 1989). An amino acid change at position 119 of the coat protein from arginine to serine produced a mutant which produced local lesions in *Chenopodium quinoa*. Coat protein could be detected in these lesions but no virions or viral RNA were present in extracts of the lesions. However infectious RNA was isolated by phenol extraction and was infectious when passaged. These results showed that coat protein and viral RNA were present in the inoculated leaves but that the RNA was not encapsidated by the mutant coat protein. In spinach plants the mutant was confined to local lesions whereas the wild-type transcript spread systemically through the plant (Quillet *et al.*, 1989).

### Dianthoviruses

The role of the red clover necrotic mosaic virus coat protein was investigated by creating insertion, deletion and frame-shift mutants (Xiong *et al.*, 1993). These mutants were all able to replicate and produce normal symptoms in the inoculated leaves of *Nicotiana benthamiana* and *Nicotiana clevelandii* at 15°C and 25°C. In addition all mutants infected *N. benthamiana* plants systemically at 15°C; the symptoms were similar to a those of a wild-type infection but took longer to appear. However none of the mutants gave systemic symptoms when the plants were held at 25°C. The ability of the mutants to produce systemic symptoms at the same rate as the wild-type virus depended on the temperature and the host genotype. The coat protein was not required for replication and cell-to-cell movement of the virus and depending on the host genotype and environmental conditions, may or may not be required for long-distance spread of the virus (Xiong *et al.*, 1993).

## Tymoviruses

Coat protein mutants of turnip yellow mosaic virus were designed to elucidate the role of the protein in infection by the virus (Bransom *et al.*, 1995). Three mutants were constructed: a deletion mutant which produced a coat protein of 71 amino acids instead of the normal 189 amino acids (amino acids 22-139 deleted), a frame-shift mutant which inserted 5 nucleotides in a central part of the gene and resulted in the production of a protein which contained the first 114 codons of the coat protein fused to 21 codons encoded by the read-through domain of the overlapping protein and a protein with a mutation in the initiation codon which prevented translation of the coat protein. All three mutants were able to replicate in protoplasts although the amount of (+)-strand RNA accumulated was less than wild-type. This indicated that the coat protein was not essential for viral replication in protoplasts although it seemed to influence the accumulation of (+)-strand RNA. When inoculated onto Chinese cabbage or turnip plants, local lesions were produced by all mutants in the inoculated leaves but no systemic symptoms appeared which suggested that the coat protein was required for long-distance movement of the virus but was not necessary for cell-to-cell movement (Bransom *et al.*, 1995).

### *3. Viruses with coat proteins not required for cell-to-cell and long-distance movement:*

#### Bipartite geminiviruses

Experiments with the bipartite geminivirus tomato golden mosaic virus where the coat protein gene was mutated to produce proteins with an insertion at the N terminus, a frame-shift mutation giving a coat protein consisting of the N terminal 63% of the protein and a deletion resulting in a protein with only the C terminal 30 amino acids were done. Results from experiments with these mutants showed that the coat protein was not

necessary for viral DNA replication, systemic spread or symptom development (Gardiner *et al.*, 1988).

A similar result was obtained with coat protein mutants of another bipartite geminivirus. Two deletion mutants and one restriction fragment inversion mutant of the coat protein of bean golden mosaic virus were constructed. These mutants replicated in inoculated bean plants and induced systemic symptoms similar to a wild-type infection. Viral DNA was isolated from mutant-infected plants but no coat protein could be detected (Azzam *et al.*, 1994).

### **Tombusviruses**

To investigate the role of the tomato bushy stunt virus coat protein, a mutant was constructed with a 50 nucleotide deletion that introduced a termination codon 9 nucleotides downstream of the deletion (Scholthof *et al.*, 1993). This had no effect on the infectivity of the mutant in protoplasts which indicated that the coat protein was not required for RNA replication. The mutated virus was able to move systemically through whole plants as virus specific RNAs were detected in non-inoculated leaves although no virions were detected. The symptoms produced on plants inoculated with the mutant virus were typical of an infection with the wild-type virus. The coat protein of tomato bushy stunt virus was therefore shown to be dispensable for cell-to-cell and long-distance movement of the virus (Scholthof *et al.*, 1993).

A coat protein mutant of cucumber necrosis virus (CNV), another tombusvirus, was constructed by deleting the 16 amino acids of the protruding (P) domain of the coat protein (McLean *et al.*, 1993). This domain was thought to be important in stabilising virus particles. When inoculated onto *Nicotiana clevelandii* plants, a systemic host of CNV, the mutant virus was able to move from cell-to-cell and systemically although the appearance of systemic symptoms was delayed compared with infections

with wild-type transcript. When leaf sap from these systemically infected plants was passaged onto other *N. clevelandii* plants, they developed local lesions and systemic symptoms which were typical of a wild-type infection. The coat protein mutant also moved systemically in *Nicotiana benthamiana* which is another systemic host of CNV. No virions, coat protein subunits, or virus coat protein specific RNA could be detected in sap from mutant-infected plants. RT-PCR was used to see what regions, if any, of the coat protein ORF remained in mutant infected plants and results showed that only the first 27 nucleotides of the coat protein gene were retained and the remainder of the coat protein coding sequence had been lost. When this RT-PCR product was cloned into the corresponding region of a wild-type CNV cDNA clone, the symptoms produced in transcript inoculated plants were indistinguishable from those produced in a wild-type infection. These results demonstrated that the complete CNV coat protein was not required for replication, cell-to-cell or long-distance transport of the virus (McLean *et al.*, 1993).

Further mutations of the CNV coat protein gene with insertions, deletions and frame-shifts were constructed. When these mutants were inoculated onto *N. clevelandii* plants they produced necrotic lesions and were able to move systemically through the plants although at a slower rate than that observed with wild-type transcripts. The coat protein coding sequences had been deleted as had been found with the P domain mutant of CNV (McLean *et al.*, 1993) and virus particles were not detected for any of the mutants. The P domain therefore was necessary for the formation of virus particles or for their stability (Sit *et al.*, 1995).

A mutation in the S domain, a portion of the coat protein essential for the maintenance of virion structure, was made in the coat protein of cymbidium ringspot virus (Dalmay *et al.*, 1992). Six amino acids were deleted from the coat protein including 2 from the S domain. This coat protein mutant produced lesions in the inoculated leaves of *N. clevelandii*

but did not infect it systemically. Inoculation onto *N. benthamiana* plants resulted in the production of local lesions and systemic symptoms which were similar to those produced on infection with wild-type transcript. Viral RNAs were isolated from inoculated leaves of *N. clevelandii* and from inoculated and systemically infected leaves of *N. benthamiana* but no virus particles were found in leaf tissue infected with the coat protein mutant. The results suggested that a mutation affecting the formation of virions did not prevent cell-to-cell movement of the virus but, depending on the host plant, had an effect on long-distance spread of the virus (Dalmay *et al.*, 1992).

### Hordeiviruses

Mutants of the hordeivirus barley stripe mosaic virus were produced with deletions and an initiation codon mutation in the coat protein gene. All of the mutants were fully infectious in barley plants. In the dicotyledonous hosts *Chenopodium amaranticolor* and *Nicotiana benthamiana*, none of the deletion mutants were infectious but the point mutation produced wild-type symptoms. No coat protein could be detected in leaves infected by the mutant which showed that the coat protein was not required for replication or systemic movement of the virus (Petty and Jackson, 1990).

### 1.2.2 THE ROLE OF VIRAL COAT PROTEINS IN VECTOR TRANSMISSION

The coat proteins of some viruses have been associated with transmission of the virus by their vectors. Monopartite geminiviruses such as beet curly top virus (BCTV) are transmitted by leafhoppers whereas bipartite geminiviruses such as African cassava mosaic virus (ACMV) are transmitted by whiteflies. When a chimeric virus was constructed by replacing the ACMV coat protein with that of BCTV, the leafhopper vector

of BCTV was able to transmit the chimeric virus and BCTV, but not ACMV. These results showed that the specificity of leafhopper transmission from the insect to the plant resided in the coat protein (Briddon *et al.*, 1990). A coat protein mutant of maize streak virus (MSV), a monopartite geminivirus, was not transmitted by its leafhopper vector. Leafhoppers fed on inoculated leaves of plants infected with a mutant lacking the coat protein did not transmit the virus which suggested that the coat protein was required for insect transmission of the virus (Lazarowitz *et al.*, 1989).

The role of the coat protein in the transmission of bean golden mosaic geminivirus (BGMV) by its whitefly vector was examined by constructing coat protein mutants. All of the mutants systemically infected bean plants when inoculated by electric discharge particle acceleration. However none of the mutants was transmitted by whiteflies to other bean plants which confirmed that the BGMV coat protein was required for whitefly transmission of the virus (Azzam *et al.*, 1994).

Determinants of aphid transmissibility in other viruses such as the potyviruses and cucumoviruses have been shown to reside in the coat protein (Atreya *et al.*, 1991; Kantrong and Sako, 1993; Chen and Francki, 1990). Experiments by Baulcombe *et al.* (1993) showed that aphid transmissibility of a potyvirus, potato aucuba mosaic virus (PAMV), lay in the DAG amino acid sequence located in the N terminal region of its coat protein. This amino acid motif had been identified in the coat proteins of potyviruses and was required for their aphid transmissibility. When the N terminal 40 amino acids of the PAMV coat protein containing the DAG sequence was substituted into PVX (a potyvirus which is not aphid borne) in place of its own N terminus, the recombinant PVX virus was then spread by aphids. These results showed clearly that the DAG motif previously

identified in potyvirus coat proteins was necessary for aphid transmission of the potyvirus PAMV (Baulcombe *et al.*, 1993).

The transmissibility of viruses by vectors other than insects has been shown to be associated with the coat protein. Some tombusviruses may be transmitted by a fungal vector the specificity of which depends on the coat protein. Cucumber necrosis virus (CNV) is transmitted by *Olpidium bornovanus* but the cherry strain of tomato bushy stunt (TBSV) is not. When reciprocal exchanges were made between the coat proteins of these viruses, TBSV virions with the CNV coat protein were transmitted by the fungus but CNV virions with a TBSV coat protein were not. These results provided evidence that the coat protein of CNV contained determinants which specify transmission of the virus by *O. bornovanus* (McClean *et al.*, 1994).

### 1.2.3 THE ROLE OF VIRUS COAT PROTEINS IN PATHOGENESIS

Interactions between viruses and their plant hosts result in the production of visible symptoms which range from mild chlorosis to severe necrosis. Little is known about the processes involved in the formation of these symptoms. The hypersensitive response (HR) manifested in the formation of local necrotic lesions may be seen as an attempt by the plant to limit the spread of the virus by confining it to the site of infection. According to the gene for gene hypothesis, some viral gene product specifically interacts with a host gene product to trigger the HR. Until recently, little was known about host resistance genes which protect against viral infection. Studies on satellite RNAs and some viruses have also provided information on which genes of these viruses are involved in determination of the host response.



Most work on the genetics of virus-host interactions has been done with two systems. One is the interaction of tobacco mosaic virus with the N' gene in tobacco and the second is the interaction of potato virus X with the Rx gene in potatoes.

### 1. TMV vs. N' resistance gene

The symptoms induced by TMV infection on some plants have been shown to be controlled by host genes called N and N'. Plants with the N gene, which is naturally found in *Nicotiana glutinosa*, can localise all strains of TMV, including the common strain, causing necrotic local lesions (Takahashi, 1956). One exception to this is the TMV strain Ob which can overcome the N gene mediated resistance (Padgett and Beachy, 1993). The N gene was recently cloned from tobacco and shown to encode a protein of 131.4K with an N terminal domain showing similarity to cytoplasmic domains of the *Drosophila* Toll protein and the interleukin-1 receptor (Whitham *et al.*, 1994). This led the authors to suggest that the product of the N gene may function as a receptor which interacts with a TMV gene product resulting in the induction of defence mechanisms. The N' gene from *Nicotiana sylvestris* allows plants containing this gene to localise most strains of TMV but not the common strain (Valleau and Johnson, 1943).

The involvement of the coat protein gene in the induction of the N' gene was demonstrated by making recombinants between two strains of TMV, one of which was the common strain (TMV-OM) capable of systemic spread in *N. sylvestris* and other plants with the N' gene and the other was the tomato strain (TMV-L) which induced the necrotic response in *N. sylvestris* and other tobacco plants with the N' gene. The coat protein gene of TMV-L was substituted into the genome of TMV-OM and the resulting recombinant was able to induce the necrotic response. This demonstrated

that the viral factor of TMV-L that induced the necrotic response in N' plants was encoded in the coat protein gene sequence (Saito *et al.*, 1987).

Further work on the coat protein gene showed that the hypersensitive response of N' plants to TMV was determined by a single nucleotide change in the coat protein gene. A local lesion mutant was obtained and parts of it were exchanged with the cloned U1 (common strain) parent virus. These hybrids were analysed to determine which sequences of the mutant were responsible for the induction of the HR. A point mutation in the coat protein gene which encoded phenylalanine in exchange for serine at position 148 of the mutant virus was found and was responsible for inducing local lesions in *N. sylvestris* (Knorr and Dawson, 1988). In order to see if other mutations in the coat protein could also be responsible for the induction of the HR, mutants were constructed with 4 separate amino acid substitutions in the coat protein. All of them were able to induce the HR in *N. sylvestris* which demonstrated that a number of point mutations could alter the virus-host relationship (Culver and Dawson, 1989a). Although all of the mutants were able to induce the HR, some of them were strong elicitors of the HR and caused rapid lesion formation, whereas others were weaker elicitors with slower forming lesions.

To determine if the HR in *N. sylvestris* was induced by the altered RNA or coat protein of the mutants, the translational start sites of the coat protein were removed from cDNA clones of one HR inducing mutant and the systemically infecting U1 strain. Transcripts from these mutants were infectious and able to move from cell-to-cell in *N. tabacum* plants with the N gene but failed to induce the HR in *N. sylvestris*. These results showed that the altered coat protein of the mutant was the elicitor of the HR in *N. sylvestris* (Culver and Dawson, 1989b). To investigate if other viral

components or replication processes were involved in inducing the HR, transgenic *N. sylvestris* plants were generated which expressed the coat protein ORF of either the HR inducing mutant or the wild-type virus that did not induce the HR. Two HR inducing mutants, one a strong elicitor of the HR and the other a weak elicitor, were chosen. Those plants which expressed the HR eliciting coat proteins developed symptoms characteristic of the N' gene HR while those which expressed the wild-type non-HR eliciting coat protein showed no symptoms. This result demonstrated that the expression of coat proteins alone elicited the N' gene HR. The phenotypic differences between the strong and weak elicitors of the HR were maintained through the expression of their coat proteins in N' gene plants (Culver and Dawson, 1991).

Further work on coat protein mutants of TMV-L showed that changing the coat protein gene affected the ability of the virus to induce the HR in plants with the N' gene. Mutants with deletions in the C terminal region of the protein were constructed and it was shown that only small deletions in the C terminus were tolerated for the virus to retain the ability to induce the HR. Chimeras of the coat protein gene were made which contained the N terminal 2/3 and C terminal 1/3 of the protein separately derived from TMV-L and TMV-OM. Transcripts from both chimeras induced necrotic local lesions in N' plants which indicated that the site responsible for eliciting the response mapped to both regions of the protein for TMV-L. These results suggested that a structural feature rather than a small part of the coat protein or RNA sequence was responsible for the HR in N' plants (Saito *et al.*, 1989).

TMV gene products other than the coat protein are also involved in overcoming host resistance to viral infection. The replicase gene of TMV-Lta1 was found to be responsible for its ability to overcome resistance

conferred by the *Tm-1* gene in tomato plants (Meshi *et al.*, 1988) and the movement protein gene of TMV-Ltb1, a resistance breaking strain of the virus was able to infect tomatoes with the *Tm-2* gene (Meshi *et al.*, 1989). The viral factor which interacts with the N gene in tobacco plants is yet to be elucidated.

## 2. PVX vs. Rx resistance gene

Potato plants carrying the dominant resistance genes Nb, Nx or Rx respond to infection by potato virus X (PVX) in different ways, and strains of the virus have been classified based on this response. Group 1 strains of the virus induce the HR in the presence of both Nb and Nx genes, group 2 strains induce the HR only in the presence of the Nb gene and those of group 3 only in the presence of the Nx gene. Group 4 strains of the virus are resistance breaking and do not induce the HR in plants with either the Nb or Nx genes. Plants with the extreme resistance gene Rx are resistant to infection by most strains of PVX although one strain PVX<sub>HB</sub>, was identified which systemically infected plants carrying the Rx gene.

In order to investigate the viral determinants involved in resistance breaking of Nx and Rx, hybrid viral genomes were constructed using PVX<sub>UK3</sub> an isolate of group 3 and PVX<sub>HB</sub> the resistance breaking strain of group 4. Hybrids which contained the coat protein gene of PVX<sub>HB</sub> showed resistance breaking properties when inoculated onto potato plants with the Nx and Rx genes. Therefore it was concluded that these resistance breaking properties resided in the coat protein gene (Kavanagh *et al.*, 1992). Coat protein mutants and hybrids were constructed to further analyse which parts of the protein were responsible for its interaction with the Rx gene. These experiments used strains PVX<sub>HB</sub> and PVX<sub>CP4</sub>, rather than PVX<sub>UK3</sub>, as PVX<sub>CP4</sub> was more closely related to PVX<sub>HB</sub> being 89% similar at the

nucleotide sequence level. Results of these experiments showed that the amino acids at positions 121 and 127 (with codon 121 being the major determinant) of the coat protein determined whether the viral isolate was sensitive to or overcame the Rx mediated resistance. PVX<sub>HB</sub> and mutants with lysine and arginine at positions 121 and 127 respectively, were able to overcome the resistance of Rx whereas, when these codons encoded threonine and arginine, the viral strains were sensitive to Rx resistance. Therefore the resistance was induced when there was a threonine residue at position 121 (Goulden *et al.*, 1993).

PVX isolates which induce resistance in potato plants carrying the Rx gene also elicit the production of necrotic lesions on *Gomphrena globosa*: the resistance breaking strain PVX<sub>HB</sub> infects the inoculated leaves of *G. globosa* without producing necrotic lesions. PVX<sub>HB</sub>/PVX<sub>CP4</sub> hybrids were designed to see if there was a local lesion determinant in the coat protein of PVX<sub>CP4</sub>. Analysis of these hybrids showed that a threonine at position 121 of the coat protein was required for the elicitation of local lesions in *G. globosa*. This result, and those of Goulden *et al.*, 1993, indicated that both potato plants containing the Rx gene and *G. globosa* plants were recognising the same feature of the virus coat protein. Following recognition by this factor, a response was induced in the host plant (Goulden and Baulcombe, 1993).

### 3. Pathogenesis determinants in other viruses

In addition to the systems mentioned above, work has been done on other viruses to see which viral determinants were involved in the production of symptoms.

Satellite RNAs of cucumber mosaic virus affect the symptoms produced in host plants by the virus. The symptoms can be attenuated or exacerbated by the satellite with attenuation of symptoms being the most common effect (Roossinck *et al.*, 1992). The effect of the satellite RNA on symptoms depends on the helper virus strain and the host plant. Inoculation of tomato plants with a particular combination of satellite RNA and helper virus produced markedly different symptoms than those produced in tobacco plants inoculated with the same combination of satellite and helper virus (Collmer and Howell, 1992).

Studies with the double stranded DNA genome of cauliflower mosaic virus showed that gene VI encoded a domain which controlled systemic spread of the virus (Schoelz *et al.*, 1986). Point mutations in gene VI changed the symptoms produced in host plants infected with cauliflower mosaic virus from systemic mosaic to local necrotic lesions (Daubert and Routh, 1990). These results showed that gene VI of CaMV determined whether the host plant reacted with a HR or allowed systemic infection by the virus.

Results from investigations on host determinants and symptom severity of monopartite and bipartite geminiviruses showed that different genes were involved for the different viruses. With beet curly top virus, the primary determinants of virulence mapped to the left side of the genome (Stenger *et al.*, 1994), which supported earlier work in which the product of ORF L4 was shown to be a major determinant of pathogenesis (Stanley and Latham, 1992). ORF L4 of another monopartite geminivirus, tomato leaf curl virus, was also shown to encode a polypeptide involved in symptom development (Rigden *et al.*, 1994). Mutants of maize streak virus in which the coat protein had been deleted did not develop classic disease symptoms in inoculated plants (Lazarowitz *et al.*, 1989), and a mutant

lacking most of the coat protein ORF of tomato golden mosaic virus produced delayed and attenuated symptoms (Gardiner *et al.*, 1988).

The coat proteins of other viruses have been shown to affect the type of symptoms produced in infection of host plants. Two strains of alfalfa mosaic virus produce different symptoms when inoculated onto Samsun NN tobacco plants. One, strain 425 (the Leiden isolate), caused mild chlorosis whereas another, yellow spot mosaic virus (YSMV) caused local necrotic lesions. Fragments were exchanged between the strains and symptom expression was found to be associated with the coat protein gene. When a glutamine residue at position 29 of the 425 coat protein was changed to arginine, the symptoms changed from mild chlorosis to severe necrosis. This suggested that a single amino acid change was sufficient to induce a hypersensitive response (Neeleman *et al.*, 1991).

The biological significance of the P (protruding) domain of the turnip crinkle virus (TCV) coat protein was investigated by site-directed mutagenesis. P-domainless mutants failed to accumulate in plants or protoplasts but revertants that elicited atypical systemic symptoms in *N. benthamiana* appeared several weeks post-inoculation. One revertant elicited milder symptoms than the wild-type virus whereas a second revertant elicited more severe symptoms. The coat proteins of the revertants differed from the wild-type in at least two amino acid positions in the hinge region which joins the S (shell) domain to the P domain. These different amino acids were engineered into the wild-type TCV background and it was found that mutations in the hinge region were sufficient to elicit the milder symptoms. Mutations in the coat protein of TCV therefore affected the symptoms expressed in *N. benthamiana* (Heaton *et al.*, 1991).

A single amino acid substitution in the coat protein of cucumber mosaic virus (CMV) affected the symptoms produced in *Nicotiana tabacum*. Some strains of CMV induce a bright yellow/white chlorosis in tobacco instead of the light green/dark green mosaic induced by most CMV strains. Recombination of Fny-CMV, a green mosaic strain, and M-CMV, a chlorotic strain, localised the chlorosis-inducing domain to a region of the coat protein in which the two strains differed by only two nucleotides. When M-CMV was altered to include the cytosine of Fny-CMV in place of uracil at nucleotide 1642, the resulting amino acid change from leucine to proline at position 129 of the coat protein produced symptoms identical to Fny-CMV in tobacco plants (Shintaku *et al.*, 1992).

#### 1.2.4 COAT PROTEIN MEDIATED RESISTANCE

The phenomenon of cross-protection, where inoculation of plants with a mild strain of a virus prevented infection with a more severe strain, had been known for a number of years before the production of the first transgenic tobacco plant expressing the TMV coat protein gene (Horsch *et al.*, 1985). These transgenic plants were resistant to TMV infection (Powell-Abel *et al.*, 1986), and since this work was reported many plants have been transformed with different coat protein genes which have enabled them to resist infection (Beachy *et al.*, 1990).

This research has been extended to include other viral genes such as the replicase gene and plants transformed with these genes have been protected against viral infection (Baulcombe, 1994). In most cases where plants have been transformed with viral genes, it is likely that most of the resistance to viral infection results from a viral RNA-based mechanism rather than from the encoded viral proteins that are produced (Baulcombe, 1994; Dougherty *et al.*, 1994).



### 1.3 TYMOVIRUS COAT PROTEIN SECONDARY STRUCTURE PREDICTION

Many viruses produce large numbers of icosahedral virions which can be easily crystallised. Those that have been crystallised and their coat proteins analysed by X-ray diffraction include tomato bushy stunt tombusvirus (TBSV) (Harrison *et al.*, 1978), southern bean mosaic sobemovirus (SBMV) (Abad-Zapatero *et al.*, 1980), turnip crinkle carmovirus (TCV) (Hogle *et al.*, 1986), satellite tobacco necrosis virus (STNV) (Liljas *et al.*, 1982), foot-and-mouth disease aphthovirus (FMDV) (Acharya *et al.*, 1989) and human rhinovirus (Rossmann *et al.*, 1985). The structures of these coat proteins were shown to be variants of an eight stranded anti-parallel  $\beta$  barrel. The only exception to this general structure of icosahedral virions was that of the coat protein of the icosahedral particles of bacteriophage MS2. When determined by X-ray crystallography MS2 virions were shown to have a distinctive structure which had no similarity to other icosahedral RNA viruses (Valegard *et al.*, 1990). Preliminary X-ray diffraction analysis of the virions of erysimum latent tymovirus suggested that the coat protein of these virions, and by inference those of all tymoviruses, have an eight stranded anti-parallel  $\beta$  barrel structure (Jose Varghese, personal communication to Adrian Gibbs).

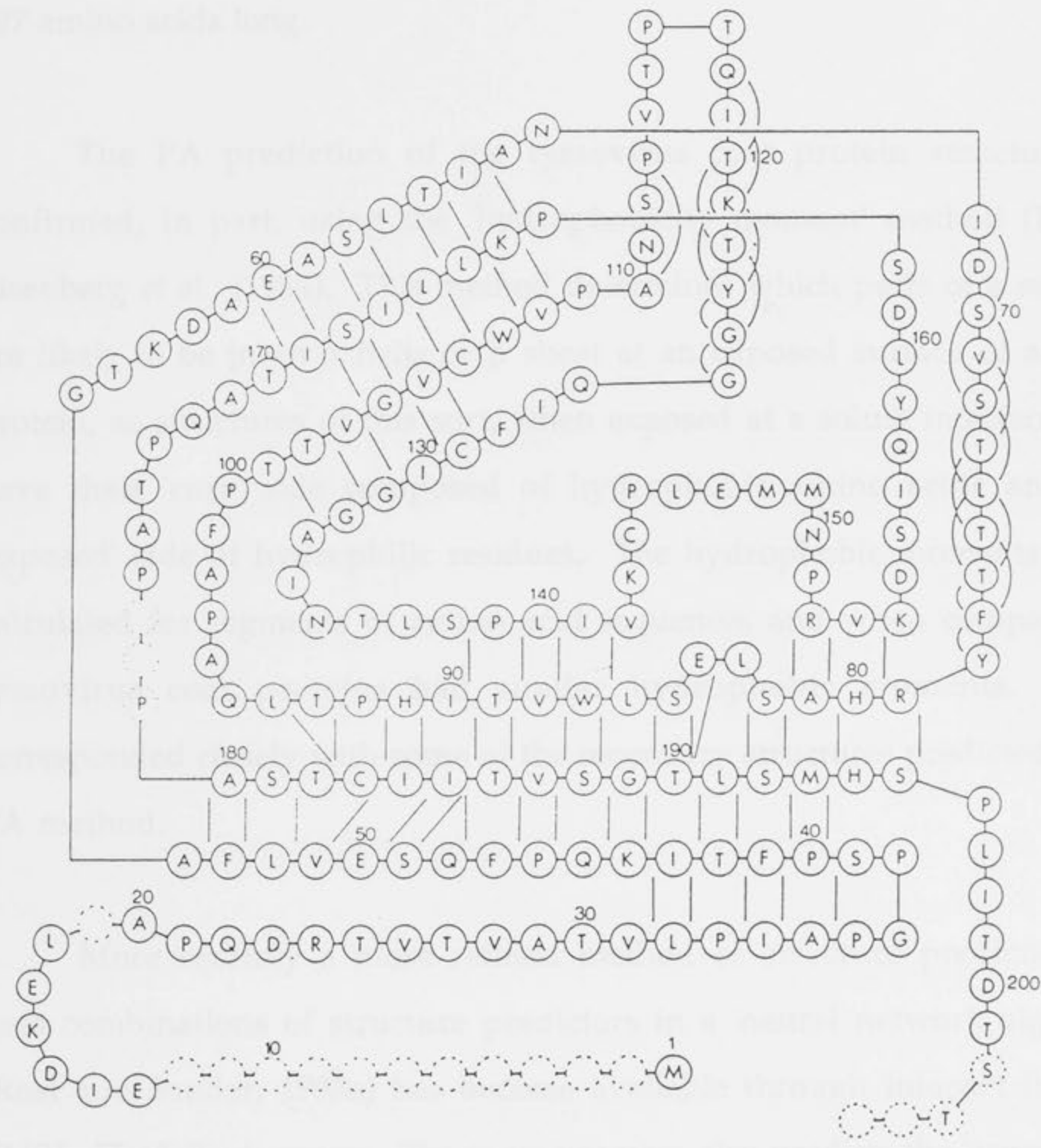
As the number of protein sequences generated by molecular methods increases much more rapidly than the number of protein structures determined by X-ray crystallography, methods have been devised to predict the structures of proteins from their sequences. A prediction of the secondary structure of the TYMV coat protein was first reported by Argos (1981). Five different methods were used to predict the structure from the amino acid sequence and it was found that the sequence was largely  $\beta$ -sheet with only one of two likely  $\alpha$  helical regions convincingly detected. Adrian Gibbs, (personal communication) between 1984-1986, used the same five

methods with five others (in a package distributed by the Leeds Protein Group) to examine all the plant viral coat proteins of known structure and he concluded from his results that these methods were ineffective and probably inaccurate.

However, when the published sequences of some  $\beta$ -barrel coat proteins of known structure were used to test the profile alignment method (PA method) of Gribskov, McLachlan and Eisenberg (Gribskov *et al.*, 1987) consistent results were obtained. In the first step of the PA method, the amino acid sequences of proteins of known structure were aligned by those structures, rather than by their sequence similarity, to produce a 'profile'. That profile was then aligned with the query sequence (the one of unknown structure) using a modified version of the dynamic programming algorithm of Needleman-Wunsch (Needleman and Wunsch, 1970) and, in this instance, the Dayhoff MDM 78 matrix of amino acid similarities. Using the PA method with a profile from three plant viral proteins of known structure (TBSV, SBMV, TCV) and carnation mottle virus (CaMV), which was also used to construct the profile as it had been included in a structural alignment used by Carrington *et al.* (1987) and using as query sequences each of ten tymovirus coat protein sequences, it was possible to establish whether there was any consistent positioning of query tymovirus sequences with the profile. It was found that, in the central two-thirds of the 'profile', where the 'profile' sequences were aligned by the  $\beta$ -sheets of the eight-stranded barrels, all ten tymovirus sequences aligned in the same relative position. Only at the N- and C-termini was there some ambiguity in the alignment of different tymovirus sequences. The consensus of these alignments was then mapped to the published structure of the four 'profile' proteins, and hence a prediction of the structure of the tymovirus coat protein was produced (Figure 1.1) (Adrain Gibbs, personal communication). This structure resembles those of the 'profile' proteins in that all the  $\beta$ -sheet

Figure 1.1 Structure of the tymovirus coat protein as predicted by Gibbs using the PA method. The empty circles correspond to gaps required to align the tymovirus coat proteins, notably the longer N terminus of *erysimum* latent tymovirus.

regions in the case of the proteins were present, but they are now separated in  
 distinct regions from each other. Thus the protein was now present in a  
 single region of the  $\beta$ -tubulin side of the tubulin dimer, and this  
 explains why tubulin dimers are only 100-200 amino acids long,  
 whereas those of the coat protein tend to contain the protein over 200-  
 300 amino acids long.



DNA. Finding errors. The same program did produce a number of  
 of different parts of an amino acid sequence. These methods were used to  
 use the protein prediction of the structure of the structure of the protein  
 made by Agmon, L. and others (1978).

The method uses a protein signature and a search algorithm system  
 which uses this profile for prediction. The algorithm is built up by two  
 steps. In the first step the SWISSPROT sequence database is searched for

regions in the core of the protein were present, but two of four superficial  $\alpha$ -helical regions were missing. Thus the tymovirus coat protein seems to be a trimmed version of the  $\beta$ -barrel folds of other similar coat proteins, and this explains why tymovirus coat proteins are only 189-202 amino acids long, whereas those of the coat proteins used to construct the 'profile' were 260-387 amino acids long.

The PA prediction of the tymovirus coat protein structure was confirmed, in part, using the 'hydrophobicity moment' method (HM) of Eisenberg *et al.*, (1984). This method determines which parts of a sequence are likely to be in an  $\alpha$  helix or  $\beta$  sheet at an exposed surface of a folded protein, as structures of this sort, when exposed at a solute interface, often have their 'core' side composed of hydrophobic amino acids and their 'exposed' side of hydrophilic residues. The hydrophobic moments can be calculated for segments of amino acid sequences and when compared, all tymovirus coat proteins had similar hydrophobic moments. These corresponded closely with some of the secondary structures predicted by the PA method.

More recently a more refined method of structure prediction that uses combinations of structure predictors in a 'neural network algorithm' (Rost and Sander, 1993a) has become available through Internet from the EMBL, Heidelberg server. The same program also predicts the 'accessibility' of different parts of an amino acid sequence. These methods were used to test the earlier predictions of the structure of the tymovirus coat protein made by Argos (1981) and Adrian Gibbs.

The method uses a profile alignment and a neural network system which uses this profile for prediction. The alignment is built up in two steps. In the first step the SWISSPROT sequence database is searched for

homologous sequences. The sequences from the database were aligned consecutively with the sequence of unknown structure. After the alignment of each sequence a profile was compiled and used to align the next sequence and so on. The profile was then used by the neural network system for predicting the structure for which the expected reliability was >82% (Rost and Sander, 1993a; 1993b; Rost *et al.*, 1993; Rost *et al.*, 1994; Rost and Sander, 1994).

The coat proteins sequences of 18 tymoviruses were sent individually to the EMBL server and the predicted structure was returned. The prediction results were recorded on a CLUSTAL alignment of the tymovirus coat protein sequences. A consensus of the 18 coat protein predictions was generated; for each position the predicted structure (helix, sheet, loop or not predicted) in 50% or more of the sequences was recorded as the consensus for that position. Figure 1.2 shows the aligned consensus sequence generated. This consensus was then mapped onto the structure predicted by Gibbs (Figure 1.3) to see how they compared.

In addition to predicting structural elements, the Protein Predict method also predicted hydration accessibility for each amino acid residue (i.e. whether it was exposed or buried). Alignments were made in the same way for accessibility information predicted for each tymovirus coat protein. Again, data for which the expected reliability was >82% was used and a consensus sequence for buried and exposed residues generated (Figure 1.4). The consensus was also mapped onto the structure predicted by Gibbs (Figure 1.5).

Results from the Protein Predict method of the coat protein secondary structure correlate well with that predicted by Gibbs. Most regions predicted to be  $\beta$  sheets, loops and  $\alpha$  helices by the two methods agree. The only

```

APLV | L-----LLL...LLLLLLLLL---LLLLLLLLLLLLL.E...EE...L...EEE..L.HHHHHHHH...EEE.LLL.LLLLLL
BMV  | L-----LL...LLLLLLLLL---LLLLLLLLLLLLL.EEE..EEEE..L...EEEE..LL...EEE.LLLLLLLLLL
CYVV | L-----LL...LLLLL.LLLL---LLLLLLLLLLLLL.EE...EE...LL...EEEE..L.HHHHHHHH...HH...LLLLLLLLLLLL
DMV  | L-----LLL...E.LLLLLLLLLL---LLLLLLLLLLLLL.EEE..EEEE...EEEE..L.HHHHHH...EEE.LLLLLLLLLL
ELV  | LLLLLLLLL.LLL..EE..LLLLLLLLL.LLLLLLLLLL.EEE..EE...LL...EEEE..LL...EEEE.LLLLLLLLLL
EMV  | L-----LL...LLLLLLLLL---LLLLLLLLLLLLL.EEE..EE...L.L...EEE...HHHHHHHHHH.H...E.LLLLLLLLLL
KYBP | L-----LL...LLLLL.LLL---LLLLLLLLLLLLL.EEE..EE...LL...EEEE..L.HH...EE.LLLLLLLLLL
KYJB | L-----LL...LLLLL.LLL---LLLLLLLLLLLLL.EEE..EE...LL...EEEE..L.HH...EE.LLLLLLLLLL
KYPD | L-----LLL...LLLLLLLLL---LLLLLLLLLLLLL.EEE..EEEE..L.L...EEEE..L.HHHHHH...EEE.LLLLLLLLLL
OkMV | L-----LLL...LLLLLLLLL---LLLLLLLLLLLLL.EE...EE...LLLLL.EEEE..LL...EEE.LLLLLLLLLL
OYMV | L-----LLL...LLLLLLLLL---LL.LLLLLLLLLL.EEE..EE...LL...EEEE..L.HHHH.H...EEEE.LLLLLLLLLL
PhyMV | L-----LLL...E.LLLLLLLLLL---LLLLLLLLLLLLL.EEE..EE...L.L...EEEE..LL.HHHH.H...EEE.LLLLLLLLLL
TYMVBL | L-----LLL.LLLLLLLLLL---...LLLLLLLLL.EEE..EEEE..L...EEEE..L...EE.LLLLLLLLLL
TYMVCa | L-----LLL.LLLLLLLLLL---LLLLLLLLLLLLL..EE..EE..L.LL.EEEE..L.H.HH...EE.LLLLLLLLLL
TYMVCL | L-----LLL.LLLLLLLLLL---L...LLLLLLLLL.EEE..EE...L.L...EEEE...EEE.LLL.LLLL
TYRoth | L-----LLL.LLLLLLLLLL---LLLLLLLLLLLLL.EEE..EE...LLL.EEEE..L.H.HH...EE.LLLLLLLLLL
TYType | L-----LLL.LLLLLLLLLL---...LLLLLLLLL.EEE..EEEE..L...EEEE..L...EE.LLLLLLLLLL
WCuMV | L-----LLL...E.LLLLLLLLLL---LLLLLLLLLLLLL.EEE..EEEE..L...EEE...HHHHHHH.HH.H...EEE.LLLLLLLLLL

Consen | L      LLL...LLLLLLLLL  LLLLLLLLLLLLL.EEE...EE...L...EEEE..L.HH...EEE.LLLLLLLLLL

APLV | .EEEEEE.LLLLLL.HHHHHH.LLL.EEE...LLL.E...LL...LLL..EE.LL..EEE..L--LLLLLLLLL.EEEEEEE.EEE.LLL..LL
BMV  | .EEEEEE.LLLLLL...HH..LL.EEEE...LLLLL.EE..LL...LL..EEE.L..EEEE..L--LLLLLLLLL.EEEEEEEEEE.LL..LL
CYVV | .EEEEEE.LLLLLL.HHHHHH.LLL.EEE...LLL..E..LLL.LL...EEEE.L..EEEE.L--LLL-LLLL.EEEEEEE.EEE.LLL..LL
DMV  | EEEEEEE.LLLLLL.HHHHHH..LL.EEEE...LLL.EE..LL...LL..EEE.L..EEEE..L--LLLLLLLLL.EEEEEEEEEE.LL..LL
ELV  | EEEEEEE.LLLLLL.HHHHHH..LL..EEE...LLL.LL..LL..LL..EE.L..EEEE.LLLLLLLLLL.EEEEEEEEEE.LLL..LL
EMV  | EEEEEEE.LLLLLL.HHHHHH.LL..EEE...LLL...LL...L...E..LL..EEE..L--LLLLLLLLL.EEEEEEEEEE.LLL..LL
KYBP | .EEEEEE.LLLLLL.HHHHHH.LL..EEE...LLL.EE..LLL.LL...EEEE.L..EEEE..L--LLLLLLLLL.EEEEEEEEEE.L.L..LL
KYJB | .EEEEEE.LLLLLL.HHHHHH.LL..EEE...LLL.EE..LLL.LL...L.EEEE.L..EEEE..L--LLLLLLLLL.EEEEEEEEEE.L...LL
KYPD | EEEEEEE.LLLLLL.HHHHHH..LL..EEE...LLL..E..LL...L...EEE.L..EEE..L--LLLLLLLLL.EEEEEEEEEE.LLL..LL
OkMV | .EEEEEE.LLLLLL.HHHHHH..LL.EEEE...LLL..E.LLLLLL...EE.L..EEEE..L--LLLLLLLLL.EEEEE..EEE.LLL..LL
OYMV | EEEEEEE.LLLLLL.HHHHHH.LL..EEE...LLL...LLL.LL...EEE.L..EEEE.L--LLLLLLLLL.EEEEEEEEEE.LLLL.LLLL
PhyMV | EEEEEEE.LLLLLL.HHHHHH.LL.EEEE...LLL..E..LL...L..EEELL..EEE..L--LLLLLLLLL.EEEEEEEEEE.LL..LL
TYMVBL | .EEEEEE.LLLLLL...LL.EEEE...LLL.LL..LL..L..EEE.L..EEEE.L--LLLLLLLLL.EEEEE.EEEE.LLL..LL
TYMVCa | .EEEEEE.LLLLLL...LL.EEEE...LLL.LL..LL..L..EEE.L.EEEEE.L--LLLLLLLLL.EEEEEEEEEE.LLL..LL
TYMVCL | .EEEEEE.LLLLLLH..H..LL.EEEE...LLL.LL..LL..LL.EEEE.L..EEEE.L--LLLLLLLLL.EEEEEEEEEE.LLL..LL
TYRoth | .EEEEEE.LLLLLL...LL.EEEE...LLL.LL..LL..L..EEE.L.EEEEE.L--LLLLLLLLL.EEEEEEEEEE.LLL..LL
TYType | .EEEEEE.LLLLLL.H.HHHH.LL.EEEE...LLL.LL..LL..L..EEE.L..EEEE.L--LLLLLLLLL.EEEEEEEEEE.LLL..LL
WCuMV | EEEEEEE.LLLLLL.HHHHHH..LL..EEE...LLL.LL..LL...EEELL..EEE..L--LLLLLLLLL.EEEEEEEEEE.LL..LL

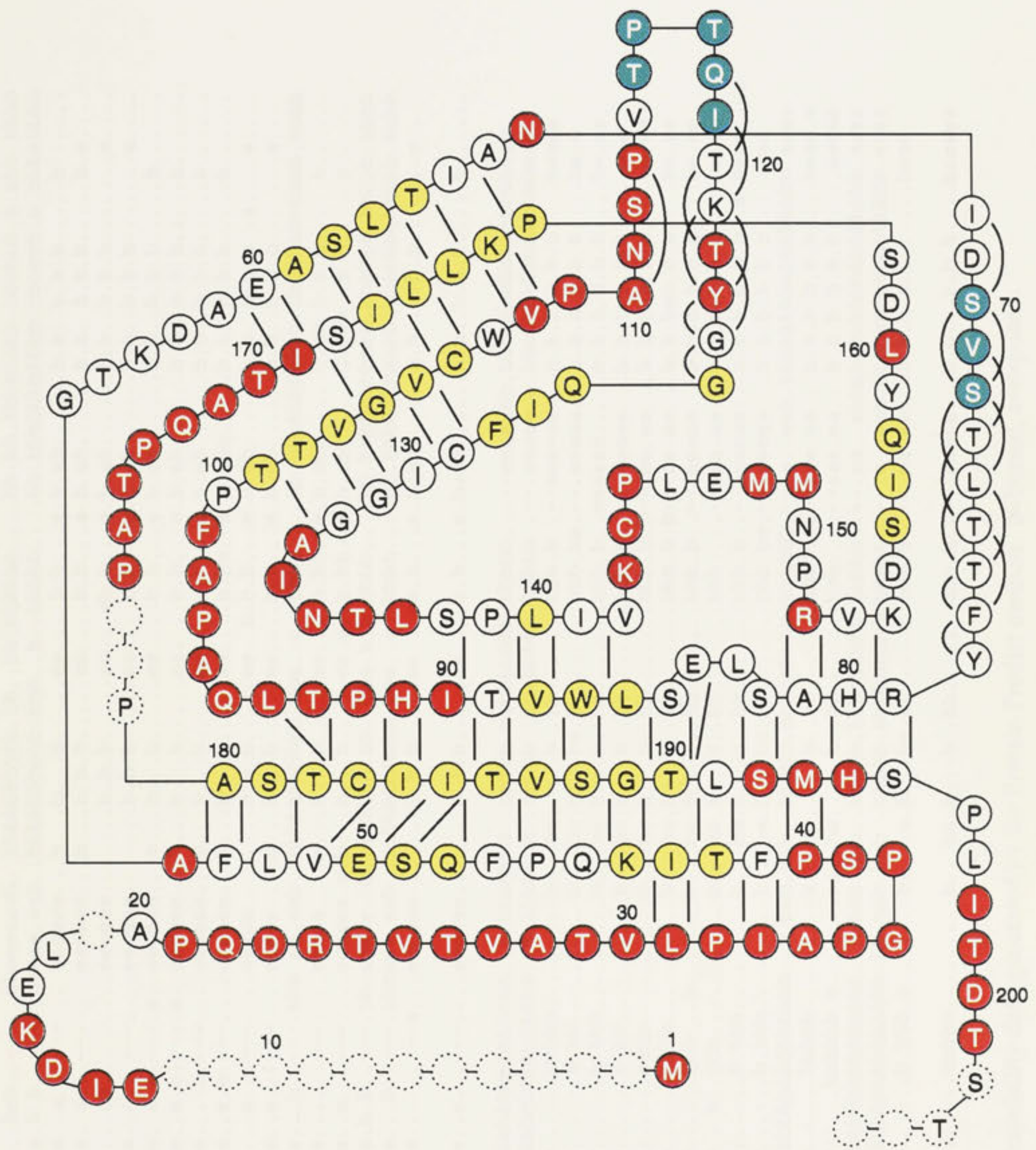
Consen | .EEEEEE.LLLLLL.HHHHHH..LL.EEEE...LLL..E..LL..LL..L...EEE.L..EEEE.L  LLLLLLLLL.EEEEEEEEEE.LLL..LLL

```

Figure 1.2 Consensus of the  $\alpha$  helix and  $\beta$  sheet predictions generated by the Protein Predict method. L=loop, E= $\beta$  sheet, H= $\alpha$  helix

Figure 1.3 The consensus of the  $\alpha$  helix,  $\beta$  sheet and loop regions recorded in Figure 1.2 mapped onto the Gibbs predicted structure of the tymovirus coat protein to show their locations.



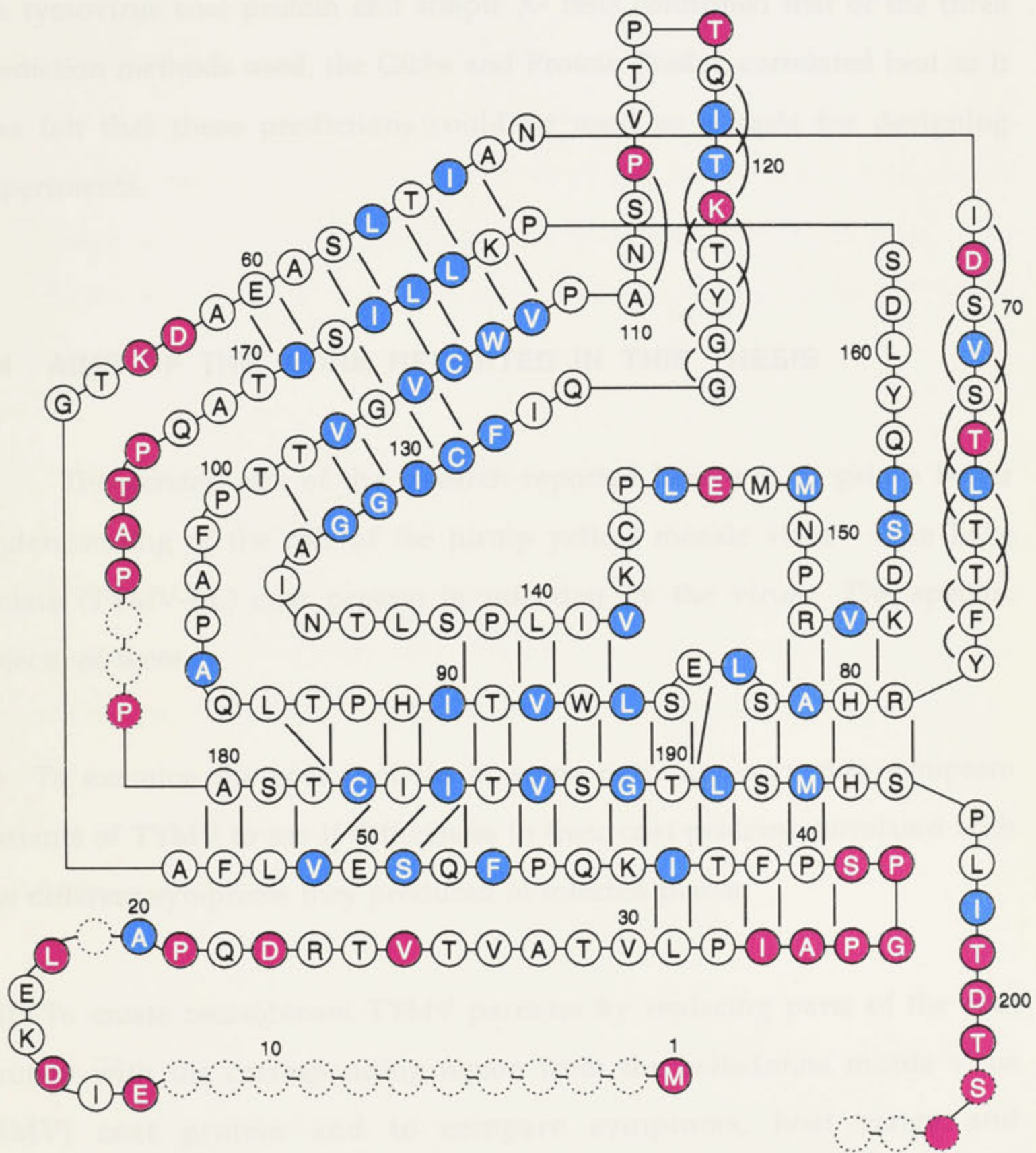


- Loop
- β sheet
- α helix

APLV	e-----ee...bbe...beb...---.bbeeeeeeb...bbbbbbbbbb.bb...bb.bbbbbb...bb.bb.bbbbbbbb.bbbbbb.b.bbb.bbbb
BMV	e-----ee...bbebe.e.b.b...---.b.eee.eeb...bbbbbbbbbb.bbb...b...bbbb...bb.bbbbbbbbbbbb.bbbbbb.b.bbbebbbb
CYVV	e-----e.e...e.e...e...---...eeeeee...b...b...b...ee...b.b...e.b.eb...b.b...b.b.b...b.....
DMV	e-----ee.e...e.e.e...e...---...eeeeee...b...b.b.b...ee...b.b...e.b.eb...b.b...b.b.b...b.e....
ELV	ee.e...e...eeeb.e...ee...eeeeee...b.b.b...ee...eb.b...e.b.eb...b.b...b.b.b...e....
EMV	e-----ee.e...e...e...---...eeeeee...b.b.b...ee...b.b...e.b.eb...b.b...b.b.b...e....
KYBP	e-----e...e...ee...e.e...---.e. ....eeeeee...b...b...b...ee...b.b...e.b.eb...b.b...b.b.b...b.....
KYJB	e-----e...e...ee...e.e...---.e. ....eeeeee...b...b...b...ee...b.b...e.b.eb...b.b...b.b.b...b.....
KYPD	e-----e.e...be.e...---...eeeeee...b...b...b...ee...b.b...e.b.eb...b.b...b.b.b...b.....
OkMV	e-----e...b...e...e.e...---...eeeeee...b...b...b...ee...b.bb...e.b.ebb...b.b...b.b.b...b.....
OYMV	e-----ee.e...ebe...e...---...eeeeee...b.b.b...ee...b.b.b...e.b.eb...b.b...b.b.b...e....e....
PhyMV	e-----ee.e...e.e.e...e...---...eeeeee...b...b.b.b...ee...b.b...e.b.eb...b.b...b.b.b...e....
TYMVBL	e-----ebbeebbe.e...b...---.bbb.ee.eeb...bbbbbbbbbb.bbe...e...bbb...bb.bbbbbbbbbbbb.bbbbbbbbbbbb.bbbb
TYMVCa	e-----e...e...e...---...eeeeee...b...b...b...ee...b.bb...e.b.ebb...b.be.b.b.b...b.....
TYMVCL	e-----e...ebb...e...e...---...eeeeee.e.b...b...b...ee.e...b.bb...e.b.eb...b.bee...b.b...b.....
TYRoth	e-----e...bb.b...e.b.b...---.bb. ....eee.e.bb.bbbbbbb.bb...bbe.b.b.bbbb...bb.bbbbbbbbbbbb.bbbbbb.bbbbbb.bbbb
TYType	e-----ebbeebb...e...b...---.bbbeee.eeb...bbbbbbbbbb.bbe...e...bbb...bb.bbbbbbbbbbbb.bbbbbbbbbbbb.bbbb
WCuMV	e-----eeee.be.e.e...---e...eeeeee...b.b.b...ee...eb.b...e.b.eb...b.b...b.b...e....
Consen	e-----e.e...ebe.e...e...---...eeeeee...b...b.b.b...ee...b.b...e.b.eb...b.b...b.b.b...b.....
APLV	bbbbbbb...be.b.bbbbbbbbbbbbbbbbbbb...b.bbbbbbb...bbbbbb.bbbbbbbbbbbbbb...--eeeeebb.bbbbbbbbbbbbbbbbee
BMV	bbbbbbb...be.b.bbbbbbbbbbbbbbbbbbb...b.bbbbbbb...bbbbbb...bbbbbbbbbbbbb...--eeeeee.b.bbbbbbbbbbbbbbbbee
CYVV	b.bbbb...e...eebbe...bbbbbb...b...be...b...bb...bbb.b...--eee-ee...b.b.b.b.b.b...beeee
DMV	b.bbbb...e...eebbe...b.bb...b...be.b...be.b...bbb.be...--eeeeee...b.b.b.b.b.b...be.e
ELV	b.bbbb...e...e.bbe...b.bbb...b...be.b...b.bb...bbb.b...eeeeee...b.b.b.b.b.b...be.ee
EMV	b.bbbb...e...eebbe...b.bb...b...be.b...b.bb...bbb.b...--eeeeee...b.b.b.b.b.b...eee
KYBP	b.bbbb...e...e.bbe...bbbb...b...be.b...b.bb...bbb.b.b--.ee.ee...b.b.beb.b.b...be.ee
KYJB	b.bbbb...e...e.bbe...bbbb...b...be.b...b.bb...bbb.b.b--.ee.ee...b.b.beb.b.b...be.ee
KYPD	b.bbbb...e...ebbe...bbb...b...b...be.b...b.bb...bbb.b...--eeeeee...b.b.b.b.b.b...be.ee
OkMV	.bbbb...e...bbe...b.bbb...b...be.b...b.bb...bbb...--eee.ee...b.b.b.b.b.b...be.ee
OYMV	b.bbbb...e...eebbe...b.bbb...b...be.b...b.bb...bbb.b...--eeeeee...b.b.b.b.b.b...e.beee...ee
PhyMV	b.bbbb...e...be.bbe...b.bbb...b...be.b...be.bb...bbb.be...--eeeeee...b.b.b.b.b.b...eee
TYMVBL	bbbbbbb...be.b.bbbb.bbbbbbbbbbbbbbb...b.bbb.bbb...bbbbbb.bbbbbbbbbbebbbbbb--.e...eeb.bbbbbbbbbbbbbbbb.eeee
TYMVCa	b.bbbb...e...e.b.e...bbbbbb...b...be...b...bb...bbb...e--eee.ee...b.b.b.b.b.b...beee.e
TYMVCL	b.bbbb...e...b.e...bbbbbb.b...be...be...b...bb...bbb...--eee.ee...b.b.b.b.b.b...eeee
TYRoth	bbbbbbb...be.b...bbb.bbbbbbbbbbbbbbb...bbbbbb.bbb...bbbbbb.bbbbbbbbbbbbbb...--ee...ebbbbbbbbbbbbbbbbb.eeee
TYType	bbbbbbb...be.b.bbbb.bbbbbbbbbbbbbbb.bb.bbb.bbb...bbbbbb.bbbbbbbbbbebbbbbb--.e...eeb.bbbbbbbbbbbbbbbb.eeee
WCuMV	b.bbbb...e...eebb...b.bbb.b...b...e.b...be.bb...bbb.b...--ee.ee...eb.b.b.b.b...beee
Consen	b.bbbb...e...e.bbe...bbbbbb...b...be.b...b.bb...bbb.b...--eeeeee...b.b.b.b.b.b...beeeee

Figure 1.4 Consensus of the accessibility data generated by the Protein Predict method. b=buried, e=exposed.

Figure 1.5 The consensus of the accessibility data listed in Figure 1.4 mapped onto the Gibbs predicted structure of the tymovirus coat protein to show the location of buried and exposed residues.



difference was in the positioning of an  $\alpha$  helix between residues 115-125. Information from the accessibility predictions showed that both the N and C termini were exposed as were parts of both  $\alpha$  helices. Both the Protein Predict and Gibbs methods predicted the same gross structural features for the tymovirus coat protein and simple  $X^2$  tests confirmed that of the three prediction methods used, the Gibbs and Protein Predict correlated best so it was felt that these predictions could be used as a basis for designing experiments.

#### 1.4 AIMS OF THE WORK REPORTED IN THIS THESIS

The general aim of the research reported here was to gain a better understanding of the role of the turnip yellow mosaic virus - Blue Lake isolate (TYMV-BL) coat protein in infection by the virus. The specific objectives were:

- (i) To examine the coat proteins of some naturally occurring symptom variants of TYMV to see if differences in their coat proteins correlated with the different symptoms they produced in infected plants
- (ii) To create recombinant TYMV particles by replacing parts of the coat protein with the corresponding region from the belladonna mottle virus (BMV) coat protein and to compare symptoms, host range and immunogenicity of these recombinants with the wild-type TYMV-BL.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 2.1 MATERIALS

##### 2.1.1 CHEMICALS

General laboratory chemicals were supplied by Sigma, Boehringer Mannheim and BDH. Agarose and acrylamide were from FMC and Boehringer respectively. Alcohols and acids were supplied by BDH and were of an AnalaR grade or purer.

##### 2.1.2 ENZYMES AND NUCLEOTIDES

Restriction and modification enzymes were from Pharmacia, Promega, Boehringer and New England Biolabs. Thermostable Vent DNA polymerase was from New England Biolabs. T7 RNA polymerase, NTPs and dNTPs were from Pharmacia. Radiolabelled nucleotides were supplied by Amersham or DuPont. Cellulase and macerase were supplied by Calbiochem.

##### 2.1.3 PRIMERS AND OTHER MATERIALS

Primers were synthesised in the Biomolecular Resource Facility at the ANU. Hybond blotting membranes were supplied by Amersham and X-ray film by Kodak. Pre-cast polyacrylamide gels were supplied by Novex. NBT, BCIP and alkaline phosphatase-conjugated second antibody were supplied by Boehringer. Bacterial cell strains and plasmids were supplied by Promega and Stratagene.

### 2.1.4 PRIMER SEQUENCES

All primer sequences were derived from the sequence of the coat protein of pBL16.

Primer name	Sequence (5'-3')
Tall Comp	CCCTCGAGT(C/T)TGAATTGCTTC
CL-3	GGTCTAGACATATGGTTCCGATGACCCTCGG
P2B	TGGTCCGATGACCCTCGGAA
CL-6	GCATGACGGTAGAAGGTGGT
CL-6Comp	ACCACCTTCTACCGTCATGC
CL-4	TCAAGGTATTGAATTGAATC
CL-4Comp	GATTCAATTCAATACCTTGA

### 2.1.5 KITS

Kit	Supplier
Altered Sites <i>in vitro</i> mutagenesis kit	Promega
Cyclist Exo <sup>-</sup> Pfu DNA sequencing kit	Stratagene
Prism ready reaction dyedeoxy terminator cycle sequencing kit	Applied Biosystems
Sequenase version 2 sequencing kit	United States Biochemical
SPOTs epitope analysis kit	Cambridge Research Biochemicals
Megaprime DNA labelling kit	Amersham
Wizard mini, maxi and PCR preps DNA purification kits	Promega

## 2.2 METHODS

### 2.2.1 PREPARATION OF PLASMID DNA

Several different methods were used to prepare small and large quantities of DNA. These methods were based on the alkaline lysis method of Birboim and Daly (1979).

Single bacterial colonies were picked and grown overnight at 37°C in LB broth (1L-10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl) with ampicillin added to give a final concentration of 80 µg/ml. The cells were pelleted and resuspended in resuspension buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg/ml RNase A). Lysis buffer (0.2 M NaOH, 1% (w/v) SDS) was added to disrupt the bacterial cell walls and neutralisation buffer (1.32 M potassium acetate pH 4.8) added to neutralise the NaOH in the lysis buffer. The cell debris was pelleted and the resulting clarified supernatant was either (a) treated with RNase, phenol/chloroform extracted and the DNA precipitated with ethanol (Promega miniprep method), (b) mixed with a resin and the plasmid DNA eluted from the resin after washing (Promega Wizard minipreps and maxipreps), or (c) precipitated with isopropanol and the resulting pellet resuspended in water before being loaded onto a cesium chloride gradient to remove bacterial chromosomal DNA (original method of Birboim and Daly). The quantity of resuspension, lysis and neutralisation buffers used and their composition varied depending on the amount of cells being pelleted and which method was followed. The recipes above are for the Promega Wizard miniprep and maxiprep methods.

### 2.2.2 RESTRICTION ANALYSIS OF PLASMID DNA

Restriction digests of DNA were done in order to generate appropriate vectors and inserts for ligation and to map recombinant plasmids.



DNA and sterile water were mixed together in a microcentrifuge tube. An appropriate amount of 10X restriction enzyme buffer was added, according to the manufacturers instructions. The restriction enzyme was then added (1-5 U/ $\mu$ g of DNA) and the contents of the tube mixed. The reaction was incubated for 1-2 hours at the required temperature (usually 37°C) and analysed by gel electrophoresis.

### **2.2.3 ELECTROELUTION OF DNA FRAGMENTS FROM AGAROSE GELS**

(Sambrook *et al.*, 1989)

Following electrophoresis through agarose gels, restriction fragments of interest were excised from the gel and the DNA eluted. This was done by placing the gel slices in dialysis tubing with sterile water and sealing the tubing. The tubing was placed in a gel tank containing a shallow layer of 0.5X TAE and an electric current of 25mA passed through the system for 30 minutes to elute the DNA from the agarose. The sterile water containing the eluted DNA was removed from the tubing and phenol/chloroform extracted. The DNA was ethanol precipitated with sodium acetate.

### **2.2.4 LIGATION OF VECTOR AND INSERT DNAs**

Ligation reactions were done in a final volume of 10  $\mu$ l. The reaction mix contained approximately 100 ng vector DNA, 20-50 ng insert DNA, 1  $\mu$ l 10X buffer C (0.5 M Tris-HCl pH 7.4, 0.1 M MgCl<sub>2</sub>), ATP to a final concentration of 1 mM, DTT to a final concentration of 10 mM and 1-2 U T4 DNA ligase. The reactions were incubated overnight at 4°C.

## 2.2.5 TRANSFORMATION OF LIGATION PRODUCTS INTO SUPERCOMPETENT BACTERIAL CELLS

(Sambrook *et al.*, 1989).

Bacterial cells were grown in 2X YT broth (1L-16g Bacto-tryptone, 10g Bacto-yeast extract, 5g NaCl) at 37°C until the OD<sub>600</sub> was 0.4-0.8. They were made competent by pelleting and resuspending in TFB (10 mM MES pH6.3, 45 mM MnCl<sub>2</sub>.4H<sub>2</sub>O, 10 mM CaCl<sub>2</sub>.2H<sub>2</sub>O, 100 mM KCl, 3 mM hexamminecobalt chloride), dimethylformamide and β-mercaptoethanol. Typically, 5 µl of the ligation mix and 200 µl of supercompetent cells were mixed gently and incubated on ice for 30 min. The cells were heat shocked at 42°C for 90 seconds and chilled on ice before being added to 800 µl of LB medium. The cultures were incubated at 37°C, with gentle shaking, to allow the cells to recover for 1 hour. 100 µl of culture was then spread on LB agar plates containing 80 µg/ml ampicillin and incubated at 37°C overnight to allow colonies to grow.

## 2.2.6 SEQUENCING OF DOUBLE STRANDED DNA

### 2.2.6(a) Cycle Sequencing

(Murray, 1989)

Cycle sequencing was done using the Stratagene Cyclist Exo<sup>-</sup> *Pfu* DNA sequencing kit as instructed by the manufacturers. Briefly, 50-100 ng template and 20-50 ng primer were combined in a total reaction volume of 30 µl. The reaction mix also contained 4 µl 10X sequencing buffer (200 mM Tris-HCl pH 8.8, 100 mM KCl, 20 mM MgSO<sub>4</sub>, 100 µM(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1% Triton, 1mg/ml BSA, 20 µM ATP, 50 µM each dCTP, dGTP, dTTP), 10 µCi <sup>35</sup>SdATP, 2.5 U Exo<sup>-</sup> *Pfu* polymerase and 4 µl DMSO. 7 µl of reaction mix was aliquoted into each of four tubes containing 3µl dideoxy NTP, either A, C, G or T (1.5 mM) and mixed thoroughly. The reactions were then cycled

through a temperature profile consisting of an initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at an appropriate temperature (depending on the primer sequence) for 30 sec and extension at 72°C for 1 min. Following the cycling reactions, 5 µl stop mix (80% formamide, 50 mM Tris-HCl pH 8.3, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added to each tube and the products of the sequencing reactions separated by electrophoresis in a sequencing gel. After passing an electrical current through the gel for the appropriate time, it was dried and exposed to X-ray film.

#### *2.2.6(b) Sequencing double stranded DNA using Sequenase (USB)*

(Tabor and Richardson, 1987)

Sequencing by this method was done according to the manufacturers instructions. Double-stranded DNA was first denatured in alkali. Approximately 1 µg DNA was mixed with 0.2 M NaOH and 0.2 mM EDTA and incubated at room temperature for 5 minutes. 0.1 volumes 3 M sodium acetate was added to neutralise the mixture and the DNA precipitated by the addition of 2.5 volumes of 100% ethanol at -20°C. The DNA was pelleted and resuspended in 3.5 µl SDW; 1µl 5X Sequenase buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl<sub>2</sub>, 250 mM NaCl) and approximately 5 ng primer were added. The primer and DNA were allowed to anneal by heating for 2 min at 65°C followed by slow cooling to room temperature. To this annealed DNA mix 0.5 µl 100 mM DTT, 1 µl labelling mix (7.5 µM each dGTP, dCTP, dTTP), 0.5 µl α<sup>35</sup>S dATP and 2 U Sequenase version 2 enzyme were added. The labelling reaction mixture was incubated at room temperature for 2-5 min after which 1.6 µl was added to each of 4 tubes containing 1.25 µl ddNTP (either A,C,G or T). The termination reactions were incubated at 37°C for 5 min and 2 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to the

tubes. The products of the sequencing reactions were then analysed by electrophoresis through a sequencing gel.

#### 2.2.6(c) *Dye terminator sequencing*

Approximately 750 ng - 1 µg DNA was mixed in a tube with 24 ng of the appropriate primer. 9.5 µl of terminator pre-mix was added with enough SDW to bring the final volume to 20 µl. The mixture was then loaded into a capillary tip and cycled 25 times through a temperature profile consisting of denaturation at 96°C for 30 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 min. The reaction mixtures were then transferred to a microcentrifuge tube and 80 µl of SDW added before being phenol:chloroform extracted twice. The mixtures were precipitated by the addition of 15 µl of 2 M sodium acetate pH 4.5 and 300 µl of 100% ethanol and centrifuged for 15 min at room temperature to pellet the reaction products. The pellets were washed with 70% ethanol and air dried. They were resuspended in formamide buffer, denatured and analysed in an Applied Biosystems 373A DNA sequencer at the Biomolecular Resource Facility at the ANU.

### 2.2.7 ISOLATION OF VIRUS PARTICLES FROM LEAF TISSUE

#### 2.2.7(a) *Large scale purification of tymovirus particles from Chinese cabbage*

(Keese *et al.*, 1989)

Fresh or frozen Chinese cabbage leaves in which the virus had been propagated for approximately 3 weeks was added to phosphate/ascorbic acid buffer (0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.1 M ascorbic acid pH 7.0) in a ratio of approximately 2:1 leaves:buffer (w:v). Following homogenisation for 3-4 min in a Waring blender, the mixture was filtered through a double layer of cheesecloth and 1/3 volume of chloroform:butanol (1:1) was added. After stirring for 30 min at 4°C the mixture was centrifuged at 4°C for 20 min

(Sorvall GSA rotor, 6,000 rpm, Sorvall RC5-C preparative centrifuge) to break the emulsion. The aqueous phase was removed, PEG and NaCl added to give final concentrations of 12% and 4% respectively and stirred at room temperature for 1 hour. The mixture was centrifuged again as above and the resulting pellets resuspended overnight at 4°C in 10 mM Tris/EDTA pH 7.0. Low speed centrifugation clarified the suspension before the supernatant was centrifuged at 4°C for 4 hours (Sorvall T865 rotor, 30,000 rpm, Sorvall OTD-B ultracentrifuge). The pellets were again resuspended overnight in buffer and clarified by low speed centrifugation. The suspension was layered onto 10-40% linear sucrose gradients in TE pH 7.0 (10 mM Tris-HCl pH 7.0, 1 mM EDTA pH 8.0) and centrifuged at 4°C for 3 hours (Sorvall SW28, 26,000 rpm, Sorvall OTD-B ultracentrifuge). The light scattering bands (top and bottom components) were removed separately from the gradients and diluted in TE pH 7.0. The virus particles were pelleted by centrifugation at 4°C for 4 hours (Sorvall T865 rotor, 30,000 rpm, Sorvall OTD-B ultracentrifuge), resuspended in TE pH 7.0 with 1 mM sodium azide and stored at 4°C.

#### **2.2.7(b) *Small scale isolation of tymovirus particles from Chinese cabbage***

(Skotnicki *et al.*, 1993a)

This method was used when only a small amount of virus was required. One to two leaves infected with virus were macerated in a leaf grinder and the sap collected in 10 ml tubes. TE pH 7.0 with 1 mM DTT was used to wet the leaves and maximise the yield of virus particles. An equal volume of chloroform/n-butanol (1:1) was added and the tube shaken vigorously to mix the two phases. Following centrifugation at 4°C in a table top centrifuge (15 min at 5000 rpm) the aqueous phase was transferred to Beckman polycarbonate tubes and the virus pelleted for 30 min at 4°C (TL-100.3 rotor, 70,000 rpm, Beckman TL-100 ultracentrifuge). The resulting pellets were resuspended overnight in a minimal volume of TE pH 7.0.

## 2.2.8 ISOLATION OF RNA

### 2.2.8(a) *Isolation of RNA from virus particles*

(Skotnicki *et al.*, 1993a)

100-200  $\mu\text{l}$  of resuspended virus particles from a small scale virus preparation or 50  $\mu\text{l}$  of purified virus was made up to 500  $\mu\text{l}$  with RNA extraction buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2% (w/v) SDS) and incubated at 56°C for 10 min. 16  $\mu\text{l}$  5 M NaCl was added to the tubes and after mixing, 800  $\mu\text{l}$  phenol/chloroform (1:1) was added to the tubes and the contents mixed well. After centrifugation for 5 min, the aqueous phase was transferred to a clean tube and the phenol/chloroform extraction repeated if there was a lot of sediment at the interface of the first extraction. RNA in the aqueous phase was precipitated twice with ethanol and sodium acetate and the OD<sub>260/280</sub> determined.

### 2.2.8(b) *Small scale isolation of total RNA from leaf tissue*

(Verwoerd *et al.*, 1989)

Fresh leaf discs were collected in microcentrifuge tubes and frozen in liquid nitrogen. The leaf tissue was ground to a fine powder using sterile glass rods pre-cooled in liquid nitrogen. 250  $\mu\text{l}$  phenol and 250  $\mu\text{l}$  extraction buffer (0.1 M LiCl, 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% (w/v) SDS) heated to 80°C were added and mixed by vortexing. 250  $\mu\text{l}$  chloroform/isoamyl alcohol (24:1) was added and following vortexing the tubes were centrifuged for 5 min in a microfuge. The aqueous phases were removed, mixed with 1 volume of 4 M LiCl and RNA allowed to precipitate overnight at 4°C. RNAs were pelleted by centrifugation in a microfuge at 4°C for 10 min and dissolved in 250  $\mu\text{l}$  SDW. A tenth of a volume of 3 M sodium acetate pH 5.2 and 2 volumes of 100% ethanol were added and the RNA precipitated at -20°C. Following centrifugation, the RNA pellets were washed with 70% ethanol, dried and resuspended in SDW.

### *2.2.8(c) Large-scale isolation of total RNA from leaf tissue*

(Chomczynski and Sacchi, 1987)

Fresh or frozen leaf tissue (-70°C) was ground to a fine powder in liquid nitrogen. A volume of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M  $\beta$  mercaptoethanol) was added (the quantity depended on the amount of tissue) and the slurry mixed thoroughly. 0.1 vol of 2 M sodium acetate pH 4, 1 vol phenol (water saturated) and 0.2 vol chloroform:isoamyl alcohol (49:1) were added with mixing after the addition of each reagent. The final suspension was incubated on ice for 15 min and centrifuged at 5000 rpm for 20 min at 4°C. The aqueous phase was transferred to a clean tube, mixed with 1 vol isopropanol and RNA precipitated at -20°C for at least 1 hour. Following centrifugation, as above, the resulting RNA pellet was dissolved in denaturing solution, transferred to a microcentrifuge tube and re-precipitated with 1 vol of isopropanol as above. The RNA was pelleted in a microcentrifuge, washed with 75% ethanol, dried and resuspended in SDW.

### *2.2.8(d) Large-scale isolation of total RNA from leaf tissue*

(Advanced Biotechnologies)

This method was similar to that of Chomczynski and Sacchi and used a ready-prepared isolation reagent. RNA was isolated following the manufacturers instructions. Briefly, leaf tissue was frozen in liquid nitrogen and ground to a fine powder. For each 100mg of tissue 1 ml of reagent was used. Following addition of the reagent the tubes containing the leaf tissue and the reagent were vortexed and stored at 4°C for 5 min. 0.2 ml of chloroform per 1 ml of reagent was added, the tubes shaken vigorously and held on ice at 4°C for 5 min. The homogenate was centrifuged (microcentrifuge, 14 000 rpm, 4°C, 15 min). The aqueous phase was transferred to a clean tube and an equal volume of isopropanol added. The RNA was precipitated at 4°C for 10 min and collected by centrifuging

(microcentrifuge, 14 000 rpm, 4°C, 10 min). The RNA pellet was washed with 75% ethanol, dried and resuspended in SDW.

### **2.2.9 FORMALDEHYDE GEL ELECTROPHORESIS OF RNA**

(Sambrook *et al.*, 1989)

A 1% agarose gel was prepared by dissolving the required amount of agarose in water. The gel solution was then cooled to 60°C and 5X formaldehyde gel running buffer (0.1 M MOPS pH 7, 40 mM sodium acetate, 5 mM EDTA pH 8) to give a final concentration of 1X was added. For a 150 ml gel, 4.5 ml of formaldehyde was also added and the gel poured and allowed to solidify in a fume hood. The RNA samples were prepared by mixing the RNA (in a volume of 4.5 µl) with 2 µl of 5X running buffer, 3.5 µl formaldehyde and 10 µl of formamide. The samples were incubated at 55°C for 5 min and chilled on ice. 2 µl of formaldehyde gel loading buffer (50% glycerol, 1 mM EDTA pH 8, 0.25% bromophenol blue, 0.25% xylene cyanol) was added and the samples loaded onto the gel. The gel was electrophoresed at 100V in 1X formaldehyde gel running buffer for ~3 hours until the bromophenol blue dye had migrated 8cm.

### **2.2.10 NORTHERN BLOTTING OF RNA FROM FORMALDEHYDE GELS**

(Sambrook *et al.*, 1989)

Following electrophoresis, gels containing RNA to be blotted were washed with several changes of SDW to remove formaldehyde. The gel was then soaked in 20X SSC (3 M NaCl, 0.3 M sodium citrate) for 45 min. The gel was placed in contact with a Hybond N<sup>+</sup> charged membrane and the RNA transferred overnight by capillary flow of the transfer buffer (20X SSC) through the gel. Following transfer, the RNA was fixed onto the membrane



by placing it on several sheets of Whatman filter paper which had been saturated with 0.05M NaOH for 5 min. The membrane was rinsed in 2X SSC, air dried and stored at 4°C.

### 2.2.11 CDNA SYNTHESIS

(Sambrook *et al.*, 1989)

5 µg total RNA and 100 ng primer were mixed together and heated to 90°C for 1 min. The tubes were chilled on ice and 5X reverse transcriptase buffer (Promega-250 mM Tris-HCl pH 8.3, 250 mM KCl, 50 mM MgCl<sub>2</sub>, 50 mM DTT, 2.5 mM spermidine), 10 mM dNTPs, RNasin and AMV reverse transcriptase added to give final concentrations of 1X, 0.5 mM, 1 U and 0.5 U respectively in a total volume of 40 µl. The tubes were incubated at 42°C for 45-60 min to allow cDNA synthesis to proceed. When the reactions were complete, the volume was made up to 100 µl with SDW and phenol/chloroform extracted. cDNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol at -20°C.

### 2.2.12 POLYMERASE CHAIN REACTION

(Sambrook *et al.*, 1989)

PCR was typically done in a total volume of 20 µl. A master mix was prepared first containing all the necessary reagents and cDNA or DNA was added last. For one reaction the quantities were as follows: 10X Vent polymerase buffer New England Biolabs (1X-10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100), 10 mM dNTPs, 100 mM MgSO<sub>4</sub>, forward and reverse primers and Vent polymerase enzyme to give final concentrations of 1X, 125 µM, 1.25 mM, 5 ng and 0.5 U respectively. 100-200 ng of DNA was added and the reactions were cycled 17 times through a profile of denaturation at 95°C for 30 sec, annealing for 30

sec at the appropriate temperature, depending on the primer combination, and extension at 72°C for 1 min. A control reaction without DNA was also included. PCR products were then examined by agarose gel electrophoresis.

### 2.2.13 SOUTHERN BLOTTING OF DNA

(Sambrook *et al.*, 1989)

DNA to be blotted was loaded onto a 1% agarose gel and electrophoresed in 1X TAE running buffer (50X-242g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA in 1L). Following electrophoresis the gel was soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min, rinsed with SDW and soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) for 30 min. The DNA was transferred overnight from the gel to a Hybond N+ membrane using 20X SSC (3 M NaCl, 0.3 M sodium citrate) as the transfer buffer. The DNA was fixed to the membrane by placing it on top of several sheets of Whatman filter paper which had been soaked in 0.4 M NaOH. The membrane was air dried and stored at 4°C.

### 2.2.14 RNA TRANSCRIPTION

(Titus, 1991 - Promega protocols)

Plasmids were linearised with *NdeI* before being transcribed into RNA. After restriction, the DNA was phenol/chloroform extracted and precipitated with 3 M sodium acetate pH 5.2 and 100% ethanol. Transcription reactions were set up at room temperature with the following being added in order: 5X transcription buffer (200 mM Tris-HCl pH 7.5, 30 mM MgCl<sub>2</sub>, 10 mM spermidine, 50 mM NaCl), 100 mM DTT, 2 mg/ml BSA, RNasin ribonuclease inhibitor, 10 mM ATP, CTP, UTP, 1 mM GTP, 5 mM cap analogue (M<sup>7</sup>G(5')ppp(5')G), 1-5 µg linearised plasmid DNA, T7 RNA polymerase and SDW to a total volume of 50 µl. The final concentrations

were 1X, 10 mM, 50 ng/ $\mu$ l, 1.5 U, 0.5 mM, 0.05 mM, 0.5 mM, 100 ng/ $\mu$ l and 30 U respectively. The reactions were incubated at 37°C for 60-90 min before the addition of 2 U RQ1 DNase. The reactions were incubated at 37°C for a further 10 min and were phenol/chloroform extracted and transcripts precipitated by the addition of 0.5 vol 7.5 M ammonium acetate and 2.5 vols of 100% ethanol. Pelleted transcripts were washed with 70% ethanol, air dried and resuspended in SDW for inoculation onto Chinese cabbage plants. Some of the transcripts (usually about 1/20) were examined by electrophoresis through polyacrylamide gels to check their integrity.

#### **2.2.15 <sup>32</sup>P LABELLING OF RADIOACTIVE RNA AND DNA PROBES AND HYBRIDISATION WITH MEMBRANES**

(Sambrook *et al.*, 1989; Feinberg and Vogelstein, 1983)

Membranes were incubated at 65°C for 4-6 hours or overnight in prehybridisation solution (6X SSC, 5% dextran sulphate, 0.1% (w/v) SDS, 10 mM EDTA, 0.5% Boehringer blocking reagent) before the radiolabelled probe was added. The solution was prepared by mixing everything except the blocking reagent and heating to 90-95°C to dissolve the dextran sulphate. The blocking reagent was then added and the solution heated at 90-95°C for 1-2 hours until the blocking reagent had dissolved completely. The solution was allowed to cool to 65°C before the membranes were added to prehybridise.

RNA probes were prepared at room temperature as follows: 5X transcription buffer (200 mM Tris-HCl pH 7.5, 30 mM MgCl<sub>2</sub>, 10 mM spermidine, 50 mM NaCl), 200 mM DTT, RNasin, minus-U nucleotide mix, [ $\alpha$ -<sup>32</sup>P]UTP, linearised template, T7 RNA polymerase and SDW were added to give final concentrations of 1X, 10 mM, 0.5 U, 1.5 mM, 0.25  $\mu$ Ci, ~50 ng and 0.5 U. SDW was used to give a final volume of 20  $\mu$ l. The reactions were

incubated at 37-40°C for 30-45 min. 2 U RQ1 DNase were added and the reactions incubated for a further 10 min. After phenol/chloroform extraction the transcripts were precipitated with 0.5 vol. ammonium acetate and 2.5 vols 100% ethanol with carrier RNA to aid precipitation. Pelleted transcripts were resuspended, denatured at 55°C for 5-10 min, added to prehybridised membranes and allowed to hybridise overnight at 65°C.

DNA probes were labelled using [ $\alpha$ -<sup>32</sup>P]dCTP and the Amersham Megaprime DNA labelling system. 25 ng DNA was labelled in a total volume of 50  $\mu$ l. 5  $\mu$ l of random nonamer primers were added to the 25 ng of template and boiled for 5 min. After the mixture had cooled to room temperature 10  $\mu$ l labelling buffer, 5  $\mu$ l [ $\alpha$ -<sup>32</sup>P]dCTP and 2  $\mu$ l (2 U) Klenow enzyme were added. The labelling reactions were incubated at 37°C for 10-30 min. The labelled DNA was precipitated with ammonium acetate and ethanol, pelleted and resuspended in SDW. After being denatured by boiling for 5 min the labelled DNA was hybridised with the Hybond N<sup>+</sup> membrane overnight at 65°C.

#### Post-hybridisation washes:

The membranes were rinsed twice at room temperature in 2X SSC, 0.1% (w/v) SDS. This was followed by washing at 65°C using buffers increasing in stringency until the background radioactivity on the membrane had been removed. These buffers were 1X SSC, 0.1% (w/v) SDS, 0.5X SSC, 0.1% (w/v) SDS and 0.1X SSC, 0.1% (w/v) SDS. The membranes were washed for 30 min in each buffer and were exposed to X-ray film at -80°C. Membranes with high radioactivity counts were exposed at room temperature.

### 2.2.16 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEIN SAMPLES

(Laemmli, 1970)

Protein samples to be analysed in polyacrylamide gels were denatured in 1X sample buffer (50 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 0.1% bromophenol blue, 10% glycerol) by boiling for 3-5 min. The samples were loaded on 12% acrylamide gels (Novex pre-cast gels) and electrophoresed at 125V using 1X SDS gel running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS) as the electrophoresis buffer until the dye front had migrated almost to the end of the gel. The gel was then stained in Coomassie Blue to detect proteins or was processed for Western blotting.

### 2.2.17 WESTERN BLOTTING OF PROTEINS

(Towbin *et al.*, 1979; Burnette, 1981)

The blotting membrane (Hybond-C), pieces of Whatman filter paper and gels to be blotted were soaked in transfer buffer (12 mM Tris, 96 mM glycine, 20% methanol). The blotting apparatus was assembled so that the current passed through the gel and deposited the proteins on the membrane. The proteins were transferred by passing a constant 30 V through the system for 1-2 hours.

### 2.2.18 IMMUNODETECTION OF MEMBRANE-BOUND PROTEINS

Following Western transfer, membranes were blocked (3% BSA in 1X Tris/NaCl [10X: 9% NaCl, 100 mM Tris-HCl pH 7.4]) for 2 hours at room temperature or overnight at 4°C. The membrane was washed 5 times for 5 min (1X Tris/NaCl, 0.1% Tween 20) and incubated with the appropriate dilution of antiserum in blocking buffer (3% BSA in 1X Tris/NaCl) for 3

hours at room temperature or overnight at 4°C. The membrane was washed again and incubated for 1 hour at room temperature or overnight at 4°C with alkaline phosphatase conjugated anti-rabbit IgG in 1X Tris/NaCl containing 1% BSA. Boehringer sheep anti-rabbit IgG conjugated to alkaline phosphatase was used at a dilution of 1:5000. The membrane was washed as above and processed with colour development solution (66 µl NBT stock, 33 µl BCIP stock, 20 ml carbonate buffer: see below). The reaction was stopped by transferring the membrane to a distilled water bath.

NBT (p-nitro blue tetrazolium chloride) stock solution was 50mg/ml in 70% dimethyl formamide. BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) stock solution was 50mg/ml in 100% dimethylformamide. Carbonate buffer: 0.1 M NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>.6H<sub>2</sub>O (pH 9.8).

#### **2.2.19 PREPARATION AND ELECTROPORATION OF CHINESE CABBAGE PROTOPLASTS**

(Weiland and Dreher, 1989; Last *et al.*, 1991)

Protoplasts were prepared from leaves of sterile plants maintained in tissue culture or from leaves of glasshouse grown plants. In the case of glasshouse material, the plants were held in the dark for ~48 hours prior to the preparation of protoplasts. Leaves were removed and sterilised by washing with sodium hypochlorite solution for ~10 min, rinsing with 70% ethanol for 5-10 sec and washing with SDW several times. These leaves or those of tissue culture grown plants were then placed in a Petri dish containing 0.55 M mannitol/0.1% MES (pH 5.7) and sterile carborundum was gently applied to the lower epidermis using sterile cotton buds. The leaves were washed with 0.55 M mannitol/0.1% MES to remove the carborundum and placed in enzyme solution (2% cellulase, 0.1% macerage, 0.1% BSA in 0.55 M mannitol/0.1% MES; filter sterilised before use). Following gentle vacuum infiltration for ~30 min, 10 µl of a 20 mg/ml stock of cefotaxime was added

as an antibiotic, the Petri dishes were sealed and incubated overnight in the dark at 25°C.

The following morning the leaves were gently disrupted to release the protoplasts which were then passed through 328, 110 and 50 µm mesh sieves. After sedimentation at low speed (100g for 5 min) the protoplasts were gently resuspended in 1:1 1 M mannitol: artificial seawater [1L-18.18g NaCl, 0.52g KCl, 4.64g MgSO<sub>4</sub>.7H<sub>2</sub>O, 3.39g MgCl<sub>2</sub>.6H<sub>2</sub>O, 1.0g CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.147g NaHCO<sub>3</sub>, 2.13g MES (pH 6.0)]. The protoplasts were then counted using a haemocytometer, pelleted gently as above and resuspended in the required volume of TBS pH9 [1L-3.63g Tris, 876mg CaCl<sub>2</sub>.2H<sub>2</sub>O, 8.78g NaCl, 50g mannitol (pH 9.0)] to give a concentration of 2 X 10<sup>6</sup> protoplasts per ml. Immediately before electroporation 100 µl of protoplasts and 100-500 ng of transcript or RNA were mixed in the electroporation chamber and three pulses of 275 V, with a pulse width of 5 ms and a delay of 100 ms, were applied between the electrodes from a 24 µF capacitor. The protoplasts were transferred to 6 well plates (wells were 3.5 cm in diameter), 10 µl of 0.5 M mannitol pH 5.0, 10 µl of 20 mg/ml cefotaxime and 900 µl culture medium were added. The plates were sealed and the protoplasts incubated in the dark at 25°C. After 2-3 days, the protoplasts were harvested by pelleting (100g for 10 min), the supernatant was removed and the protoplasts stored at -70°C.

The TBS pH 9 and culture medium used in these experiments were prepared by Jenny Gibson, CSIRO Division of Plant Industry, Canberra.

### 2.2.20 *IN VITRO* MUTAGENESIS

*in vitro* mutagenesis was done using the Promega Altered Sites system.

#### 2.2.20(a) *Construction of recombinant TYMV coat protein genes*

##### 2.2.20(a)i Cloning of the pTYMV-BL16 coat protein gene into the pSelect-1 vector and preparation of single stranded DNA:

The *Pst*I/*Xba*I 900bp fragment containing the coat protein gene (Fig. 2.1) was excised from the full-length virus clone in pGEM 7Zf(+) and ligated into the pSelect-1 vector which had been digested with *Pst*I and *Xba*I (see 2.2.4). The products of the ligation were used to transform JM109 competent cells (2.2.5) and plated onto LB agar plates containing 15 µg/ml tetracycline, 0.5 mM IPTG and 40 µg/ml X-Gal. White colonies containing recombinant plasmids were picked and grown up for plasmid preps (2.2.1). The plasmid DNA was checked by digestion with *Pst*I and *Xba*I (2.2.2).

Single stranded DNA was produced for use in the mutagenesis reactions as outlined in the manual provided by the manufacturer. The helper phage R408 was used to infect an overnight culture of cells containing the recombinant pSelect plasmid and single stranded DNA recovered by PEG precipitation.

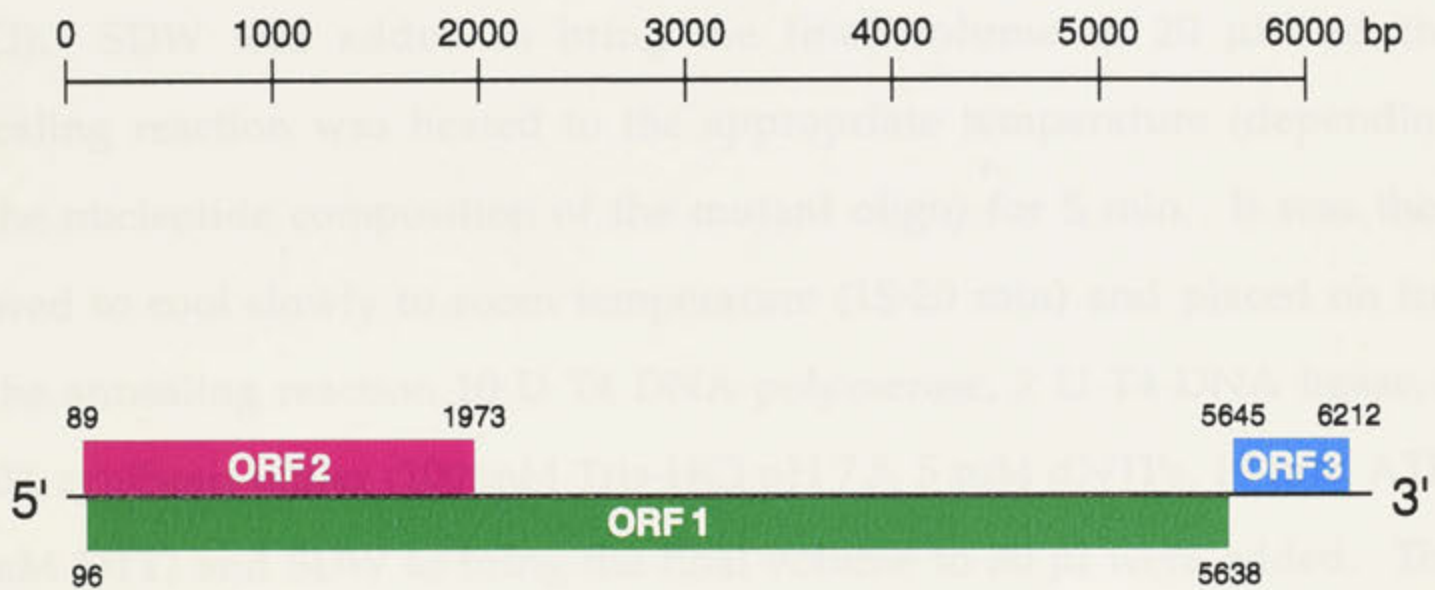
The recombinant pSelect plasmids used in this work were prepared by Anne Mackenzie.



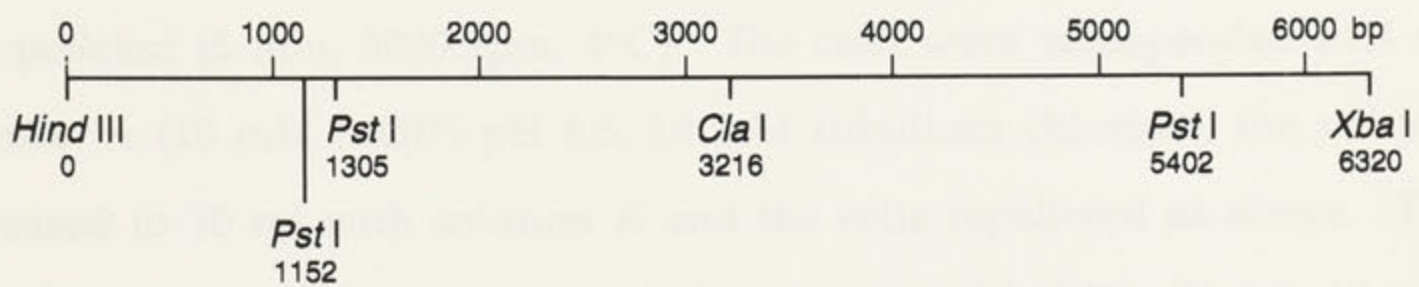
Figure 2.1(a) Map of the TYMV-BL genome showing the positions of the three open reading frames. ORF 1 encodes the replicase protein and initiates at position 96 in the RNA genomic sequence ending at position 5638. ORF 2 encodes the movement protein; it initiates at position 89 and ends at position 1973 in the RNA genomic sequence. ORF 3 encodes the coat protein from a sub-genomic RNA which corresponds to the genomic RNA between positions 5645 and 6212.

(b) Restriction map of the pBL-16 cDNA clone showing the positions of the commonly used restriction enzymes used in sub-cloning procedures.

A) genome map of *TYMV-BI*



B) Restriction map of *pBI-16*



### 2.2.20(a)ii Annealing of the mutagenic oligonucleotides to the single stranded recombinant pSelect plasmid and synthesis of the mutant strand:

The mutagenesis reactions involved annealing of both the mutant and ampicillin repair oligonucleotides to the single stranded template. Annealing was done by mixing 0.05 pmol single stranded DNA, 0.25 pmol amp repair oligonucleotide, 1.25 pmol mutagenic oligonucleotide and 2  $\mu$ l 10X annealing buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl<sub>2</sub>, 500 mM NaCl). SDW was added to bring the final volume to 20  $\mu$ l and the annealing reaction was heated to the appropriate temperature (depending on the nucleotide composition of the mutant oligo) for 5 min. It was then allowed to cool slowly to room temperature (15-20 min) and placed on ice. To the annealing reaction 10 U T4 DNA polymerase, 2 U T4 DNA ligase, 3  $\mu$ l 10X synthesis buffer (100 mM Tris-HCl pH 7.5, 5 mM dNTPs, 10 mM ATP, 20 mM DTT) and SDW to bring the final volume to 30  $\mu$ l were added. The reaction was incubated at 37°C for 90 min to allow mutant strand synthesis.

### 2.2.20(a)iii Transformation into BMH 71-18 mut S competent cells:

Competent cells were prepared according to the manufacturers instructions. Briefly, BMH cells were grown in LB medium until the OD<sub>600</sub> was 0.13-0.15 and pelleted (5 min, 3000 rpm, 4°C). The cells were resuspended in 1 ml solution A (10 mM MOPS pH 6.5, 10 mM rubidium chloride), the volume increased to 10 ml with solution A and the cells repelleted as above. The cells were resuspended in 1 ml solution B (10 mM MOPS pH 6.5, 10 mM rubidium chloride, 50 mM calcium chloride) and the volume increased to 10 ml with solution B. The cells were incubated on ice for 30 min and repelleted as above. They were finally resuspended in 2 ml solution B. 3  $\mu$ l DMSO was added to 200  $\mu$ l BMH competent cells and, after mixing, the synthesis reaction mix was also added. The cells were incubated on ice for

30 min after which 4 ml LB was added and the cells allowed to recover at 37°C for 1 hour. 1 ml of cells was removed and spread onto LB agar plates containing 125 µg/ml ampicillin. The plates were incubated at 37°C overnight. To the remaining 3 ml of culture, ampicillin was added to give a final concentration of 125 µg/ml and the cultures incubated at 37°C with shaking overnight.

#### 2.2.20(a)iv Plasmid preparation and transformation into JM109 competent cells:

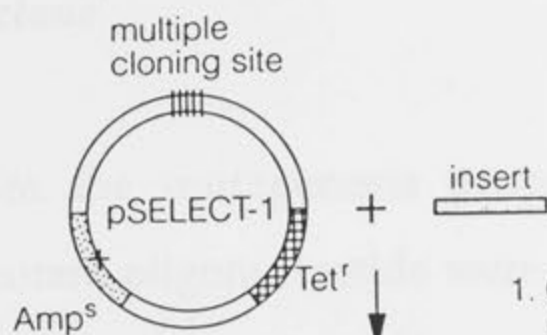
BMH cells were harvested from an overnight culture by centrifugation and plasmid DNA prepared as outlined (2.2.1). If the overnight culture didn't grow and colonies were present on the LB plates, these colonies were grown up overnight and DNA prepared as above.

JM109 competent cells were prepared using the same method as that for BMH cells. 200 µl cells were transformed with ~100 ng plasmid DNA (isolated from BMH cells). Following the incubation on ice for 30 min, 2 ml LB was added to the cells and they were incubated at 37°C for 1 hour with very gentle shaking to allow them to recover. The culture was divided into 2 microfuge tubes, the cells were pelleted and resuspended in 50 µl LB. The cells were plated onto LB agar plates containing 125 µg/ml ampicillin and incubated at 37°C overnight.

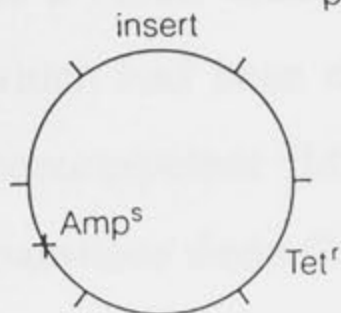
In general, 10 colonies from the JM109 transformation were picked and plasmid DNA prepared as outlined (2.2.1). Four of these plasmids were then sequenced using appropriate primers to determine if the mutant oligonucleotide had been incorporated into the coat protein gene (2.2.6).

The mutagenesis procedure is outlined in Figure 2.2.

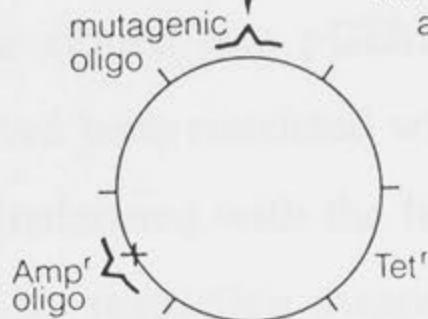
Figure 2.2 Outline of the mutagenesis procedure used to construct coat protein recombinants of TYMV-BL. (From: "Promega Protocols and Applications Guide", E.D. Titus ed.)



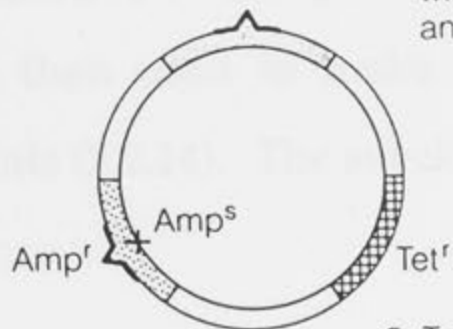
1. Clone insert into pSELECT-1 vector.
2. Infect with helper phage, grow in media + Tet, purify ssDNA.



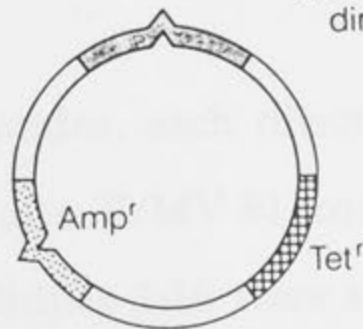
3. Anneal mutagenic oligo and ampicillin repair oligo.



4. Synthesize mutant strand with T4 DNA polymerase and ligate.



5. Transform BMH 71-18 mut S. Grow in media + ampicillin.
6. Prepare mini-prep DNA.
7. Transform JM109. Select mutants on ampicillin plates.
8. Screen for mutants by direct sequencing.



### 2.2.20(b) *Subcloning of recombinant coat protein genes into the full-length virus cDNA clone*

Plasmids from the mutagenesis procedure which had been shown to contain the mutant oligonucleotide were digested with *Pst*I and *Xba*I to yield the 900 bp fragment containing the mutant coat protein gene. The eluted fragment was cloned into a vector containing the 3' *Cla*I/*Xba*I fragment of the virus cDNA clone which had been digested with *Pst*I and *Xba*I. After transformation into supercompetent JM109 cells (2.2.5) 10 colonies were picked and plasmid preparations done (2.2.1). These plasmid were digested with *Cla*I and *Xba*I to yield the 3.1kb 3' half of the virus cDNA clone. This eluted fragment was cloned into pGEM7 Zf(+) with the full-length virus cDNA clone which had been restricted with *Cla*I and *Xba*I. Supercompetent JM109 cells were transformed with the ligation products and colonies were picked and checked by restriction mapping using *Pst*I and *Xba*I. Plasmids containing the recombinant coat protein genes in the full-length virus cDNA clones were then used to make transcripts for inoculation onto Chinese cabbage plants (2.2.14). The subcloning procedure is summarised in Figure 2.3.

### 2.2.21 **EPITOPE ANALYSIS OF THE TYMV-BL COAT PROTEIN**

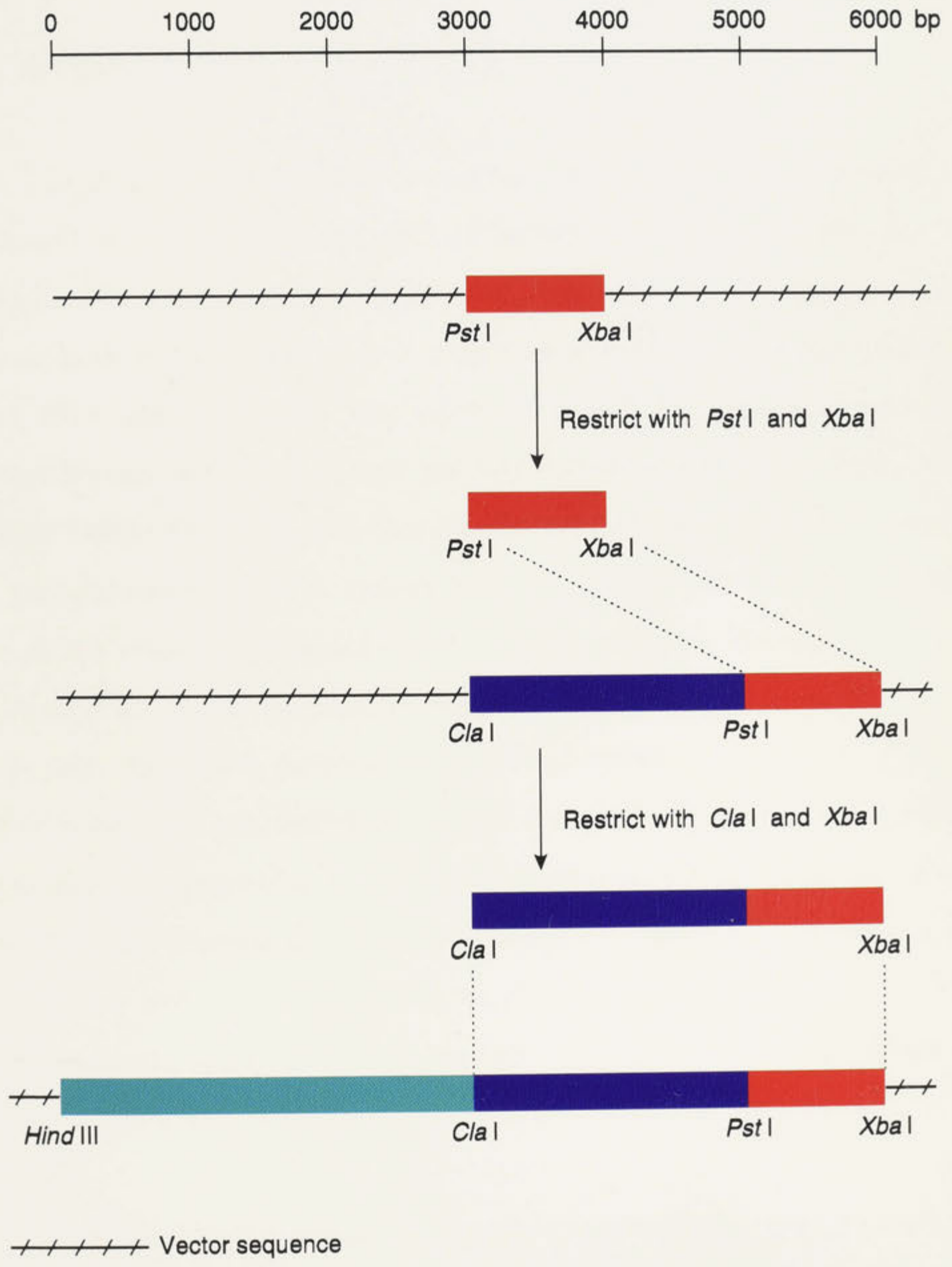
The SPOTs epitope analysis kit from Cambridge Research Biochemicals was used and the manufacturers' instructions were followed.

Overlapping 10-mer peptides, each offset from the adjacent peptide by 2 residues covering the entire TYMV-BL coat protein, were synthesised on a membrane support. Residues 1-10 were synthesised on spot no. 1, 3-12 on spot no. 2, etc.. The peptides were synthesised one amino acid at a time and when complete the peptides were acetylated and the side-chains deprotected.

Figure 2.3 Outline of the sub-cloning procedures used to construct the recombinant viruses. The coat protein gene containing the desired recombination was excised from the pSelect plasmid used for mutagenesis by digesting with *Pst*I and *Xba*I. This fragment was then cloned into a plasmid containing the *Cla*I/*Xba*I 3' fragment of the cDNA clone which had been digested with *Pst*I and *Xba*I. The 3' fragment of the virus cDNA clone containing the recombinant coat protein was then cloned into another plasmid containing the 5' fragment of the clone which had been digested with *Cla*I and *Xba*I. In this way a full-length clone of the virus containing the recombinant coat protein was constructed.



The SPOTs were analysed in the first instance using the secondary antibody to determine any antigenic regions of the protein with the antibody. The SPOTs were then prepared and analysed with several test systems.



The SPOTs were analysed in the first instance using only the secondary antibody to determine any non-specific reactions of the peptides with the antibody. The SPOTs membrane was then regenerated and analysed with several test antisera.

### 3.1 INTRODUCTION

The coat proteins of some viruses have been shown to be involved in cell-to-cell and long distance spread, therefore playing a key role in virulence and pathogenicity. Even for highly virulent viruses which spread from cell-to-cell and over long distances in the infected plant as naked RNA, changes to the coat protein affected the symptoms seen in infected plants and also restricted the host range of the virus (Della and Johnson, 1979). The coat protein of the tobacco golden mosaic virus (TMV) coat protein mutants which spread from cell-to-cell and systemically but were unable to cause systemic symptoms and induced disease symptoms (Kardos et al., 1982). The cucumber mosaic virus also has a coat protein responsible for local and systemic movement of the virus, but a coat protein lacking the protruding domain of the coat protein showed delayed appearance of symptoms in *Nicotiana glauca* plants (Della et al., 1983). The coat proteins of other viruses such as alfalfa mosaic virus (AMV) and tobacco etch virus (TEV) have been shown to play a role in the symptoms reported in plants infected with these viruses (Johnson et al., 1981; Della et al., 1983; Johnson et al., 1983) (see Chapter 2).

The most well-characterized involvement of the coat protein in symptom induction is the hypersensitive response between the tobacco etch virus (TEV) coat protein and tobacco plants with the *N* resistance gene and between the potato virus X (PVX) coat protein and potato plants with the *R*<sub>1</sub> resistance gene (Chapter 2). The ability of TMV and PVX virus

## CHAPTER THREE

### ANALYSIS OF NATURALLY OCCURRING SYMPTOM VARIANTS OF TYMV

#### 3.1 INTRODUCTION

The coat proteins of some viruses have been shown to be involved in cell-to-cell and long distance spread; therefore playing a key role in virulence and pathogenesis. Even for barley stripe mosaic virus, which spreads from cell-to-cell and over long distances in the infected plant as naked RNA, changes to the coat protein affected the symptoms seen on infected plants and also affected the host range of the virus (Petty and Jackson, 1990). This was also true for tomato golden mosaic geminivirus coat protein mutants which spread from cell-to-cell and systemically but some of the mutants produced delayed and attenuated disease symptoms (Gardiner *et al.*, 1988). The tombusvirus cucumber necrosis virus also has a coat protein dispensible for local and systemic movement of the virus, but a mutant lacking the protruding domain of the coat protein showed delayed appearance of symptoms in *Nicotiana clevelandii* plants (McLean *et al.*, 1993). The coat proteins of other viruses such as alfalfa mosaic virus, turnip crinkle virus and cucumber mosaic virus have been shown to affect the symptoms expressed in plants inoculated with these viruses (Neeleman *et al.*, 1991; Heaton *et al.*, 1991; Shintaku *et al.*, 1992) (see Chapter 1).

The most well-characterised involvement of the coat protein in symptom formation is the hypersensitive response between the tobacco mosaic virus (TMV) coat protein and tobacco plants with the N' resistance gene and between the potato virus X (PVX) coat protein and potato plants with the Rx resistance gene (Chapter 1). The ability of TMV and PVX virus

isolates to overcome the HR and spread from local lesions to systemically infect plants was mapped to the coat protein. This demonstrated the importance of the coat protein in pathogenesis of these viruses. Differences in symptoms produced by viruses have also been mapped to other regions of the viral genome including the 3' untranslated region of tobacco vein mottling potyvirus (Rodriguez-Cerezo *et al.*, 1991).

The results of other workers demonstrated the involvement of the coat protein in pathogenesis for some viruses so it was decided to check whether there were correlations between symptoms and differences in the coat proteins of natural variants of TYMV. Symptom variants of TYMV were isolated from their native host *Cardamine lilacina* growing around Blue Lake in the Mt. Kosciusko National Park (New South Wales, Australia). These variants were isolated from separate plants growing in an area of 50m along the south-facing shore of the lake. The symptoms produced by them ranged from very mild to extreme mosaic and were consistent through several passages in *C. lilacina* and Chinese cabbage plants grown in the glasshouse. In order to determine whether there was a genetic basis for the differential virulence of these TYMV isolates to *C. lilacina* and Chinese cabbage, six variants were chosen for coat protein sequence analysis. The variants were named as follows; D5 and F39 which produced mild symptoms, P1 which produced intermediate symptoms and F41, N37 and Q18 which all produced severe symptoms. Figure 3.1 shows the symptoms produced in Chinese cabbage plants infected with the six variants.



Figure 3.1 Photographs of Chinese cabbage plants infected with virus particles from each of the six TYMV symptom variants originally isolated from the native host *Cardamine lilacina*. D5 and F39 produce mild symptoms in inoculated plants, P1 produces intermediate symptoms and F41, N37 and Q18 all produce severe symptoms. Photographs were taken 14 days post inoculation.





## 2.2 RESULTS

Various particles were isolated from Chinese cabbage plants infected with the tobacco etch virus (TEV) and used for the preparation of the particles (2.2.1). The various isolates were originally prepared from single virus isolations and therefore were genetically pure. The following section describes the purification of the particles.



... of the virus ... in 1977 ... and ...



### 3.2 RESULTS

Virus particles were isolated from Chinese cabbage plants infected with the six variants and TYMV-BL (2.2.7b) and viral RNA extracted from the particles (2.2.8a). The variant isolates were originally passaged from single viral lesions and therefore were genetically pure. The coat protein genes of the variants were amplified by RT-PCR (this method involves synthesising cDNA from viral RNA using the 3' CL-3 primer and amplifying the coat protein gene using the virus specific primers Tall Comp and CL-3 (2.1.4)). PCR products were purified from excess dNTPs and primer using the Promega Wizard PCR preps system and sequenced using dye terminators (2.2.6c). The coat protein genes of all variants were sequenced 4 times, twice in each direction, using a series of primers as outlined in Figure 3.2. The sequenced coat protein genes were compared with that of TYMV-BL as were the deduced amino acid sequences and the results are summarised in sequence alignments (Figures 3.3 and 3.4) and Tables 3.1 and 3.2.

All of the variants differed from TYMV-BL in their nucleotide and encoded amino acid sequences although the number of differences varied from one variant to another. In all variants the number of silent nucleotide changes was greater than non-silent and the resulting amino acid changes were both conservative and non-conservative. F39 had the greatest number of amino acid changes with 12 different to TYMV-BL, D5 and Q18 had 7 differences, N37 had 5 differences and F41 and P1 both had 3 differences. All variants except F41 had a threonine in position 22 instead of isoleucine and in N37 this was the only non-conservative amino acid change. All of the amino acids which differed from TYMV-BL in N37 and Q18 (severe variants) were also present in F39 (mild variant). This suggested that the mild symptoms were due to amino acid changes in F39 not present in N37

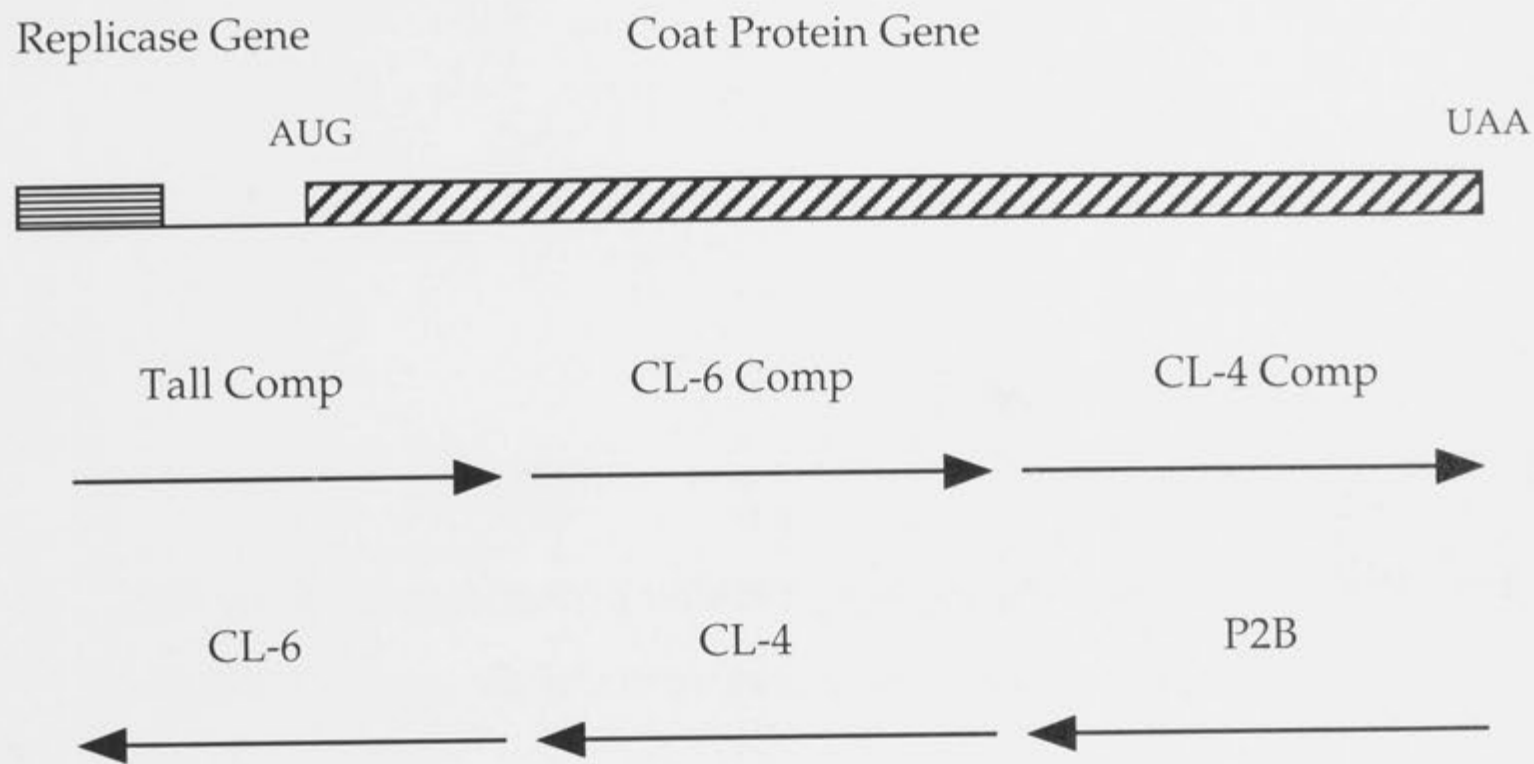


Figure 3.2 Map showing the locations of the primers used to sequence the coat protein genes of the symptom variants. The coat protein genes were sequenced completely in both directions using these primers.

Figure 3.3 Nucleotide sequences of the coat proteins genes of the six symptom variants compared with the sequence of TYMV-BL.

```

          10      20      30      40      50      60
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  ATGGAAATCGACAAAGAACTCGCCCCCAAGACCGCACCAGTCACCGTCGCCACCGTTTTA
D5  .....G.....A.....
F39 .....A.....
F41 .....
N37 .....A.....
P1  .....T.....T.....
Q18 .....A.....
*****
          70      80      90      100     110     120
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  CCAATTGCCCCCGGCCCTCACCTTTCACCATCAAACAACCGTTCAGTCTGAAGTTCTG
D5  ...C..T..T.....C.....
F39 ..G.C..T.....C.....
F41 .....
N37 ..G.C..T.....C.....
P1  ...C..T.....
Q18 ..G.C..T.....C.....
** * ** * ** *****
          130     140     150     160     170     180
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  TTTGCTGGGACCAAAGATGCCGAGGCCTCTCTCACCATCGCCAACATCGACAGCGTTTCC
D5  ....G..A.....T....G.....
F39 ....G.....G.....T.....G.....
F41 .....T.....G.....
N37 .....
P1  .....
Q18 .....T.....G.....
**** * ** *****
          190     200     210     220     230     240
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  ACCCTTACCACCTTCTACCGTCATGCCTCTCTGGAATCACTCTGGGTCACCATCCATCCT
D5  ....C.....C.....
F39 ....C.....T.....
F41 .....TA.....
N37 ....C.....T.....
P1  .....T.....C.....
Q18 ....C.....T.....C..C
*****
          250     260     270     280     290     300
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  ACCTTGCAAGCCCCGGCTTCCCAACCACGGTTGGCGTTTGCTGGGTACCCGCCAACTCC
D5  .....A.....G.....C..T.....
F39 .....A.....G.....C.....
F41 .....C.....
N37 .....A.....G.....C.....
P1  .....G.....C.....
Q18 .....A.....G.....C.....
*****
          310     320     330     340     350     360
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  CCAGTCACTCCCACCCAGATCACCAAGACCTACGGCGGCCAGATCTTCTGCATTGGAGGC
D5  .....
F39 ...A.....A.....
F41 .....
N37 ...A.....A.....
P1  .....
Q18 ...A.....A.....T.....G
*** *****

```

```

          370      380      390      400      410      420
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  GCCATCAACACTCTCTCACCCCTCATCGTCAAGTGCCCACTTGAAATGATGAACCCCGG
D5  .....T.....
F39 .....T.....
F41 .....
N37 .....T.....
P1  .....A.....
Q18 .....T.....
*****
          430      440      450      460      470      480
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  GTCAAAGATTCAATTCAATACCTTGACTCGCCAAACTCCTCATCTCCATCACCGCTCAA
D5  .....
F39 .....T...G.....
F41 .....
N37 .....
P1  .....
Q18 .....
*****
          490      500      510      520      530      540
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  CCCACCGCTCCCCCGCATCGACCTGCATAATAACTGTATCAGGAACTCTCTCGATGCAC
D5  .....
F39 .....A.....G.....
F41 .....
N37 .....
P1  .....
Q18 .....
*****
          550      560      570      580      590      600
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  TCTCCGCTCATCACGGACACTTCCACCTAAGTTCTCGATCTTTAAAATCGTTAGCTCGCC
D5  .....
F39 .....
F41 .....
N37 .....
P1  .....
Q18 .....
*****
          610      620      630      640      650      660
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  AGTTAGCGAGGTCTGTCCCCACACGACAGATAATCGGGTGCAACTCCCGCCCCTTTCCG
D5  .....
F39 .....
F41 .....
N37 .....
P1  .....
Q18 .....
*****
          670      680      690      700      710      720
-----+-----X-----+-----X-----+-----X-----+-----X-----+-----X
BL  AGGGTCATCGGAACCA
D5  .....
F39 .....
F41 .....
N37 .....
P1  .....
Q18 .....
*****

```

```

          10          20          30          40          50          60
-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x
BL  MEIDKELAPQDRTVTVATVLPVPIAPGPSPTIKQPFQSEVLFAGTKDAEASLTIANIDSVS
D5  .....A.....I..TV.....L.....G.....G.....
F39 .....I..TV.....L.....G.....G.....V...R..
F41 .....V...R..
N37 .....I..TV.....L.....
P1  .....TV.....
Q18 .....I..TV.....L.....V...R..
*****.*****.***.*****.*****.*****.*****.*****.*****.*****.*****.*****
          70          80          90          100         110         120
-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x
BL  TLTTYRHASLESLEWVTIHPTLQAPAFPTTVGVCWVPANSPVTPPTQITKTYGGQIFCIGG
D5  .....
F39 .....I.....
F41 .....Y.....
N37 .....I.....
P1  .....
Q18 .....I.....
*****.*****.*****.*****.*****.*****.*****.*****.*****.*****.*****
          130         140         150         160         170         180
-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x
BL  AINTLSPLIVKCPLEMMNPRVKDSIQYLDSPKLLISITAQPTAPPASTCIITVSGTLSMH
D5  .....
F39 .....A.....D..G.....
F41 .....
N37 .....
P1  .....H.....
Q18 .....
*****.*****.*****.*****.*****.*****.*****.*****.*****.*****.*****
          190         200         210         220         230         240
-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x-----+-----x
BL  SPLITDTST
D5  .....
F39 .....
F41 .....
N37 .....
P1  .....
Q18 .....
*****

```

Figure 3.4 Amino acid sequences of the coat proteins of the six symptom variants compared with that of TYMV-BL.

Variant Name	Symptoms	<u>Nucleotide Changes</u>		<u>Amino Acid Changes</u>	
		Silent	Non-silent	Conserv.	Non-conserv.
D5	Mild	9	7	3	4
F39	Mild	8	12	6	6
F41	Severe	2	3	1	2
N37	Severe	8	5	4	1
P1	Intermediate	6	3	1	2
Q18	Severe	12	7	5	2

Table 3.1 Summary of the sequence differences of the coat proteins of the six symptom variants. The variant sequences were compared with TYMV-BL and the number of nucleotide and amino acid changes are listed. The differences in the nucleotide sequences were located mainly in the 5' two-thirds of the coat protein gene with fewer in the 3' one-third and none in the non-coding region at the 3' end of the virus genome. (Conserv. = conservative amino acid change).



<u>Variants</u> <u>Name</u>	<u>Symptoms</u>	<u>Amino Acid Changes</u>
D5	Mild	<b>T-A(13)</b> , V-I(19), <b>I-T(22)</b> , A-V(23), F-L(29), <b>A-G(42)</b> , <b>A-G(49)</b>
F39	Mild	V-I(19), <b>I-T(22)</b> , A-V(23), F-L(29), <b>A-G(42)</b> , <b>A-G(49)</b> , A-V(54), <b>S-R(58)</b> , V-I(102), P-A(151), <b>A-D(163)</b> , <b>A-G(166)</b>
F41	Severe	A-V(54), <b>S-R(58)</b> , <b>I-Y(78)</b>
N37	Severe	V-I(19), <b>I-T(22)</b> , A-V(23), F-L(29), V-I(102)
P1	Intermediate	<b>I-T(22)</b> , A-V(23), <b>L-H(134)</b>
Q18	Severe	V-I(19), <b>I-T(22)</b> , A-V(23), F-L(29), A-V(54), <b>S-R(58)</b> , V-I(102)

Table 3.2 Differences in the coat protein amino acids of TYMV-BL and the symptom variants. Non-conservative changes are highlighted and the positions of the changes are listed in brackets. The single letter code for the amino acids is used.

and Q18. D5 and F39 had two amino acid differences in common which were not present in any of the other variants and therefore might explain the mild symptoms they produced in infected plants. These changes were non-conservative and both involved an alanine to glycine change at positions 42 and 49 of the coat protein. However both these variants also contained amino acid changes which were unique to them alone. P1 contained three amino acid changes, one of which (leucine to histidine), was not present in any of the other variants and may explain the intermediate symptoms it produced; the other two amino acid changes were present in both the mild and severe variants and hence may not be involved in the production of intermediate symptoms.

### 3.3 DISCUSSION

The extent to which the changed amino acids in the coat proteins of the symptom variants affect their phenotype is difficult to determine. TYMV-BL produces an extreme mosaic in infected plants so the variants which caused severe symptoms were probably closest to the wild-type virus. Therefore the amino acid changes in the variants which produced mild and intermediate symptoms in plants are of interest in investigating which residues are important in the severity of symptoms expressed.

Previous work on TYMV located a single amino acid substitution in the 69K movement protein which increased the symptom severity of the virus (Tsai and Dreher, 1993) and the results reported here suggest that some amino acid substitutions in the coat protein may be involved in attenuating the severe symptoms of TYMV-BL. However, to investigate whether or not the glycine residues at positions 42 and 49 in the coat proteins of the mild variants are involved in attenuating severe symptoms a more detailed analysis would need to be done. One approach would be to

substitute these glycine residues into the TYMV-BL coat protein by *in vitro* mutagenesis and to inoculate plants with transcript of the mutant. The plants could then be monitored for the appearance of symptoms. The same approach could be taken to check the involvement of the leucine to histidine change in the P1 coat protein in the production of intermediate symptoms.

It is, of course, likely that other viral proteins may be involved in the production of symptoms in plants. In cauliflower mosaic virus, single amino acid substitutions in the gene VI-encoded protein affected the symptoms expressed by the virus in *Datura stramonium* plants (Daubert and Routh, 1990) whereas in the monopartite geminiviruses, tomato leaf curl (TLCV) and beet curly top (BCTV), the protein product of ORF C4 was shown to be a determinant of pathogenesis (Rigden *et al.*, 1994; Stanley and Latham, 1992). The  $\alpha$  gene of barley stripe mosaic hordeivirus was shown to encode a protein involved in symptom development by the virus in oat plants (Weiland and Edwards, 1994), whereas the protein encoded by RNA $\gamma$  was shown to affect the symptoms in *Chenopodium amaranticolor* (Petty *et al.*, 1994). These last two results suggest that symptom expression is a complex process which involves different viral proteins in different plant hosts. Differences in pathogenicity can also result from nucleotide changes in the viral genome that do not alter the encoded amino acids. In barley stripe mosaic hordeivirus, a nucleotide change in the 5' non-coding region of RNA $\gamma$  was shown to be a determinant of pathogenesis in *Nicotiana benthamiana* (Petty *et al.*, 1990), and in maize streak geminivirus silent nucleotide changes in gene VI affected the symptoms expressed by this virus (Boulton *et al.*, 1991). The possibility that silent nucleotide changes may be involved in the pathogenicity of TYMV variants therefore cannot be ruled out. Indeed, nucleotide changes that give rise to changes in the amino acid



## CHAPTER FOUR

### ANALYSIS OF TYMV-BL COAT PROTEIN RECOMBINANTS

#### 4.1 INTRODUCTION

Experimental mutagenesis of viral coat proteins has been used by several workers to investigate the role(s) of the coat protein in viral infection. Results from such experiments showed that coat proteins of some viruses were required for long distance movement, coat proteins of others were required for both long distance and cell-to-cell movement while other viruses did not require the coat protein for either. Those viruses for which the coat protein was required for long distance movement included tobacco mosaic virus (TMV), southern bean mosaic virus (SBMV), rice yellow mottle virus (RYMV), brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV), turnip yellow mosaic virus (TYMV) (Dawson *et al.*, 1988, Saito *et al.*, 1990, Fuentes and Hamilton, 1993, Brugidou *et al.*, 1995, Sacher and Ahlquist, 1989, Allison *et al.*, 1990; Bransom *et al.*, 1995). Viruses which required the coat protein for both long distance and cell-to-cell movement included potato virus X (PVX), tobacco etch potyvirus (TEV), cowpea mosaic virus (CMV), and turnip crinkle virus (TCV) (Chapman *et al.*, 1992; Dolja *et al.*, 1994; 1995; Wellink and van Kammen, 1989; Laakso and Heaton, 1993; Hacker *et al.*, 1992). Other viruses which did not need the coat protein for either systemic or local spread included cucumber necrosis virus (CNV), barley stripe mosaic virus (BSMV) and tomato golden mosaic virus (TGMV) (Sit *et al.*, 1995; Petty and Jackson, 1990; Gardiner *et al.*, 1988). In alfalfa mosaic virus the coat protein was required for genome activation, accumulation of plus-strand RNA and cell-to-cell movement but the virus did not move as stable virions (Van der Vossen *et al.*, 1994). The investigation of the roles of the coat proteins of the above viruses involved using mutagenesis to produce insertions, deletions and frame-shifts to

change chosen parts, for example the N and C termini and amino acids that had been shown to be important as binding sites or in forming salt bridges. A more detailed account of this work is presented in Chapter 1.

While much work had been done on the non-structural proteins of TYMV involving investigation of the size, composition, expression and roles of the replicase and overlapping proteins, (Morch and Benicourt, 1980; Morch *et al.*, 1989; Weiland and Dreher, 1989; Bransom *et al.*, 1991; Bozarth *et al.*, 1992; Tsai and Dreher, 1993; Bransom and Dreher, 1994 and Bransom *et al.*, 1995), little was known about the role of the tymovirus coat protein in infection of host plants until a recent report in which the TYMV coat protein was mutagenised and shown to be necessary for long distance movement of the virus (Bransom *et al.*, 1995) (Chapter 1). Some earlier work based on immunochemical experiments had shown the existence of three antigenic determinants in the coat protein. One of these was at the N terminus of the protein and encompassed amino acids 1-12, one was at the C terminus (amino acids 182-189) and a third was localised to residues 57-64 (Pratt *et al.*, 1980; Quesniaux *et al.*, 1983a, 1983b). Other work using crosslinking methods had identified three regions of the coat protein in close contact with the viral RNA. However one of these regions corresponded to the N terminus which was later suggested to be exposed on the surface of the protein (Quesniaux *et al.*, 1983a). The other regions thought to be in contact with the RNA were located between amino acids 33-45 and 132-152. Within these regions amino acids 42-45 and 141-142 bound strongly to the RNA (Ehresmann *et al.*, 1980).

As the mutagenesis methods of other workers had been successful in elucidating the roles of coat proteins, it was decided to use mutagenesis to investigate the roles of different parts of the tymovirus coat protein. To determine whether the tymovirus coat protein not only affected processes

such as viral replication, but also more subtle characters such as viral host range, recombinants were designed to replace sections of the turnip yellow mosaic virus-Blue Lake isolate (TYMV-BL) coat protein gene with the corresponding regions of the belladonna mottle virus (BMV) gene. Although these experiments differed from those done with other viruses in that most involved constructing recombinants rather than inserting amino acids, deleting amino acids or frame shifting the coat protein, this approach was taken because the amino acid sequences of TYMV-BL and BMV are similar yet their host ranges are different. Thus recombinants of this sort might reveal which amino acids of the TYMV-BL coat protein were involved in host range determination. Also, those regions essential for infection by the virus could be identified.

Table 4.1 shows the amino acid changes introduced into the TYMV-BL coat protein for each of ten recombinants (R1-R10), Table 4.2 shows the corresponding nucleotide sequences. The various regions that were changed were chosen using the predicted structure of the TYMV-BL coat protein (Chapter 1) and using the results of published immunochemical studies of the TYMV coat protein.

(1) Recombinants no. 2, 3 and 4 (R2, R3 and R4) produced coat proteins with changes at the N terminus and were constructed as the predicted structure and the accessibility data from the EMBL PHD protein prediction programs inferred that the N terminus of the coat protein was probably exposed at a surface of the particle and not buried within the protein.

(2) Recombinant 10 (R10), which produced a coat protein with changes at the C terminus of the protein, was made for the same reason.

<u>Recombinant</u> <u>Number</u>	<u>Positions changed</u>	<u>Amino Acid Changes</u>
1	(amino acid 1)	TYMV M * (Termination)
2	(amino acids 1-12)	TYMV MEIDKELAPQDR BMV MDESKIIVTVKQP
3	(amino acids 13-20)	TYMV TVTVAT-V BMV SISAPGFT
4	(amino acids 1-20)	TYMV MEIDKELAPQDR TVTVAT-V BMV MDESKIIVTVKQPSISAPGFT
5	(amino acids 57-67)	TYMV IDSVSTLTTFY BMV SDSLAKLTSGY
6	(amino acids 106-114)	TYMV TQITKTYGG BMV SKILSIYGG
7	(amino acids 140-142)	TYMV PRV BMV AMI
8	(amino acid 143)	TYMV K N (point mutation)
9	(amino acids 144-153)	TYMV DSIQYLDSPK BMV DSTIYTDSPK
10	(amino acids 181-189)	TYMV HSPLITDTST BMV SAPLLQAL*

Table 4.1 The positions of the 10 recombinant TYMV coat proteins and the amino acid sequence changes made.



Recombinant Number	Nucleotide Changes Made	
1	TYMV-BL	ATG TAA (Termination)
2	TYMV-BL	ATG GAA ATC GAC AAA GAA CTC --- GCC CCC CAA GAC CGC
	BMV	ATG GAT GAA TCA AAA ATT GTC ACC GTC AAG CAA CCC AGC
3	TYMV-BL	ACC GTC ACC GTC GCC ACC GTT
	BMV	ATC TCT GCT CCC GGC TTT ACT
4	TYMV-BL	ATG GAA ATC GAC AAA GAA CTC --- GCC CCC CAA GAC CGC ACC GTC ACC GTC GCC ACC GTT
	BMV	ATG GAT GAA TCA AAA ATT GTC ACC GTC AAG CAA CCC AGC ATC TCT GCT CCC GGC TTT ACT
5	TYMV-BL	ATC GAC AGC GTT TCC ACC CTT ACC ACC TTC TAC
	BMV	TCT GAC TCC CTG GCC AAA CTC ACC TCT GGG TAT
6	TYMV-BL	ACC CAG ATC ACC AAG ACC TAC GGC GGC
	BMV	TCC AAA ATT CTC TCC ATC TAT GGC GGC
7	TYMV-BL	CCC CGG GTC
	BMV	GCC ATG ATC
8	TYMV-BL	AAA AAC (Point Mutation)
9	TYMV-BL	GAT TCA ATT CAA TAC CTT GAC TCG CCC AAA
	BMV	GAT TCA ACC ATT TAC ACC GAC TCG CCC AAA
10	TYMV-BL	CAC TCT CCG CTC ATC ACG GAC ACT TCC ACC TAA
	BMV	TCT GCT CCC CTC TTG CAG GCT CTA TAA

Table 4.2 TYMV-BL nucleotide sequences with the BMV sequences that replaced them in each recombinant.

(3) Recombinants 5 and 6 (R5 and R6) produced coat proteins with the predicted alpha helical parts of the TYMV-BL coat protein replaced with the corresponding regions of BMV. The predicted coat protein structure had indicated that parts of both helices were exposed at a surface of the virus particle, and might therefore be involved in the interaction of the virus with the host and hence with the infection process.

(4) Recombinant 7 (R7) produced a coat protein with changes in the region of the TYMV coat protein where cross-linking studies had shown the protein to interact with the viral RNA (Ehresmann *et al.*, 1980).

(5) Recombinant 9 (R9) produced a coat protein with a change in a region of the coat protein that had been shown to have strong antigenic activity in inhibition tests (Pratt *et al.*, 1980). These tests had also shown regions modified by R2, R4 and R5 to be important antigenically.

(6) Recombinants 1 and 8 (R1 and R8) were designed to produce recombinant viruses with point mutations introduced into their coat proteins. R1 produced a recombinant with a termination codon in place of the methionine at the N terminus of the protein, and R8 had a highly conserved lysine residue changed to asparagine. This lysine residue is present in all tymovirus coat proteins sequenced to date and hence it is possibly an important amino acid in enabling the virus to produce stable virions. Asparagine was chosen as it was encoded by changing only one nucleotide in the third position from adenosine to cytidine.

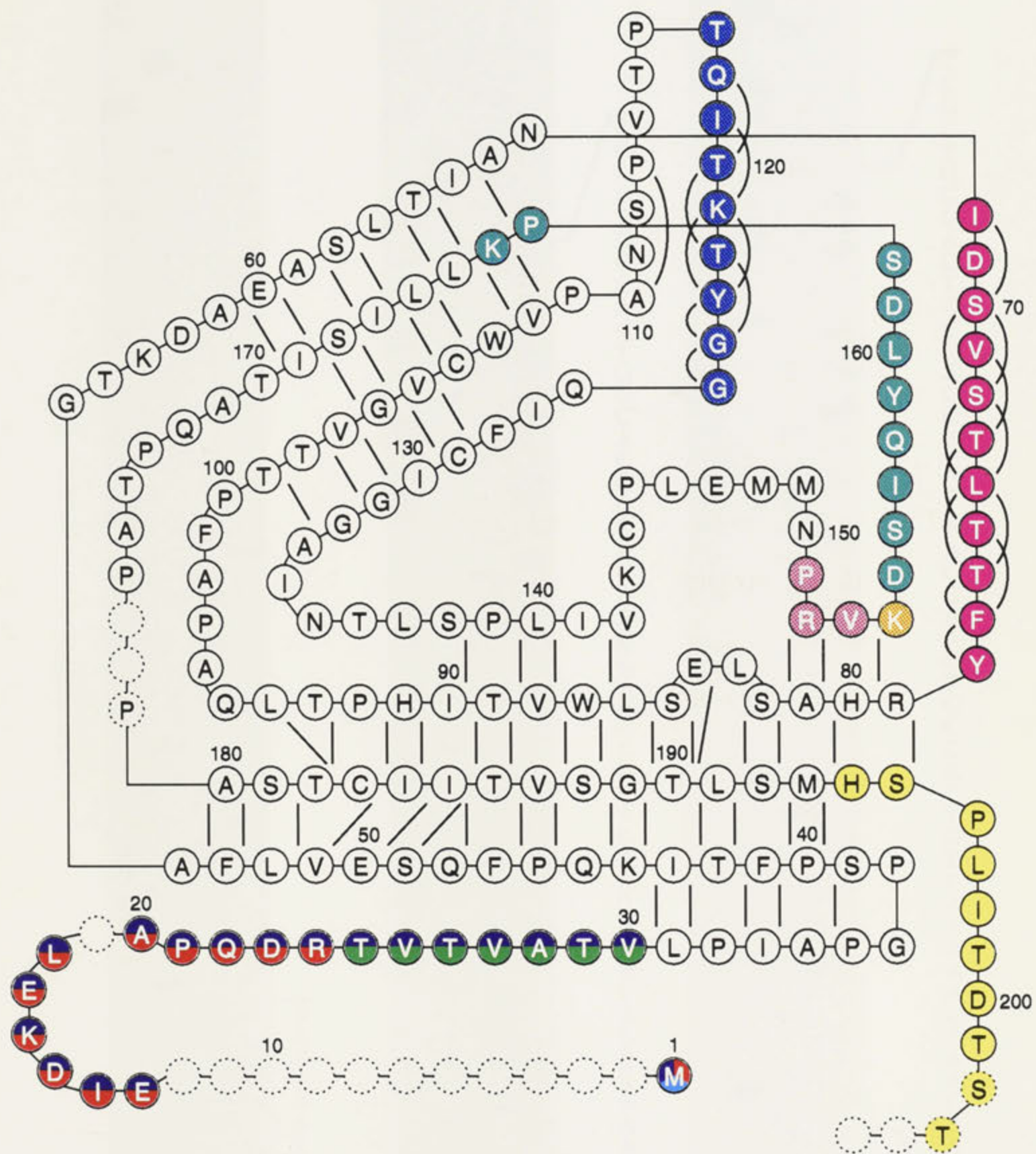
Figure 4.1 shows the positions of the changes produced in these recombinants on a diagram representing the predicted structure of the TYMV coat protein.

## 4.2 RESULTS

### 4.2.1 CONSTRUCTION, CLONING AND VERIFICATION OF THE GENOMIC SEQUENCES OF THE RECOMBINANTS

The pBL-16 coat protein recombinants were constructed using the Altered Sites *in vitro* mutagenesis kit from Promega. The coat protein gene was cloned into a specialised plasmid and oligonucleotides with the desired nucleotide sequences were used to incorporate changes into the coat protein gene. These coat protein genes were sequenced to ensure that the correct nucleotides had been included in the coat protein gene. Figure 4.2 shows the sequences of the recombinant genes compared with the corresponding regions of those of the wild-type pBL-16. The coat protein genes were then sub-cloned back into the full-length pBL-16 clone of the TYMV-BL virus. The resulting recombinant virus genomes were checked by digestion with *Pst*I and *Xba*I to ensure that the correct restriction pattern was obtained. The restriction pattern of the recombinants should be identical to that of the wild-type pBL-16 clone which would indicate that the recombinant clones had been assembled correctly. Results of these analyses are shown in Figure 4.3. When the genomic integrity of the recombinants had been verified, 5 $\mu$ g of recombinant plasmid was linearised by digestion with *Nde* I and transcribed into full length RNA for inoculation onto Chinese cabbage plants as outlined in Chapter 2 (2.2.14). Figure 4.4 shows full length transcripts for all the recombinants. This indicated that changes to the coat protein genes which had been introduced by the recombinants did not affect transcription of the cDNA clones to RNA. Therefore an absence of

Figure 4.1 Diagrammatic representation of the backbone folding of the TYMV coat protein showing the positions of the different recombinant amino acids; the amino acids shown in the diagram are those of TYMV-BL



- |  |  |
|--|--|
| <span style="color: blue;">●</span> Primer 1     | <span style="color: darkblue;">●</span> Primer 6 |
| <span style="color: red;">●</span> Primer 2      | <span style="color: pink;">●</span> Primer 7     |
| <span style="color: green;">●</span> Primer 3    | <span style="color: yellow;">●</span> Primer 8   |
| <span style="color: darkblue;">●</span> Primer 4 | <span style="color: teal;">●</span> Primer 9     |
| <span style="color: pink;">●</span> Primer 5     | <span style="color: yellow;">●</span> Primer 10  |

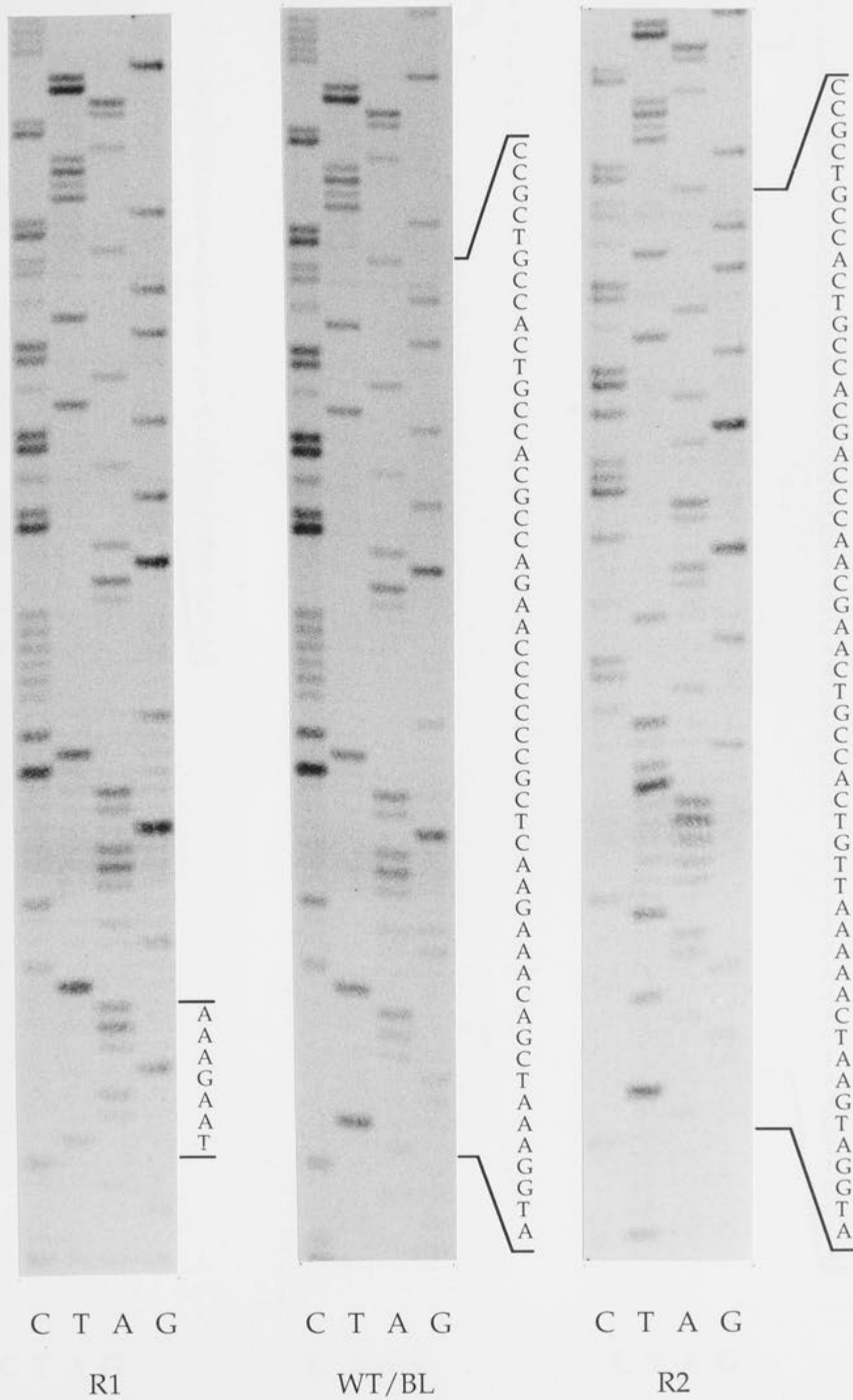
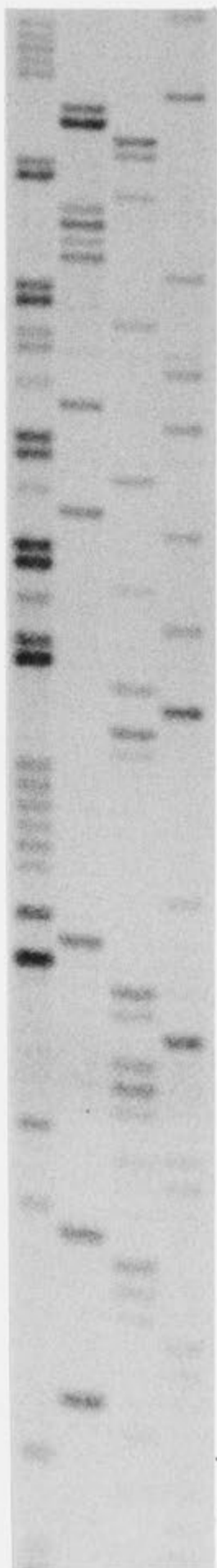


Figure 4.2 Photographs of autoradiographs showing sequences of recombinant coat protein genes and the corresponding sequences in the wild-type (TYMV-BL) coat protein gene.



A  
T  
T  
T  
C  
A  
T  
T  
T  
C  
G  
G  
C  
C  
C  
T  
C  
G  
T  
C  
T  
C  
T  
A  
C  
G  
C  
C  
A  
G  
A  
A  
C  
C  
C

C T A G  
 R3



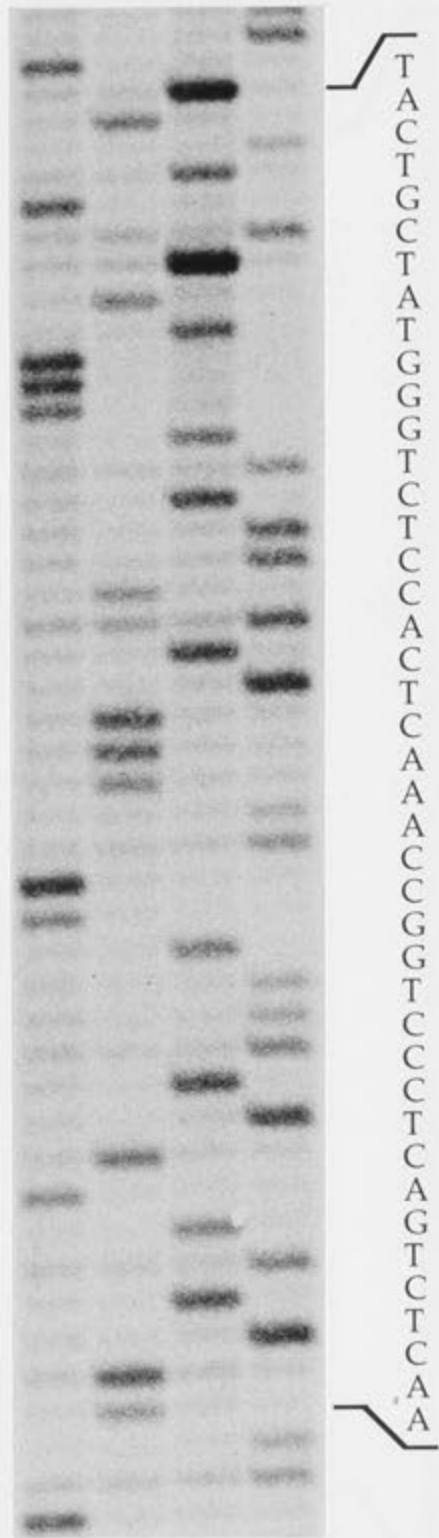
A  
T  
T  
T  
T  
G  
C  
C  
A  
C  
C  
G  
C  
T  
G  
C  
C  
A  
C  
T  
G  
C  
C  
A  
C  
G  
C  
C  
A  
G  
A  
A  
C  
C  
C  
C  
C  
G  
C  
T  
C  
A  
A  
G  
A  
A  
A  
C  
A  
G  
C  
T  
A  
A  
A  
G  
G  
T  
A

C T A G  
 WT/BL



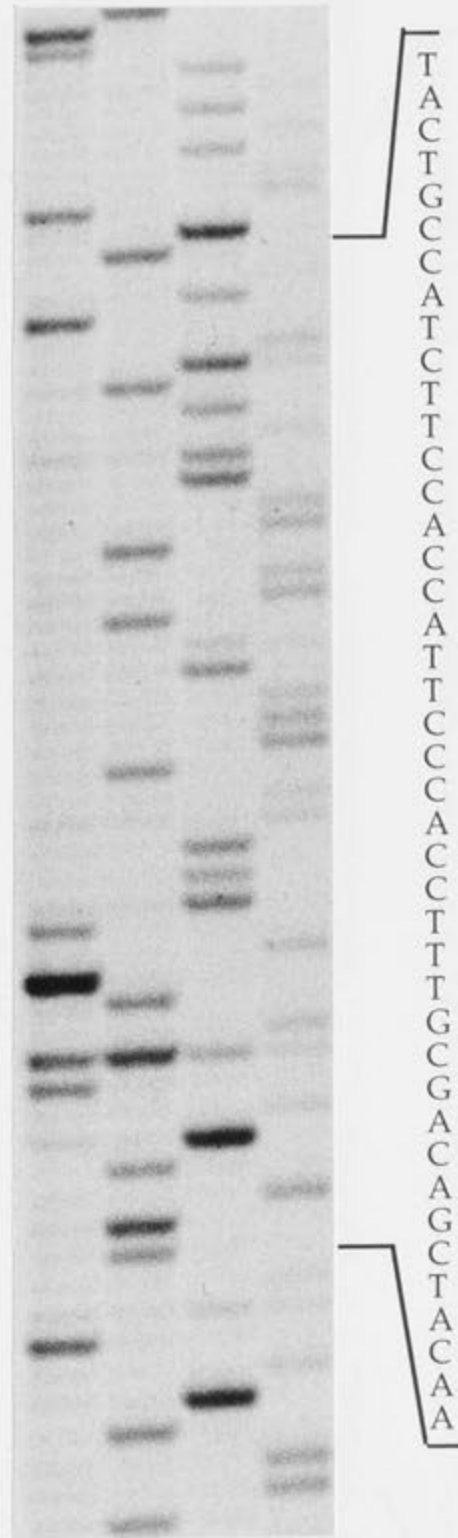
A  
T  
T  
T  
C  
A  
T  
T  
T  
C  
G  
G  
C  
C  
C  
T  
C  
G  
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A  
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G  
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A  
A  
A  
A  
C  
T  
A  
A  
G  
T  
A  
G  
G  
T  
A

C T A G  
 R4



G A T C

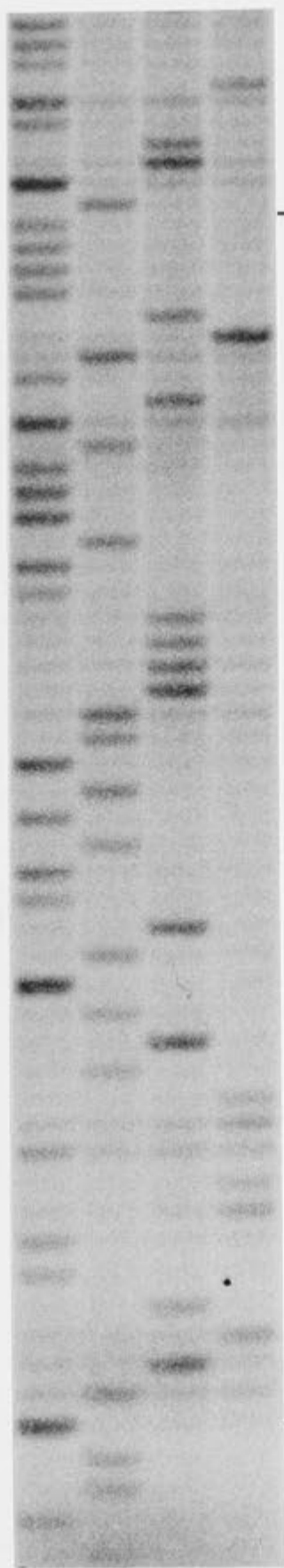
R5



G A T C

WT/BL

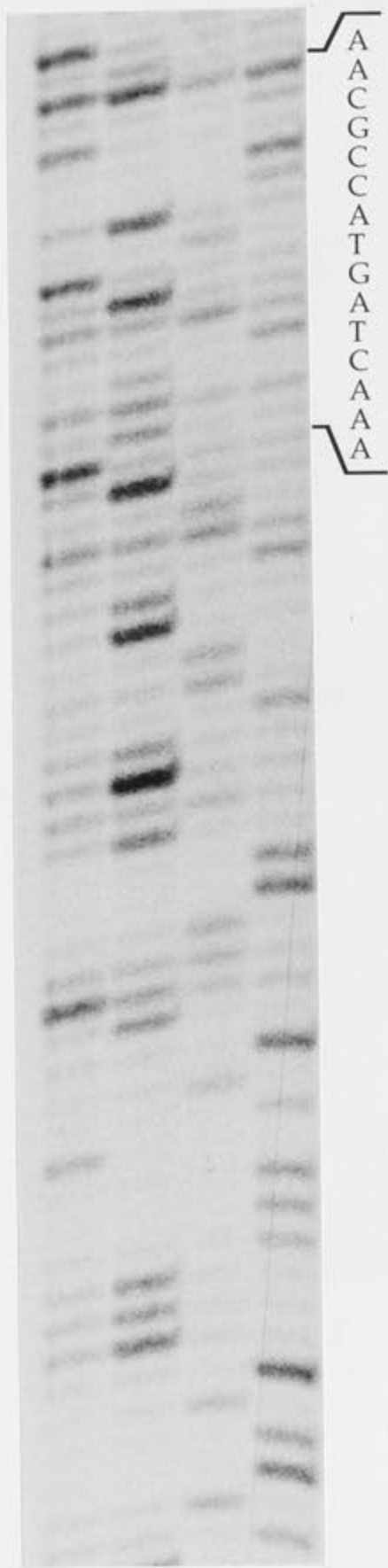




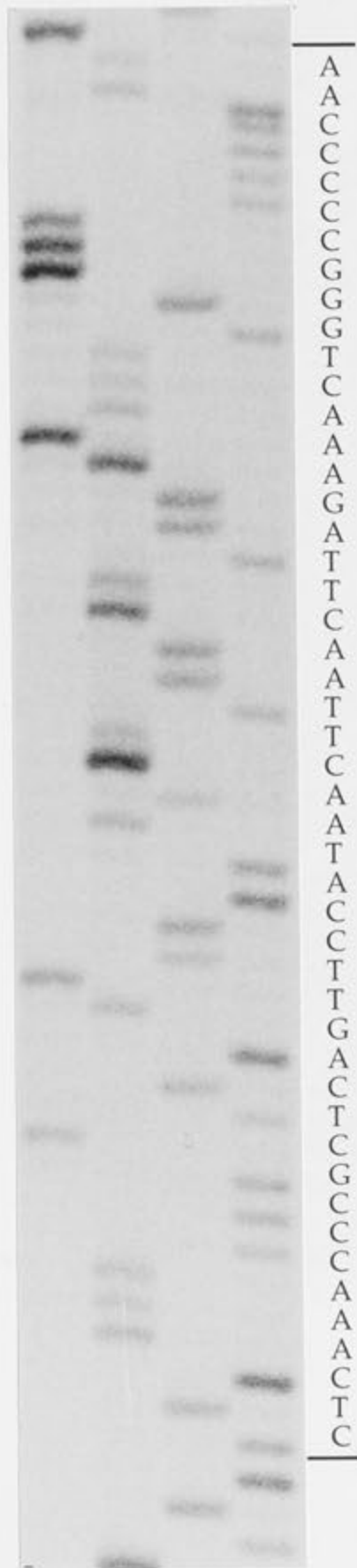
C T A G  
R6



C T A G  
WT/BL



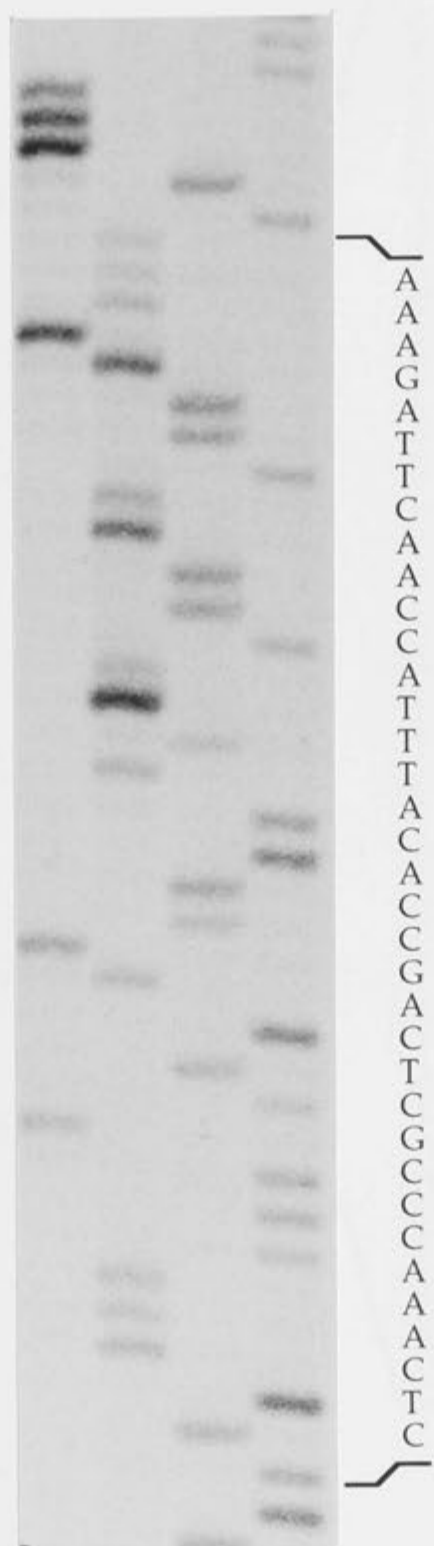
G A T C  
R7



G A T C  
WT/BL

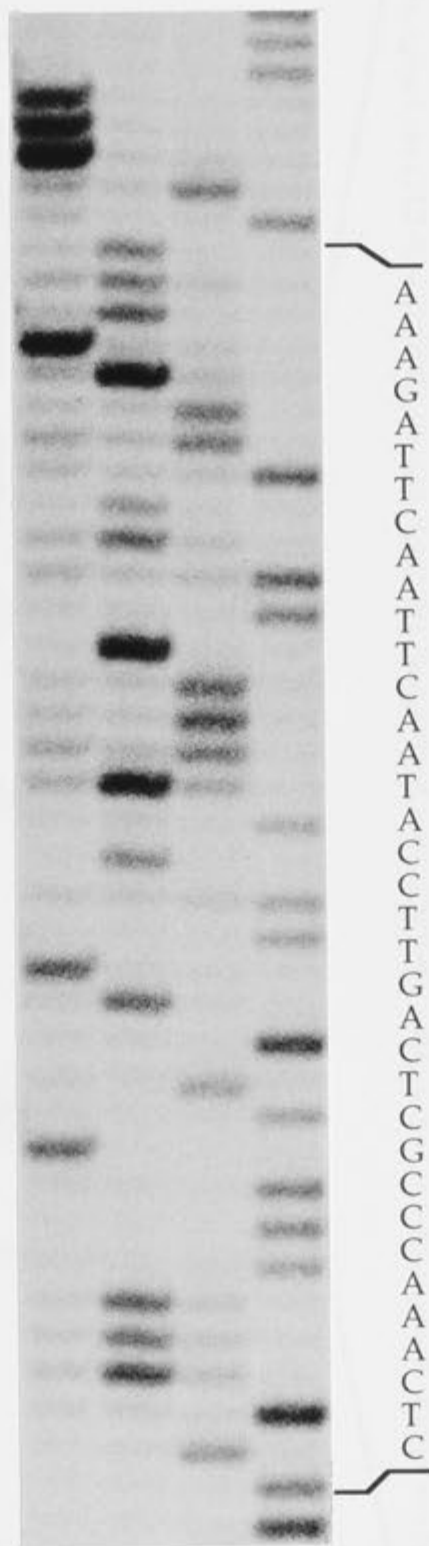


G A T C  
R8



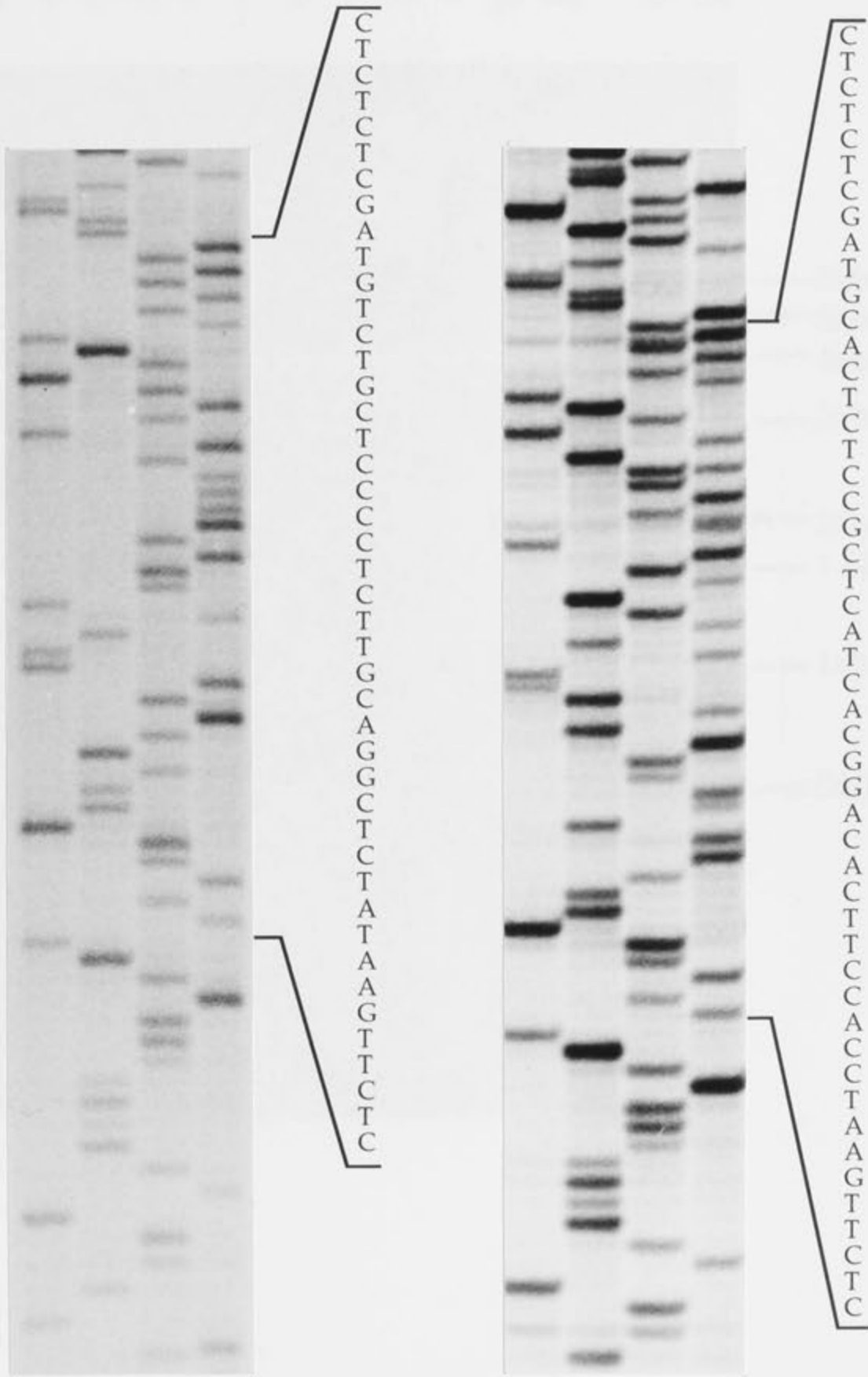
G A T C

R9



G A T C

WT/BL



G A T C

R10

G A T C

WT/BL

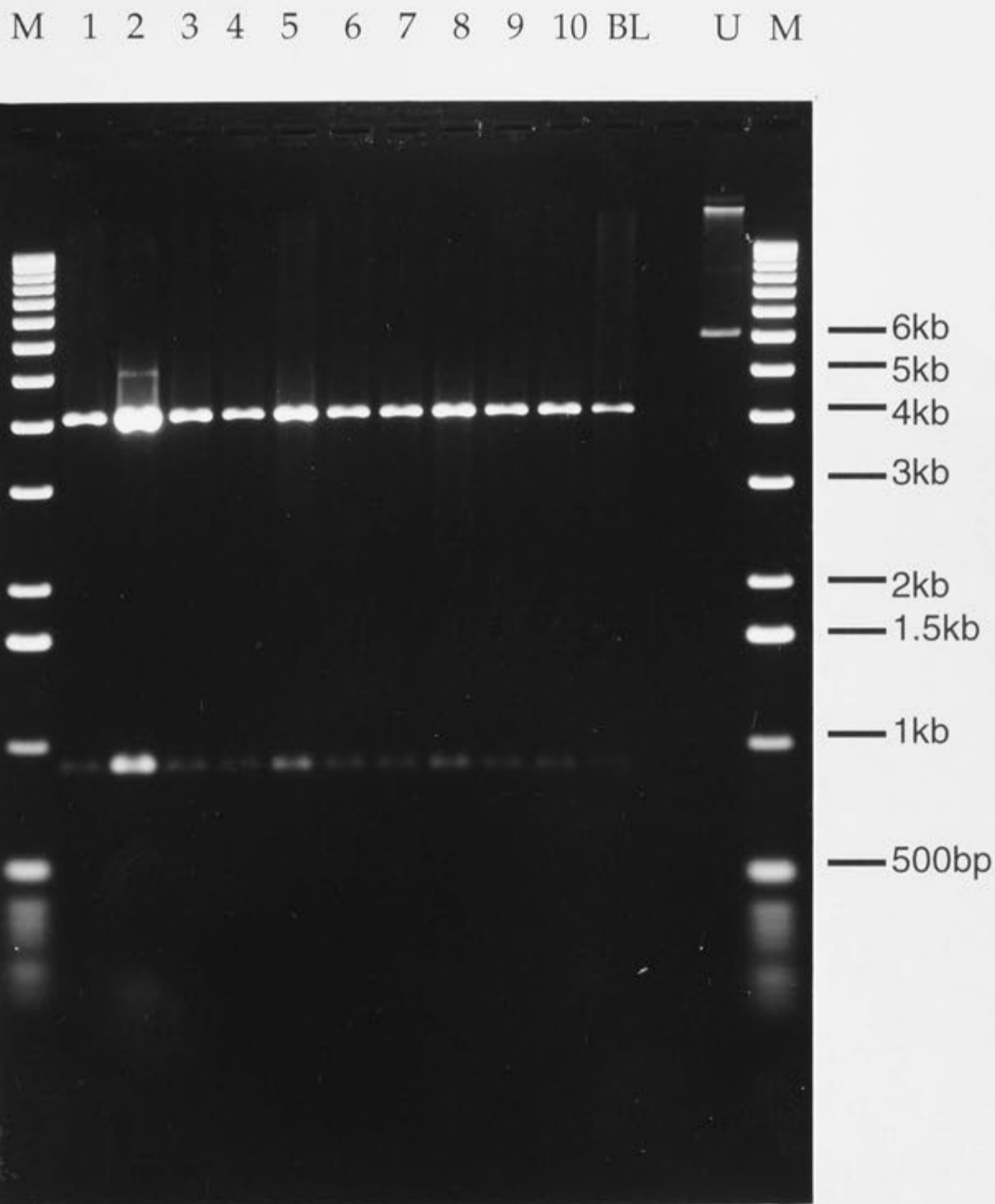


Figure 4.3 Restriction endonuclease analysis of all recombinants with *Pst*I and *Xba*I restriction enzymes. Plasmids containing the recombinant virus clones were incubated with the enzymes at 37°C for 2-4 hours before electrophoresis through 1% agarose gels in 1XTAE buffer overnight. BRL 1Kb ladder was used as molecular weight markers (lanes M) and the sizes are shown along the side. Recombinants 1-10 were loaded in order with digested pBL-16 as a control. Uncut pBL-16 was also included.

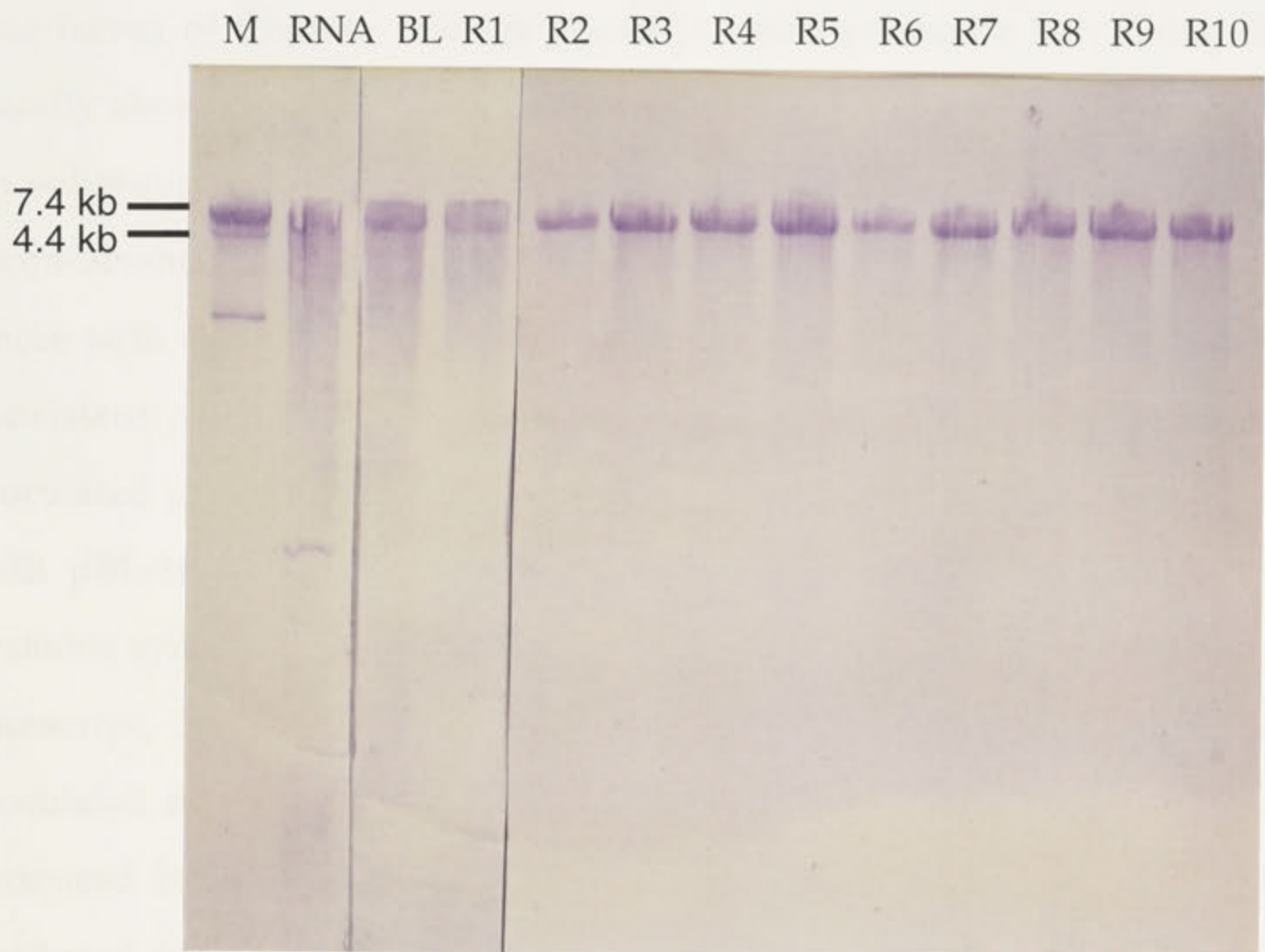


Figure 4.4 Gel fractionation of transcripts obtained from each recombinant. Following transcription a small quantity of each transcript was subjected to electrophoresis in a 3.5% polyacrylamide gel to check its size and integrity. The gel was stained with 0.04% toluidine blue and destained in water before being dried onto paper. RNA molecular weight markers and total viral RNA isolated from particles of TYMV-BL were included in lanes 1 and 2 respectively. The sizes of the markers and the location of the subgenomic RNA are indicated along the side. Recombinants 1-10 were loaded as shown.

symptoms on inoculated plants would not be due to transcripts being less than full-length.

#### 4.2.2 INOCULATION OF TRANSCRIPTS ONTO CHINESE CABBAGE PLANTS

Transcripts were manually inoculated onto the first two expanded true leaves of Chinese cabbage plants (*Brassica pekinensis* var. Wong Bok), usually about two weeks old, and checked for the appearance of symptoms on subsequent days. Photographs of plants inoculated with the different recombinants and showing systemic symptoms are shown in figure 4.5. Those with changes at the N terminal end of the coat protein (R2 and R3 ) consistently gave systemic symptoms, although the symptoms on R3 inoculated plants were different from those produced on plants inoculated with pBL-16 (wild-type) transcript. Plants inoculated with R2 showed systemic symptoms at the same time as those inoculated with the wild-type transcript, whereas the appearance of systemic symptoms in plants inoculated with R3 was delayed by approximately 5-7 days. The symptoms produced by R3 were consistently and significantly different from those produced by R2 and wild-type transcript. Infection with pBL-16 or R2 produced a systemic mosaic which spread from the base of the leaf through the leaf veins to cover the entire leaf. With R3, yellow patches appeared over the leaf veins but these did not spread and cover the entire leaf.

Recombinants 4 and 5 which produced coat proteins altered at the N-terminal end and  $\alpha$  helix A respectively, systemically infected approximately 50% of the time and gave symptoms indistinguishable from a wild-type infection. R7 and R9 gave systemic symptoms about 10% of the time but the appearance of symptoms was delayed by up to 3 weeks and sometimes longer compared with the wild-type virus. The appearance of



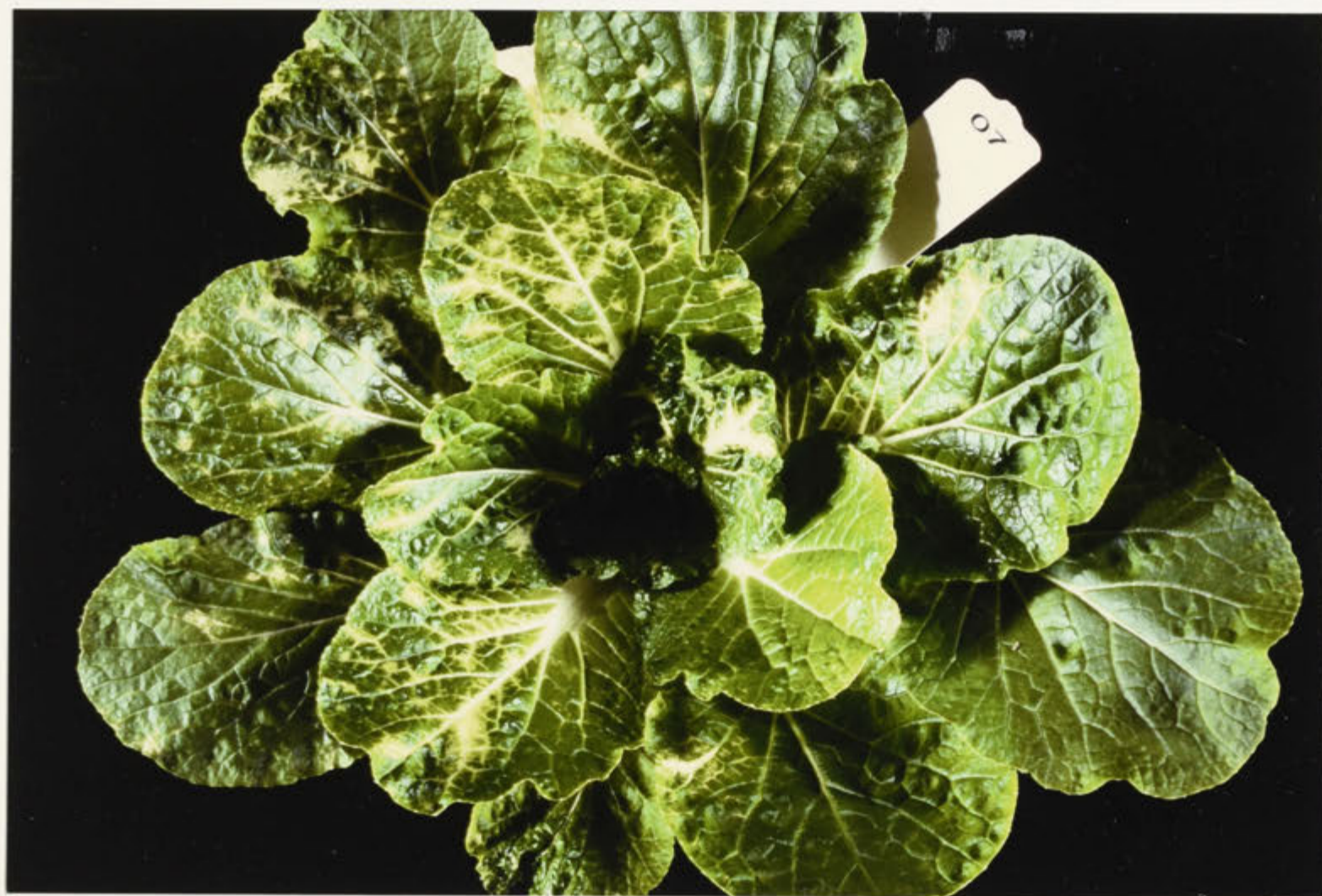
Figure 4.5 Photographs of Chinese cabbage plants infected with transcript RNA from each of the recombinants. The labels in the pots show which recombinants were used to inoculate the plants. In the case of R5 plants showing lesions on the inoculated leaves are also included as these were the symptoms produced by the recombinant 50% of the time. Lesions like these were also produced by R4 50% of the time. R1 inoculated plants are not shown as they, like R10 inoculated plants, did not develop any symptoms. Photographs were taken 7-21 days post inoculation depending on which recombinant was used for inoculation. A photograph of a control plant inoculated with transcript of pBL-16 is included for comparison of symptoms.













systemic symptoms was also delayed compared with the wild-type virus when sap containing virus particles of these recombinants was passaged onto fresh Chinese cabbage plants but they were not as delayed as when transcript RNA was used as inoculum. The symptoms produced were similar to those seen in plants infected with R3. R6 and R8 gave local lesions on the inoculated leaves and, when passaged, gave local lesions on the inoculated leaves.

The other recombinants, R1 and R10, never produced systemic symptoms or local lesions. The above results are summarised in Table 4.3.

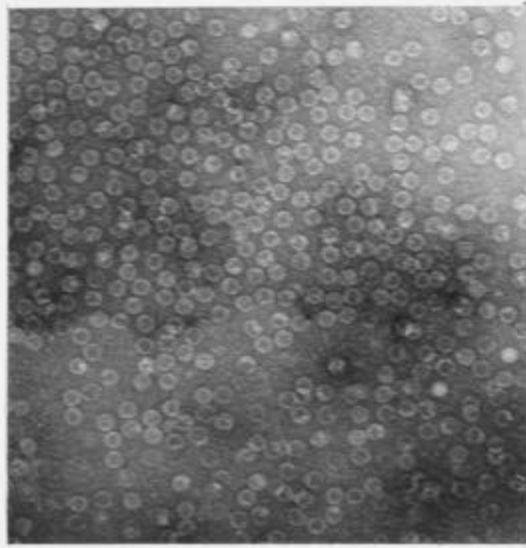
The ability of the recombinants to produce systemic symptoms on some occasions but not others may have been caused by differences in glasshouse conditions and differences in individual plants.

The presence of the recombinant virus in the systemically infected leaves was checked by extracting virus particles from the leaves and examining them in the electron microscope. Only those recombinants which produced systemic symptoms contained virus particles (Figure 4.6). Plants were also checked for the presence of virus by RT-PCR using the coat-protein specific primers Tall Comp and CL-3 (primer sequences are listed in Chapter 2 [2.1.4]). Figure 4.7 shows the PCR products which were subsequently sequenced and the presence of the recombinant virus verified. In all cases where there was a systemic infection that produced symptoms, only the recombinant virus with which the plant was inoculated was recovered from the leaves showing symptoms.

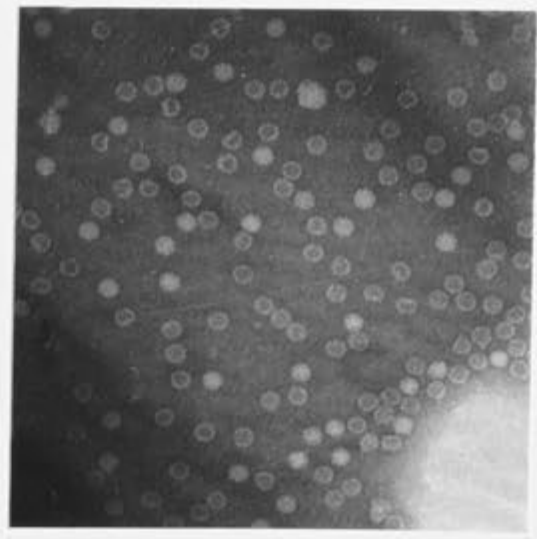
Tests were also done to see if some of the recombinants spread without causing systemic symptoms. Leaf discs were taken from the inoculated and tip leaves of plants infected with each of the 10 recombinants. Total RNA was isolated and cDNA synthesised for PCR. Only those recombinants that produced clear symptoms in inoculated or tip

<u>Recombinant No.</u>	<u>Symptoms produced in inoculated plants</u>	<u>Delay in appearance of systemic symptoms compared to wild-type</u>
1	none	n/a
2	systemic (100%), identical to WT	none
3	systemic (100%), different to WT	5-7 days
4	systemic (50%), identical to WT	none
5	systemic (50%), identical to WT	none
6	local lesions	n/a
7	systemic (10%), different to WT	up to 21 days
8	local lesions	n/a
9	systemic (10%), different to WT	up to 21 days
10	none	n/a

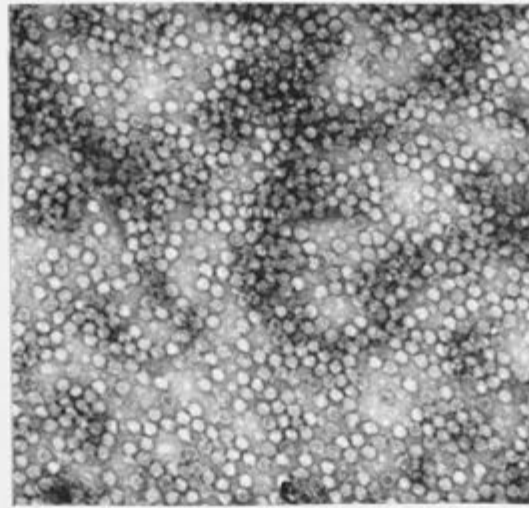
Table 4.3 Summary of the type of symptoms produced by the different recombinants and the timing of their appearance in comparison with the wild-type control. Experiments were done on at least 15 separate occasions and the numbers in brackets indicate the percentage of times systemic symptoms were produced by these recombinants. WT= wild-type



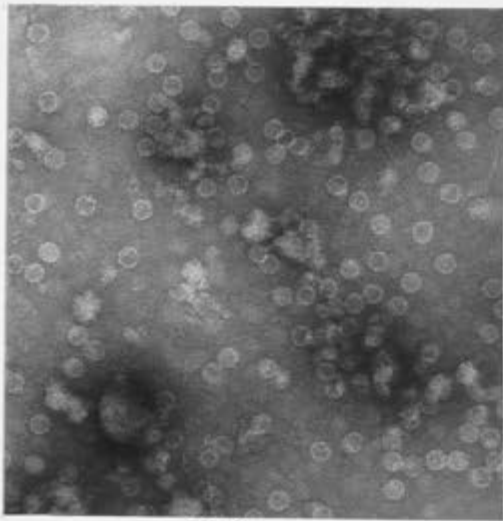
R2



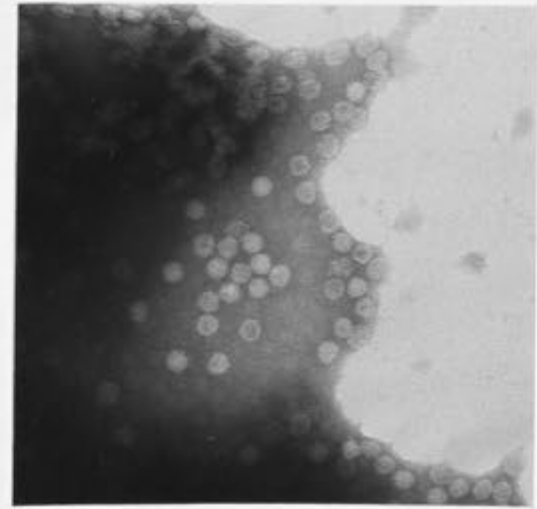
R3



BL



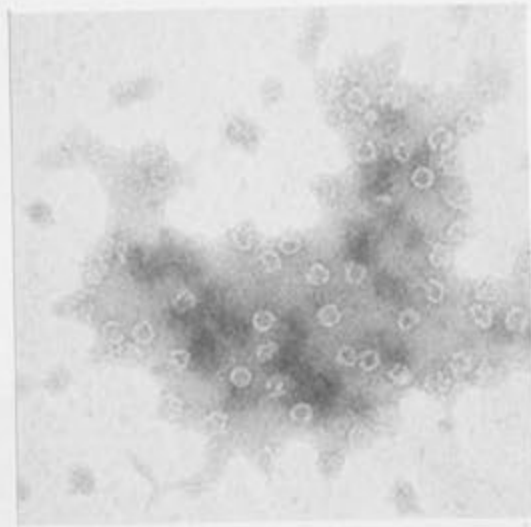
R4



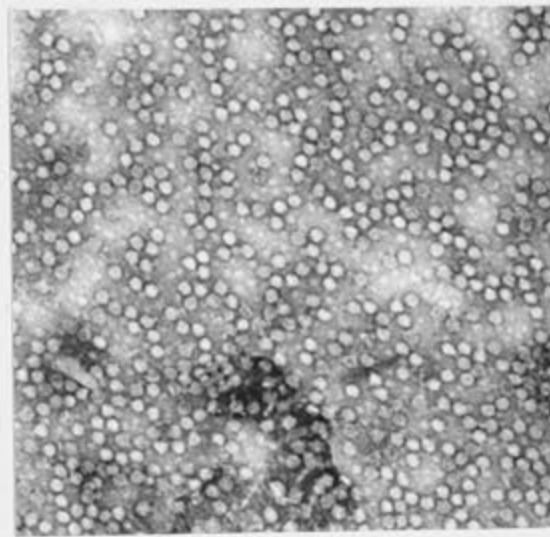
R5

Figure 4.6 Photographs of electron micrographs showing the particles of each of the systemically infecting recombinants and TYMV-BL. Virus particles were stained with ammonium molybdate and photographed at a magnification of 40,000.

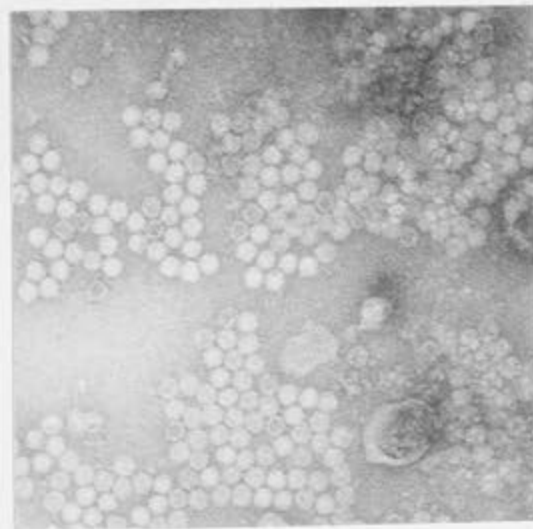




R7



BL



R9

Figure 17. Electron micrograph of gel-purified RNA products amplified by RT-PCR from plants inoculated with each of the genetically engineered *Neovirus*. Total RNA was isolated from leaves of selected plants and used for RT-PCR analysis. The products were purified and analyzed by agarose gel electrophoresis and ethidium bromide staining. The RT-PCR products were electrophoresed on agarose gels containing 0.5% agarose, 0.5% formaldehyde and stained with ethidium bromide. The products were visualized by UV light. The products were purified and analyzed by agarose gel electrophoresis and ethidium bromide staining.

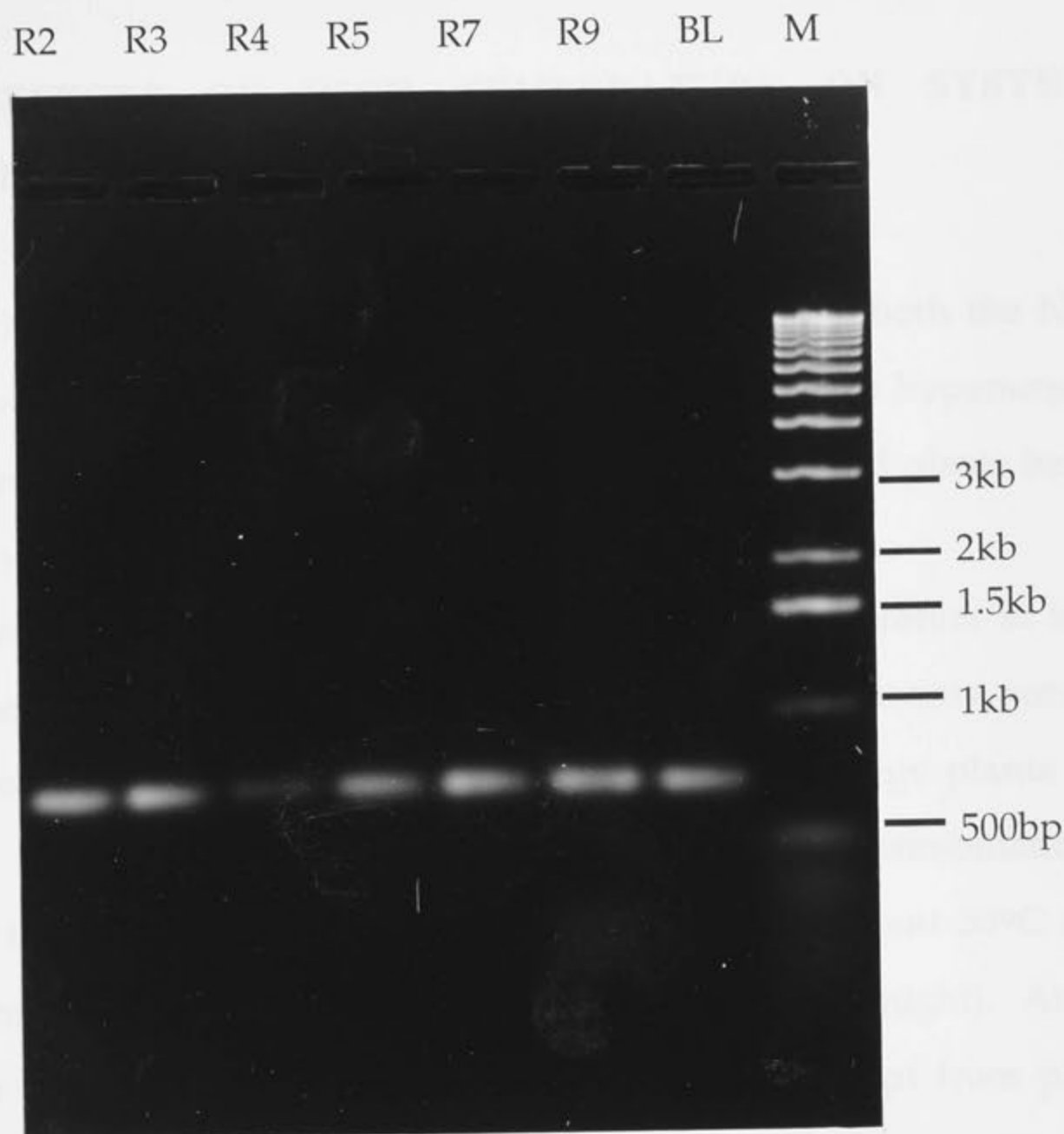


Figure 4.7 Photograph of gel fractionated PCR products amplified by RT-PCR from plants inoculated with each of the systemically infecting recombinants. Total RNA was isolated from leaves of infected plants and used for cDNA synthesis. The coat protein genes were then amplified from the cDNA using Tall Comp and CL-3 primers (primer sequences, 2.1.4). The PCR products were electrophoresed through a 1% agarose gel with 1XTAE as running buffer and 1kb ladder as marker. A 700bp fragment corresponding to the coat protein gene amplified for each recombinant.

leaves gave any PCR products using the TYMV coat protein specific primers. No PCR products were amplified for recombinants that only produced lesions.

#### 4.2.3 EFFECTS OF HIGH TEMPERATURE ON SYSTEMIC INFECTION BY TYMV-BL RECOMBINANTS

In *Nicotiana tabacum* and *Nicotiana glutinosa* plants, both the N and N' genes which localise tobacco mosaic virus (TMV) in the hypersensitive response are inhibited at high temperatures (above 30°C) and plants become systemically infected by TMV (Van Loon, 1975; Fraser, 1983).

Experiments were done to see if elevating the temperature at which TYMV inoculated plants were held would induce recombinants normally localised to lesions to spread systemically. Chinese cabbage plants were inoculated with recombinant transcripts and, for each recombinant, one plant was then held at a constant 20°C, another at a constant 30°C and a third in normal glasshouse conditions (25°C day and 18°C night). At 20°C and under normal glasshouse conditions both the transcript from pBL-16 and R2 produced systemic symptoms. However, at 30°C none of the plants, including those inoculated with transcript of pBL-16, showed systemic symptoms. This result suggested that recombinants restricted to lesions in inoculated leaves were probably movement-deficient, and not restricted to lesions due to the hypersensitive response of the host.

Another experiment was done to check whether the lack of infectivity resulted from high temperatures after inoculation and also to check the temperature range at which TYMV replicates. Plants were inoculated in the glasshouse with equal amounts of purified TYMV-BL virions and held for different time periods; 0, 1, 4 and 6 hours in the glasshouse before transferring them to constant temperatures of 20°C, 28°C

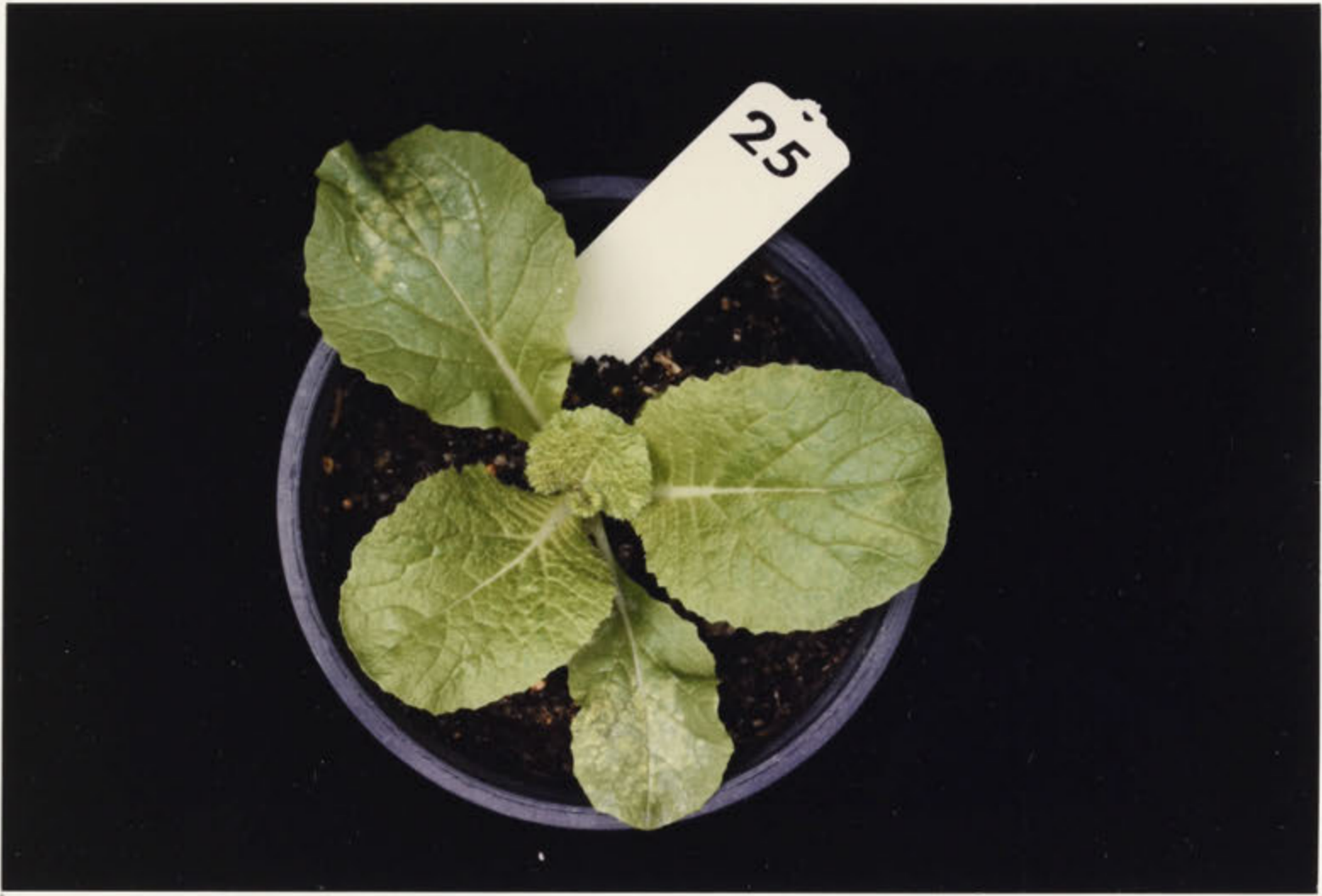
and 30°C. In addition, inoculated plants were held at 25°C day/20°C night and under normal glasshouse conditions, as described above. The results showed that the virus spread systemically in plants held at 20°C and 25°C/20°C but replication was inhibited at higher temperatures. There was no difference in the symptoms shown by plants held for different time periods in the glasshouse prior to incubation at the chosen temperatures. The inoculated leaves of plants held at 28°C and 30°C developed yellow lesions indicating that the virus was accumulating in the inoculated leaves but was prevented from spreading systemically at those temperatures. Figure 4.8 shows representative plants from these experiments.

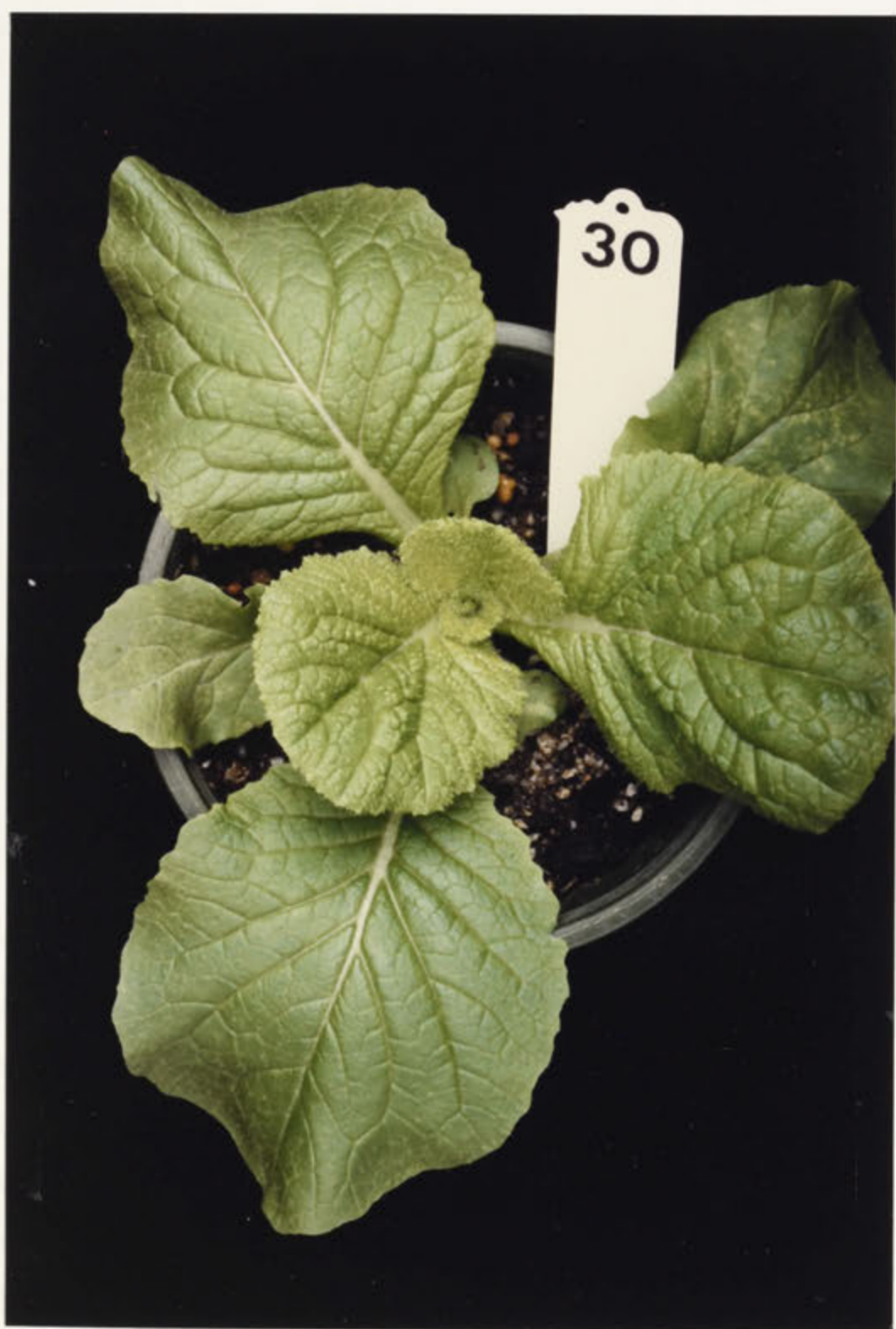
#### 4.2.4 INOCULATION OF CHINESE CABBAGE PROTOPLASTS WITH RECOMBINANT TRANSCRIPTS

The recombinants were further analysed to investigate whether those not producing systemic infections had an impaired ability to form stable virions or were unable to move from cell-to-cell. Chinese cabbage protoplasts were prepared and electroporated with transcript for each of the 10 recombinants (Chapter 2 [2.2.19]). Total RNA was isolated from the protoplasts and analysed by electrophoresis in a formaldehyde gel before being blotted onto a nylon membrane (Chapter 2 [2.2.9; 2.2.10]). The blot was probed with a radiolabelled transcript of the *Pst*I/*Xba*I fragment of the pBL-16 clone which included the coat protein gene (Figure 2.1). Electroporation experiments were repeated many times but only once was infection detected by Northern blotting. Figure 4.9 shows a photograph of the resulting autoradiograph. The results show that only those recombinants which infected whole plants and produced symptoms in them replicated in protoplasts. This suggested that those recombinants which did not infect whole plants were not just unable to spread from cell-to-cell but were unable to replicate. However, R4 and R9 did not replicate in protoplasts



Figure 4.8 Photographs of Chinese cabbage plants inoculated with purified TYMV-BL virus particles and held at different temperatures: 20°C, 25°C, 28°C and 30°C. Plants held at 25°C and below developed systemic symptoms while those held above 25°C developed chlorotic lesions on the inoculated leaves indicating that at higher temperatures the virus was restricted to the inoculated leaves. Plants were photographed 7 days post inoculation.





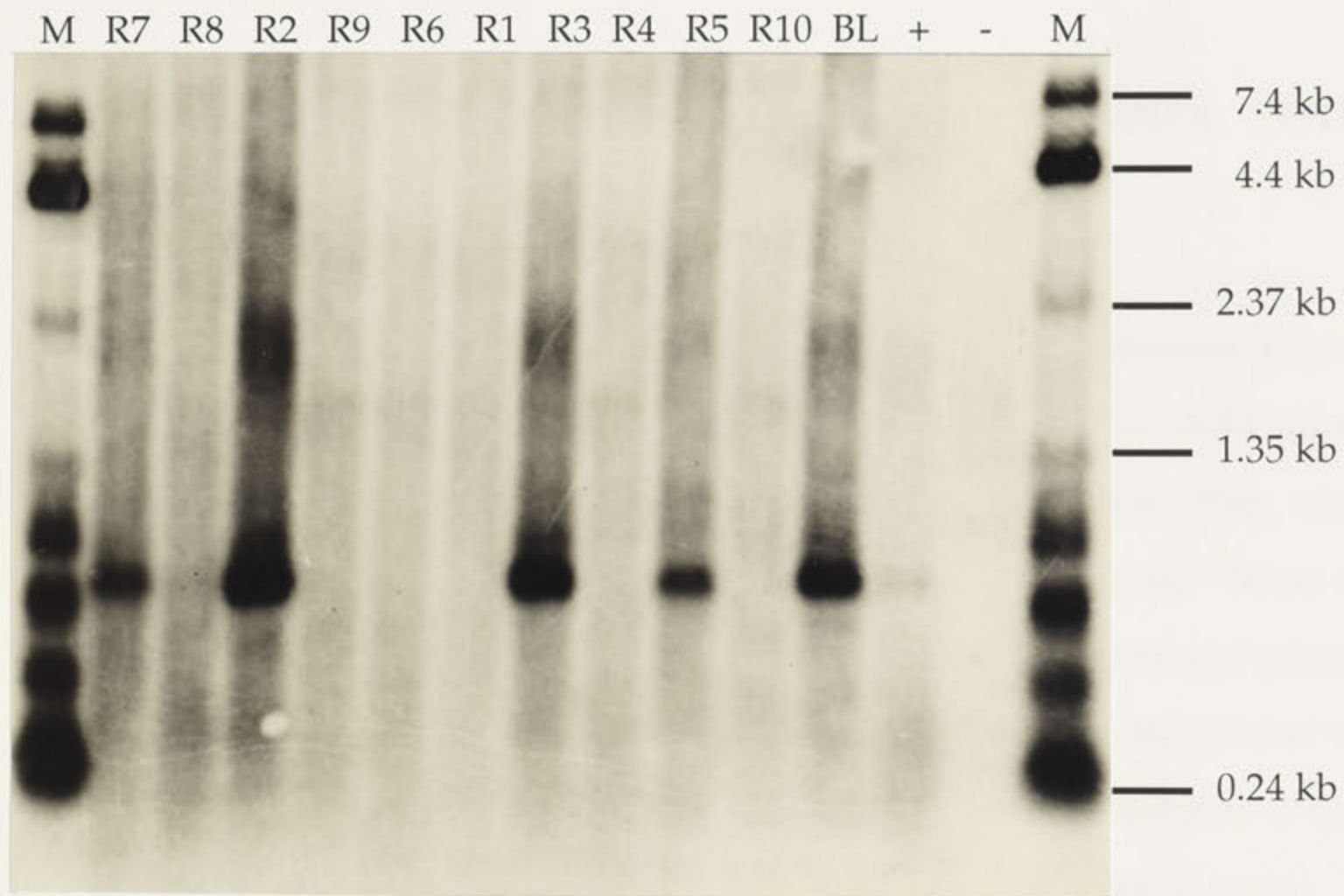


Figure 4.9 Northern blot of total RNA isolated from protoplasts electroporated with 5 $\mu$ g transcript for each recombinant. The blot was hybridised with a  $^{32}$ P labelled transcript of the coat protein to detect the (+) sense RNA and the RNA markers were detected with random primer  $^{32}$ P labelled lambda DNA. The recombinants with which the protoplasts were electroporated are indicated at the top and the sizes of the fragments in the RNA ladder are indicated along the side. The lane denoted + contained RNA from protoplasts electroporated with viral RNA and lane - contained protoplast RNA only. Subgenomic RNA was detected in samples electroporated with transcript of R7, R2, R3, R5, pBL-16 and viral RNA (+). No genomic RNA could be detected in the samples but the dark smears in these lanes are probably due to degraded genomic RNA.



which was unexpected as they systemically infected whole plants. As this experiment could not be reproduced, the most probable explanation for this unexpected result was that the transcripts were degraded before they were electroporated into the protoplasts and were thus unable to replicate. Degradation of transcript could also explain the lack of replication of R1, R6, R8 and R10.

#### 4.2.5 INOCULATION OF TYMOVIRUS HOST PLANTS WITH RECOMBINANT TRANSCRIPTS

Belladonna mottle tymovirus infects a range of solanaceous plants such as tobacco cultivar SR1. To see if the TYMV recombinants could infect SR1 as a result of the presence of parts of the BMV coat protein, they were inoculated onto SR1 plants with BMV RNA as a positive control. None of the plants except those inoculated with BMV RNA showed any symptoms and no TYMV coat protein gene could be amplified from the inoculated or tip leaves by RT-PCR. This demonstrated that the TYMV recombinants had not acquired the ability to infect a known host of BMV as a result of containing parts of the BMV coat protein and in turn that the ability of BMV to infect SR1 was not determined by the amino acid sequence of those parts of its coat protein incorporated into TYMV.

The recombinants were also inoculated onto *Arabidopsis thaliana* plants (Cruciferae), which is a host of TYMV (Mary Skotnicki, personal communication). Those recombinants which gave systemic symptoms in Chinese cabbage gave similar symptoms in *Arabidopsis* plants and while the presence of virions was not determined, the coat protein gene was amplified from infected leaves using RT-PCR. This was as expected as *Arabidopsis* and Brassicas, such as cabbage, are both members of the Cruciferae family. In addition, R8 which gave local lesions on Chinese cabbage systemically

infected *Arabidopsis*. The possible reasons for this are discussed at the end of this chapter.

To see if the coat protein recombinants had altered host ranges, known host plants for all tymoviruses were inoculated with transcripts of each of the 10 recombinants. Table 4.4 lists the plants used and the tymoviruses which infect them. Plants were inoculated with transcript from 5 $\mu$ g of linearised plasmid and monitored for the appearance of symptoms. No symptoms were seen on any of the plants and no virus was detected by RT-PCR in the inoculated or tip leaves except in the positive controls (Chinese cabbage plants inoculated with transcript of pBL-16).

### 4.3 DISCUSSION

The results of experiments using coat protein recombinants indicated that the tymovirus coat protein was necessary for cell-to-cell and long distance movement of the virus. The role of the coat protein in replication of the virus was not determined because of problems with protoplast electroporation experiments. Only those recombinants which produced virus particles could systemically infect whole plants or replicate in protoplasts. For R2, R4 and R5 which systemically infected plants, the replacement of amino acids did not affect the ability of the virus to replicate and move locally or over long distances in plants. The symptoms produced in plants and the time taken for the symptoms to appear were the same as those in plants infected with wild-type transcripts. The N terminus of the coat protein (R2 and R4) seemed therefore to be flexible in the amino acid sequences that could be inserted into it - the amino acid sequence of the wild-type protein seemed not to be involved in determining its ability to form stable virions and as such was probably not important in subunit interactions. This result was in agreement with the prediction of the coat

**Test Plants****Tymoviruses which give systemic infection**

<i>Brassica chinensis</i>	TYMV, ELV
<i>Chenopodium quinoa</i>	ELV, APLV, CoYMV
<i>Cucumis sativus</i>	WCuMV, OkMV
<i>Cucurbita pepo</i>	WCuMV
<i>Gomphrena globosa</i>	-----
<i>Nicotiana clevelandii</i>	EMV, APLV, PhyMV, BMV, DMV, OYMV, PIMV, OkMV
<i>Nicotiana glutinosa</i>	EMV, APLV, BMV, DMV
<i>Petunia hybrida</i>	EMV, APLV, BMV, DMV, PIMV
<i>Phaseolus vulgaris</i>	DeYMV
<i>Pisum sativum</i>	OYMV, ScrMV, PIMV, KYMV

Table 4.4 A list of the host plants and the tymoviruses which produce systemic infection when inoculated onto them.

Abbreviations: **EMV**: eggplant mosaic virus, **APLV**: Andean potato latent virus, **PhyMV**: Physalis mosaic virus, **BMV**: belladonna mottle virus, **DMV**: dulcamara mottle virus, **OYMV**: ononis yellow mosaic virus, **ScrMV**: scrophularia mottle virus, **PIMV**: plantago mottle virus, **WCuMV**: wild cucumber mosaic virus, **TYMV**: turnip yellow mosaic virus, **KYMV**: kennedyia yellow mosaic virus, **CoYMV**: cocoa yellow mosaic virus, **DeYMV**: desmodium yellow mottle virus, **OkMV**: okra mosaic virus, **ELV**: erysimum latent virus. Information from: "Handbook of plant virus infections comparative diagnosis." E. Kurstak ed.

protein secondary structure and the accessibility data for the protein (Chapter 1), both of which suggested the N terminus may be exposed. Immunochemical studies had shown the N terminus to be exposed on the surface of the protein as was the region encompassed by R5 (amino acids 57-67). Results with R5 support the location of this region on the surface of the protein and changing the amino acid sequence did not abolish infectivity by the virus.

The fact that some of the recombinants, especially R3, R7 and R9, produced symptoms that were different and delayed compared with transcript from pBL-16 indicated that some of the altered amino acids were involved in efficient replication or movement of the virus. Changing them affected the ability of the virus to move systemically through the plant in that the symptoms were less severe and delayed, although other parts of the infection process could also have been affected. The recombinants replicated in protoplasts and whole plants but it was not clear if the different symptoms were caused by less efficient replication of the virus or less efficient translocation.

For R6 and R8 where infection with transcripts resulted in the formation of local lesions, the amino acid changes eliminated the ability of the virus to move systemically through the plant. No viral nucleic acid was detected in protoplasts although it would be expected that the recombinants should be able to replicate as they formed local lesions on the inoculated leaves of Chinese cabbage plants. However as already stated, infection was only detected once by Northern blotting of protoplast RNA despite repeating the experiment a number of times. It is possible that by optimising conditions for protoplast electroporations, infection could be detected for R6 and R8. In the case of R8 where a lysine residue found in all tymoviruses was changed to an asparagine, the conserved lysine must be important for

the functioning of the coat protein in infecting Chinese cabbage as this recombinant was confined to lesions in Chinese cabbage but was able to infect *Arabidopsis* systemically. It was not transmitted by sap from *Arabidopsis* to Chinese cabbage which seemed to suggest a role in virus/host interaction. The lysine residue may interact with surrounding amino acids in folding the coat protein into a particular conformation. However the lysine residue is conserved in all tymoviruses and the group as a whole infects a wide range of host plants so it must be involved in more than a specific TYMV-Chinese cabbage interaction. The lysine residue may be involved in RNA binding which would be important in all tymoviruses irrespective of host range but this does not explain why R8 could systemically infect *Arabidopsis* and not Chinese cabbage, as RNA binding would still be important in forming stable virions in an infection of *Arabidopsis*.

Recombinant 1 gave no symptoms when inoculated onto Chinese cabbage and this was as expected. This recombinant had a point mutation that replaced methionine at the start of the coat protein with a termination codon. This recombinant did not replicate in protoplasts or in whole plants. Without the coat protein, the other non-structural proteins of TYMV did not move from cell-to-cell or over long distances in whole plants. This was proven by the fact that the probe for Northern blots included part of the replicase gene which encodes the putative replicase protein (Weiland and Dreher, 1989). This gene was not detected in blots of RNA isolated from R1 electroporated protoplasts which suggested it was unable to replicate in protoplasts without the coat protein. The result for R1 is not in agreement with those of Bransom *et al.* (1995). These workers constructed TYMV coat protein gene mutants which expressed shortened coat proteins or no coat protein. Their mutants were able to replicate in protoplasts indicating the

dispensability of the coat protein for replication and local movement of the virus.

Recombinant 10 which had an altered C terminus of the TYMV-BL coat protein, shortened the C terminus by 2 amino acids and did not replicate in plants or protoplasts. These amino acids must be necessary for infection by the virus and may be involved in coat protein subunit interactions or interactions with the host plant. Bransom *et al.* (1995) found that the particles produced by a coat protein with a short extension at the C terminal end were less stable than the wild-type virus. Thus the results obtained with R10 agree with those of Bransom *et al.* (1995) in defining the C terminal amino acids as crucial and apparently involved in virion stability.

Overall, this set of experiments showed that certain regions in the coat protein were important for certain functions. Amino acids at the N terminus could be changed without affecting systemic infection of Chinese cabbage plants although changing some of them (amino acids 13-20) resulted in less efficient replication or movement which was manifested as a delay in the appearance of systemic symptoms. Changes in amino acids in the putative  $\alpha$  helix also did not affect systemic infection although this recombinant systemically infected only 50% of the time as did R4 with the long N terminal replacement. Other recombinants such as R7 and R9 systemically infected only 10% of the time with delayed appearance of symptoms. Changes in other regions of the protein restricted the recombinants to the inoculated leaves of the plants with the formation of local lesions. This demonstrated the role of the coat protein in long-distance movement of the virus. Finally, changing amino acids at the C terminus or introducing a termination codon in place of the methionine at the N terminus of the protein abolished cell-to-cell and long distance movement of the virus. This indicates a role for the protein in cell-to-cell

movement in addition to long-distance movement in contrast to the results reported by Bransom and colleagues, but may reflect differences in the types of changes introduced into the coat protein. Their coat protein-less mutant was constructed by changing the initiating methionine to leucine and positioning a termination codon 5 amino acids downstream while their C terminal mutant extended the coat protein by 5 amino acids. In contrast, R10 had changes in the actual C terminal amino acids and did not infect plants or protoplasts. This suggested that the TYMV amino acid sequence was required at the C terminus for infection by the virus. The fact that some parts of the TYMV coat protein can be replaced without affecting the virus while other parts cannot suggested that corresponding parts of the TYMV and BMV coat proteins, while being genetically homologous, are not always functionally homologous; parts of the coat protein function in their parental viruses but will not necessarily perform the function of the corresponding region of another virus. Therefore, alterations in symptoms produced by recombinants are probably due to changes in protein-protein interactions resulting from the insertion of BMV peptides in place of the wild-type sequences.

## CHAPTER FIVE

### FURTHER ANALYSIS OF INFECTIOUS RECOMBINANTS

#### 5.1 INTRODUCTION

Antibodies raised against particular antigens are specific for those antigens and bind to them at sites known as epitopes or antigenic determinants. These epitopes may be continuous, composed of short linear fragments of amino acids which are contiguous in the primary sequence, or discontinuous, where the site of antibody binding consists of amino acids distant in the linear sequence but brought together by folding (Van Regenmortel *et al.*, 1993). Because of their sensitivity, antibodies are useful tools for studying fine details of virus structure and also for assessing the degree of similarity between viruses. It has been proposed that the antigenicity of specific polypeptides is due to their exposure on the surface of proteins where they are able to interact with antibody domains (Novotny *et al.*, 1986).

Studies on the structure of the tymovirus coat protein using antibodies were first done by Pratt *et al.* (1980). Inhibition of complement fixation tests were used, and peptides corresponding to different regions in the coat protein were tested for their ability to inhibit the reaction of antibodies with virus particles and denatured coat protein. These workers found that two peptides, one corresponding to residues 1-12 at the N terminus of the coat protein and the second corresponding to amino acids 46-67 in the coat protein inhibited the reaction of viral protein with antibodies raised against it. However, only the second peptide (residues 46-67) strongly inhibited the reaction between virus particles and their specific



antiserum. This suggested that at least part of this region lay at the surface of the coat protein subunit and was exposed in the capsid.

Further experiments were done by Quesniaux and colleagues (1983a, 1983b) using inhibition of complement fixation, radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA). They identified three antigenic determinants in residues 1-12, 57-64 and 183-189. When peptides covering the N terminal 1-12 amino acids were tested in an assay using virus particles and antibodies raised against them, there was no inhibition of complement fixation. However, the authors suggested that the N terminus of the protein (amino acids 1-12) was exposed on the surface of the capsid but this conclusion was based on the assumption that parts of the protein inside the capsid were not immunogenic and that the virus particles remained intact following adsorption to the solid phase used for RIA and ELISA. It is possible that these assumptions are wrong as cross-linking and immunological analysis using monoclonal antibodies to study the coat protein of another tymovirus, physalis mottle virus (PhMV), showed that the N terminus was cross-linked to the viral RNA and buried within the particle (Kekuda *et al.*, 1993). Cross-linking studies with TYMV also showed that three regions of the coat protein including amino acids 1-12 at its N terminus interacted with the viral RNA (Ehresmann *et al.*, 1980). The other regions which were in contact with the RNA were between amino acids 33-45 and 132-152. Taken as a whole, results from immunological and cross-linking analyses of the tymovirus coat protein suggested that the N terminus of the protein was buried and interacted with the viral RNA whereas the part of the coat protein between residues 46-67 was exposed on the surface of virus particles.

No further serological analysis of the TYMV coat protein had been done so in an effort to confirm the results of the above workers and to gain

more insight into which regions of the coat protein were most antigenically active, an epitope analysis of the TYMV coat protein was done. The availability of a peptide synthesis kit based on the work of Geysen (Geysen *et al.*, 1984) provided the opportunity to synthesise peptides which could be probed with TYMV specific antibodies. The original Pepsan system (Geysen *et al.*, 1984) used polyethylene rods on which overlapping hexapeptides covering the 213 amino acids of the coat protein of foot-and-mouth disease virus were synthesised. The hexapeptides were then analysed by ELISA using a variety of antisera including some raised against whole virus particles. It was found that residues at certain positions in the protein were essential for reaction with antisera raised against whole particles; therefore the system was able to map epitopes on viral antigens to a resolution of a single amino acid (Geysen *et al.*, 1984). Since then this system, or a modification of it (SPOTs) using a cellulose membrane (Frank, 1992) in place of polyethylene coated rods, has been used by a number of workers to map epitopes on coat proteins of both plant and animal viruses.

Overlapping octapeptides covering the coat protein of Johnsongrass mosaic potyvirus were probed with polyclonal and monoclonal antisera raised against intact virus particles. Epitopes recognised by monoclonal antibodies also reacted with polyclonal antibodies and were located in the exposed N terminus of the protein (Shukla *et al.*, 1989). Overlapping hexapeptides of the potato leafroll virus coat protein were tested against both polyclonal and monoclonal antibodies. Several epitopes were recognised in the protein which included regions of the putative S domain and the N terminus (Torrance, 1992).

An analysis of previously identified epitopes of beet necrotic yellow vein furovirus using overlapping decapeptides and monoclonal antisera showed that not all amino acids in the epitopes were equally important for

antibody binding. In addition, replacement studies with alanine showed that amino acids outside the linear epitope sequence influenced antibody binding (Commandeur *et al.*, 1994). The coat protein of another furovirus, potato mop-top virus, was analysed using the SPOTs system. Overlapping octapeptides corresponding to the coat protein were synthesised and probed with monoclonal antibodies. Four of the six epitopes recognised were in regions predicted by computer algorithms to be antigenic, and when the epitopes were mapped onto a predicted structure for the coat protein, they were located on or close to the surface of virus particles (Pereira *et al.*, 1994).

Mapping of epitopes in antigenic regions of animal virus coat proteins has also been done using the Pepscan system. Peptide scanning was used to identify regions of the poliovirus coat proteins that bound human antisera (Roivainen *et al.*, 1991) and to identify amino acids involved in binding monoclonal antibodies to bluetongue virus (du Plessis *et al.*, 1994). Neutralisation epitopes of the pre-S envelope protein of duck hepatitis B virus (DHBV) were investigated by using monoclonal antibodies and overlapping octapeptides in order to identify residues critical for binding of the antibodies (Chassot *et al.*, 1993). Further studies on DHBV using the same techniques identified which amino acids at the N terminus of the pre-S protein were important in activating the immune response of adult ducks (Chassot *et al.*, 1994).

In the experiments discussed in this Chapter the SPOTs system was used to analyse epitopes on the coat protein of TYMV using antisera against virus particles and particles of R2 and R3. Wild-type and R2 antisera were also used to analyse particles of all infectious recombinants in Western blots. The electrophoretic mobility of particles of infectious recombinants was compared with that of wild-type particles as the isoelectric points of some recombinants had been changed by the inserted BMV amino acids.

Finally, as results with R2, R3 and R4 had shown that the viral coat protein could tolerate changes in the amino acid sequence at the N terminus without losing its capability to systemically infect host plants, recombinants were designed in which the N terminus of TYMV was replaced with an epitope from the merozoite of *Plasmodium falciparum*. Because antibodies raised against R2 seemed to be specific to the changed part of the N terminus it was possible that inserted foreign epitopes at the N terminus of the coat protein would be presented during the immune response and specific antibodies raised against it. Using a system like this, plants infected with viruses containing such foreign epitopes could be used as vaccines. There have been several reports on using carrier molecules to which epitopes of animal pathogens had been fused to stimulate an immune response. These carrier molecules include the core (Stahl and Murray, 1989; Clarke *et al.*, 1987) and surface (Delpeyroux *et al.*, 1986) antigens of hepatitis B virus, yeast Ty protein (Adams *et al.*, 1987), poliovirus (Burke *et al.*, 1988; Dedieu *et al.*, 1992) and viruses of bacteria (Greenwood *et al.*, 1991; Mastico *et al.*, 1993 ). In all cases the chimeric viruses formed stable particles and stimulated the production of antibodies which were specific for the inserted peptides.

Cowpea mosaic virus has also been used as a carrier molecule and it was shown that foreign sequences could be inserted into the exposed  $\beta\text{B}-\beta\text{C}$  loop of the small (S) coat protein. These sequences included parts of VP-1 of foot-and-mouth disease virus (FMDV), parts of VP-1 of human rhinovirus 14 (HRV-14) and the "Kennedy epitope" of gp41 of human immunodeficiency virus 1 (HIV-1). All three chimeras gave wild-type symptoms on the inoculated leaves and both the HRV and HIV chimeras produced systemic symptoms similar to CPMV. Virus particles could be easily purified from systemically infected HRV and HIV leaves and both possessed the antigenic properties of the inserted sequence. In the case of

HRV, chimeric virions were immunogenic in rabbits and able to raise antibodies which recognised VP-1 of HRV-14. Immunological analysis of the HIV chimera was not reported. The results led the authors to conclude that cowpea mosaic virus had significant potential as a peptide presentation system (Usha *et al.*, 1993; Porta *et al.*, 1994).

## 5.2 METHODS

### 5.2.1 SYNTHESIS OF PEPTIDES ON SPOTS MEMBRANE AND IMMUNISATION OF RABBITS FOR ANTIBODY PRODUCTION

The SPOTs epitope analysis kit from Cambridge Research Biochemicals was used to synthesise overlapping peptides covering the entire TYMV coat protein. An overlapping series of peptides, each 10 amino acids long, were synthesised to scan the coat protein; adjacent spots on the membrane overlapped by 8 amino acids. The peptides were synthesised, following manufacturers instructions, and were then probed with several polyclonal antisera which had been raised in rabbits. These antisera were raised against purified virions of wild-type TYMV, recombinant 2 and recombinant 3. Recombinants 2 and 3 were chosen to raise antisera as they always gave a systemic infection when inoculated onto Chinese cabbage plants; the changes to their coat proteins did not affect their ability to infect and it was of interest to see how antisera raised to their virions would react with the wild-type peptides on the spots membrane.

Pre-immune serum was taken from rabbits (two for each immunogen) two weeks before injection of the virus. 1 mg virus in a total volume of 1 ml was injected intravenously into the ear for each immunogen and after two weeks, early bleed antisera were collected from each rabbit. Following recovery for 7-10 days, rabbits were injected sub-

cutaneously at four sites with 1mg of virus particles in Freund's complete adjuvant. Late bleed antisera were collected after 4 weeks. Rabbits were boosted by an intravenous injection with 1 mg virus in a total volume of 500  $\mu$ l three and a half months later and antisera collected from the rabbits 3 weeks after the booster injection was called late bleed 2 antisera. All antisera collected were frozen at  $-20^{\circ}\text{C}$ . This immunisation schedule was followed as previous work had shown that early bleed antisera to intact particles of Johnsongrass mosaic virus contained antibodies directed to the surface exposed N terminus. Late bleed antisera also contained antibodies to the core region of the particles (Hewish *et al.*, 1993). It was thought that early bleed antisera raised against TYMV would recognise surface exposed regions of the coat protein and that late bleed antisera would be directed to less exposed regions. Using these antisera in conjunction with the SPOTs peptides it was hoped to identify the immunologically active regions of the protein and to compare them with the predicted secondary structure of the coat protein (Chapter 1).

Antisera were also used for immunodetection on Western blots of the coat proteins from all systemically infecting recombinants (2.2.16-2.2.18).

### **5.2.2 ELECTROPHORETIC MOBILITY OF RECOMBINANT PARTICLES**

The isoelectric points of the BMV amino acid sequences inserted in place of the TYMV-BL amino acids were calculated using the GCG 'Isoelectric' program and are shown in table 5.1. It was of interest to see if the particles of the recombinants had a changed electrophoretic mobility when compared with the wild type particles. A difference in electrophoretic mobility would suggest that the BMV amino acids inserted into the TYMV coat protein were located on the surface of the virus particles. Virus particles of the infectious recombinants (R2, R3, R4, R5, R7 and R9) and

TYMV-BL were stained, stained through a 1% agarose gel in 0.05 M Tris-HCl, 25 mM EDTA, pH 7.0 at 200 V for 2-3 hours. The gel was stained with Coomassie Blue and destained in a methanol/acetic acid solution to visualize the virus particles.

### 5.13 CONSTRUCTION OF A COAT PROTEIN RECOMBINANT CONTAINING MA A15 ANTIGEN AMINO ACIDS IN THE N TERMINUS

#### Isoelectric points of peptides changed in the recombinants

The amino acids changed in the N terminus of the TYMV coat protein formed part of the sequence in the major coat protein subunit.

	Replaced Sequence TYMV-BL	Inserted Sequence BMV
R2	4.17	6.52
R3	6.06	6.06
R4	4.17	6.52
R5	3.73	6.31
R7	10.53	6.06
R9	4.11	4.11

Table 5.1 Isoelectric points of the TYMV-BL coat protein recombinant amino acids compared with the wild-type amino acids

Table 5.2(a) shows the amino acid sequence of the foreign peptide inserted at the N terminus of the TYMV coat protein and Table 5.2(b) shows the coat protein sequence. When the primers for mutagenesis were being designed, the codons used were chosen according to the codon usage of the TYMV-BL coat protein.

TYMV-BL were electrophoresed through a 1% agarose gel in 100 mM Tris-HCl, 25 mM EDTA pH 7.0 at 100 V for 2-3 hours. The gel was stained with Coomassie Blue and destained in a methanol/acetic acid solution to visualise the virus particles.

### 5.2.3 CONSTRUCTION OF A COAT PROTEIN RECOMBINANT CONTAINING MALARIA ANTIGEN AMINO ACIDS IN THE N TERMINUS

The malaria antigen chosen to replace the N terminus of the TYMV coat protein formed part of the precursor to the major merozoite surface antigen (PMMSA 2) of the Papua New Guinea isolate FC27. Sequence analysis of the PMMSA gene of different isolates showed that the gene consisted of blocks of sequence that were either conserved or variable between different isolates with the variable sequences falling into two distinct types. The most variable region was at the 5' end and contained repeats that were different in independent cloned isolates. Monkeys immunised with preparations of the PMMSA protein were protected against homologous challenge and partly protected against heterologous challenge. In addition, immunisation with peptides derived from the PMMSA protein also partially protected monkeys from heterologous challenge (Peterson *et al.*, 1988). Because of its ability to protect against infection it had been suggested that this antigen would be a good candidate for a malaria vaccine.

Table 5.2(a) shows the amino acid sequence of the foreign peptide inserted at the N terminus of the TYMV coat protein and Table 5.2(b) shows the nucleotide sequence. When the primer for mutagenesis were being designed, the codons used were chosen according to the codon usage of the TYMV-BL coat protein.



**A**

Mutant	Amino Acid Changes	
--------	--------------------	--

<b>E71</b>	TYMV-BL (WT)	MEIDKELAP
	TYMV-BL/E71	MSNTFINNA

**B**

Mutant	Nucleotide Changes	
--------	--------------------	--

<b>E71</b>	(WT)	ATG GAA ATC GAC AAA GAA CTC GCC CCC
	(E71)	ATG TCG AAC ACC TTC ATC AAC AAC GCC

Table 5.2 (A) Amino acid sequence and (B) nucleotide sequence of E71 which was used to replace the N terminus of TYMV-BL

## PRIMER SEQUENCE

E71 5'- GAC GGT GCG GTC TTG GGC GTT GTT GAT GAA GGT GTT CGA CAT GTC GGG  
GCT GAT -3'

*in vitro* mutagenesis (2.2.20) was used to replace the N terminal 8 amino acids in the malaria antigen mutant. Subcloning of the mutant coat protein into the full-length virus clone, sequencing of the insert, transcription into RNA and inoculation of Chinese cabbage plants was as previously described (Chapter 2).

## 5.3 RESULTS

### 5.3.1 SPOTS ANALYSIS WITH TYMV AND RECOMBINANT ANTISERA

Initially the SPOTs membrane was probed with the secondary antibody ( $\beta$ -galactosidase conjugated sheep anti-rabbit IgG) only to determine if there were any non-specific interactions between the peptides and the secondary antibody. Spots 14, 32 and 52-54 reacted with the secondary antibody and as a result were ignored in subsequent reactions. The membrane was stripped and regenerated following this, and all other tests with antisera, according to the manufacturers' instructions. In total the membrane was probed with 5 more antisera; three containing antibodies raised against TYMV virus particles and two containing antibodies raised against particles of recombinants 2 and 3. Only late bleed antisera were used as the early bleed TYMV antiserum did not react with the peptides. One possible explanation for this was that the early bleed antisera may have been predominantly IgM and this was not detected by the secondary antibody.

However, when undiluted early bleed antiserum was used to detect protein on a Western blot, a sharp band corresponding to the coat protein was detected with alkaline phosphatase conjugated second antibody. This indicated that there was IgG present in the antisera but at a low concentration.

The results of experiments with the TYMV antisera are shown in Figures 5.1 - 5.3 and are summarised in Table 5.3. The photographs show the spots on the membrane which reacted clearly with the antisera. The diagrams show the positions of the peptides on each spot mapped onto the structure of the coat protein to show their locations. Late bleed 2 antiserum reacted with spot no. 14 which had previously been detected with the secondary antibody alone and, as this may have been a non-specific reaction between the spot and the secondary antibody, it was ignored.

Results from SPOTs analysis with TYMV antisera showed that several regions of the coat protein were antigenic and bound antibodies. For the late bleed and late bleed 2 antisera (both from the same animal) the only difference was that an additional region of the N terminus was antigenic and had elicited the production of antibodies in the late bleed 2 antiserum. With the exception of the N terminus, both antisera recognised the same peptides on the membrane (Table 5.3, Figure 5.2).

The third TYMV antiserum was a gift from Alan Brunt and was raised against the type strain of TYMV ~20 years ago. This antiserum reacted with different peptides on the SPOTs membrane in addition to the peptides at the N and C termini of the coat protein (Table 5.3, Figure 5.3).

Figures 5.4 and 5.5 show results of tests using antisera raised against recombinants 2 and 3 respectively. R2 antiserum reacted with spot no. 54

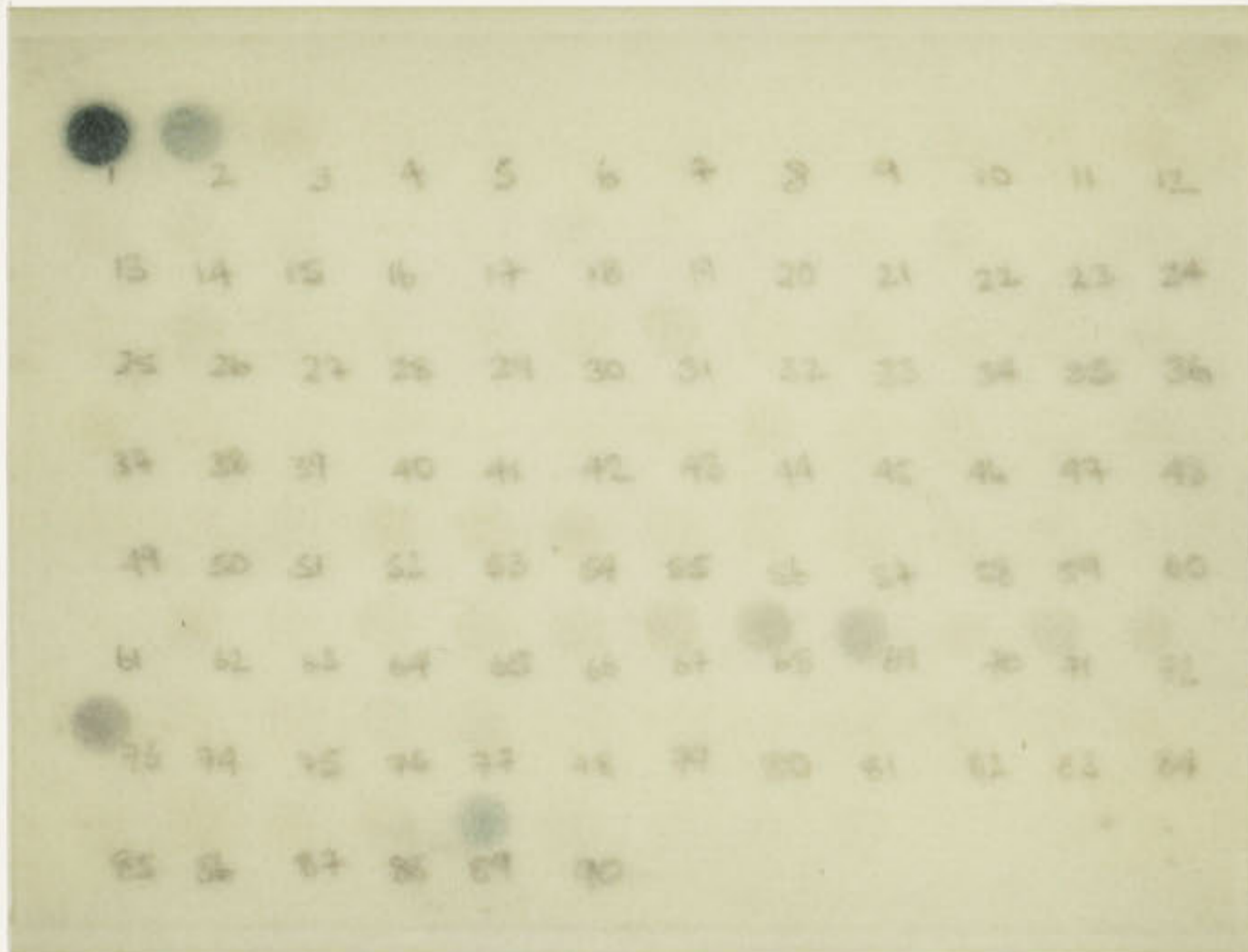
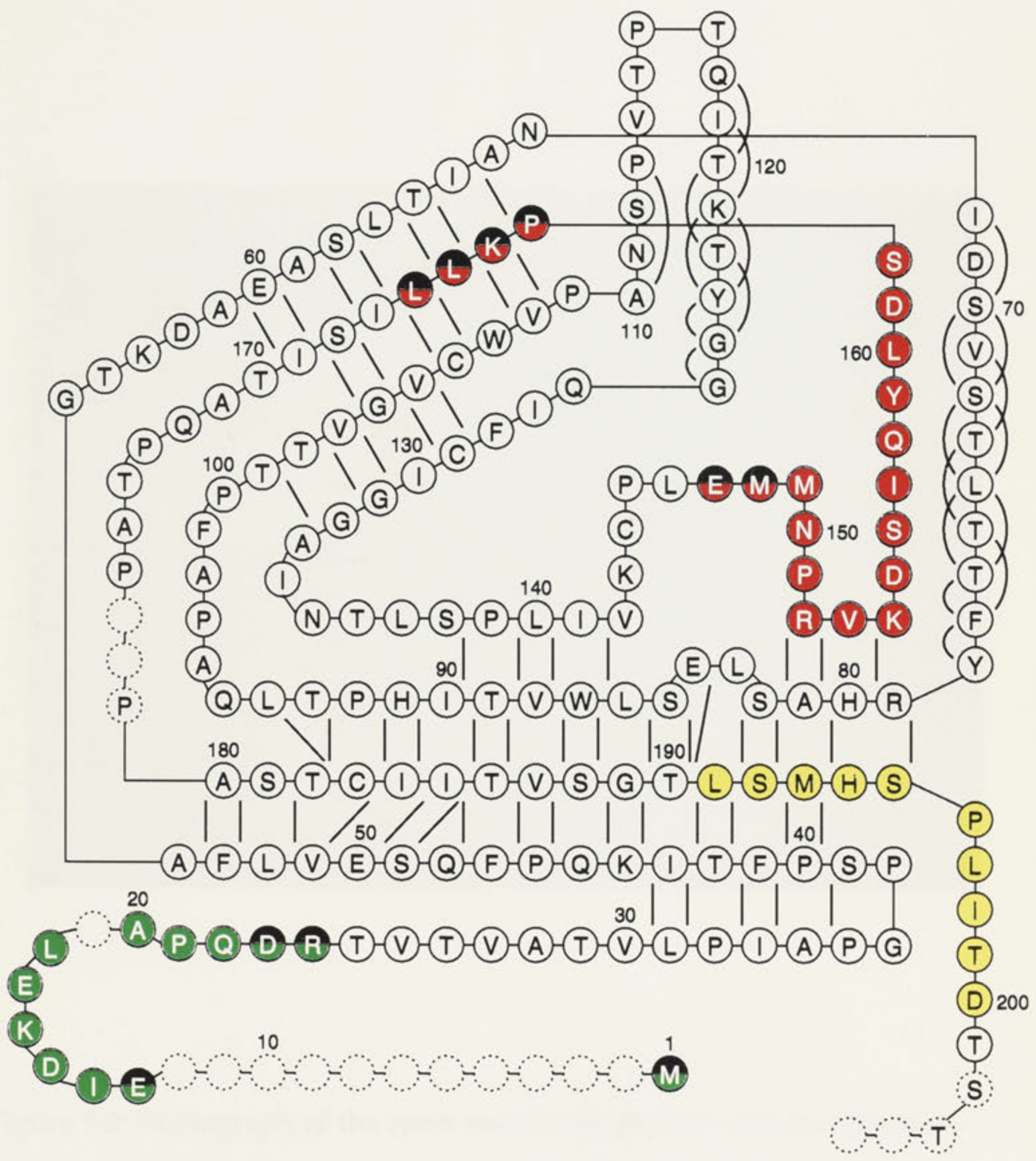


Figure 5.1 Photograph of the spots membrane probed with late bleed antibodies to purified particles of TYMV-BL (1:2000 dilution). Overleaf: spots peptides which reacted with late bleed antibodies to TYMV-BL mapped onto the predicted structure of the TYMV coat protein. Amino acids which are half coloured are those which were present in only one peptide on the membrane whereas the other coloured amino acids were present in more than one of the overlapping peptides. This is also the case in Figures 5.2-5.5.



- Spots No. 1, 2
- Spots No. 68, 69, 71, 73
- Spot No. 89

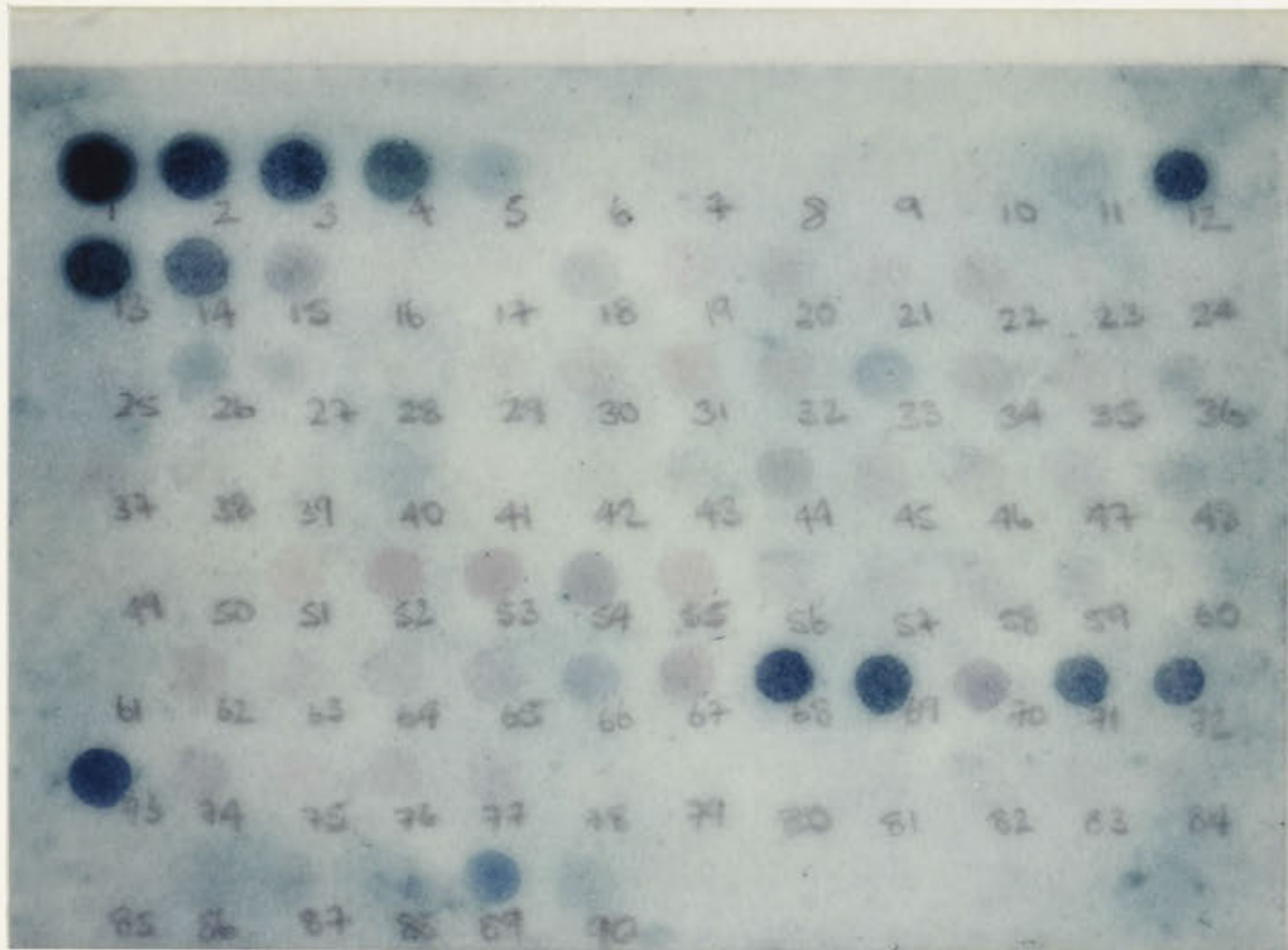
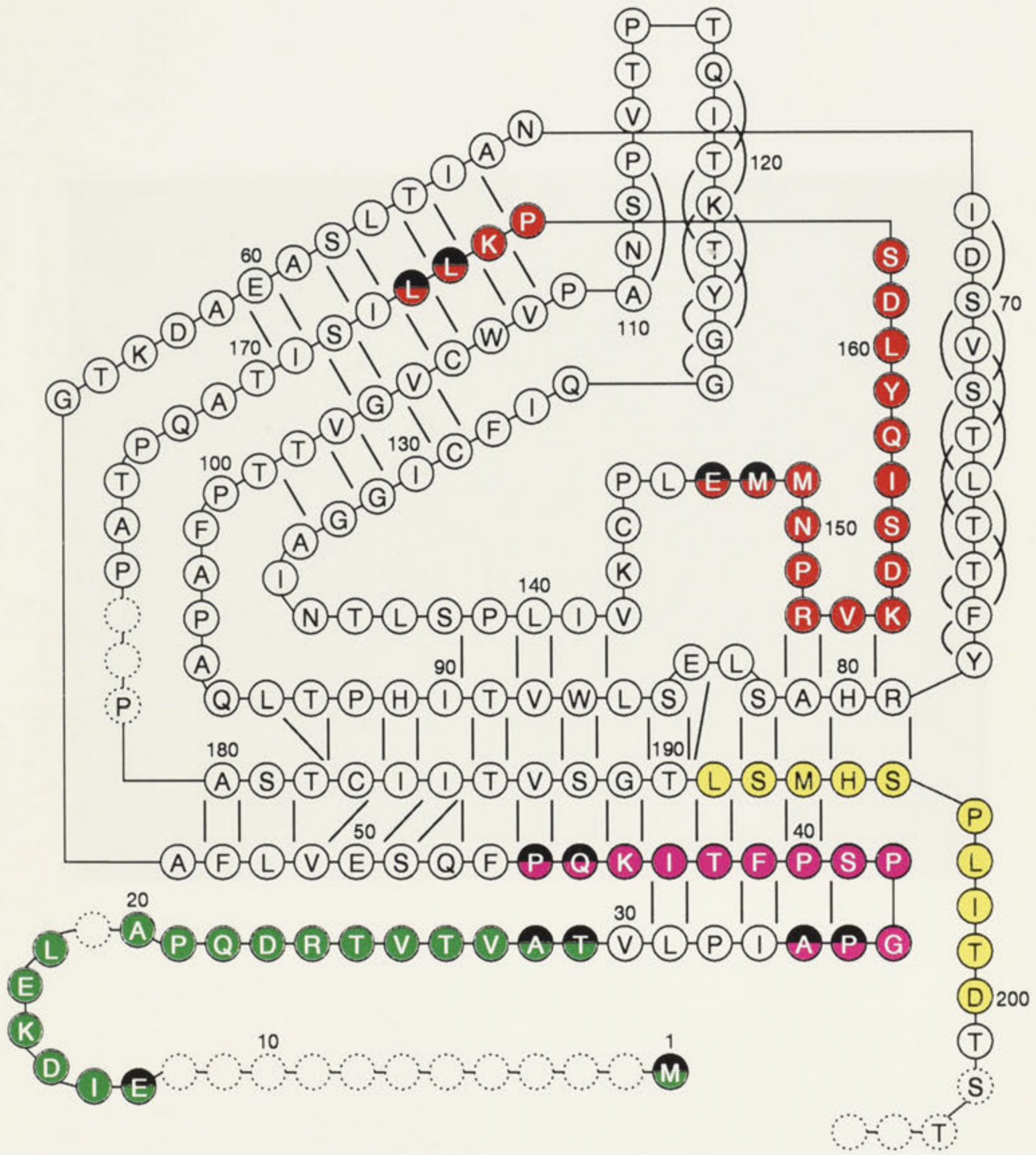


Figure 5.2 Photograph of the spots membrane probed with late bleed 2 antibodies to purified particles of TYMV-BL (1:50 dilution). Overleaf: spots peptides which reacted with late bleed 2 antibodies to TYMV-BL mapped onto the predicted structure of the TYMV coat protein. These antibodies and those in Fig. 5.1 were from the same animal but were from a later bleed.



- Spots No. 1, 2, 3, 4, 5
- Spots No. 12, 13
- Spots No. 68, 69, 71, 72, 73
- Spot No. 89

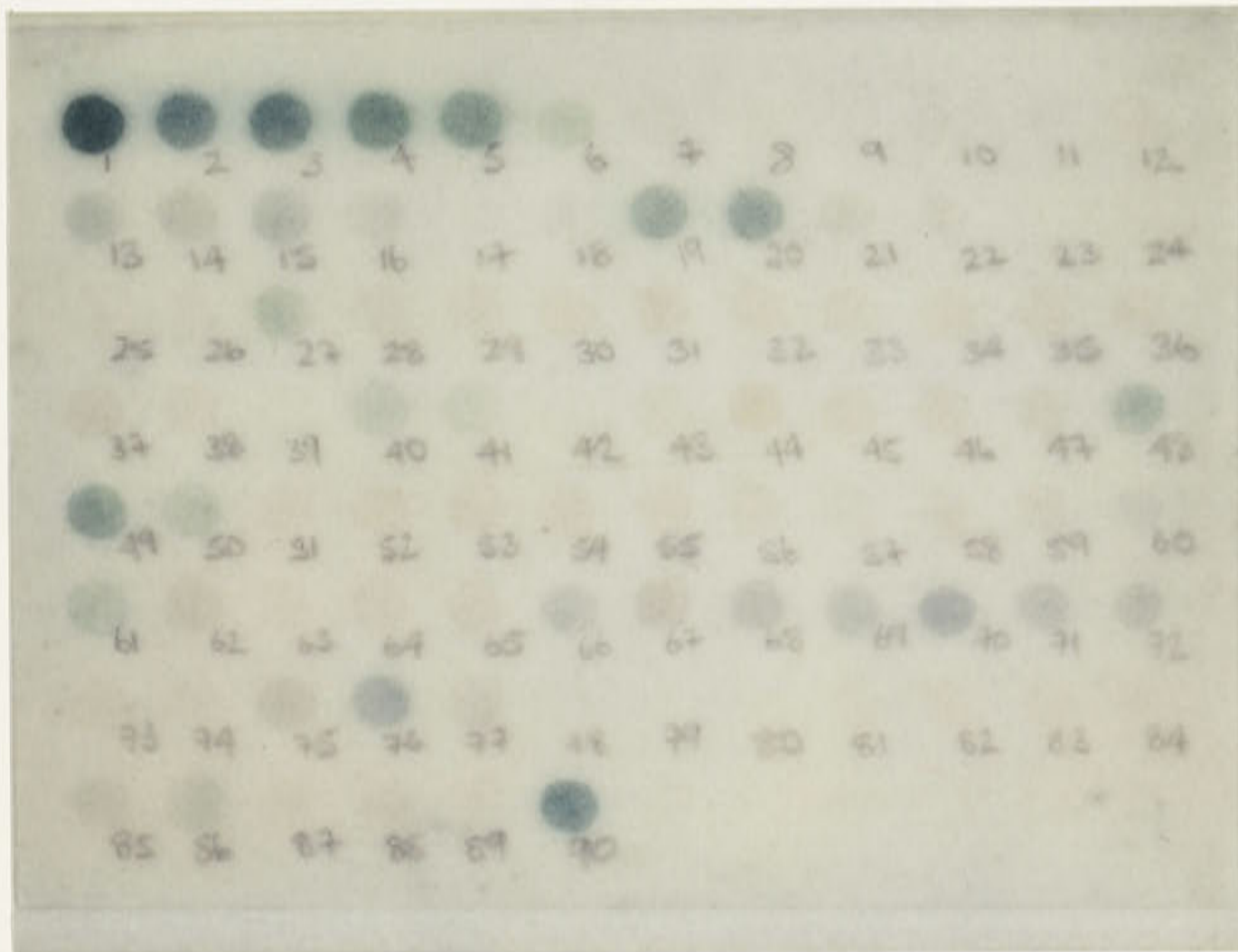
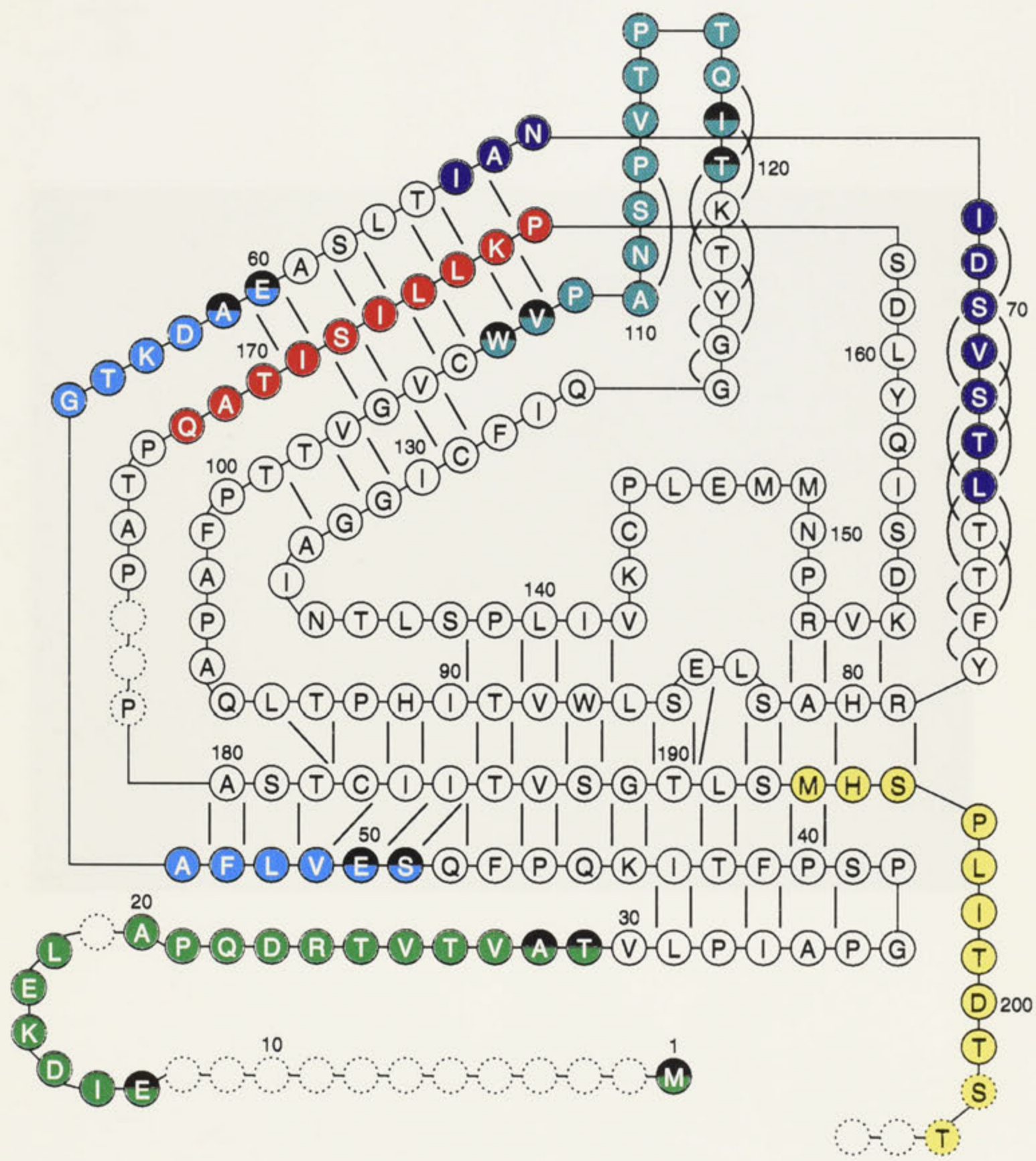


Figure 5.3 Photograph of the spots membrane probed with antibodies raised against purified particles of TYMV-type (1:200 dilution). Overleaf: spots peptides which reacted with antibodies to TYMV-type mapped onto the predicted structure of the TYMV coat protein.





- |  |  |
|--|--|
| <span style="color: green;">●</span> Spots No. 1, 2, 3, 4, 5 | <span style="color: teal;">●</span> Spots No. 48, 49, 50 |
| <span style="color: blue;">●</span> Spots No. 19, 20         | <span style="color: red;">●</span> Spot No. 76           |
| <span style="color: darkblue;">●</span> Spot No. 27          | <span style="color: yellow;">●</span> Spot No. 90        |

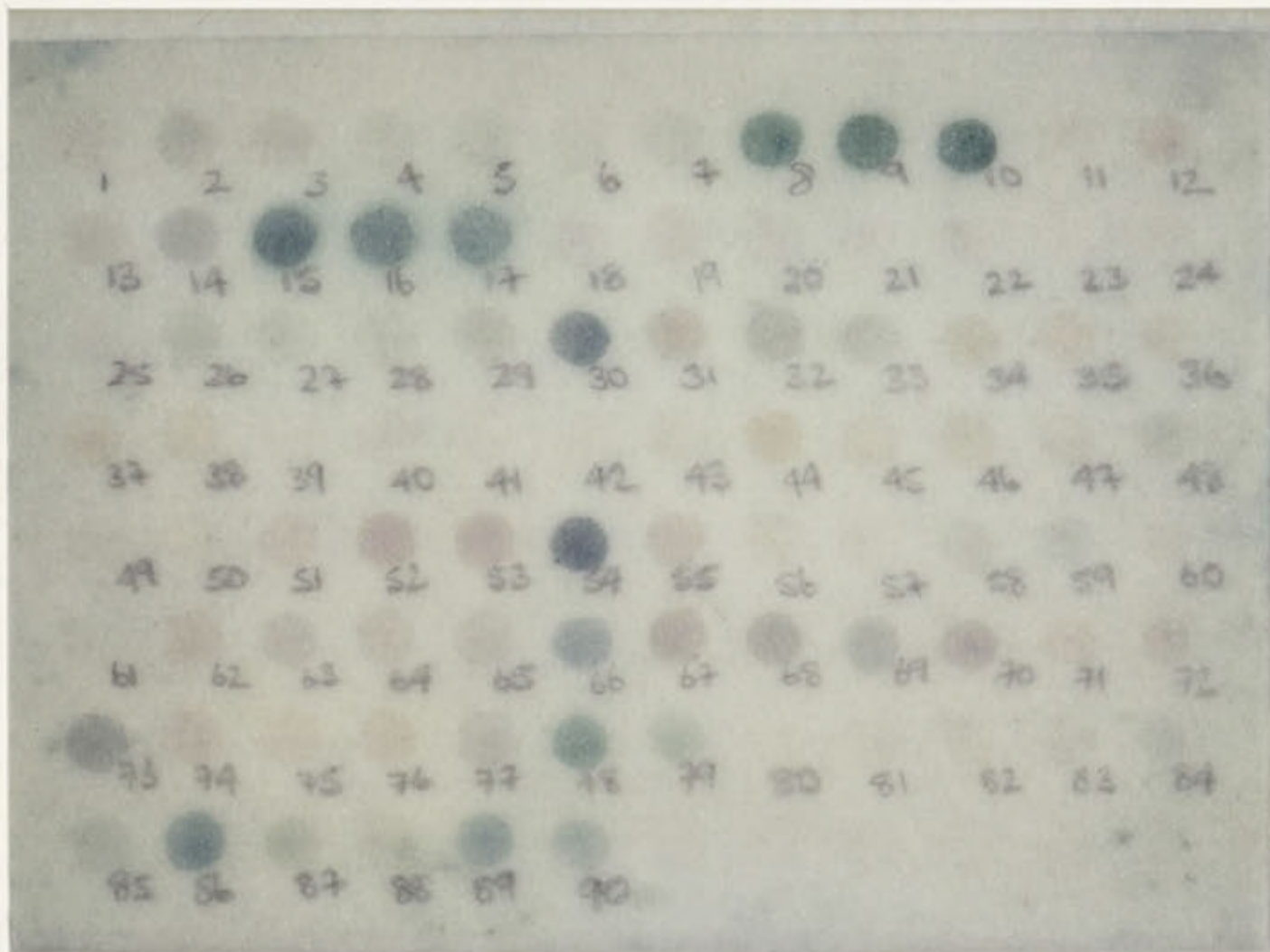
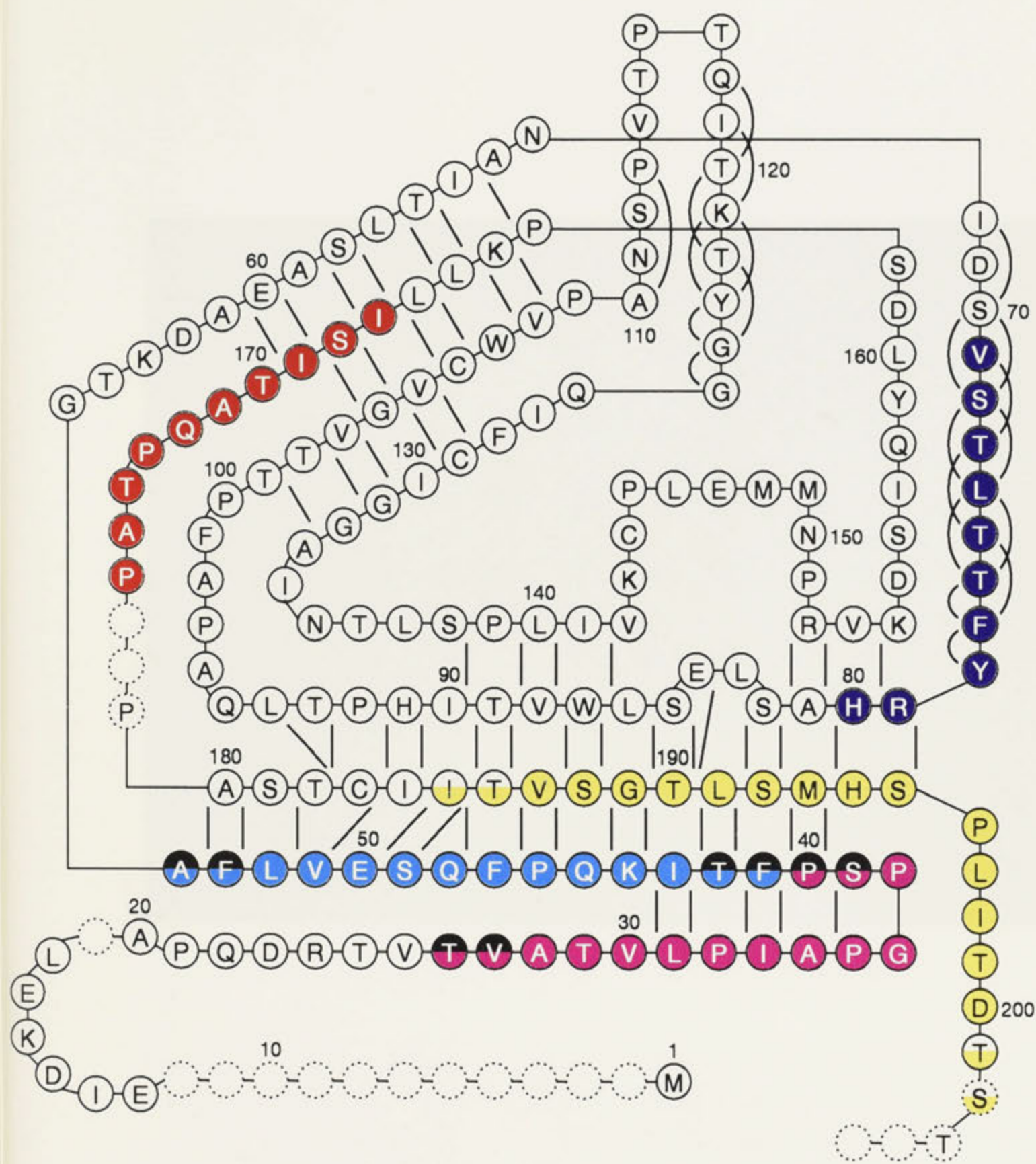


Figure 5.4 Photograph of the spots membrane probed with antibodies raised against purified particles of Recombinant 2 (R2) (1:200 dilution).

Overleaf: spots peptides which reacted with antibodies to R2 mapped onto the predicted structure of the TYMV coat protein.



● Spots No. 8, 9, 10

● Spot No. 78

● Spots No. 15, 16, 17

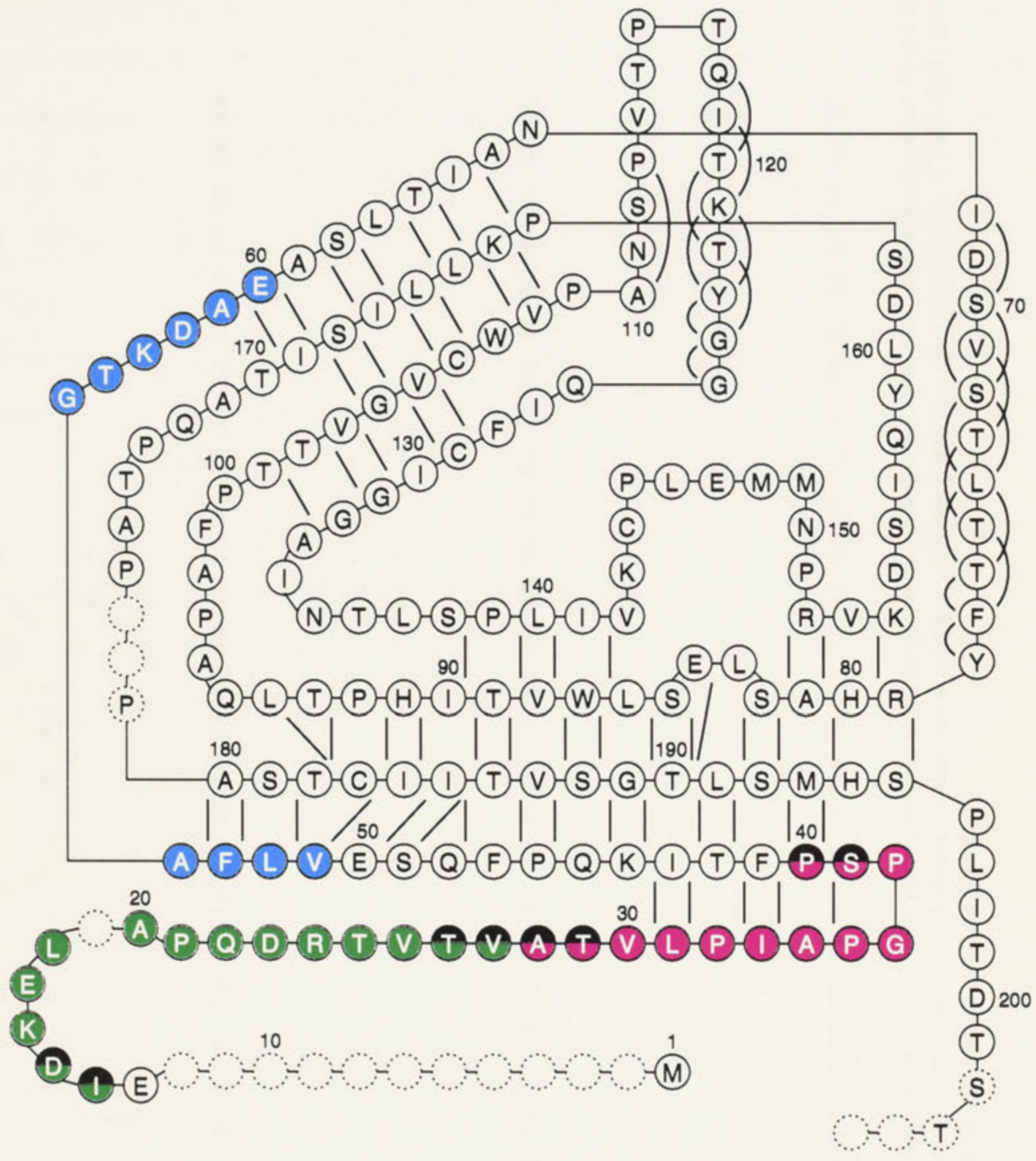
● Spots No. 86, 87, 89, 90

● Spot No. 30



Figure 5.5 Photograph of the spots membrane probed with antibodies raised against purified particles of Recombinant 3 (R3) (1:100 dilution).

Overleaf: spots peptides which reacted with antibodies to R3 mapped onto the predicted structure of the TYMV coat protein.



- Spots No. 2, 3, 4
- Spots No. 9, 10
- Spot No. 20

R3		+	+	+						+	+									+										
R2								+	+	+					+	+	+													+
TYMV Type	+	+	+	+	+																+	+							+	
TYMV-BL(2)	+	+	+	+	+							+	+																	
TYMV-BL(1)	+	+																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

R3																														
R2																														
TYMV Type																							+	+	+					
TYMV-BL(2)																														
TYMV-BL(1)																														
	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60

R3																															
R2																															
TYMV Type																															
TYMV-BL(2)																															
TYMV-BL(1)																															
	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	

Table 5.3 Summary of the spots peptides which reacted with each antibody used to probe the membrane. The five antibodies used are listed along the side and the peptide numbers are listed along the bottom. + denotes a reaction with the particular spot or peptide. TYMV-BL(1) antibodies are late bleed and TYMV-BL(2) antibodies are late bleed 2.

which, like spot no. 14, bound non-specifically to the secondary antibody and was therefore ignored.

When the SPOTs peptides were probed with R2 antiserum, several peptides reacted with the antibodies but the most notable difference from the wild-type TYMV antisera was that the R2 antibodies did not react with the N terminal 14 amino acids. Recombinant 2 contained the N terminus of BMV in place of TYMV (amino acids 1-12) so it appeared that antibodies raised against this recombinant did not recognise the wild-type TYMV N terminal amino acids. Other amino acids in the coat protein were also recognised by the antibodies (Table 5.3).

When the peptides were probed with an antiserum raised against recombinant 3 which had amino acids 13-20 replaced by those of BMV but with the wild-type amino acids in positions 1-12, amino acids 2-28 at the N terminus were recognised by the antisera. In addition, other epitopes were recognised by antibodies in recombinant 3 antiserum (Table 5.3).

### **5.3.2 WESTERN BLOTTING OF RECOMBINANT PARTICLES WITH ANTIBODIES**

Particles were purified from leaves systemically infected with recombinants 2, 3, 4, 5, 7 and 9 and wild-type TYMV-BL. The amount of protein in each sample was quantified and 1 $\mu$ g of each was loaded onto a 12% polyacrylamide gel. Following electrophoresis the gel was stained with Coomassie Blue, destained and photographed (Figure 5.6). It can be seen that for each sample there was a protein band of ~20Kd, which is the size of the coat protein.

M R2 R3 R4 R5 R7 R9 BL M

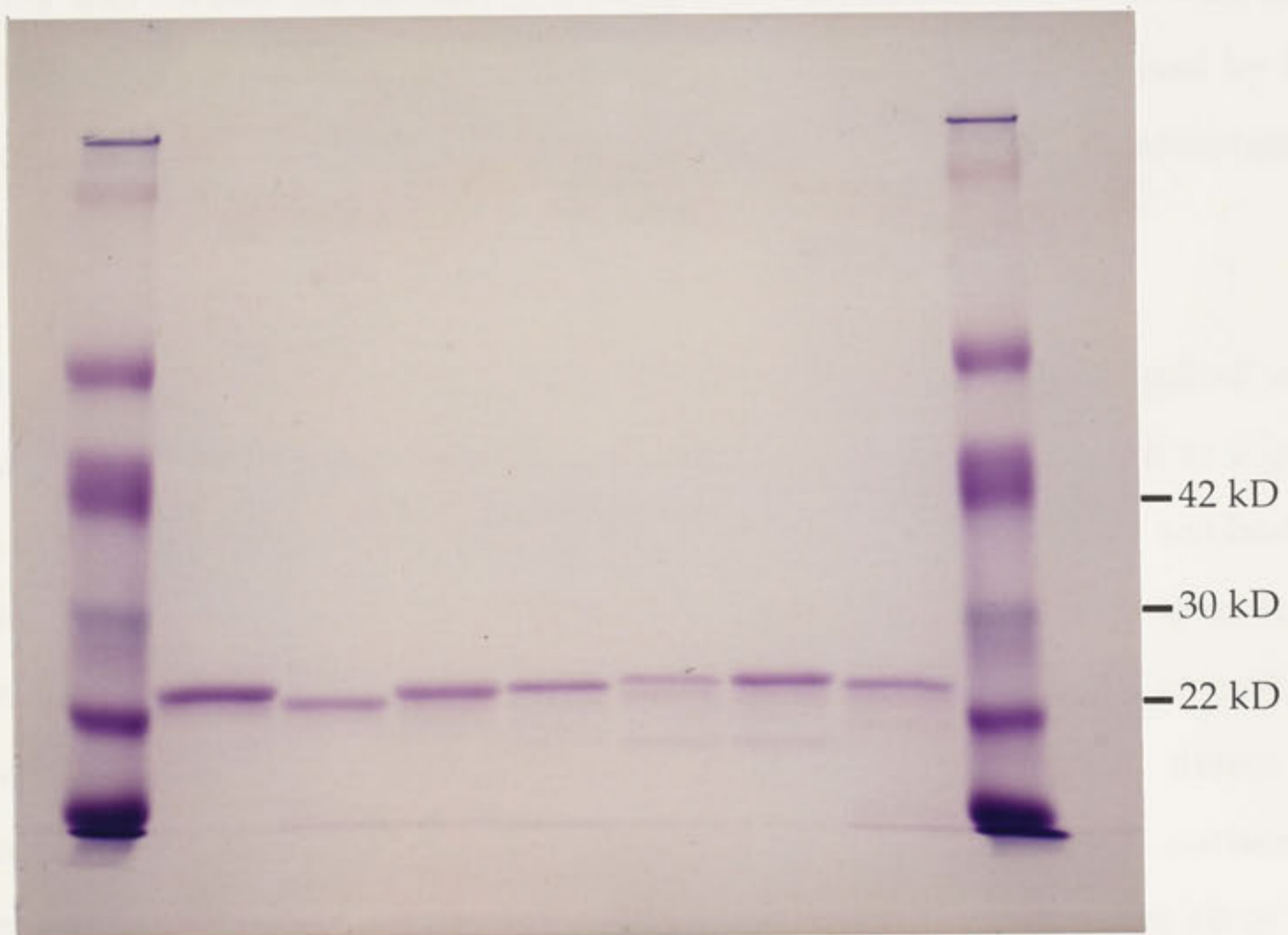


Figure 5.6 12% SDS-polyacrylamide gel loaded with virus particles of infectious recombinants. 1 $\mu$ g total protein was loaded for each sample. The gel was stained with Coomassie Blue and desaturated in a methanol/acetic acid solution. Protein molecular weight markers were from Novex and the contents of each lane is indicated on top. The sizes of the molecular weight markers are shown along the side.



A gel identical to the one above was prepared; virions from infectious recombinants were subjected to SDS-PAGE, blotted onto nitrocellulose and probed with both TYMV and R2 antisera (2.2.16-2.2.18). Figure 5.7 shows the proteins which were detected with the TYMV-BL antibodies and demonstrates that coat proteins of all infectious recombinants except R2 and R4 reacted with the TYMV-BL antiserum. This result suggested that most of the antibodies in the TYMV-BL antiserum reacted with the N terminal 1-12 amino acids of the TYMV coat protein which were absent in R2 and R4. This was further supported by the fact that recombinant 3, with wild-type TYMV amino acids 1-12 present at the N terminus, was recognised by the antibodies on a Western blot. All of the other infectious recombinants reacted with the TYMV antibodies.

Another gel, prepared as above, was electroblotted and probed with antibodies raised against recombinant 2. In this analysis, shown in Figure 5.8, all recombinant coat proteins were detected indicating that antibodies must have been elicited by R2 against more than the BMV specific N terminus as this region was not present in the other infectious recombinants which had wild-type N termini. In order to detect all infectious recombinants and TYMV-BL in Western blots the R2 antiserum must have contained antibodies raised against regions common to all of the recombinants and the wild-type protein as suggested by results with the SPOTs test using R2 antibodies where several regions of the coat protein reacted with the antibodies.

All gels blotted onto nitrocellulose for probing with antibodies were stained following electroblotting. Residual protein which had not transferred from the gel to the membrane was present in all samples. This proved that there was protein in all lanes of the gel and that negative results

M R2 R3 R4 R5 R7 R9 BL M

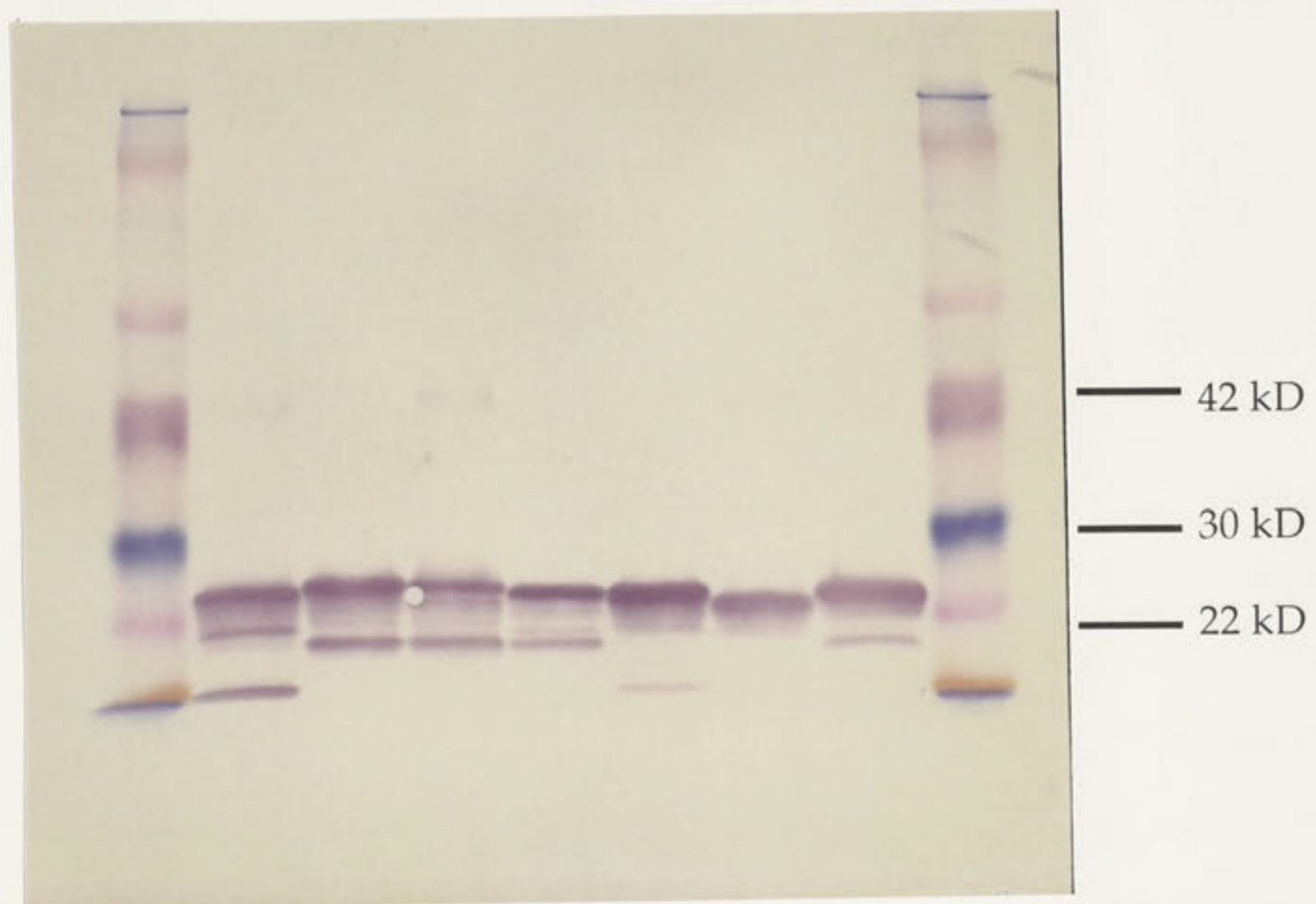


Figure 5.7 Western blot of a polyacrylamide gel as described in Figure 5.6. The blot was probed with polyclonal antibodies raised against purified particles of TYMV-BL. (early bleed, 1:100 dilution). Lanes 1 and 9 contain rainbow markers from Novex and the contents of the other lanes are indicated along the top. The sizes of the molecular weight markers are shown along the side.

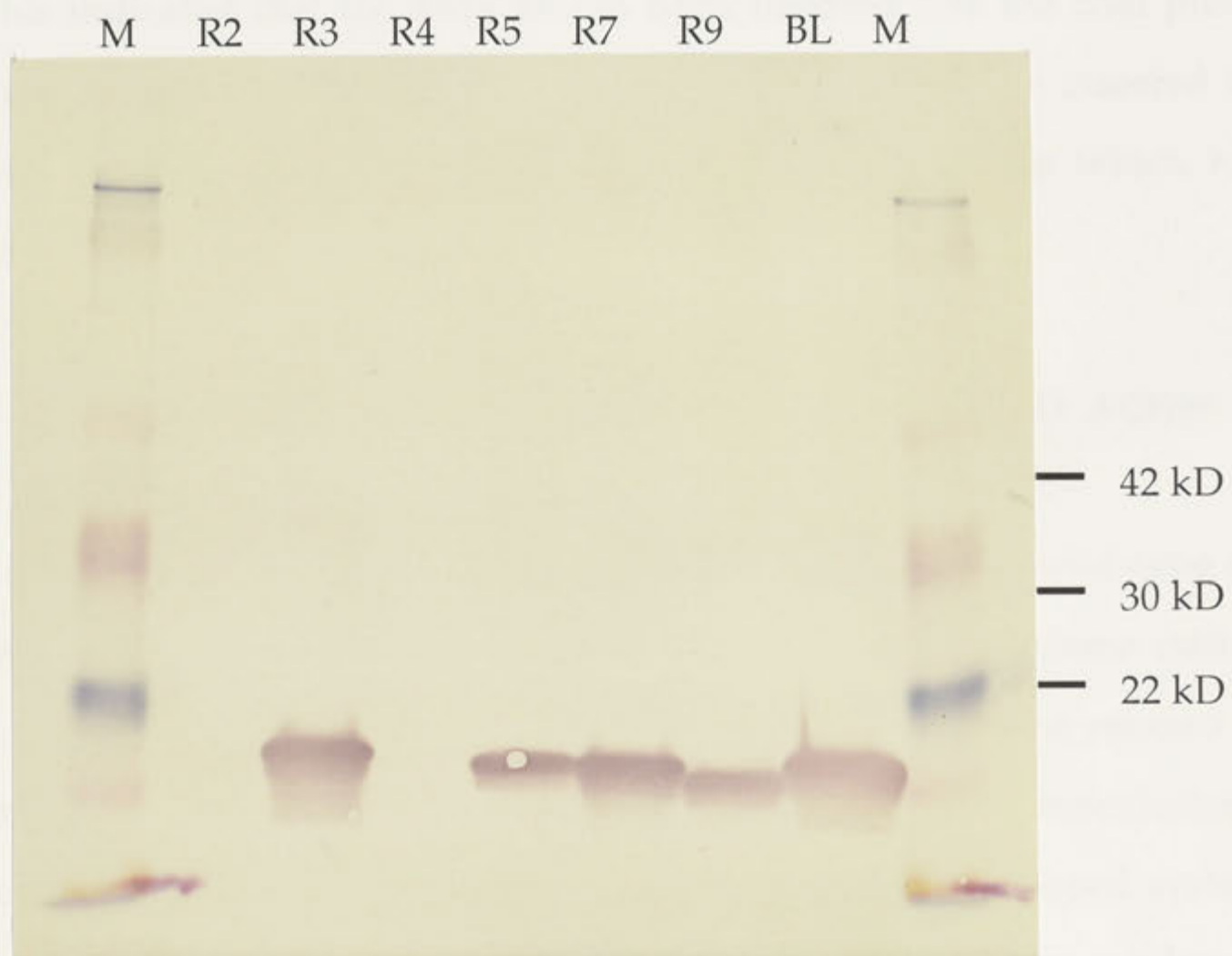


Figure 5.8 Western blot of a polyacrylamide gel as described in Figure 5.6. The blot was probed with polyclonal antibodies raised against purified particles of R2. (early bleed, 1:100 dilution). Lanes 1 and 9 contain rainbow markers from Novex and the contents of the other lanes are indicated along the top. The sizes of the molecular weight markers are shown along the side.

with antibody probing were not due to lack of protein on the membranes but due to non-binding of antibodies to antigen on the membrane.

### 5.3.3 ELECTROPHORETIC MOBILITY OF VIRUS PARTICLES

Figure 5.9 shows the positions of the particles of the different recombinants in the gel and it is clear that all of the recombinant particles except those of R5 had the same electrophoretic mobility as those of TYMV-BL. This indicated that the BMV amino acids inserted into the coat protein of R5 were exposed on the surface of the virus whereas those inserted into the coat proteins of the other recombinants, including those which have changed isoelectric points, were not surface exposed.

### 5.3.4 N TERMINAL MUTANTS WITH NON-VIRAL AMINO ACIDS

Figure 5.10 shows the sequences of the E71 mutant and wild-type coat protein genes which were transcribed and inoculated onto Chinese cabbage plants. The non-viral sequence was present at the 5' end of the mutant E71 coat protein gene in the clones used for transcription and inoculation of plants. Plants inoculated with control pBL-16 transcript developed systemic symptoms 8 days post inoculation (dpi) but the E71 mutant was confined to small yellow lesions until 11 dpi when it spread to give systemic infection. Figure 5.11 shows photographs of plants with symptoms resulting from inoculation with the mutant.

Total RNA was isolated from lesions on inoculated leaves of E71 and from tissue systemically infected with E71 and the pBL-16 control (2.2.8b). The RNA was used for RT-PCR to amplify virus specific products. A primer specific for the E71 sequence inserted was designed so that the presence of the mutation at the 5' end of the gene could be checked quickly without the

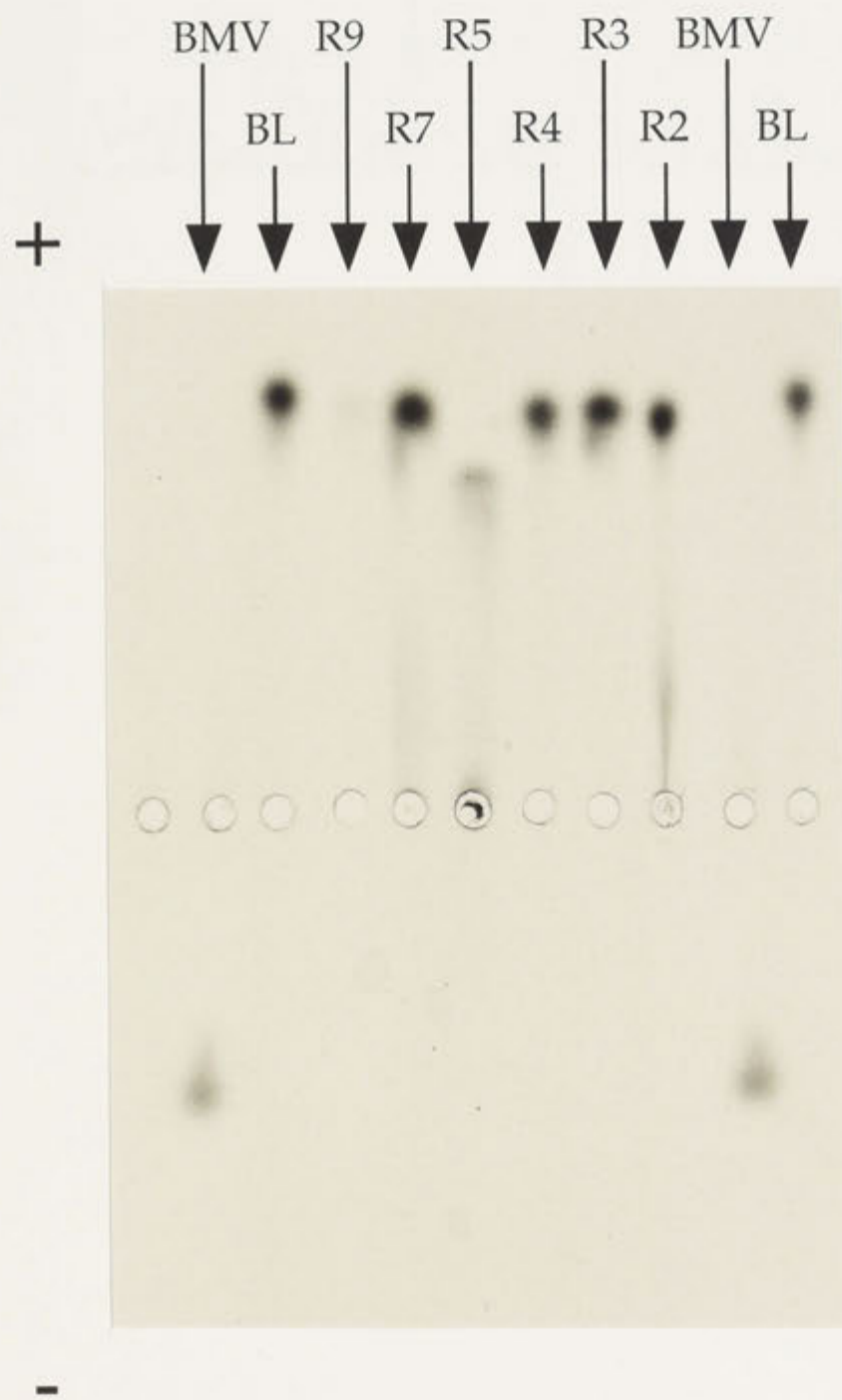
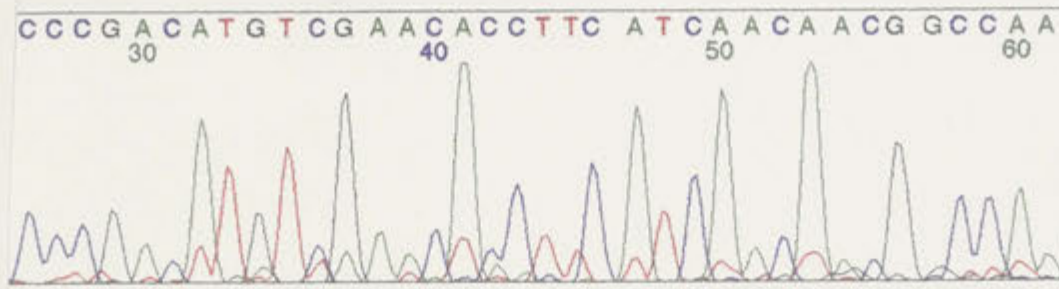
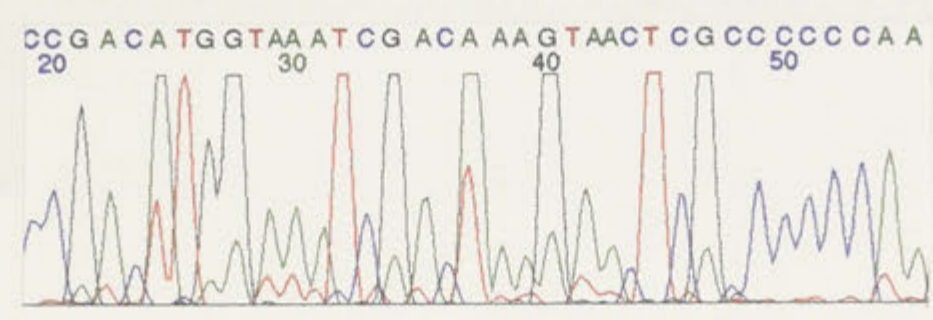


Figure 5.9 Electrophoretic mobility of infectious recombinant particles compared with that of TYMV-BL at pH 7.0. Particles were electrophoresed through a 1% agarose gel and visualised by staining with Coomassie Blue. The positions of the different particles are indicated; the amount of particles loaded for R5 and R9 was less than for the other recombinants. The positions of the anode and cathode are indicated.



E71



WT

Figure 5.10 Sequence of the E71 malaria antigen mutant compared with the wild-type virus. The samples were sequenced using dye terminators and Tall Comp primer.



Figure 5.11 Photographs of Chinese cabbage plants infected with transcript of the E71 mutant and the wild-type virus. At 11 days post inoculation systemic symptoms were established in the E71 inoculated plant but the symptoms were not as severe as those in the wild-type inoculated plant. A photograph taken 17 days post inoculation shows the development of symptoms in the E71 inoculated plants (3) compared with the wild-type inoculated plant (1).





necessity for sequencing. The sequence of the primer (E71 SEQ) was as follows: 5'-TCG AAC ACC TTC ATC AAC AAC GCC-3' and Figure 5.12 shows a photograph of the PCR products amplified using primer combinations CL-3/Tall Comp and CL-3/E71SEQ (primer designed by, and PCR done by, Anne Mackenzie). When the primers CL-3 and Tall Comp were used, the coat protein was amplified from E71 lesions, E71 systemically infected tissue and from the pBL-16 control infected tissue. When the second primer combination of CL-3 and E71SEQ was used, coat protein was only amplified from E71 lesions and systemically infected tissue but not the pBL-16 control. This result showed that the E71 sequence was present and that the virus was able to systemically infect plants with the insertion of a non-viral sequence in the 5' end of the coat protein gene.

#### 5.4 DISCUSSION

When results using TYMV-BL antibodies (late bleed 2) in the SPOTs test and on Western blots are compared, they do not agree in that several epitopes were detected in the SPOTs test but Western blotting results suggested that the N terminus was the dominant epitope involved in eliciting the immune response. With R2 and R4 their N termini were changed by the insertion of BMV amino acids and antibodies raised against the wild-type protein failed to recognise them on Western blots. It is not known why the other epitopes besides the N terminus detected by the SPOTs test were not also detected on Western blots of R2 and R4 proteins. One possible explanation may be that the epitopes involved in stimulating the immune response were discontinuous and reflected a structural feature which was destroyed when the protein was denatured in SDS-PAGE. These epitopes may have remained intact on the SPOTs membrane due to interactions between the amino acids in each peptide which may have formed structural features recognised by the antibodies. The

	TALL COMP			E71.SEQ			
	S	L	S	S	L	S	
M	BL	E71	E71	BL	E71	E71	M

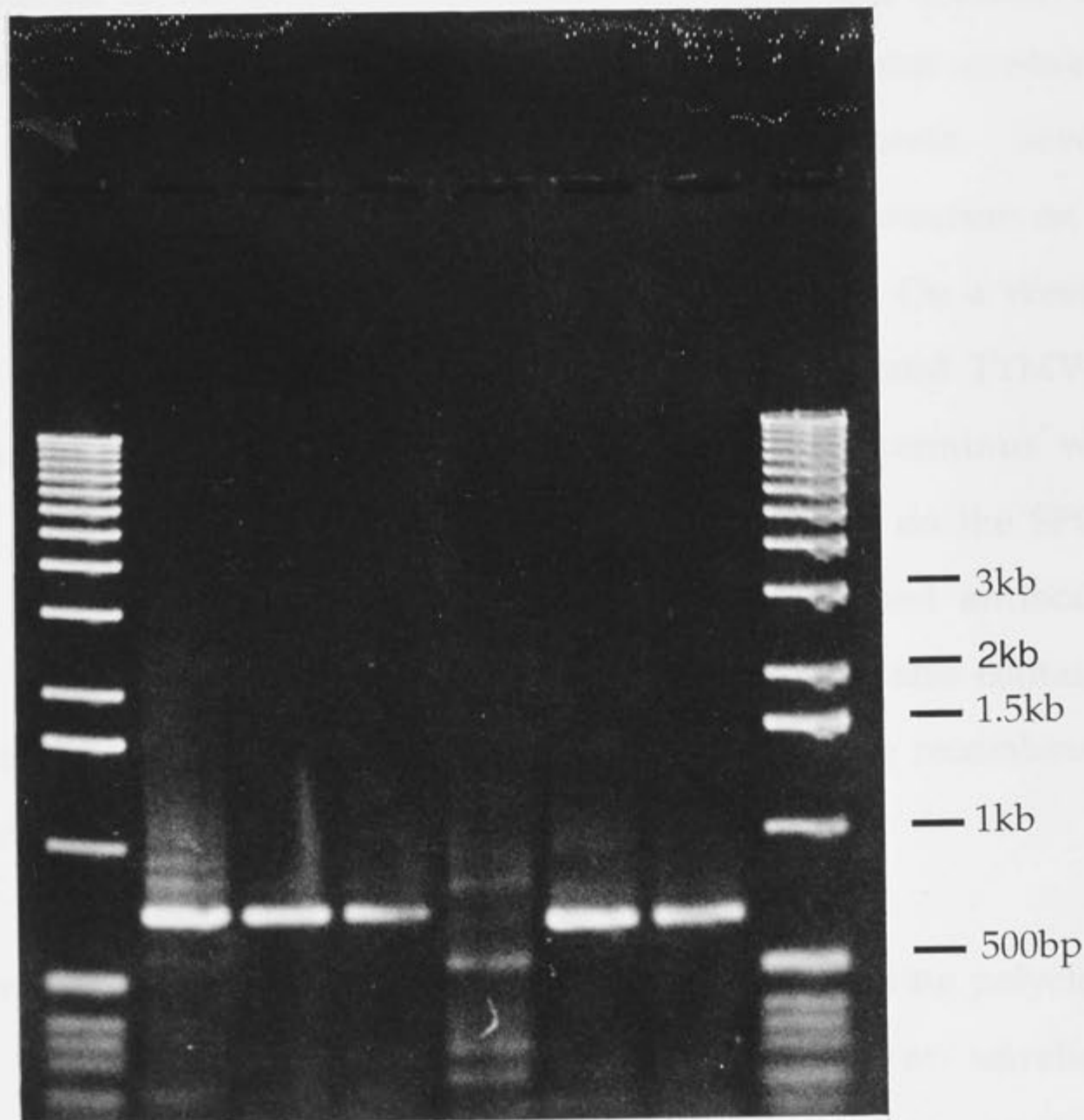


Figure 5.12 RT-PCR products from lesions infected with E71 and from tissue systemically infected with E71 and wild-type transcript. PCR was done using the primer combinations Tall Comp/CL-3 and E71SEQ/CL-3. The first combination of primers amplified coat protein from all three samples whereas the second combination, with an E71 specific primer, only amplified coat protein from the lesions and tissue systemically infected with E71. The latter PCR products are slightly smaller than those amplified with the Tall Comp/CL-3 combination; this is due to the position of the E71SEQ primer in the coat protein gene sequence.

immunodominance of the N terminal 1-12 amino acids was reflected by the fact that R3, which had amino acids 13-20 replaced by BMV but had TYMV-BL amino acids in positions 1-12, was detected on Western blots by the wild-type antibodies as were all the other infectious recombinants.

In contrast to the above, results of the SPOTs test and Western blot with antiserum against R2 suggested that this antiserum contained antibodies raised against several epitopes on the coat protein. Several regions of the TYMV-BL coat protein were detected by this antiserum on the SPOTs membrane but these did not include the N terminus. On a Western blot the R2 antiserum detected all infectious recombinants and TYMV-BL protein which suggested that epitopes other than the N terminus were recognised. The fact that the N terminus was not detected on the SPOTs membrane by the R2 antiserum suggested that it contained antibodies specific for the BMV 1-12 amino acids inserted. However, it also contained antibodies against other epitopes which were detected in the recombinants on the Western blot.

The results reported here and those of other workers using polyclonal antibodies with the SPOTs system suggest that they are an unreliable combination for detecting surface-exposed areas on proteins. Electrophoretic mobility experiments with particles of infectious recombinants showed that R5, with the predicted  $\alpha$  helix a of TYMV-BL changed to that of BMV, was the only one with a changed mobility at pH 7.0. The other recombinants, some of which had isoelectric points which differed from the wild-type protein due to the inserted BMV amino acids (R2, R4, R7), had the same mobility as the TYMV-BL particles (Figure 5.9). This suggested that the inserted BMV amino acids in these recombinants were not exposed and did not affect the mobility of the particles. This result agreed with earlier work on the N terminal amino acids of PhMV and

TYMV where amino acids 1-12 had been shown to cross-link the viral RNA (Kekuda *et al.*, 1993; Ehresmann *et al.*, 1980). This, and the fact that particles of N terminal recombinants did not react with BMV antibodies in immunodiffusion tests, suggested that these amino acids were probably buried within the particle. The predicted accessibility data from EMBL (Figures 1.5, 1.6) indicated that parts of the N terminus were exposed and parts were buried but the results reported here suggest that it is buried. The discrepancy may be explained by the fact that the predicted accessibility data was for an accuracy of >82% but the prediction was not 100% accurate.

Particles of R5 probably had the BMV amino acids exposed on the surface as their mobility was affected. The location of  $\alpha$  helix a on the surface of the capsid was also demonstrated by the fact that in immunodiffusion tests particles of R5 were the only ones which reacted with BMV antibodies. The surface location of R5 is in agreement with earlier results as R5 encompasses amino acids 57-67 which were shown to be exposed on the capsid (Pratt *et al.*, 1980). In the SPOTs analyses only 2 of the 5 antibodies tested (antibodies against R2 and TYMV-type) detected peptides corresponding to  $\alpha$  helix a of the wild-type protein. This reflected the heterogeneity of the immune systems of individual rabbits where different regions of the protein were recognised and involved in eliciting an immune response and also that exposed parts of a protein were not necessarily antigenic. A lack of recognition of surface regions was also seen with Johnsongrass mosaic virus where peptides corresponding to the C terminus were not detected by antibodies in the SPOTs system even though it had been previously shown to be surface located (Shukla *et al.*, 1989).

Various peptides corresponding to regions in the TYMV-BL coat protein including the C terminus were detected by the different antibodies on the spots membrane which again reflects differences in the immune

responses of individual rabbits. The majority of reports in the literature using the SPOTs or pepsan systems involved studies with monoclonal antibodies to identify epitopes and the amino acids which specifically reacted with monoclonal antibodies in neutralisation epitopes. The use of monoclonal antibodies to TYMV in conjunction with overlapping peptides on a SPOTs membrane may be more useful as a means to precise mapping and size determination of epitopes as was shown with beet necrotic yellow vein virus (Commandeur *et al.*, 1994).

Results with the E71 mutant showed that the coat protein of TYMV-BL can tolerate the insertion of non-viral amino acids and retain the ability to systemically infect plants. The mutant virus is stable and the foreign amino acids are retained through passage of the virus. The next step in assessing the suitability of TYMV-BL as a system for presentation of foreign epitopes for vaccine development is to prepare pure virions from infected plants and raise antibodies against them. The antibodies can be checked for specificity to the foreign amino acids by Western blotting with the merozoite surface antigen. If there is a positive reaction, which is likely given the immunodominance of the N terminus, then the coat protein of TYMV-BL has significant potential as a peptide presentation system. TYMV-BL coat protein mutants containing other foreign amino acids will be tested to check constraints such as the size limit and type of amino acids which can be tolerated by the virus.

## CHAPTER SIX

### DISCUSSION

While little is known about the host range specificity of plant viruses, many studies (see Chapter 1) have shown that changes to parts of the viral genome, in particular to the coat protein gene, affect the symptoms produced by the virus. Skotnicki *et al.* (1993) attempted to delineate which tymovirus genes were important in determining host range by constructing hybrid viruses. Full-length clones of eggplant mosaic virus (EMV), ononis yellow mosaic virus (OYMV) and turnip yellow mosaic virus - Blue Lake isolate (TYMV-BL) were used and hybrids were constructed by exchanging the coat protein gene between EMV or OYMV and TYMV-BL. These three viruses have different host ranges but when the hybrids were inoculated onto host plants of the parental viruses, no symptoms were seen in inoculated leaves and no viral RNA was detected. Experiments were also done by Skotnicki *et al.* (1992) to investigate which parts of the TYMV genome were involved in the production of symptoms in inoculated plants and involved exchanging parts of two TYMV clones (TYMV-BL and TYMV-CL; both full-length clones of two Australian isolates of the virus) which produced different symptoms in plants. However, symptoms produced in plants inoculated with the hybrids could not be linked to the exchanged parts of the genome.

The experiments reported here were a further attempt to discover the host range and symptom determinants of tymoviruses (in particular TYMV) but in contrast to earlier studies they focussed on the coat protein alone. The results of these experiments have more clearly defined the role of the tymovirus coat protein in infection of its hosts and have also assigned locations to parts of the protein in the virus particle. Two approaches were

taken to this study: in the first, naturally occurring symptom variants of TYMV-BL were isolated and their coat protein genes sequenced to see if there were any changes in the protein sequence which correlated with the different symptoms. When the coat protein sequences of the symptom variants were compared with that of TYMV-BL there were only two amino acid differences which correlated with the type of symptoms produced in inoculated plants. To determine if these different amino acids are the cause of attenuated symptoms, they would have to be inserted into the wild-type coat protein and the resulting recombinant inoculated onto plants to see if the symptoms were altered.

In the second approach, recombinants were constructed by inserting parts of the BMV coat protein into the corresponding part of the TYMV-BL coat protein. The effects of changing the coat protein in this way were investigated by inoculating Chinese cabbage plants with transcripts of the recombinants and monitoring the resulting symptoms.

Results with the coat protein recombinants showed that viable hybrids could be produced by replacing parts of the TYMV-BL coat protein with homologous parts from the BMV coat protein. However, none of the recombinants had a host range that differed from TYMV-BL suggesting that the regions examined were not involved in host range determination and other parts of the virus need to be examined to gain an understanding of this complex process. Some of the recombinants produced different symptoms when inoculated onto plants and confirmed the role of the coat protein in both cell-to-cell and long-distance movement of TYMV-BL in its host. Information provided by experiments on recombinant particles helped to confirm previous results on the location of certain parts of the protein in the capsid structure and epitope analysis of the coat protein located a major antigenic epitope of TYMV-BL which can be replaced with non-viral amino acids. This indicates that TYMV may be a viable epitope

presentation system for the production of vaccines and work is underway to test the immunogenicity of the non-viral peptide.

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