



PATHOLOGY AND MOLECULAR COMPARISON OF A RANGE OF PEA SEED-BORNE MOSAIC VIRUS ISOLATES

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SUMMARY

This thesis describes: the development of serological and nucleic acid based diagnostic methods for pea seed-borne mosaic virus (PSbMV); isolate specific effects on infected pea plants; the collection and biological comparison of new PSbMV isolates from Pakistan; the cloning and sequencing of specific parts of the genome of selected isolates; nucleotide and amino acid sequence comparisons between selected isolates; and, the development of a ribonuclease protection assay (RPA) for identifying genomic differences among the PSbMV isolates.

The dot-immunobinding assay (DIBA) was modified to detect PSbMV. For virus, the DIBA detection threshold was 1 ng per μl and in sap extracted from infected leaf the dilution endpoint was 1/4096. DIBA was used for the detection of PSbMV in samples collected from the field, dry pea seed and in seedlings germinated in the glasshouse. Nucleic acid-based hybridisation assays using dot-blot and tissue-print formats were developed to detect PSbMV. These assays used randomly primed cDNA probes (^{32}P - and Digoxigenin-labelled) representing the full genome of PSbMV. For virus, the dot blot hybridisation assay detection threshold was 160 pg per μl and in sap extracted from infected leaf the dilution endpoint was 1/3125. For RNA, the dot blot hybridisation assay detection threshold was 51 fg per μl and in total nucleic acid extracted from infected leaf the dilution endpoint was 1/3125. PSbMV RNA was detected in leaf and embryo squashes by tissue-print hybridisation assay.

To study the pathogenesis of PSbMV, the South Australian isolate S6 and the North American type isolate US were classified by pathotype specific pea genotypes and used as representatives of the PSbMV pathotypes P-4 and P-1 respectively. Under glasshouse conditions, Dundale pea plants inoculated mechanically with S6 showed severe mosaic

symptoms on leaves, the premature emergence of axillary shoots at 21 days post inoculation (at the 11-12 node plant stage), delayed flowering, delayed pod set, delayed pod maturation, and the senescence of plants was delayed by 14 days. Seed yield was reduced by 82% and the virus was transmitted to 31% of seedlings. Isolate US showed mild leaf rolling of leaves, the emergence of axillary shoots at 28 days post inoculation (at the 19-20 node plant stage) and senescence of plants was delayed by 7 days. Seed yield was reduced by 35% and the virus was transmitted to 8% of seedlings. Axillary shoots were produced in healthy plants from 35 days at the 22-23 node stage when the plants had already started to flower. Both viruses showed petal colour break. Plants infected via seed with S6 were symptomless, they senesced 7 days late, seed yield was reduced by 52% and 12% of their seed contained virus. Some of the plants infected via seed with US showed mild leaf rolling, there was no significant effect on yield and 7% of seed contained virus.

During the early growing season of 1995, a survey was made for PSbMV in the major pea-growing areas of North West Frontier Province (NWFP) of Pakistan. Of 713 samples collected from 62 commercial pea fields or experimental plots, 11 samples were positive by DIBA to PSbMV with an average incidence of 1.5 %. PSbMV was also detected in 1 to 5% of dry seed from five of the 12 Pakistani pea varieties tested and in 8 to 20% of seedlings raised from seed of three of these varieties. Twelve Pakistani PSbMV isolates (PK1-PK12) were recovered from the field samples, DIBA positive seed extracts and from seedlings germinated in the glasshouse. These isolates were compared with isolates S6 and US and were not distinguishable by serology, capsid protein size, RNA size or dot blot hybridisation using randomly primed cDNA probes to isolates S6 and US. The ability of isolates PK1-PK12, S6 and US to infect a standard range of pathotype specific pea differential genotypes showed that they could be classified into 4 distinct groups. One group contained isolates US, PK2, PK3, PK6, PK7 and PK10 and was classified as a pathotype P-1 group. Another group contained

isolates S6, PK8 and PK12 and was classified as a pathotype P-4 group. The remainder could not be classified by the existing PSbMV pathotyping system and were tentatively placed into two other groups named U-1 (unknown 1) and U-2 (unknown 2). Isolates PK1, PK4, PK5 and PK11 were placed in the U-1 group and PK9 was placed in the U-2 group.

Nucleotide and amino acid sequences of the 3' and 5' parts of the RNA of isolates S6 (P-4), US (P-1) and PK9 (U-2) were compared. The 3' part included some of the NIb gene, the full coat protein (CP) gene and the untranslated region (UTR). The 5' part included some of the P1-Pro gene and the full helper component (HC-Pro) gene. Both parts were amplified by the reverse transcription polymerase chain reaction (RT-PCR) using specific primers designed from the published sequences of the P-1 DPD1 and P-4 NY isolates of PSbMV. All PCR products were cloned into the pGEM[®]-T transcription vector and sequenced in both directions. Nucleotide and amino acid sequence alignments and phylogenetic relationships among the PSbMV isolates S6, US and PK9 were compared with the previously fully sequenced (P-1 DPD1 and P-4 NY isolates) and partially sequenced {P-1 NZ (New Zealand); CAN (Canada) and GER (Germany)} isolates of PSbMV. Phylogenetic comparisons based on nucleotide sequences divided these isolates into two main groups. The first group contained isolates CAN, DPD1, GER, NZ, PK9 and US. However, much diversity was found among the isolates in this first group, and three clusters of isolates were observed. The clusters included isolates DPD1, NZ, and PK9, isolates CAN and US, and the third cluster contained only isolate GER. The second group contained isolates S6 and NY.

Increased discrimination between isolates was achieved by the ribonuclease protection assay (RPA). A comparison of all of the 14 PSbMV isolates (S6, US and PK1-PK12) quantified their genomic heterogeneity within defined 3' parts of isolate S6 (1737 nt), US (1739 nt) and PK9 (1366 nt). The RPA banding patterns obtained for cRNA probes

from isolates S6, US and PK9 were used for a phylogenetic analysis which placed the 14 PSbMV isolates into three distinct groups. The first group contained isolates US, PK5, PK6 and PK7. The second group contained isolates S6, PK8 and PK12. The third group contained isolates PK1, PK2, PK3, PK4, PK9, PK10, and PK11.

This is the first comparison of a range of geographically different isolates of PSbMV on the basis of both biological and molecular properties. The biological comparison has put the Pakistani PSbMV isolates into four groups and RPA into three distinct groups. Identification of a previously undescribed cluster of isolates of PSbMV including representatives from Pakistan only, may be evidence for a centre of evolution of PSbMV in or near Pakistan, an area which is also considered to be near the centre of origin of pea, the principal host of PSbMV.

PUBLICATIONS

(arising from this thesis)

1. Ali, A. and Randles, J. W. (1995). Some characteristics of a pathotype 4 isolate of pea seed-borne mosaic virus from South Australia. (Abstr.) *10th Biennial Australasian Plant Pathology Conference*, Christchurch, New Zealand. p84.
2. Ali, A. and Randles, J. W. (1997). Early season survey of pea viruses in Pakistan and the detection of two new pathotypes of pea seed-borne mosaic potyvirus. *Plant Disease* **81**, 343-347.
3. Ali, A. and Randles, J. W. (1997). Yield loss due to a South Australian pathotype 4 isolate of pea seed-borne mosaic virus. (Abstr.) *Third International Food Legume Conference*, Adelaide, Australia. p169.
4. Ali, A. and Randles, J. W. (1997). 3' terminal sequence of the RNA genome of the South Australian pathotype 4 isolate of pea seed-borne mosaic virus. (Abstr.) *11th Biennial Australasian Plant Pathology Conference*, Perth, Australia. p105.
5. Ali, A., Randles, J. W. and Hodgson, R. A. J. (1998). Sensitive detection of pea seed-borne mosaic potyvirus by dot blot and tissue print hybridisation assays. *Australian Journal of Agricultural Research* **49**, 191-197.
6. Ali, A. and Randles, J. W. (1998). An evaluation of the effects of infection with two pathotypes of pea seed-borne mosaic virus on the morphology and yield of pea. *Australasian Plant Pathology* **27**, 226-233.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this thesis being made available for photocopying and loan when deposited in the University Library.

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ABBREVIATIONS

A	absorbance
ATP	adenosine triphosphate
AMV	alfalfa mosaic virus
AP 7.5	alkaline phosphatase buffer (pH, 7.5)
AP 9.5	alkaline phosphatase buffer (pH, 9.5)
aa	amino acid
APS	ammonium persulphate
BBMV	broad bean mottle virus
BBTMV	broad bean true mosaic virus
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLRV	bean leaf roll virus
BSA	bovine serum albumin
BWYV	beet western yellows virus
BYMV	bean yellow mosaic virus
Cs ₂ SO ₄	caesium sulphate
CaCl ₂	calcium chloride
CAN	Canada
CP	coat protein
CCMV	cowpea chlorotic mottle virus
cDNA	complementary deoxyribonucleic acid
cm, mm, nm, µm	centimetre, millimetre, nanometre, micrometre
CMV	cucumber mosaic virus
cpm	counts per minute
cRNA	complementary ribonucleic acid
°C	degree celsius
DPD1	Danish plant directorate 1
dATP	2'- deoxyadenosine 5'-triphosphate
dCTP	2'- deoxycytosine 5'-triphosphate
dGTP	2'- deoxyguanosine 5'-triphosphate
dTTP	2'- deoxythynosine 5'-triphosphate
dNTP	mixture of deoxynucleoside-triphosphates in equimolar
DEPC	diethyl pyrocarbonate
DBHA	dot blot hybridisation assay
DIBA	dot-immunobinding assay

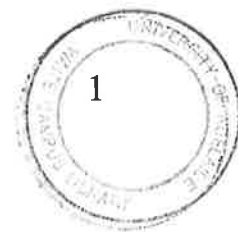
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EM	electron microscope
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediamine <i>tetra</i> acetic acid
EtBr	ethidium bromide
FGRB	formaldehyde gel running buffer
FGSB	formaldehyde gel sample buffer
<i>g</i>	gravitational force
g, mg, μ g, ng, pg, fg	gram, milligram, microgram, nanogram, picogram, femtogram
G1	generation 1
G2	generation 2
G3	generation 3
GER	Germany
HCl	hydrochloric acid
HC-Pro	helper component protein
hr	hour
IPTG	<i>iso</i> -propyl- β -D-thiogalactopyranoside
kb	kilo base
kDa	kilo dalton
L-1	pathotype L-1
LiCl	lithium chloride
LB	Luria-Bertani medium
LBA	Luria-Bertani agar medium
LMV	lettuce mosaic virus
mmole, nmole, pmole	millimole, nanomole, picomole
M, mM, μ M	molar, millimolar, micromolar
MMuLV	Moloney murine leukaemia virus
MgCl ₂	magnesium chloride
μ Ci	micro curie
min	minute
ml, μ l	millilitre, microlitre
MnCl ₂	manganese chloride
MOPS	3-N-{morpholino}propane sulfonic acid

MW	molecular weight
NBT	nitro blue tetrazolium
NIb	nuclear inclusion b protein
nt	nucleotide
NY	New York
NZ	New Zealand
OD	optical density
ORF	open reading frame
P-1	pathotype 1
P-4	pathotype 4
PI	plant introduction
P1-Pro	P1 protein
PAGE	poly acrylamide gel
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
KAc	potassium acetate
KCl	potassium chloride
K-PTA	potassium phosphotungstic acid
PEMV	pea enation mosaic virus
PK1-PK12	Pakistani isolate 1-Pakistani isolate 12
PMV	peanut mottle virus
PSbMV	pea seed-borne mosaic virus
PSNV	pea stem necrosis virus
PSV	peanut stunt virus
rATP	adenosine 5'-triphosphate
rCTP	cytidine 5'-triphosphate
rGTP	guanosine 5'-triphosphate
rUTP	uridine 5'-triphosphate
RNA	ribonucleic acid
RNase A	ribonuclease A
RNase T1	ribonuclease T1
RNasin [®]	ribonuclease inhibitor
RPA	ribonuclease protection assay
RT	reverse transcription
NaOAc	sodium acetate
SeqEd [®]	sequence editing

ss	single stranded
S	sedimentation coefficient
SDS	sodium dodecyl sulphate
SDDW	sterile double distilled water
TPA	tissue print hybridisation
TAE	tris-acetate
TBE	tris-borate
TE	tris-EDTA
TEMED	N, N, N'- N'- tetramethylethylenediamine
TES	tris-EDTA-SDS
TMV	tobacco mosaic virus
UV	ultra violet
U-1	unknown 1
U-2	unknown 2
UTR	untranslated region
V	voltage
v/v	volume: volume ratio
w/v	weight: volume ratio
X-Gal	5-bromo-4-chloro-3-indolyl- β -galactopyranoside

Chapter 1

GENERAL INTRODUCTION



1.1 The pea crop

Pea (*Pisum sativum* L.) is the fourth most important grain legume crop world-wide (Davies, 1993). Green pea production is estimated to be 4.8 million tonnes grown on 0.76 million hectares and dry pea production is estimated to be 16.3 million tonnes on 9.5 million hectares (Maury and Khetarpal, 1992; Davies, 1993). The primary centre of origin of peas is the mountainous region of Southwest Asia, particularly Afghanistan and India, while the secondary centre is the Mediterranean region (Makasheva, 1983).

In Australia, peas represent the largest proportion of the grain legume crop, contributing to both export and local market economies. Green pea is grown on 9,203 hectares with a total production of 36,286 tonnes, while dry peas are grown on 382,000 hectares with a total annual production of 456,000 tonnes (Anon. 1993).

1.2 Pea viruses

As shown in Table 1.1, pea has been reported to be susceptible to more than 50 viruses that infect pea either naturally or experimentally (Hagedorn, 1984; Maury *et al.*, 1987; Buchen-Osmond *et al.*, 1988; Boswell and Gibbs, 1983; Fletcher, 1993; Phillips and Chandrashekar, 1994; Murphy *et al.*, 1995). Among these viruses, pea seed-borne mosaic virus (PSbMV) has gained the most global attention due to its continuing threat to pea production and the associated processing industry (Kraft and Hampton, 1980).

1.3 Discovery of PSbMV

PSbMV was first discovered in Czechoslovakia (Musil, 1966) and given the name pea leaf rolling virus (Kvicala and Musil, 1967). In Japan it was found in 1967 and named pea seed borne mosaic virus (Inouye, 1967). In the USA, the virus was detected in

Table 1.1 Viruses infecting pea naturally (field) or experimentally (glasshouse)

Virus genus	Virus name	Transmission			
		A	F/N	M	S
Alfamovirus	- alfalfa mosaic virus	+		+	+
Bromovirus	- broad bean mottle virus			+	
Carlavirus	- pea streak virus	+			
	red clover vein mosaic virus	+			+
Carmovirus	- pea stem necrosis virus				
Comovirus	- broad bean stain virus	+		+	+
	broad bean true mosaic virus	+		+	+
	cowpea severe mosaic virus				+
	glycine mosaic virus			+	
	red clover mottle virus				
Cucumovirus	- cucumber mosaic virus	+		+	+
	peanut stunt virus	+			+
Dianthovirus	- red clover necrotic mosaic virus		F	+	
Faba virus	- broad bean wilt virus	+		+	
Ilarvirus	- tobacco streak virus				+
Luteovirus	- bean (pea) leafroll virus	+			
	beet western yellows virus	+			
	chickpea stunt virus	+			
	soybean dwarf virus	+			
	subterranean clover red leaf virus	+			
Nanovirus	- milk vetch dwarf virus	+			
	subterranean clover stunt virus	+			
Necrovirus	- tobacco necrosis virus				
Nepovirus	- lucerne symptomless virus			+	+
	tomato black ring virus		N		+
Pea enation mosaic virus	- pea enation mosaic virus	+		+	+
Potexvirus	- clover yellow mosaic virus				+
	white clover mosaic virus			+	+
Potyvirus	- bean common mosaic virus	+		+	+
	bean yellow mosaic virus	+		+	+
	beet mosaic virus	+		+	
	clover yellow vein virus	+		+	
	cowpea aphid borne mosaic virus	+		+	+
	lettuce mosaic virus	+		+	+
	passionfruit woodiness virus	+		+	
	pea mosaic virus	+		+	
	pea seed-borne mosaic virus	+		+	+
	peanut mottle virus	+		+	+
	soybean mosaic virus	+		+	+
	turnip mosaic virus	+		+	
	watermelon mosaic virus	+		+	
	zucchini yellow mosaic virus	+		+	
Sobemovirus	- subterranean clover mottle virus			+	
Tobravirus	- pea early browning virus		N		+
Tomato spotted wilt virus	- tomato spotted wilt virus	+		+	
Tymovirus	- desmodium yellow mottle virus				
	kennedya yellow mosaic virus			+	
	plantago mottle virus				
Unassigned viruses	- clover blotch virus				
	plantago mosaic virus				
	pea dwarf mosaic virus				
	pea pimple pod virus	+		+	
	pisum virus				
	soybean mild mosaic virus				

A = aphids, F/N= fungus/nematode, M = mechanical, S = seed, + = transmit, Blank = not reported

Hagedorn, 1984; Maury *et al.*, 1987; Fletcher, 1993; Buchen-Osmond *et al.*, 1988; Boswell and Gibbs, 1983; Phillips and Chandrashekar, 1994; Murphy *et al.*, 1995

different places in the same year but was described by different names; in Oregon as pea fizzle top virus (Hampton, 1972), in Washington State as a seed borne virus of pea (Mink *et al.*, 1969), and in Wisconsin as a new seed borne virus of pea (Stevenson and Hagedorn, 1969). In the Netherlands it was named pea leaf-rolling mosaic virus (Bos, 1970). The virus was then renamed pea leaf rolling mosaic virus to distinguish it from pea leafroll and broad bean leafroll viruses (Musil, 1970). Later, on the basis of its seed transmitted nature observed by different workers, the name pea seed-borne mosaic virus was adopted internationally (Hampton and Mink, 1975).

1.4 Geographical distribution

PSbMV was first reported from Czechoslovakia in 1966 (Musil, 1966) and was soon reported in a number of countries, as shown in Table 1.2. It is believed that Northern India is an original epicentre of PSbMV (Maury and Khetarpal, 1992) from where it spread through infected seed of breeding lines to Western Europe and subsequently from Europe to other parts of the world.

In Australia, PSbMV was first detected in pea seeds imported from Sweden to Tasmania (Munro, 1978). Later, it was observed in field peas in Queensland showing fizzle top symptoms. In South Australia, a virus resembling PSbMV was detected by biological indexing and indirect enzyme-linked immunosorbent assay (ELISA) in 9 out of 83 field samples and in 2 out of 12 certified lines tested (Ligat *et al.*, 1991).

1.5 Agricultural importance

PSbMV is one of the economically important pathogens due to its pathogenicity and high rate of seed transmission in pea and other food legumes such as chickpea (*Cicer arietinum*), fababean (*Vicia faba*), and lentil (*Lens culinaris*) (Chiko and Zimmer, 1978;

Table 1.2 Distribution of PSbMV and year of first report in different countries

Year	Country	Reference
1966	Czechoslovakia	Music, (1966)
1967	Japan	Inn, (1967)
1968	West Germany	Thottappilly & Schmutter, (1968)
1968	Oregon (USA)	Hampton, (1969)
1968	Washington (USA)	Mink <i>et al.</i> , (1969)
1968	Wisconsin (USA)	Stevenson & Hagedorn, (1969)
1970	Netherlands	Bos, (1970)
1975	Poland	Kowalska, (1979)
1976	Canada	Zimmer & Ali-Khan, (1976)
1977	Yugoslavia	Milicic & Grbelja, (1977)
1978	Australia	Munro, (1978)
1978	East Germany	Karl & Schmidt, (1978)
1978	Sweden	Munro, (1978)
1978-79	New Zealand	Fry & Young, (1980)
1980	Switzerland	Pelet, (1980)
1981	United Kingdom	Matthews <i>et al.</i> , (1981)
1984	India	Thakur <i>et al.</i> , (1984)
1988	Morocco	Fortass & Bos, (1991)
1991	Brazil	Dusi <i>et al.</i> , (1993)
1985-92	Algeria, Egypt Ethiopia Jordan Lebanon Libya Sudan Tunisia Turkey	Makkouk <i>et al.</i> , (1993)
1990-92	Belgium	Gofflot and Verhoyen, (1994)
1993	Pakistan	Bashir <i>et al.</i> , (1993)

Ovenden and Ashby, 1981; Maury and Khetarpal, 1992; Makkouk *et al.*, 1993). As the name suggests, the main threat from PSbMV comes from its transmission through the seed (Inouye, 1967), followed by secondary transmission by aphids. As a result, PSbMV has a broad geographical distribution (Table 1.2) and has probably been spread throughout the world by infected seed (Matthews *et al.*, 1981; Alconero and Hoch, 1989). In commercial seed lots of pea, rates of seed transmission up to 90% have been reported from the USA (Mink *et al.*, 1969; Knesek and Mink, 1970) and Switzerland (Pelet, 1980). The seed transmission of the virus has also been reported in pea germplasm collections from Canada (Ali-Khan and Zimmer, 1979), USA (Hampton and Braverman, 1979), New Zealand (Fry and Young, 1980), and the UK (Matthews *et al.*, 1981).

Previous research shows that 2 week old pea seedlings considered healthy after checking by ELISA for PSbMV can give positive ELISA reactions 5 to 6 weeks after emergence (Khetarpal and Maury, 1990). Such infected plants usually remained symptomless and rarely exhibited any symptoms. Similarly, symptomless infection with PSbMV in vegetative tissues of pea was also observed by Ligat and Randles (1993). Such infection might be a limiting factor at quarantine check points where biological indexing and serological methods are currently used as a control procedure for the identification of virus in seed stocks (Khetarpal and Maury, 1987).

1.6 Yield losses

PSbMV has caused serious concern among growers due to its devastating effects on the yield of pea and other legume crops. Yield losses in peas and other legumes infected with PSbMV depend on the cultivar, severity of the strain and developmental stage of the plant at ^{infection} inoculation. When plants were inoculated at the 3 node stage, the yield (seed weight) was reduced 11% in pea cv Trapper, 36% in cv Century (Chiko and

Zimmer, 1978), 33% in cv Pamaro (Ovenden and Ashby, 1981), 16% in cv Belinda, 28% in cv Amino (Khetarpal *et al.*, 1988), and 15% in commercial pea cultivars in France (Maury and Khetarpal, 1992). In the UK, pea plants inoculated with PSbMV in glasshouse trials before flowering reduced pod yield up to 8% and seed yield by up to 36% while plants infected through the seed showed a 63% reduction in pod number and an 84% reduction in seed yield (Anon. 1985).

Recently in West Asia and North Africa, PSbMV infection reduced yield in food legumes such as chickpea by up to 66%, pea up to 49%, lentil up to 45%, faba bean up to 41%, some forage legumes such as *Lathyrus ochrus* up to 36%, *L. sativus* up to 12%, *Vicia narbonensis* up to 32%, and *V. sativa* up to 8% (Makkouk *et al.*, 1993).

1.7 Pathotypes

PSbMV is classified into three pathotypes: P-1 and P-4 from pea and L-1 from lentil germplasm accessions. These can be distinguished only by pathotype specific pea genotypes (Table 1.3) (Alconero *et al.*, 1986). The isolates were obtained from seed-borne infections of single plants in the germplasm accessions PI 179458 (isolate recovered was labelled P-1), PI 471128 (isolate recovered was labelled P-4) and PI 432112 (isolate recovered from lentil was labelled L-1). All the three isolates P-1, P-4, and L-1 had similar morphology, host range, symptomatology, seed transmission capacity and serological reactions to other isolates of PSbMV. They differed however, in their infection of the specific range of pea genotypes (Table 1.3) and are therefore considered as representative of 3 pathotypes of PSbMV.

Resistance to PSbMV was first discovered in USDA *Pisum* plant introduction (PI) accessions (Stevenson and Hagedorn, 1971). Since then four recessive resistance-conferring genes, *sbm-1*, *sbm-2*, *sbm-3* and *sbm-4* have been described in pea. Gene

sbm-1 confers resistance to pathotype P-1 isolates of PSbMV (Gritton and Hagedorn, 1975). Genes *sbm-2* and *sbm-3* confer resistance to L-1 (Provvidenti and Alconero, 1988a) and *sbm-4* confers resistance to P-4 (Provvidenti and Alconero, 1988b). Genes *sbm-1*, *sbm-3* and *sbm-4* are closely linked on chromosome 6 (Gritton and Hagedorn, 1975; Provvidenti and Alconero, 1988a and 1988b) whereas *sbm-2* is located on chromosome 2 (Provvidenti and Alconero, 1988a).

Table 1.3 Reactions of pea to P-1, P-4, and L-1 pathotypes of PSbMV (Alconero, *et al.*, 1986)

Accession	P-1	P-4	L-1
PI 193586	R	R	R
PI 193835	R	R	R
PI 269774	R	S	R
PI 269818	R	S	R
PI 343305	S	S	S
PI 347329	S	R	R
PI 347422	S	R	R
PI 347464	S	R	R

R = resistant, S = susceptible

1.8 Purification

To obtain information on many biochemical and physical properties, to produce antisera for serological studies, and to characterise and identify new viruses, virus particles need to be partially purified.

As mentioned before, PSbMV is transmitted mechanically and thus can be easily

multiplied in pea seedlings. The first purification of virus particles from plants infected with PSbMV was reported in 1967, when some organic solvents for the clarification of extracts (carbon tetrachloride-ether combination, butanol and chloroform) were tested and it was observed that all these solvents except chloroform destroyed the infectivity of PSbMV-infected extracts (Inouye, 1967). Over the last 30 years, many improvements in the purification methodology have resulted in the possibility of several-fold increases in the yields of PSbMV. The most important improvements involve: use of high molarity buffer, chloroform, and low speed centrifugation for initial clarification of pea extracts (Stevenson and Hagedorn, 1973); use of 10 mM sodium diethyldithiocarbamate (NaDIECA) with 10 mM cysteine (for root tissues) or with cysteine containing 10 mM EDTA (for leaf tissues), followed by clarification in 0.5 volume of chloroform (Knesek *et al.*, 1974) and use of extraction buffer containing 100 mM Tris-HCl, pH 9.0 with 2 volumes of 0.2% 2-mercaptoethanol, 1 volume of carbon tetrachloride, and 1 volume of chloroform (Hamilton and Nichols, 1978).

The use of sucrose density gradients has led to the successful purification of several potyviruses with a high degree of purity and without significant aggregation or change in particle size distribution (Moghal and Francki, 1976). Recently, PSbMV isolates have been purified successfully using either isopycnic density gradients of Nycodenz[®] (Ligat *et al.*, 1991) or caesium sulphate (Wang *et al.*, 1992).

1.9 Biological properties

1.9.1 Symptoms of PSbMV infection

PSbMV induces either symptomless infection or slight mosaic of leaves, leaf roll or whole plant necrosis. Variation in symptoms is due to factors such as different strains, and the influence of temperature. In temperate regions, symptoms of PSbMV are not

conspicuous, and this is often a limiting factor for detecting the disease in the field. However, under suitable conditions the symptoms of PSbMV are visible both in the field and glasshouse (Maury and Khetarpal, 1992).

1.9.1.1 On leaf

Under suitable conditions in the glass house or growth chamber, pea plants infected with PSbMV exhibit various symptoms, including downward lateral rolling of leaflets, vein clearing, swelling of veins, mottling, shortening of internodes, tight curling of tendrils, rosetting and stunting (Mink *et al.*, 1969; Stevenson and Hagedorn, 1969; Hampton, 1972). The mosaic symptoms may or may not be clear (Hampton and Mink, 1975). However, plants infected in the field often develop transitory symptoms or fail to express symptoms under certain growing conditions (Hampton *et al.*, 1976). Infected plants may show retarded maturity and produce no or few distorted flowers which give rise to distorted pods containing fewer ^{and} abnormal seeds (Hampton, 1972).

1.9.1.2 On seed

PSbMV may produce symptoms on seed. Pea seeds having a necrotic line pattern on the seed coat were found to be ELISA -positive for PSbMV, suggesting that such seeds had originated from infected plants (Khetarpal and Maury, 1987). However, Maury and Khetarpal (1992) later reported that a necrotic line pattern on the seed coat is not a symptom of seed transmission because only a few such seeds can transmit PSbMV. On the other hand, seed without symptoms may also transmit PSbMV (Stevenson and Hagedorn, 1970).

1.9.1.3 On infected seedlings

The symptoms produced on seedlings raised from infected seeds are highly variable. Infected seeds may give an irregular emergence with either no symptoms or mild symptoms which are expressed late in the growth cycle. The virus may be present without inducing symptoms in 5 to 10% of the plants from infected seed lots (Hampton, 1972). On the other hand, symptoms may be visible within four to five days of germination in the form of severe leaf shortening, rolling and stunting of the seedlings.

1.9.2 Intracellular symptoms

Virus infection may induce inclusion bodies in infected cells. They are characteristic of infection by certain viruses and are visible by electron microscopy. All potyviruses induce the formation of characteristic pinwheel or scroll-shaped cylindrical inclusion bodies in the cytoplasm of infected cells. PSbMV also produces characteristic inclusions, such as pinwheels, rings, circles, tubes or bundles in the cytoplasm of PSbMV-infected plant cells (Purcifull and Edwardson, 1967). Eleven PSbMV isolates were compared by thin-section electron microscopy to observe cytological abnormalities among isolates. Nine isolates uniformly induced formation of typical pinwheel inclusions, 2 of them also induced tonoplast aggregates (TA) in a few cells. Other cytological abnormalities induced by one or more PSbMV isolates included extensive masses of convoluted endoplasmic reticulum, aggregates of virus like particles and crystalline inclusions consisting of ultrastructural hexagons. Root parenchyma cells from PSbMV-infected plants contained few inclusions, but instead contained extensive masses of virus-like particles that occupied most of the cytoplasmic volume in some cells (Hampton *et al.*, 1973).

1.9.3 Host range

PSbMV has a wide host range but *Pisum* spp. are considered to be the natural host of the virus. PSbMV can infect 51 species of plants belonging to 12 dicotyledonous families and most of the non-leguminous hosts are infected without producing symptoms (Aapola *et al.*, 1974; Maury and Khetarpal, 1992). Among the various plant species that can be infected by PSbMV, the diagnostic species are Perfection type pea cultivars, *Vicia faba* var. minor (systemic host), *Chenopodium amaranticolor* Coste & Reyn (necrotic local lesion host) and *C. quinoa* Wild (chlorotic local lesion host) (Hampton and Mink, 1975).

1.9.4 Transmission

Viruses of the family *Potyviridae* are transmitted by many aphid species (genus *Potyvirus*) in a non-persistent, non-circulative manner, by eriophyid mites (genus *Rymovirus*), and by ^afungus (genus *Bymovirus*). However, 7.3% of the potyviruses and 25% of the rymoviruses and bymoviruses are also transmitted through seed (Mink, 1993). PSbMV is transmitted mechanically, through seed (Inouye, 1967; Mink *et al.*, 1969) and by aphids (Kvicala and Musil, 1967; Aapola and Mink, 1973).

1.9.4.1 Seed transmission

As PSbMV is seed-borne in pea (Mink *et al.*, 1969; Alconero and Hoch, 1989), PSbMV-infected seeds rather than alternate host species are considered to be the inoculum reservoir and to play a pivotal role in the dissemination of PSbMV from one locality to another. The virus is transmitted through the seed to the germinating seedlings which act as the primary inoculum for the secondary spread of PSbMV by aphids to nearby healthy peas in the field (Khetarpal and Maury, 1987).

The rate of seed transmission of PSbMV is variable and depends on the interaction between the virus strain, host plant, the temperature of plant growth and stage of plant development at the time of inoculation. For example, PSbMV has been found to be seed transmitted in 148 of 165 pea varieties in the world collection, with seed infection rates of 1-5% in 74 varieties, 5-10% in 39, 10-20% in 24, 30% in 7 and more than 30% in 4 of them. In the USA, seed transmission of PSbMV in some pea introductions was recorded as 100% (Maury and Khetarpal, 1992), in Australia 90% (Ligat *et al.*, 1991) while in Japan the rate of seed transmission of PSbMV varied from 10 to 30% depending on the cultivar (Inouye, 1967).

PSbMV is also seed transmitted in other grain legumes including lentil up to 44% (Hampton and Muehlbauer, 1977), faba bean up to 3% (Musil, 1980) and at a low percentage (<1%) in seeds of several forage legume species including *Lathyrus annuus*, *L. ochrus*, *L. sativus*, *Vicia articulata*, *V. ervilia*, *V. narbonensis*, *V. palaestina*, *V. panonnica*, and *V. sativa* (Hampton and Mink, 1975; Makkouk *et al.*, 1992).

Khetarpal and Maury (1990) reported that seed transmission of PSbMV varied considerably at different temperatures. The seed transmission recorded in pea cv Belinda was 26, 53, 88, 50, and 39% at 21, 25, 28, 30 and 32°C respectively. Seed transmission of PSbMV was also affected when plants were inoculated at different growth stages. The percent seed transmission varied from 7% to 29% in pea cv A.45 when plants were inoculated at 2 and 3 weeks after emergence, respectively (Kraft and Hampton, 1980). Similarly, in France, the seed transmission of PSbMV varied from 0.4 to 12% in nine commercial pea cultivars mechanically inoculated with PSbMV before the flowering stage (Maury and Khetarpal, 1992).

1.9.4.2 Aphid transmission

Aphid transmission of potyviruses depends on the properties of two viral proteins, the non structural helper component protein (Govier and Kassanis, 1974) and capsid protein (Atreya *et al.*, 1990). Aphid transmission plays a very important role in the spread of PSbMV within the field. PSbMV was reported to be transmitted by 22 aphid species in a non-persistent manner with an optimal acquisition period of 10-90 seconds (Gonzalez and Hagedorn, 1970). However, it was also observed that *Myzus persicae* transmitted the virus from infected to healthy plants following acquisition feeding periods of 1-2 min, 10 min, 3 hr and even 2 days. Transmission of the virus was more efficient following acquisition periods of 10 min and 3 hr (Chiko and Zimmer, 1978). In the USA, it was found that *Macrosiphum euphorbiae*, the potato aphid, tended to be a more efficient vector than *Myzus persicae*, the green peach aphid which in turn was more efficient than *Acyrtosiphon pisum*, the pea aphid (Maury and Khetarpal, 1992).

1.10 Serological properties

PSbMV is a moderately good immunogen. Antisera obtained from rabbits given 5 intravenous injections of 0.1 mg virus had a homologous titre of 1/1024 in micro precipitin tests (Knesek *et al.*, 1974) and also reacted specifically with purified PSbMV in gel diffusion tests. No reaction was observed with healthy controls. Hamilton and Nichols (1978) reported that antisera produced from purified virus (1.2-1.8 mg/injection) injected intravenously into rabbit^s, were comparable in dilution end point (1: 2,048) in the micro precipitin test with purified virus preparation (0.1 mg/ml). So far the PSbMV isolates reported (Hampton *et al.*, 1981; Alconero *et al.*, 1986; Ligat *et al.*, 1991; Ali and Randles, 1997) are indistinguishable on the basis of serology.

1.11 Physical properties

Particles of potyviruses are flexuous filaments, 680-900 nm long and 11-13 nm in diameter, with a S_{20w} of 150-160, density in CsCl 1.31 g/cm^3 , $E^{0.1\%}_{1\text{cm}, 260 \text{ nm}} = 2.4-2.7$ (Shukla *et al.*, 1991). PSbMV particles are slightly flexuous filamentous rods measuring $770 \times 12 \text{ nm}$. Preparations usually contain one sedimenting component with a sedimentation coefficient (S_{20w}) of 154 S and a buoyant density in CsCl of 1.329 g/cm^3 and $E^{0.1\%}_{1\text{cm}, 260 \text{ nm}} = 2.5$ (Huttinga, 1975). Some isolates appear highly susceptible to breakage unless fixed with 3.5% glutaraldehyde (Hampton *et al.*, 1974).

In crude sap extracted from roots, PSbMV remained infective for more than 96 hr, whereas undiluted leaf extracts became non-infective within 24 hr at room temperature. However, the virus remained infective for more than 96 h when the leaf extract was diluted 10^{-1} or more. The dilution-end-point was between 10^{-3} and 10^{-4} while the thermal inactivation point was 55°C after 10 min (Hampton and Mink, 1975). On the other hand Chiko and Zimmer (1978) while working on PSbMV in Manitoba, recorded the dilution-end-point as 10^{-4} - 10^{-5} , the thermal inactivation point was 55 - 60°C after 10 min and longevity in vitro of undiluted sap was up to 8 days at 20°C . However, sap diluted to 10^{-2} remained infectious after 15 days.

1.12 Chemical properties

Particles of potyviruses contain a single protein, Mr $30-47 \times 10^3$ in size and a single molecule of linear, positive sense single stranded RNA, about 9.7 kb in size (Mr $3.0-3.5 \times 10^6$) with 5% RNA by weight. The particles have helical symmetry with a pitch of about 3.4 nm and do not show any sub-structure. RNA molecules have a poly (A) tail at the 3' terminus. A genome linked protein (VPg) of about 24 kDa is covalently linked at the 5'-terminus (Murphy *et al.*, 1995).

The genome of PSbMV consists of RNA representing $5.3 \pm 1\%$ of the particle weight with a base ratio of adenine 44.0%, guanine 22.8%, cytidylic acid 17.6% and uridylic acid 15.6% (Knesek *et al.*, 1974). Coat protein is about 94% of the particle weight and the sub unit molecular mass is 34 kDa (Huttinga, 1975). The protein coat contains aspartic acid and glutamic acid in greatest amounts.

1.13 Molecular properties

1.13.1 Genome structure and organisation

The genome of PSbMV like other potyviruses contains a single-stranded positive-sense RNA that is almost 10 kb in length (Johansen *et al.*, 1991). It has a single open reading frame (ORF) of 9816 nucleotides with the potential to encode a large single polypeptide of molecular mass 364 kDa that is post-translationally autocatalytically cleaved into ten mature proteins as shown in Fig. 1.1. The 5' end of the genome is attached to a viral genome linked protein (VPg) followed by a 5' untranslated region (5' UTR). The 3' end of the genome is flanked by a 3' untranslated region (3' UTR) followed by a polyadenylated tail (poly A).

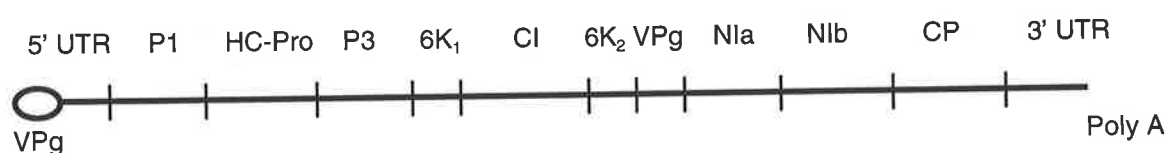


Fig. 1.1 Structure and organisation of PSbMV single stranded RNA genome (Johansen *et al.*, 1991)

1.13.2 Gene products and functions

The functions of different gene products into which potyviral polyprotein is cleaved are described below in order from the 5' end (N-terminus of the polyprotein) of the genome to the 3' end (C-terminus of the polyprotein) (Riechmann *et al.*, 1992):

The first protein gene (P1) encoding a protease;

The helper component protease gene (HC-Pro; P2) is a multifunctional protein. The N-terminal region is required for aphid transmission, efficient genome amplification and symptom expression, the central region is required for systemic movement in plants and the C-terminal domain is a cysteine-type proteinase that autocatalytically cleaves between itself and the P3 protein (Kasschau and Carrington, 1995; Maia *et al.*, 1996). Recently, it has been reported that the 5' UTR, the P1 protease and the N'-terminal two-thirds of the HC-Pro have a major effect on seed transmission (Johansen *et al.*, 1996b);

The third protein gene (P3) -function unknown;

(6K₁) encoding a putative 6K peptide which may be involved in replication;

The cylindrical inclusion gene (CI) encoding an RNA helicase and a protein that forms pinwheel structures in the cytoplasm of infected cells;

(6K₂) encoding a second putative 6K peptide which like 6K₁ may be involved in replication;

The VPg protein has been found in potyviral genomes to be covalently linked by a phosphodiester bond to the 5' terminus of the genome. In the case of PSbMV it has been shown recently by Keller *et al.* (1998) that the VPg determines PSbMV pathotype-

specific virulence in pea.

The nuclear inclusion gene (NIa) encoding a third protease and the VPg;

which cleaves between the NIa?

The nuclear inclusion b gene (NIb) encoding an RNA dependent RNA polymerase;

The coat protein gene (CP) is also a multifunctional protein. It is responsible for the encapsidation of viral RNA, cell-to-cell and systemic movement (Dolja *et al.*, 1994 and 1995). A conserved three amino acid motif, Asp-Ala-Gly (termed the DAG motif), located near the N-terminus of the potyviral coat protein and exposed on the surface of virus particles has been shown to play a key role in aphid transmission (Atreya *et al.*, 1995; Maia *et al.*, 1996).

The 5' untranslated region (UTR) of different potyviruses is rich in adenine residues and has a few guanine residues. The 5' UTR of different potyviruses contains conserved nucleotide blocks which suggests that they could play an important role in the virus life cycle, for example in the processes of encapsidation, translation or replication (Lain *et al.*, 1989; Turpen, 1989; Riechmann *et al.*, 1992). In the case of tobacco etch virus (TEV) the 5' UTR can function as an enhancer of translation (Carrington *et al.*, 1990).

The 3' untranslated region (UTR) is heterogeneous in size and sequence among the different potyviruses. This region contains AU-rich segments and is predicted to be responsible for secondary structures. The 3' UTR is highly diverse among the different potyviruses but among the different strains of the same potyvirus it is more conserved than for example the CP gene (Wetzel *et al.*, 1991; Riechmann *et al.*, 1992).

The poly (A) tail of potyviruses is also heterogeneous in length (Lain *et al.*, 1988; Turpen, 1989). In plum pox virus (PPV), the poly (A) tail is rapidly recovered upon

replication in plants of PPV infectious in vitro transcripts and might have some functional significance (Riechmann *et al.*, 1990 and 1992).

1.13.3 The coat protein gene

The 3'-terminal sequences covering all or part of the coat protein gene together with the untranslated region (UTR) have been determined for several PSbMV isolates (Table 1.4). Timmerman *et al.*, (1990) determined the nucleotide sequence of a 1346 nt cDNA representing the 3'-terminal sequences of PSbMV New Zealand isolate (pathotype P-1), which contained a single long open reading frame (ORF) of 1189 nucleotides ending with a single TAA termination codon. Downstream from the ORF was an untranslatable region of 189 nucleotides followed by eight nucleotides of polyadenylate. The probable location of the PSbMV coat protein codon within the long ORF was determined by comparing the inferred amino acid sequence with other potyviral coat protein sequences and by examining the sequences for a potyviral poly protein cleavage cassette sequence. Direct chemical sequencing of the PSbMV coat protein revealed it to be blocked at its amino terminus. A partial amino acid sequence representing the N-terminus of the protease-resistant core of the coat protein was determined, however. Alignment of the PSbMV coat protein sequence and the sequences of 7 other potyviral coat proteins revealed significant homology, ranging from 53.7% for potato virus Y strain D to 43.2% for tobacco vein mottling virus.

1.14 Sequenced pathotypes

The complete nucleotide sequences of the RNA genome of two PSbMV pathotypes (P-1 DPD1 isolate and P-4 NY isolate) have been determined from cloned cDNA and by direct sequencing of viral RNA (Johansen *et al.*, 1991 and 1996a). As shown in Table 1.4, the genome of P-1 DPD1 is composed of a ss-RNA molecule of 9924 nucleotides

excluding the poly (A) tract while the P-4 NY isolate RNA is 9860 nucleotides long. Other PSbMV isolates which have been partially sequenced at the 3' region (covering CP gene and 3' UTR) are listed in Table 1.4.

1.15 Scope of this thesis

This thesis describes an early season survey of pea viruses in the NWFP of Pakistan and the detection of 12 PSbMV isolates. These Pakistani PSbMV isolates together with a South Australian isolate and a type isolate from the USA were grouped by pathotyping but it is shown that some of them did not fit into the existing pathotype classification system. They were compared by serology, their capsid protein size and RNA, cDNA probes hybridisation, nucleotide sequences of coat protein gene and 3' untranslated region and also by ribonuclease protection assay (RPA). On the basis of RPA, three distinct PSbMV groups were observed having been compared with the 2 previously reported based on pathotypes 1 and 4.

This thesis also reports an improved diagnostic method dot immunobinding assay for PSbMV, followed by the development of dot blot and tissue print hybridisation assays. These diagnostic methods were efficiently used when a study on pathogenesis was done to determine the agronomic effects of the South Australian isolate and the type isolate from the USA in a single commercial pea cultivar.

Table 1.4 Sequences of genomic RNA of a range of PSbMV isolates

Name of isolate	Origin	Pathotype	Sequence length (bp)	Part of genome sequenced	Accession numbers	Source
DPD1 isolate	Denmark	P-1	9924	Full genome	D10930	Johansen <i>et al</i> , 1991
NZ isolate	New Zealand	P-1	1346	Full CP and 3' UTR	D10453	Timmerman <i>et al.</i> , 1990
NY isolate	New York	P-4	9860	Full genome	X89997	Johansen <i>et al</i> , 1996a
CAN isolate	Canada	-	1273	Partial CP and 3' UTR	Z48508	Gen Bank
GER isolate	Germany	-	1273	Partial CP and 3' UTR	Z48509	Gen Bank
U16215	Unknown	-	879	Partial CP	U16215	Gen Bank

- Not classified into pathotypes

Chapter 2

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

All chemicals used in this thesis were analytical reagent or molecular biology grade. Solutions were prepared with double distilled water, filtered through a 0.22 μm sterile filter (Schleicher and Schuell) or autoclaved where appropriate.

2.1.1 Seed sources

Names and seed sources of indicator plant species, pathotype specific or host specific pea genotypes, and Pakistani pea varieties used in this work are listed in Table 2.1.

2.1.2 Virus sources

Cultures of PSbMV isolate S6 (obtained from field peas in South Australia) and a type isolate US (originally obtained from R. O. Hampton, Oregon University, U. S. A) (Ligat *et al.*, 1991), alfalfa mosaic alfamovirus (AMV), bean yellow mosaic potyvirus (BYMV), and cucumber mosaic cucumovirus (CMV) were obtained from the Waite Campus collection. Bean leafroll luteovirus (BLRV) was kindly supplied by Dr. M. Schwinghamer, Department of Agriculture, NSW. Twelve PSbMV isolates (PK1-PK12) (see Chapter 6, Table 6.3) were obtained from Pakistan (Ali and Randles, 1997).

2.1.3 Antisera

All polyclonal antisera used for the PSbMV isolates (S6, US) and other viruses (see Table 6.1) were obtained from the Waite Campus collection except for BLRV antiserum which was from the collection of Dr. K. Izadpanah, Shiraz University, Iran. Goat anti-rabbit-IgG-alkaline phosphatase conjugate (Sigma) and anti-digoxigenin-alkaline phosphatase (Boehringer Mannheim) were purchased commercially.

Table 2.1 List of indicator hosts, pea genotypes, pea varieties and their sources

Name	Source
<u>Indicator hosts</u>	
<i>Chenopodium amaranticolor</i> Coste & Reyn	Waite Campus Collection
<i>C. quinoa</i> L.	
<u>Pathotype specific pea genotypes</u>	
193586	Dr. J. D. Fletcher, Crop and Food Research,
193835	New Zealand
269774	
347329	
347422	
<u>Host specific pea genotypes</u>	
272146	A. D. McIntyre, Australian Temperate Fields Crops
269804	Collection, Victorian Institute for Dry Land Agriculture
272171	
269777	
<u>Pea varieties</u>	
Arkeei Ap-1	Seed market in Haripur, Hazara, NWFP Pakistan
Hazara Local	
Climax	Agric. Res. Station, Swat, NWFP Pakistan
Dasana	
Mashang	
Nauvana	
Rondo	
VIP	
Peshawar Local-I	Farmers
Peshawar Local-II	
Swabi Local	
PF-400	Seed market in Haripur, Hazara, NWFP Pakistan
Dundale	Waite Campus Collection

2.1.4 Synthetic oligodeoxyribonucleotides

Synthetic oligodeoxyribonucleotides (primers) used for reverse transcription (RT), polymerase chain reaction (PCR) and sequencing were selected from known sequences and designed by the PCR Amplify[®] program. Stability and primability of the primers were checked by the Oligo[™] program. The selected primers were prepared either on an Applied Biosystem (USA) Model 380B DNA synthesiser, Department of Plant Science, University of Adelaide or ordered from GENSET KK, Tokyo, Japan. All primers were unphosphorylated and purified from premature termination products by Mono Q column, high pressure liquid chromatography (HPLC) by the manufacturer and resuspended in sterile water. Primers for sequencing (T7 and SP6 promoter) and random hexamers for synthesis of radioactive and non-radioactive cDNA probes were purchased from Promega (USA). The nucleotide sequences of each primer used in this study are listed in Table 2.2.

2.1.5 Bacterial plasmid system for cloning

Escherichia coli strain DH5 α was used for all routine cloning work in this thesis. Routine cloning was carried out using the pGEM[®]-T vector system I (Promega), a plasmid carrying ampicillin resistance and with promoter sequences for T7 and SP6 RNA polymerases flanking the polylinker.

2.1.6 Biochemicals, gels, media, stains and solvents

The enzymes, nucleotides, molecular weight markers, biochemicals and other chemicals used in this study are listed in Appendix A. Composition and preparation of agarose gels, polyacrylamide gels, bacterial growth media, staining solutions and solvents are listed in Appendix B.

Tabel 2.2 List of primers used in this thesis

Primers	Size (nt)	Primer sequences from 5' to 3'
<u>Labelling-primer</u>		
Random hexamer	6	(NNN NNN)
<u>RT-primers</u>		
AK1	31	TTTTCTCCAGAAAGCCCTACTGCCACCAAAC
AK3	25	TTCTGAGTGCCTCCAACCTATACA
<u>PCR-primers</u>		
AK2	31	CCGGTACCAGAAAGATGTTTGGTTTATGTCA
AK4	27	ACCACGGAATATAAAGATGAAAGCTGA
P4F1	25	TAGAAGCAGGGAATTCTCTCACAGA
P4F2	27	TACACAGAAATCATTTACACTCCAATA
<u>Sequencing-primers</u>		
T7 Promotor	20	TAATACGACTCACTATAGGG
S6 Promotor	19	GATTTAGGTGACACTATAG
AK5	24	TACCTGGGTATGTACACTTCTGTT
AK6	24	AGCTCAGGAAGGAAAAGCACCATA
AK7	25	ATTCAGTGACGTAGCGGAGGCGTA
AK8	25	TTCCGAATGTGCTGCAAAAGTTCAT
AK9	22	AAATGCTGCAGACTAATCGCCA
AK10	23	AATTGCAACAATTCGTTCTTCTT
AK11	23	AAGTTTTGGTATCAGAATTCCAT
AK12	21	ATGAAGGTGGCGATGGATCAA
AK13	24	AACACAAAGTGAACCTTGAGAAATA
AK14	23	ATAAATGGGATGTGGACAATGAT
AK15	21	TAATAAGGTAGCATGGCACAA
AK16	23	TGTGTTTCCAATCACCAAATGAT
AK17	22	TGGCGTTTTTCAGACACTTAAAT
AK18	21	TTAATTTGAGCTTTGGCAGAA

2.2 METHODS

All methods were carried out as described in Sambrook *et al.* (1989) unless otherwise stated.

2.2.1 Plant culture

UC (University of California) potting mix consisting of four parts washed river sand and three parts (dry volume) pea moss and nutrient supplements was prepared by Plant Growth Services, University of Adelaide, Waite Campus, and steam-sterilised at 100°C for 30 min before use. Plants were grown in 150 mm diameter plastic pots in an insect free air conditioned glasshouse at 18-25°C or in a growth room (20°C) under continuous light at 2,000 lx.

2.2.2 Virus inoculation

Inoculum was made with virus infected leaf tissue or seed crushed in 50 mM sodium borate buffer pH 8.1 at the rate of 2 ml per gram (w/v) of infected tissue (Ligat *et al.* 1991). Inoculum was rubbed gently onto the young Carborundum (500-mesh) dusted leaves of test plants.

2.2.3 Quantification of virus and nucleic acids

Virus concentration was calculated assuming that $A^{0.1\%}_{260\text{ nm}} \sim 3.3$. DNA or RNA purity was estimated from the $A_{260/280\text{ nm}}$ ratio.

2.2.4 Electron microscopy

Preparations of virus were examined by electron microscopy to assess their purity, degree of fragmentation and aggregation. Grids (400 mesh) with a plastic film or carbon coat were first made hydrophilic with a spark tester *in vacuo* (Edwards, England) and then placed on the droplet of purified virus preparation for 30-60 sec. The grids were drained with filter paper, negatively stained on a droplet of 2% K-PTA (pH 6.8) solution for 10 sec and examined with a Phillips EM 400 electron microscope.

2.2.5 Western blot analysis

Proteins were electrophoresed in a 10% SDS-PAGE resolving gel (section 2.2.11.3) using a mini vertical gel electrophoresis unit (Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane (0.45 μ m pore diameter, BA 85, Schleicher and Schuell, Dassel) with Towbin transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, pH 8.3; Towbin *et al.*, 1979) using a semi-dry transfer cell (Bio-Rad Transblot® SD). The membrane was air dried and developed by the reagents as for dot-immunobinding assay (DIBA) (section 3.2.5.4).

2.2.6 Avoiding ribonuclease (RNase) contamination

During RNA preparation, the following measures were adopted to avoid RNase contamination (Sambrook *et al.*, 1989). Glassware was baked at 180°C for 10 hr and plasticware was rinsed with 2 M KOH. Electrophoresis tanks used for RNA electrophoresis were rinsed thoroughly with diethyl pyrocarbonate (DEPC) treated water (0.1% DEPC in water) before use. All the solutions were prepared either in DEPC treated water using RNase free glassware or in sterile water and then treated with 0.1% DEPC for at least 12 hr at room temperature and then autoclaved.

2.2.7 Phenol:chloroform extraction and ethanol precipitation

DNA or RNA solutions were extracted once with an equal volume of phenol:chloroform (1:1) (Appendix B) and once with an equal volume of chloroform:isoamylalcohol (24:1) (Appendix B). The nucleic acids were precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 3 volumes of chilled ethanol followed by incubation at -70°C for 2 hr or -20°C overnight. The pellet of nucleic acid was recovered by centrifugation at $14,000 \times g$ for 15 min, washed in 70% ethanol, dried in a vacuum and resuspended in sterile water or TE buffer (Table 2.3).

2.2.8 Isolation of RNA

2.2.8.1 From virus particles

Purified virus particles were treated with RQ1 DNase (12 units DNase/mg virus) for 1 hr at 37°C, followed by RNase (100 µg/ml) for 30 min at 37°C. The mixture was then digested with proteinase K (200 µg/ml) for 4 hr at 37°C. The reaction mix was extracted with phenol:chloroform and precipitated as described above (section 2.2.7). The final RNA pellet was resuspended in DEPC treated water. The concentration of RNA was calculated spectrophotometrically (section 2.2.3).

2.2.8.2 From plant tissue

Leaf: For total nucleic acid extraction, infected or healthy leaf tissue was ground to a powder in liquid nitrogen and then mixed with one volume of TES buffer (Table 2.3). The mixture was incubated at 37°C for 45 min, extracted with phenol:chloroform and precipitated as above (section 2.2.7). The final pellet of total nucleic acid was resuspended in DEPC treated water.

Table 2.3 Compositions of some common solutions/buffer used in this thesis

Solutions/buffers	Compositions
<u>DIBA buffers</u>	
Phosphate buffered saline (PBS), pH 7.4	137 mM NaCl, 3 mM KCl, 8 mM Na ₂ HPO ₄ , 1 mM KH ₂ PO ₄
Alkaline phosphatase buffer (AP 7.5)	100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl ₂ , 0.05 % Triton X-100
Alkaline phosphatase buffer (AP 9.5)	100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl ₂
<u>Electrophoresis buffers</u>	
Agarose gel sample buffer (6 x)	30 % glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol
Formaldehyde gel sample buffer (FGSB)	50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol
Formaldehyde gel running buffer (1x FGRB)	20 mM MOPS, pH 7.0, 8 mM sodium acetate, 1 mM EDTA
SDS-gel sample buffer	50 mM Tris-HCl pH 6.8, 100 mM mercaptoethanol, 10% glycerol, 2% SDS and 0.01% bromophenol blue
SDS-gel running buffer	25 mM Tris-HCl, 250 mM glycine pH 8.3, 0.01% SDS
Urea PAGE sample buffer	80% formamide, 2 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol
Tris-acetate (1 x TAE)	40 mM Tris-acetate, pH 8.0, 1 mM EDTA
Tris-borate (1 x TBE)	90 mM Tris-borate, pH 7.6, 1 mM EDTA
<u>Enzyme digestion buffers</u>	
RQ1 DNase buffer	40 mM Tris-HCl, 10 mM NaCl, 6 mM MgCl ₂ and 1 mM CaCl ₂
Proteinase K buffer	10 mM Tris-HCl pH 8.1, 5 mM EDTA and 0.5% SDS
RNase digestion buffer	10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA
<u>Hybridisation buffers</u>	
Denhardtts solution (1X)	0.02% BSA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone
Pre-hybridisation buffer	6x SSPE, 5x Denhardtts solution, 0.5% SDS, 100 ug/ml boiled salmon sperm DNA, and 50% formamide
RPA hybridisation buffer	80% formamide, 40 mM PIPES pH, 6.4, 400 mM NaCl, and 1 mM EDTA
SSPE (1x)	150 mM NaCl, 10 mM Na ₂ H ₂ PO ₄ , 1 mM EDTA
SSC (1x)	150 mM NaCl, 15 mM sodium citrate, pH 7.0
<u>Other buffers</u>	
RNA PAGE elution buffer	500 mM ammonium acetate, 1 mM EDTA, and 0.1% SDS
TES buffer	100 mM Tris-HCl pH 8.3, 2 mM EDTA, and 2% SDS
TE buffer	10 mM Tris-HCl pH 7.6 or 8.0 and 1 mM EDTA

Seed: Seeds harvested from infected or healthy plants were soaked overnight in sterile water. Individual seeds were crushed with 5 volumes (w/v) of TES buffer. The mixture was incubated at 37°C for 45 min. Total nucleic acid was extracted from each seed individually as described above.

2.2.9 Preparation of cDNA probes

2.2.9.1 ³²P-labelled cDNA probes

The method of Randles and Rohde (1990) was modified to prepare randomly primed cDNA (³²P-labelled) probes. Purified RNA (1 µg) was added to the 30 µl reaction mix containing random hexanucleotide primers (25 ng/µl) (Table 2.2), 10 mM MgCl₂, 50 mM Tris-HCl, pH 8.3, 50 µM each dATP, dGTP and dTTP, 30 µCi [³²P] dCTP (3000 Ci/ mmol), 7 mM-2-mercaptoethanol, and 200 units of MMuLV reverse transcriptase. The mixture was incubated for 5 hr at 37°C and then chilled on ice. The transcribed probe was purified as follows.

2.2.9.1.a Purification

The probe was fractionated by Sephadex[®] G-50 chromatography (Appendix B). The fractions of the labelled cDNA probe were collected from the column and stored at -20°C.

2.2.9.1.b Measurement of radioactivity

The radioactivity of the ³²P-labelled cDNA probes (Chapter 4) or ³³P-labelled cRNA probes (Chapter 9) was determined by liquid scintillation counting (LS 5000 TD, Beckman[®]).

2.2.9.2 DIG-labelled cDNA probes

The randomly primed cDNA probes (DIG-labelled) were prepared using a DIG DNA labelling kit (Boehringer Mannheim). Purified RNA (1 µg) was incubated in the presence of random hexanucleotides (6.25 units/ml), 10 mM MgCl₂, 50 mM-Tris-HCl, pH 7.2, 100 µM dithiothreitol (DTT), 200 µg/ml BSA, 100 µM each of dATP, dCTP, dGTP, 65 µM dTTP, 35 µM DIG-11-dUTP and 400 units of MMuLV reverse transcriptase. The 20 µl reaction mix was incubated at 37°C for 20 hr. The reaction was stopped by the addition of 2 µl of 200 mM EDTA. The transcribed DIG-labelled cDNA was precipitated with 0.1 volume of 4 M LiCl and 2.5 volume of chilled ethanol at -20°C for 1 hr or overnight. The final DIG-labelled cDNA pellet was resuspended in 50 µl TE (pH 8.0) buffer and stored at -20°C.

2.2.10 Nucleic acid hybridisation

2.2.10.1 Dot blot hybridisation

One µl dots from purified virus, RNA, sap or total nucleic acid extracted from virus infected and healthy pea plants or seed were applied to a nylon membrane (Zeta-probe®). Nucleic acid was fixed under UV light (125 mJoule) (GS gene linker™ UV chamber, BioRad).

2.2.10.2 Tissue print hybridisation

Leaflet samples from infected or healthy pea plants were squashed by firmly pressing onto the surface of a nylon membrane (Navot *et al.*, 1989). Seeds from infected and healthy pea plants were first soaked overnight in sterilised water, dissected and the embryos separated. Embryos and cotyledons were squashed directly onto a nylon

membrane as above. The membrane was presoaked with 0.5% SDS and 100 µg/ml proteinase K (to prevent degradation of RNA by plant ribonucleases). Nucleic acid squashes were fixed by UV light (section 2.2.10.1).

2.2.10.3 Northern blot hybridisation

Formaldehyde denaturing 1% agarose gel electrophoresis (section 2.2.11.1) was used to fractionate viral RNA. The gel was washed with sterile distilled water to remove excess ethidium bromide and 25 mM NaOH was added. The RNA transfer sandwich consisted of the following layers (from bottom to top): an absorbing pad (sponge), 3 sheets of Whatman 3 MM filter paper prewetted with 25 mM NaOH, washed gel, and the nylon membrane cut to the gel size. A sheet of plastic cut to the gel size was layered on the top of the nylon membrane to mask the gel. Three sheets of pre-wet Whatman 3 MM filter paper were layered and the bubbles were removed with a glass rod. A 3-4 cm thick layer of dry paper towel was placed on the top and a weight of 500-1,000 g was applied. The transfer was completed in 4-12 hr and the sandwich was disassembled. The nylon membrane was checked under UV light to determine whether the RNA had been transferred. RNA was then fixed with the UV light (section 2.2.10.1). Membranes were stored at 4°C.

2.2.10.4 Southern blot hybridisation

PCR products or DNA restriction fragments were separated by non-denaturing 1% agarose minigels (section 2.2.11.1). Following electrophoresis, the gel was washed in sterile distilled water and denatured in 1.5 M NaCl, 25 mM NaOH for 10 min. DNA was transferred from the gels to nylon membrane as above (section 2.2.10.3). The membrane was checked under UV light to monitor the transfer of DNA from gel to nylon membrane. DNA was fixed as above and stored at 4°C.

2.2.10.5 Prehybridisation and hybridisation assay

Membranes with dot blots were washed in $2 \times$ SSPE (Table 2.3) containing 0.1% SDS for 1 hr before prehybridisation. Prehybridisation and hybridisation were carried out according to Sambrook *et al.* (1989). The membranes were prehybridised at 42°C for 8 hr in 10 ml of pre-hybridisation buffer (Table 2.3). Hybridisation was done in the same buffer but with either ^{32}P -labelled or DIG-labelled cDNA probe. Hybridisation was done for 20 hr at 42°C. The membrane was washed twice in $2 \times$ SSPE containing 0.1% SDS at 55°C for 30 min. Higher stringency washes were done in $1 \times$ SSPE containing 0.1% SDS for 30 min at 65°C.

2.2.10.6 Detection

The membranes probed with ^{32}P -labelled cDNA probe were autoradiographed at -70°C. Membranes probed with DIG-labelled cDNA probe were treated as follows. After hybridisation and post-hybridisation washes, membranes were equilibrated in Buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 1 min. Membranes were soaked in Buffer 2 (1% blocking reagents in Buffer 1, DIG DNA labelling kit, Boehringer Mannheim) for 30 min followed by incubation in Buffer 2 containing anti-DIG-alkaline phosphatase (1: 5000) for 30 min. Membranes were washed twice in Buffer 1 for 15 min each followed by equilibration in Buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl_2) for 2 min. Membranes were incubated in 10 ml Buffer 3 containing 45 μl nitroblue tetrazolium (NBT) and 35 μl 5-bromo-4-chloro-3-indolyl phosphate (X-Phosphate) for 30-60 min to develop the dark bluish colour. The reaction was stopped by adding Buffer 1 for 5 min.

2.2.11 Gel electrophoresis

2.2.11.1 Agarose gel electrophoresis

Non-denaturing agarose mini gels were used to fractionate DNA fragments or total nucleic acid extracts from plants. Minigels containing 0.8-1% of agarose (Type II: medium EEO, Sigma) were prepared (Appendix B) in $1 \times$ TAE (Table 2.3). The agarose was dissolved by heating in an autoclave, cooled to 40°C and ethidium bromide (0.5 µg/ml) was added before pouring into the gel former. Appropriate gel combs were used for making wells in the gel. DNA samples were loaded onto a submerged gel in the presence of $1 \times$ agarose gel sample buffer (Table 2.3). The gel was electrophoresed at approximately 8 V/cm until the bromophenol blue had migrated 3/4 of the length of the gel. DNA was visualised under UV light and photographed.

Formaldehyde denaturing agarose gels were used for the analysis of ss-RNA as described by Sambrook *et al.* (1989). Agarose (1%) was dissolved in DEPC-treated water, cooled to 60°C and mixed with pre-warmed formaldehyde gel running buffer (FGRB) (Table 2.3). The gel was pre-run at 7 V/cm for 30 min. The 20 µl mixture (containing 4.5 µl RNA sample, 2.0 µl of $5 \times$ FGRB, 3.5 µl (@ 2.2 M) formaldehyde and 10 µl deionised formamide) was incubated at 65°C for 15 min, and cooled on ice. The mixture was centrifuged briefly, 2 µl of formaldehyde gel sample buffer (FGSB) (Table 2.3) was added and subsequently loaded on a pre-run gel with an appropriate RNA molecular weight marker. The gel was run in $1 \times$ FGRB buffer at 7 V/cm for 3-4 hr. The gel was rinsed with water for 10 min to remove formaldehyde and stained with ethidium bromide containing 0.1 M ammonium acetate for 1-2 hr. RNA was visualised under UV light and photographed.

2.2.11.2 Polyacrylamide gel electrophoresis (PAGE)

Urea denaturing polyacrylamide gels were used for the analysis of transcribed complementary RNA (cRNA) probes and ribonuclease protection assays (RPA) (Chapter 9). The PAGE gels (5%, thickness) (Appendix B) were prepared, allowed to polymerise for 60 min and then pre-electrophoresed for 30 min at 200 V until the gel temperature was stabilised at 50°C. Temperature was maintained at 50°C by using a water reservoir in contact with the gel plates (Bio-Rad). Samples (10-20 µl) were mixed with an equal volume of 2 × urea PAGE sample buffer (Table 2.3), denatured by heating for 3-4 min at 95°C and loaded immediately. Gels were electrophoresed at 50°C, in 1 × TBE (Table 2.3) for 3 hr at 200 V. After electrophoresis, gels were dried in a gel dryer (Model 583, BioRad) for 45 min at 80°C and autoradiographed at -70°C.

2.2.11.3 Sodium dodecyl sulphate-PAGE (SDS-PAGE)

Discontinuous SDS-PAGE (Laemmli, 1970; Sambrook *et al.* 1989) was used for the analysis of protein. Gels were prepared (Appendix B) with a 4% polyacrylamide stacking layer (buffered in 50 mM Tris-HCl pH 6.8 and 0.1% SDS) and a 10% polyacrylamide resolving layer (buffered in 150 mM Tris-HCl pH 8.8 and 0.1% SDS). Protein samples were heated at 95°C for 5 min in SDS-gel sample buffer (Table 2.3) and electrophoresed in SDS-gel running buffer (Table 2.3) at 10 V/cm until the bromophenol blue dye had reached the bottom of the resolving gel.

2.2.11.3.a Staining

SDS-PAGE gels containing protein were stained for 1-4 hr in five volumes of the 0.25% Coomassie brilliant staining solution (Appendix B). The gel was destained with several changes of destaining solution (Appendix B) overnight or until the bands were

clearly visible. The gel was photographed and stored in water containing 20% (v/v) glycerol in a sealed plastic bag (Sambrook *et al.* , 1989).

2.2.12 Isolation of DNA from agarose gels

DNA bands of interest were cut from agarose gels with a sterile razor blade under UV light. DNA was eluted from the gel slices using a Jetsorb gel extraction kit (Appendix A). Each excised gel slice was placed in a 1.5 ml tube and weighed. For each 100 mg gel slice 300 μ l buffer A₁ and 10 μ l Jetsorb suspension was added and mixed by vortexing. The mix was incubated at 50°C for 15 min to melt the gel completely. DNA was pelleted together with Jetsorb suspension by centrifuging at $10,000 \times g$ for 30 sec. The pellet was resuspended once with 300 μ l (1:3 w/v) of buffer A₁ and centrifuged as before. The supernatant was removed and the pellet was washed twice with 300 μ l (1:3 w/v) of buffer A₂. After the second wash, the Jetsorb pellet was dried by air or under vacuum, resuspended in TE buffer or water and incubated for 5 min at 50°C. After centrifugation as before, the supernatant containing DNA was transferred into a new tube and stored at -20°C.

2.2.13 Polymerase chain reaction (PCR)

2.2.13.1 Reverse transcription (RT)

Purified viral RNA or total nucleic acids (1-2 μ g) from virus infected plants were mixed with the appropriate reverse primer, kept at 65°C for 10 min, cooled on ice then added to a reaction mix containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.6 mM of each dNTP, 40 units of RNAsin[®], and 200 units of MMuLV reverse transcriptase in a final volume of 25 μ l. The reaction mix was incubated at 37°C for 1 hr.

2.2.13.2 Standard PCR assay

PCR was done in a volume of 15 μ l which contained 1-5 μ l incubated RT reaction mix (section 2.2.13.1), 200 nmol each of a set of primers, 200 μ M of each dNTP, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, and 0.3 units of *AmpliTaq* DNA polymerase. PCR was done with a single denaturing step, 30-35 cycles of denaturing, annealing, extension and a final extension step in a Perkin-Elmer thermal cycler (GeneAmp PCR system 2400). After the PCR, products were analysed on a 1% agarose gel (section 2.2.11.1).

2.2.14 Cloning of PCR products

2.2.14.1 Ligation of cDNA into plasmid vector

Approximately 10-25 ng of the plasmid DNA (pGEM[®]-T vector) was ligated with the PCR amplified DNA at a molar ratio of 1:3 (vector : insert) in the presence of ligation buffer (30 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT and 1 mM ATP) and T4 DNA ligase (3 Weiss units/ μ l) in a reaction volume of 10 μ l. The reaction was incubated overnight at 16°C. An aliquot of 1-5 μ l of the ligation mix was used for transformation.

2.2.14.2 Preparation of competent bacterial cells

To prepare competent bacterial cells, a culture of *E. coli* DH5 α stored in glycerol at -70°C was plated on an LB agar (LBA) (Appendix B) plate and incubated at 37°C overnight. A single colony was selected and inoculated into 3-5 ml of LB medium containing ampicillin (1 μ g/ μ l). The culture was incubated at 37°C overnight with continuous shaking. The overnight culture was diluted 100 times with SOB (Appendix

B) medium (0.5 ml culture in 50 ml SOB) and incubated at 37°C until the OD 600 of the SOB medium was 0.5-0.9.

The bacterial cells were placed on ice for 15 min and transferred to pre-cooled 50 ml centrifuge tubes and centrifuged in a SS 34 rotor at $1,000 \times g$ at 4°C for 15 min. The supernatant was removed carefully and the cells were drained well. The cells were gently resuspended in 7.5 ml of RF₁ buffer [0.1 M RbCl, 50 mM MnCl₂, 30 mM KAc, 10 mM CaCl₂ and 15% (v/v) of glycerol, pH 5.8 with (0.2 M CH₃.COOH)] and left on ice for 30 min. The cells were centrifuged as before and the supernatant was removed. The cells were drained well and gently resuspended in 4 ml of RF₂ buffer [10 M RbCl, 75 mM CaCl₂, 10 mM MOPS and 15% (v/v) glycerol, pH 6.8 (with NaOH)]. The cells were kept on ice for 30 min and dispensed as 100-200 µl aliquots into sterile 1.5 ml tubes. The competent cells were frozen in liquid nitrogen and stored at -70°C.

2.2.14.3 Transformation of competent bacterial cells with recombinant plasmids

2.2.14.3.a Electroporation

Transformation of bacterial cells with plasmid was performed by electroporation using a Gene-Pulser (Bio-Rad). The competent bacterial cells were thawed on ice and centrifuged briefly if necessary. To 45 µl of competent cells was added 1 µl of ligation mix (containing 5 ng of plasmid DNA or 60 ng of ligated DNA). The mixture was transferred to an ice-cold, disposable electroporation cuvette (0.1 cm electrode gap, Bio-Rad) and subjected to electroporation at 1.8 kV, 125 µFD and 200 Ω. Following electroporation, the cells were mixed immediately with 1 ml LB medium and incubated at 37°C for 1 hr. Aliquots of 100-200 µl of the cell suspension were cultured on LBA plates containing ampicillin, IPTG and X-gal (Appendix B) at 37°C overnight.

2.2.14.3.b Heat shock

Transformation by heat shock was done according to Sambrook *et al.* (1989) with minor modifications. Aliquots of 100 μ l of the competent cells were thawed on ice and transferred to pre-cooled tubes and incubated on ice for 10 min. A ligation mix of 1-5 μ l was added, mixed gently by pipetting and kept on ice for 30 min. Cells were heat shocked at 42°C for 45 sec in a water bath and cooled on ice for 2 min. Pre-warmed SOC (Appendix B) medium (0.8 ml) was added and the mixture was incubated at 37°C for 1 hr on a shaker. Aliquots of 100-200 μ l of the cell suspension were cultured as above (section 2.2.14.3.a).

2.2.14.4 Selection of clones:

As mentioned by Sambrook *et al.* (1989) white bacterial colonies were selected for minipreparations of plasmid DNA. Restriction enzyme digestion analysis was used to further confirm the presence or the absence of the inserted DNA fragment.

2.2.15 Preparation of cloned plasmid DNA

2.2.15.1 Minipreparation of plasmid DNA

Plasmid DNA was isolated by a modification of the alkaline lysis procedure of Sambrook *et al.* (1989). A single white colony was picked with a sterile toothpick and transferred into 3 ml of LB containing ampicillin (150 μ g/ml). The culture was grown overnight at 37°C with vigorous shaking. Bacterial culture of 1.5 ml was taken in a tube and centrifuged at $14,000 \times g$ for 1 min at room temperature. The supernatant was removed and the pelleted bacterial cells were resuspended by vortexing in 100 μ l of ice-cold GTE solution (50 mM glucose, 25 mM Tris-HCl pH, 8.0 and 10 mM EDTA).

Lysozyme 10 μ l (10 mg/ml in 10 mM Tris-HCl pH, 8.0) was added before placing on ice for 5 min. 200 μ l of 0.2 M NaOH, 1% SDS (freshly prepared) was added and the solution was mixed by inversion. After placing on ice for 5 min, 160 μ l of 3 M Na acetate, pH 5.2 was added. The contents of the tubes were mixed by inversion and again incubated on ice for 5 min. The mixture was centrifuged at $14,000 \times g$ for 10 min at room temperature to pellet the cell debris. Plasmid DNA was precipitated from the supernatant by adding 2 volumes of ice-cold ethanol (95%) and pelleted by centrifugation for 15 min as before. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 20 μ l of TE (pH 8.0) or water. Plasmid DNA was stored at -20°C .

2.2.15.2 Midipreparation of plasmid DNA

Bacterial cells were pelleted from 50 ml of overnight culture by centrifugation in a Sorvall GSA rotor at $10,000 \times g$ at 4°C for 10 min. The supernatant was discarded and the bacterial pellet was resuspended in 3 ml of GTE solution by vigorous vortexing. The suspension was mixed with 500 μ l of lysozyme (10 mg/ml in 10 mM Tris-HCl pH, 8.0) and with 6 ml of freshly prepared 0.2 M NaOH, 1% SDS. The mixture was gently inverted several times and incubated on ice for 5 min. The mixture was neutralised by the addition of 4.5 ml of ice cold 3 M Na acetate (pH, 5.2). The contents of the tube were gently mixed by inversion and then incubated on ice for 10 min. Cellular debris and chromosomal DNA was sedimented by centrifugation at $10,000 \times g$ at 4°C for 25 min and the supernatant was extracted once with an equal volume of phenol:chloroform (1:1 v:v). The nucleic acid was recovered from the aqueous phase by isopropanol (0.6 volume) precipitation and centrifugation at $10,000 \times g$ at 4°C for 20 min. The pellet was washed with 1 ml ice-cold 70% ethanol and dried under vacuum. DNA was resuspended in 2.5 ml TE buffer or water. The plasmid DNA was further treated with DNase-free RNase (@20 μ g/ml) to remove bacterial RNA and protein. The solution

was extracted twice with phenol:chloroform and once with chloroform. Plasmid DNA was precipitated from the solution by the addition of an equal volume of 13% PEG (MW 8,000) and 1.6 M NaCl, followed by incubation at room temperature for 5 min. DNA was recovered by centrifugation at $14,000 \times g$ at 4°C for 15 min. DNA was precipitated with 100% ethanol, washed as above and the pellet was resuspended in 100 μl TE or sterile water.

2.2.16 Analysis of insert in recombinant plasmid DNA

DNA samples were digested with restriction endonucleases in buffer systems recommended or supplied by the manufacturer and incubated at the appropriate temperatures for the required period of time. In analytical digests, 1-5 μg of DNA was incubated with 2-10 units each of the appropriate restriction enzymes in a reaction volume of 50 μl for a minimum of 3 hr. A small quantity (1-5 μl) of the digested reaction mix was analysed on a 1% agarose gel.

2.2.17 DNA sequencing

DNA sequencing was done using automated DyeDeoxyTM Terminator Cycle Sequencing procedure (Applied Biosystems Model 373 DNA sequencing system, with *AmpliTaq* DNA polymerase) at the Department of Haematology, Flinders Medical Centre, Adelaide, Australia. Plasmid DNA (5 μl) purified by the minipreparation (section 2.2.15.1) or midipreparation procedure (section 2.2.15.2) was used in each sequencing reaction.

Chapter 3

PURIFICATION AND SEROLOGICAL DETECTION OF PSbMV

3.1 INTRODUCTION

Partially or highly purified virus preparations are essential to obtain information on chemical, physical, biochemical, and biological properties and to produce antisera for serological studies. In addition, virus purification gives information on the purity of virus preparation with respect to (i) presence of virus like particles with different sedimentation properties; (ii) presence of a contaminating virus; (iii) presence of contaminating host macromolecules and (iv) state of aggregation of the virus (Matthews, 1991). Purified preparations of most plant viruses are effective antigens when injected into suitable animals and stimulate the production of antibodies that can be used in various serological assays. Serological assays can be used both to identify viruses and to study the relationship between virus isolates or strains (Walkey, 1985).

Antisera against PSbMV were first developed by Musil (1970) and by Stevenson and Hagedorn (1973) and have been used in many serological assays (Khetarpal and Maury, 1987). PSbMV is a moderately good immunogen (section 1.10).

PSbMV indexing is done mainly by recognising symptoms in the field followed by infectivity assays on pea cultivars and indicator hosts (Maury and Khetarpal, 1992). As symptoms of PSbMV infection vary from severe to symptomless, screening programs based on symptoms will not be satisfactory for detecting infected plants (Hamilton and Nichols, 1978). Biological indexing has the disadvantages of requiring a number of different hosts and insect vectors, large amounts of space in the glasshouse and long experimental times.

Serological assays provide rapid and easy methods for the diagnosis of plant viruses either in purified preparations or plant extracts. Serological assays have many advantages (Matthews, 1991): (i) the specificity of the reaction allows virus to be

measured in the presence of host material or other impurities; (ii) results are obtained in a few hours or overnight compared with days for infectivity assays; (iii) some serological detection and assay procedures are more sensitive than infectivity measurements; (iv) serological tests are particularly useful with viruses that have no good local lesion host or that are not sap transmissible; (v) antisera can be stored and comparable tests made over a period of years and in different laboratories; (vi) virus concentration can be determined.

This chapter describes an improved method for purifying PSbMV particles and the subsequent extraction of the viral genomic RNA (Ali *et al.*, 1998). Also an improved dot-immunobinding assay (DIBA) has been developed for PSbMV diagnosis (Ali and Randles, 1997).

3.2 MATERIALS AND METHODS

3.2.1 Virus isolates and propagation

The PSbMV isolates S6 and US (section 2.1.2) were propagated in pea (cv Dundale) (section 2.2.2). The inoculated plants were kept in a growth room at a constant temperature (25°C) with continuous supplementary light at 2,000 l ×. The infected seedlings were harvested 18-25 days after inoculation.

3.2.2 Purification of PSbMV particles

PSbMV was purified from infected pea seedlings by a modification of the method of Wang *et al.* (1992).

All the steps were carried out in a cold room at 4°C. Infected tissues (120-420 g),

containing whole leaf, midribs, tendrils and stipules were transferred to a stainless steel Waring blender and homogenised in 480-1,680 ml of buffer A (100 mM ammonium acetate, pH 7.2, 20 mM EDTA, 1% Na₂SO₃ and 1 M urea). The resulting slurry was filtered through 4 layers of cheese cloth and clarified by adding Triton X-100 to 2.5% with continued vigorous stirring for 30 min. After centrifugation in a Sorvall GSA rotor at $11,000 \times g$ for 10 min, the virus enriched fraction was precipitated from the aqueous phase by adding 0.1 M NaCl and 4% polyethylene glycol (MW 6000). The mixture was stirred for 50 min and centrifuged as above. The supernatant was discarded and pellets were resuspended by stirring for 1.5 hr in 50 ml of buffer B (10 mM sodium borate buffer, pH 8.2) containing 1 mM EDTA and then left at 4°C overnight. The suspension was centrifuged in a Sorvall SS-34 rotor at $8,200 \times g$ for 10-15 min and the pellet was discarded. The supernatant was layered over a 30 % (w/v) sucrose cushion (5 ml) and centrifuged in a Beckman Ti-60 rotor (tube capacity 38.5 ml) at $86,000 \times g$ for 2 hr. Each pellet was resuspended in 1 ml of buffer B, and pooled. Cs₂SO₄ (3.75 g) was added to the suspension and the final volume was adjusted to 12 ml. A density gradient was formed by centrifuging in a SW-65 rotor (tube capacity 4.4 ml) at $137,000 \times g$ for 17 hr. The virus bands were collected with a syringe using an 18 gauge needle and subsequently applied to another Cs₂SO₄ gradient. Finally the virus bands were collected, pooled and dialysed against 3 changes of buffer B. Virus particles were concentrated in a TLA-100.3 rotor at $86,000 \times g$ for 2 hr. Final virus pellets were resuspended in 500 µl of buffer B.

3.2.3 Virus quantitation and storage

The concentration of purified preparations of PSbMV was determined as in section 2.2.3. Purified virus preparations that were used in a few hours were kept at 4°C while preparations intended for future use were mixed with 50% glycerol and stored at -20°C.

3.2.4 Extraction of RNA from PSbMV particles

RNA was isolated from purified preparations of isolates S6 and US (section 2.2.8.1). The size of the RNA was estimated in formaldehyde denaturing 1% agarose gels (section 2.2.11.1) using RNA molecular weight marker (Appendix A).

3.2.5 Detection of PSbMV by dot-immunobinding assay (DIBA)

The method of Ligat *et al.* (1991) was modified and used for serological detection of PSbMV. All incubations were done at room temperature with gentle agitation. All the antibody solutions contained glucose and mannose at a concentration of 600 mM each to avoid non-specific binding of antibodies to plant lectins.

3.2.5.1 Preparation of sample

Tissues from root, leaf, flower, immature green pod and whole green seed were crushed in plastic bags (75 × 140 mm) with one volume (w/v) of PBS (Table 2.3). Whole dry seed was soaked overnight in PBS before crushing as above. The extract from the crushed tissues was placed in 1.5 ml tube and centrifuged at $10,000 \times g$ for 5 min. Aliquots of 1 μ l were taken from the supernatant and dotted onto nitrocellulose membranes. The membrane was air dried and kept at room temperature or 4°C.

3.2.5.2 Preparation of blocking solution L

Solution L was prepared by homogenising healthy pea leaves (cv Dundale) in 9 volumes (w/v) of PBS buffer. The extract was centrifuged in a Sorvall SS-34 rotor at $10,000 \times g$ for 5 min before use. The supernatant was taken and both glucose and mannose were added to a final concentration of 600 mM. Skim milk (2.6%) and BSA

(1%) were also added to blocking solution L.

3.2.5.3 Preparation of blocking solution S

Solution S was prepared by soaking healthy pea seeds overnight in PBS, before grinding in 9 volumes (w/v) of PBS. The extract was centrifuged as above. Glucose and mannose were added to the supernatant to a final concentration of 600 mM. Skim milk (2.6%) and BSA (1%) were also added to blocking solution S.

3.2.5.4. DIBA procedure

3.2.5.4.a Blocking of membrane

Membranes dotted with leaf samples were blocked by immersing in solution L (section 3.2.5.2) whereas membranes dotted with seed samples were blocked with solution S (3.2.5.3) for 1 hr.

3.2.5.4.b Specific antibody binding

The blocking solution was removed and replaced with PSbMV isolate S6 or US polyclonal antisera diluted 1:2000 (v/v) in 10 ml PBS buffer. After incubation for 1 hr, the membrane was washed 3 times for 20 min each in AP 7.5 (Table 2.3).

3.2.5.4.c Conjugate antibody binding

Membranes were incubated for 1 hr in the presence of goat anti-rabbit IgG-alkaline phosphatase conjugate, diluted 1:2000 (v/v) in PBS, washed as above (section 3.2.5.4.b) and then given a quick wash in AP 9.5 (Table 2.3).

3.2.5.4.d Substrate

Membranes were incubated in the dark for 5-15 min in AP 9.5 containing 0.33 mg/ml nitro blue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma). Reactions were stopped with 10 mM Tris-HCl, pH 7.5, containing 5 mM EDTA. Results were recorded visually by the intensity of colour in comparison with a dilution series of positive controls.

3.2.6 Comparison by DIBA

To compare isolates S6 and US on the basis of DIBA, an experiment was done as follows. Sap was extracted (section 3.2.5.1) either from isolate S6 or US infected plants and diluted 10-fold (1/1 to 1/1000,000). One μ l dots were dotted from each dilution onto a nitrocellulose membrane and air dried. The pattern was replicated on 7 membranes. Each membrane was developed by DIBA (section 3.2.5.4) with 2-fold dilutions of isolate S6 antiserum in the range of 1/1000 to 1/64,000). Membranes prepared in a similar way were developed with the same range of dilutions of isolate US antiserum.

3.2.7 Western immunoblotting

For western immunoblot analysis (section 2.2.5) purified PSbMV particles or whole leaf sap were mixed with an equal volume of $2 \times$ SDS-gel sample buffer (Table 2.3), heated at 100°C for 5 min (to denature and solubilise proteins) and fractionated on a 10% SDS-polyacrylamide gel (section 2.2.11.3).

3.3 RESULTS

3.3.1 Virus purification

PSbMV isolates S6 and US were successfully purified by the same protocol (section 3.2.2). A single light scattering band was obtained in the Cs_2SO_4 gradients (Fig. 3.1). Isolate S6 yielded 2.5 to 3.5 mg of virus and isolate US yielded 1.7 to 2 mg of virus per 100 g of infected tissues.

3.3.2 Size measurement of PSbMV particles

All preparations of PSbMV isolates showed flexuous rod shaped particles. The lengths of particles ranged from 700 to 785 nm (Fig. 3.2). Electron micrographs of purified particles showed no visible contamination, significant fragmentation or aggregation of the virus particles.

3.3.3 Infectivity assay

Purified virus preparations of both isolates S6 and US produced necrotic local lesions when inoculated to *Chenopodium amaranticolor* (Fig. 3.3), indicating that the virion are infectious.

3.3.4 Size of the PSbMV RNA

As shown in Fig. 3.4, a single RNA band of 9.5-10 kb was obtained from isolates S6 and US. No difference was observed in the size of their RNA. However, virus preparations which were not treated with DNase and RNase before protein digestion of the virus particles, showed two bands above the viral RNA band (Fig. 3.5). The

uppermost band was removed by DNase while the middle band was removed with RNase treatment of the virus particles before the nucleic acid extraction step.

3.3.5 Development of a reliable DIBA for diagnosis of pea viruses

High backgrounds typically obtained with DIBA of pea seed samples (Ligat *et al.*, 1991) were overcome by incubating membranes in blocking solution S containing both glucose and mannose. Skim milk (2.6%) and BSA (1%) had no additional effect on reducing background when added to the blocking solution and were ineffective when used alone as a blocking agent (data not shown). The optimised DIBA procedure (section 3.2.5.4) consistently gave similar results when a range of samples were tested for PSbMV and also for other pea viruses (Chapter 6).

3.3.6 Sensitivity of DIBA

Using either PSbMV-S6 or US antiserum, DIBA detected down to 1 ng/ μ l of both isolates of purified PSbMV (Fig. 3.6 A) and the dilution end point of sap from infected plants was 1/4096 (Fig. 3.6 B). All the blocking and antibody solutions could be used for at least 5 sequential membrane incubations with no apparent loss in sensitivity of detection. Membranes probed with one virus antiserum and stored for 2-3 months at room temperature could be successfully reprobed with another virus antiserum. Groups of 4 to 5 membranes (100 squares/membrane) dotted with different samples were also successfully blocked and probed together with each antiserum.

3.3.7 Comparison by DIBA

As shown in Fig. 3.7 A and B, no difference was observed between isolates S6 and US on the basis of DIBA when their infected sap was detected in a 10-fold dilution series.

3.3.8 Size of the coat protein of PSbMV

SDS-PAGE showed a band of ca. 36 kDa (Fig. 3.8 A) when either purified virus preparation or infected sap of isolates S6 and US was loaded on the gel. These bands were further confirmed to be the coat protein of PSbMV by western blot analysis (Fig. 3.8 B). No protein band of the same size was observed for samples from healthy control pea plants.

3.4 DISCUSSION

The purification protocol described here (section 3.2.2) was used to purify both isolates S6 and US, which are typical pathotype P-4 and P-1 isolates of PSbMV respectively (Ali and Randles, 1997). This indicates that the protocol has broad spectrum applicability in purifying different PSbMV isolates. The purified virus preparation did not show any visible contamination or significant aggregation when checked under the electron microscope. However, following RNA isolation from virus particles, it was observed by agarose gel electrophoresis that some DNA and RNA bands were always seen in addition to the band of the size expected to originate from virus particles. These DNA and RNA bands migrated more slowly than the viral RNA and were successfully removed by DNase and RNase treatment of the virus preparation (section 2.2.8.1) before the proteinase treatment. This phenomenon has not been reported before. In light of the above results, it is suggested that purified virus preparation of PSbMV should be predigested with DNase and RNase to remove contaminating nucleic acids before injecting into a rabbit for antiserum production.

PSbMV isolates S6 and US are not distinguishable by serology (Fig. 3.6 and 3.7). Also no difference was observed in the size of their RNA genomes (Fig. 3.4) or coat protein sizes (Fig. 3.8) which suggests that these criteria are not suitable to distinguish these

*Is this a good
test to distinguish
between
isolates?*

isolates of PSbMV.

DIBA has about the same sensitivity as enzyme-linked immunosorbent assay (ELISA) in microtitre plates (Ligat *et al.*, 1991) but it has the additional advantages of simplicity, speed, and economy. Moreover, prepared membranes can be stored, sent by mail, and reprobed with different antisera. DIBA of pea seed is sometimes affected by high backgrounds (Ligat *et al.*, 1991) but this has been overcome by further modifying DIBA for pea virus diagnosis in field plants, and for detecting PSbMV in dry seed and germinated seedlings. The DIBA was improved by using a high concentration of glucose and mannose (600 mM each) and different blocking solutions such as solution L (section 3.2.5.2) when membranes were dotted with leaf samples, and solution S (section 3.2.5.3) when membranes were dotted with seed samples. The test gave consistent results with known positive samples of 5 viruses indicating that it can probably be used for the routine indexing of pea viruses merely by varying the test antiserum in the procedure. The DIBA procedure developed here was used successfully for the detection of PSbMV and other viruses during a survey in Pakistan (Chapter 6).

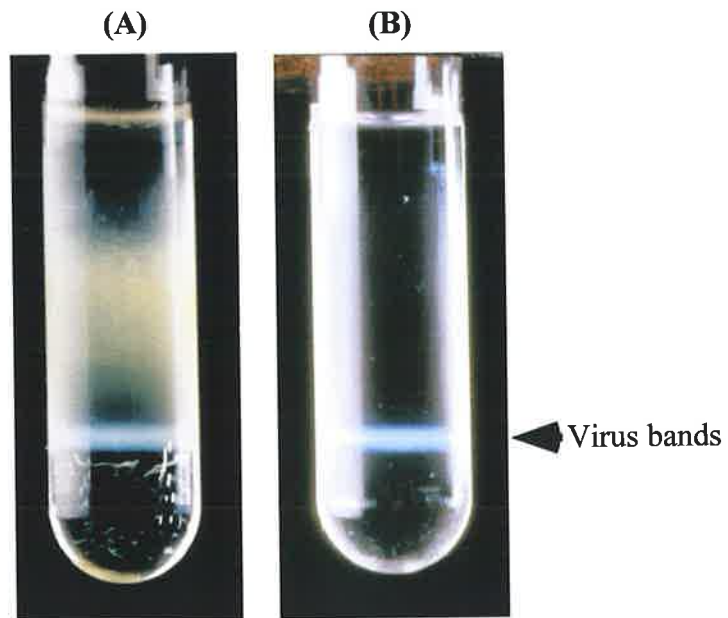


Fig. 3.1 Light scattering band of PSbMV (arrowed) in Cs_2O_4 gradient. **(A)** virus band after first cycle of gradient with plant material on the top, **(B)** second cycle of gradient with only virus band.

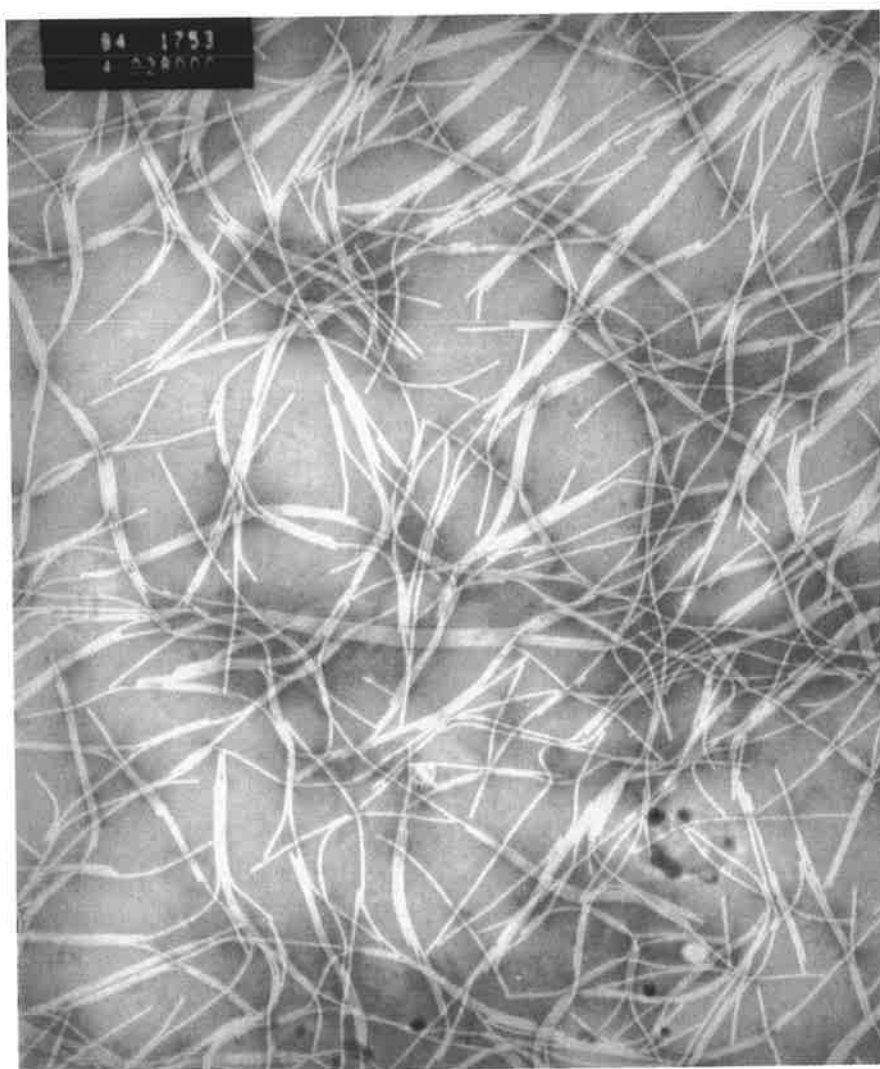


Fig. 3.2 Electron micrograph of purified preparation of isolate S6 of PSbMV.



Fig. 3.3 Necrotic local lesions induced by a purified PSbMV-S6 preparation mechanically inoculated on *Chenopodium amaranticolor* leaves after 7 days post inoculation.

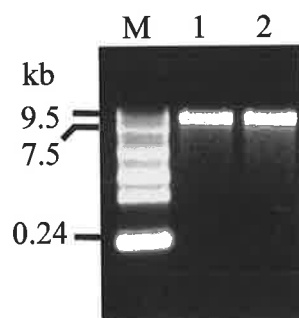


Fig. 3.4 Formaldehyde denaturing 1% agarose gel electrophoresis showing RNA extracted from a purified PSbMV preparation. RNA marker (M), isolates S6 RNA approximately 9.5 kb (lane 1), isolate US RNA approximately 9.5 kb (lane 2).

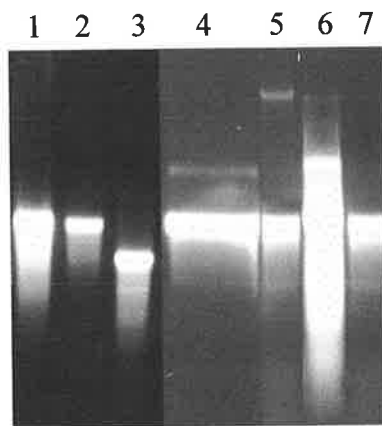


Fig. 3.5 Formaldehyde denaturing 1% agarose gel electrophoresis showing RNA extracted from a purified PSbMV preparation. Isolate S6 RNA (lane 1), isolate US RNA (lane 2), and TMV RNA used as a standard control (lane 3), PSbMV-S6 particles treated with DNase (lane 4), S6 particles treated with RNase (lane 5), S6 particles not treated with DNase and RNase (lane 6), S6 particles treated with DNase and RNase (lane 7).

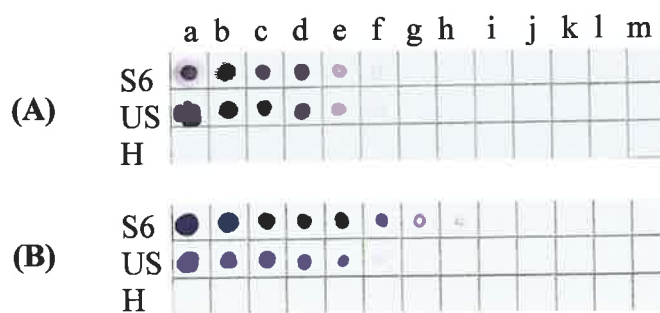
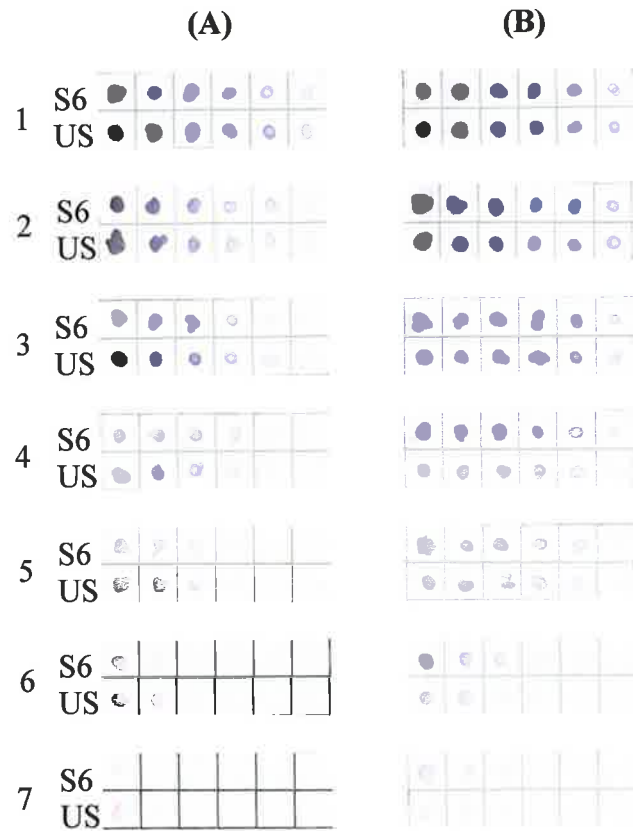


Fig. 3.6 Detection of a range of amounts of virus by DIBA using PSbMV-US antiserum diluted (1:2000). **(A)** 1 µl aliquots of purified PSbMV-S6 and -US, a = 1.2 µg, b = 600 ng, c = 300 ng, d = 100 ng, e = 10 ng, f = 1 ng, g = 100 pg, h = 10 pg, i = 1 pg, j = 100 fg, k = 10 fg, l = 1 fg, **(B)** Dilution endpoint of 1 µl aliquots from infected sap of PSbMV-S6 and -US isolates ranging from 1/2 to 1/8,192 in a 2-fold dilution series. Row H indicates a 2-fold dilution series of healthy pea.



too many just

Fig. 3.7 Comparison of PSbMV isolates S6 and US by DIBA. 10-fold dilution series (1/1 to 1/1000,000) from infected sap of isolates S6 and US. 1 μ l dots were applied from each dilution on each membrane. **(A)** Membranes were developed by DIBA treated with PSbMV-S6 antiserum diluted as follows: membrane 1= 1:1000, 2 = 1:2000, 3 = 1: 4000, 4 = 1:8000, 5 = 1:16,000, 6 = 1:32,000, 7 = 1:64,000, **(B)** Membranes were developed with PSbMV-US antiserum diluted as for isolate S6.

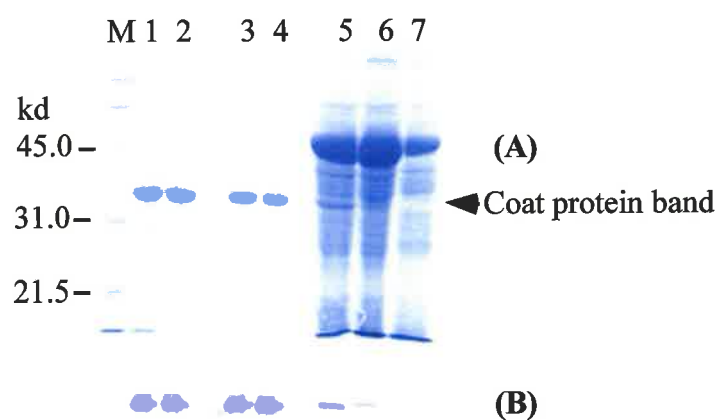


Fig. 3.8 Analysis of PSbMV capsid protein by **(A)** SDS-PAGE and **(B)** western blotting. Protein marker (M), purified isolate S6 preparation (lane 1-2), purified isolate US preparation (lane 3-4), sap from isolate S6 infected pea plant (lane 5), sap from isolate US infected pea plant (lane 6), sap from healthy pea plant (lane 7).

Chapter 4

DIAGNOSIS OF PSbMV BY NUCLEIC ACID HYBRIDISATION

4.1 INTRODUCTION

Plant virus diseases can be detected by bioassay on suitable plant cultivars. The assay is sensitive, but is laborious, expensive and time consuming. Modern detection techniques for plant viruses are based on the identification of the specific molecular component (s) of the causal agent in samples. Virus specific protein can be detected by appropriate serological technique including (ELISA, DIBA or immunofluorescence). The genetic material of the pathogen (nucleic acid) can be detected by nucleic acid hybridisation assay. The nucleic acid hybridisation assay is useful for some plant virus infections, when virus coat protein is not produced and such infections cannot be identified with serological techniques (Harrison and Robinson, 1982; Harrison *et al.*, 1983). Nucleic acid hybridisation is also used to detect virus satellite RNAs and viroids which are not detectable by serological methods (Kaper and Waterworth, 1977; Francki, 1985).

Nucleic acid hybridisation techniques have been applied to detect viruses using complementary DNA probes which are ^{32}P -labelled (Boulton *et al.*, 1986; Palukaitis, 1986; Varveri *et al.*, 1987; Baulcombe and Fernandez-Northcote, 1988), biotin-labelled (Chan *et al.*, 1985; Eweida *et al.*, 1990; LeClerc *et al.*, 1992) or digoxigenin (DIG)-labelled (Crespi *et al.*, 1991; Harper and Creamer, 1995; Singh and Singh, 1995). The molecular hybridisation technique has the advantage of detecting the viral genome in all its forms (single- or double-stranded, encapsidated or not), thereby providing a specific and sensitive method for early detection (Roy *et al.*, 1988).

As mentioned in section 1.5, PSbMV gave symptomless infection in vegetative tissues of pea and could not be detected by ELISA (Khetarpal and Maury, 1990; Ligat and Randles, 1993). Such infection might be a limiting factor at quarantine check points where biological indexing and serological methods are currently used as a control procedure for the identification of virus in seed stocks (Khetarpal and Maury, 1987).

Therefore, to detect PSbMV RNA rather than its coat protein, nucleic acid-based assays using dot-blot and tissue print formats were developed (Ali *et al.*, 1998) in this chapter which use uncloned randomly primed cDNA probes (^{32}P -labelled and DIG-labelled) produced by reverse transcription of PSbMV RNA.

4.2 MATERIALS AND METHODS

4.2.1 Viral RNA preparation

PSbMV RNA was isolated from purified preparations of isolate S6 and US as described in section 2.2.8.1. Purified RNA of isolates S6 and US (Fig. 3.4) was used for the synthesis of cDNA probes as below. Total RNA from plant tissues was prepared as in section 2.2.8.2.

4.2.2 Synthesis of radioactive and non-radioactive cDNA probes

Radioactive (^{32}P -labelled) cDNA probes were synthesised as in section 2.2.9.1 while non-radioactive (DIG-labelled) cDNA probes were prepared as in section 2.2.9.2. For both types of probes, RNA of either isolate S6 or US was reverse transcribed to produce randomly primed cDNA probes representing the full genome of PSbMV RNA. Both radioactive and non-radioactive cDNA probes were incubated at 85°C for 5-10 min before adding to the hybridisation buffer. Radioactive cDNA probe was added at a concentration of $0.1\text{-}0.6 \times 10^6$ cpm/ml.

4.2.3 Size measurement of cDNA probes

Aliquots of both radioactive and non-radioactive cDNA probes were run in a native 1% agarose gel (section 2.2.11.1) to measure the size of the transcribed probes. Agarose gel

with the ^{32}P -labelled cDNA probe was dried under vacuum for 2 hr at 80°C and exposed directly to X-ray film at -70°C . Agarose gel with DIG-labelled cDNA probes were either stained with ethidium bromide and viewed by UV fluorescence in the UV transilluminator or transferred to a nylon membrane (section 2.2.10.4) and identified by colorimetric detection (section 2.2.10.6).

4.2.4 Sample preparation for dot blot hybridisation

Purified virus preparations (section 3.3.1) or the RNA (section 3.3.4) of isolates S6 and US prepared earlier were used for dot blotting.

Total nucleic acid from leaf tissue (50-100 mg) or individual seeds was extracted as in section 2.2.8.2. The final pellet of total nucleic acid was dissolved in 20 μl of sterile water.

Sap from PSbMV-infected and healthy pea plants was extracted in TES buffer (Table 2.3) by crushing the leaves (w/v 1:1) and centrifuging at $10,000 \times g$ for 3 min. The supernatant was taken and used for dot blotting.

4.2.5 Dot blotting

All dilutions were carried out in $12 \times \text{SSC}$ (Table 2.3) and 6% formaldehyde buffer. Aliquots of 1 μl containing purified virus or RNA diluted in a 5-fold series (100 ng to 10.2 fg), were dotted on nylon membranes. Total nucleic acid or sap (extracted from leaves or seed) was diluted 5-fold (11 times) (Figs. 4.2 and 4.3) and then aliquots of 1-2 μl were dotted from each dilution on nylon membranes. Nucleic acid was fixed under UV light (section 2.2.10.1). The membrane was washed in $2 \times \text{SSPE}$ (Table 2.3) containing 0.1% SDS for 1 h before prehybridisation.

4.2.6 Tissue printing

Tissue prints of leaflets or seed taken from pea plants infected with either isolate S6 or US were done as described in section 2.2.10.2. Tissue prints from leaflets of pea plants infected with pea mosaic potyvirus (PMV), bean yellow mosaic potyvirus (BYMV) and healthy pea plants were also done as positive and negative controls. Preparation of tissue prints from the testa of seeds was not successful because testas were dry. The tissue blots were fixed under UV light (section 2.2.10.2). The membranes were washed in $2 \times$ SSPE (Table 2.3) containing 0.1% SDS for 1 h before prehybridisation.

*positive for what
all of these
are negative
controls for
PSbMV.*

4.2.7 Hybridisation and detection assays

Dot, Northern, Southern or tissue blotted membranes were prehybridised and hybridised (section 2.2.10.5) with either radioactive or non-radioactive cDNA probes and detected as in section 2.2.10.6.

4.3 RESULTS

4.3.1 Purity of RNA and size of the cDNA probes

Agarose gel electrophoresis of viral RNA (Fig. 3.4) and hybridisation with ^{32}P -labelled cDNA probes (data not shown) revealed a single band of approximately 9.5-10 kb. This suggests that the purified RNA of PSbMV isolates was a suitable template for the production of cDNA probes. PSbMV RNA was successfully extracted from infected pea leaves as shown in Fig. 4.1. The randomly primed cDNA probes (radioactive and non-radioactive) prepared by reverse transcription of PSbMV RNA were predominantly 0.2-0.4 kb in length (Fig. 4.2)

4.3.2 Detection by radioactive probes

Purified PSbMV and its RNA were detected to a concentration of 800 pg and 51 fg respectively with their respective homologous ^{32}P -labelled cDNA probes (Fig. 4.3 A I and II). However, the limit of detection with the heterologous probe was 20 ng of purified virus and 160 pg of purified RNA. PSbMV RNA was also detected in total nucleic acids (Fig. 4.3 B, I and II) and sap from infected leaves (Fig. 4.3 C, I and II) when both were diluted at 1: 625. However, the heterologous hybridisation end point was one order of magnitude less than the homologous hybridisation end point. Total nucleic acid and sap from healthy controls (Fig. 4.3 B and C, I and II) gave no non specific reaction.

4.3.3 Detection by non-radioactive probes

The sensitivity of non-radioactive (DIG-labelled) randomly primed cDNA probes was slightly higher than that of radioactive probes. The limit of detection by non-radioactive probes for homologous virus and RNA was 160 pg and 51 fg respectively (Fig. 4.4 A, I and II). The heterologous hybridisation end point for purified virus was 4 ng and for RNA was 32 pg. The S6 cDNA probe detected its homologous RNA at 1:3125 dilution in total nucleic acid or infected sap while heterologous RNA was detected at 1:625 dilution in total nucleic acid or infected sap (Fig. 4.4 B and C, I). However, the US cDNA probe was able only to detect its homologous RNA in total nucleic acid or infected sap at 1:625 dilution and heterologous RNA at 1:125 (Fig. 4.4 B and C, II). No reaction was obtained for the healthy control indicating that the test was specific and reliable.

4.3.4 Detection by tissue print hybridisation

Leaflet from pea plants infected with PSbMV (isolates S6 and US), PMV, BYMV and healthy were squashed onto nylon membranes. The membranes were hybridised with ^{32}P -labelled S6 (Fig. 4.5 A) and US (Fig. 4.5 B) randomly primed cDNA probes. Duplicate membranes were also hybridised with DIG-labelled random primed cDNA probes of S6 (Fig. 4.6 A) and US (Fig. 4.6 B). PSbMV RNA was detected directly in squashes of the tested tissues. A strong signal was obtained with S6 infected leaflets when hybridised with S6 cDNA probes and vice versa. No signal was obtained for squashes of PMV, BYMV (data not shown) and healthy control (Fig. 4.5 and 4.6) confirming the specificity of cDNA probes. Similar sensitivity was obtained when DIG-labelled cDNA probes (Fig. 4.6 A and B) were used instead of ^{32}P -labelled cDNA probes.

4.3.5 Detection of PSbMV in dry seed

As PSbMV is seed-borne and is transmitted through the embryo from generation to generation, 72 seeds were tested either by dot blot or tissue print hybridisation using radioactive or non-radioactive cDNA probes. PSbMV was readily detected in dot blots (Fig. 4.7 A) and direct squashes from embryo (Fig. 4.7 B). No virus was detected in cotyledon squashes. There was no non-specific reaction with healthy seed samples.

4.4 DISCUSSION

Over 75% of plant viruses have plus-strand RNA genomes, and the most common probe is cDNA made to viral RNA by reverse transcription (Hull and Al-Hakim, 1988). Randomly primed cDNA probes made directly by reverse transcription of viral RNA offer a simple, sensitive, flexible approach to the diagnosis of plant viruses and are able

to detect any part of the plant virus genome. Randomly primed cDNA probes represent the whole genome of the virus rather than any specific part of the genome. Different regions of the genome are often conserved among related viruses to different extents and would be expected to affect the specificity of the test (Koenig *et al.*, 1988; Robinson and Romero, 1991). A particular advantage of the cDNA probes in this study was that the probe made to isolate S6 (pathotype P-4) of PSbMV can detect a biologically different isolate US (pathotype P-1) of the same virus and vice versa. This result indicates that cDNA probes made to any pathotype of PSbMV are likely to detect the same or the other pathotype in field or seed lots and could provide a broad spectrum assay for routine testing.

The sensitivity of radioactive and non-radioactive cDNA probes for PSbMV detection was almost the same for dot blot and tissue print hybridisation assays. But as radioactive probes have a short shelf life and involve health hazards during use and disposal, a modified method using DIG-labelled random primed cDNA probes was developed. DIG-labelled cDNA probes would be more useful due to their long shelf life, lack of health hazards and ability to be reused several times. For example, the DIG-labelled cDNA probe was used 10 months after preparation without significant loss of sensitivity (Ali *et al.*, 1998).

In this study, both dot blot and tissue print hybridisation assays, using either cDNA probe, were sensitive, specific, and reliable tests for the detection of PSbMV. These assays were fast and easy to perform and can be used to detect the PSbMV genome either in total nucleic acids, in crude sap or directly in squashed tissues. Thus early viral infection, where only viral RNA is synthesised, can be detected before any measurable amount of virus protein is produced (Sela *et al.*, 1984). The dot blot hybridisation assay using either cDNA probe can detect as little as 51 fg of purified PSbMV RNA (Fig. 4.3 and 4.4). The detection limit (1:3125 fold dilution) in total nucleic acid and infected

crude sap was almost the same for both radioactive and non-radioactive probes. This detection limit corresponds to less than 1 µg of tissue dotted per spot, which suggests that this amount of tissue contains at least 51 fg PSbMV RNA.

Tissue print hybridisation (TPH) is also a simple and rapid assay for directly detecting viral RNA in squashed tissues. It can be used to simply and quickly screen whole plants as well as most plant tissues for the location and distribution of the virus at different stages of infection, particularly during symptomless infection. It detected PSbMV with either cDNA probe in as little as 5 mg infected tissue. Tissue print hybridisation also successfully detected PSbMV in squashes from embryo (Fig. 4.7). PSbMV in the mature seed testa is not infectious to the next generation of seedlings but retains its infectivity in the embryo only during seed transmission and storage (Masmoudi *et al.*, 1994). On the basis of these results, we believe that the assay could be applied for large scale screening of young seedlings or embryos. This assay can also be used during a survey by squashing the collected samples directly onto membranes rather than bringing samples to the laboratory for processing.

In conclusion, both dot blot and tissue print hybridisation assays were found to be specific and reliable for the detection of PSbMV. These assays were used to detect the PSbMV genome in purified virus or RNA preparation, in total nucleic acid and in crude sap or directly in squashed tissues. These assays can be adopted as simple, and rapid tools in diagnosing PSbMV in quarantine screening, study^{ies} on epidemiology and field surveys for all PSbMV isolates or pathotypes.

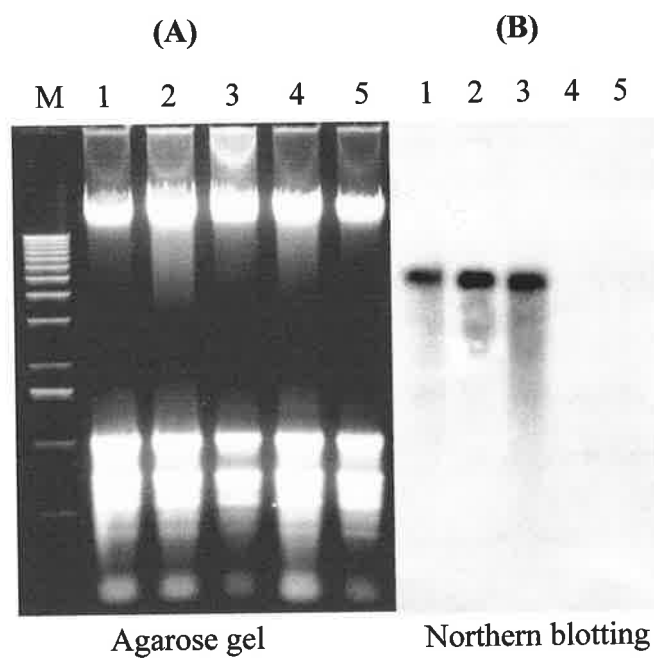


Fig. 4.1 1% electrophoretic agarose gel fractionation of total nucleic acids extracted from pea leaves. (A) PSbMV-S6 infected (lane 1-3), healthy (lane 4-5), DNA marker (M), (B) Northern blot analysis of total nucleic acids with a ^{32}P -labelled cDNA probe prepared from isolate S6.

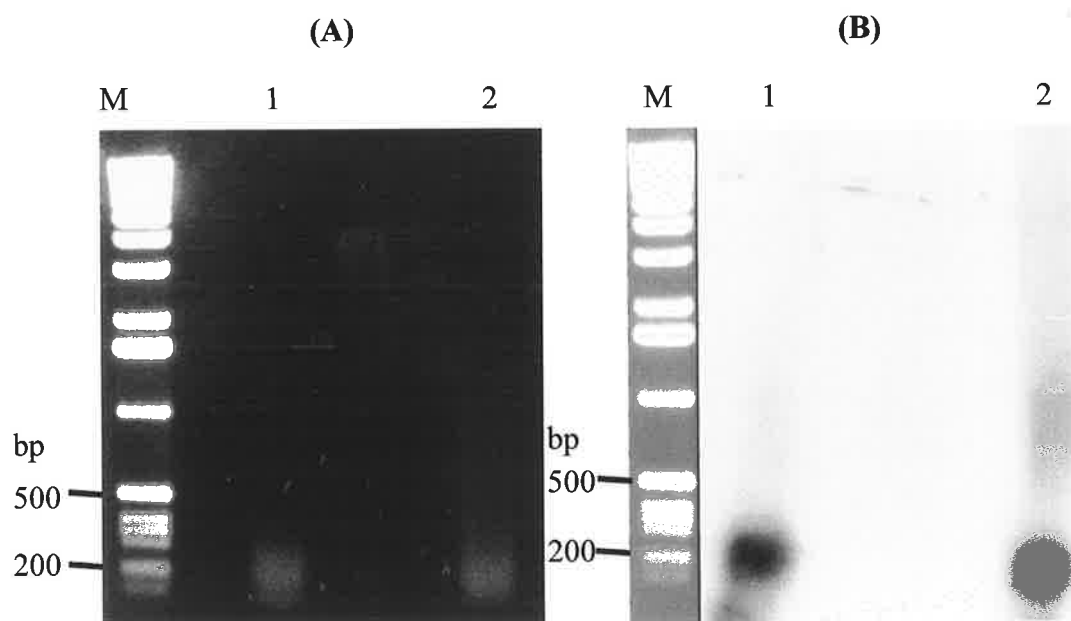


Fig. 4.2 Size measurement of randomly primed cDNA probes by non-denaturing 1% agarose gel electrophoresis. **(A)** DIG-labelled cDNA probes, DNA marker (M), cDNA probes of isolate S6 (lane 1) and isolate US (lane 2), **(B)** ^{32}P -labelled cDNA probes, DNA marker (M), cDNA probes of isolate S6 (lane 1) and isolate US (lane 2).

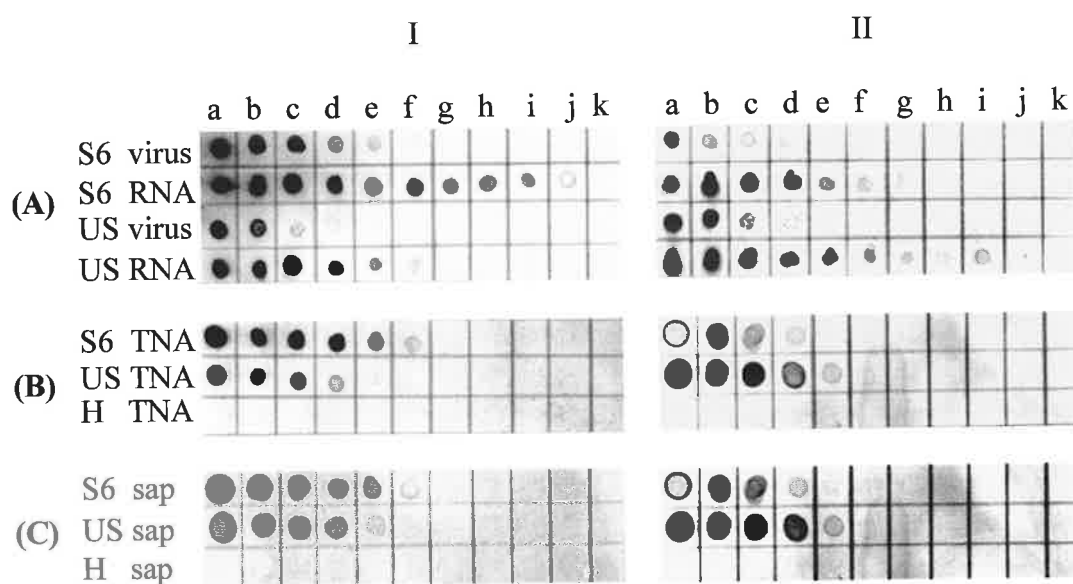


Fig. 4.4 Dot-blot hybridisation with an uncloned randomly primed DIG-labelled cDNA probe to isolate S6 (I) and the isolate US (II). **(A)** Purified virus and RNA from isolates S6 and US were dotted as 1 μ l per spot: a = 100 ng (1:1), b = 20 ng (1:5), c = 4 ng (1:25), d = 800 pg (1: 125), e = 160 pg (1: 625), f = 32 pg (1: 3125), g = 6.4 pg (1: 15625), h = 1.28 pg (1:78125), i = 256 fg (1: 390625), j = 51 fg (1: 1953125), k = 10.2 fg (1: 9765625), **(B)** Total nucleic acid (TNA), **(C)** Sap from S6 and US infected plants, and healthy (H) pea plants dotted as 1 μ l per spot respectively, a = 1:1, b= 1: 5, c = 1: 25, d = 1: 125, e = 1: 625, f = 1: 3125, g = 1: 15625, h = 1: 78125, i = 1: 390625, j = 1: 1953125, k = 1: 9765625.

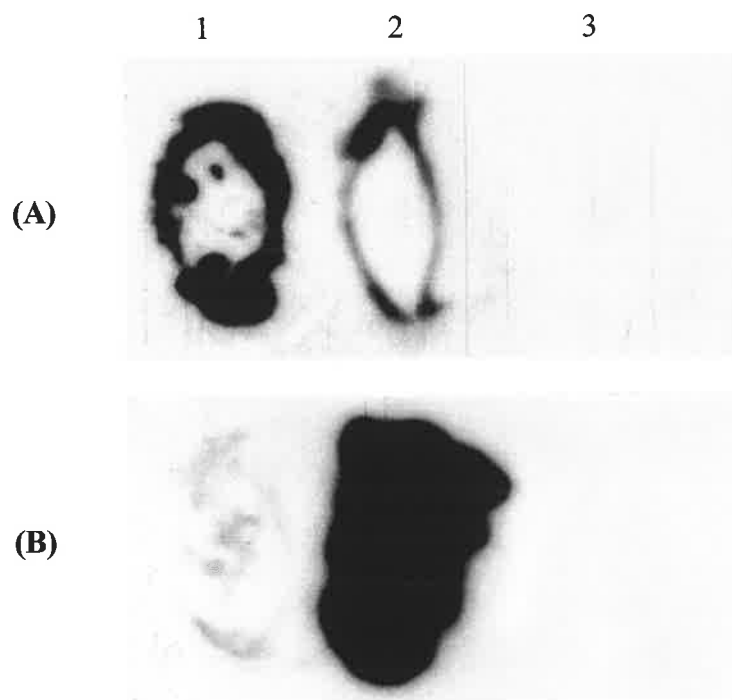


Fig. 4.5 Autoradiograph of infected leaflets squashed on nylon membranes. **(A)** Membrane hybridised with ^{32}P -labelled cDNA probe to the isolate S6, **(B)** Membrane hybridised with ^{32}P -labelled cDNA probe to the isolate US. 1 = Leaflet infected with the isolate S6, 2 = Leaflet infected with the isolate US, and 3 = Healthy pea leaflet.

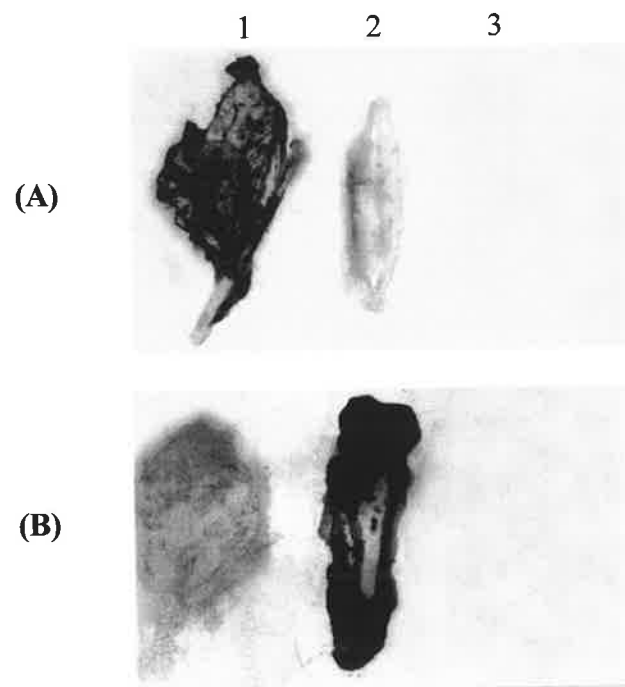


Fig. 4.6 Colorimetric detection of infected leaflets squashed on nylon membranes. **(A)** Membrane hybridised with DIG-labelled cDNA probe to the isolate S6, **(B)** Membrane hybridised with DIG-labelled cDNA probe to the isolate US. 1 = Leaflet infected with the isolate S6, 2 = Leaflet infected with the isolate US, and 3 = Healthy pea leaflet.

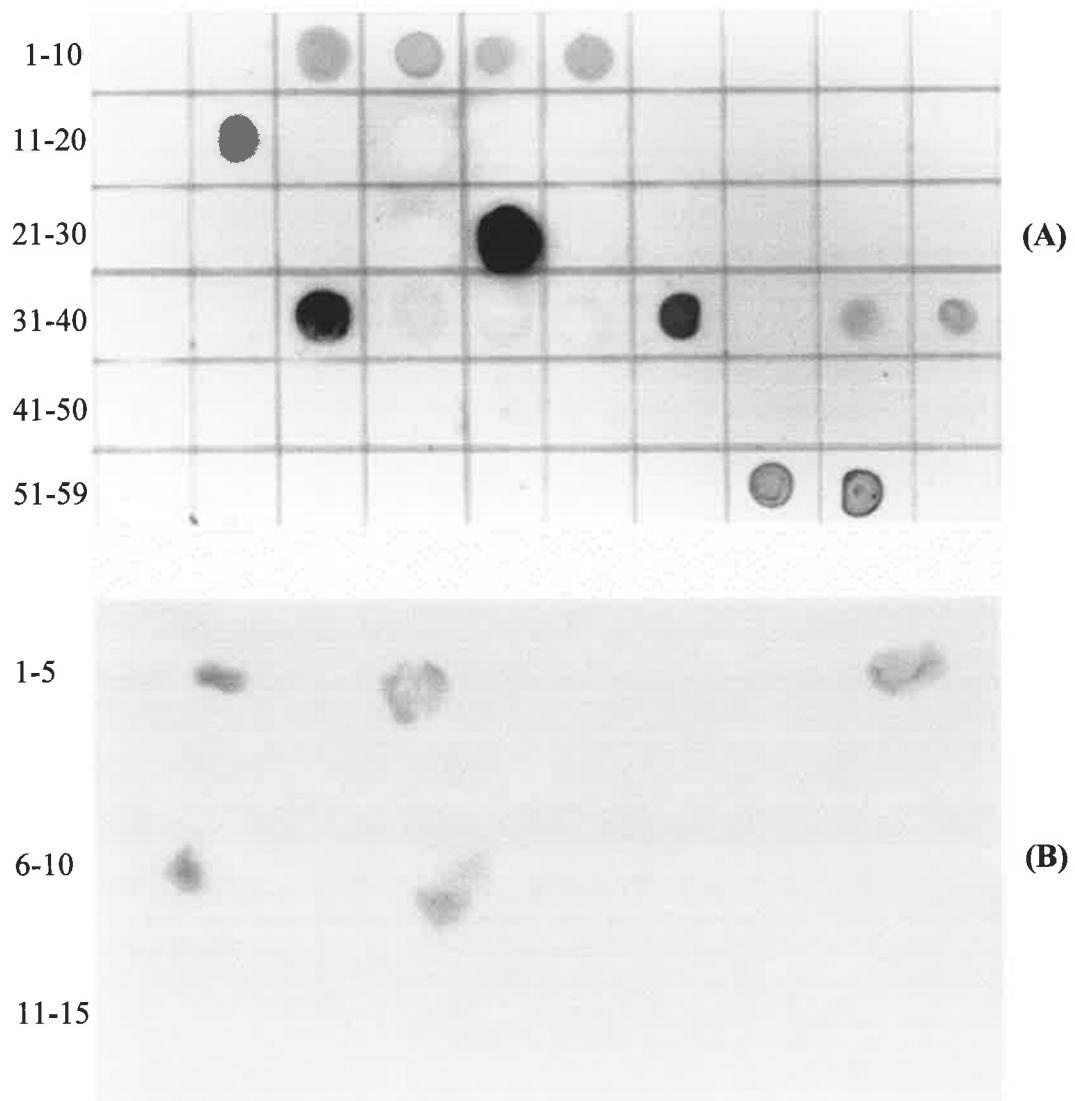


Fig. 4.7 Detection of PSbMV infection in pea seed by (A) dot blot and (B) tissue print hybridisation using DIG-labeled cDNA probe to the isolate S6. (A) Samples 1-50 are total nucleic acids extracted from the seed of plants infected with PSbMV, 51-57 from healthy pea seeds, and 58-59 are PSbMV infected leaves as a positive control, (B) Samples 1-10 are imprints of embryos from PSbMV infected seeds, while 11-15 contains imprints of embryos from healthy pea seeds.

Chapter 5

PATHOLOGY OF PSbMV

5.1 INTRODUCTION

Plant virus diseases have been reported to cause losses of approximately US \$ 60 billion per year worldwide, and viruses come second after fungi with respect to the disease losses they cause (Matthews, 1991). Plant viruses cause considerable yield losses in many crops depending on the virus, host and environmental conditions. Measurement of yield losses provides important information for that particular virus or isolate.

PSbMV is economically important because it reduces the yield of pea and other food legumes such as lentil, faba bean and chickpea (section 1.6). Yield losses in peas infected with PSbMV depend on the host species, cultivar, the virus isolate and age of the plant at the time of infection (Kraft and Hampton, 1980; Khetarpal *et al.*, 1988; Anonymous, 1991; Maury and Khetarpal, 1992; Kumari *et al.*, 1993).

Several reports have been published on the yield losses due to PSbMV but they are specific to particular isolates and localities (section 1.6). Three pathotypes of PSbMV have been reported (section 1.7) but no data are available on the losses caused by specific pathotypes of PSbMV in commercial pea lines and no analysis of the factors leading to effects on yield have been reported.

In Australia, although PSbMV was first reported in 1978 (Munro, 1978) and has since been observed in field peas in Queensland and South Australia (Ligat *et al.*, 1991), no information is available on the effect on yield of pea of any PSbMV isolates reported from Australia.

This chapter describes an analysis of the effects of isolate S6 (P-4) in three sequential generations of the commercial field pea cv Dundale and compares them with those of isolate US (P-1) of PSbMV. In addition, symptom severity, and rate of seed

transmission are reported for isolates S6 and US (Ali and Randles, 1998).

5.2 MATERIALS AND METHODS

5.2.1 Plant growth and virus culture

Single seeds of pea cv Dundale were germinated in UC soil mix in 150 mm pots (section 2.2.1). Seedlings were grown in an insect-free air conditioned glasshouse, at day/night temperatures of 25 and 18°C respectively. Cultures of isolates S6 and US (section 2.1.2) were maintained in mechanically inoculated pea (cv Dundale).

5.2.2 Detection of PSbMV

All tissue samples were indexed for PSbMV by DIBA (section 3.2.5.4) or dot blot hybridisation assay (DBHA) (section 4.2.4).

5.2.3 Assay of sample

Individual seedlings were tested weekly for PSbMV infection by taking tissue discs 3 mm in diameter from leaflets at each node, petals and immature green pods. Whole green seed or mature dry seed were also tested. The tissue discs collected at each time from each seedling were pooled. The discs were placed in a 1.5 ml tube, frozen in liquid nitrogen, crushed and mixed with two volumes (w/v) of TES buffer (Table 2.3) at 22°C. Dry seeds were soaked overnight in sterile water before crushing individually in a plastic bag with two volumes (w/v) of TES buffer. Whole green seeds was directly crushed as above without soaking in water. Aliquots of 1 µl were dotted immediately onto nitrocellulose membrane and air dried. Membranes were tested immediately by DIBA or stored at 4°C.

The remaining extract of each of the above samples was extracted with one volume of 0.1 M Tris-HCl saturated phenol (pH 7.6) followed by chloroform extraction (section 2.2.7). Total nucleic acid was precipitated from the aqueous phase using 0.1 volume of 3 M Na acetate (pH 5.2) and 3 volumes of chilled ethanol and storing at -70°C for 30 min. The final pellet of total nucleic acid was dissolved in 20 μl sterile water and 1 μl dots were applied to nylon membranes and air dried. Nucleic acid was fixed under UV light (section 2.2.10.1) and membranes were either analysed immediately by dot blot hybridisation or stored at 4°C .

5.2.4 Generation 1 (G1) plants

A randomised block design was set up with 30 replicates (Fig. 5.1) of the following three treatments; seedlings inoculated with isolate S6; seedlings inoculated with isolate US; and uninoculated controls. Test seedlings were shown to be free of PSbMV before inoculation by DIBA of tip leaves. One week after emergence, healthy pea seedlings (cv Dundale) dusted with carborundum (500-mesh) were mechanically inoculated with an extract of either isolate S6 or US infected leaves in 50 mM sodium borate buffer, pH 8.1 (1:1 w/v) (Ali and Randles, 1997). Two weeks after inoculation, each inoculated plant was tested by DIBA for the presence of PSbMV. Seedlings were grown to maturity and were called generation 1 (G1) plants. Data were recorded weekly of symptom severity, and growth and yield components of each seedling as listed in Table 5.1. After maturity, dry seeds were collected from both infected and healthy controls plants and stored at 4°C .

5.2.5 Generation 2 (G2) plants

To determine the levels of seed transmission of isolates S6 and US and their effects on plants infected through the seed, 60 seeds from each treatment in G1 were selected at random, weighed and their colour recorded. They were sown to produce generation 2

(G2) plants. Germination data were recorded at one and two weeks after sowing. Visual observations for symptom expression were made weekly on each seedling until maturity. Two weeks after germination, seedlings were tested individually for PSbMV either by DIBA or DBHA (section 5.2.2). Eight weeks after maturity, plants were retested. Data were recorded for the same parameters as for G1 (Table 5.1). After maturation dry seeds were collected and stored at 4°C.

5.2.6 Generation 3 (G3) plants

Forty one seeds produced by plants infected with isolate S6 and 39 seeds produced by plants infected with isolate US in G2 were sown to produce generation 3 (G3) plants. A similar number of seeds was sown at random from G2 healthy plants as a control. Before sowing, seeds were weighed and their colour recorded. Germination data were recorded at one and two weeks after sowing. All germinated seedlings were tested weekly for PSbMV by DIBA and DBHA (section 5.2.2). Seeds which did not germinate were also tested. Plants were grown to maturity and data were recorded as for G1. In addition, 50 seeds each from plants negative to isolates S6 and US in G2 (Table 5.2) were germinated to test for the presence of virus in generation 3 (G3).

5.3 RESULTS

5.3.1 Symptoms in G1 and G2 plants

In G1, isolate S6 induced systemic vein clearing, vein banding, severe mosaic, leaf distortion, abnormal tendril curling and stunting of plants (Fig. 5.2 A). Symptoms were observed on all compound leaves and stipules of inoculated plants from six days after inoculation until plants senesced. In contrast, isolate US induced mild leaf rolling and plant stunting (Fig. 5.2 B) which was visible from 13 to 56 days post inoculation (Table

5.1). Later emerging leaves were symptomless. Both isolates induced a colour break in petals (Fig. 5.2 C).

In G2, all plants infected via seed with isolate S6 were symptomless, whereas some of the plants infected with isolate US showed mild leaf rolling (Table 5.1).

5.3.2 Changes in plant development and habit

As shown in Table 5.1, in G1, isolate S6 induced early emergence of axillary shoots 21 days after inoculation. Plants were then at the 11-12 node stage, and this produced a bushy appearance. Isolate US induced axillary shoot emergence at 28 days after inoculation when plants were at the 19-20 node stage. There were also fewer axillary shoots on the plants infected with isolate US than plants infected with isolate S6. Axillary shoots were produced in healthy plants from 35 days at the 22-23 node stage when the plants had already started to flower. Infected plants also showed a delay in their growth and development. In G1, isolate S6 delayed flowering, pod setting, pod maturation and plant maturity (Fig. 5.3 B) by about 14 days. Plants infected with isolate US showed a delay of 7 days in maturity.

In G2, the only change observed in plant development was that plants infected with isolate S6 showed a 7 day delay in maturity.

5.3.3 Effects on growth components

As shown in Table 5.1, infection with both isolates affected most of the growth components quantitatively. For example, in G1 the number of nodes was increased, but plant height was reduced (Fig. 5.3 A, B) and the internodes were shorter. The number of flowers was not affected, but for S6 the number of pods and number of seeds per pod

were reduced. Pods of infected plants (Fig. 5.4 A) were much shorter than healthy pods (Fig. 5.4 B) and also contained fewer seeds. Therefore, the number of seeds per plant was reduced in plants infected with isolates S6 or US. No significant effects were observed in these parameters in G2.

5.3.4 Effects on yield

In G1, dry shoot weight was higher in plants infected with isolate S6 or US than in healthy control plants (Table 5.1). Dry seeds harvested from infected plants (Fig. 5.5 B and C) were deformed, crinkled, smaller in size and lighter in weight than those of healthy plants (Fig. 5.5 A). Dry seed weight was reduced by 82% in plants infected with isolate S6 and by 35% in plants infected with isolate US.

In G2, dry seed weight was reduced in plants infected with isolate S6 by 52% and seeds were deformed and crinkled whereas dry seed weight and structure of plants infected with isolate US ^{was} not significantly affected.

5.3.5 Effects on germination

In G2, the germination of seeds collected from plants infected with isolates S6 and US was reduced to 78% and 91%, respectively, and was delayed by up to one week when compared to healthy seeds (Table 5.2). In G3, germination of seeds was reduced to 73% from plants infected with isolate S6 and 88% from plants infected with isolate US and was delayed by up to one week. Thus the rate of germination was about the same for seed collected from G1 and G2. Germination of seeds collected from plants (G2) negative to S6 and US was reduced to 89% and 91% respectively but no delay in germination was observed (Table 5.2).

5.3.6 Rate of seed transmission

As shown in Table 5.2, seed transmission was observed for both S6 and US in G2 and G3. In G2, 31% of seedlings were infected with isolate S6 and 8% were infected with isolate US. In G3, none of the seedlings raised from the seed of plants infected with either isolate S6 or US (G2) were virus positive. However, some of the ungerminated seeds dug up 2 weeks after sowing were found to contain S6 (12%) and US (7%) when tested by dot blot hybridisation (Table 5.2). Seedlings raised from the seed of plants negative to isolate S6 or US (G2) were also virus negative. No correlation was observed between seed colour and weight and the transmission of the S6 and US isolates through the seed.

5.4 DISCUSSION

Yield loss is an important criterion for determining the pathogenicity of viruses. The present results showed that the South Australian isolate S6 is more severe than isolate US in terms of symptom severity, effects on growth, seed yield and rate of seed transmission in pea (cv Dundale).

Isolate S6 which produced severe symptoms in mechanically inoculated G1 plants, did not produce symptoms after its transmission through seed to G2 plants. This resembles the sub-liminal infection previously reported by Ligat and Randles (1993). However isolate US produced mild symptoms in G1 and produced symptoms after its transmission through seed to G2 plants. This symptom variation and expression might be associated with different pathotypes of PSbMV (Maury and Khetarpal, 1992). The effects of isolate S6 on growth and yield of pea were greater than for isolate US in G1 as well as G2 plants (Table 5.1). These differences in the effects showed that isolate S6 (pathotype 4) is more severe than isolate US (pathotype 1). In addition, the symptomless infection by

only one
isolate with
pathotype

isolate S6 in G2 would hinder detection of the virus and may lead to a high incidence in the field which could be expected to reduce crop yield and produce a high level of seed infection in the subsequent generation.

It was interesting to observe that in G1 plants infected with either isolate S6 or US, the number of nodes was increased as compared to healthy controls. However, the plant height was reduced and internodes were shorter. Similarly, dry shoot weight was higher in isolates S6 and US infected plants than the healthy control (Table 5.1) probably due to the production of excessive axillary shoots.

It was found that G1 plants infected mechanically were more severely affected by isolates S6 and US than G2 plants infected via seed. Seed infection may have little effect on growth and yield of pea plants but would play a role in the form of infected seedlings which will act as an initial source of inoculum for the secondary spread of the virus by aphids in the field. It is likely that aphid inoculated plants would respond to infection similarly to G1 plants in this trial. Infected seed will also act as a carrier of the virus from one generation to the next and in dissemination of the virus from one locality to another with the movement of infected seed. Seed collected from infected plants were inferior in quality and quantity (Fig. 5.5) as compared to healthy control, and also showed delayed and reduced germination (Table 5.2).

The observation that isolate S6 produced more infected seed than isolate US in both generations may be determined by the virus isolate. However, it is unlikely that differences in the rate of seed transmission generally are due to the virus isolate only. Other factors which may influence seed transmission of a virus include host cultivars, environmental factors and plant age at infection (Bennett, 1969; Geering and Randles, 1994). The rate of seed transmission in pea varieties originating from Pakistan for isolate S6 ranged from 0 to 16% and for isolate US from 0 to 56%, which indicates that host

variety affects this parameter (Ali and Randles, 1997).

*This could also be
used for resistance to
seed transmission*

Apart from the actual losses in seed yield, an additional effect described here is the delay in maturity of infected plants which may lead to greater losses in the field if harvesting is done at first crop maturity. Likewise, this delay in plant maturity would result in a corresponding delay in marketable pea production. In conclusion, these results suggest that the agronomic effects of PSbMV-S6 would be a result of delayed maturity and reduced seed yield of infected plants rather than reduction in total dry matter production. Such an effect would make it difficult to recognise the importance of PSbMV infection in crops by field inspections.

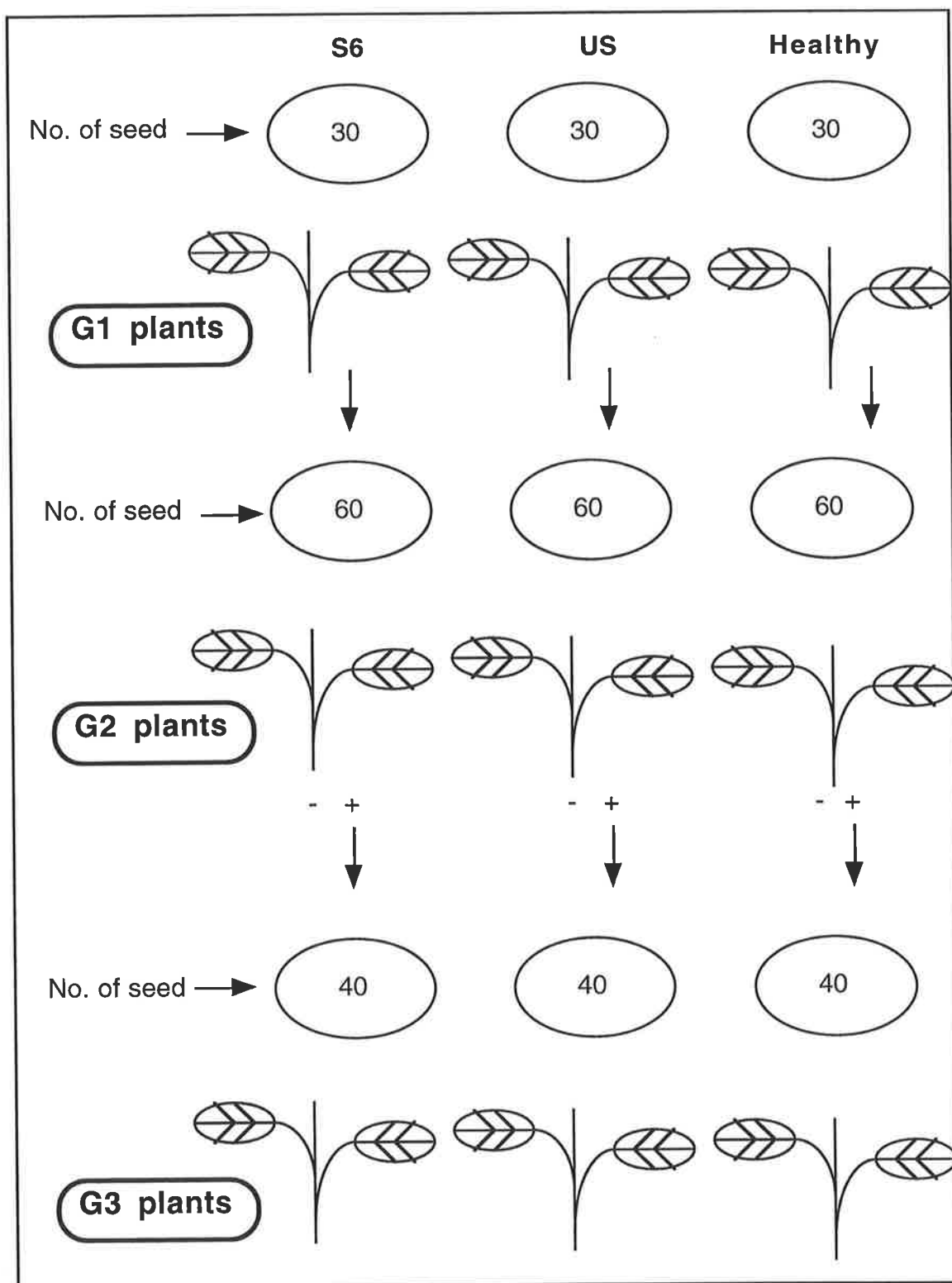


Fig. 5.1 Systematic representation of the study on pathogenesis conducted for isolates S6 and US of PSbMV in three sequential generations of pea cv Dundale.

(A)



(B)



(C)



Fig. 5.2 Systemic symptoms induced by PSbMV in inoculated Dundale pea. (A) isolate S6 showing severe mosaic, vein clearing and interveinal chlorosis, (B) isolate US showing leaf rolling, (C) isolate S6 showing colour break in petals, compared with healthy flower (left).

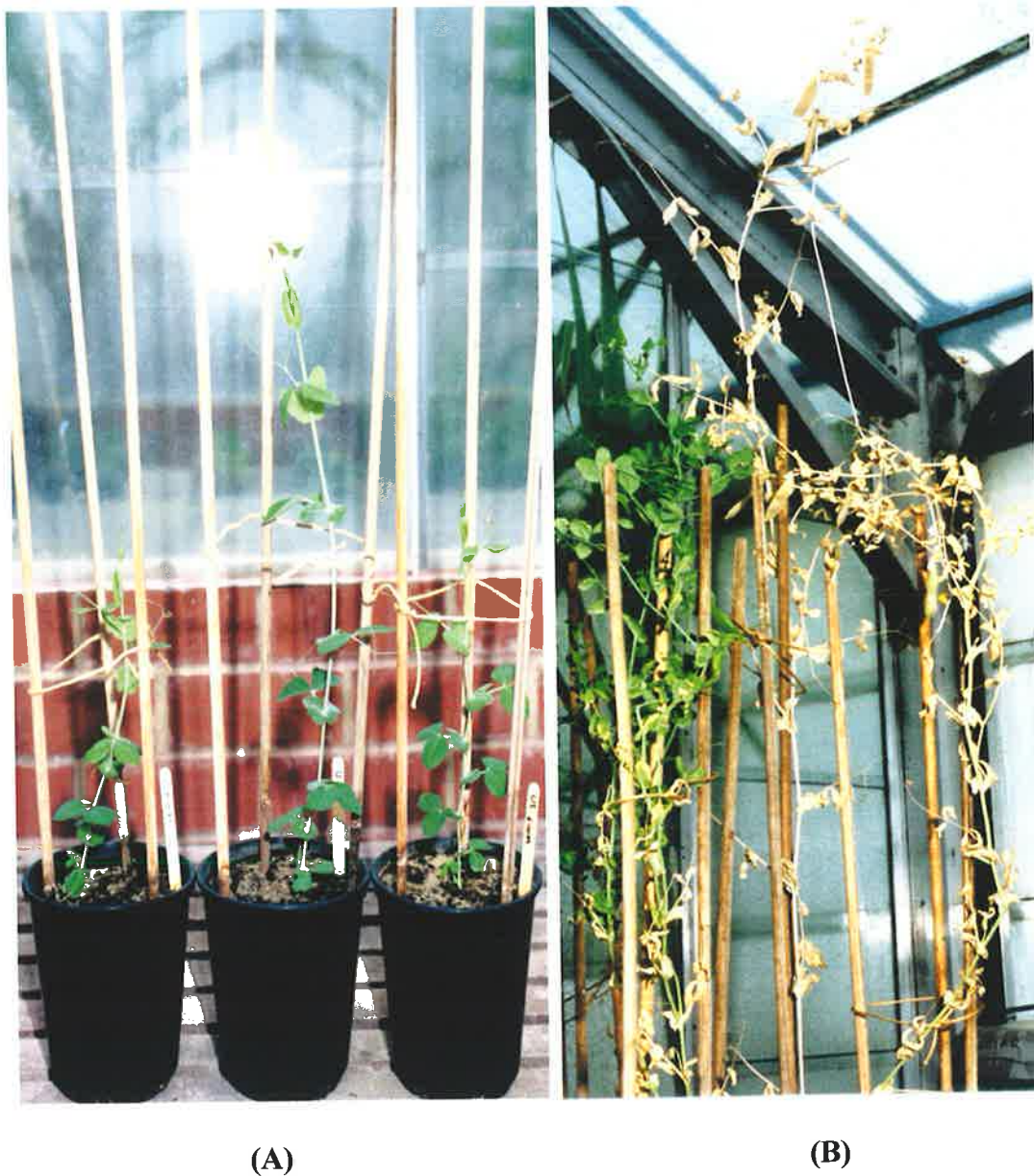


Fig. 5.3 Effect of PSbMV on plant height and plant maturity in generation 1. **(A)** Pea seedlings 2-weeks after inoculation with isolate S6 (left), and isolate US (right) compared with healthy (middle), **(B)** Plant infected with S6 (left) showing green leaves, whereas US infected (right) and healthy control plants (middle) have matured.



(A)



(B)

Fig. 5.4 Effect of PSbMV on pod size in generation 1. (A) PSbMV-infected plants showing short pods with few seeds, (B) healthy plant with normal size showing high number of seeds per pod.

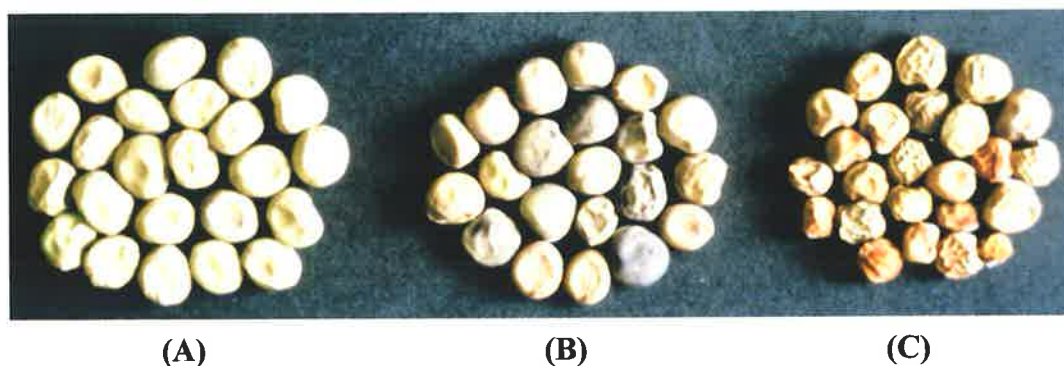


Fig. 5.5 Dry seed collected from (A) healthy plants, (B) US infected and (C) S6 infected plants in generation 1.

Table 5.1 Effects of PSbMV isolates S6 and US on growth and yield of pea in two sequential generations (G1-G2)

Character	G1 ^A			G2 ^B		
	Healthy	US infected	S6 infected	Healthy	US infected	S6 infected
<i>Development of plant</i>						
Time to first symptom (days)		13 ^C	6		12 ^D	NE
" " first detection of virus		14	14		14	56
" of symptom retention		56	98		56	N
" to first axillary bud	35	28	21	42	42	42
" " first flower	35	35	49	42	42	42
" " first pod set	42	42	56	49	49	49
" " first pod maturation	56	56	70	63	63	63
" " first plant maturity	77	84	91	84	84	91
<i>Habit of plant</i>						
Nodes on main stem	24.7 a ^F	27.3 b (1.11) ^G	31.2 c (1.26)	30.5 a	29.2 a	27.6 a
Nodes on axillary stems	5.1 a	6.6 a	9.4 b (1.8)	3.5 a	4.0 a	4.3 a
Axillary stems/plant	2.5 a	3.0 a	4.2 b (1.7)	2.1 a	2.4 a	2.3 a
Flowers/plant	5.6 a	5.4 a	5.2 a	3.4 a	3.0 a	2.8 a
Pods/plant	4.5 a	4.3 a	2.4 b (0.53)	2.4 a	2.1 a	2.2 a
Seeds/plant	14.0 a	11.1 b (0.79)	3.7 c (0.26)	7.3 a	6.3 a	5.9 a
<i>Growth (cm)</i>						
Plant height	139 a	124 b (0.89)	126 b (0.91)	173 a	176 a	166 a
Pod length	5.2 a	4.2 b (0.81)	3.1 c (0.60)	5.2 a	4.9 a	4.5 a
<i>Yield (g)</i>						
Top dry weight/plant	4.2 a	4.4 ab (1.05)	4.9 b (1.17)	4.8 a	4.6 a	4.3 a
Dry seed weight/plant	1.7 a	1.1 b (0.65)	0.3 c (0.18)	0.9 a	0.7 ab	0.4 b (0.48)

A Plants infected mechanically in G1
 B Plants infected via seed in G2
 C Days post inoculation
 D Days after emergence
 E Symptomless infection
 F Means within the rows followed by different letters are significantly different at P = 0.05
 G Ratio of infected: healthy

Table 5.2 Seed transmission and effect on germination of PSbMV isolates S6 and US in three sequential generations (G1-G3) of pea

Treatments	G 1		G2				G3				
	No. plants inoculated	%B infection	No. seed sown	% germination 7 days 14 days		% seedling ^E infection	No. seed sown	% germination 7 days 14 days		% seedling ^E infection	% ungerminated ^E seed containing virus
Isolate S6	30	100	60 ^C	70	78	31	41 ^F	65	73	0	12
							50 ^G	86	89	0	0
Isolate US	30	100	60 ^C	85	91	8	39 ^F	82	88	0	7
							50 ^G	90	91	0	0
Control	30 ^A		60 ^D	97	97		41 ^H	95	95		

A Uninoculated control plants
 B Seedlings tested by DIBA
 C Seed collected from S6 and US infected plants in G1
 D Seed collected from healthy control plants in G1
 E Seedlings or seed tested by DIBA and dot blot hybridisation
 F Seed collected from S6 and US infected plants in G2
 G Seed collected from S6 and US negative plants in G2
 H Seed collected from healthy control plants in G2

Chapter 6

SURVEY OF PSbMV IN PAKISTAN

6.1 INTRODUCTION

PSbMV has gained global attention due to its worldwide occurrence and seed transmitted nature. Since its discovery in Czechoslovakia in 1966 (Musil, 1966), PSbMV has been reported from more than 35 countries (Table 1.2) in the last 32 years.

In Pakistan, green pea is the second most important food legume crop after chickpea (*Cicer arietinum* L.), but production figures of 70,800 tons grown on an area of 141,400 ha (Anonymous, 1994) are well below the world average. Peas are grown in all provinces as a winter crop, but in the North West Frontier Province (NWFP) they are also grown as an autumn crop .

Epidemics of virus-like diseases are prevalent in winter grown peas in Pakistan but the causal viruses have not been identified. More than fifty viruses have been reported to infect peas naturally or experimentally (Hagedorn, 1984). Of these, PSbMV is one of the most important because it can reach a high incidence through combined seed and aphid transmission (Hampton and Mink, 1975; Hampton and Muehlbauer, 1977; Musil, 1980; Makkouk *et al.*, 1992; Ligat and Randles, 1993) and cause high yield losses (Anonymous, 1985; Anonymous, 1991). In Pakistan, a severe disease in lentil (*Lens culinaris* Med.) was attributed to PSbMV in 1992 (Aftab *et al.*, 1992), but the virus was first reported in experimental pea crops in 1993 (Bashir *et al.*, 1993).

This chapter describes a limited survey carried out for the first time for viruses in early winter grown pea crops in NWFP of Pakistan. In particular, it reports the detection of four pathotypes of PSbMV, two of which do not fit into the existing classification and thus represent previously undescribed pathotypes (Ali and Randles, 1997).

6.2 MATERIALS AND METHODS

6.2.1 Seed, antisera and virus sources

Seeds of indicator plants, pea genotypes and Pakistani pea varieties are listed in Table 2.1. Sources of antisera used for pea viruses are mentioned in section 2.1.3. PSbMV isolates and other pea viruses are described in section 2.1.2.

6.2.2 Survey area

Sixty-two commercial pea fields or experimental plots from 45 locations in 8 districts of the major pea growing areas of NWFP Pakistan were surveyed for pea viruses during November and December, 1995. Three of the districts, Abbottabad, Haripur and Swat (Table 6.1) were in the hilly region, the remainder were on the plains.

6.2.3 Sampling

Leaf samples comprising 2-3 leaflets were collected from plants using a systematic random crop sampling procedure (Barnett, 1986). Symptomatic plants observed during the survey were sampled further by also taking flowers and pods containing green seed. Samples were placed separately in plastic bags, transported on ice to the laboratory, extracted and applied to the membrane (see below) within 24 hr of collection. The crops sampled varied in time of sowing, developmental stage, cultivar and site. Samples were collected and tested serologically for 14 different viruses: alfalfa mosaic alfamovirus (AMV), bean leafroll luteovirus (BLRV), beet western yellows luteovirus (BWYV), bean yellow mosaic potyvirus (BYMV), broad bean mottle bromovirus (BBMV), broad bean true mosaic comovirus (BBTMV), cucumber mosaic cucumovirus (CMV), cowpea chlorotic mottle bromovirus (CCMV), lettuce mosaic potyvirus (LMV), pea

enation mosaic enamovirus (PEMV), peanut mottle potyvirus (PMV), peanut stunt cucumovirus (PSV), PSbMV and pea stem necrosis virus (PSNV). Species of both aphids and weeds within the pea field or adjacent to it were recorded and some weeds were sampled. Leaf samples from serologically virus positive plants were dried over calcium chloride for transfer to the Waite Campus.

6.2.4 Sample preparation

Leaf tissue samples of 0.5 g were placed in individual plastic bags (75 × 140 mm) and extracted by crushing with one volume (w/v) of PBS (Table 2.3). One hundred seeds of each variety collected during the survey (Arkeel AP-1, Climax, Dasana, Hazara Local, Mashang, Nauvana, Peshawar Local-I, Peshawar Local-II, PF-400, Rondo, Swabi Local, and VIP) were soaked overnight in 10 ml PBS before extracting individually as above. In addition, 40 seeds of each of the above 12 varieties were germinated and grown in a glass house held at 20-25°C. At five weeks after emergence, a 0.5 g sample comprising leaflets from the youngest compound leaf of each seedling was extracted as above. All extracts were centrifuged at 10,000 × *g* for 5 min. Aliquots of 1 µl were dotted onto nitrocellulose membrane. The membrane was air dried and analysed either immediately or stored at 4°C. Residual extracts of the seed samples were stored at -20°C for 6 days before inoculation onto test host species. Positive controls comprising sap from AMV, BLRV, BYMV, CMV or PSbMV infected leaves were dotted onto membranes at dilutions of 1/1, 1/10 and 1/100.

6.2.5 Detection by DIBA

DIBA (section 3.2.5.4) was used to test 713 samples (Table 6.1) collected from the field during the survey, 1200 individual seeds (Table 6.2) of 12 Pakistani pea varieties (100 seeds/variety) and 301 seedlings (Table 6.2) from the germinated seeds of the Pakistani

pea varieties.

6.2.6 Bioassay of PSbMV isolates and determination of pathotype

PSbMV isolates positive by DIBA in field samples, seed extracts or seedlings were transferred by mechanical inoculation (section 2.2.2) to *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* L. and pea cv Dundale. Each selected isolate was also inoculated to three seedlings of the pathotype (Tables 6.4) (Alconero *et al.*, 1986) and host specific (Table 6.5) (Hampton *et al.*, 1981) pea differential genotypes.

6.2.7 Screening of Pakistani pea varieties for susceptibility to PSbMV isolates S6 and US

Pea varieties collected from Pakistan during the survey were tested for susceptibility to the isolates S6 and US of PSbMV. Four or five seedlings of each variety were mechanically inoculated with infected sap (diluted 1:2 w/v in 50 mM sodium borate buffer, pH 8.1) of isolates S6 and US. Symptoms were recorded at 2 weeks after inoculation and each plant was tested by DIBA. The plants were grown to maturity and the seeds also tested by DIBA for seed transmissibility of the two isolates S6 and US.

6.3 RESULTS

6.3.1 Detection of BYMV, CMV and PSbMV in surveyed crops

The results of the survey for viruses present in pea detected by DIBA (Fig. 6.1) are shown in Table 6.1. Eighty two samples were positive for at least one of the three viruses, BYMV, CMV and PSbMV. None of the remaining 11 viruses (AMV, BLRV, BWYV, BBMV, BBTMV, CCMV, LMV, PEMV, PMV, PSV, PSNV) were detected in

any of the samples tested. Of the three viruses, BYMV had the highest incidence occurring in 67 samples at 4 sites, followed by PSbMV in 11 samples at 5 sites and CMV in 4 samples at 2 sites. All three viruses were detected in samples taken from plants at the pre-flowering, flowering and post flowering stages but no 2-3 week old plants were virus positive. Weed species found were *Amaranthus* sp., *Brassica* spp., *Chenopodium* spp. (including *C. amaranticolor* Coste and Reyn.), *Cucumis sativus* L., *Datura stramonium* L., *Lens culinaris*, *Phaseolus lathyroides* L., *Spinacia oleracea* L., and *Vicia faba* L. Of these only *Vicia faba* was positive for BYMV. Pea aphids (*Acyrtosiphon pisum* Harris) (Fig. 6.2 B) were observed on BYMV and PSbMV infected plants in the hilly areas. Only one sample had a mixed infection and contained BYMV and PSbMV. A variety of symptoms was observed in the field including severe mosaic, transient vein clearing, stunting, rosetting, and mild leaf rolling. Most of the plants showing severe mosaic and transient vein clearing were positive for BYMV. Of the 11 samples positive for PSbMV by DIBA, three showed mild leaf rolling and slight mosaic (Fig. 6.2 A, B) while eight showed stunting and apical rosetting.

6.3.2 Detection of PSbMV in seed and seedlings

Seeds from 5 of the 12 commercial and local varieties from Pakistan tested were infected with PSbMV (Table 6.2). The highest incidence was found in variety Rondo (5%). Seedlings from three of the above 12 pea varieties were infected with PSbMV (Table 6.2 and Fig. 6.3). When observations were made on seedlings one week after emergence, one seedling each of Dasana and Rondo varieties showed severe leaf rolling. These infected seedlings remained stunted with short internodes and few flowers up to maturity. After five weeks the number of symptomatic seedlings increased to 2 for Dasana, 3 for Rondo and one for Swabi Local and all showed mild leaf rolling, reduction of the leaf lamina and vein banding. When tested by DIBA the

number of positive seedlings was 3 for Dasana and 5 for Rondo, indicating that symptomless infection occurred (Table 6.2). Seeds and seedlings of the varieties: Arkeel AP-1, Hazara Local, Mashang, Nauvana, Peshawar Local-II, and PF-400 were negative for PSbMV. All the varieties showed poor emergence except for VIP as shown in Table 6.2. None of the seeds or seedlings were positive for BYMV.

6.3.3 Pakistani PSbMV isolates

Twelve Pakistani PSbMV isolates (PK1-PK12) were isolated from pea seedlings, positive seed extracts and the field during the survey (Table 6.3). PSbMV isolate PK1 was obtained from a seedling of variety Swabi Local; isolates PK2 and PK3 were from Dasana seedlings; isolates PK4 and PK5 were from Rondo seedlings; isolates PK6, PK7, PK9 and PK10 were from DIBA positive seed extracts of Rondo; and isolate PK8 was from a positive seed extract of variety VIP. Isolates PK11 and PK12 were collected from field plants during the survey.

All of the 12 isolates produced necrotic local lesions on *C. amaranticolor*, chlorotic local lesions on *C. quinoa*, and systemic leaf rolling and mosaic in Dundale pea (Fig. 6.4). However, none of the DIBA positive seed extracts from Climax, Dasana, and Peshawar Local-I were infectious to the above indicators.

All of the 12 Pakistani PSbMV isolates gave a positive serological reaction to PSbMV-S6 antiserum when five-fold dilution series of each isolate was tested by DIBA (Fig. 6.5). When the capsid protein was analysed and compared by SDS-PAGE and western blot analysis, no differences were observed within the 12 PSbMV isolates and with isolates S6 and US of PSbMV (Fig. 6.6). Among the 12 Pakistani PSbMV isolates, isolates PK4 and PK9 (Tables 6.4) were purified (Fig. 6.7) (section 3.2.2) and RNA was isolated (Fig. 6.8) (section 2.2.8.1). No difference was observed in the purified

particles or RNA of isolates PK4 and PK9 when compared with isolates S6 and US.

6.3.4 Reaction of Pakistani PSbMV isolates on pea differential genotypes

The 12 PSbMV isolates (PK1-PK12) were classified into pathotypes by the reactions induced on the standard pathotype specific pea differential genotypes (Alconero *et al.*, 1986). None of the isolates infected lines 193586 or 193835 (Table 6.4) which are reported to be resistant to PSbMV. Isolates PK2, PK3, PK6, PK7 and PK10 were typical of pathotype P-1 as shown by their ability to infect lines 347329 and 347422. Isolates PK8 and PK12 were typical of pathotype P-4 by infecting only line 269774 (Alconero *et al.*, 1986). Isolates PK1, PK4, PK5 and PK11 were not typical of either P-1 or P-4 due to their inability to infect lines 347329 and 269774 and were classified as unknown 1 (U-1). Isolate PK9 differed from all of the other isolates, in infecting both the P-1 and P-4 resistant lines. This was named as unknown 2 (U-2).

In addition, the host group specific pea differential genotypes (Hampton *et al.*, 1981) were used to distinguish the 12 Pakistani PSbMV isolates. All the isolates (Table 6.5) uniformly caused rapid whole necrosis of line 272146 (Host group I) confirming their identity as PSbMV. None of the isolates infected line 269777 (Host group IV) which was previously reported to be infected by PSbMV (Hampton *et al.*, 1981).

6.3.5 Screening of pea varieties for resistance to isolates S6 and US

Of the 12 pea varieties from Pakistan, nine were susceptible to either isolate S6 or US (Table 6.6). The varieties VIP, Climax and Swabi Local were not susceptible to either pathotype as shown by absence of symptoms and negative reactions by both DIBA and indexing on *C. amaranticolor*. Isolate S6 produced mild to severe mosaic and stunting in the three varieties it infected at only one week after inoculation while isolate US

produced typical mild leaf rolling in all nine varieties that were infected at 2 weeks after inoculation. In susceptible varieties the seed transmission of isolate S6 ranged from 0 to 16% and from 0 to 56% for isolate US. Examples of symptomless infection were obtained for the varieties Rondo, Arkeel AP-1, and Peshawar Local-II (Table 6.6) as shown by testing with DIBA.

6.4 DISCUSSION

This survey is the first done for pea viruses in Pakistan and showed that natural infection with BYMV, CMV, and PSbMV occurred in the field. The survey results indicated that of these three viruses, PSbMV was the most widespread, occurring in 5 of the 8 districts surveyed. The incidence of PSbMV in sampled crops ranged from 0 to 8%, varying with respect to variety, seed source, locality and possibly with migratory aphid activity. The incidence of PSbMV in dry seed (0 to 5%) was similar to that detected for field samples, suggesting that there had been little secondary spread of PSbMV up to the time of the survey (early winter). The absence of infection in sampled weeds (data not shown) also supports the view that they were not a source of virus, nor had PSbMV spread into them. Seed is therefore considered to be the main reservoir of PSbMV for survival between seasons.

Some of the plants showing virus-like symptoms failed to react with any of the virus antisera tested. These plants may be infected with viruses other than those tested for in this study or may have been exposed to other pathogens or environmental stresses leading to the production of virus-like symptoms. In the Abbottabad and Charsada (Table 6.1) districts where no virus infection was found, sample size would have been too small to detect infection levels below 10%.

The incidence of PSbMV in dry seed from the 5 virus positive varieties was 1 to 5%

(Table 6.2) and was similar to the incidence of virus (0 to 4.3%) on the basis of symptoms in one week old glasshouse seedlings raised from the same varieties. However, at 5 weeks post-emergence, the percentage of plants infected with PSbMV was determined to be between 0 and 12% by symptoms and between 0 and 20% by DIBA. This increase in PSbMV incidence in young plants with time may be explained by delayed movement of PSbMV from infected embryos (Khetarpal and Maury, 1990). This implies that seed transmission should be evaluated on 5 week old plants rather than seed or young seedlings. The observed differences between seed and seedling levels of infection are presumably due to the different rates of infection of the testa and embryo (Masmoudi *et al.*, 1994).

PSbMV isolates were grouped into the existing pathotype classification (Alconero *et al.*, 1986) by their ability to infect the range of standard pea differential genotypes. Isolates which did not fit into the existing classification and were therefore tentatively placed in groups U-1 and U-2 could possibly be renamed as pathotypes P-5 and P-6 but further characterisation of these is needed to define their biological properties. Chapter 8 and 9 report on molecular properties of these isolates. On the basis of host specific genotypes, symptom variation was observed among the isolates, indicating that there is marked biological variability between isolates within the same pathotype. None of the 12 Pakistani isolates infected line 269777 (Table 6.5) which was reported to be infected by the PSbMV isolates reported by Hampton *et al.* (1981). It will be important to investigate and compare why the Pakistani isolates can not infect this line while others do.

In the pea varietal screening, the varieties Climax, Swabi Local and VIP remained uninfected after mechanical inoculation by either US or S6. However, Swabi Local and VIP were not immune to PSbMV because they had yielded isolate PK1 (U-1) and isolate PK8 (P-4) (Table 6.4) respectively. The DIBA positive seed extract from Climax

was not classified because it did not infect indicator hosts. The virus in this variety may have been limited to the testa whereas in the other varieties PSbMV probably infected the embryo and would therefore have been transmissible to the next generation. Further resistance testing should include transmission of US and S6 by aphids (Aapola *et al.*, 1974). It is also possible that isolates PK1 (U-1) and PK8 (P-4) are biologically different from US and S6 of Ligat *et al.* (1991) and are able to overcome the resistance of these varieties. Further research is reported in chapter 8 and 9 on their molecular properties.

It is interesting to speculate that this wide variation among the PSbMV isolates from Pakistan is related to the long association of *Pisum* with this geographical region of the world (Makasheva, 1983). Perhaps the centre of diversity of the crop can be correlated with high diversity in a seed-borne virus such as PSbMV.

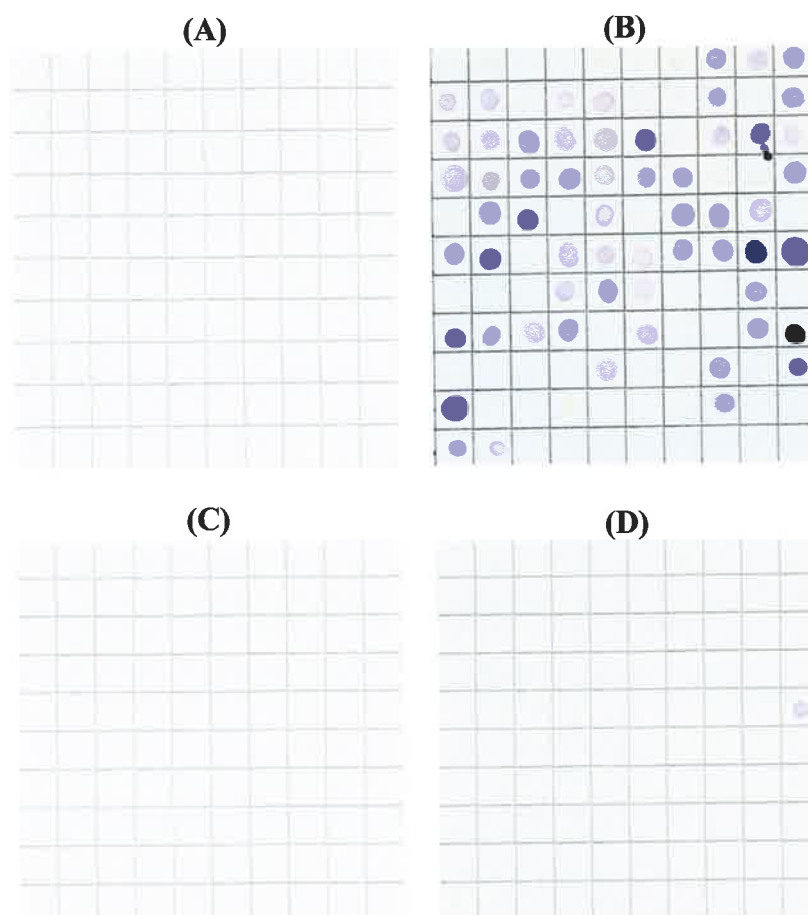


Fig. 6.1 Example of the results of DIBA for leaf samples collected in a survey in Pakistan. Each membrane was dotted with 100 samples in 4 replicates. **(A)** Membrane was probed with AMV antiserum, **(B)** membrane with BYMV antiserum, **(C)** membrane with CMV antiserum and **(D)** membrane probed with PSbMV antiserum.

(A)



(B)



Fig. 6.2 Field symptoms of PSbMV. (A) Leaf of a sample collected during the survey in Pakistan which was positive by DIBA to PSbMV antiserum and showed a slight mosaic and leaf rolling, (B) PSbMV DIBA positive field sample infested with pea aphids (*Acythosiphon pisum*).

(A)



(B)



Fig. 6.3 Infected seedlings (5 weeks old) from Pakistani pea varieties (A) Dasana and (B) Rondo, germinated in the glasshouse at the Waitc Campus showing leaf rolling symptoms attributed to PSbMV.

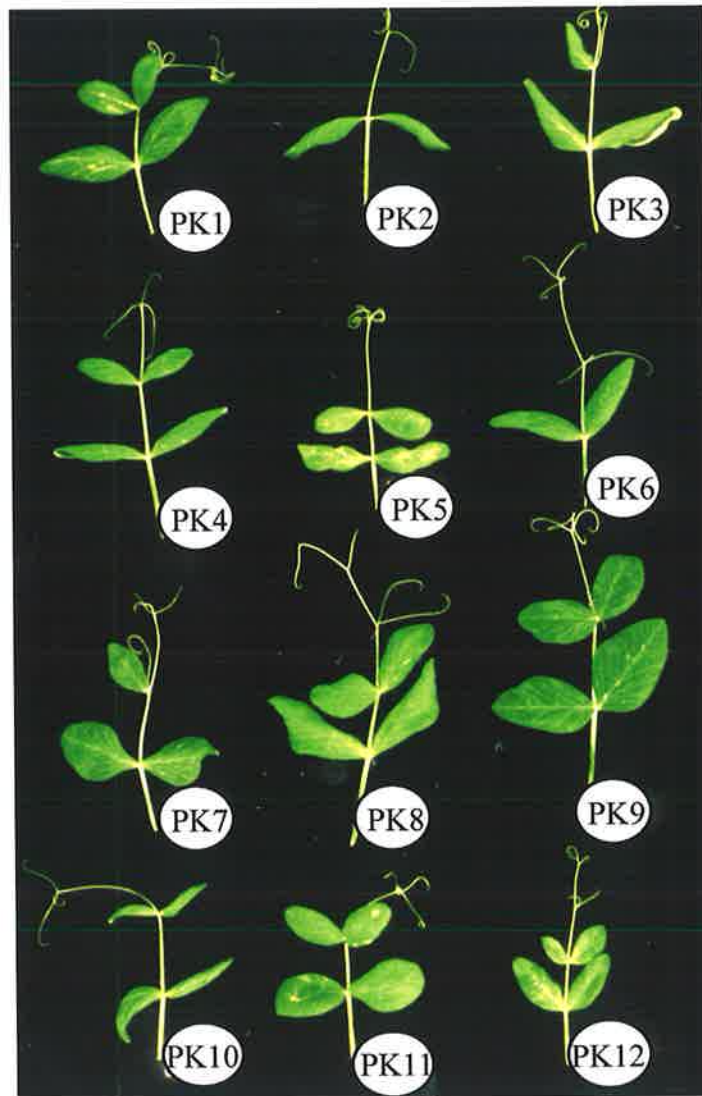


Fig. 6.4 Symptoms of the Pakistani PSbMV isolates (PK1-PK12) on leaf (nodes 6-7) of pea cv Dundale.

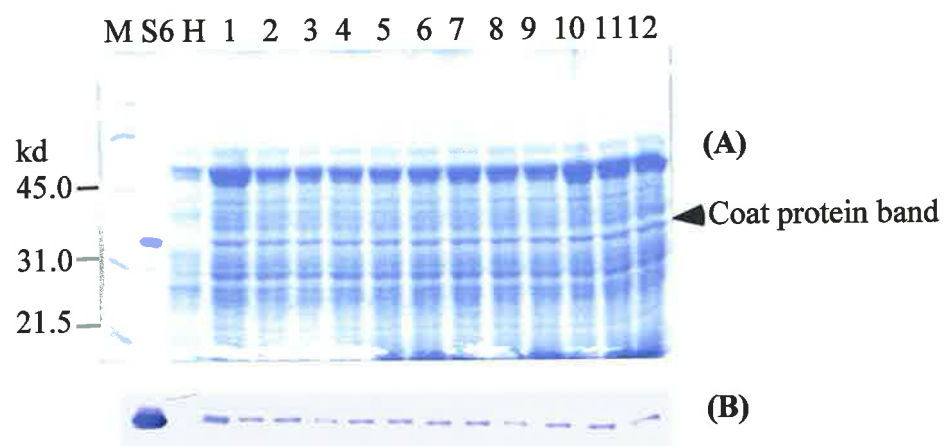
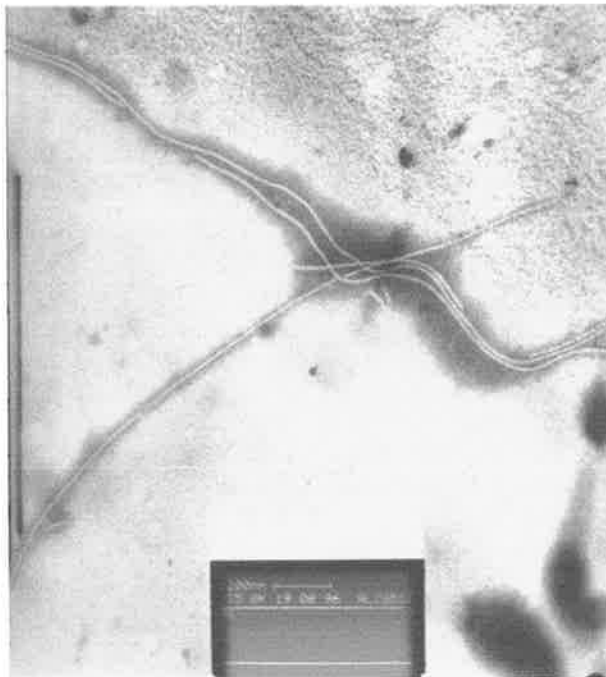


Fig. 6.6 Analysis of capsid protein of Pakistani PSbMV isolates by (A) SDS-PAGE and (B) western blotting. Protein marker (M), purified preparation of isolate S6 (lane 2), healthy sap (lane 3), isolates PK1-PK12 (lane 4-15).

(A)



(B)



Fig. 6.7 Electron micrograph of purified preparation of PSbMV isolates. (A) Isolate PK4 and (B) isolate PK9.

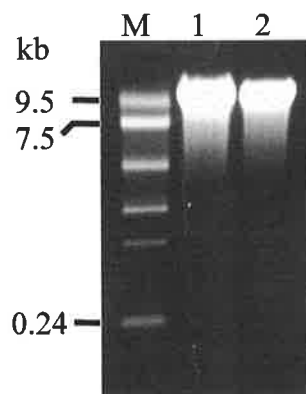


Fig. 6.8 Formaldehyde denaturing 1% agarose gel electrophoresis showing RNA extracted from purified PSbMV preparations. RNA marker (M), isolate PK4 RNA approximately 9.5 kb (lane 1), and isolate PK9 RNA approximately 9.5 kb (lane 2).

Table 6.1 Incidence of viruses in pea crops in North West Frontier Province of Pakistan as determined by DIBA

District	No. of locations	No. of fields	No. of samples	No. of plants positive for		
				BYMV	CMV	PSbMV
Abbottabad	1	1	18	0	0	0
Charsada	1	1	11	0	0	0
Haripur	7	14	184	62	0	2
Mardan	4	5	50	2	0	0
Malakand Agency	2	4	41	0	0	1
Peshawar	17	22	240	2	2	2
Swabi	7	9	112	0	2	2
Swat	6	6	57	1	0	4
Total	45	62	713	67 (9.4%)	4 (0.57%)	11 (1.5%)
The above collected samples were also tested against the antisera of the following viruses but all of them were negative.						
Alfalfa mosaic virus (AMV)		Cowpea chlorotic mottle virus (CCMV)				
Bean leafroll virus (BLRV)		Lettuce mosaic virus (LMV)				
Beet western yellow virus (BtWYV)		Pea enation mosaic virus (PEMV)				
Broad bean mottle virus (BMV)		Peanut mottle virus (PMV)				
Broad bean true mosaic virus (BBTMV)		Pea stunt virus (PSV) and Pea stem necrosis virus (PSNV)				

We have these positive samples for all viruses

Dr. P. A. B.

Table 6.2 Detection of PSbMV by dot immunobinding assay in dry seed and seedlings germinated from seeds of Pakistani pea varieties

Variety	No. of seeds ^a		No. of seedlings ^a			Percent Infection
	tested	positive	tested	symptomatic ^b	positive	
Arkeel AP-1	100	0	25	0	0	0
Climax	100	1	32	0	0	0
Dasana	100	2	23	2	3	13
Hazara Local	100	0	26	0	0	0
Mashang	100	0	21	0	0	0
Nauvana	100	0	23	0	0	0
Peshawar Local-I	100	2	25	0	0	0
Peshawar Local-II	100	0	25	0	0	0
PF-400	100	0	23	0	0	0
Rondo	100	5	25	3	5	20
Swabi Local	100	0	13	1	1	8
VIP	100	1	40	0	0	0

^a All seed and seedling were also tested to BYMV but none of them was positive

^b Five weeks after emergence

Table 6.3 Names and sources of Pakistani PSbMV isolates

Isolate name	Source	Variety	Symptoms produced	DIBA detection
PK1	Glasshouse seedlings	Swabi Local	VC, lr	+
PK2	Glasshouse seedlings	Dasana	LR	+
PK3	Glasshouse seedlings	Dasana	LR	+
PK4	Glasshouse seedlings	Rondo	lr	+
PK5	Glasshouse seedlings	Rondo	lr	+
PK6	Seed extract	Rondo	lr	+
PK7	Seed extract	Rondo	lr	+
PK8	Seed extract	VIP	M+lr	+
PK9	Seed extract	Rondo	M	+
PK10	Seed extract	Rondo	LR	+
PK11	Pea field	Peshawar Local-I	lr	+
PK12	Pea field	Peshawar Local-I	M+VC	+
Symptom symbols: VC= vein clearing; LR, lr = leaf roll (severe, mild); M, m = mosaic (severe, mild)				

Pathotype ^a specific genotypes	Pakistan PSbMV Isolates													
US ^b	S6 ^b	PK1	PK2	PK3	PK4	PK5	PK6	PK7	PK8	PK9		PK10	PK11	PK12
193586	R	R	R	R	R	R	R	R	R	R		R	R	R
193835	R	R	R	R	R	R	R	R	R	R		R	R	R
269774	R	S	R	R	R	R	R	R	S (M)	S(M+IC)		R	R	S (M)
347329	S	R	R	S (lr)	S (lr)	R	R	S (lr)	S (LR)	R		S (lr)	R	R
347422	S	R	S (lr) ^c	S (lr)	S (lr)	S (lr)	S (lr)	S (lr)	S (lr)	R		S (lr)	S (lr)	R
Pathotype grouping	P-1	P-4	U-1	P-1	P-1	U-1	U-1	P-1	P-1	P-4	U-2	P-1	U-1	P-4

^a Alconero *et al.* (1986)

^b Ligat *et al.* (1991)

^c Symptom symbols: LR, lr = leaf roll (severe, mild); M, m = mosaic (severe, mild); IC = interveinal chlorosis; R = resistant as indicated by absence of symptoms and negative DIBA; S = susceptible

^b Ligat *et al.* (1991)

C Symptom symbols: LR, lr = leaf roll (severe, mild); M, m = mosaic (severe, mild); IC = interveinal chlorosis; R = resistant as indicated by absence of symptoms and negative DIBA; S = susceptible

Table 6.5 Reactions of Pakistani PSbMV isolates on host specific pea differential genotypes 2 weeks after mechanical inoculation

		Pakistani PSbMV Isolates													
Host group	Host specific ^a genotypes	US	S 6	PK 1	PK 2	PK 3	PK 4	PK 5	PK 6	PK 7	PK 8	PK 9	PK 10	PK 11	PK 12
I	272146	NI ^b	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
III	269804	lr	M	LR	LR	lr	LR	lr	lr	LR	M+lr	M	LR	LR	M+VC
III	272171	I	M	lr	lr	lr	VB	lr	lr	lr	M+lr	M	lr	LR	M
IV	269777	NT	NT	I	I	I	I	I	I	I	I	I	I	I	I
V	269774	I	M	I	I	I	I	I	I	I	M	M+IC	I	I	M

^a Hampton *et al.* (1981)

^b Symptom symbols: NI = rapid whole plant necrosis and death; LR, lr = leaf roll (severe, mild); M = severe mosaic; VB= vein banding and leaf size reduction; VC= vein clearing; IC = interveinal chlorosis; NT = not tested; I= immune as indicated by absence of symptoms and negative DIBA

Table 6.6 Susceptibility of pea varieties to infection with PSbMV isolates US and S6

Varleites	Proportion symptomatic		Proportion of seedlings positive by DIBA		Proportion of seed ^a positive by DIBA		% seed transmission	
	U S	S 6	U S	S 6	U S	S 6	U S	S 6
Arkeel Ap-1	2/4 (lr)	0/4	4/4	0/4	8/17		47	
Climax	0/5	0/5	0/5	0/5				
Dasana	4/4 (lr) ^b	0/4	4/4	0/4	7/19		37	
Hazara Local	3/4 (lr)	0/4	3/4	0/4	2/23		9	
Mashang	4/4 (lr)	2/4 (M)	4/4	2/4	0/15	0/8	0	0
Nauvana	4/4 (lr)	0/4	4/4	0/4	10/18		56	
Peshawar Local-I	2/3 (lr)	0/4	2/3	0/4	5/27		19	
Peshawar Local-II	1/4 (lr)	2/3 (M)	2/4	2/3	2/30	4/25	7	16
PF-400	4/5 (lr)	0/4	4/5	0/4	4/8		50	
Rondo	1/4 (lr)	1/4 (m)	2/4	1/4	1/8	1/25	13	4
Swabi Local	0/3	0/3	0/3	0/3				
VIP	0/5	0/5	0/5	0/5				

^a Seed collected from infected plants

^b Symptom symbols: M, m = mosaic (severe, mild); Lr, lr = leaf rolling (severe, mild)

Numerator = number symptomatic or positive, denominator = number observed or tested

Chapter 7

PCR AMPLIFICATION AND CLONING OF REGIONS OF THE PSbMV GENOME

7.1 INTRODUCTION

The polymerase chain reaction (PCR) was first developed by Saiki *et al.* (1985). PCR rapidly amplifies DNA sequences or transcripts in vitro with very high specificity using oligonucleotide primers and *Taq* DNA polymerase in a simple automated reaction. PCR has the ability to amplify a specific DNA sequence from as short as 50 nt to over 10,000 nt in length, more than a million fold in a few hours through repetitive cycles of DNA synthesis. Currently, PCR can be applied in the areas of disease diagnosis, detection of pathogens, DNA comparisons, cloning of genomic sequences and gene sequencing (Hadidi *et al.*, 1993).

From the results obtained in previous Chapters, isolates S6, US and 12 Pakistani (PK1-PK12) PSbMV isolates were not distinguishable by a serological comparisons but showed biological differences. To differentiate these PSbMV isolates at the nucleotide level, PCR was applied to amplify specific gene products using PSbMV specific primers. Genome segments including either of two genes, the coat protein (CP) or the helper component (HC-Pro) were targeted for this study.

The primary functions of the CP are to encapsidate viral RNA and to determine aphid transmissibility (section 1.13.2). The CP is the most extensively characterised potyviral gene product in terms of sequence data. The interest in CP comes mainly from its usefulness in taxonomic and evolutionary studies, in diagnosis and CP-mediated resistance (Riechmann *et al.*, 1992). The high variability of amino acids observed in the surface exposed N-terminal region of potyvirus CP suggests that it is involved in virus specific functions or host-vector-virus interactions. The CP could for example, affect host range and symptom induction (Ward and Shukla, 1991) while the conserved DAG motif at the N-terminal end of CP is involved in virus transmission by aphids (section 1.13.2).

The HC-Pro is responsible for aphid transmission and polyprotein processing, symptom expression, virus replication and movement (section 1.13.2). Recently, Johansen *et al.*, (1996b) demonstrated that the N-terminal two thirds of the HC-Pro of PSbMV has an active role in seed transmission.

This chapter describes the amplification of the 3' region of isolates S6, US, PK4 and PK9, and the 5' region of isolate S6 by PCR and their cloning in a transcription vector. The 3' region covers a part of the Nlb gene, full CP gene and UTR of the PSbMV genome excluding the poly (A) tail while the 5' region covers a part of the P1-Pro gene and the full HC-Pro gene. As shown in Fig. 7.2, the PCR product of the 3' region for isolate S6 is named S6 1.7, for isolate US it is US 1.7, for isolate PK4 it is PK4 1.3, for isolate PK9 it is PK9 1.3 and for the 5' region PCR product for isolate S6 it is named S6 1.8.

7.2 MATERIALS AND METHODS

7.2.1 Reverse transcription (RT) and PCR primers

RT and PCR primers are listed in Table 2.2. All the primers were designed and prepared (section 2.1.4) from the published sequences of PSbMV isolates DPD1 and NY (Table 1.4). The melting temperature, hairpin structure and duplex formation of each of the primers was analysed by Oligo™. The primer with highest primability and stability, with least hairpin and duplex formation were selected for PCR. An example is shown in Fig. 7.1.

7.2.2 First strand cDNA synthesis by reverse transcription

First strand cDNA was synthesised by reverse transcription (section 2.2.13.1). From 1

to 2 µg of PSbMV RNA was mixed with 100 pmole of the reverse primer (Table 2.2) in a total reaction volume of 25 µl.

7.2.3 Second strand cDNA synthesis and PCR amplification

Second strand cDNA was synthesised and amplified by PCR (section 2.2.13.2) (Fig. 7.2). PCR was optimised and done using the following temperature regime: 1 cycle of denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 sec, annealing from 65 to 70°C for 30 sec, and extension at 72°C for 1 min. An extension period of 7 min was applied after the 30th cycle, then the product was held at 4°C.

7.2.4 Analysis, elution and cloning of PCR products

PCR reaction products were fractionated on a 1.0% agarose gel (section 2.2.11.1) and bands of the expected size were excised and purified from the gel (section 2.2.12). The excised fragments were directly ligated (section 2.2.14.1) into the T-tailed transcription vector pGEM®-T (section 2.1.5). The resulting plasmids were used to transform *E. coli* DH5α either by electroporation (section 2.2.14.3.a) or heat shock (section 2.2.14.3.b). The plasmid DNA was prepared from transformed bacteria (section 2.2.15). The recombinant plasmid DNA was further checked for the presence of insert by restriction digestion (section 2.2.16)

7.3 RESULTS

7.3.1 Amplification of the 3' region

Analysis of the PCR reaction products showed a single band of ca. 1.7 kb for isolate S6 (Fig. 7.3 A) and isolate US (Fig. 7.4 A) when the primer pair AK1 and P4F2 (Table 2.2)

were used (section 7.2.3) at an annealing temperature of 70°C. The same 1.7 kb product was also obtained for both isolates S6 and US using an annealing temperature of 65-69°C but additional non-specific bands of different sizes were observed (data not shown).

The above primer pair (AK1 and P4F2) did not amplify a 1.7 kb product at the 3' region for isolate PK4 but it did amplify a 1.7 kb product for isolate PK9 (data not shown). The primer set AK1 and P4F1 (Table 2.2) amplified ca 1.3 kb of the 3' region (Fig. 7.5 A) for both isolates PK4 and PK9 of PSbMV. PCR was done at the same temperature regimes as above except that annealing was at 65°C. No PCR product was observed for the negative controls.

7.3.2 Amplification of the 5' region

Agarose gel electrophoresis of the PCR reaction products showed a single band of ca 1.8 kb for isolate S6 (Fig. 7.6 A) using primer pair AK3 and AK4 (Table 2.2). PCR was done using temperature regimes as in section 7.2.3 except that the annealing temperature was 65°C. This primer pair did not amplify the 5' region from the RNA of isolates US, PK4 or PK9.

7.3.3 Cloning of the 3' region and the 5' region

The PCR products of the 3' region for isolates S6 (Fig. 7.3 A), US (Fig. 7.4 A), PK4 and PK9 (Fig. 7.5 A) and the 5' region of isolate S6 (Fig. 7.6 A) were eluted (section 2.2.12) and cloned (section 2.2.14.1) in the pGEM[®]-T vector. The PCR product of the 3' region for isolate PK4 was not cloned in several attempts. Recombinant plasmids containing inserts of either the 3' region of isolates S6, US and PK9 or the 5' region of isolate S6 were double digested with *Pst* I and *Nco* I endonucleases. The presence of cDNA

inserts was determined by agarose gel electrophoresis and are shown in Fig. 7.3 B (isolate S6), Fig. 7.4 B (isolate US), Fig. 7.5 B (isolate PK9) and Fig. 7.6 B (isolate S6).

7.3.4 PCR of the 3' region of PSbMV isolates PK1-PK12

Reverse transcription (section 2.2.13.1) and PCR (section 2.2.13.2) was done from total nucleic acids obtained from pea leaves (section 2.2.8.2) infected with the twelve PSbMV isolates (PK1-PK12). A range of different primer pairs was used in PCR. However, none of them amplified the 3' region except the primer pair of AK1 and AK2 (Table 2.2). This primer pair amplified 1.4 kb of the 3' region of isolates PK1, PK2, PK4, PK8, PK11, and PK12 at annealing temperature of 65°C as shown in Fig. 7.7 A. However, non-specific bands were obtained when PCR was done at 65°C from total nucleic acid using primers pair AK1 and AK2. The primer pair also amplified isolates S6 and US which were used as a positive control. No product was obtained from the healthy plants used as a negative control. The PCR products were identified by Southern blotting (2.2.10.4) with a ³²P-labelled S6 cDNA probe (Fig. 7.7 B).

7.3.5 PCR of the 5' region of PSbMV isolates PK1-PK12

The primer pair AK3 and AK4 (Table 2.2) used for isolate S6 was tested to amplify the 5' region from total nucleic acid of 12 Pakistani PSbMV isolates. No PCR product was obtained for isolates PK1-PK12 (data not shown) except for isolate S6 which was used as a positive control.

7.4 DISCUSSION

The primer pair AK1 and PF2 which was designed to amplify the 3' region of the genome from a common region shared by the PSbMV isolates DPD1 and NY

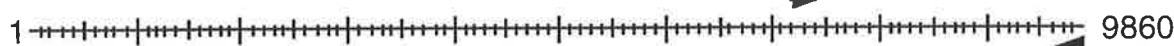
successfully amplified fragments of isolates S6 and US. This shows that the primer regions of isolate S6 and US are closely similar. However, the Pakistani isolates PK4 and PK9 were not amplified by the above primer pair indicating that they probably differed in this region from isolates S6 and US. Similarly, other primer pairs were unable to amplify the 3' region for all the Pakistani PSbMV isolates as tested in Fig. 7.7. It is concluded that some sequence variability also exists among the Pakistani PSbMV isolates in the 3' region represented by PCR primers AK1 and PF2 (Table 2.2).

In the case of the 5' region, again the primer was designed from a region common to PSbMV isolates DPD1 and NY. However, the primer was able to amplify the 5' region for isolate S6 only and not for isolate US. The same primer was also unable to amplify the 5' region from any of the Pakistani isolates (PK1-PK12). These results suggest that there is much sequence diversity in the 5' region of the genome. On the basis of these results, it will be necessary to clone and sequence the PCR-amplified products to determine the percent nucleotide sequence similarities among the biologically different PSbMV isolates.

Many attempts were made to clone the 3' region PCR product of isolate PK4 but without success, even though the same cloning procedure was successful for cloning the PCR products of isolates S6, US and PK9. It seems possible that isolate PK4 has unique properties which prevent it from being cloned in the pGEM[®]-T vector.

(A)

PSbMV genome P-4 NY



1737

Fragment of length 1737 (including primers)

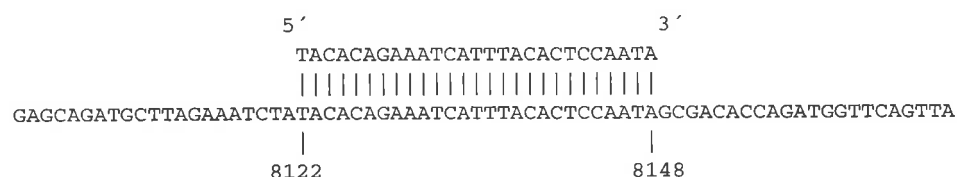
Left Primer: P4F2, Right Primer: AK1

Target sequence 8148 to 9827 GC content = 42.8%

Primer: P4F2

Primability of Match = 100%

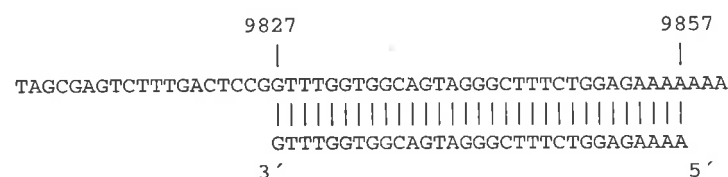
Stability of Match = 65%



Primer: AK1

Primability of Match = 100%

Stability of Match = 75%



(B)

Melting data for primer P4F2

Current Oligo, 27-mer [1]:

Td = 64.3° [nearest neighbor method]

Tm = 68.6° [%GC method]

Tm = 70° [2°*(A+T) + 4°*(G+C) method]

Mr = 8.3 k (one strand)

Mr = 16.7 k (two strands)

μg/OD = 49.0 (dsDNA)

Base	Number and %
A	12 [44.4]
C	7 [25.9]
G	1 [3.7]
T	7 [25.9]
A + T	19 [70.4]
G + C	8 [29.6]

DNA Melting Temperature in Various Salt and Formamide Concentrations [°C]				
[mM]	xSSC	0%	10%	50%
1	(0.006)	18.8	12.3	-13.7
10	(0.06)	35.4	28.9	2.9
50	(0.3)	47.0	40.5	14.5
165	(1)	55.6	49.1	23.1
330	(2)	60.6	54.1	28.1
500	(3)	63.6	57.1	31.1
1000	(6)	68.6	62.1	36.1

Tm [°C] = 81.5 + 16.6*log[Na] + 0.41*(%GC) - 675/length - 0.65*(%formamide) - (% mismatch)

(C)

Hairpin structure of the primer P4F2

Current Oligo, 27-mer [1]:
Current+ Oligo: the most stable 3'-dimer: 2 bp, -1.0 kcal/mol

```
5' TACACAGAAATCATTTACACTCCAATA 3'
   |||      |||      |||      |||
3' ATAACCTCACATTTACTAAAGACACAT 5'
```

Current- Oligo: the most stable 3'-dimer: 2 bp, -1.0 kcal/mol

```
5' TATTGGAGTGTAATGATTTCGTGTGA 3'
   |||      |||      |||      |||
3' ATGTGTCCTTTAGTAAATGTGAGGTTAT 5'
```

Current+ Oligo: the most stable dimer overall: 4 bp, -5.3 kcal/mol

```
TACACAGAAATCATTTACACTCCAATA 3'
      |||      |||      |||
3' ATAACCTCACATTTACTAAAGACACAT 5'
```

Hairpin: $\Delta G = 0.7$ kcal/mol, Loop = 8 nt, $T_m = 7^\circ$

```
5' TACACAGAAATCATTTACA
      |||      |||
3' ATAACCTC
```

(D)

Duplex formation of the primer P4F2

1. Duplex Length = 3 bp; $\Delta G = 0.7$ kcal/mol; Loop = 8 nucleotides
15 TTA 13 5' TACACAGAAATCATTTACA 24 AAT 26 3' ATAACCTC
2. Duplex Length = 3 bp; $\Delta G = 1.4$ kcal/mol; Loop = 3 nucleotides
8 AAA 10 5' TACACAGAAAT 16 TTT 14 3' ATAACCTCACATTTA
3. Duplex Length = 2 bp; $\Delta G = 2.2$ kcal/mol; Loop = 8 nucleotides
15 TT 14 5' TACACAGAAATCATTTACA 24 AA 25 3' ATAACCTC
4. Duplex Length = 2 bp; $\Delta G = 2.2$ kcal/mol; Loop = 7 nucleotides
16 TT 15 5' TACACAGAAATCATTTACA 24 AA 25 3' ATAACCTC
5. Duplex Length = 2 bp; $\Delta G = 2.5$ kcal/mol; Loop = 5 nucleotides
8 AA 9 5' TACACAGAAAT 16 TT 15 3' ATAACCTCACATTTA
6. Duplex Length = 2 bp; $\Delta G = 2.6$ kcal/mol; Loop = 4 nucleotides
9 AA 10 5' TACACAGAAATC 16 TT 15 3' ATAACCTCACATTTA
7. Duplex Length = 2 bp; $\Delta G = 2.6$ kcal/mol; Loop = 4 nucleotides
8 AA 9 5' TACACAGAAAT 15 TT 14 3' ATAACCTCACATTTAC

Fig. 7.1 (A) Testing of primers AK1, and P4F2 by PCR amplify programe to give the expected size product, (B) Calculating of P4F2 primer melting data, (C) hairpin structutre, (D) and duplex formation by Olido™ computer programe.

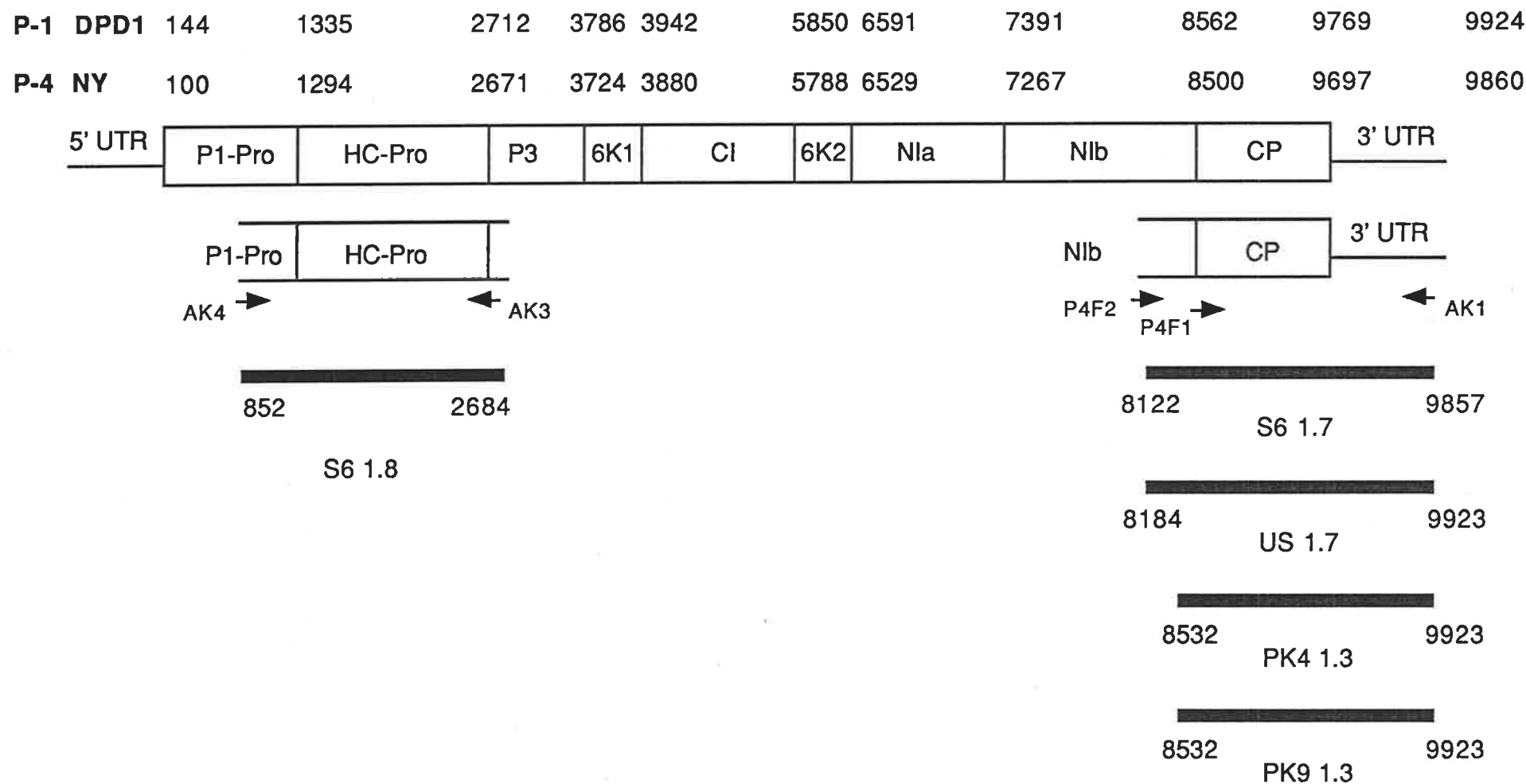


Fig. 7.2 Location in the PSbMV genome of the segments which were amplified by PCR with specific primers, cloned and sequenced. PCR amplified fragments of PSbMV isolate S6 (S6 1.7 and S6 1.8), isolate US (US 1.7), isolate PK4 (PK4 1.3), and isolate PK9 (PK9 1.3) are shown as dark horizontal lines. Nucleotide numbers listed above represent the beginning of each coding region of pathotypes P-1 and P-4 of PSbMV genome. Sequences of primers AK1, AK3, AK4, P4F1 and P4F2 are listed in Table 2.2.

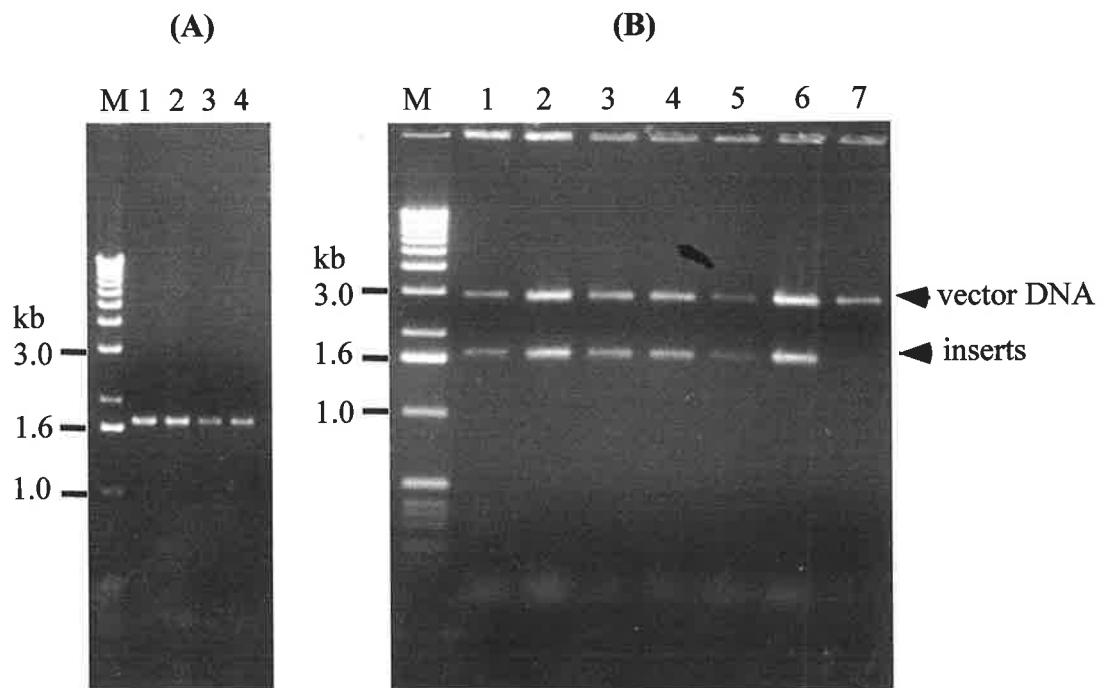


Fig. 7.3 (A) PCR amplified products (1.7 kb) of the 3' region of isolate S6 (lane 1-4) from purified RNA using primer pair AK1 and P4F2, (B) Cloning of the PCR product and double digestion of recombinant plasmid DNA with restriction enzymes showing the 1.7 kb insert (lane 1-6), plasmid DNA with no insert (lane 7), DNA marker (M).

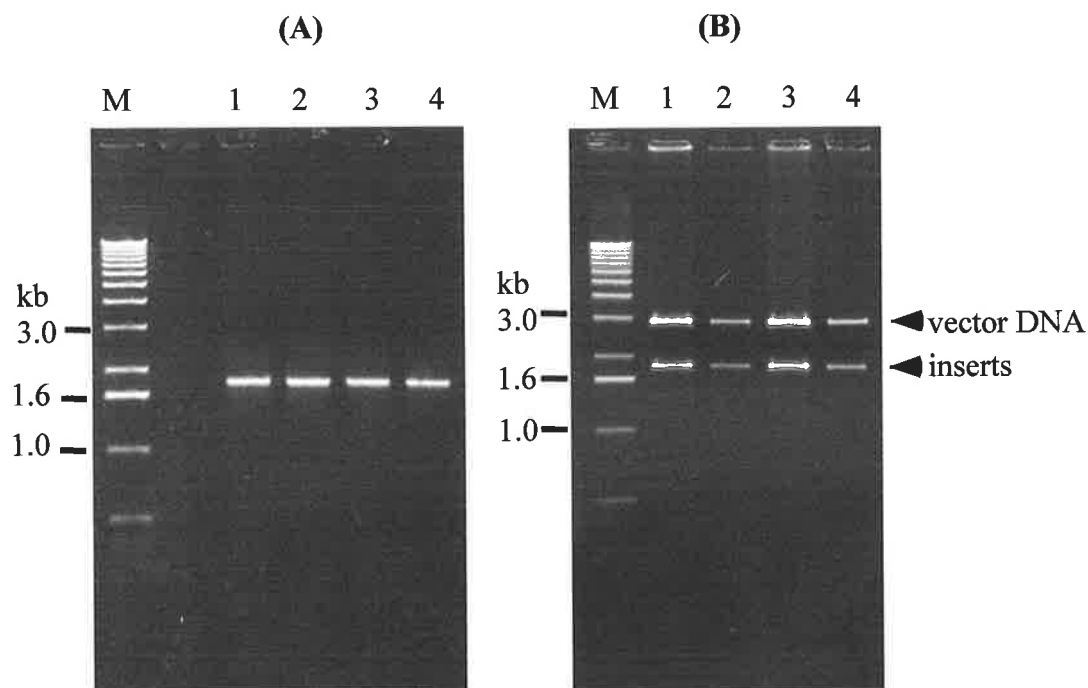


Fig. 7.4 (A) PCR amplified products (1.7 kb) of the 3' region of isolate US (lane 1-4) from purified RNA using primer pair AK1 and P4F2, (B) Cloning of PCR product and double digestion of recombinant plasmid DNA with restriction enzymes showing the 1.7 kb insert (lane 1-4), DNA marker (M).

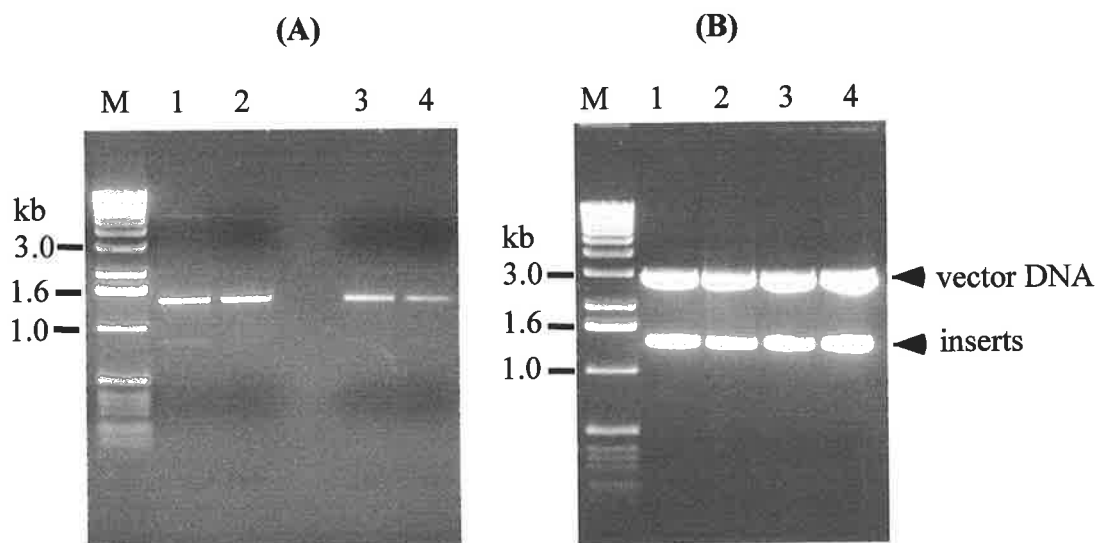


Fig. 7.5 (A) PCR amplified products (1.3 kb) of the 3' region of isolate PK4 (lane 1-2) and isolate PK9 (lane 3-4) from purified PK4 and PK9 RNA using primer pair AK1 and P4F1, **(B)** Cloning of the PCR product and double digestion of recombinant plasmid DNA with restriction enzymes showing the 1.3 kb insert of isolate PK9 (lane 1-4), DNA marker (M).

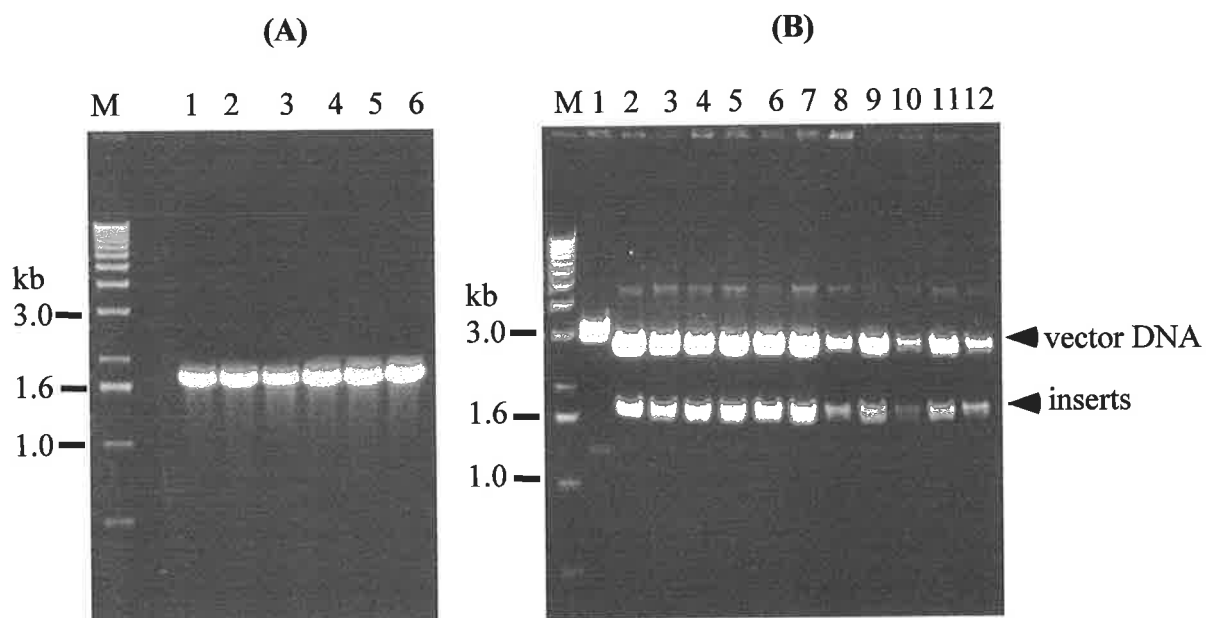


Fig. 7.6 (A) PCR amplified products (1.8 kb) of the 5' region of isolate S6 (lane 1-6) from purified RNA using primer pair AK3 and AK4, **(B)** Cloning of the PCR product and double digestion of recombinant plasmid DNA with restriction enzymes showing the 1.8 kb insert (lane 2-12), plasmid DNA with no insert (lane 1), DNA marker (M).

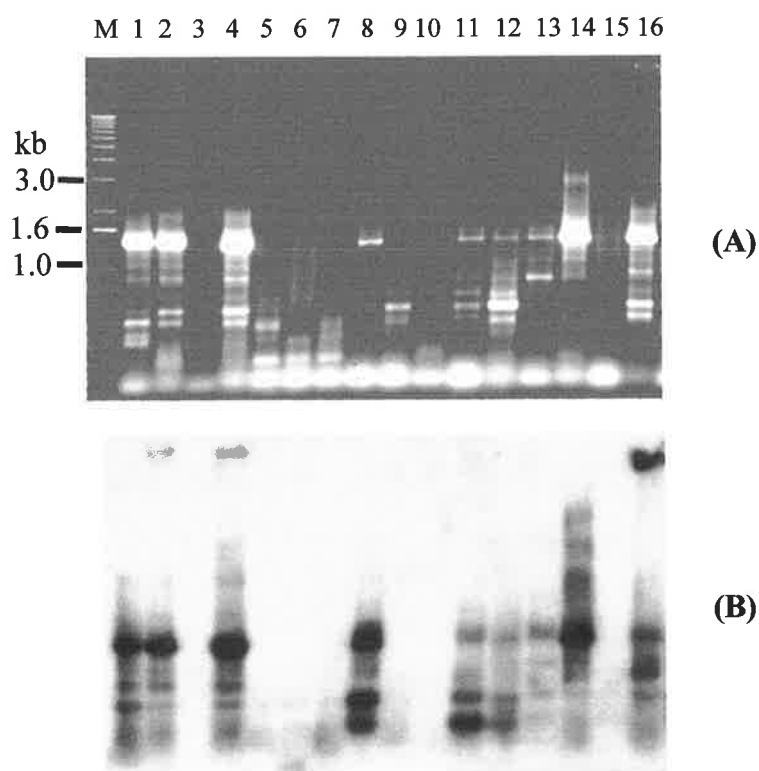


Fig. 7.7 (A) PCR amplified products (1.4 kb) of the 3' region of Pakistani PSbMV isolates PK1-PK12 (lane 1-12) isolate US (lane 13), isolate S6 (lane 14), from total nucleic acid using primer pair AK1 and AK2. Total nucleic acids from healthy tissues (lane 15) and purified isolate S6 RNA (lane 16) was used as negative and positive control respectively , **(B)** Southern blot of the PCR products with ^{32}P -labelled randomly primed cDNA probe of isolate S6, DNA marker (M).

Chapter 8

SEQUENCE COMPARISONS OF CLONED REGIONS FROM PSbMV

8.1 INTRODUCTION

Nucleotide sequencing of a virus nucleic acid fragment gives fundamental information about the genetic information in that fragment. Sequencing provides a direct way to identify and characterise a virus by comparing it with other sequences in genome data bases. In diagnosis, sequencing is the only way to be absolutely sure of the identity of the nucleic acid fragment. There are several methods of sequencing (Sambrook *et al.*, 1989) but the most commonly used method is automated nucleic acid sequencing (DyeDeoxy™ Terminator Cycle Sequencing) because it is rapid, simple, reactions are done in single tubes, µg amounts of template are required and it is convenient for processing large number_λ³ of samples.

This chapter describes the sequences of the PCR-derived clones (Fig. 7.2) of PSbMV isolates S6, US and PK9. Differences among their partial nucleotide sequences and deduced amino acid sequences are described. They are compared at the genomic level to identify features which are related to their biological diversity (Ali and Randles, 1997). The partial sequences of isolates S6, US and PK9 are also compared with published sequences of other isolates of PSbMV (Table 1.4).

As shown in Table 1.4, the genomes of two PSbMV isolates DPD1 and NY have been fully sequenced and the sequences of coat protein genes of other isolates are known. The sequences described in this chapter have been used to study phylogenetic relationships at the nucleotide sequence and amino acid sequence levels among the PSbMV isolates S6, US, PK9, DPD1, NZ, CAN, GER and NY.

8.2 MATERIALS AND METHODS

8.2.1 Sequencing primers

All the internal sequencing primers listed in Table 2.2 were designed from the previously published sequences of PSbMV isolates DPD1 and NY (Table 1.4) as in section 7.2.1. Sequencing primers T7 and SP6 used as external sequencing primers were obtained commercially (Table 2.2).

8.2.2 Clones sequenced

The PSbMV DNA inserts (Fig 7.2) cloned in the pGEM[®]-T vector representing the 3' region of isolate S6 (S6 1.7); US (US 1.7); PK9 (PK9 1.3) and the 5' region of isolate S6 (S6 1.8) were selected for sequencing.

8.2.3 Preparation of plasmid DNA for sequencing

The recombinant plasmid DNA for sequencing was prepared as described in section 2.2.15.2.

8.2.4 DNA sequencing strategy

Double-stranded DNA inserts in the pGEM[®]-T vector representing the PCR-derived clones were sequenced as in section 2.2.17. 5 µl of the plasmid DNA (250 ng/µl) with 2 µl of the primer (3.2 pmol/µl) was used in each sequencing reaction. Each viral DNA insert in the pGEM[®]-T vector was sequenced in both orientations using the T7 and SP6 primers as well as internal primers (Table 2.2) shown schematically together with their directions of sequencing in Fig. 8.1.

8.2.5 DNA sequencing analysis using computer programs

DNA sequence data were analysed using a number of different sequence analysis computer programs. The sequence data were first edited with SeqEdTM software (Applied Biosystems, Inc., USA). The nucleotide and amino acid sequences were compared with genome data bases by MPsrchTM (Oxford Molecular Ltd, UK) in the Internet site of DISC Homology Search: (<http://www.dna.affrc.go.jp/htdocs/homology/homology.html>) (Gotoh, 1982; Lipman and Pearson, 1985).

The alignment of the cloned sequences and previously published PSbMV sequences were done with the Clustal W program (Version 1.4; Thompson *et al.*, 1994) using Web ANGIS (Australian National Genome Information Service, The University of Sydney, NSW, Australia). Selected nucleotide sequences of PSbMV isolates were used as inputs to generate unrooted phylogenetic trees using the EDNADIST or EPRODIST followed by EFITCH program in the evolutionary analysis program of Web ANGIS.

8.3 RESULTS

8.3.1 The genomic sequences of PSbMV clones (S6 1.7, US 1.7, PK9 1.3 and S6 1.8)

The regions of PSbMV RNA cloned from isolates S6, US and PK9, were sequenced as represented schematically together with their directions of sequencing, in Fig. 8.1. The use of synthetic oligonucleotides primers designed for sequencing (Table 2.2), enabled each clone to be sequenced in one direction for up to 400 or 450 nucleotides.

As shown in Fig. 8.2, the complete nucleotide sequence of the 3' region for isolate S6 (clone S6 1.7) was 1737 nucleotides covering part of the Nib gene (378 nt), the full CP gene (1197 nt) and the 3' UTR (162 nt) of the PSbMV RNA genome. The length of the

3' region sequence for isolate US (clone US 1.7) was 1739 nucleotides covering part of the NIb gene (377 nt), the full CP gene (1200 nt) and the 3' UTR (162 nt) while for isolate PK9 (clone PK9 1.3), the sequence length was 1366 nucleotides which covered a short segment of NIb gene (6 nt), the full CP gene (1198 nt) and UTR (162 nt). The nucleotide sequence of the 5' region (clone S6 1.8) was 1833 nucleotides covering the partial P1-Pro gene (541 nt) and the full HC-Pro gene (1292 nt). Nucleotide sequences of all the clones (S6 1.7, US 1.7, PK9 1.3 and S6 1.8) were compared to each other and with 5 other PSbMV isolates (Table 1.4) as follows while nucleotide sequence alignment is listed in Appendix C.

8.3.2 Comparison of the partial NIb sequence

Percent nucleotides and amino acid sequence similarities in partial NIb are shown in Table 8.1. At the nucleotide level isolate US is more closely related to isolate DPD1 than to isolate NY while isolate S6 is more closely related to NY than to DPD1. These isolates can be divided into two groups which loosely fit the pathotype classification of P-1 and P-4. Thus P-1 would contain isolates DPD1 and US while P-4 contains isolates NY and S6. In contrast, the similarity (86.5 % nt; 94.4 % aa) between DPD1 and NY was greater than the similarity (84.7 % nt; 92.8 % aa) between the US and S6 isolates of PSbMV (Table 8.1). As shown in Fig. 8.3, the alignment of amino acids of the partial NIb gene showed variable regions among the PSbMV isolates except that amino acids at position 58, 60, 82 and 113 which showed identity within pathotypes and differences between isolates of P-1 group and P-4 groups. Mutation within the P-1 group isolates (DPD1 and US) was observed in 6 amino acids compared to one for the P-4 group isolates (NY and S6).

8.3.3 Comparison of the complete CP sequence

Percent comparisons of nucleotide and amino acid sequences of the full coat protein gene for several PSbMV isolates are listed in Table 8.2. Percent nucleotide sequence similarities again divided the isolates loosely into two groups, the P-1 group (isolates DPD1, PK9, NZ, US, CAN and GER) and the P-4 group (isolates NY and S6). The looseness of the classification is illustrated by the observation that the isolate PK9 has 98.7 % similarity with DPD1 and 86.8 % with NY thus placing it within the P-1 group (Table 8.2). The nucleotide sequence similarity within the isolates of the P-1 group ranged from 100 to 93.3%. The range was 100 to 98.9% for the isolates of the P-4 group.

On the basis of percent amino acid sequence similarities, the PSbMV isolates could again be divided into two broad groups (P-1 and P-4) (Table 8.2). An amino acid sequence alignment of these PSbMV isolates showed that mutations were more frequently observed in the N'-terminus than the C'-terminus of the CP gene (Fig. 8.4). The conserved amino acids at positions 78, 86, 88, 92, 97, 99, 101, 103, 117, 129, 133 and 142 in the alignment (Fig. 8.4) was observed which showed identity within the pathotypes and differences between isolates of the P-1 group and P-4 groups. However, variation at the amino acid level was also observed within the isolates of P-1 and P-4 groups, for example in the case of isolate PK9 (Fig. 8.4).

8.3.4 Phylogenetic relationship on the basis of CP sequence

The examination of a phylogenetic tree based on the alignment of the full CP gene nucleotide sequences (Fig. 8.5) or amino acid sequences (Fig 8.6) of 8 isolates clearly puts these isolates into two main groups: P-1 and P-4. The P-1 group contains isolates DPD1, PK9, NZ, US, CAN, GER while the P-4 group contains isolates NY and S6.

However, the phylogenetic tree showed that isolates in the P-1 group were diverse and put them into three sub groups (DPD1, NZ, PK9) (CAN, US) (GER). On the basis of the CP gene comparison, it was evident that isolate S6 is related to pathotype P-4 group isolate and isolates US and PK9 are in this broader pathotype P-1 group.

8.3.5 Comparison of the CP amino acids by hydropathy plots

As shown in Fig 8.7, the CP amino acid sequences of isolates S6, US and PK9 were compared by hydropathy plots to further confirm the effect of variation in the CP amino acid on the structure of the coat protein. It was observed that various structural changes occur in the coat protein of isolates S6, US and PK9 as indicated in Fig 8.7.

8.3.6 Comparison of the UTR sequence

A comparison of the 3' untranslated region of 162 nucleotides for eight PSbMV isolates showed that this part of the genome is more conserved than the CP gene (Table 8.3). It was evident that the similarities among the isolates were greater in the UTR than in the CP gene. For example, 100 % similarities were observed in some of the P-1 group isolates (DPD1, PK9, US and CAN) (Table 8.3) which is in contrast to the comparison made on the basis of CP gene where diversity was found within the group P-1 isolates. Isolates NY and S6 were again distinguishable from the members of the P-1 group. The examination of the phylogenetic tree based on the alignment of the UTR nucleotide sequences of 8 isolates shows two main groups: P-1 and P-4 groups (Fig 8.8). However, the P-1 group was diverse and contains two subgroups: (CAN, DPD1, NZ, PK9 and US). The P-4 group contain^s isolates S6 and NY.

8.3.7 Comparison of the partial P1-Pro sequence

When the 541 nucleotide long partial P1-Pro sequence of isolate S6 was compared with that of isolate DPD1 and NY, nucleotide sequence similarity of 97.2% was observed with isolate NY and 76.9% with DPD1 (Table 8.4). Similarly, on the basis of the amino acid sequence, 97.8% similarity was observed with isolate NY and 79.4% with DPD1. It is concluded from the percentage similarity that the partial sequence of P1-Pro shows major sequence differences between isolates S6 and DPD1. As shown in Fig. 8.9, an alignment of the P1-Pro amino acid sequences shows the large number of substitutions between isolates S6 and DPD1 and very small difference between isolates S6 and NY.

8.3.8 Comparison of the complete HC-Pro sequence

To further confirm the similarity of isolate S6 to P-4 group isolate at the nucleotide sequence level, the HC-Pro gene (1292 nucleotide long) which is considered to be the variable region of the genome (Maia *et al.*, 1996) was aligned with isolate DPD1 and NY. The nucleotide sequence similarity observed was 82.0 % with isolate DPD1 and 98.5 % with isolate NY. Amino acid similarity of isolate S6 with isolate DPD1 was 92.1 % and with isolate NY was 98.1%. The percentage similarities of both nucleotide and amino acid sequence showed that S6 is almost identical to the NY (P-4 isolate) of PSbMV. The amino acid sequence alignment (Fig. 8.9) shows that variation occurred throughout the HC-Pro gene between isolates S6 and DPD1 but there was little variation between isolates S6 and NY.

8.4 DISCUSSION

The sequencing results obtained in this chapter confirm that isolate S6 is closely related to ^{the} P-4 group isolate while isolate US is distantly related to P-1 group isolates based on

the nucleotide sequences of both the 3' region and the 5' region of the PSbMV genome. These results are similar to those obtained on the basis of pathotype specific pea genotypes (Table 6.4) (Ali and Randles, 1997) which also put isolate S6 in the P-4 group and isolate US in the P-1 group.

Isolate PK9 is neither P-1 nor P-4 on the basis of pathotyping but it is in the P-1 group on the basis of its CP gene sequence. It is possible that isolate PK9 differs from other P-1 isolates mainly in the so far undefined region of the genome which is responsible for pathogenicity but on the basis of nucleotide sequences of the 3' region of the genome, it is related to P-1 group isolates. However, in the P-1 group isolate PK9 is in a separate subgroup from isolate US which indicates the diversity of isolate PK9 from isolate US and other P-1 group isolates.

Variation was observed in the total length of the CP gene sequence among the PSbMV isolates S6, US and PK9. The total length of the CP gene for isolate US was 1200 nt, followed by PK9 (1198 nt) and then S6 (1197 nt). However, the length of the UTR was the same (162 nt) for all the three (S6, US and PK9) isolates.

It was found from the alignment of the nucleotide and amino acid sequence and also from the hydropathy plots of CP amino acids that the N-terminal region of the coat protein gene showed high variability, which could be involved in host range and symptom expression (Riechmann *et al.*, 1992). The UTR was conserved among the PSbMV isolates and is not suitable to use for differentiation of isolates.

The 5' region which covers the partial P1-Pro and full HC-Pro also showed high variability between isolates S6 and DPD1. The HC-Pro of isolate US was not sequenced in this study but from the variation between isolates S6 and DPD1, it seems that there might be high variability between isolate S6 and US as well. This supports the previous

results that the primers (AK3 and AK4, Table 2.2) designed for the amplification of the HC-Pro from the common sequence of isolates DPD1 and NY, could only amplify isolate S6 and not isolate US. As demonstrated recently by Johansen *et al.* (1996b), the 5' 2.5 kb (which cover 5' UTR, P1-Pro and two thirds of HC-Pro) of the PSbMV genome had a major influence on the rate of seed transmission. In view of this reference, it seems possible that the difference between the rate of seed transmission of isolate S6 and US (Ali and Randles, 1998) might be due to variability in the sequence of partial P1 and HC-Pro gene of isolates S6 and US.

It is clear from the percentage similarity that the partial sequence of P1-Pro shows major sequence differences between isolates S6 and DPD1. As shown in Fig. 8.9, an alignment of the P1-Pro amino acid sequences shows the large number of substitutions between isolates S6 and DPD1 and very small difference between isolates S6 and NY.

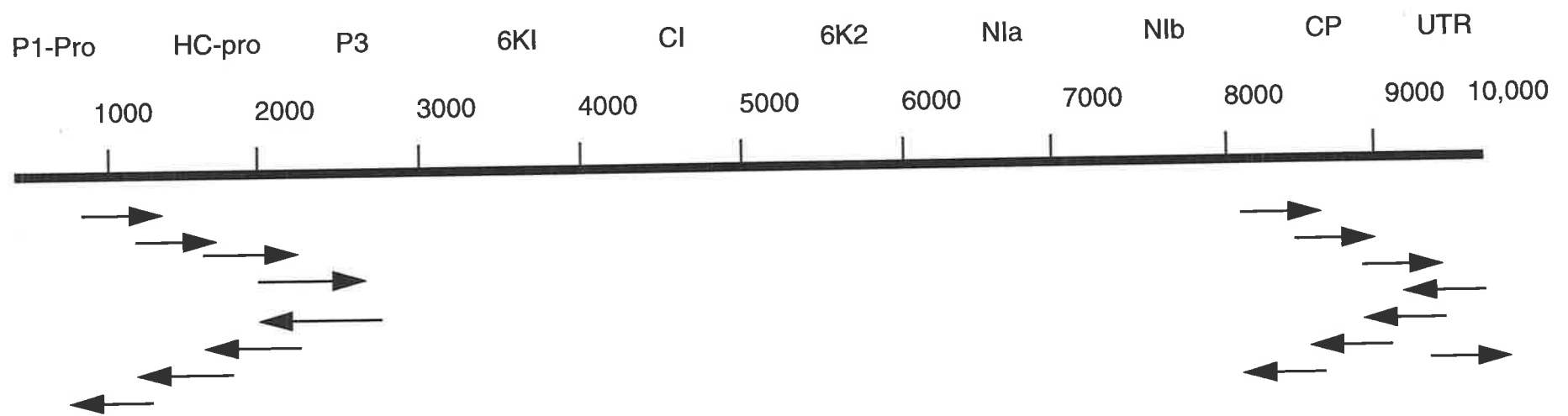


Fig. 8.1 Schematic representation of DNA clones sequenced from the PSbMV genome. The orientation of the sequencing primers is indicated by the direction in which they lie.

Fig. 8.2 Complete nucleotide sequence of the PSbMV clones (S6 1.7, US 1.7, PK9 1.3 and S6 1.8) sequenced in this study.

S6 1.7 clone (covers a part of Nib gene, full CP gene and UTR)

TACACAGAAATCATTTTACACTCCAATAGCGACACCAGATGGTTCAGTTATAAAGAAAACAAAAGGAAATAACAGTGGCCAACCATCAACT	90
GTCGTCGATAACACATTAATGGTAATTCTCGCAATGCAGTACAGCTTGCACTTACTAGGTGGATTTTGAAACTCAGGATGACGTCATT	180
CGCTATTTTCGCAAATGGTGATGATCTCCTCATAGCTGTGCGTCCAGATTGTGAATTCGTGCTCGAAGGTTTAGAGAAACATTTCTCAAAC	270
CTAGGGTTAAACTACGACTTTTCGGCACGGCATCATGATAAGAAAGACGTTTGGTTTATGTCAACAAAAGGCATTTTAAGAGATGGTATT	360
CTAATACCAAAGCTCGAAGAGGAACGAATCGTTGCAATTCTGGAATGGGATAGAAGCAGGAATTCCTCACAGACTCGATGCGATATGT	450
GCAGCAATGATTGAAGCTTGGGGTTACGATGAACTTTTCGAGCACATTCGGAATTCATTATTGGCTGCTAGAACAGGAGCCATATAGA	540
AGTATAGCTCAGGAAGGCAAGCACCATATATTGCAGAAACAGCTCTTCGACACTTATACACAAATGCTGTGCAACACAAAGTGAATTA	630
GAAAGGTACATAGAGCAATTGATCAGCACTACAATGAGGACGGTAATGGTCCGATTAAAGTGAGGTTGCAAGCTGGTGATGAGACCAAA	720
GATGAAGAGAAAACGAGAAAAGAAGAGGAGGATAGAAAGAGACGAGAGAGAATATCGACGCAAGTCAATTTGATTTCGAGTCGAGACAAC	810
AAAAAGAACAAAAACAAAGAGAGTGATACATCGAACAAAGCCATAGTGAAATCAGATCGAGATGTTGATGCAGGAACATCAGGAACCATC	900
ACAGTGCCTAGACTCGAAAAGATTTTCAGCAAAAATTAGAATGCCGAAGCACAAAGTGCGGTGGCGATTAGTCTGCAGCATTTGGTTGAC	990
TACAACCCAGCACAGGTTGACATATCAAACTCGCGCAACACAGAGCCAAATTGATAATTGGTGGAGAGCAGTGTCTCAAGAGTATGGG	1080
ATTGGTGACAGCGAGATGCAAGTGTTAGCAAGTGGTTGATGGTGTGGTATTTGAAAACGGAACATCGCCTAACATAAATGGGATGTGG	1170
ACAAATGATGGACGGGAGGAACAGGTTGAGTACCCCTAAAACCAAGTGTGGATAATGCGCGTCCAACTTTTAGACAAATAATGGCGCAT	1260
TTCAAGTGACGTAGCGGAGGCGTACATTGAAAAGAGAACTCAACAGAAGTGATACACCCAGGTATGCTTTACAACGCAATTTAAGGGAC	1350
CCGAGTCTAGCGAGATATGGTTTCGATTTTACGAAATCACAGCCAAGACACCTGTGAGGGCAAGAGAGGCACACTTTCAAATGAAAGCA	1440
GCAGCAATCAGAGGAAAATCCAACAGCCTATTGGCTTGGACGGGAACGTTGGGACACAGGAGGAAAACCGGAGAGGCACACAGCAGAA	1530
GATGTCAATCAGAATATGCACAACCTTCTTGGAATGAGAGCCATGTAAACCGTATGTATTTTCAGTACTGTGCATGCAATTTTCGTAAATT	1620
CAGTGTGCCTTTGACACCATGTTTAATAGCATTATGTATCTAGGGTTCTATTATCATCAATTCATAGCGAGTCTTTGACTCCGGTTTG	1710
GTGGCAGTAGGGCTTTCTGGAGAAAAA	1737

US 1.7 clone (covers a part of Nib gene, full CP gene and UTR)

TACACAGAAATCATTTTACACTCCAATAGCAACACCAGATGGTTCAGTCATAAAGAAGACGAAAGGAAATAACAGTGGTCAACCATCAACA	90
GTGTTGATAACACATTGATGGTGGTTCTTGCTATGCAATATAGCCTACAGTTACTTGGAGTTGATTTTGAGAAACAGGATGAAGTCGTT	180
CGCTATTTTCGCAAATGGTGATGATCTATTGATAGCTGTGCGCCAGACTGTGAGTTTGTGCTGAAAGGCTTGGAGGCACATTTCTCTAAT	270
CTGGGGTTAAATCATGATTTTCTGCGCGGCACCATGACAAGAAAGATGTTTGGTTTATGTCAACAAGGGGCATTTTGAGAGATGGAATTC	360
TGATACCAAACTTGAAGAAGAACGAATTGTTGCAATTTTGAATGGGATAGAAGTAGAGAATTTTCACATAGGCTTGATGCTATATGTG	450
CAGCAATGATTGAAGCTTGGGGTTACGATGAGCTTTTCGAGCATATTCGGAATTCATTATTGGTTATTAGAACAGGAACCATACAGGA	540
GCATAGCGCAGGAAGGAAAAGCACCATACATCGCAGAGACAGCGCTCCGGCACCTGTACACAAATGCCATGGCAACACAAAGTGAACCTTG	630

AGAAATACACGGAAGCAATCAATCAGCATTACAACGATGAAGGTGGCGATGGATCAATCAAGGTTTCGGTTGCAAGCTGGTGACGAAACTA	720
AGGATGACGAAAGAAGAAGGAAAGAGGAGGAGACAGAAAGAAAGAGAGGAGATATCGATACGAGCCAGTTTCGGTTGCAATCGTGACA	810
ACAAGAAAAACAAAACAATGAGAGCGATGCTCCTAATAGATCAATAGTGAAATCTGATCGAGACGTGGATGCAGGAACTTCAGGCACAA	900
TTATAGTGCCAAGGCTTGAGAAGATTTAGCCAAAATTAGGATGCCAAAACACAAGGGTGGAGTAGCTATTAGCTTGCAACATTTAGTTG	990
ACTACAATCCAGCACAAAGTTGACATCTCAAATACTCGAGCAACGCAGAGCCAGTTTCGATAACTGGTGGAGGGCAGTGTCACAAGAGTACG	1080
GGGTGGAGACAATGAAATGCAAGTTTGGCAAGTGGTTTGATGGTATGGTGCAATTGAAAATGGAACATCGCCTAACATAAATGGGATGT	1170
GGACAATGATGGACGGGAAGAGCAAGTTGAGTACCCCTAAAGCCAGTGATGGATAATGCGCGTCCAACTTTCAGACAGATAATGGCGC	1260
ATTTTCAGTGACGTAGCGGAGGCGTACATTGAAAAGAGAACTCAACAGAGGTGTACATACCCAGGTACGCTCTACAACGCAATTTAAGGG	1350
ACCCGAGTCTTGCAAGATATGGTTTCGACTTCTACGAAATCACAGCAAAGACACCTGTGAGGGCAAGAGAGGCACACTTTCAGATGAAAG	1440
CAGCAGCAATCAGAGGAAAATCCAACAGCCTATTTGGCTTGGATGGGAACGTTGGGACACAGGAGGAGAACACGGAGAGGCACACAGCAG	1530
AAGATGTCAATCAGAACATGCACAATCTTCTTGAATGAGAGCCATGTAATCCGTATGTATTTTAGTACTGTCATACATTTTCGTTAAA	1620
TTTCAGTGTGTCTTTGACACCATGTTTAATAGCATTATGTATTTCTAGGGTTCTATTATCATCAATTCCATAGTGAGTCTTTGACTTCGGT	1710
TTGGTGGCAGTAGGGCTTTCTGGAGAAAA	1739

PK9 1.3 clone (covers a short part of N1b gene, full CP gene and UTR)

TTGATTAAAAACGAATTGTTGCAATTTTGAATGGGATAGAAGTAAAGAATTTTCACATAGGCTTGATGCCATATGTGCAGCAATGATTG	90
AAGCTTGGGGTTACGACGCGCTTTTGATGACATATTCGTAAACTCTATTATTGGTTGTTAGAACAGGAACCATACAGGAGCATAGCTCAGG	180
AAGGAAAAGCACCATACATCGCAAAGACAGCGCTTCGGCACCTGTACACAAATGCCATGGCAACACAAAGTGAGCTTGAGAAAATACCGG	270
AAGCAATCAATCAGCATTACAATGATGAAGGTGGCGATGGATCAATCAAGGTTTCGATTGCAAGCTGGTGACGAAACCAAGGATGATGAAG	360
GAAGAAGGAAAGAGGAGGAGGACAGAAAGAAAAGAGAGGAGAGTTTCGATGCGAGCCAGTTTGGTTCGAATCGTGACAATAAGAAAAACA	450
AAAAATAAGAGAGTGACACATCAAAACAATTAATAGTGAAGTCTGATCGAGATGTTGATGCAGGATCTTCAGGCACAATCACAGTACCAA	540
GGCTTGAAAAGATCTCAGCAAAGATTAGGATGCCAAAACACAAAGCGGAGTAGCTATCAGCTTGCAACATTTAGTTATTACAATCCAGC	630
ACAAGTTGACATTTCAAACACTCGAGCAACGCAGAGCCAGTTTCGATAACTGGTGGAGGGCAGTGTCGCAAGGTACGGGGTTGGAGACAA	720
TGAAATGCAAGTTTGGCAAGTGGTTTGATGGTATGGTGCAATTGAAAATGGAACATCGCCTAACATAAATGGGATGTGGACAATGATGGA	810
CGGGGAAGAACAGGTTGAGTACCCCTAAAGCCAGTGATGGATAATGCGCGTCCAACTTTCAGACAGATAATGGCGCATTTTCAGTGACGT	900
AGCGGAGGCGTACATTGAAAAGAGAACTCAACAGAGGTGTACATACCCAGGTACGCTCTACAACGCAATTTAAGGGACCCGAGTCTTGC	990
AAGATATGGTTTCGACTTCTACGAAATCACAGCAAAGACACCTGTGAGGGCAAGAGAGGCACACTTTCAGATGAAAGCAGCAGCAATCAG	1080
AGGAAAATCCAATAGCCTATTTGGCTTGGATGGGAACGTTGGGACACAGGAGGAGAACACGGAGAGGCACACAGCAGAAGATGTCAATCA	1170
GAATATGCACAATCTTCTCGAATGAGAGCCATGTAATCCGTATGTATTTTAGTACTGTCATACATTTTCGTTAAATTTTCAGTGTGTCT	1260
TTGACACCATGTTTAATAGCATTATGTATTTCTAGGGTTCTATTATCATCAATTCCATAGTGAGTCTTTGACTTCGGTTTGGTGGCAGTAG	1350
GGCTTTCTGGAGAAAA	1366

S6 1.8 clone (covers a part of P1-Pro gene and full HC-Pro gene)

ACCACGAAATATAAAGATGAAAGCTGAGTCCATCGACGCTTTAATTGACCAGGTTGTGACTTGCGCGGGGAAGCATGCTAAACAAGTTTT 90
TCTAATTGACAAGCAAAAGGCAAAACAAAGTCTGGATAAAGCGAGTCAATGATATTCGCTTGTGCAAGTCGAGACAAAACACCACAGAGG 180
GAAAATTAGTCAAAGGGATGTTTTGCTGAATGATCTCACTAAGAGGTTGCTAGATATTCGCGAGCAAAACAACACATGTTACCCGTC 270
AAGCAGCATCACACATGGACACAGCGCGTAGTCTTTTTGCGTGAGAATATAGATGGCAACAAGTCATACAGCAGTGATGATTTGTTTGT 360
TGTTAGAGGAAAAGAAATGGAATGATGGAAGGTCGTAATAAGGTAGCATGGCACAAGATGTTCCAGATAAACCACTTTTCTAGTGT 450
CGGACTTAAATCTGGAATGCGTTTGACGCGAATATGTGAAATTGAGAGATGAGTCAACTAGTGACCACGAGTGTTGGCGGGATCAC 540
ACCAGAAGAATGTGGAATTATTGCCGCGCACATACTACGAGTTTTCTATCCGTGTTGGCGCATAACTTGCACAAAATGCATTTCAACTTG 630
GTTGAGTAAGCCAACAAGTGAGCAAAATTGAACACATATATGTTGAGGACATTTGGCAATACAGGATTTGAATTGGAATAATCCAGCAGC 720
GCATCATGTGGCTCATATGATTGAGCTATTGCGGCAAGGATTAAAAACACAACATTCAACATGGAACAAACACAAAAGTCCATGAATT 810
AATTGGTCACCGGCAAGATGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGCGTTTGGCGGCGCATGGTTCTAACACAATCGAATG 900
GGAGAGCATGAATGAGAGTTTGCTAGAACTAGCAGCTGGCACAATAGGAGGACCGAGTCAATTGCAGGTGGCGGCATCTCAAGTTTCCG 990
TAACAAGGTTTCTGCCAAAGCTCAAATTAATTTGCGGTGATGTGCGACAATCAGTTGGATACGAACGGAAATTTCTGTGGGGCGAACG 1080
TGGATATCATGCGAAACGTTTCTTTTCAGAGTATTTTACAAAGATTGATCCAAAAGATGGGTACACTCACCATGCTGTTAGAACAAACACC 1170
GACAGGAGTTAGGCATCTGGCAGTAGGAACTTGATCATACCAGGCGATCTCCAAAACTTAGGGAAAACTTGAAGGTGTGTCAATCAC 1260
AACAGTGGACATCTCAGAAAAGTGCCTCAGTAGGCGGAATGGAGATTTTGTATTCCCTTGCTCATGTGTAACACTTGAAAATGGAAAACC 1350
AGCCATGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAACACAGGCGATCCAAAGTTGGTTGATTACCTAAGACTGA 1440
AGCTGGTGGAATGTGGATCGCTAAAGAAGGATATTGTTACATCAACATATTCTTTGCAATGCTTGTAACGTTAGTGAGAAGGATGCTAA 1530
GGACTTCACAAAATTTGTAAGGGATGAAATCATGCCACAACCTGGGAAATGGCCAACATATGATGGATGTAGCAACTGCATGTTATAAATT 1620
AGCAATAATTTATCTGACGTGCGTGATGCACAGCTACCGAGAATACTAGTTGATCACTCAGAGCAAACTTTTCATGTAATAGATTCTATA 1710
TGGTCCATGACTACAGGATATCACATCTTGAAAGCAGGACAGTTTCCAGTTGATAAGCTTTGCGCATGGTGCTTTGGTGGGTGAGAT 1800
GAAGATGTATAGAGTTGGAGGCACTCAGAAAAT 1833

Fig. 8.3 Alignment of amino acids sequence of partial N1b gene of four PSbMV isolates. Identical amino acids are shown by dots (.) within the alignment, and dashes (-) indicate gaps or deletion within the alignments. Isolates underlined are sequenced in this study.

DPD1	2681	YTEIIYTP IATPDG S VIKKTKGNNSGQPSTVVDNTLMVILAMQYSLQLLGVDFETQDEVV	2740
<u>US</u>	1	V.....K..E.V	60
<u>S6</u>	1	I.....T..D.I	60
NY	2675	I.....T..D.I	2734

DPD1	2741	RYFANGDDLLIAVRPDCEFLKGLEIHFSNLGLNYNFSARHHDKKDVWFMSTRGILRDGI	2800
<u>US</u>	61	K...A.....HD.-.....R.I.....	120
<u>S6</u>	61	E...K.....YD.S.....K.I.....	120
NY	2735	E...K.....YD.S.....K.M.....	2794

DPD1	2801	LIPKLE	2806
<u>US</u>	121		126
<u>S6</u>	121		126
NY	2795		2800

Fig. 8.4 Alignment of amino acid sequences of the coat protein gene of eight PSbMV isolates. Dots (.) indicate amino acids identical to each other, blanks show regions unsequenced and gaps or deletions are shown as dashes (-) within the alignments. The conserved amino acid motif (DAG) responsible for aphid transmission in potyviruses is indicated as bold and underline. Conserved amino acid block presumably specific for the P-1 and P-4 groups are indicated as bold.

DPD1	2807	EERIVAILEWDRSREFSHRLDAICAAMIEAWGYDELLQHIRKFYYWLLEQEPYRSIAQEG	2866
<u>PK9</u>	1	IK.....K.....I.....A.-...L.....	59
NZ	1	EE.....R.....I.....E..Q...F.....	60
<u>US</u>	1	EE.....R.....I.....E..Q...F.....	60
CAN	1	E..Q...F.....	31
GER	1	E..Q...F.....	31
<u>S6</u>	1	EE.....R.....V.....E..Q...F.....	60
NY	2801	EE.....R.....I.....E..Q...F.....	2860
DPD1	2867	KAPYIAETALRHLYTNAMATQSELEKYTEAINQHYNDEGGDGSIKVRLQAGDETKDDERR	2926
<u>PK9</u>	60	K.....M.....K.T..N...D.G.D.S.....DEGR	119
NZ	61	E.....M.....K.T..N...D.G.D.S.....DERR	120
<u>US</u>	61	E.....M.....K.T..N...D.G.D.S.....DERR	120
CAN	32	E.....M.....K.T..N...D.G.D.S.....DERR	91
GER	32	E.....M.....K.T..N...D.G.D.S.....DERK	91
<u>S6</u>	61	E.....V.....R.I..D...-D.N.P.....EEKR	119
NY	2861	E.....V.....R.I..D...-D.N.P.....ERET	2919
DPD1	2927	RKEEEDRKKREESIDASQFGSNRDNKKKNKNKESDTPNKLIVKSDRDV DAG SSGTITVPRL	2986
<u>PK9</u>	120	.K.....K...SF.A...G.N.....K...TS.KLIV.....S....T....	179
NZ	121	.K.....K...SI.A...G.N.....K...TS.KLIV.....S....T....	180
<u>US</u>	121	.K.....K...SI.T...G.N.....N...AP.RSIV.....T....I....	180
CAN	92	.K.....K...SI.A...G.N.....K...AP.RSIA.....T....I....	151
GER	92	.R.....K...SI.A...G.N.....K...TS.KLVV.....S....T....	151
<u>S6</u>	121	.K.....R...NI.A...D.S.....K...TS.KAIV.....T....T....	179
NY	2920	.K.....R...NI.A...G.S.....K...TS.KAIV.....T....T....	2979

DPD1	2987	EKISAKIRMPKHKGGVAISLQHLVDYNPAQVDISNTRATQSQFDNWWRAVSQEYGVGDNE	3046
<u>PK9</u>	180	A.....V..N.	238
NZ	181	D.....R.....V..N.	240
<u>US</u>	181	D.....A.....V..N.	240
CAN	152	D.....A.....V..N.	211
GER	152	D.....A.....I..S.	211
<u>S6</u>	181	D.....A.....I..S.	239
NY	2980	D.....A.....I..S.	3039

DPD1	3047	MQVLASGLMVWCIENGTSPPNINGMWTMMDGEEQVEYPLKPVMDNARPTFRQIMAHFSDVA	3106
<u>PK9</u>	239		298
NZ	241		300
<u>US</u>	241		300
CAN	212		271
GER	212		271
<u>S6</u>	241		299
NY	3040		3099

DPD1	3107	EAYIEKRNSTEVYIPRYALQRNLRDPSLARYGDFYETAKTPVRAREAHFQMKAAAIRG	3166
<u>PK9</u>	299	YIPR.A.....	358
NZ	301	---.A.....	356
<u>US</u>	301	YIPR.A.....	360
CAN	272	YIPR.A.....	331
GER	272	YIPR.T.....	331
<u>S6</u>	301	YIPR.A.....	359
NY	3100	YIPR.A.....	3159

DPD1	3167		3206
<u>PK9</u>	359		398
NZ	357		396
<u>US</u>	361		400
CAN	332		371
GER	332		371
<u>S6</u>	361		399
NY	3160		3199

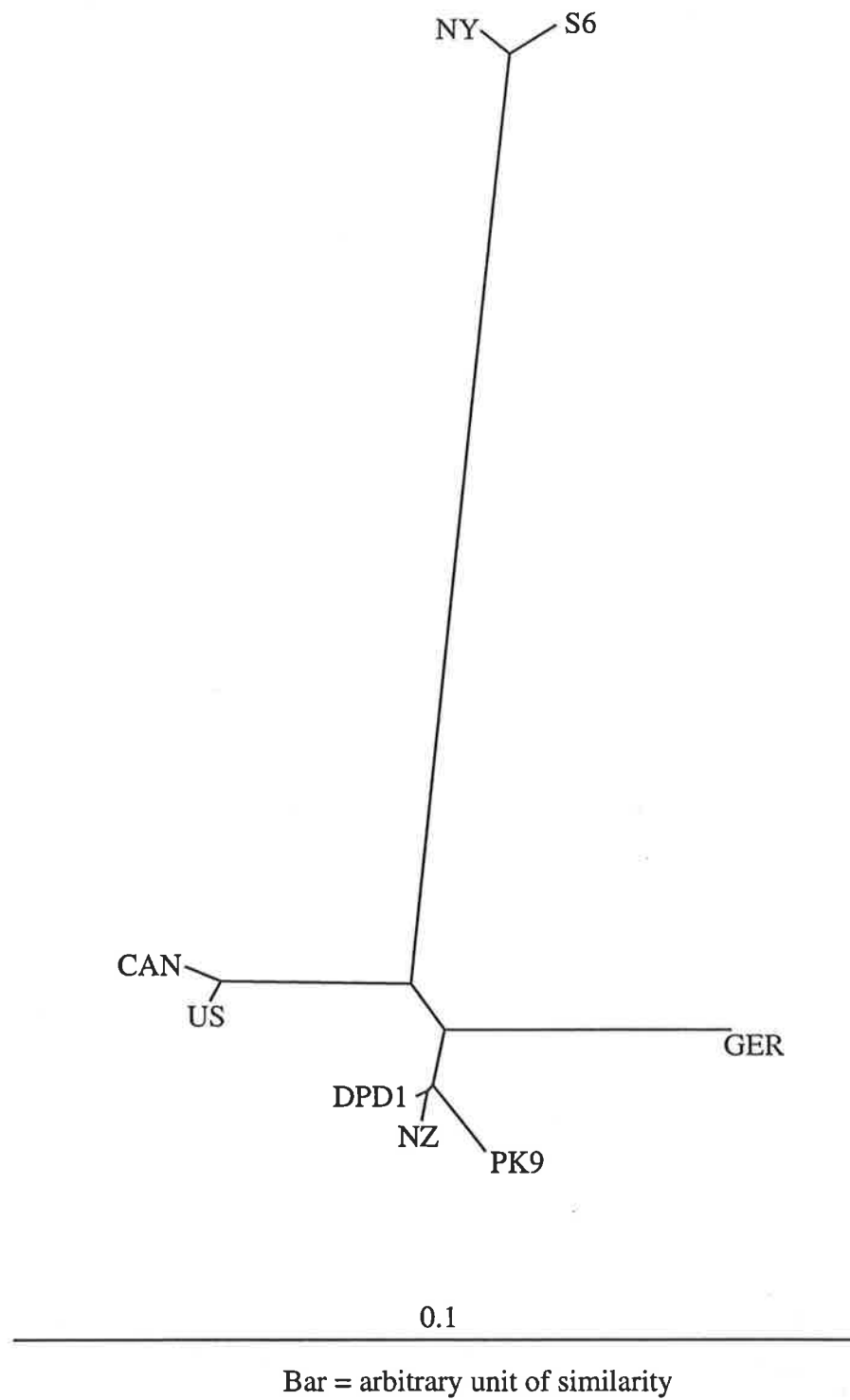


Fig. 8.5 Unrooted phylogenetic tree obtained from the alignment of CP nucleotide sequences using EDNADIST followed by EFITCH in the EVOLUTIONARY ANALYSIS program of Web Angis

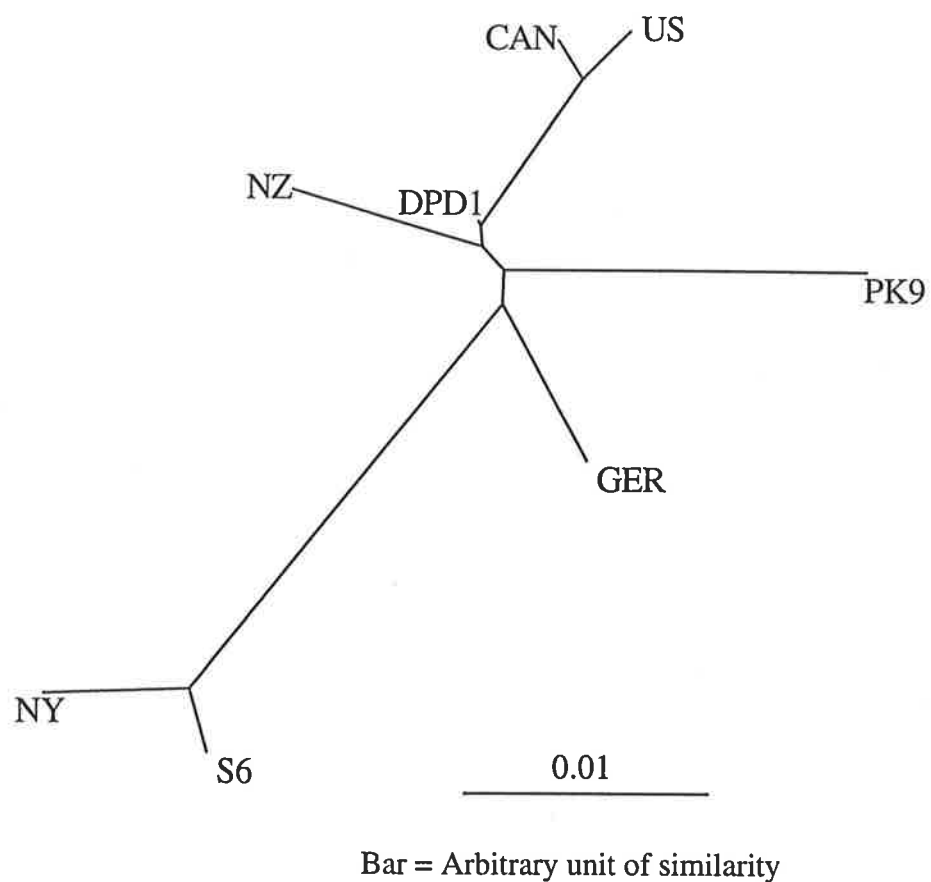
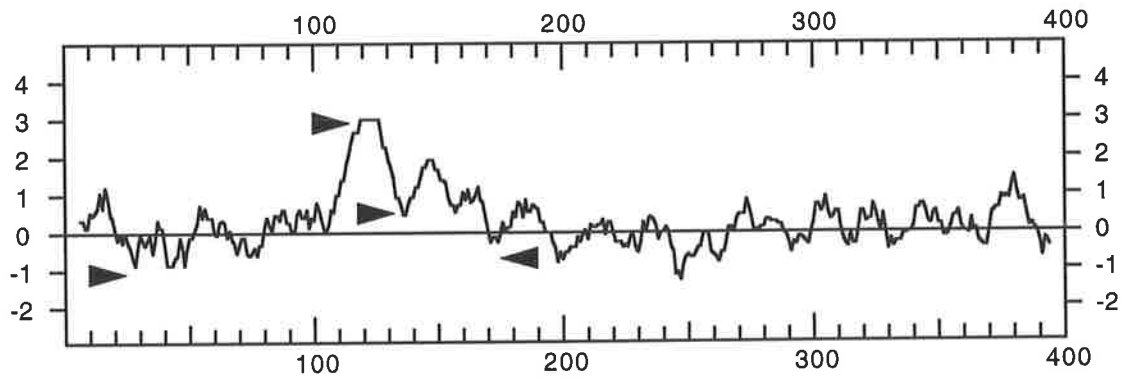
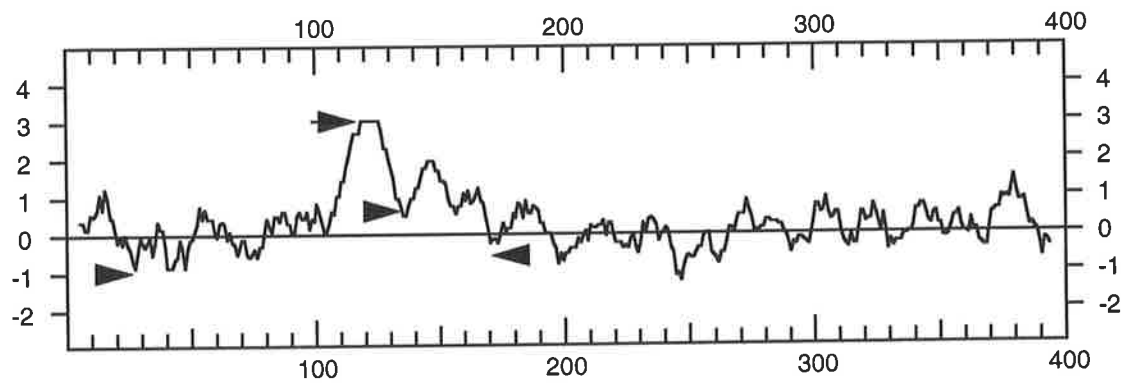


Fig. 8.6 Unrooted phylogenetic tree obtained from the alignment of CP amino acid sequences using EPRODIST followed by EFITCH in theEVOLUTIONARY ANALYSIS program of Web Angis

S6 hydropathy plot



US hydropathy plot



PK9 hydropathy plot

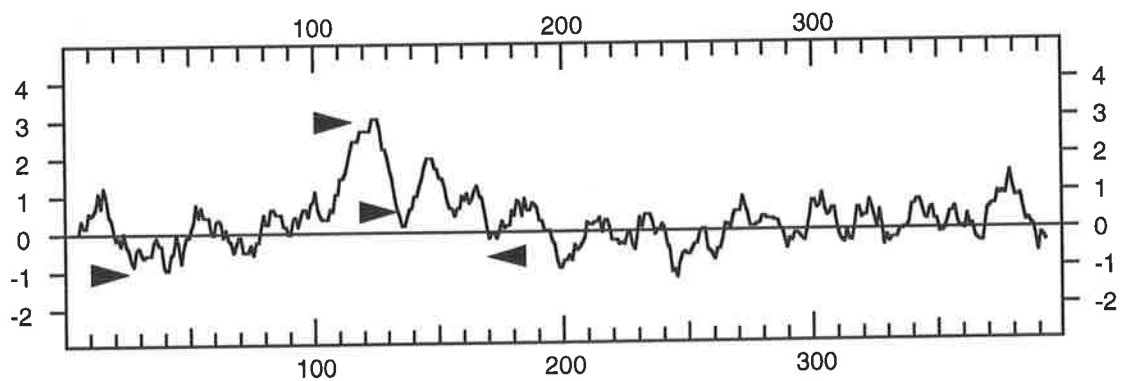


Fig. 8.7 Comparison of the hydropathy plots of the amino acids of the coat protein gene of isolates S6, US and PK9. Arrows indicate the structural changes in CP due to the variation in amino acid sequences.

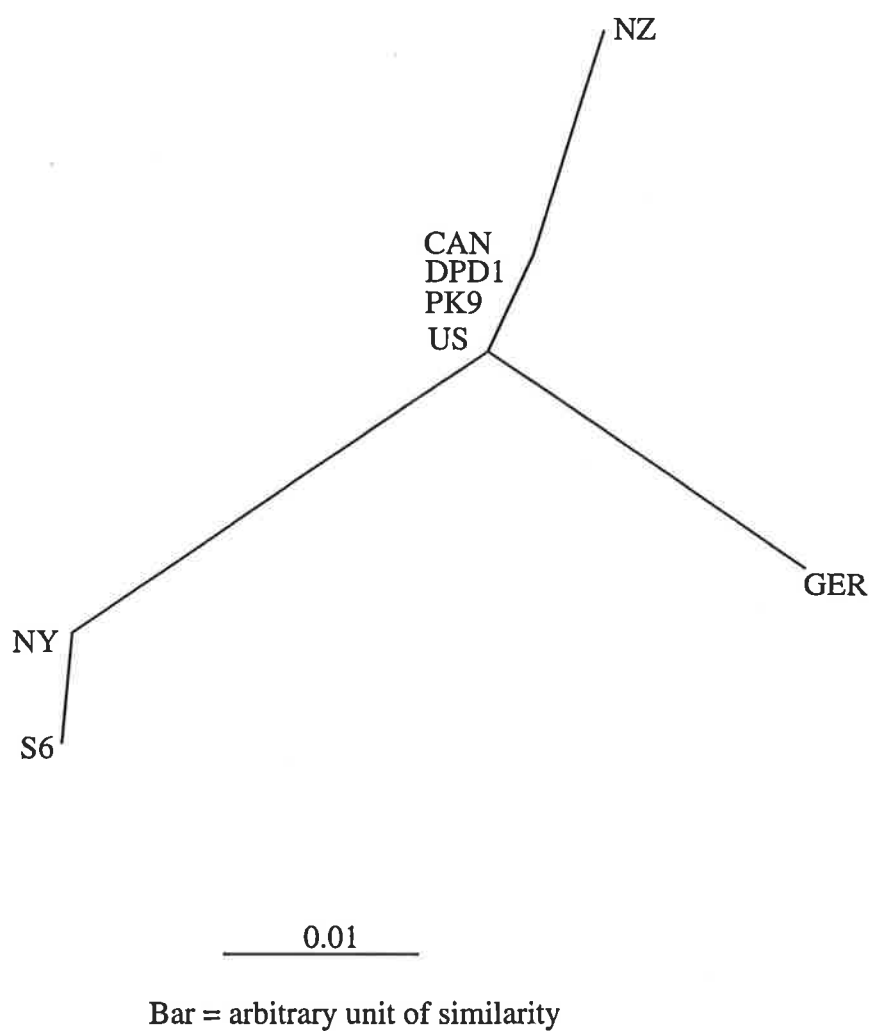


Fig. 8.8 Unrooted phylogenetic tree obtained from the alignment of UTR nucleotide sequences using EDNADIST followed by EFITCH in the EVOLUTIONARY ANALYSIS program of Web Angis

Fig. 8.9 Alignment of amino acids sequences of partial P1-Pro and full HC-Pro genes of three isolates of PSbMV.

P1-Pro

DPD1	251	PQSLKMKTESIDVLEQVMTIAGKHAKQVTLIDKQKTNRVWIRRVNGVRLQLQVETKHHKG	310
S6	1	.RNI...A...A..D..V.C.....F.....A.K..IK...DI.....R.	60
NY	252	.RNI...A...A..D..V.C.....F.....A.K..VK...DI.....R.	311
DPD1	311	IISQKDASLNNLTKRVARHFARKTAYIHPSDSITHGHSGVVFLRANISGSKSYSIDDLFV	370
S6	61	K...R.VL..D.....Y..S..THV...S.....E..D.N....S.....	120
NY	312	K...R.VL..D.....Y..S..THV...S.....E..D.N....S.....	371
DPD1	371	VRGKRNGKLMESRNKVAWRKMFQIDHFSIVGIKIWNFAEYVKLRDESVSDHDCVGGIT	430
S6	121	G.....H.....N...S..L..N.....T...E.....	180
NY	372	G.....H.....D...S..L...S.....T...K.....	431

HC-Pro

DPD1	431	PEECGILAAQILRVFYPCWRITCTKCISNWLKPTSEQIEHIYERGNLAIQDLNKRIPSA	490
S6	181	I..H.....T.....V..H.....WK..A.	240
NY	432	L..H.....T.....V..H.....RK..A.	491
DPD1	491	HHVTQMVELLRQRIKNTTFDMGNNTKVHELIGHRQDGVFRHLNRLNNSILAANGSSTIEW	550
S6	241	...AH.I.....N.E.....CV...H..N....	300
NY	492	...TH.I.....N.E.....CV...H..N....	551
DPD1	551	ESMNESLLELARWHNKRTESTIASGGISSFRNKISAKAQINFALMCDNQLDTNGNFVWGER	610
S6	301	R.....G.....V.....	360
NY	552	R.....G.....V.....	611

DPD1 611 GYHAKRFFSEFFTKIDPKDGYSHHTVRATPTGVRHLAIGNLIIPGDLQKLREKLEGVSIT 670
S6 361 Y.....T..A..T.....V..... 420
NY 612 Y.....T..A..T.....I..... 671

DPD1 671 AVGISEKCVSRRNGDFVYPCSCVTSENGKPVLSDVILPTRNHLVIGNTGDPKLVLDLPKTE 730
S6 421 T.D.....L.....AM..... 480
NY 672 T.D.....L.....AM..... 731

DPD1 731 TGRMWIAKEGYCYINIFFAMLVNVSEKDAKDFTKFVRDEIMPQLGKWPTMMDVATACYKL 790
S6 481 A.G.....GK..... 540
NY 732 A.G.....KR..... 791

DPD1 791 AIIYPDVRDAQLPRIIVDHSEQIFHVIDSYGSMTTGYHILKAGTVSQLISFAHGALLGEM 850
S6 541 T.....S.....V... 600
NY 792 T.....N.....A... 851

DPD1 851 KMYRVGGTQK 860
S6 601 610
NY 852 861

Table 8.1 Percentage nucleotide (plain) and amino acid (bold) sequence similarities in the partial sequences of NlB gene of four PSbMV isolates. Isolates sequenced in this study are underlined

	DPD1	<u>US</u>	<u>S6</u>	NY
DPD1	100 100			
<u>US</u>	95.8 95.2	100 100		
<u>S6</u>	86.0 95.2	84.7 92.8	100 100	
NY	86.5 94.4	85.2 92.1	98.9 99.2	100 100

Table 8.2 Percentage nucleotide (plain) and amino acid (bold) sequence similarities in the coat protein gene of eight PSbMV isolates. Isolates sequenced in this study are underlined

	DPD1	<u>PK9</u>	NZ	<u>US</u>	CAN	GER	<u>S6</u>	NY
DPD1	100							
<u>PK9</u>	98.7 97.3	100 100						
NZ	98.5 98.5	97.6 96.3	100 100					
<u>US</u>	96.2 98.3	95.2 95.5	94.8 96.8	100 100				
CAN	95.9 98.4	95.1 96.5	94.5 96.8	99.3 99.2	100 100			
GER	95.7 98.1	95.1 96.5	94.6 97.0	93.3 96.2	93.4 96.5	100 100		
<u>S6</u>	87.3 94.8	86.5 93.0	86.3 93.8	86.7 94.0	86.7 94.1	86.4 94.6	100 100	
NY	87.4 94.8	86.8 93.0	86.4 93.8	86.8 93.8	86.8 93.5	86.5 94.3	98.9 98.7	100 100

Table 8.3 Percentage nucleotide sequence similarities in the 3' UTR of eight PSbMV isolates. Isolates sequenced in this study are underlined

	DPD1	<u>PK9</u>	NZ	<u>US</u>	CAN	GER	<u>S6</u>	NY
DPD1	100							
<u>PK9</u>	100	100						
NZ	98.7	98.7	100					
<u>US</u>	100	100	98.7	100				
CAN	100	100	98.7	100	100			
GER	97.5	97.5	96.2	97.5	97.5	100		
<u>S6</u>	95.7	95.0	94.3	95.0	95.6	94.3	100	
NY	96.3	96.3	94.9	96.3	96.2	94.9	99.4	100

Table 8.4 Percentage nucleotide (plain) and amino acids (bold) sequence similarities in the partial P1-Pro and full HC-Pro genes of three PSbMV isolates. Isolates sequenced in this study are underlined

	P1-Pro			HC-Pro		
	DPD1	<u>S6</u>	NY	DPD1	<u>S6</u>	NY
DPD1	100			100		
<u>S6</u>	76.9 79.4	100		82.0 92.1	100	
NY	76.9 78.9	97.2 97.8	100	81.7 92.1	98.5 98.1	100

Chapter 9

IDENTIFICATION OF GENOMIC HETEROGENEITY USING THE RIBONUCLEASE PROTECTION ASSAY

9.1 INTRODUCTION

The ribonuclease protection assay (RPA) was first developed in 1983 (Zinn *et al.*, 1983) and has become a standard technique for RNA analysis. It is an extremely sensitive technique (10 to 50 times more sensitive than northern blots) for the quantitation of specific RNA (Melton *et al.*, 1984, Sambrook *et al.*, 1989). The RPA is based on the hybridisation of the analysed RNA to a radioactively labelled RNA probe with subsequent digestion of unhybridised RNA with single strand-specific nucleases, typically a combination of RNases A and T1. The portion of the probe which hybridises with a homologous RNA sequence is protected from ribonuclease digestion and can be visualised on a denaturing polyacrylamide gel electrophoresis followed by autoradiography.

The RPA has been widely used to study various aspects of RNA and its structure, such as identification and quantitation of specific RNA transcripts (Winter *et al.*, 1985; Trudel *et al.*, 1987; Kurath and Palukaitis, 1989a), localisation of 5' transcription initiation sites (Shimada *et al.*, 1987), localisation of splicing boundary sites (Solnick and Lee, 1987), localisation of 3' polyadenylation sites (Hart *et al.*, 1985), and variation in the RNA sequence of a population particularly by identifying point mutations (Myeres *et al.*, 1985; Lopez-Galindez *et al.*, 1988; Kurath and Palukaitis, 1989b).

PSbMV isolates found in different parts of the world (Maury and Khetarpal, 1992) vary in symptoms, host range, rate of seed transmission, pathogenicity and nucleotide sequence (Table 1.4). In addition, PSbMV isolates S6, US and the Pakistani isolates (PK1-PK12) in this study also showed variation in symptoms, host range, rate of seed transmission, pathogenicity and nucleotide sequencing. Differentiation of PSbMV isolates by expression of pathotype specific pea differentials does not give detailed information on the genome of that particular isolate and is also time consuming. It is

also laborious and expensive to clone and sequence every PSbMV found in field populations in order to compare genomes.

RPA is shown here to be useful for studying the genomic heterogeneity among the isolates of PSbMV described in this thesis. It has been optimised and used to determine the level of variation in the 3' and 5' regions of viral RNA. Isolates S6 and US were used as [✓]representatives of pathotypes P-4 and P-1 in this RPA. The data have been used for a phylogenetic analysis which shows that isolates can be placed in three distinct groups on the basis of the genetic variation identified by RPA.

9.2 MATERIALS AND METHODS

9.2.1 Isolation of PSbMV RNA

RNA was isolated from purified PSbMV particles (section 2.2.8.1) or from PSbMV infected pea leaves (section 2.2.8.2). The final pellet of RNA was dissolved in 29 µl of RPA hybridisation buffer (Table 2.3) and stored at -20°C.

9.2.2 Source of clones

The recombinant clones S6 1.7, US 1.7, PK9 1.3 and S6 1.8 (section 8.2.2) were used to synthesise the minus-sense ³³P-labelled cRNA transcripts.

9.2.3 In vitro transcription of plasmid clones by RNA polymerase

9.2.3.1 Preparation of template DNA

Recombinant plasmid DNA (section 8.2.2) was linearised by digesting with restriction

endonucleases in the buffer recommended by the supplier (section 2.2.16). The recombinant clones S6 1.7 and S6 1.8 were linearised with *Nco* I to make minus-sense radioactively labelled transcripts using SP6 RNA polymerase. The recombinant clones of isolates US 1.7 and PK9 1.3 were linearised with *Spe* I to make minus-sense radioactively labelled transcripts using T7 RNA polymerase. The digested DNA mixture was run on a 1% agarose gel and the linearised DNA was eluted from the gel (section 2.2.12). It was precipitated by the addition of 0.1 volume of 3 M Na acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol (section 2.2.7). The DNA was resuspended in sterile water and stored at -20°C. The final concentration of the DNA was adjusted to 0.5-1 µg / µl.

9.2.3.2 Preparation of radiolabelled cRNA probes

Linearised DNA (1-2 µg) was mixed with 40 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 unit /µl RNasin, 0.5 mM each of rATP, rCTP and rGTP, 12 µM rUTP, 50-100 µCi α-³³P-UTP (Bresatec), and 20-40 units of T7 or SP6 RNA polymerase (Promega) as appropriate. The mixture was incubated at 37°C for 1 hr.

9.2.3.3 Removal of template DNA

The DNA template was destroyed by the addition of 1 unit of RNase-free DNase and incubation at 37°C for 15 min. The reaction was stopped by the addition of 1 µl of 0.5M EDTA.

9.2.3.4 Purification and elution of full length cRNA transcripts

At the end of the DNase incubation, an equal volume of 2 × urea PAGE sample buffer

(Table 2.3) was added to the reaction, and heated for 5 min at 90-95°C. The product was fractionated on a 0.75 mm thick 5% polyacrylamide gel containing 8 M urea (section 2.2.11.2). After electrophoresis the gel was covered with Glad Wrap™ and exposed to X-ray film at room temperature for 2-30 min. The film was autoradiographed and the area of the gel containing full length labelled cRNA transcript was excised with a sterile razor blade and transferred with clean forceps to a 1.5 ml tube. The gel fragment was submerged in about 400 µl of elution buffer (0.5 M NH₄ acetate, 1 mM EDTA, 0.1% SDS) and incubated at 37°C overnight with gentle agitation.

9.2.3.5 Precipitation and storage of cRNA probe

After overnight incubation the tube was centrifuged briefly and the supernatant containing the eluted probe was carefully removed from the gel fragment. The probe was precipitated with 2.5 M ammonium acetate and 2.5 volume of 100% ice cold ethanol at -20°C overnight. The pellet was washed twice with 70% ethanol and resuspended in 10-20 µl RPA hybridisation buffer. The radioactivity of the probe was measured by scintillation counting (section 2.2.9.1.b) and stored at -20°C.

9.2.4 Hybridisation of cRNA probe and sample RNA

Hybridisation was done in a 1.5 ml tube by mixing 29 µl of sample RNA and 1 µl of ³³P-labelled cRNA probe (1×10^4 to 1×10^5 cpm). The contents of the tube were mixed gently, denatured at 95°C for 5 min and immediately transferred to a water bath at 45-50°C. Hybridisation was done for 18-22 hr.

9.2.5 Ribonuclease treatment of RNA:RNA hybrid

After hybridisation the mixture was cooled to room temperature and added to 300 μ l of RNase digestion buffer (Table 2.3) containing RNase A (40 μ g/ml) and RNase T1 (20 μ g/ml). RNA digestion was done at 30-37°C for 30-90 min. The solution which now contained double stranded RNA fragments was deprotenized with 15 μ l of proteinase K (10 mg/ml) and 20 μ l of 10% SDS (w/v) at 37°C for 2-3 hr. Carrier yeast RNA (Type III, Sigma) of 1 μ l (10 mg/ml) was added and the RNase protected fragments were extracted with an equal volume of phenol:chloroform (section 2.2.7). The final RNA pellet was resuspended in 10-15 μ l of DEPC water.

9.2.6 Separation and detection of protected fragments by PAGE

The RNA pellet obtained in section 9.2.5 was mixed with an equal volume of 2 x urea PAGE sample buffer (Table 2.3). The samples were heat denatured at 95°C for 5 min and fractionated on a 8 M urea denaturing 5% polyacrylamide gel (section 2.2.11.2). After electrophoresis, the gel was transferred onto a sheet of filter paper (Whatman 1), covered with Glad Wrap™ and dried in a gel dryer (Model 583, Bio-Rad) at 80°C for 45 min. The dry gel was exposed to X-ray film in the presence of intensifying screens at -70°C for 1-15 days.

9.2.7 Optimised procedure for RPA

The RPA procedure optimised for routine use was as follows. For each reaction, 10,000-100,000 cpm of ³³P-labelled cRNA probe was mixed with 20-50 μ g of total RNA in 30 μ l of RPA hybridisation buffer. Mixtures were heated at 95°C for 5 min and then annealed by incubation at 50°C for 20 hr. After incubation, 300 μ l RNase digestion buffer containing RNase A (40 μ g/ml) and RNase T1 (20 μ g/ml) was added and the

mixtures were incubated for 90 min. Twenty microlitres of 10% SDS and 15 µl of proteinase K (10 mg/ml) were then added and reactions were incubated at 37°C for 2 hr. After adding 10 µg soluble RNA from yeast (Type III, Sigma), the protected RNA fragments were extracted with phenol:chloroform and ethanol precipitated (section 2.2.7). The final RNA pellets were resuspended in 10 µl of DEPC water. RNA samples were heated for 5 min at 95°C, cooled on ice and loaded onto 5% polyacrylamide, 8 M urea gels and electrophoresed at 50°C at 180-200 mA for 2 hr. After electrophoresis the gel was covered with polythene film, dried and exposed to X-ray film at -70°C for 1-15 days.

The RPA banding pattern obtained with either cRNA probe (S6 1.7, US 1.7, PK9 1.3 and S6 1.8) used in this thesis were repeated at least twice to confirm the results.

9.2.8 Phylogenetic analysis of RPA fragments data

For each RPA, the number of fragments (Table 9.1) was recorded (irrespective of the relative intensities of the fragment bands). They were considered to be taxonomically informative characters and used as input data for the phylogenetic relationship analysis among the PSbMV isolates. The number of RPA fragments obtained was analysed by $d_{ij} = (m_i + m_j - 2 m_{ij})/N$ (Aranda, *et al.*, 1993) where d_{ij} is the number of nucleotide substitutions per site between any two isolates (i and j), m_i and m_j are the number of RPA fragments for i and j , m_{ij} is the number of RPA fragments common to i and j , and N is the length of the cRNA probe in nucleotides. For example, the analysis of RPA banding pattern in Fig 9.1 showed for isolate US the presence of $m_i = 10$ fragments (Table 9.1) and for isolate PK1 the presence of $m_j = 15$ fragments (Table 9.1); 8 fragments (Table 9.2) were common to both isolates (m_{ij}). The length (N) of the cRNA probe (S6 1.7) used was 1737 nucleotides. Thus, for isolates US and PK1 the $d_{ij} = 10 + 15 - 2 \times 8 / 1737 = 0.0052$ (Table 9.3).

Estimated d_{ij} values were used to construct a distance matrix which was analysed using PAUP (Beta test version) to assess phylogenetic relationships among the PSbMV isolates. User input distances were analysed as substitution restrictions to create a neighbour joining starting tree (Saitou and Nei, 1987) for a heuristic search by tree bisection-reconnection (Idris and Brown, 1998).

9.3 RESULTS

9.3.1 Comparisons using the S6 1.7 cRNA probe

When the S6 1.7 cRNA probe was used in RPA and annealed to the total RNA of PSbMV isolates, the pattern of protected fragments following RNase digestion (Fig. 9.1) divided the 14 isolates into three groups. Group I contained isolates US, PK5, PK6, PK7; group II contained isolates S6, PK8 and PK12; and group III contained isolates PK1, PK2, PK3, PK4, PK9, PK10, and PK11. These groupings were further confirmed by the d_{ij} values (Table 9.2 and 9.3) obtained from the RPA banding pattern (Fig 9.1) and used in the phylogenetic relationship in Fig. 9.2. As shown in Fig 9.2, variation within the isolates of the same group was observed.

9.3.2 Comparisons using the US 1.7 cRNA probe

The d_{ij} values (Tables 9.4 and 9.5) obtained from the RPA banding pattern using US 1.7 (Fig. 9.3) also divided the above PSbMV isolates into three groups (Fig 9.4) but the relationship among the isolates within the groups was different. For example, in group I isolates PK5, PK6 and PK7 varied in the banding pattern (Fig 9.3) but were similar when S6 1.7 cRNA probe was used (Fig 9.1). Similarly, in group II isolates S6 and PK12 were similar but varied from isolate PK8 (Fig. 9.3). This differs from the pattern for group II when S6 1.7 cRNA was used (Fig. 9.1). In group III, isolates PK1, PK2,

PK3, PK4, PK10, and PK11 were similar to each other whereas isolate PK9 varied in the number of fragments from the rest of the isolates in the group (Fig 9.3).

9.3.3 Comparisons using the PK9 1.3 cRNA probe

The RPA pattern obtained from the cRNA probe of PK9 1.3 (Fig. 9.5), also divided the PSbMV isolates into three groups (I, II, III) giving a result that was almost the same as that of the S6 1.7 grouping. However, the RPA pattern and the number of fragments obtained by using PK9 1.3 (Fig. 9.5) was different from that of the S6 1.7 (Fig. 9.1). The phylogenetic relationship of the PSbMV isolates obtained from the d_{ij} values (Table 9.6 and 9.7) is shown in Fig. 9.6.

9.3.4 Comparisons using the S6 1.8 cRNA probe

When the S6 1.8 cRNA probe was used, only two types of pattern were observed (Fig. 9.7). Isolates S6, PK8, and PK12 were similar to each other and different from isolates PK2, PK3 and PK6 which gave a similar pattern to each other. The rest of the isolates including US, PK1, PK4, PK5, PK7, PK9, PK10, and PK11 did not produce any banding pattern presumably due to greater variation among the sequences. It was therefore not possible to use this probe to study phylogenetic relationships among all the isolates. Nevertheless, this probe showed a clear distinction between a group containing isolates S6, PK8 and PK12 and a group containing isolates US, PK1, PK4, PK5, PK7, PK9, PK10, and PK11.

9.4 DISCUSSION

For detecting and comparing RNA sequences, RPA is a technique which provides more information than nucleic acid blot hybridisation but less information than nucleic acid

sequencing. However, the RPA is much less labour intensive than cloning and sequencing of nucleic acid samples. Thus information on sequence variation in RNA populations can be gathered much more rapidly using an RPA than by sequencing but differences are not defined at the specific nucleotide level.

The results obtained in this chapter showed genomic differences among the isolates in the 3' region that contains the partial NIB, full coat protein and UTR. According to the RPA pattern obtained in Fig. 9.1, 9.3 and 9.5, isolates of PSbMV were divided into three groups: P-1, P-4 and a third group which has not been reported previously. Using RPA, it was also possible to differentiate the PSbMV isolates within each group which is otherwise difficult to achieve by biological criteria.

The cRNA probes of the 3' region clones (S6 1.7, US 1.7 and PK9 1.3) which cover the partial NIB gene, the full coat protein gene and the UTR classified the PSbMV into three groups. However, the cRNA probe of the S6 1.8 clone which covered the partial P1-Pro and full HC-Pro was found to be the most variable region of the genome and a different RPA pattern was obtained (Fig. 9.7). For example, the S6 1.8 cRNA probe put isolates PK2, PK3 and PK6 in one group but the S6 1.7 cRNA probe put these isolates in separate groups.

Using either of the above three cRNA probes (S6 1.7, US 1.7 and PK9 1.3), the Pakistani isolates were divided into three major groups. Further variation was shown among the isolates within the group. A comparison of these RPA results with that of pathotyping (Chapter 6) and sequencing (Chapter 8), showed that there were some differences in the grouping of these isolates. For example, by pathotyping in Table 6.4, isolates PK2, PK3 and PK10 were typical P-1 but on the basis of RPA they were in the unknown group. Similarly, isolate PK5 is a typical U-1 by pathotyping but on the RPA it was put closely to isolate US. One possible explanation is that these isolates might

have sequence similarities in the 3' region used in RPA but differs from each other in the part of the genome responsible for pathogenicity. It is not known that 3' region is involved in pathogenicity or not.

RPA seems to be sensitive for detecting minor differences in the 3' region such as variation among the group isolates of the same group and major variation such as in the 5' region. The 5' region (S6 1.8 kb) was too large and reported to be highly variable (Maia *et al.*, 1996) and is not suitable for comparison by RPA. Therefore a sequence comparison is needed for this region in the PSbMV isolates.

From these results, it is assumed that the RPA developed for PSbMV isolates in this study will be a quick method to identify a pathotype of PSbMV at the genomic level before cloning or sequencing is used to characterise any isolate. It will also be useful for identifying new isolates during surveys or epidemiological studies and to find new isolates or pathotypes in the field or in imported pea seed.

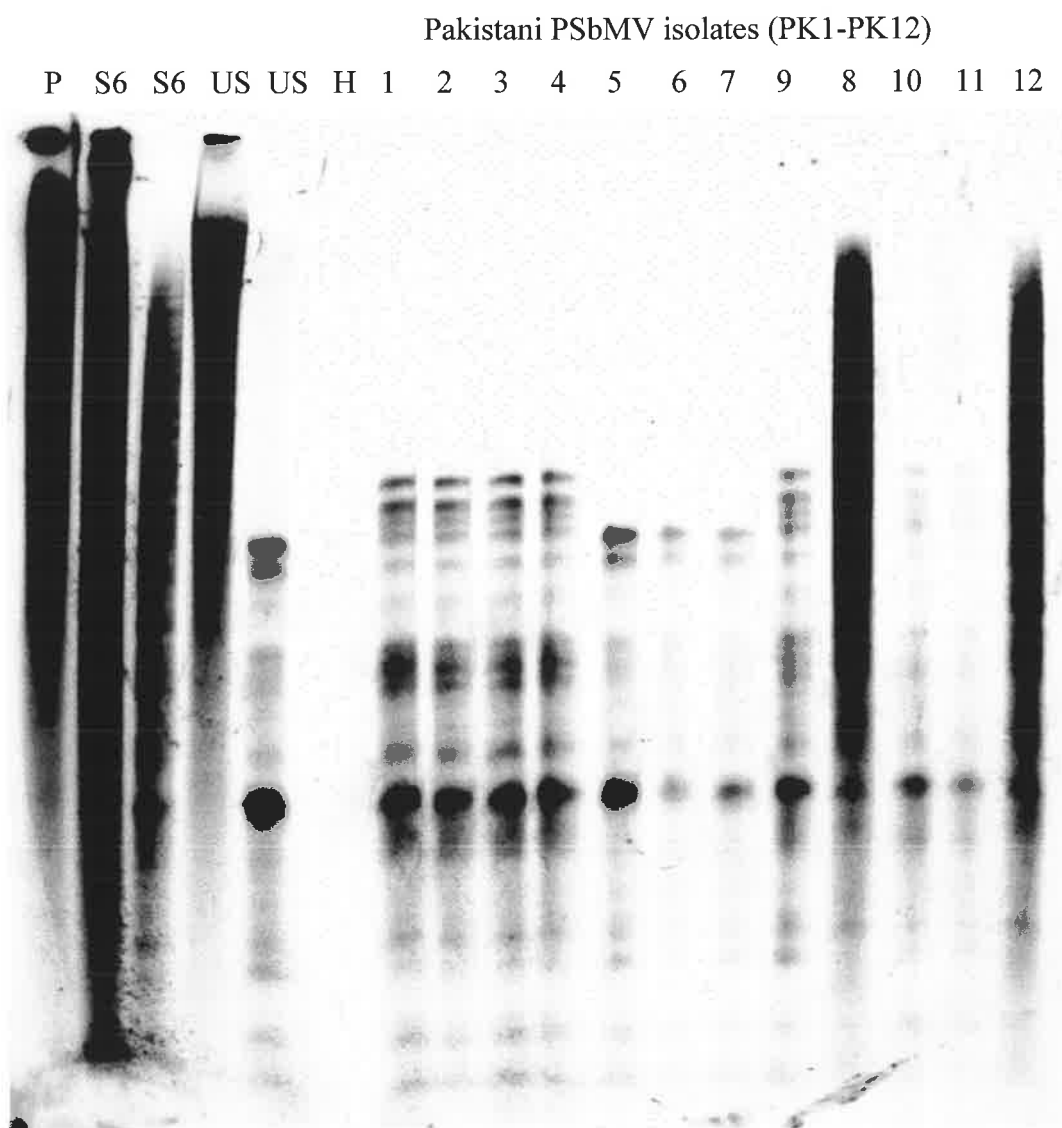


Fig. 9.1 RPA pattern of PSbMV isolates using the S6 1.7 cRNA probe. cRNA probe only (P), hybrids of cRNA with RNA of isolate S6 and US respectively and not digested with RNases (lane 2 and 4), hybrids of cRNA with RNA of isolate S6 and US respectively (lane 3 and 5), of healthy control (lane 6), of isolates PK1-PK12 (lane 7-18) and digested with RNases.

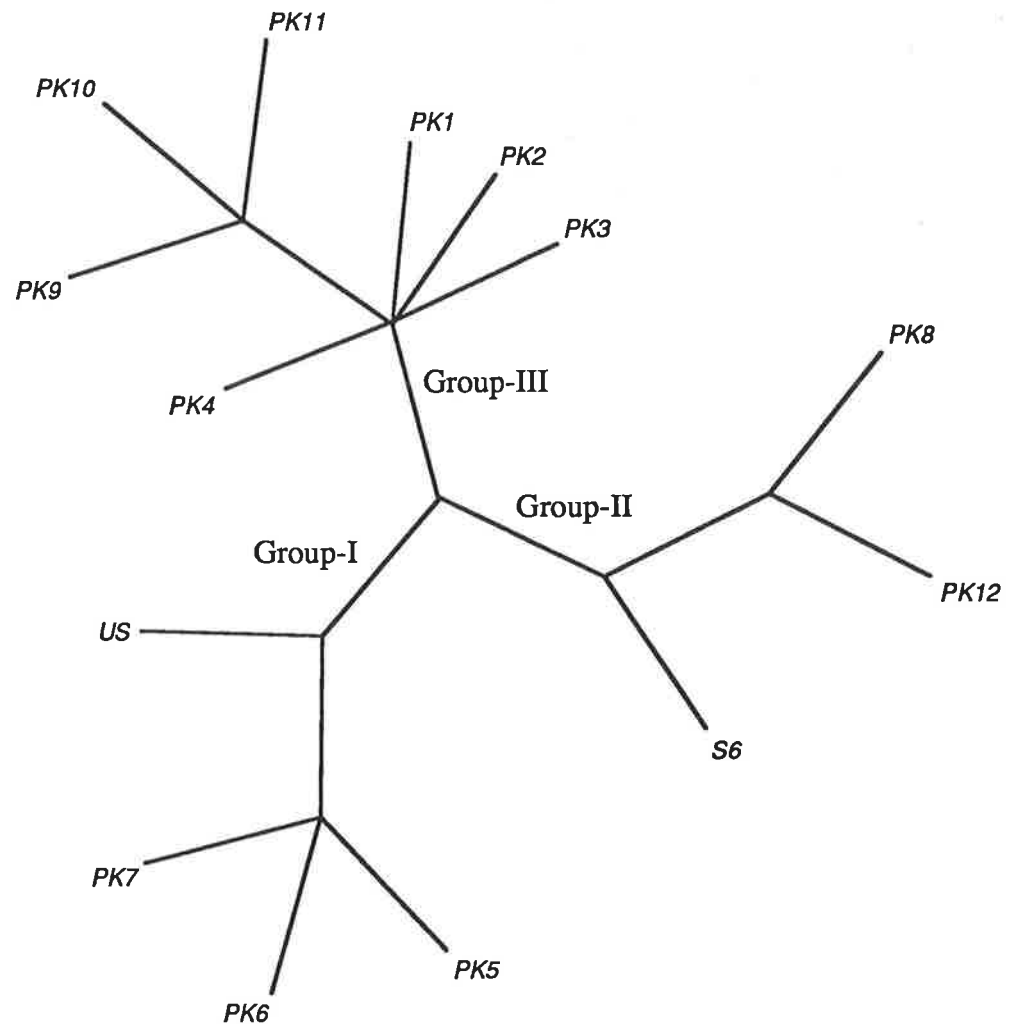


Fig. 9.2 Phylogenetic tree constructed from d_{ij} values (Table 9.3) obtained from RPA pattern using the S6 1.7 cRNA probe (Fig 9.1). Phylogenetic tree was obtained using PAUP (Beta test version) by the heuristic search method by tree bisection-reconnection (Idris and Brown, 1998).

Pakistani PSbMV isolates (PK1-PK12)

P S6 S6 US US H 1 2 3 4 5 6 7 8 9 10 11 12

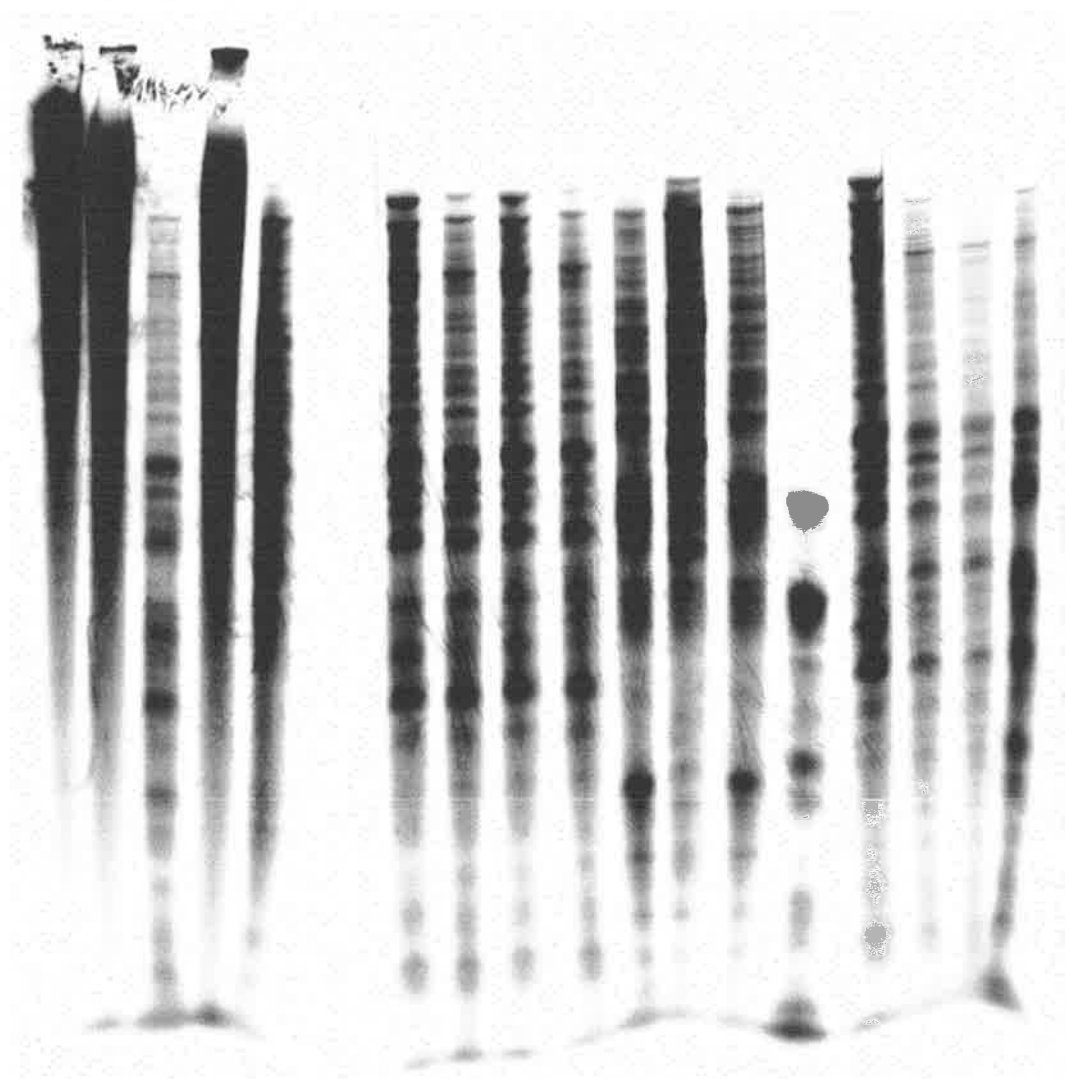


Fig. 9.3 RPA pattern of PSbMV isolates using the US 1.7 cRNA probe. cRNA probe only (P), hybrids of cRNA with RNA of isolate S6 and US respectively and not digested with RNases (lane 2 and 4), hybrids of cRNA with RNA of isolate S6 and US respectively (lane 3 and 5), of healthy control (lane 6), of isolates PK1-PK12 (lane 7-18) and digested with RNases .

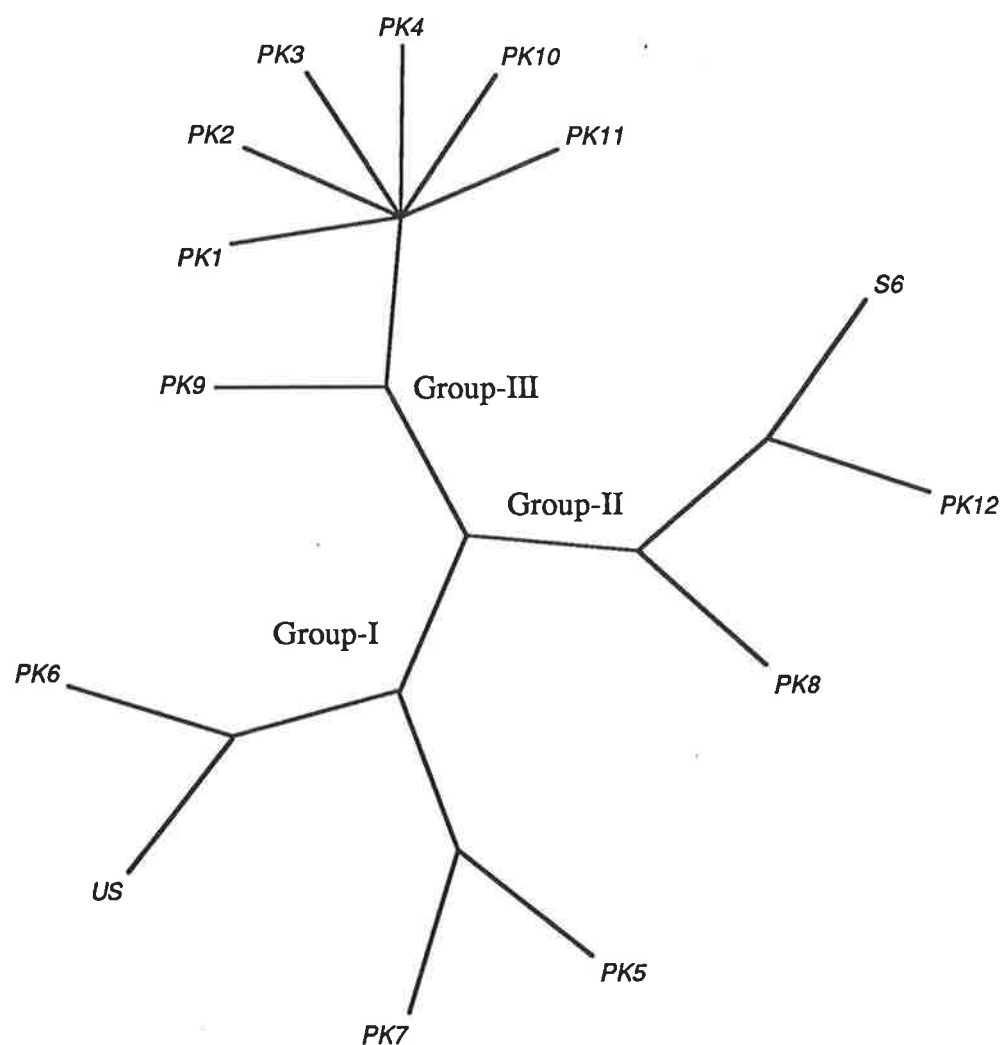


Fig. 9.4 Phylogenetic tree constructed from d_{ij} values (Table 9.5) obtained from RPA pattern using the US 1.7 cRNA probe (Fig 9.3). Phylogenetic tree was obtained using PAUP (Beta test version) by the heuristic search method by tree bisection-reconnection (Idris and Brown, 1998).

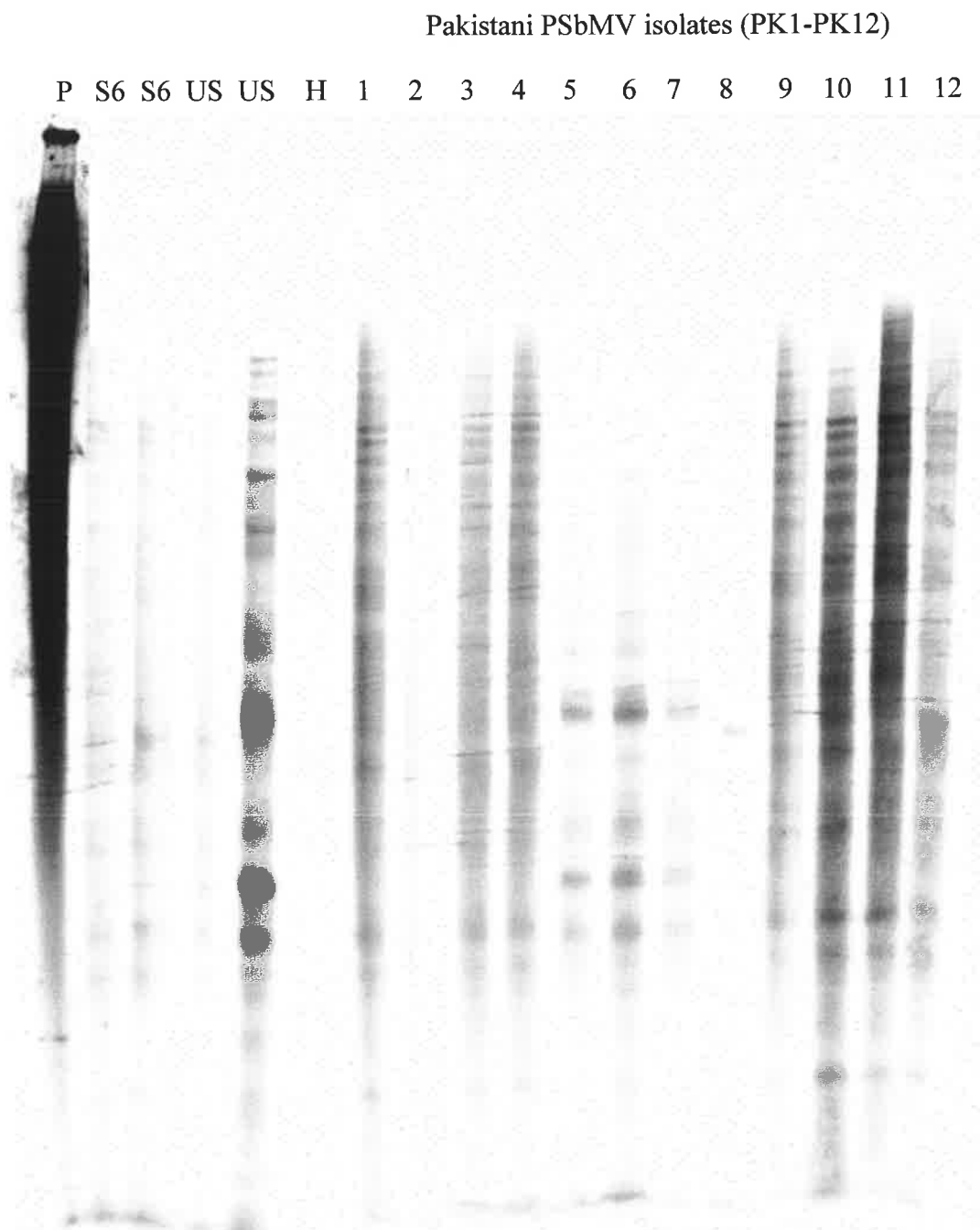


Fig. 9.5 RPA pattern of PSbMV isolates using the PK9 1.3 cRNA probe. cRNA probe only (P), hybrids of cRNA with RNA of isolate S6 and US respectively and not digested with RNases (lane 2 and 4), hybrids of cRNA with RNA of isolate S6 and US respectively (lane 3 and 5), of healthy control (lane 6), of isolates PK1-PK12 (lane 7-18) and digested with RNases .

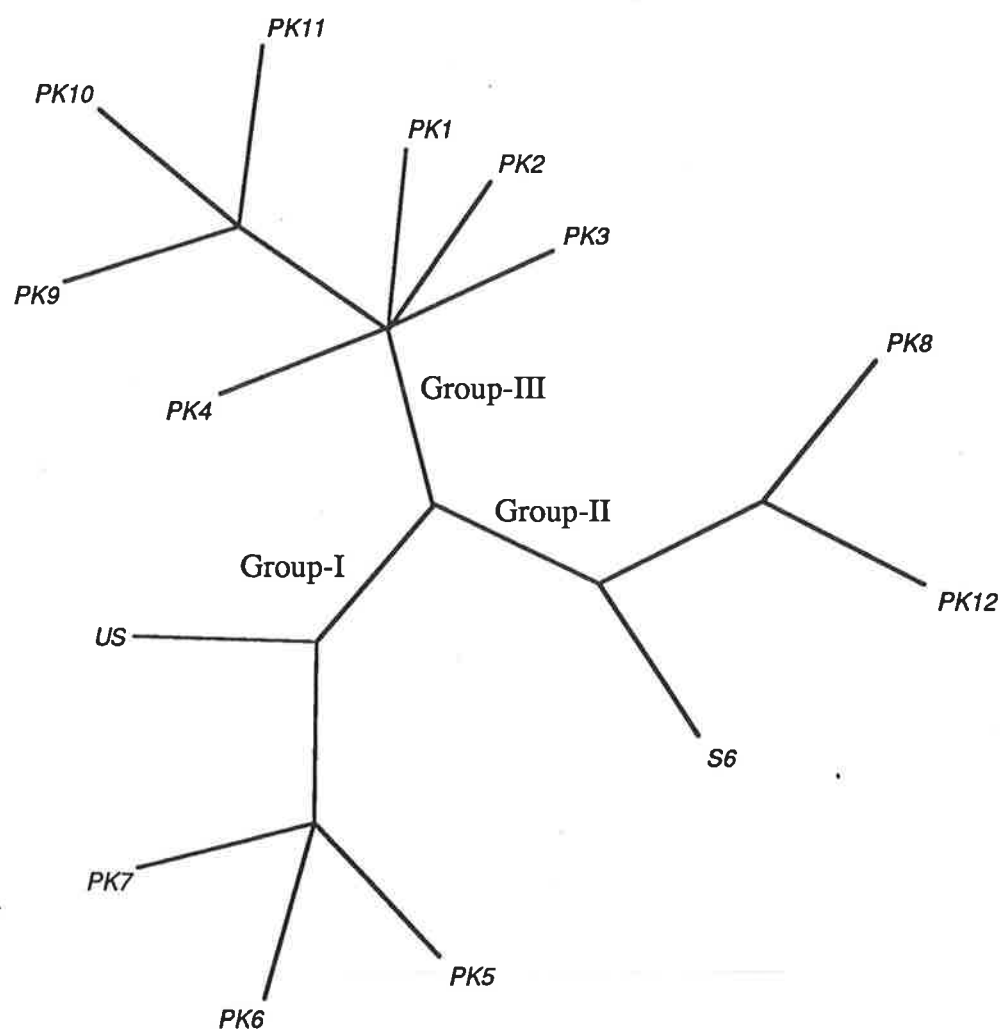


Fig. 9.6 Phylogenetic tree constructed from d_{ij} values (Table 9.7) obtained from RPA pattern using the PK9 1.3 cRNA probe (Fig 9.5). Phylogenetic tree was obtained using PAUP (Beta test version) by the heuristic search method by tree bisection-reconnection (Idris and Brown, 1998).

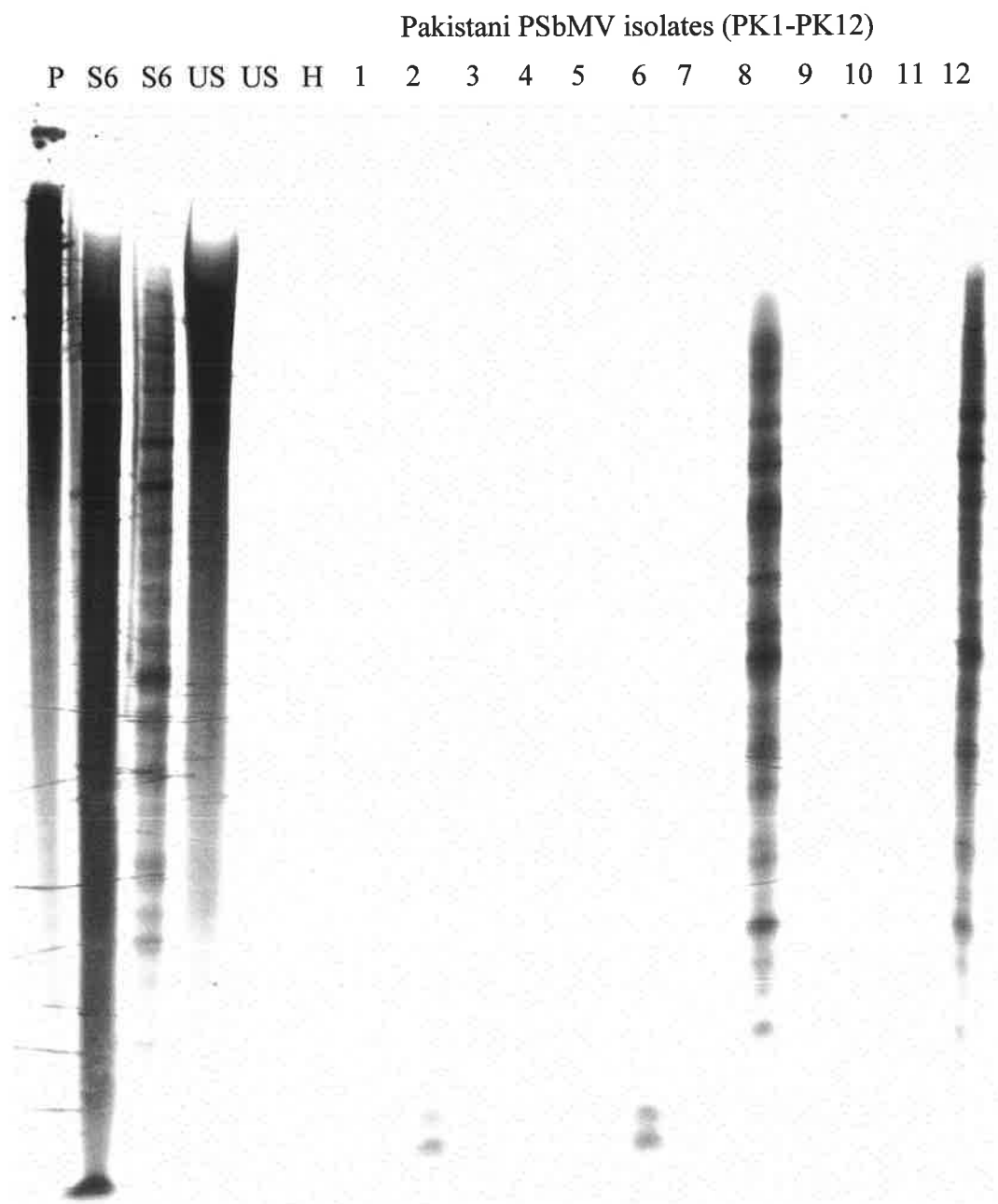


Fig. 9.7 RPA pattern of PSbMV isolates using the S6 1.8 cRNA probe. cRNA probe only (P), hybrids of cRNA with RNA of isolate S6 and US respectively and not digested with RNases (lane 2 and 4), hybrids of cRNA with RNA of isolate S6 and US respectively (lane 3 and 5), of healthy control (lane 6), of isolates PK1-PK12 (lane 7-18) and digested with RNases.

Table 9.1 Number of RPA fragments obtained from their RPA pattern for each isolate using cRNA probes of clone S6 1.7, US 1.7 and PK9 1.3

Isolate	cRNA probes		
	S6 1.7	US 1.7	PK91.3
S6	18	27	11
US	10	23	18
PK1	15	25	22
PK2	15	25	22
PK3	15	25	22
PK4	15	25	22
PK5	10	22	17
PK6	10	24	17
PK7	10	22	17
PK8	18	10	5
PK9	15	28	22
PK10	15	25	22
PK11	15	25	22
PK12	18	26	11

Table 9.2 Number of RPA fragments common among the PSbMV isolates using cRNA probe S6 1.7

cRNA probe S6 1.7														
	S6	US	PK1	PK2	PK3	PK4	PK5	PK6	PK7	PK8	PK9	PK10	PK11	PK12
S6	-													
US	2	-												
PK1	3	8	-											
PK2	3	8	15	-										
PK3	3	8	15	15	-									
PK4	3	8	15	15	15	-								
PK5	2	10	8	8	8	8	-							
PK6	2	10	8	8	8	8	10	-						
PK7	2	10	8	8	8	8	10	10	-					
PK8	17	1	1	1	1	1	1	1	1	-				
PK9	3	8	15	15	15	15	8	8	8	1	-			
PK10	3	8	15	15	15	15	8	8	8	1	15	-		
PK11	3	8	15	15	15	15	8	8	8	1	15	15	-	
PK12	17	1	1	1	1	1	1	1	1	17	1	1	1	-

Table 9.3 Genomic distances as expressed by *dij* values obtained by using RPA data from Table 9.2

cRNA probe S6 1.7														
	S6	US	PK1	PK2	PK3	PK4	PK5	PK6	PK7	PK8	PK9	PK10	PK11	PK12
S6	-													
US	0.0138	-												
PK1	0.0155	0.0052	-											
PK2	0.0155	0.0052	0.0000	-										
PK3	0.0155	0.0052	0.0000	0.0000	-									
PK4	0.0155	0.0052	0.0000	0.0000	0.0000	-								
PK5	0.0138	0.0000	0.0052	0.0052	0.0052	0.0052	-							
PK6	0.0138	0.0000	0.0052	0.0052	0.0052	0.0052	0.0000	-						
PK7	0.0138	0.0000	0.0052	0.0052	0.0052	0.0052	0.0000	0.0000	-					
PK8	0.0012	0.0155	0.0184	0.0184	0.0184	0.0184	0.0156	0.0156	0.0156	-				
PK9	0.0155	0.0052	0.0000	0.0000	0.0000	0.0000	0.0075	0.0075	0.0075	0.0184	-			
PK10	0.0155	0.0052	0.0000	0.0000	0.0000	0.0000	0.0075	0.0075	0.0075	0.0184	0.0000	-		
PK11	0.0155	0.0052	0.0000	0.0000	0.0000	0.0000	0.0075	0.0075	0.0075	0.0184	0.0000	0.0000	-	
PK12	0.0012	0.0144	0.0173	0.0173	0.0173	0.0173	0.0144	0.0144	0.0144	0.0012	0.0173	0.0173	0.0173	-

Table 9.4 Number of RPA fragments common among the PSbMV isolates using cRNA probe US 1.7

cRNA probe US 1.7														
	S6	US	PK1	PK2	PK3	PK4	PK5	PK6	PK7	PK8	PK9	PK10	PK11	PK12
S6	-													
US	5	-												
PK1	12	3	-											
PK2	12	3	25	-										
PK3	12	3	25	25	-									
PK4	12	3	25	25	25	-								
PK5	6	20	2	2	2	2	-							
PK6	6	22	2	2	2	2	21	-						
PK7	6	20	2	2	2	2	22	22	-					
PK8	8	0	0	0	0	0	0	0	0	-				
PK9	12	3	25	25	25	25	2	2	2	0	-			
PK10	12	3	25	25	25	25	2	2	2	0	25	-		
PK11	12	3	25	25	25	25	2	2	2	0	25	25	-	
PK12	25	4	12	12	12	12	3	3	3	10	12	12	12	

Table 9.5 Genomic distances as expressed by *dij* values obtained by using RPA data from Table 9.4

cRNA probe US 1.7														
	S6	US	PK1	PK2	PK3	PK4	PK5	PK6	PK7	PK8	PK9	PK10	PK11	PK12
S6	-													
US	0.0230	-												
PK1	0.0161	0.0242	-											
PK2	0.0161	0.0242	0.0000	-										
PK3	0.0161	0.0242	0.0000	0.0000	-									
PK4	0.0161	0.0242	0.0000	0.0000	0.0000	-								
PK5	0.0213	0.0029	0.0247	0.0247	0.0247	0.0247	-							
PK6	0.0224	0.0017	0.0259	0.0259	0.0259	0.0259	0.0023	-						
PK7	0.0213	0.0029	0.0247	0.0247	0.0247	0.0247	0.0000	0.0012	-					
PK8	0.0121	0.0189	0.0201	0.0201	0.0201	0.0201	0.0184	0.0196	0.0184	-				
PK9	0.0178	0.0259	0.0017	0.0017	0.0017	0.0017	0.0265	0.0276	0.0265	0.0219	-			
PK10	0.0161	0.0242	0.0000	0.0000	0.0000	0.0000	0.0247	0.0259	0.0247	0.0201	0.0017	-		
PK11	0.0161	0.0242	0.0000	0.0000	0.0000	0.0000	0.0247	0.0259	0.0247	0.0201	0.0017	0.0000	-	
PK12	0.0017	0.0236	0.0155	0.0155	0.0155	0.0155	0.0242	0.0253	0.0242	0.0092	0.0166	0.0155	0.0155	-

Table 9.6 Number of RPA fragments common among the PSbMV isolates using cRNA probe PK9 1.3

cRNA probe PK9 1.3														
	S6	US	PK1	PK2	PK3	PK4	PK5	PK6	PK7	PK8	PK9	PK10	PK11	PK12
S6	-													
US	3	-												
PK1	4	4	-											
PK2	4	4	22	-										
PK3	4	4	22	22	-									
PK4	4	4	22	22	22	-								
PK5	3	17	4	4	4	4	-							
PK6	3	17	4	4	4	4	17	-						
PK7	3	17	4	4	4	4	17	17	-					
PK8	4	0	0	0	0	0	0	0	0	-				
PK9	3	4	22	22	22	22	4	4	4	0	-			
PK10	3	4	22	22	22	22	4	4	4	0	22	-		
PK11	3	4	22	22	22	22	4	4	4	0	22	22	-	
PK12	10	3	6	6	6	6	3	3	3	4	6	6	6	-

Table 9.7 Genomic distances as expressed by *dij* values obtained by using RPA data from Table 9.6

cRNA probe PK9 1.3														
	S6	US	PK1	PK2	PK3	PK4	PK5	PK6	PK7	PK8	PK9	PK10	PK11	PK12
S6	-													
US	0.0168	-												
PK1	0.0183	0.0234	-											
PK2	0.0183	0.0234	0.0000	-										
PK3	0.0183	0.0234	0.0000	0.0000	-									
PK4	0.0183	0.0234	0.0000	0.0000	0.0000	-								
PK5	0.0161	0.0007	0.0227	0.0227	0.0227	0.0227	-							
PK6	0.0161	0.0007	0.0227	0.0227	0.0227	0.0227	0.0000	-						
PK7	0.0161	0.0007	0.0227	0.0227	0.0227	0.0227	0.0000	0.0000	-					
PK8	0.0058	0.0168	0.0198	0.0198	0.0198	0.0198	0.0161	0.0161	0.0161	-				
PK9	0.0183	0.0234	0.0227	0.0000	0.0000	0.0000	0.0227	0.0227	0.0227	0.0198	-			
PK10	0.0183	0.0234	0.0227	0.0000	0.0000	0.0000	0.0227	0.0227	0.0227	0.0198	0.0000	-		
PK11	0.0183	0.0234	0.0027	0.0000	0.0000	0.0000	0.0227	0.0227	0.0227	0.0198	0.0000	0.0000	-	
PK12	0.0015	0.0168	0.0154	0.0154	0.0154	0.0154	0.0161	0.0161	0.0161	0.0059	0.0154	0.0154	0.0154	-

Chapter 10

GENERAL DISCUSSION

10.1 Diagnosis

The ability to detect virus infection in plants is important for predicting and monitoring plant virus epidemics. Various procedures are being used for the detection and diagnosis of plant viruses but to effectively detect and control the spread of viruses, it is necessary that the method of detection be sensitive, specific, reliable and easy to perform (Matthews, 1991; Chia *et al.*, 1995). Plant virus diseases can be detected by bioassay on suitable plant cultivars which is a sensitive assay but laborious, expensive and time consuming. Modern rapid methods of plant virus detection are based on the identification of a specific molecular component of the casual agent in tested samples. Virus-specific protein can be detected by serological techniques while the genetic material of the pathogen (nucleic acid) can be detected by a nucleic acid hybridisation assay (Nikolaeva, 1995).

The diagnosis of PSbMV by symptomatology, biological indexing and serology is affected by a range of factors including virus strain, host cultivar and temperature of plant growth. For example, under field conditions symptoms of PSbMV infection are not conspicuous and can escape detection in the field (Maury and Khetarpal, 1992). In addition, several workers have reported symptomless infection of PSbMV where the virus cannot be detected by serology (Khetarpal and Maury, 1990; Ligat and Randles, 1993). At quarantine check points biological indexing and serological methods are currently used for the identification of virus in seed stocks (Khetarpal and Maury, 1987). For such purposes, it is necessary to develop efficient, sensitive and reliable methods for the diagnosis of PSbMV in the field and seed stock.

This thesis reports a modified dot-immunobinding assay (DIBA) for the detection of PSbMV. DIBA was modified by using specific blocking solutions (section 3.2.5) for leaf and seed samples ^{with} and a high concentration of mannose and glucose. Thus non-

specific binding of the antibody with plant lectins (Higgins *et al.*, 1983) was avoided. Extracts of healthy leaf sap or healthy seed extracts in combination with mannose and glucose were shown to be better blocking agents than BSA or skim milk. DIBA was simple and rapid and could detect a minimum amount of about 1 ng of virus (Ali and Randles, 1997). DIBA was found to be consistent when positive controls comprising sap from PSbMV infected leaves (dotted at dilutions of 1/1, 1/10, 1/1000) were included in all tests. DIBA was not only used for PSbMV detection during the survey in Pakistan by testing 713 field samples, 1200 individual dry seeds and 301 seedlings germinated in the glasshouse but also for other viruses like AMV, BYMV and CMV (Table 6.1) which showed that the assay was reliable and specific.

Non-specific results obtained during serology may sometimes be due to antibodies to plant protein in the antiserum as a result of the injection of host materials with virus particles. Purified virus preparations may not be completely free of host contaminants. For example, in this study, virus bands (Fig 3.1) obtained from the virus purification procedure contained not only PSbMV-RNA (section 3.3.4) but also two bands of DNA and RNA which were always seen in association with viral RNA by agarose gel electrophoresis (Fig 3.5). These extra DNA and RNA bands may be antigenic and give non-specific results in DIBA. They might also have host proteins associated with them which could be antigenic. The appearance of such DNA and RNA bands in association with virus particles has not been previously reported. Therefore, it is suggested that the nature of these bands should be identified as well as effects on the specificity of antiserum should be tested.

The need for higher sensitivity for the detection of virus prompted the development of detection methods based on nucleic acid probes (Goodwin and Nassuth, 1993). More sensitive detection and examination of relationships among virus isolates may be achieved by nucleic acid hybridisation. Dot blot and tissue-print nucleic acid based

assays have been developed (Chapter 4) which use randomly primed cDNA probes (^{32}P -labelled or DIG-labelled) representing the entire genome of PSbMV. These probes were specific and no non-specific hybridisation was observed when BYMV and PMV were tested (section 4.3.4). The probes also showed different sensitivity in the dilution endpoint of homologous and heterologous RNA templates of isolates S6 (P-4) and US (P-1). The sensitivity of dot blot hybridisation was higher than DIBA as it could detect as little as 160 pg/ μl of virus and in sap to a dilution endpoint of 1:3125 while 51 fg/ μl of RNA and in total nucleic acid to a dilution endpoint of 1:3125 (Ali *et al.*, 1998). An example of the utility of the dot blot hybridisation assay was that PSbMV or its RNA could be extracted from 50 mg of infected tissue, concentrated by centrifugation, dissolved in 20 μl and 1 μl (equivalent to 2.5 mg of tissue) could be assayed.

Tissue-print hybridisation assay was used for the direct detection of PSbMV in squashed tissue from pea, however not all plants have tissues that can be easily squashed. It should be noted that the sensitivity of tissue printing could not be measured and therefore negative tests with the procedure may not be reliable.

10.2 Pathogenesis of PSbMV

Yield loss is an important criterion for determining the virulence of a pathogen in a particular crop. Yield losses caused by PSbMV infection in pea and other legumes have been reported throughout the world (Makkouk *et al.*, 1993) and are highly variable. These losses have been determined only for local unclassified virus isolates and refer mainly to seed yield of pea. Nothing has been reported for pathotype specific losses and their effects on the growth and yield components of pea (Maury and Khetarpal, 1992).

One of the aims of this thesis was to determine how two distinct pathotypes of PSbMV affected the growth and production of pea. The isolates S6 and US which were

representatives of pathotypes P-4 and P-1 respectively (Ali and Randles, 1997) were used to study their effects in the commercially grown pea cv Dundale. Comprehensive observations of PSbMV infection were made in three sequential generations of pea in order to compare the effects on plants infected mechanically and via seed. It was interesting that the mechanically inoculated plants (Generation 1, G1) were more severely affected than the plants infected via seed (Generation 2, G2). Although smaller effects on yield components were observed in plants infected via seed, seed infection is still important because it can serve as an inoculum to new localities as well as providing primary infection sources for secondary spread by aphids.

The P-4 S6 isolate was shown to be more severe than the P-1 US isolate in its symptomatology, effects on growth, yield components and rate of seed transmission (Ali and Randles, 1998). This was in contrast to the previous reports for another P-4 NY (New York isolate) of PSbMV which showed slight reduction in growth, low seed yield and seed weight and low seed transmission (0.3%) when compared to a P-1 DPD1 (Denmark isolate) of PSbMV which produced vein clearing, leaf rolling and internode shortening, high seed yield and seed weight and high seed transmission (24%) in pea cv 549 (Johansen *et al.*, 1996a). As discussed in chapter 6, the maximum seed transmission rate of P-4 S6 in 12 Pakistani pea varieties was 16% in comparison to 56% for P-1 US (Ali and Randles, 1997). Results in this thesis therefore show that there is variation between the pathogenicities of pathotypes P-4 S6 and P-4 NY, and between P-1 DPD1 and P-1 US. Thus pathotyping does not predict disease severity.

The symptoms of colour break in flower petals and the induction of excessive axillary shoots due to isolates P-4 S6 and P-1 US found in this work have not been reported previously for any PSbMV isolate. This colour break and excessive axillary shoot production might be useful signs for identifying PSbMV infection in the field.

Because mechanically inoculated plants were more severely affected by both PSbMV isolates than the plants infected via seed infection, it is suggested that a number of pea varieties should be inoculated by aphids, mechanically and via seed in both glasshouse and field trials, to further evaluate the effect of mode of inoculation, environment and pathotypes on pathogenesis of PSbMV.

10.3 Ecology of PSbMV

Although PSbMV was first reported from Czechoslovakia and has been reported from a number of countries where peas are grown, it is believed that northern India could be the centre of origin of PSbMV (Maury and Khetarpal, 1992) from where it has spread through infected seed to other parts of the world including Czechoslovakia. This view is supported by evidence that the primary centre of origin of peas is the mountainous region of Southwest Asia, in particular Afghanistan and India. This indicates that PSbMV might have had a long association with pea growing areas of these countries. At present however, very little is known there about the incidence of PSbMV or its isolates.

This is the first report of the natural infection of PSbMV in the major pea growing areas of NWFP of Pakistan, an area close to Afghanistan (Ali and Randles, 1997). PSbMV was recorded from field peas but also from the dry seeds of local pea varieties which are considered to be the main source of inoculum multiplication. Survey results indicated that some of the pea fields and pea varieties were apparently free of PSbMV infection. Several explanations may be considered. It is possible that the incidence of PSbMV was below the level of detection. Another reason may be that farmers of NWFP have grown peas for many centuries with repeated monoculture cultivation and they multiply their own seed locally. Plants for seed production are selected by their good phenotypic appearance and yield. Therefore, selection against infection with

severe isolate of PSbMV may have occurred. Again, it is possible that these apparently healthy plants could have a mild infection which might cross protect against severe isolates of PSbMV (Matthews, 1991). This last explanation seems unlikely as all apparently healthy plants were negative by DIBA, whereas they should be positive by DIBA if harbouring a mild strain of PSbMV. It is also possible that these apparently healthy plants and seeds contain a natural source of resistance which has not been identified before (see section 10.5).

10.4 Variation

As for other organisms, viruses show variation between individual isolates and they may be classified as distinct strains. Criteria such as biological, serological and structural properties and nucleic acid and protein composition are used for the recognition of strains (Matthews, 1991). The study of variability is one of the most important aspects of plant ^{virology} viruses because strains vary in disease severity, can mutate to overcome crop plant resistance and create a problem for classification and identification. Therefore, it is important to characterise the isolates of a virus to the strain level and identify variation among them. RNA viruses are believed to exist as mixtures of related genomes known as quasi-species (Domingo, 1992; Smith *et al.*, 1997). The sequence variants probably arise from the high mutation rate associated with viruses with RNA genomes (usually 10^{-4} - 10^{-5} per base) (Holland *et al.*, 1992) which in turn seems to be a function of the lack of a proof-reading activity in the viral replicase (Steinhauer *et al.*, 1992). The molecular basis of variation is mutation arising from recombination, deletions, additions, nucleotide sequence rearrangements and reassortment of multiple genomes.

Since the discovery of PSbMV in 1966 in Czechoslovakia, it has been reported in a number of countries but so far the only system of classifying PSbMV isolates has been

by biological characterisation on the pathotype specific pea genotypes developed by Alconero *et al.* (1986). According to this classification system, PSbMV isolates have been grouped into three pathotypes: pathotypes P-1 and P-4 from peas, and pathotype L-1 from lentil. Resistance to these pathotypes is reported to be controlled by 4 recessive genes (*sbm-1*, *sbm-2*, *sbm-3* and *sbm-4*) identified in *Pisum sativum* lines homozygous for the recessive gene resistance. Gene *sbm-1* confers resistance to pathotype P-1 isolates (Gritton and Hagedorn, 1975); *sbm-2* and *sbm-3* to pathotype L-1 (Provvidenti and Alconero, 1988a) and *sbm-4* to pathotype P-4 (Provvidenti and Alconero, 1988b). So far examples of pathotypes P-1 (DPD1) (Johansen *et al.*, 1991) and P-4 (NY) (Johansen *et al.*, 1996a) have been fully sequenced while the CP genes of some others including unclassified PSbMV isolates have been partially sequenced (Table 1.4). The sequence of pathotype L-1 isolate has not been reported. No reports have been published describing variation among PSbMV isolates at the molecular level.

A major part of this thesis was to compare a range of methods for evaluating variation among these PSbMV isolates. The 14 PSbMV isolates (S6, US and the Pakistani isolates PK1-PK12) were shown to be similar when compared by serology, capsid protein size, RNA size and nucleic acid hybridisation using randomly primed cDNA probes. When these PSbMV isolates were classified into pathotypes according to the existing system of Alconero *et al.* (1986), some of the Pakistani isolates (PK1, PK4, PK5, PK9 and PK11) were not P-1, P-4 or L-1. As reported in chapter 6, they were grouped tentatively into two previously undescribed pathotypes: U-1 (isolates PK1, PK4, PK5, PK11) and U-2 (isolate PK9) (Ali and Randles, 1997).

The 3' region which covers part of the NIb gene, the full coat protein (CP) gene and the untranslated region of the RNA genome of PSbMV was used to study the molecular variation among isolates S6 (P-4), US (P-1), PK4 (U-1) and PK9 (U-2). The CP gene of potyviruses (section 1.13.2) is multifunctional. It is involved in encapsidation of viral

RNA, aphid transmission, cell-to-cell and systemic movement, as well as showing variation by serology and providing transgenic resistance (Riechmann *et al.*, 1992). These isolates showed molecular variation when compared both by polymerase chain reaction (PCR) and nucleotide sequencing. For example, the primers designed from a common region in the P-1 DPD1 and P-4 NY isolates (Johansen *et al.*, 1991 and 1996a) were used in PCR to amplify the 3' region for isolates S6, US, PK4 and PK9. The first evidence for significant variation was that these primer pairs only amplified the expected region for isolates S6 and US. Therefore, another primer pair was used to amplify the 3' region for the Pakistani isolates PK4 and PK9. This sequence variation among isolates S6 (P-4), US (P-1) and PK9 (U-2) was further characterised by cloning and sequencing the PCR products of the 3' region. However, the PCR product of isolate PK4 (U-1) failed to be cloned in this work, although the same procedure was adopted as used for other isolates. The reason for this failure of cloning is not known. In future work, conditions for the cloning of PCR product of the isolate PK4 should be optimised to allow comparison of the nucleotide sequences with other known PSbMV isolates.

The nucleotide sequences of the 3' regions of isolates S6, US and PK9 were not only compared with each other but were also compared with the sequences of known classified (P-1 DPD1, P-1 NZ and P-4 NY) and unclassified (CAN and GER) (Table 1.4) isolates of PSbMV. A comparison of the CP nucleotide sequences of isolates S6, US, PK9 and the known isolates CAN, DPD1, GER, NZ and NY showed that isolates S6 and US were related to isolates in the P-4 and P-1 groups respectively. In contrast, isolate PK9 fell into the broader P-1 group. However, it was noticed that the P-1 group contained three subgroups or clusters (Table 10.1) and PK9 was in a separate cluster from isolate US. Isolate PK9 showed variation in 12 amino acids when the CP amino acid was aligned with the representative isolate DPD1 in the P-1 group (Fig 8.3). Thus, although PK9 was in the P-1 group it still varied from the other P-1 isolates. As a nucleotide sequence similarity comparison was made only in the CP gene, it might be

possible that isolate PK9 is related to P-1 group isolates on the basis of CP gene sequence but differs in the region of the genome which is responsible for pathogenicity. It is not known whether the CP gene is involved in the pathogenicity of these PSbMV isolates. Isolate PK9 was shown not to be a pathotype L-1 isolate because unlike isolate L-1 it infected both the P-1 and P-4 susceptible pea genotypes in the assay to determine pathotype (Table 1.3 and 6.4). Similarly isolates CAN, and GER (previously unclassified) were placed in the P-1 group on their partial CP nucleotide sequence similarities but were in clusters separate from isolate PK9 thus indicating that P-1 group contains a diverse group of isolates.

Although nucleotide sequencing is widely used for genetic analysis of RNA viruses and has provided insights into molecular variation, sequencing of large viral fragments or genomes is currently a major task (Lopez-Galindez *et al.*, 1988). In addition, failure to clone any isolate (such as isolate PK4, Chapter 7) may not give the required comparison among the isolates. In contrast, the ribonuclease protection assay (RPA) has been used to identify variation in the RNA sequences of a population of many animal and plant viruses (Kurath and Palukaitis, 1989b). It is less labour intensive than cloning and sequencing and information on sequence variation in RNA population can be gathered much more rapidly. Specific applications of the RPA in plant virology include (a) detecting the expression of viral sequences in transgenic plants, (b) quantitation of viral sequences present in nucleic acid extracts, (c) detecting and mapping variation in strains or isolates of virus, and (d) detecting and mapping heterogeneity in RNA populations; for example, in studies involving virus evolution and virus epidemiology (Palukaitis *et al.*, 1994).

The RPA method has been optimised and used for identifying molecular variation among the 14 PSbMV isolates. Using the cRNA probes transcribed from the cloned PCR products of the 3' region of isolates S6 (1737 nt), US (1739 nt) and PK9 (1366 nt),

the RPA data used in phylogenetic analysis divided the 14 PSbMV isolates into three discrete groups. Isolates S6, US and PK9 were representatives of their respective groups. In comparison, the cRNA probe transcribed from the cloned PCR products of the 5' region of isolate S6 (1833 nt; which covers part of the P1-Pro and the full HC-Pro) used in the RPA did not give discrete grouping of these 14 isolates. Only isolates PK8 and PK12 which were classified as P-4 by pathotyping showed similar banding patterns to isolate S6 (P-4) and isolates PK2, PK3 and PK6 clustered as another group (Table 10.1). The remaining isolates including isolate US did not give any banding pattern and it was not possible to use this RPA data for phylogenetic analysis. This suggests that this region of the genome is too sensitive and variation between sequences is too great for this assay to be used. This variation in the 5' region confirms the previous results for these isolates when PCR was used to amplify 5' region where no product was obtained for these isolates except isolate S6 (section 7.3.5). Therefore, it can be assumed that this region of the genome is more variable than the 3' region and is not suitable for RPA comparison. It is further suggested that apart from 3' and 5' regions other regions of the genome should be selected for RPA comparisons in order to identify further variation among these PSbMV isolates and compare the consistency of RPA with that of 3' region results.

As shown in Table 10.1, a comparison of all the three methods (pathotyping, nucleotide sequencing and RPA) was used for grouping of the 14 PSbMV isolates. For example, isolates S6 (P-4), US (P-1) and PK9 (U-2) were placed in separate groups by pathotyping and RPA whereas the nucleotide sequence comparisons (see above) put isolates US and PK9 together in a broader P-1 group but in different sub groups within the P-1 group. In the case of the other 11 isolates (Table 10.1) which were compared only by pathotyping and RPA, the grouping of isolates PK6, PK7, PK8 and PK12 was similar by both methods but the grouping of the remaining isolates (PK1, PK2, PK3, PK4, PK5, PK10, PK11) was different when compared by pathotyping and RPA.

Table 10.1 The grouping of PSbMV isolates by pathotyping, nucleotide sequencing of the CP gene and RPA of two regions of the genome.

Groups	Pathotyping	Nucleotide sequencing	RPA	
		CP gene	3' region (NIb,CP, UTR)	5' region (P1-Pro, HC-Pro)
P-4	S6 PK8 PK12	S6 NY	S6 PK8 PK12	S6 PK8 PK12
P-1	US PK2 PK3 PK6 PK7 PK10	US CAN PK9 DPD1 NZ	US PK5 PK6 PK7	- - - PK2 PK3 PK6
U-1	PK1 PK4 PK5 PK11	GER	PK9 PK1 PK2 PK3 PK4 PK10 PK11	- - - - -
U-2	PK9			

- no bands were protected

Explanation for this includes the following. Pathotyping is a biological method and only determines the pathogenicity of the isolate. Although cRNA probes transcribed from 3' region of the genome was used in RPA, the involvement of either this 3' region or any other region of the genome in pathogenicity is not known. So it is not necessary that isolates placed in a particular group by pathotyping must be in the same group when compared by RPA using a cRNA probe representing only the 3' region of the genome. Another possibility is that isolates PK1, PK4, PK5, and PK11 which were in the U-1 group by pathotyping (Table 10.1) and were placed with the PK9 isolate by RPA might have nucleotide sequences similar to isolate PK9 in the 3' region but may differ in the region of the genome responsible for pathogenicity. This may also apply to isolates PK2, PK3 and PK10 which were placed in the P-1 group by pathotyping.

One important outcome from the RPA grouping was that variation ^{among} between the isolates which was shown by RPA analysis could not be observed by pathotyping. For example, using the US cRNA probe (Fig 9.3) isolates PK5, PK6 and PK7 were placed in the P-1 group, while isolate PK6 showed variation in the banding pattern from isolate PK5 and PK7. This variation was evident from the phylogenetic analysis shown in Fig 9.4. Similarly variation was observed (Fig 9.2 and 9.4) within the group of isolates PK9, PK1, PK2, PK3, PK4, PK10, PK11 which were grouped together by RPA. This detection of variation within the group of isolates can be considered to be an additional advantage of the RPA for comparing isolates. ✓

Symptomatology also varied even if pathotyping did not show as much.

On the basis of the results obtained from the RPA, it is concluded that apart from the pathotype P-1, P-4 and L-1 groups described previously (Alconero *et al.*, 1986), at least one other group of PSbMV exists and is represented only among the Pakistani PSbMV isolates used in this thesis. This new PSbMV group can be named the PK9 group from the representative of the group characterised in this thesis. The evolution of the PK9 group might be the result of natural variation occurring among the Pakistani PSbMV

isolates due to their association with the centre of diversity of the principal host plant “pea”.

10.5 Resistance

Breeding resistance to viruses is generally regarded as being the best long term control measure (Hull and Davies, 1992).

It was observed that some of the Pakistani pea varieties (Climax, Swabi local and VIP) were immune to inoculation with P-4 S6 and P-1 US. However, these varieties were susceptible to Pakistani PSbMV isolates since isolate PK1 (U-1) was isolated from VIP and isolate PK8 (P-4) was isolated from Swabi local. The DIBA positive seed extract from Climax did not infect indicator hosts. It seems that these varieties might be a good source of resistance against PSbMV but further screening against the P-4 NY and P-1 DPD1 isolates, and other available isolates from the new group is recommended.

10.6 Conclusions

The major contribution of this work has been the collection and identification of a new group of PSbMV isolates from Pakistan. In addition, a molecular method (RPA) was found to be useful for classifying these new isolates which otherwise could not be classified by other systems. The ability of RPA to detect microsequence heterogeneity may be extended to diagnostics, epidemiology and evolutionary studies of PSbMV in particular, and other RNA plant viruses in general. The diagnostic methods like DIBA and dot blot hybridisation assays developed were suitable tools to detect and confirm the identities of these new isolates of PSbMV.

APPENDIX A

APPENDIX A

ENZYMES

Lysozyme

MMuLV-reverse transcriptase

Nco I

Pst I

Proteinase K (DNase and RNase free)

RNase A and RNase T1

RQ1 DNase (RNase free)

Spe I

Taq DNA polymerase

T4 DNA ligase

T7 RNA polymerase

SP6 RNA polymerase

SOURCES

Sigma, USA

Gibco BRL, USA

Boehringer Mannheim, Germany

Boehringer Mannheim, Germany

Boehringer Mannheim, Germany

Boehringer Mannheim, Germany

Promega, USA

Boehringer Mannheim, Germany

Promega, USA

Promega, USA

Promega, USA

Promega, USA

NUCLEOTIDES & RADIONUCLEOTIDES

Deoxynucleotide triphosphates (dNTPs)

dATP (2'-deoxyadenosine 5'-triphosphate, sodium salt)

dCTP (2'-deoxycytosine 5'-triphosphate, sodium salt)

dGTP (2'-deoxyguanosine 5'-triphosphate, sodium salt)

dTTP (2'-deoxythymosine 5'-triphosphate, sodium salt)

Promega, USA

Ribonucleotide triphosphates (rNTPs)

rATP (adenosine 5'-triphosphate, disodium salt)

rCTP (cytidine 5'-triphosphate, disodium)

rGTP (guanosine 5'-triphosphate, disodium)

rUTP (uridine 5'-triphosphate, disodium)

Promega, USA

Radionucleotides triphosphates (NTPs) α -³²P-dCTP (3000 Ci/mmol) α -³³P-dUTP (1500 Ci/mmol)

DIG-labelled-dUTP, alkali-labile

Promega, USA

Bresatec, Australia

Bresatec, Australia

Boehringer Mannheim, Germany

KITS

Jet sorb gel extraction kit

Jet sorb plasmid mini kit

Jet sorb plasmid midi kit

Astral Scientific, Australia

Astral Scientific, Australia

Astral Scientific, Australia

MOLECULAR WEIGHT MARKERS

1 Kb DNA ladder

RNA ladder (0.24-9.5 Kb, 1 μ g/ μ l)

Gibco BRL, USA

Gibco BRL, USA

BIOCHEMICALS

Ampicillin

RNAsin[®] Ribonuclease inhibitor

Salmon sperm DNA

Boehringer Mannheim, Germany

Promega, USA

Sigma, USA

CHEMICALS

2-mercaptoethanol

Acrylamide

Agar, purified

Agarose (Type II: medium EEO)

SOURCES

Sigma, USA

Bio-Rad Laboratories, USA

Oxoid Ltd., England

Sigma, USA

Ammonium acetate	BDH Chemical, England
Ammonium persulphate	Bio-Rad Laboratories, USA
Ammonium sulphate	BDH Chemical, England
Bacto-tryptone	Oxoid Ltd., England
Bacto-yeast extract	Oxoid Ltd., England
BCIP	Sigma, USA
Boric acid	BDH Chemical, England
Bromophenol blue	BDH Chemical, England
BSA	Sigma, USA
Caesium sulphate	BDH Chemical, England
Calcium chloride	BDH Chemical, England
Chloroform	BDH Chemical, England
D-Glucose	BDH Chemical, England
D-Mannose	Sigma, USA
DEPC	Sigma, USA
di-Sodium hydrogen orthophosphate	BDH Chemical, England
Sodium thiosulphate	BDH Chemical, England
Dimethyl formamide	Sigma, USA
EDTA	BDH Chemical, England
Ethidium bromide	Sigma, USA
Ethanol	BDH Chemical, England
Ficoll [®] 400	Pharmacia Biotech, Sweden
Formaldehyde	Ajax Chemicals Ltd., Australia
Formamide	BDH Chemical, England
Glacial acetic acid	BDH Chemical, England
Glycerol	BDH Chemical, England
Hydrochloric acid	BDH Chemical, England
IPGT	Boehringer Mannheim, Germany

Iso-amyle alcohol	BDH Chemical, England
Magnesium chloride	BDH Chemical, England
Magnesium sulphate	Ajax Chemicals Ltd., Australia
Manganese chloride	BDH Chemical, England
MOPS	Sigma, USA
N, N' -Methylene-bis-acrylamide	Bio-Rad Laboratories, USA
NBT	Sigma, USA
Phenol	BDH Chemical, England
Polyethylene glycol 8000	Sigma, USA
Polyvinyl pyrrolidone (40,000)	Sigma, USA
Potassium acetate	BDH Chemical, England
Potassium chloride	BDH Chemical, England
Potassium dihydrogen orthophosphate	BDH Chemical, England
Resin, analytical grade mixed bed {AG [®] 501-X8 (D)}	Bio-Rad Laboratories, USA
Rubidium chloride	Sigma, USA
SDS	BDH Chemical, England
Sephadex [®] G-50, fine	Pharmacia Biotech, Sweden
Silver nitrate	BDH Chemical, England
Skim milk	Coles market
Sodium acetate	BDH Chemical, England
Sodium chloride	BDH Chemical, England
Sodium hydroxide	BDH Chemical, England
TEMED	Sigma, USA
Triton X-100	Sigma, USA
Trizma base	Sigma, USA
Urea	BDH Chemical, England
X-gal	Promega, USA
Xylene cyanol F.F.	Ajax Chemicals Ltd., Australia

APPENDIX B

APPENDIX B

AGAROSE GELS

Non-denaturing:

0.8-1.0 g of agarose was mixed with 100 ml of TAE buffer and dissolved by heating in microwave oven for 3-4 min. The agarose was cool down to 50°C in a water bath and 16 µl EtBr (10 mg/ml) was added before pouring in the gel plate.

Denaturing:

0.5 g of agarose was dissolved as above in 31.7 of DEPC-treated water. Cool down to 50°C in a water bath and add 10 ml of 5 × FGRB and 8.27 ml of formamide (preheated at 50°C) to make the final volume 50 ml. Poured the mixture in minigel (13 cm in length) up to 40 ml.

POLYACRYLAMIDE GELS

Acrylamide solution: (30%)

29 g of acrylamide and 1 g of bis-acrylamide were mixed together, dissolved in SDDW and the volume was adjusted to 100 ml. The polyacrylamide solution was sterilised with a syringe filter (45 µm). The sterilised solution was stored in a light protected bottle at 4°C and used for 6-8 months.

Acrylamide solution: (40%)

39 g of acrylamide and 1 g of bis-acrylamide were dissolved together in 100 ml of SDDW. The solution was filter sterilised and stored as above.

5% denaturing (8 M urea) polyacrylamide gel:

<u>Reagents</u>	<u>Volume (30 ml)</u>
SDDW	22.5
Urea	14.4 g
40% acrylamide solution	4.5 ml
10 × TBE	3.0
10% APS	0.210
TEMED	0.032

10% SDS-polyacrylamide gel:

<u>10% Resolving gel</u>	<u>(10 ml)</u>	<u>4% Stacking gel</u>	<u>(5ml)</u>
SDDW	4 ml		3 ml
1.5 M Tris (pH 8.8)	2.5	0.5 M Tris (pH 6.8)	1.25
30% acrylamide solution	3.3		0.83
10% SDS	0.1		0.05
10% APS	0.05		0.05
TEMED	0.005		0.005

BACTERIAL GROWTH MEDIA**LB medium:**

<u>Reagents</u>	<u>volume 1 litre</u>
bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g
SDDW	800 ml

Dissolve the solutes and adjust the pH to 7.0 with 10 N NaOH. Adjust the volume of the solution up to 1 litre with SDDW. Sterilise by autoclaving at 15 lb/sq.in. on liquid cycle for 20 min.

LBA medium:

Take LB liquid medium and add bacto agar (@ 15 g/litre). Sterilise by autoclaving at 15 lb/sq.in. on liquid cycle for 20 min.

LBA medium for transformation:

Take LB liquid medium and add bacto agar (@ 15 g/litre). Sterilise by autoclaving at 15 lb/sq.in. on liquid cycle for 20 min. Before pouring into petridishes, add 200 µl of ampicillin (50 mg/ml), 200 µl of IPGT (25 mg /ml) and 500 µl of X-gal (20 mg /ml).

SOB medium:

<u>Reagents</u>	<u>volume 1 litre</u>
bacto-tryptone	20 g
bacto-yeast extract	5.0 g
NaCl	0.5 g
SDDW	800 ml

Dissolve the solutes and adjust the pH to 7.0 with 10 N NaOH. Adjust the volume of the solution up to 1 litre with SDDW. Sterilise by autoclaving as above. Store at room temperature. Add 10 ml of filter sterilised 1 M MgCl_2 and 10 ml of filter sterilised 1 M MgSO_4 .

SOC medium:

Take SOB medium (100 ml) and add 2 ml of 1 M filter sterilised glucose solution prior to use.

STAINING SOLUTIONS2% Phosphotungstic acid used for electron microscopy:

2.0 g of $\text{H}_3\text{PO}_4 \cdot 12 \text{WO}_3 \cdot x \text{H}_2\text{O}$ was dissolved in 100 ml of sterile water and the pH 6.8 of the solution was maintained with 1 N NaOH.

0.25% Coomassie brilliant blue used for SDS-PAGE:

0.25 g of Coomassie brilliant blue R250 was dissolved in 90 ml of 45% methanol and 10% of glacial acetic acid. The solution was filter sterilised and stored in a light protected bottle.

SDS-PAGE destaining solution:

100 ml destaining solution was by mixing 45 ml of concentrated methanol, 10 ml of glacial acetic acid and 45 ml sterile water.

SOLVENTSChloroform solution:

24 volumes of chloroform were mixed with 1 volume of *iso*-amyl alcohol and stored in a light protected bottle.

Formamide:

Formamide was mixed with a mixed bed resin (AG 501-X8) (10 g/100 ml) to deionise by stirring for 1 hr and then filtered with two layers of Whatmann filter paper No.1. The deionised formamide was stored at -20°C.

Phenol:

Solid phenol was melted in water bath at 60°C. Equal volume of 0.5 M Tris-HCl (pH 7.5) was added and stirred for 15 min. The upper phase was removed after 2 min. Then

equal volume of 0.1 M Tris-HCl (pH 7.5) was added and stirred as above. Finally 0.1 volume of 0.1 M Tris-HCl (pH 7.5) was added and stored in a light proof bottle.

Phenol:Chloroform solution:

The above phenol solution was mixed with an equal volume of chloroform (24:1) and shake for 1-2 min. The solution was stored at 4°C.

Sephadex® G-50

Sephadex® G-50 fine resin of 2.5 g was mixed (@ 10 g/160 ml) with 40 ml of sterile water. The solution was shaken for 1-2 min and then left on the bench for 25 min until the beads were sit at the bottom. The upper aqueous phase was removed and this process was repeated 3 times. Finally 30 ml of 1 × TAE (pH 7.6) was added and then sterilised by autoclaving at 15 lb/sq.in. on liquid cycle for 20 min. The solution was stored at room temperature.

APPENDIX C

APPENDIX C

(a) Nucleotide sequence alignment of partial Nib gene of 4 PSbMV isolates

DPD1	8184	TACACAGAAATCATTTCACCTCCAATAGCAACACCAGATGGCTCAGTCATAAAGAAGACG	8243
<u>US</u>	1	A.....T.....C.....G..G	60
<u>S6</u>	1	G.....T.....T.....A..A	60
NY	8122	G.....T.....T.....A..A	8181
DPD1	8244	AAAGGAAATAATAGTGGACAACCATCAACAGTTGTTGATAACACATTGATGGTGATTCTT	8303
<u>US</u>	61	T.....A..T..T.....G....GG...T	120
<u>S6</u>	61	C.....T..C..C.....A....AA...C	120
NY	8182	C.....T..T..C.....A....AA...C	8241
DPD1	8304	GCTATGCAGTATAGCCTACAGCTACTTGGAGTAGATTTTGAAACACAGGATGAAGTCGTT	8363
<u>US</u>	121	..T....A..T...C.A...T...T..A..T.....G AA.....A...G..	180
<u>S6</u>	121	..A....G..C...T.G...T...A..T..G.....A.CT.....C...A..	180
NY	8242	..A....G..C...T.G...T...A..T..G.....A.CT.....C...A..	8301
DPD1	8364	CGCTATTTTGCAAATGGTGATGATCTATTGATAGCTGTGCGGCCAGACTGTGAGTTTGTG	8423
<u>US</u>	181	T.....AT.G.....G.....C.....G..T...	240
<u>S6</u>	181	C.....CC.C.....T.....T.....A..C...	240
NY	8302	C.....CC.C.....T.....T.....A..C...	8361
DPD1	8424	CTCAAAGGCTTGGAGATACATTTCTCTAATCTGGGGTTAAATTATAACTTTTCTGCGCGG	8483
<u>US</u>	241	..GA...C..GG..GC.....T..T..G.....TC.TG.-.....T..G...	299
<u>S6</u>	241	..CG...T..AG..AA.....A..C..A.....CT.CG.C.....G..A...	300
NY	8362	..CG...T..AG..AA.....A..C..A.....CT.CG.C.....G..A...	8421
DPD1	8484	CACCATGACAAGAAAGATGTTTGGTTTATGTCAACAAGAGGCATTTTGAGAGATGGAATT	8543
<u>US</u>	300	..C.....C.....T.....GG.....T..G.....A...	359
<u>S6</u>	301	..T.....T.....C.....AA.....T..A.....T...	360
NY	8422	..C.....T.....C.....AA.....G..A.....T...	8481
DPD1	8544	CTGATACCAAACTTGAA	8561
PK9	1	T..T	5
<u>US</u>	360	..G.....AC.T..A	377
<u>S6</u>	361	..A.....GC.C..A	378
NY	8482	..A.....AC.C..A	8499

(b) Nucleotide sequence alignment of complete coat protein gene of 8 PSbMV isolates

DPD1	8562	GAAGAACGAATTGTTGCAATTTTGAATGGGATAGAAGTAGAGAATTTTCACATAGGCTT	8621
<u>PK9</u>	1	T.AA.....T.....A...T.....T.AA.....T..A..T..G..T	60
NZ	1	G.AG.....T.....A...T.....T.GA.....T..A..T..G..T	60
<u>US</u>	1	G.AG.....T.....A...T.....T.GA.....T..A..T..G..T	60
CAN			
GER			
<u>S6</u>	1	G.GG.....C.....A...C.....C.GG.....C..T..C..A..C	60
NY	8500	G.AG.....C.....G...C.....C.GG.....C..T..C..A..C	8559
DPD1	8622	GATGCCATATGTGCAGCAATGATTGAAGCTTGGGGTTACGACGAGCTTTTGCAGCATATT	8681
<u>PK9</u>	61	CA.....T.....C.....C..C.CG.....T...T...	120
NZ	61	CA.....C.....C.....C..C.AG.....C...T...	120
<u>US</u>	61	TA.....T.....C..T.AG.....C...T...	120
CAN	1	T..T.AG.....C...T...	35
GER	1	T..C.AA.....C...T...	35
<u>S6</u>	61	AG.....T.....C..T.AA.....C...C...	120
NY	8560	AA.....T.....C..T.AA.....C...C...	8619
DPD1	8682	CGGAAATTCTATTATTGGTTGTTAGAACAGGAACCATACAGGAGCATAGCTCAGGAAGGA	8741
<u>PK9</u>	121	..T...C.C.....T.GT.....A.....C..G..C.....T.....A	180
NZ	121	..G...T.C.....T.GT.....A.....C..G..C.....T.....A	180
<u>US</u>	121	..G...T.C.....T.AT.....A.....C..G..C.....G.....A	180
CAN	36	..G...T.T.....T.AT.....A.....C..G..C.....G.....A	95
GER	36	..G...T.C.....T.AT.....A.....C..G..C.....T.....A	95
<u>S6</u>	121	..G...T.C.....C.GC.....G.....T..A..T.....T.....C	180
NY	8620	..G...T.C.....C.GC.....G.....T..A..T.....T.....C	8679
DPD1	8742	AAAGCACCATACATCGCGGAGACAGCGCTTCGGCACCTGTACACAAATGCCATGGCAACA	8801
<u>PK9</u>	181	..A.....C..C..AA.G.....G..T..G...C.G..C.....CA.G.....	240
NZ	181	..A.....C..C..AG.G.....G..T..G...C.G..C.....CA.G.....	240
<u>US</u>	181	..A.....C..C..AG.G.....G..C..G...C.G..C.....CA.G.....	240
CAN	96	..A.....C..C..AG.G.....G..A..G...C.G..C.....CA.G.....	155
GER	96	..A.....C..C..AG.G.....G..C..G...C.G..T.....CA.G.....	155
<u>S6</u>	181	..G.....T..T..AG.A....T..T..A...T.A..C.....TG.C.....	240
NY	8680	..G.....T..T..AG.A....T..T..A...T.A..C.....TG.C.....	8739
DPD1	8802	CAAAGTGAAGTTGAGAAATACACGGAAGCAATCAATCAGCATTACAATGATGAAGGTGGC	8861
<u>PK9</u>	241	GC.T..G.AA...CG.....CA.....T....TGA...A.GT..C	300
NZ	241	AC.T..G.AA...CG.....CA.....T....TGA...A.GT..T	300
<u>US</u>	241	AC.T..G.AA...CG.....CA.....T....CGA...A.GT..C	300
CAN	156	AC.T..G.AA...CG.....CA.....T....CGA...A.GT..C	215
GER	156	AC.C..G.AA...CG.....CA.....T....CGA...A.GT..C	215
<u>S6</u>	241	AT.A..A.GG...TA.....TG.....C.....---G.AC..T	297
NY	8740	AC.A..A.GG...TA.....TG.....C.....---G.AC..T	8796

DPD1	8862	GATGGATCAATCAAGGTTTCGATTGCAAGCTGGTGACGAAACCAAGGATGATGAAAGAAGA	8921
<u>PK9</u>	301	G...AT.A..C..G..TC.A.....C..A..C..G.....TGAAGG.AGA	360
NZ	301	G...AT.A..C..G..TC.A.....C..A..C..G.....TGAAAG.AGA	360
<u>US</u>	301	G...AT.A..C..G..TC.G.....C..A..T..G.....CGAAAG.AGA	360
CAN	216	G...AT.A..C..G..TC.G.....C..A..T..G.....CGAAAG.AGA	275
GER	216	G...AT.A..C..G..TC.G.....C..A..C..G.....CGAAAG.AAA	275
<u>S6</u>	301	A...TC.G..T..A..GA.G.....T..G..C..A.....AGAGAA.CGG	357
NY	8800	A...TC.G..T..A..GA.G.....T..G..C..A.....AAGAGA.ACG	8856
DPD1	8922	AGGAAAGAGGAGGAGGACAGAAAGAAAAGAGAGAGAGTATCGATGCGAGCCAGTTTGGT	8981
<u>PK9</u>	361	..G.A...G.....C.....A.A.....G.T...TG.G..C..G..T.G.	420
NZ	361	..G.A...G.....C.....A.A.....G.A...TG.G..C..G..T.G.	420
<u>US</u>	361	..G.A...G.....C.....A.A.....G.A...TA.G..C..G..C.G.	420
CAN	276	..G.A...G.....C.....A.A.....G.A...TG.G..C..G..C.G.	335
GER	276	..G.G...G.....C.....A.A.....G.A...TG.G..C..G..T.G.	335
<u>S6</u>	361	..A.A...A.....T.....G.C.....A.A...CG.A..T..A..T.A.	417
NY	8860	..A.A...A.....T.....G.C.....A.A...CG.A..T..A..T.G.	8916
DPD1	8982	TCGAATCGTGACAATAAGAAAAACAAAAATAAAGAGAGTGACACACCAAACAAATTAATA	9041
<u>PK9</u>	421	..G.A...T.....T..G..A.....T..A.....T..CA.AT.A..C.A.TTAA..	480
NZ	421	..G.A...T.....T..G..A.....T..A.....T..CA.AT.A..C.A.TTAA..	480
<u>US</u>	421	..G.A...T.....C..G..A.....C..T.....C..TG.TC.T..T.G.TCAA..	480
CAN	336	..G.A...T.....C..G..A.....C..A.....T..TG.TC.T..T.G.TCAA..	395
GER	336	..A.A...T.....T..G..A.....T..A.....T..CA.AT.A..C.A.CTAG..	395
<u>S6</u>	421	..G.G...A.....C..A..G.....C..A.....T..TA.AT.G..C.A.GCCA..	477
NY	8920	..G.G...A.....C..A..G.....C..A.....T..TA.AT.G..C.A.GCCA..	8976
DPD1	9042	GTGAAGTCTGATCGAGATGTTGATGCAGGATCTTCAGGCACAATCACAGTACCAAGGCTT	9101
<u>PK9</u>	481	.T...G..T.....T..T.....T.T.....C..A..C.C...A..A..G..T	540
NZ	481	.T...G..T.....T..T.....T.T.....C..A..C.C...A..A..G..T	540
<u>US</u>	481	.T...A..T.....C..G.....A.T.....C..A..T.T...G..A..G..T	540
CAN	396	.C...A..T.....C..G.....A.T.....C..A..T.T...G..A..G..T	455
GER	396	.T...G..T.....T..T.....T.T.....T..A..C.C...A..A..G..T	455
<u>S6</u>	481	.T...A..A.....T..T.....A.A.....A..C..C.C...G..T..A..C	537
NY	8980	.T...A..A.....T..T.....A.A.....A..C..C.C...G..T..A..C	9036
DPD1	9102	GAAAAGATCTCAGCAAAGATTAGGATGCCAAAACACAAAGGCGGAGTGGCTATCAGCTTG	9161
<u>PK9</u>	541	..A.....C.....A..G..T..G.....A..A.....A..C..A..A..T..C..CT.G	600
NZ	541	..A.....C.....A..G..T..G.....A..A.....A..C..A..A..T..C..CT.G	600
<u>US</u>	541	..G.....T.....C..A..T..G.....A..A.....G..T..A..A..T..T..CT.G	600
CAN	456	..G.....T.....C..A..T..G.....A..A.....G..T..A..A..T..T..CT.G	515
GER	456	..A.....C.....A..G..C..G.....A..A.....A..C..A..A..G..C..C..CT.A	515
<u>S6</u>	541	..A.....T.....A..A..T..A.....G..G.....A..T..G..G..G..T..TC.G	597
NY	9040	..A.....T.....A..A..T..A.....G..G.....A..T..G..G..G..T..TC.G	9096
DPD1	9162	CAACATTTAGTTGATTACAATCCAGCACAAGTTGACATTTCAAACACTCGAGCAACGCAG	9221
<u>PK9</u>	601	..A.....A...-..T.....T.....A..A.....C..T.....C.....A.....G..G	659
NZ	601	..A.....A...G.T.....T.....A..A.....C..T.....C.....A.....G..G	660
<u>US</u>	601	..A.....A...G.C.....T.....A..A.....C..C.....T.....A.....G..G	660
CAN	516	..A.....A...G.C.....T.....A..A.....C..C.....T.....A.....G..G	575
GER	516	..G.....A...G.C.....T.....G..A.....T..T.....C.....A.....G..A	575
<u>S6</u>	601	..G.....G...G.C.....C.....A..G.....C..A.....C.....C.....A..G	657
NY	9100	..G.....G...G.C.....C.....A..G.....C..A.....C.....C.....A..G	9156

DPD1	9222	AGCCAGTTCGATAACTGGTGGAGGGCAGTGTGCGCAAGAGTACGGGGTTGGAGACAATGAA	9281
<u>PK9</u>	660	G..C.....C.....GGC.....G.....G..C...G....A..C.AT..A	719
NZ	661	G..C.....C.....GCG.....G.....G..C...G....A..C.AT..A	720
<u>US</u>	661	G..C.....C.....GGC.....A.....G..C...G....A..C.AT..A	720
CAN	576	G..C.....C.....GGC.....A.....G..C...G....A..C.AT..A	635
GER	576	G..C.....C.....GGC.....G.....A..C...A....A..T.GT..A	635
<u>S6</u>	661	A..T.....T.....AGC.....T.....G..T...A....T..C.GC..G	717
NY	9160	A..T.....T.....AGC.....C.....G..T...A....T..C.GC..G	9216
DPD1	9282	ATGCAAGTTTTGGCAAGTGGTTTGATGGTATGGTGCATTGAAAATGGAACATCGCCTAAC	9341
<u>PK9</u>	720	T..G.....A.....C.....T.....G.....	779
NZ	721	T..G.....A.....C.....T.....G.....	780
<u>US</u>	721	T..G.....A.....C.....T.....G.....	780
CAN	636	T..G.....A.....C.....T.....G.....	695
GER	636	T..G.....A.....C.....C.....A.....	695
<u>S6</u>	721	G..A.....G.....T.....C.....G.....	777
NY	9220	G..A.....G.....T.....C.....G.....	9276
DPD1	9342	ATAAATGGGATGTGGACAATGATGGACGGGGAAGAGCAGGTTGAGTACCCCCTAAAGCCA	9401
<u>PK9</u>	780	C.....A..A..G.....C.A..G...	839
NZ	781	C.....A..G..G.....C.A..G...	840
<u>US</u>	781	C.....A..G..A.....C.A..G...	755
GER	696	T.....G..A..G.....T.G..G...	755
<u>S6</u>	781	C.....G..A..G.....C.A..A...	837
NY	9280	C.....G..A..G.....C.A..A...	9336
DPD1	9402	GTGATGGATAATGCGCGTCCAACCTTTCAGACAGATAATGGCGCATTTCAGTGACGTAGCG	9461
<u>PK9</u>	840	T..C.....G.....	899
NZ	841	T..C.....G.....	900
<u>US</u>	841	T..C.....G.....	900
CAN	756	T..C.....G.....	815
GER	756	C..T.....G.....	815
<u>S6</u>	841	T..T.....A.....	897
NY	9340	T..T.....A.....	9396
DPD1	9462	GAGGCGTACATTGAAAAGAGAAACTCAACAGAGGTGTACATACCCAGGTACGCTCTACAA	9521
<u>PK9</u>	900	G.....ATACCCAGGTACG..C.....	959
NZ	901	G.....-----G..C.....	948
<u>US</u>	901	G.....ATACCCAGGTACG..C.....	960
CAN	816	G.....ATACCCAGGTACG..C.....	875
GER	816	G.....ATACCCAGGTACA..C.....	875
<u>S6</u>	901	A.....ATACCCAGGTATG..T.....	957
NY	9400	A.....ATACCCAGGTATG..T.....	9456
DPD1	9522	CGCAATTTAAGGGACCCGAGTCTTGCAAGATATGGTTTCGACTTCTACGAAATCACAGCA	9581
<u>PK9</u>	960	T..A..A..T..T..C..C..C.....A	1019
NZ	949	T..A..A..T..T..C..C..C.....A	1008
<u>US</u>	961	T..A..A..T..T..C..C..C.....A	1020
CAN	876	T..A..A..T..T..C..C..C.....A	935
GER	876	T..A..G..C..C..T..T..T.....A	935
<u>S6</u>	961	A..G..A..T..T..C..T..T.....C	1017
NY	9460	A..A..A..T..T..C..T..T.....C	9516

DPD1	9582	AAGACACCTGTGAGGGCAAGAGAGGCACACTTTCAGATGAAAGCAGCAGCAATCAGAGGA	9641
<u>PK9</u>	1020	G.....	1079
NZ	1009	G.....	1068
<u>US</u>	1021	G.....	1080
CAN	936	G.....	995
GER	936	G.....	995
<u>S6</u>	1021	A.....	1077
NY	9520	A.....	9576

DPD1	9642	AAATCCAATAGCCTATTTGGCTTGGATGGGAACGTTGGGACACAGGAGGAGAACACGGAG	9701
<u>PK9</u>	1080	T.....T.....G.....	1139
NZ	1069	T.....T.....G.....	1128
<u>US</u>	1081	C.....T.....G.....	1140
CAN	996	C.....T.....G.....	1055
GER	996	T.....C.....G.....	1055
<u>S6</u>	1081	C.....C.....A.....	1137
NY	9580	C.....C.....A.....	9636

DPD1	9702	AGGCACACAGCAGAAGATGTCAATCAGAATATGCACAATCTTCTCGGAATGAGAGCCATG	9761
<u>PK9</u>	1140	T.....T.....C.....	1199
NZ	1129	T.....T.....C.....	1188
<u>US</u>	1141	C.....T.....T.....	1200
CAN	1056	C.....T.....T.....	1115
GER	1056	C.....T.....T.....	1115
<u>S6</u>	1141	T.....C.....T.....	1197
NY	9640	T.....C.....T.....	9696

(c) Nucleotide sequence alignment of the UTR of 8 PSbMV isolates

DPD1	9762	TAATCCGTATGTATTTT	TAGTACTGTCATACATTTTCGTTAAATTT	CAGTGTGTCTTTGA	9821	
<u>PK9</u>	1	...TC.....	T.....	A.....	T....GT.T.....	60
NZ	1	...TC.....	T.....	A.....	T....TG.T.....	60
<u>US</u>	1	...TC.....	T.....	A.....	T....GT.T.....	60
CAN	1	...TC.....	T.....	A.....	T....GT.T.....	60
GER	1	...TT.....	T.....	G.....	T....GT.T.....	60
<u>S6</u>	1	...AC.....	C.....	G.....	-...GT.C.....	59
NY	9697	...AC.....	C.....	G.....	-...GT.T.....	9755

DPD1	9822	CACCATGTTTAATAGCATTATGTATTCTAGGGTTCTATTATCATCAATTCCATAGTGAGT	9881	
<u>PK9</u>	61	A.....	T....	120
NZ	61	A.....	T....	120
<u>US</u>	61	A.....	T....	120
CAN	61	A.....	T....	120
GER	61	T.....	T....	120
<u>S6</u>	61	A.....	C....	119
NY	9757	A.....	C....	9815

DPD1	9882	CTTTGACTTCGGTTTGGTGGCAGTAGGGCTTTCTGGAGAAAA	9923	
<u>PK9</u>	121	T.....	T....AAAA	162
NZ	121	T.....	T....	158
<u>US</u>	121	T.....	T....AAAA	162
CAN	121	T.....	T....	158
GER	121	T.....	A....	158
<u>S6</u>	121	C.....	T....AAAAA	162
NY	9817	C.....	T....AAAAAAA	9860

(d) Nucleotide sequence alignment of partial P1-Pro gene and complete HC-Pro gene of 3 PSbMV isolates

P-1 Pro gene

DPD1	893	ACCACAGAGTTTAAAAATGAAAACCTGAATCCATAGATGTGCTAATCGAGCAGGTCATGAC	952
<u>S6</u>	1	GA.A.A....G.....G....G....C..C.CTT....T..C.....TG.....	60
NY	852	GG.A.A....G.....G....G....C..C.CTT....T..C.....TG.....	911
DPD1	953	CATTGCAGGAAAGCATGCAAAACAAGTAACTTTGATTGACAAACAGAAAACCAACAGAGT	1012
<u>S6</u>	61	TTGC..G..G.....T.....TTT.C.A.....G..A..GG.A....A...	120
NY	912	TTGC..G..A.....T.....TTT.C.A.....G..A..GG.A....A...	971
DPD1	1013	CTGGATAAGGCGAGTAAATGGAGTCCGTTTATTGCAAGTTGAAACGAAGCATCATAAAGG	1072
<u>S6</u>	121	A...A.....C....ATA.T..C..G.....C..G..A..A..C..C.G...	180
NY	972	G...A.....C....ATA.T..C..G.....C..G..A..A..C..C.G...	1031
DPD1	1073	GATTATCAGCCAAAAAGACGCTTCTCTGAACAATCTTACAAAAAGAGTTGCTAGACATTT	1132
<u>S6</u>	181	..AA..T..T....GG..T.T..TG.....TGA...C..T..G..G.....T....	240
NY	1032	..AG..T..T....GG..T.T..TG.....TGA...C..A..G..G.....T....	1091
DPD1	1133	TGCAAGGAAAACAGCATATATACACCCATCAGACAGTATCACACATGGACACAGCGGAGT	1192
<u>S6</u>	241	C..G..C.....A..C..G.T....G...AG...C.....A.....C...	300
NY	1092	C..G..C.....A..C..G.T....G...AG...C.....G.....C...	1151
DPD1	1193	CGTCTTCTTGCGCGGAACATCAGTGGCAGCAAGTCATACAGTATTGATGACTTATTCGT	1252
<u>S6</u>	301	A.....T....T.A...T..AGA...C.A.....C.G.....T..G..T..	360
NY	1152	A.....T....T.A...T..AGA...T.A.....C.G.....T..G..T..	1211
DPD1	1253	TGTTAGGGGCAAAAGAAATGGGAAATTAATGGAAAGCCGTAACAAAGTTGCGTGCGCAA	1312
<u>S6</u>	361	A..A.....T..A.....G.....G.T....T..G..A..A....A...	420
NY	1212	A..A.....C..A.....G.....G.T....T..G..A..A....A...	1271
DPD1	1313	GATGTTTCAAATTGACCACTTTTCTATTGTTGGAATCAAGATTGGAACGCTTTTCGATGC	1372
<u>S6</u>	421	C..G..AA.....G...C...C.T..A..C....AT..G..T..C..	480
NY	1272	T..G..AG.....G...C...C.T..A..C....GT..G..T..C..	1331
DPD1	1373	AGAATATGTTAAATTGAGAGATGAGTCTGTAGTGACCATGACTGTGTTGGAGGAATCAC	1432
<u>S6</u>	481	G.....A.....AAC.....CG.G.....C..G.....	540
NY	1332	G.....G.....AAC.....CA.G.....A..A.....	1391

HC-Pro gene

DPD1	1433	ACCAGAAGAATGTGGCATTCTTGCTGCACAAATATTACGTGTTTTCTACCCTTGTTGGCG	1492
<u>S6</u>	541	A...A....C..G..C...C...A.....T..G.....	600
NY	1392	A...C....C..G..C...C...A.....T..G.....	1451

DPD1	1493	CATAACTTGTACGAAGTGCATTTTCGAACTGGTTGAGTAAACCAACAAGTGAGCAGATCGA	1552
<u>S6</u>	601	C..A..A.....A.CT.....G.....A..T..	660
NY	1452	C..A..A.....A.CT.....G.....A..T..	1511
DPD1	1553	ACACATTTATGAGCGAGGGAACTTAGCCATACAAGATTTGAATAAAAGAATTCCATCAGC	1612
<u>S6</u>	661	A....TT.....AC.TT.G..A.....G..T.....TGG.A.....G....	720
NY	1512	A....TT.....AC.TC.G..A.....G..C.....CGG.A.....G....	1571
DPD1	1613	ACATCATGTAACCTCAGATGGTTGAACTCCTACGTCAGAGGATCAAAAACACAACATTTGA	1672
<u>S6</u>	721	G.....GG....TA..A....G..AT.G..G..A.....T.....CA.	780
NY	1572	G.....GA....TA..A....G..AT.G..G..A.....T.....CA.	1631
DPD1	1673	CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGATGGAGTTTTTCAG	1732
<u>S6</u>	781	A.....A.....C..T....A..T..T....G.....C.....	840
NY	1632	A.....A.....C..T....A..T..T....G.....C.....	1691
DPD1	1733	GCATTTGAACCGACTCAACAATAGTATTTTAGCGGCAAATGGTTCAAGCACAATTGAGTG	1792
<u>S6</u>	841	A..C..A..T..G....T...T.CG....G....GC.T....T.A.....C..A..	900
NY	1692	A..C..A..T..G....T...T.CG....G....GC.C....T.A.....C..A..	1751
DPD1	1793	GGAAAGCATGAATGAGAGTCTATTAGAGTTAGCGCGCTGGCATAACAAGCGAACTGAATC	1852
<u>S6</u>	901	...G.....T.GC....AC....A.....C..T.G.A.G..C..G..	960
NY	1752	...G.....T.GC....AC....A.....C..T.G.A.A..C..G..	1811
DPD1	1853	TATAGCAAGTGGAGGTATTTCTAGCTTTTCGGAACAAAATATCTGCCAAAGCCCAAATCAA	1912
<u>S6</u>	961	A..T...G....C..C..C..A..T..C..T.....GG.T.....T....T..	1020
NY	1812	A..T...G....C..C..C..A..T..C..T.....GG.T.....T....T..	1871
DPD1	1913	TTTTGCACTAATGTGTGATAATCAACTGGACACAAACGGAAATTTTGTGTTGGGGCGAACG	1972
<u>S6</u>	1021	...C..GT.G....C..C.....GT....T..G.....C..G.....	1080
NY	1872	...C..GT.G....C..C.....GT....T..G.....C..G.....	1931
DPD1	1973	TGGCTACCATGCAAAGCGTTTCTTTTCTGAGTTTTTCACGAAGATTGATCCGAAGGATGG	2032
<u>S6</u>	1081	...A..T.....G..A.....A...A..T..A.....A..A.....	1140
NY	1932	...A..T.....G..A.....A...A..T..A.....A..A.....	1991
DPD1	2033	ATACTCTCATCACACTGTTAGGGCCACACCAACAGGAGTCAGGCACCTAGCAATTGGAAA	2092
<u>S6</u>	1141	G...A....C..TG.....AA.A.....G.....T....T..G...G.A.....	1200
NY	1992	T...A....C..TG.....AA.A.....G.....T....T..G...A.A.....	2051
DPD1	2093	TCTGATCATTCAGGGGATCTACAGAAATTAAGAGAGAACTTGAAGGGGTGTCAATCAC	2152
<u>S6</u>	1201	CT.....A.....C.....C..A...C.T..G..A.....T.....	1260
NY	2052	CT.....A.....C.....C..A...C.T..G..A.....T.....	2111
DPD1	2153	AGCAGTGGGCATCTCGGAGAAGTGTGTTAGCAGACGGAATGGTGACTTTGTGTATCCTTG	2212
<u>S6</u>	1261	.A.....A.....A..A.....C..C..T..G.....A..T.....T.....	1320
NY	2112	.A.....A.....A..A.....C..C..T..G.....A..T.....T.....	2171

DPD1	2213	CTCATGCGTGACATCTGAGAACGGAAAACCAGTTTGTGTCAGATGTTATTTTACCTACAAG	2272
<u>S6</u>	1321	T..A...CT...A..T.....CCA....T..C..A.....G..G..	1380
NY	2172	T..C...CT...A..T.....CCA....T..C..A.....G..G..	2231
DPD1	2273	GAATCATTGTTATTGGGAATACAGGGGATCCAAAGTTAGTTGACTTACCCAAACTGA	2332
<u>S6</u>	1381	A.....G.....A..C.....C.....G.....T.....T..G.....	1440
NY	2232	A.....G.....A..C.....C.....A.....T.....T..G.....	2291
DPD1	2333	AACTGGTAGGATGTGGATTGCAAAAGAGGGATATTGTTACATAAACATATTCTTCGCAAT	2392
<u>S6</u>	1441	.G.....G.A.....C..T.....A.....C.....T.....	1500
NY	2292	.G.....G.A.....C..T.....A.....C.....T.....	2351
DPD1	2393	GCTTGTGAACGTCAGCGAAAAGGACGCCAAGGATTTACAAAGTTTGTAGGGATGAAAT	2452
<u>S6</u>	1501	A..C..T..T..G.....T..T.....C.....A.....A.....	1560
NY	2352	A..T..T..T..G.....T..T.....C.....A.....A.....	2411
DPD1	2453	CATGCCACAGCTTGGGAAATGGCCAACAATGATGGATGTAGCAACTGCTTGCTATAAATT	2512
<u>S6</u>	1561	A...GGG.A.....T.....A..T.....	1620
NY	2412	A...AAA.G.....T.....A..T.....	2471
DPD1	2513	AGCAATAATCTACCCTGATGTTAGAGATGCCCAGCTGCCGAGAATTTTGGTTGATCACTC	2572
<u>S6</u>	1621	T..T.....C..GC.T.....A.....A.....AC.A.....	1680
NY	2472	T..T.....C..GC.T.....A.....A.....AC.A.....	2531
DPD1	2573	AGAACAAATCTTCCATGTGATCGACTCGTATGGTTCCATGACTACAGGTTATCATATCTT	2632
<u>S6</u>	1681	...G....CT..T.....A..A..T..A.....C.....A.....C.....	1740
NY	2532	...G....CT..C.....A..A..T..A.....C.....A.....C.....	2591
DPD1	2633	AAAAGCAGGAACAGTTTCTCAGTTAATAAGTTTTGCGCATGGTGCATTGTTGGGTGAGAT	2692
<u>S6</u>	1741	G.....G.....C.....G....GC.....T...GT.....	1800
NY	2592	G.....G.....C.....G....AC.....T...GC.....	2651
DPD1	2693	GAAAATGTATAGAGTTGGAGGCACTCAGAAAAT	2725
<u>S6</u>	1801	...G.....A.....	1833
NY	2652	...G.....T.....	2684

REFERENCES

- Aapola, A. A. and Mink, G. I. (1973). Potential aphid vectors of pea seed-borne mosaic virus in Washington. *Plant Disease Reporter* **57**, 552.
- Aapola, A. A., Knesek, J. E. and Mink, G. I. (1974). The influence of inoculation procedure on the host range of pea seed-borne mosaic virus. *Phytopathology* **64**, 1003-1006.
- Aftab, M., Iqbal, S. M. and Rauf, C. A. (1992). Effect of lentil strain of pea seed-borne mosaic virus on lentil. *LENS Newsletter* **19**, 51-53.
- Alconero, R., Provvidenti, R. and Gonsalves, D. (1986). Three pea seed-borne mosaic virus pathotypes from pea and lentil germplasm. *Plant Disease* **70**, 783-786.
- Alconero, R. and Hoch, J. G. (1989). Incidence of pea seed-borne mosaic virus pathotypes in the US National Pisum germplasm collection. *Annals of Applied Biology* **114**, 311-315.
- Ali, A. and Randles, J. W. (1997). Early season survey of pea viruses in Pakistan and the detection of two new pathotypes of pea seed-borne mosaic potyvirus. *Plant Disease* **81**, 343-347.
- Ali, A. Randles, J. W. and Hodgson, R. A. J. (1998). Sensitive detection of pea seed-borne mosaic potyvirus by dot blot and tissue print hybridisation assays. *Australian Journal of Agricultural Research* **49**, 191-197.
- Ali, A. and Randles, J. W. (1998). An evaluation of the effects of infection with two pathotypes of pea seed-borne mosaic virus on the morphology and yield of pea. *Australasian Plant Pathology* **27** (4) 226-233.
- Ali-Khan, S. T. and Zimmer, R. C. (1979). Screening of field pea breeding lines for pea seed-borne mosaic virus. *Canadian Journal of Plant Science* **59**, 171-175.
- Anonymous. (1985). Diseases of legumes, *Rothamsted Experimental Station Report for 1985*, 123-124.
- Anonymous. (1991). ICARDA (International Centre for Agricultural Research in the Dry Areas) *Annual Report*. 29-30.

- Anonymous. (1993). Summary of crops Australia, *Australian Bureau of Statistics*, 5-9.
- Anonymous. (1994). Agricultural statistics of Pakistan (1991-92). Government of Pakistan, Ministry of Food, Agriculture and Co-operation. Food and Agriculture Division, Economic Wing, Islamabad.
- Aranda, M. A., Fraile, A. and Garcia-Arenal, F. (1993). Genetic variability and evolution of the satellite RNA of cucumber mosaic virus during natural epidemics. *Journal of Virology* **67**, 5896-5901.
- Atreya, C. D., Raccach, B. and Pirone, T. P. (1990). A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. *Virology* **178**, 161-165.
- Atreya, P. L., Lopez-Moya, J. J., Chu, M., Atreya, C.D. and Pirone, T. P. (1995). Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids. *Journal of General Virology* **76**, 265-270.
- Barnett, O. W. (1986). Surveys for plant viruses: design and considerations. Pages 147-189 in: Plant Virus Epidemics, Monitoring, Modelling and Predicting Outbreaks. G.D. McLean, R. G. Garrett and W. G. Ruesink, eds. Academic Press, Sydney.
- Bashir, M., Khalid, S., Asif, M. and Banaras, M. (1993). First report of pea seed-borne mosaic virus in Pakistan. (Abstr.). International working group on legume viruses, p25.
- Baulcombe, D. C. and Fernandez-Northcote, E. N. (1988). Detection of strains of potato virus X and of a broad spectrum of potato virus Y isolates by nucleic acid spot hybridisation (NASH). *Plant Disease* **72**, 307-309.
- Bennett, C. W. (1969). Seed transmission of plant viruses. *Advances in Virus Research* **14**, 221-261.
- Bos, L. (1970). The identification of three new viruses isolated from Wisteria and Pisum in the Netherlands and the problem of variation within potato virus Y group. *Netherlands Journal of Plant pathology* **76**, 8-46.
- Boulton, R. E., Jellis, G. J., Baulcombe, D. C. and Squire, A. M. (1986). The application of complementary DNA probes to routine virus detection with

- particular reference to potato viruses. Pages 41-53 in: Development and Application in Virus Testing. R. A. C. Jones and L. Torrance, eds. Association of Applied Biologists, Wellesbourne, U. K.
- Boswell, K. F. and Gibbs, A. J. (1983). Viruses of legumes 1983. Descriptions and keys from VIDE. ANU research school of biological sciences, Canberra.
- Buchen-Osmond, C., Crabtree, K., Gibbs, A. and McLean, G. (1988). Viruses of plants in Australia. ANU research school of biological sciences, Canberra.
- Carrington, J. C., Freed, D. D. and Oh, C. S. (1990). Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing. *EMBO Journal* **9**, 1347-1353.
- Chan, V. T. W., Fleming, K. A. and McGee, J. O. D. (1985). Detection of subpicogram quantities of specific DNA sequences on blot hybridisation with biotinylated probes. *Nucleic Acid Research* **13**, 8083-8092.
- Chia, T. F., Chan, Y. S. and Chua, N. H. (1995). Tissue-print hybridisation for the detection and localisation of plant viruses. Pages 145-149 in: Molecular Methods in Plant Pathology. R. P. Singh and U. S. Singh, eds. CRC Press, Inc.
- Chiko, A. W. and Zimmer, R. C. (1978). Effect of pea seed-borne mosaic virus on two cultivars of field pea grown in Manitoba. *Canadian Journal of Plant Science* **58**, 1073-1080.
- Crespi, S., Accotto, G. P., Caciagli, P. and Gronenborn, B. (1991). Use of digoxigenin-labeled probes for detection and host range studies of tomato yellow leaf curl gemini virus. *Research Virology* **142**, 283-288.
- Davies, D. R. (1993). The pea crop. Pages 1-12 in: Pea genetics, Molecular Biology and Biotechnology. R. Casey., and D.R. Davies, eds. John Innes Institute, Norwich.
- Dolja, V. V., Haldeman, R., Robertson, N. L., Dougherty, W. G. and Carrington, J.C. (1994). Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. *EMBO Journal* **13**, 1482-1491.
- Dolja, V. V., Haldeman-Cahill, R., Montgomery, A. E., Vandenbosch, K. A. and

- Carrington, J. C. (1995). Capsid protein determinants involved in cell-to-cell and long distance movement of tobacco etch potyvirus. *Virology* **206**, 1007-1016.
- Domingo, E. (1992). Genetic variation and quasispecies. *Current Opinion in Genetics and Development* **2**, 61-63.
- Dusi, A. N., Nagata, T. and Iizuka, N. (1993). First report of pea seed-borne mosaic virus in Brazil. *Plant Disease* **77**, 1264.
- Eweida, M., Xu, H., Singh, B. P. and AbouHaidar, M. G. (1990). Comparison between ELISA and biotin-labelled probes from cloned cDNA of potato virus X for the detection of virus in crude tuber extracts. *Plant Pathology* **30**, 623-629.
- Fletcher, J. D. (1993). Surveys of virus diseases in pea, lentil, dwarf and broad bean crops in South Island, New Zealand. *New Zealand Journal of Crop and Horticultural Science* **21**, 45-52.
- Fortass, M. and Bos, L. (1991). Survey of faba bean (*Vicia faba* L.) for viruses in Morocco. *Netherlands Journal of Plant Pathology* **97**, 369-380.
- Francki, R. I. B. (1985). Plant virus satellites. *Annual Review of Microbiology* **39**, 151-174.
- Fry, P. R. and Young, B. R. (1980). Pea seed-borne mosaic virus in New Zealand. *Australasian Plant Pathology* **9**, 10-11.
- Geering, A. D. W. and Randles, J. W. (1994). Interactions between a seed-borne strain of cucumber mosaic cucumovirus and its lupine host. *Annals of Applied Biology* **124**, 301-314.
- Gofflot, A. and Verhoyen, M. (1994). Identification of pea seed-borne mosaic virus (PSbMV) in pea seeds in Belgium. *Review of Plant Pathology* **73**, 882.
- Gonzalez, L. C. and Hagedorn, D. J. (1970). Aphid transmission of pea seed-borne mosaic virus. *Phytopathology* **60**, 1293.
- Goodwin, P. H. and Nassuth, A. (1993). Detection and characterisation of plant pathogens. Pages 303-319 in: *Methods in Plant Molecular Biology and Biotechnology*. B. R. Glick and J. E. Thompson, eds. CRC Press, Inc.

- Gotoh, O. (1982). An improved algorithm for matching biological sequences. *Journal of Molecular Biology* **162**, 705-708.
- Govier, D. A. and Kassanis, B. (1974). A virus-induced component of plant sap needed when aphids acquire potato virus Y from purified preparations. *Virology* **61**, 420-426.
- Gritton, E. T. and Hagedorn, D. J. (1975). Linkage of the genes *sbm* and *wlo* in peas. *Crop Science* **15**, 447-448.
- Hadidi, A., Montasser, M.S., Levy, L., Goth, R. W., Converse, R. H., Madkour, M. A. and Skrzeczkowski, L. J. (1993). Detection of potato leafroll and strawberry mild-yellow-edge luteoviruses by reverse transcription-polymerase chain reaction amplification. *Plant Disease* **77**, 595-601.
- Hagedorn, D. J., ed. (1984). Diseases caused by viruses. Pages 31-37 in: Compendium of Pea Diseases. American Phytopathology Society, St. Paul, MN.
- Hamilton, R. I. and Nichols, C. (1978). Serological methods for detection of pea seed-borne mosaic virus in leaves and seeds of *Pisum sativum*. *Phytopathology* **68**, 539-543.
- Hampton, R. O. (1969). Characteristics of virus particles associated with seed-borne pea fizzle top disease. *Plant Disease Reporter* **54**, 355-358.
- Hampton, R. O. (1972). Dynamics of symptom development of the seed-borne pea fizzle top virus. *Phytopathology* **62**, 268-272.
- Hampton, R. O., Phillips, S., Knesek, J. E. and Mink, G. I. (1973). Ultrastructural cytology of pea leaves and roots infected by pea seed-borne mosaic virus. *Archiv für die gesamte Virusforschung* **42**, 242-253.
- Hampton, R. O., Knesek, J. E. and Mink, G. I. (1974). Particle-length variability of the pea seed-borne mosaic virus. *Phytopathology* **64**, 1358-1363.
- Hampton, R. O. and Mink, G. I. (1975). Pea seed-borne mosaic virus, *Association of Applied Biologists. Descriptions of plant viruses*, Commonwealth Mycological Institute. No. 146

- Hampton, R. O., Mink, G. I., Hamilton, R. I., Kraft, J.M. and Meuhlbauer, F. J. (1976). Occurrence of pea seed-borne mosaic virus in North American pea breeding lines, and procedures for its elimination. *Plant Disease Reporter* **60**, 455-459.
- Hampton, R. O. and Muehlbauer, F. J. (1977). Seed transmission of pea seed-borne mosaic virus in lentils. *Plant Disease Reporter* **61**, 235-238.
- Hampton, R. O. and Braverman, S. W. (1979). Occurrence of pea seed-borne mosaic virus immune germplasm in the plant introduction collection of *Pisum sativum*. *Plant Disease Reporter* **63**, 95-99.
- Hampton, R. O., Mink, G. I., Bos, L., Inouye, T., Musil, M. and Hagedorn, D.J. (1981). Host differentiation and serological homology of pea seed-borne mosaic virus isolates. *Netherlands Journal of Plant Pathology* **87**, 1-10.
- Harper, K. and R. Creamer. (1995). Hybridisation detection of insect-transmitted plant viruses with Digoxigenin-labeled probes. *Plant Disease* **79**, 563-567.
- Harrison, B. D. and Robinson, D. J. (1982). Genome reconstitution and nucleic acid hybridisation as methods of identifying particle-deficient isolates of tobacco rattle virus in potato plants with stem-mottle disease. *Journal of Virological Methods* **5**, 255-265.
- Harrison, B. D., Robinson, D. J., Mowat, W. P. and Duncan, G. H. (1983). Comparison of nucleic acid hybridisation and other tests for detecting tobacco rattle virus in narcissus plants and potato tubers. *Annals of Applied Biology* **102**, 331-338.
- Hart, R. P., Medevitt, M. A., Ali, H. and Nevins, J. R. (1985). Definition of essential sequences and functional equivalence of elements downstream of the adenovirus E2A and the early simian virus 40 polyadenylation sites. *Molecular and Cellular Biology* **5**, 2975-2983.
- Higgins, T. J. V., Chandler, P. M., Zurawski, G., Button, S. C. and Spencer, D. (1983). The biosynthesis of primary structure of pea seed lectin. *Journal of Biological Chemistry* **258**, 9544-9549.
- Holland, J. J., De La Torre, J. C. and Steinhauer, D. A. (1992). RNA virus population

- as quasispecies. *Current Topics in Microbiology and Immunology* **176**, 1-20.
- Hull, R. and Al-Hakim, A. (1988). Nucleic acid hybridisation in plant virus diagnosis and characterisation. *Trends in Biotechnology* **6**, 213-218.
- Hull, R. and Davies, J. W. (1992). Approaches to nonconventional control of plant virus diseases. *Critical Reviews in Plant Sciences* **11**, 17-33.
- Huttinga, H. (1975). Properties of viruses of the potyvirus group. A comparison of buoyant density, S value, particle morphology, and molecular weight of the coat protein subunit of 10 viruses and virus isolates, *Netherlands Journal of Plant Pathology* **81**, 58-63.
- Idris, A. M. and Brown, J. K. (1998). Sinaloa tomato leaf curl geminivirus: Biological and molecular evidence for a new subgroup III virus. *Phytopathology* **88**, 648-657.
- Inouye, T. (1967). A seed-borne mosaic virus of pea. *Annual Phytopathological Society of Japan* **33**, 38-42.
- Johansen, E., Rasmussen, O. F., Heide, M. and Borkhardt, B. (1991). The complete nucleotide sequence of pea seed-borne mosaic virus RNA. *Journal of General Virology* **72**, 2625-2632.
- Johansen, I. E., Keller, K. E., Dougherty, W. G. and Hampton, R. O. (1996a). Biological and molecular properties of a pathotype P-1 and P-4 isolate of pea seed-borne mosaic virus. *Journal of General Virology* **77**, 1329-1333.
- Johansen, I. E., Dougherty, W. G., Keller, K. E., Wang, D. and Hampton, R. O. (1996b). Multiple viral determinants affects seed transmission of pea seed-borne mosaic virus in *Pisum sativum*. *Journal of General Virology* **77**, 3149-3154.
- Kaper, J. M. and Waterworth, H. E. (1977). Cucumber mosaic virus associated RNA 5: casual agent for tomato necrosis. *Science* **196**, 429-431.
- Karl, E. and Schmidt, H. E. (1978). Untersuchungen zum Artenspektrum der Aphidenvektoren des Erbsenblattrollmosaik-virus (pea leaf rolling mosaic virus), *Arch. Phytopath. Pflanzensch., Berlin* **14**, 367-372.

- Kasschau, K. D. and Carrington, J. C. (1995). Requirement for HC-Pro processing during genome amplification of tobacco etch potyvirus. *Virology* **209**, 268-273.
- Keller, K. E., Johansen, I. E., Martin, R. R. and Hampton, R. O. (1998). Potyvirus genome-linked protein (VPg) determines pea seed-borne mosaic virus pathotypes-specific virulence in *Pisum sativum*. *Molecular Plant Microbe Interactions* **11**, 124-130.
- Khetarpal, R. K. and Y. Maury. (1987). Pea seed-borne mosaic virus: a review, *Agronomie* **7**, 215-224.
- Khetarpal, R. K., Bossennec, J. M., Burghofer, A., Cousin, R. and Maury, Y. (1988). Effect of pea seed-borne mosaic virus on yield of field pea. *Agronomie* **8**, 1811-1815.
- Khetarpal, R. K. and Y. Maury. (1990). Seed transmission of pea seed-borne mosaic virus in peas: early and late expression of the virus in the progeny, *Journal of Phytopathology* **129**, 265-270.
- Kneseck, J. E. and Mink, G. I. (1970). Incidence of a seed-borne virus in peas grown in Washington and Idaho. *Plant Disease Reporter* **54**, 497-498.
- Kneseck, J. E., Mink, G. I. and Hampton, R. O. (1974). Purification and properties of pea seed-borne mosaic virus. *Phytopathology* **64**, 1076-1081.
- Koenig, R., An, D. and Burgermeister, W. (1988). The use of filter hybridisation techniques for the identification, differentiation and classification of plant viruses. *Journal of Virological Methods* **19**, 57-68.
- Kowalska, C. (1979). Viruses infecting pea (*Pisum* L.) in Poland, *Genet. Pol.* **20**, 211-215.
- Kraft, J. M. and Hampton, R. O. (1980). Crop losses from pea seed-borne mosaic virus in six processing pea cultivars. *Plant Disease* **64**, 922-924.
- Kumari, S. G., Makkouk, K. M. and Ismail, I. D. (1993). Survey of seed-borne viruses in lentil in Syria and their effects on lentil yield. *Arab Journal of Plant Protection* **11**, 28-32.

- Kurath, G. and Palukaitis, P. (1989a). RNA sequence heterogeneity in natural populations of three satellite RNAs of cucumber mosaic virus. *Virology* **173**, 231-240.
- Kurath, G. and Palukaitis, P. (1989b). Satellite RNAs of cucumber mosaic virus: recombinants constructed *in vitro* reveal independent functional domains for chlorosis and necrosis in tomato. *Molecular Plant-Microbe Interaction* **2**, 91-96.
- Kvicala, B. A. and Musil, M. (1967). Transmission of pea leaf rolling virus by aphids. *Biologia (Bratislava)* **22**, 10-16.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lain, S., Riechmann, J. L., Mendez, E. and Garcia, J. A. (1988). Nucleotide sequence of the 3' terminal region of plum pox potyvirus RNA. *Virus Research* **10**, 325-342.
- Lain, S., Riechmann, J. L. and Garcia, J. A. (1989). The complete nucleotide sequence of plum pox potyvirus RNA. *Virus Research* **13**, 157-172.
- LeClerc, D., Eweida, M., Singh, R. P. and AbouHaidar, M.G. (1992). Biotinylated DNA probes for detecting virus Y and aucuba mosaic virus in leaves and dormant tuber of potato. *Potato Research* **33**, 173-182.
- Ligat, J. S., Cartwright, D. and Randles, J. W. (1991). Comparison of some pea seed-borne mosaic virus isolates and their detection by dot-immunobinding assay. *Australian Journal of Agricultural Research* **42**, 441-451.
- Ligat, S. J. and Randles, J. W. (1993). An eclipse of pea seed-borne mosaic virus (PSbMV) in vegetative tissue of pea following repeated transmission through seed. *Annals of Applied Biology* **122**, 39-47.
- Lipman, D. J. and Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. *Science* **227**, 1435-1441.
- Lopez-Galindez, C., Lopez, J. A., Melero, J. A., Fuente, L. D. L., Martinze, C., Ortin, J. and Perucho, M. (1988). Analysis of genetic variability and mapping of point mutation in influenza virus by the RNase A mismatch cleavage method. *Proc.*

- Natl. Acad. Sci. USA* **85**, 3522-3526.
- Maia, I. G., Haenni, A. L. and Bernardi, F. (1996). Potyviral HC-Pro: a multifunctional protein. *Journal of General Virology* **77**, 1335-1341.
- Makasheva, R. K. (1983). Economic importance. Page 67 in: *The Pea*. Kolos Publishers, New Delhi.
- Makkouk, K. M., Kumari, S. G. and Shehadeh, A. (1992). Seed transmission of pea seed-borne mosaic virus in *Lathyrus* and *Vicia* forage legume species. *Journal of Plant Disease and Protection* **99**, 561-563.
- Makkouk, K. M., Kumari, S. G. and Bos, L. (1993). Pea seed-borne mosaic virus occurrence in faba bean (*Vicia faba*) and lentils (*Lens culinaris*) in West Asia and North Africa, and further information on host range, transmission characteristics, and purification. *Netherlands Journal of Plant Pathology* **99**, 115-124.
- Masmoudi, K., Suhas, M., Khetarpal, R. K. and Maury, Y. (1994). Specific serological detection of the transmissible virus in pea seed infected by pea seed-borne mosaic virus. *Phytopathology* **84**, 756-760.
- Matthews, P., Dawson, J. R. O. and Hills, G. H. (1981). Pea seed-borne mosaic virus (PSbMV), *John Innes Seventy-first Annual Report 1980*, 31-32.
- Matthews, R. E. F. (1991). Pages 12-648 in: *Plant Virology* (Third edition). Academic Press, Inc.
- Maury, Y., Bossennic, J. M., Boudazin, G., Hampton, R. O., Pietersen, G. and Maguire, J. (1987). Factors influencing ELISA evaluation of transmission of pea seed-borne mosaic virus in infected pea seed: seed group size and seed decortication. *Agronomie* **7**, 225-230.
- Maury, Y. and Khetarpal, R. K. (1992). Pea seed-borne mosaic virus. Pages 74-92 in: *Plant Disease of International Importance, Diseases of Vegetables and Oil Seed Crops*. H. S. Chaube, U. S. Singh, A. N. Mukhopadhyay and J. Kumar, eds. Vol II, Prentice Hall Inc, New Jersey.
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R.

- (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridisation probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acid Research* **12**, 7035-7056.
- Milicic, D. and Grbelja, J. (1977). Pea seed-borne mosaic virus in some pea cultivars in Yugoslavia. *Zastila Bilja* **28**, 147-154.
- Mink, G. I., Kraft, J., Knesek, J. and Jafri, A. (1969). A seed-borne virus of pea. *Phytopathology* **59**, 1342-1343.
- Mink, G. I. (1993). Pollen and seed transmitted viruses and viroids. *Annual Review of Phytopathology* **31**, 375-402.
- Moghal, S. M. and Francki, R. I. B. (1976). Studies on some potyviruses with special reference to those infecting legumes in Australia. *PhD Thesis*, The University of Adelaide, South Australia.
- Munro, M. (1978). Pea seed-borne mosaic virus in quarantine. *Australian Plant Pathology Society Newsletter* **7**, 10.
- Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A. and Summers, M. D. (1995). Virus Taxonomy: Classification and Nomenclature of Viruses. *Sixth Report of the International Committee on Taxonomy of Viruses*. New York: Springer-Verlag.
- Musil, M. (1966). Über das Vorkommen des Virus des Blattrollens der Erbse in der Slowakei (Vorläufige Mitteilung). *Biologia (Bratislava)* **21**, 133-138.
- Musil, M. (1970). Pea leaf rolling mosaic virus and its properties. *Biologica (Bratislava)* **25**, 379-392.
- Musil, M. (1980). Seed transmission of pea leaf rolling mosaic virus. Sameniübertragbarkeit des Blattrollmosaik Virus der Erbse **184**, 345-352.
- Myeres, R. M., Larin, Z. and Maniatis, T. (1985). Detection of single base substitutions by ribonuclease cleavage at mismatch in RNA:DNA duplexes. *Science* **230**, 1242-1246.
- Navot, N., Ber, R. and Czosnek, H. (1989). Rapid detection of tomato yellow leaf curl

- virus in squashes of plants and insect vectors. *Phytopathology* **79**, 562-568.
- Nikolaeva, O. V. (1995). Nucleic acid hybridisation methods in diagnosis of plant viruses and viroids. Pages 133-144 in: *Molecular Methods in Plant Pathology*. R. P. Singh and U. S. Singh, eds. CRC Press, Inc.
- Ovenden, G. E. and Ashby, J. W. (1981). The effect of pea seed-borne mosaic virus on yield of peas. *Proceeding of Agronomy Society of New Zealand* **11**, 16.
- Palukaitis, P. (1986). Preparation and use of cDNA probes for detection of viral genomes. *Methods in Enzymology* **118**, 723-742.
- Palukaitis, P., Roossinck, M. J. and Garcia-Arenal, F. (1994). Applications of ribonuclease protection assay in plant virology. *Methods in Molecular Genetics* **4**, 237-250.
- Phillips, D. and Chandrashekar, M. (1994). Summary review of seedborne diseases for 24 crop genera in Australia. A review commissioned by the Australian quarantine and inspection services. AQIS, Canberra.
- Pelet, F. (1980). Pea seed-borne mosaic virus (La mosaïque héréditaire du pois), Rev. Suisse Vitic. Arboric. *Hortic.* **12**, 281.
- Provvidenti, R. and Alconero, R. (1988a). Inheritance of resistance to a lentil strain of pea seed-borne mosaic virus in *Pisum sativum*. *Journal of Heredity* **79**, 45-47.
- Provvidenti, R. and Alconero, R. (1988b). Inheritance of resistance to a third pathotype of pea seed-borne mosaic virus in *Pisum sativum*. *Journal of Heredity* **79**, 76-77.
- Purcifull, D. E. and Edwardson, J. R. 1967. Watermelon mosaic virus: Tubular inclusions in pumpkin leaves and aggregates in leaf extracts. *Virology* **32**, 393-401.
- Randles, J. W. and Rohde, W. (1990). *Nicotiana velutina* mosaic virus: evidence for a bipartite genome comprising 3 kb and 8 kb RNAs. *Journal of General Virology* **71**, 1019-1027.
- Riechmann, J. L., Lain, S. and Garcia, J. A. (1990). Infectious in vitro transcripts from a plum pox potyvirus cDNA clone. *Virology* **177**, 710-716.

- Riechmann, J. L., Lain, S. and Garcia, J. A. (1992). Highlights and prospects of potyvirus molecular biology. *Journal of General Virology* **73**, 1-16.
- Robinson, D. J. and Romero, J. (1991). Sensitivity and specificity of nucleic acid probes for potato leafroll luteovirus detection. *Journal of Virological Methods* **34**, 209-219.
- Roy, B. P., AbouHaidar, M. G., Sit, T. L. and Alexander, A. (1988). Construction and use of cloned cDNA biotin and ³²P-labelled probes for the detection of papaya mosaic potexvirus RNA in plants. *Phytopathology* **78**, 1425-1429.
- Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985). Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* **230**, 1350-1354.
- Saitou, N. and Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning, 2nd Ed., Cold Spring Harbor Laboratory Press.
- Sela, I., Reichman, M. and Weissbach, A. (1984). Comparison of dot molecular hybridisation and enzyme-linked immunosorbent assay for detecting tobacco mosaic virus in plant tissues and protoplasts. *Phytopathology* **74**, 385-389.
- Shimada, T., Inokuchi, K. and Nienhuis, A. W. (1987). Site-specific demethylation and normal chromatin structure of human dihydrofolate reductase gene promoter after transfection into CHO cells. *Molecular and Cellular Biology* **7**, 2830-2837.
- Shukla, D. D., Frenkel, M. J. and Ward, C. W. (1991). Structure and function of the potyvirus genome with special reference to the coat protein coding region. *Canadian Journal of Plant Pathology* **13**, 178-191.
- Singh, M. and Singh, R. P. (1995). Digoxigenin-labelled cDNA probes for the detection of potato virus Y in dormant potato tubers. *Journal of Virological Methods* **52**, 133-143.

- Smith, D. B., McAllister, J., Casino, C. and Simmonds, P. (1997). Virus 'quasispecies' making a mountain out of a molehill?. *Journal of General Virology* **78**, 1511-1519.
- Solnick, D. and Lee, S. I. (1987). Amount of RNA secondary structure required to induce an alternative splice. *Molecular and Cellular Biology* **7**, 3194-3198.
- Steinhauer, D. A., Domingo, E. and Holland, J. J. (1992). Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. *Gene* **122**, 281-288.
- Stevenson, W. R. and Hagedorn, D. J. (1969). A new seed-borne virus of peas. *Phytopathology* **59**, 1051-1052.
- Stevenson, W. R. and Hagedorn, D. J. (1970). Effect of seed size and condition on transmission of pea seed-borne mosaic virus. *Phytopathology* **60**, 1148.
- Stevenson, W. R. and Hagedorn, D. J. (1971). Reaction of *Pisum sativum* to the pea seed-borne mosaic virus. *Plant Disease Reporter* **55**, 408-410.
- Stevenson, W. R. and Hagedorn, D. J. (1973). Partial purification of pea seed-borne mosaic virus. *Phytopathology* **63**, 1346-1352.
- Thakur, V. S., Thakur, M. S. and Khurana, S. M. P. (1984). The pea seed-borne mosaic virus of pea in Himachal Pradesh. *Indian Journal of Plant Pathology* **2**, 156-160.
- Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Research* **22**, 4673-4680.
- Thottappilly, G. E. and Schmutter, H. (1968). Zur Kenntnis eines mechanisch Samen-, Pilz- und Insektenübertragbaren neuen Virus der Erbse. *Z. Pflanzenkr. Pflanzen Pathol. Pflanzensch* **74**, 1-8.
- Timmerman, G. M., Calder, V. L. and Bolger, L. E. A. (1990). Nucleotide sequence of the coat protein gene of pea seed-borne mosaic potyvirus. *Journal of General Virology* **71**, 1869-1872.

- Towbin, H., Staehelin, T. and Gordon, T. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
- Trudel, M., Magram, J., Brudckner, L. and Costantini, F. (1987). Upstream G γ -globin and downstream β -globin sequences required for stage-specific expression in transgenic mice. *Molecular and Cellular Biology* **7**, 4024-4029.
- Turpen, T. (1989). Molecular cloning of a potato virus Y genome: nucleotide sequence homology in non-coding regions of potyviruses. *Journal of General Virology* **70**, 1951-1960.
- Varveri, C., Ravelonandro, M. and Dunez, J. (1987). Construction and use of a cloned cDNA probe for the detection of plum pox virus in plants. *Phytopathology* **77**, 1221 -1224.
- Walkey, D. G. A. (1985). Pages 123-152 in: Applied Plant Virology. John Wiley and Sons, Inc., New York.
- Wang, D., Hayes, I. M. and Maule, A. J. (1992). Procedures for the efficient purification of pea seed-borne mosaic virus and its genomic RNA. *Journal of Virological Methods* **36**, 223-230.
- Ward, C. W. and Shukla, D.D. (1991). Taxonomy of potyviruses: current problems and some solutions. *Intervirology* **32**, 269-296.
- Wetzel, T., Candresse, T., Ravelonandro, M., Delbos, R. P., Mazyad, H., Aboul-Ata, A. E. and Dunez, J. (1991). Nucleotide sequence of the 3' terminal region of the RNA of the E1 Amar strain of plum pox potyvirus. *Journal of General Virology* **72**, 1741-1746.
- Winter, E., Yamamoto, F., Almoguera, C. and Perucho, M. (1985). A method to detect and characterise point mutations in transcribed genes: Amplification and overexpression of the mutant c-Ki-ras allele in human tumour cells. *Proc. Natl. Acad. Sci. USA* **82**, 7575-7579.
- Zimmer, R. C. and Ali-Khan, S. T. (1976). New seed-borne virus of field peas.

Canadian Agriculture **21**, 6-8.

Zinn, K., DiMaio, D. and Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human β -interferon gene. *Cell* **34**, 865-879.

Ali, A. & Randles, J. W. (1998). The effects of two pathotypes of pea seed-borne mosaic virus on the morphology and yield of pea. *Australasian Plant Pathology*, 27(4), 226-233.

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