BIOLOGICAL AND MOLECULAR VARIATION AMONG ISOLATES OF PEA SEED BORNE MOSAIC VIRUS

Valeria Anna Torok

BSc (Hons)

University of Adelaide, Australia

Department of Applied and Molecular Ecology,

Waite Campus, University of Adelaide

South Australia

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ABSTRACT

Pea seedborne mosaic virus (PSbMV) has been identified in Western Australia, South Australia and Victoria from pea and faba bean field trial material, pea breeder's germplasm and in seed from commercially available pea cultivars. Sixteen PSbMV isolates were collected between 1995 and 1998. These isolates were biologically distinct yet serologically indistinguishable. These isolates were placed into the pathotypes P-1 and P-4 using specific pea differential genotypes.

Sequences obtained from 17 *HC-Pro* and 12 *CI/6K$_2$/VPg* regions for Australian, Pakistani and typical pathotype P-1, P-2 and P-4 isolates, showed a range of 88.6% to 100% amino acid sequence similarity within the HC-Pro and 84.4% to 100% amino acid sequence similarity within the CI/6K$_2$/VPg regions. Phylogenetic analysis of both genomic regions placed isolates into groups represented by pathotype P-1 and pathotype P-4. One isolate alone alternated between the two groups depending on the genomic region analysed. Within the VPg region a variable region of 15 amino acids was identified at position 105-119 which was specific for either pathotype P-1 or pathotype P-4 of PSbMV. The VPg has been previously identified as an avirulence determinant in other potyviruses.

Two RT-PCR assays, targeting the *HC-Pro* and the *CI/6K$_2$/VPg* region of PSbMV RNA were developed for detecting all of the Australian, as well as a range of Pakistani and typical pathotype P-1 and pathotype P-4 isolates. The specificity of the *HC-Pro* RT-PCR for PSbMV was demonstrated in tests with seven legume infecting potyviruses. RFLP analysis of the *CI/6K$_2$/VPg* RT-PCR amplicons placed PSbMV isolates into pathotype P-4 or pathotype P-1. This assay has potential use in pea breeding programs where genetically controlled resistance to PSbMV is pathotype specific. Alternatively, RFLP analysis of the *HC-Pro* RT-PCR amplicons allowed the PSbMV isolates to be placed into eight distinct groups. The Australian PSbMV isolates showed less variability than the Pakistani isolates. This grouping was not pathotype specific, but is potentially useful for identifying new variants of the virus in quarantine and epidemiological research.

The RT-PCR and nucleic acid hybridisation assays developed for PSbMV detection, were 10 to 20 times more sensitive than the dot immunobinding assay (DIBA). A duplex PSbMV/TMV RT-PCR assay was developed to detect false negative RT-PCR results, because of the requirement for testing for freedom from PSbMV in diagnostic assays. The duplex RT-PCR was shown to be template specific and did not adversely affect the

sensitivity of the PSbMV detection assay. This duplex RT-PCR was developed for batch testing of dry pea seed and shown to detect infection of the embryo with PSbMV.

It is concluded that PSbMV is widespread and occurs at a low incidence in Australia. There is, therefore, an opportunity to control the virus by the development of sensitive and specific diagnostic assays particularly for seed testing. This thesis reports sequence information on new isolates of PSbMV which has allowed genomic regions to be identified which distinguish PSbMV pathotypes and isolates and led to the development of PSbMV nucleic acid hybridisation and RT-PCR assays. The duplex RT-PCR diagnostic assay developed for seed testing will aid in the identification and elimination of seed infection from germplasm collections with applications in breeding, certification and control.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma an any university of other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Valeria Anna Torok

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ABBREVIATIONS

%	Percent
α	Alpha
A	Absorbance
AMV-RT	Avian myeloblastosis virus reverse transcriptase
APS	Ammonium persulphate
ATP	Adenosine 5'- triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCMV	Bean common mosaic virus
Bis	N,N'-methylene bis-acrylamide
bp	Base pairs
BSA	Bovine serum albumin
BYMV	Bean yellow mosaic virus
C	Concentration
cDNA	Complementary DNA
ClYVV	Clover yellow vein virus
cm	Centimetre
cpm	Count per million
Cs_2SO_4	Caesium sulphate
CTAB	N-Cetyl-N,N,N-trimethyl-ammoniumbromide
CTP	Cytidine 5'- triphosphate
dATP	2'-Deoxyadenosine, 5'-Triphosphate
dCTP	2'-Deoxycytidine, 5'-Triphosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxygenin
dGTP	2'-Deoxyguanosine, 5'-Triphosphate
DNA	Deoxyribonucleic acid
dNTP	Mixture of deoxynucleotide-triphosphates in equimolar amounts
DTT	Dithiothreitol
dTTP	2'-Deoxythimidine, 5'-Triphosphate
E	Specific extinction coefficient
EDTA	Ethylenediaminetetra-acetic acid disodium salt
EtBr	Ethidium bromide

f-	Femto- (10^{-15})
γ	Gamma
g	Gram
g	Gravity
GTP	Guanosine 5'- triphosphate
H_2O	18 mΩ water (Milli-Q ultrapure water system)
hrs	Hours
I	Deoxyinosine
IPTG	*Iso*-Propyl-β-D-thiogalactopyranoside
kb	Kilobase; kilobase pairs
L	Litre
LMV	Lettuce mosaic virus
μ-	Micro- (10^{-6})
M	Molar
m-	Milli- (10^{-3})
mA	Milli Ampere
m	Metre
min	Minutes
MMLV-RT	Moloney murine leukemia virus reverse transcriptase
MOPS	3-(N-morpholino)propanesulfonic acid
MW	Molecular weight
N	Normal
n-	Nano- (10^{-9})
NBT	Nitro blue tetrazolium
^{o}C	Degrees Celsius
OD	Optical density
p-	Pico- (10^{-12})
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PeMV	Peanut mottle virus
PMV	Pea mosaic virus
PNK	Polynucleotide kinase
PSbMV	Pea seedborne mosiac virus

PVP	Polyvinylpyrrolidone
RE	Restriction endonuclease
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcription
SARDI	South Australian Research & Development Institute
SDDW	Sterile 18 mΩ water
SDS	Sodium dodecyl sulphate
sec	Seconds
TAE	Tris-acetate
TBE	Tris-borate
TEMED	N N N' N'-Tetramethylethylenediamine
T_m	Melting temperature
TMV	Tobacco mosaic virus
TNA	Total nucleic acid
Tris	Tris(hydroxymethyl)aminomethane
TuMV	Turnip mosaic virus
U	Unit
UTP	Uridine 5'- triphosphate
UV	Ultra violet
V	Voltage
v/v	Volume:volume ratio
vol	Volumes
w/v	Weight:volume ratio
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactosidase

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Pea seed-borne mosaic virus (PSbMV) is an economically important seed-transmitted potyvirus infecting pea (*Pisum sativum* L) that has been commonly found in pea germplasm collections of many countries. The virus is suspected to have spread world-wide via the exchange of infected germplasm material. Due to the secondary spread of the virus in the field by aphid vectors, a low level of seed infection can lead to a high disease incidence in subsequent generations.

Management and control of PSbMV requires sensitive, specific and reliable detection systems for PSbMV, and knowledge of the biology, structure and composition of the virus. This will allow the development of the optimal sampling and detection strategies. This chapter reviews current information on the biological effects and molecular composition of PSbMV.

1.2 Australian grain legume industry

Pea is grown world-wide and as a food source ranks next to cereals in importance (Annonymous, 1994). The value of Australian grain legumes has grown from $74 million in 1984/85 to $320 million in 1991/92: an increase of 330%. Export, as a percent of production, has increased from 39% to 70% over the same period, representing an increase in export earnings from $12 million to $223 million (Douglas, 1995).

There is significant regionalization in grain legume production in Australia due to variations in climate. Lupins (*Lupinus spp.*), field peas, faba beans (*Vicia faba*) and chickpeas (*Cicer aerietinum*) are grown in southern Australia, with field peas representing the largest proportion of the grain legume crop within this region (Douglas, 1995).

1.3 Viral diseases of *Pisum sativum*

Twenty-nine viruses can naturally infect *Pisum sativum*, while another eight are capable of infecting peas under experimental conditions (Boswell and Gibbs, 1983; Hagedorn, 1985; Phillips and Chandrashekar, 1994; Murphy *et al.*, 1995 (Table 1.1). All these viruses contain single stranded RNA genomes. Of the twenty-nine naturally infecting pea viruses

only fifteen have been reported in Australia (Buchen-Osmond *et al.*, 1988). The majority of the viruses are transmitted by aphid vectors and are not seed transmitted.

Only two of the seed transmitted viruses, PSbMV and *Pea early browning virus* (PEBV), are reported to be transmitted at significant frequencies through seed (Khetarpal and Maury, 1987; Wang and Maule, 1992; Kohnen *et al.*, 1995; Ali and Randles 1998), of which only PSbMV has been recorded in Australia (Phillips and Chandrashekar, 1994).

PSbMV belongs to the genus *Potyvirus* in the family *Potyviridae* (van Regenmortel *et al.*, 2000). Additional members of this group that naturally infect *P. sativum* are: *Pea mosaic virus* (PMV); *Bean yellow mosaic virus* (BYMV); *Clover yellow vein virus* (ClYVV); *Lettuce mosaic virus* (LMV); *Peanut mottle virus* (PeMoV); *Turnip mosaic virus* (TuMV); and *Watermelon mosaic 2 virus* (WMV-2) (Hollings and Brunt, 1981).

1.4 Biological properties of PSbMV

1.4.1 Geographic distribution

PSbMV was first isolated from pea in Czechoslovakia by Musil in 1966 and named pea leaf rolling virus (Musil, 1970). Subsequently, the virus was reported in Japan, Germany, the United States of America and The Netherlands under the synonyms of pea seed-borne mosaic virus, false pea leaf rolling virus and pea fizzle top virus (Maury and Khetarpal, 1992). To date, PSbMV has been reported to occur in Australia, Canada, Czechoslovakia, Germany, India, Japan, The Netherlands, New Zealand, Pakistan, Peru, Poland, Sweden, Switzerland, USA, UK and Yugoslavia (Munro, 1978; Ligat *et al.*, 1991; Maury and Khetarpal, 1992; Zimmer and Lamb, 1993;Wang *et al.*, 1993; Ali and Randles, 1997).

The virus is suspected to have spread world-wide through the international exchange of infected pea germplasm. It is believed that PSbMV initially spread from India to Western Europe via infected seed in breeders lines and subsequently to other parts of the world (Maury and Khetarpal, 1992). PSbMV was first reported in Australia in 1978 in pea seed imported from Sweden (Munro, 1978).

Table 1.1: Viruses infecting *Pisum sativum*

Viruses that naturally infect *Pisum sativum*			
Virus	Genus	Vector	Seed transmissible‡
Alfalfa mosaic virus	Alfalfa mosaic virus group	*Aphididae*	yes *
Bean (pea) leaf roll virus	*Luteovirus*	*Aphididae*	no
Bean yellow mosaic virus	*Potyvirus*	*Aphididae*	yes *
Beet western yellows virus	*Luteovirus*	*Aphididae*	no *
Broad bean wilt virus	Broad bean wilt virus group	*Aphididae*	no
Chickpea stunt virus	*Luteovirus*	*Aphididae*	no
Clover yellow mosaic virus	*Potexvirus*	unknown #	yes
Clover yellow vein virus	*Potyvirus*	*Aphididae*	no *
Cucumber mosaic virus	*Cucumovirus*	*Aphididae*	yes *
Lettuce mosaic virus	*Potyvirus*	*Aphididae*	yes *
Milk vetch dwarf virus	*Luteovirus*	*Aphididae*	no
Pea early browning virus	*Tobravirus*	*Trichodoridae*	yes
Pea enation mosaic virus	Pea enation mosaic virus group	*Aphididae*	yes
Pea mosaic virus	*Potyvirus*	*Aphididae*	no
Pea pimple pod	Ungrouped	*Aphididae*	no *
Pea seed-borne mosaic virus	*Potyvirus*	*Aphididae*	yes *
Pea streak virus	*Carlavirus*	*Aphididae*	no
Peanut mottle virus	*Potyvirus*	*Aphididae*	yes *
Red clover mottle virus	*Comovirus*	unknown	unknown
Red clover necrotic mosaic virus	*Dianthovirus*	unknown	no *
Red clover vein mosaic virus	*Carlavirus*	*Aphididae*	yes
Soybean dwarf virus	*Luteovirus*	*Aphididae*	no
Subterranean clover stunt	Ungrouped	*Aphididae*	no *
Soybean dwarf virus	*Luteovirus*	*Aphididae*	no *
Tomato black ring virus	*Nepovirus*	*Dorylamidae*	yes
Tomato spotted wilt virus	Tomato spotted wilt virus group	Thysanoptera	no
Turnip mosaic virus	*Potyvirus*	*Aphididae*	no *
Watermelon mosaic virus 2	*Potyvirus*	*Aphididae*	no *
White clover mosaic virus	*Potexvirus*	unknown #	yes *
Viruses that infect *P. sativum* under experimental conditions			
Virus	Genus	Vector	Seed transmissible‡
Bean common mosaic virus	*Potyvirus*	*Aphididae*	yes *
Beet mosaic virus	*Potyvirus*	*Aphididae*	no *
Cowpea aphid borne mosaic virus	*Potyvirus*	*Aphididae*	yes *
Desmodium yellow mottle virus	*Tymovirus*	unknown	unknown
Passionfruit woodiness virus	*Potyvirus*	*Aphididae*	no *
Peanut stunt virus	*Cucumovirus*	*Aphididae*	yes
Soybean mosaic virus	*Potyvirus*	*Aphididae*	yes *
Zucchini yellow mosaic virus	*Potyvirus*	*Aphididae*	no *

‡ Seed transmissibility refers to viruses that have been reported to be transmitted through seed but not necessarily through seed of *P. sativum* .
* Viruses that have been reported to occur in Australia.
# These viruses are believed to be transmitted by vectors, yet unidentified, and mechanically by grazing animals.
(Boswell and Gibbs, 1983; Buchen-Osmond *et al.*, 1988; Hagedorn 1985; Phillips and Chandrashekar, 1994; Murphy *et al.*, 1995)

1.4.2 Symptomatology

Symptoms induced by PSbMV in peas range from: an inconspicuous mosaic; shortening of the internodes, often producing a terminal rosette; downward rolling of the leaflets; transient vein clearing and vein swelling; distortion of flowers and pods; splitting of seed coats; failure to set pods in some pea cultivars; and chlorosis and stunting of the plants (Hampton and Mink, 1975).

Symptoms can fade as plants approach flowering, and vary with both the pea cultivar infected and the strain of PSbMV (Maury and Khetarpal, 1992). Symptomless PSbMV infections have also been reported (Munro, 1978; Hampton, 1972; Maury *et al.*, 1987; Ligat and Randles, 1993).

PSbMV has been reported to be detectable by serological methods without inducing symptoms in 5-10% of plants from infected seed lots (Hampton, 1972). Maury *et al.* (1987) observed that 54% of pea plants testing positive for PSbMV infection by the enyme linked immunosorbent assay (ELISA) escaped detection on the basis of symptomatology. 9% of PSbMV infected pea seedlings detected by ELISA were symptomless 28 days after emergence (Ding *et al.*, 1992). Ligat and Randles (1993) observed that primary inoculated plants were symptomatic, however only a small proportion of seedlings raised from seed of the first generation were symptomatic. Yet, symptomless plants from the second generation showed a high incidence of infection in the seed by the dot immunobinding assay (DIBA).

1.4.3 Host range

P. sativum cultivars are the main natural hosts of PSbMV, but the virus can also naturally infect lentil (*Lens culinaris*), faba bean (*Vicia faba*) and chickpea (*Cicer arietinum*) (Khetarpal and Maury, 1987; Alconero *et al.*, 1996; Makkouk *et al.*, 1993a). PSbMV can infect 47 species in 12 dicotyledonous families (Aapola *et al.*, 1974). Most non-leguminous hosts are infected without producing symptoms.

1.4.4 Secondary spread by vectors

PSbMV is easily transmitted (i) by mechanical inoculation and (ii) by several aphid species in a non-persistent or semi-persistent manner. There are nine natural aphid vectors of

PSbMV: *Acyrthosiphon pisum*, *Aphis craccivora*, *Aphis fabae*, *Dactynotus escalanti*, *Macrosiphum euphorbiae*, *Macrosiphum rosae*, *Myzus persicae*, *Ovatus crataegarius*, and *Rhopalosiphum padi* (Aapola and Mink, 1973; Hampton and Mink, 1975). Experimentally, the virus can be transmitted by 22 species in 13 genera (Maury and Khetarpal, 1992). Different PSbMV isolates are aphid transmitted at different rates. PSbMV NY pathotype P-4 is aphid transmitted at lower rates (4-16%) than PSbMV DPD1 pathotype P-1 (11-40%) and have distinct virus acquisition optima (Kohnen *et al.*, 1995).

1.4.5 Yield losses caused by PSbMV in pea crops

PSbMV infection in commercial pea cultivars has resulted in yield losses of 11-36% (Maury and Khetarpal, 1992). Yield reductions of 35% and 82% have been reported in *P.sativum* cv Dundale infected with PSbMV US and S6 isolates respectively (Ali and Randles, 1998). PSbMV infection also delays flowering, pod formation and plant maturation (Ali and Randles, 1998) and reduces seed size and, hence seed weight per 1000 seeds by 10-28% (Maury and Khetarpal, 1992). PSbMV may also induce necrotic symptoms on seed coats impacting directly on pea seed quality (Hampton and Mink, 1975). The presence of PSbMV has a direct affect on the marketability of pea seed and infected stocks are either destroyed, converted to stock feed, or exported at greatly reduced returns (Maury and Khetarpal, 1992).

1.4.6 Rates of seed transmission

Seed transmission of viruses results in a complex interaction between the host and the virus, which may be influenced by a variety of environmental factors. These include: occurrence of variants that are not seed transmitted; different genotypes of the same host species can differ in their efficiencies of transmission of a single isolate; and effect of temperature and daylength on transmission efficiencies (Wang and Maule, 1992). The main mode of spread of PSbMV is through infected pea seed. Efforts have been made to eliminate PSbMV from 2700 Plant Introduction accessions of *P. sativum* in the USA (Hampton *et al.*, 1993).

Rates of seed transmission ranging from 0.3% to 100% have been reported, depending on the pea cultivar and the strain of the virus (Maury and Khetarpal, 1992; Hampton *et al.*, 1993). PSbMV US and S6 have been reported to be seed transmitted at 8% and 31% respectively in *P. sativum* cv Dundale (Ali and Randles, 1998). Seed transmission studies

of the PSbMV DPD1 pathotype P-1 and pathotype NY P-4 isolates in 10 pea cultivars indicated that PSbMV NY was seed transmitted at lower levels (0-0.7%) than PSbMV DPD1 (0-32%) (Kohnen *et al.*, 1995). Seed transmission rates of PSbMV-28 varied from 0% to 74% depending on the pea cultivar tested (Wang and Maule, 1992).

PSbMV has been reported in pea and lentil germplasm collections in Canada, France, India, New Zealand, the United Kingdom and the United States of America (Khetarpal and Maury, 1987). PSbMV has also been reported to be seed transmitted in lentil at rates of 32% to 45% (Hampton and Muehlbaeur, 1977; Makkouk *et al.*, 1993a), in faba bean at rates of 41% and chickpea at rates of 66% (Makkouk *et al.*, 1993a). PSbMV is also seed transmitted through a low percentage of seeds of *Vicia articulata*, *V. narbonesis*, *V. pannonica*, and *V. sativa* (Hampton and Mink, 1975; Maury and Khetarpal, 1992).

1.4.7 Mode of seed transmission

For most seedborne viruses, establishment in host embryonic tissues is essential for their transmission in seeds. Two routes for embryo invasion by viruses have been recognised: (i) direct invasion of host embryo after fertilisation; (ii) and indirect invasion of zygotic embryos through the fusion of infected gametes during fertilisation (Johansen *et al.*, 1994).

Analysis of PSbMV incidence and concentration in pea seed at different developmental stages demonstrated that in a cultivar with a high incidence of seed transmission, PSbMV directly invaded the immature embryo, multiplied in the embryonic tissues and persisted, retaining its infectivity, during seed maturation and storage (Wang and Maule, 1992). In contrast, pea cultivars without seed transmission did not show invasion of the immature embryo by the virus or evidence for virus multiplication or persistence during embryo development and seed maturation (Wang and Maule, 1992). PSbMV has been shown to invade the pea embryo via the suspensor to the tip of the developing radicle (Wang and Maule, 1994). PSbMV RNA replication in pea cotyledonary tissues is restricted to a zone of cells close to the infection front and transiently suppresses the expression of host genes (Wang and Maule, 1995).

PSbMV seed transmission is influenced by multiple viral determinants and seed transmission frequency does not correlate with accumulation of virus in vegetative tissue (Johansen *et al.*, 1996a). The 5′ 2.5kb of the 10 kb PSbMV genome has a major influence

on the seed transmission frequency. The 5'NTR and HC-Pro are major determinants of seed transmission with the P1 exerting no measurable influence (Johansen *et al.*, 1996a).

1.4.8 Distribution of PSbMV in infected plants

Pollen transmission of the PSbMV-28 isolate did not occur in five susceptible cultivars of *P. sativum*, and pollen grains from infected plants showed no detectable PSbMV by ELISA or electron microscopy (Wang *et al.*, 1992). However, ELISA detected PSbMV in viable pollen from PSbMV DPD1 pathotype P-1 infected plants but not PSbMV NY pathotype P-4 plants (Kohnen *et al.*, 1992; Kohnen *et al.*, 1995).

PSbMV infection has been detected in floral tissues (sepals, petals, anther and carpel) but not in ovules prior to fertilisation (Wang and Maule, 1992). PSbMV RNA has been detected in nucleic acid extracts from infected leaves, roots, petals, and embryo axes from immature and mature seeds (Kohnen *et al.*, 1992; Kohnen *et al.*, 1995).

The testa from PSbMV infected seed has been shown by ELISA to contain PSbMV antigen (Kohnen *et al.*, 1992, Kohnen *et al.*, 1995; Wang and Maule, 1992), although virus from the testa was not infectious when bioassayed on *Chenopodium amaranticolor*. RT-PCR with PSbMV pathotype P-1 specific primers also failed to detect PSbMV DPD1 in testa samples, suggesting that the extracts did not contain functional PSbMV RNA (Kohnen *et al.*, 1992, Kohnen *et al.*, 1995). However, Phan *et al* (1997) reported detection of PSbMV RNA in testa by IC-PCR.

When examining whole plants infected with PSbMV the highest concentration of PSbMV P1 protein was found in the youngest leaves, although the PSbMV CP concentration remained the same throughout the plant (Albrechtsen & Borkardt, 1994).

1.4.9 PSbMV Pathotypes

Three pathotypes of PSbMV (P-1, P-4 and L-1) have been characterised on the basis of their ability to infect specific pea genotypes (Alconero *et al.*, 1986). A pathotype is defined as an entity of a given virus that is controlled by a specific genetic factor in a given plant species or family (Alconero *et al.*, 1986). However, a pathotype may include a range of variants differing in virulence (Provvidenti and Alconero, 1988a). The three pathotype isolates were obtained from seed-borne infections of single plants in the following

germplasm accessions; PI 432112 (isolated from *Lens culinaris* and labelled L-1), PI 179458 (isolated from *P. sativum* and labelled P-1), and PI 471123 (isolated from *P. sativum* and labelled P-4) (Alconero *et al.*, 1986). The PSbMV pathotype L-1 is also referred to as pathotype P-2 (Kasimor *et al.*, 1997)

Hampton *et al.* (1981) investigated the use of nineteen *P. sativum* lines as PSbMV strain differentials. These plant introduction (PI) accessions were placed into five host groups (I-V) based on range of response to PSbMV infection. Reactions to the PSbMV isolates were best characterised by host groups I, III and V (Hampton *et al.*, 1981). PIs previously reported to distinguish PSbMV isolates (Hampton *et al.*, 1981) have also been shown to distinguish between PSbMV P-1, P-4 and L-1 pathotypes on the basis of symptom severity (Alconero *et al.*, 1986). Other PIs have been reported to distinguish between the three pathotypes on the basis of infection (Alconero *et al.*, 1986) (Table 1.2).

Table 1.2: Reactions of plant introductions of *P. sativum* to P-1, P-4 and L-1 pathotypes of PSbMV

Accession	Virus pathotypes		
	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
PI 347466	R	R	R
PI 347467	R	R	R
PI 347470	R	R^b	R^b
PI 347492	R	R	R
PI 347494	R	R^b	R^b

R= resistant, S= susceptible, R^b= these lines include some susceptible plants (Alconero *et al.*, 1986).

Additional pathotypes have also been proposed. The Pi and Pv pathotypes have been proposed since they break all known PSbMV resistance in *P. sativum* (Khetarpal *et al.*, 1990). Two other pathotypes U-1 and U-2 have also been proposed based on their

deviation from reported reactions on specific pea differential genotypes (Ali and Randles, 1997).

1.4.10 Resistance to PSbMV in P. sativum

Resistance to PSbMV in *P. sativum* was initially proposed to result from a single recessive gene in the homozygous state designated *sbm* (Hagedorn and Gritton, 1973). Resistance to PSbMV pathotypes P-1, P-4 and L-1 are pathotype specific in *P. sativum*, with each genetic factor capable of controlling only a specific pathotype of the virus. PSbMV resistance in *P. sativum* is controlled by four recessive genes; *sbm-1*, *sbm-2*, *sbm-3*, and *sbm-4* (Alconero *et al.*, 1986; Provvidenti and Alconero, 1988a; Provvidenti and Alconero, 1988b).

Resistance to PSbMV pathotype P-1 is conferred by the *sbm-1* gene, identified in PI 193586 and PI 193835 (Gritton and Hagedorn, 1975). Resistance to PSbMV pathotype L-1 is conferred by two independently inherited single recessive genes, *sbm-2* and *sbm-3* (Provvidenti and Alconero, 1988a). The resistance gene, *sbm-2,* identified in *P. sativum* cv Bonneville is closely linked to *mo*, which in the homozygous state conditions resistance to BYMV and WMV-2. The second PSbMV pathotype L-1 resistance gene, *sbm-3,* was identified in PI 347492, a BYMV-susceptible line from India. Resistance to PSbMV pathotype P-4 is conferred by *sbm-4*, identified in PI 347492 (Provvidenti and Alconero, 1988b).

The *sbm-1*, *sbm-3* and *sbm-4* genes are clustered on chromosome 6 (Provvidenti and Alconero, 1988a; Provvidenti and Alconero, 1988b; Khetarpal *et al.*, 1990; Timmerman *et al.*, 1993) and the *sbm-2* gene is located on chromosome 2 of *P. sativum* (Provvidenti and Alconero, 1988a) The *sbm-1* gene is most closely linked to the restriction fragment length polymorphic (RFLP) marker GS185, being separated by a distance of approximately 8 centi-morgans (Timmerman *et al.*, 1993). This marker for *sbm-1* has potential value in plant breeding programs for identifying PSbMV P-1 resistant material. Resistance to PSbMV in lentils is conditioned by a single recessive gene designated *sbv* (Haddad *et al.*, 1978).

1.5 The family *Potyviridae*

The family *Potyviridae* is the largest of the 34 plant virus groups and families (Matthews, 1982). It contains over 180 definitive and possible members which cause significant losses

in agricultural, pasture, horticultural, and ornamental crops (Ward and Shukla, 1991; Murphy *et al.*, 1995). The *Potyviridae* includes six genera: Potyvirus (vectored by aphids); Rymovirus (vectored by mites); and Bymovirus (vectored by fungi); Ipomovirus (vectored by whitefly); Macluravirus (vectored by aphids); and Tritimovirus (vectored by mites) (van Regenmortel *et al.*, 2000).

1.6 The genus *Potyvirus*

1.6.1 Morphology

The virions of the potyviruses are monopartite, flexuous filaments, containing no envelope, with particle modal lengths of 650-900 nm and diameter of 11-15 nm (van Regenmortel *et al.*, 2000). Potyviral virions are composed of approximately 2,000 copies of a single structural coat protein (CP) subunit (Mr 30-47K) arranged in a helical manner, with a pitch of approximately 3.4 nm, around a single copy of viral RNA (Dougherty and Carrington, 1988; Matthews, 1982; Milne, 1988).

Potyviruses have a single molecule of plus sense single stranded RNA, 8.5-10 kb in size (Mr 3.0×10^6) (Hollings and Brunt, 1981; Murphy *et al.*, 1995). A viral protein genome (VPg) of approximately 24 kDa is covalently linked to the 5' terminus of the RNA genome (Murphy *et al.*, 1990) and a polyadenylated tract of 20-160 adenosine residues is present at the 3' terminus (Hari *et al.*, 1979; Dougherty *et al.*, 1985). (Regenmortel *et al.*, 2000).

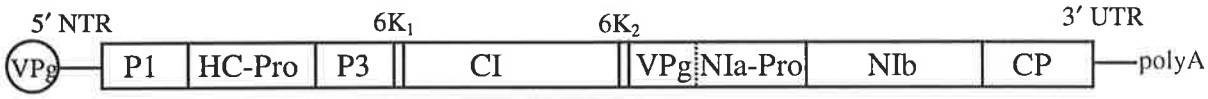
1.6.2 Cytopathology

Members of the potyvirus group share common morphological and cytopathological features. All definitive and possible members of the potyvirus group induce the formation of characteristic cylindrical inclusions in the cytoplasm of infected cells (Lesemann, 1988). These inclusions are formed by a single virus encoded protein, known as the cylindrical inclusion (CI) protein, which aggregate to form cytoplasmic pinwheel-shaped inclusion bodies. Many potyviruses also induce cytoplasmic amorphous inclusion bodies, composed of a single protein related to the HC-Pro. Some potyviruses form nuclear inclusions composed of two virus encoded proteins, NIa and NIb, found in equimolar amounts in the nucleoplasm of infected cells (Lesemann, 1988).

1.6.3 Genome organisation

The potyviral RNA contains one long open reading frame (ORF) which is translated into a large polyprotein, with an Mr of approximately 340-368K, that is subsequently cleaved into at least eight functional proteins (Riechmann *et al.*, 1992).

The different gene products into which the potyviral polyprotein is cleaved are, proceeding from the N-terminus to the C-terminus of the polyprotein: first protein/protease (P1); the helper component/protease (HC-Pro); third protein (P3); a putative 6K peptide ($6K_1$); the cylindrical inclusion protein with RNA helicase activity (CI); a second 6K peptide ($6K_2$); the nuclear inclusion "a" protein (NIa) composed of the VPg and a protease (Pro); the nuclear inclusion "b" protein (NIb) with presumed RNA polymerase activity; and the coat protein (CP) (Riechmann *et al.*, 1992), The generalized potyviral genome organisation is shown in Figure 1.1.



(5′ NTR) 5′ non-translated region; (3′ UTR) 3′ untranslated region

Figure 1.1: Potyvirus genome organisation (Riechmann *et al.*, 1992).

1.6.4 Gene functions

1.6.4.1 Proteases

Three virus-encoded proteases, P1 (Verchot *et al.*, 1991), HC-Pro (Carrington *et al.*, 1989), and NIa (Ghabrial *et al*, 1990) have been identified. These proteases process the large precursor viral polyprotein, both co- and post-translationally.

The P1 cleaves at its own C-terminus (Carrington *et al.*, 1990; Verchot *et al.*, 1991) and is related to the serine proteases (Verchot *et al.*, 1991). Mutations debilitating the P1 proteolytic activity of TEV render the virus non-viable (Verchot & Carrington, 1995).

The HC-Pro protease, which closely resembles members of the cysteine-type family of proteases, autocatalytically cleaves at its own C terminus (Carrington *et al.*, 1989).

The NIa protease is required for proteolytic maturation of the majority of viral proteins (Carrington *et al.*, 1988; Carrington and Dougherty 1987a; Carrington and Dougherty 1987b; Garcia *et al.*, 1989; Hellmann *et al.*, 1988) and is related to the trypsin-like family of cellular serine proteases (Gorbalenya *et al.*, 1989). This enzyme exhibits *cis*-preferential proteolytic activity *in vitro* at several sites, including the CI/$6K_2$, $6K_2$/NIa and NIa/NIb cleavage sites, and *trans* proteolytic activity at the P3/CI and NIb/CP protein sites. Additionally, NIa catalyzes cleavage at an internal site between the VPg and Pro domains (Dougherty and Parks, 1991). Processing at the NIa internal site is inefficient due to a suboptimal cleavage site motif flanking the scissile bond. Internal cleavage site inactivating mutations, as well as mutations resulting in accelerated internal cleavage, are amplification debilitated (Carrington *et al.*, 1993; Schaad *et al.*, 1996), suggesting that the slow processing characteristic of the internal site is an important regulatory feature.

1.6.4.2 Genes involved in the replication module

A set of core replication proteins catalyses the essential enzymatic steps during RNA synthesis. These proteins include the CI helicase, the NIa and the NIb RNA dependent RNA polymerase (Reichmann *et al.*, 1992). The CI, NIa and NIb potyviral proteins show homology with proteins considered to be involved in the replication and expression of picorna-, nepo- and comovirus RNA (Domier *et al.*, 1987; Goldbach and Wellink, 1988). The N- terminus of the CI protein is proposed to contain a nucleotide-binding motif (NTBM) with RNA dependent ATPase helicase activity (Robaglia *et al.*, 1989; Gorbalenya and Koonin, 1989; Lain *et al.*, 1990). The NIb protein is a potyviral RNA-dependent RNA polymerase containing conserved motifs, including the GDD motif, characteristic of these enzymes (Domier *et al.*, 1987; Robaglia *et al.*, 1989; Poch *et al.*, 1989).

Potyviral replication proceeds by the copying of the plus strand to the complementary minus strand intermediate, followed by plus strand synthesis. This occurs in the membrane associated cytoplasmic fraction (Martin and Garcia, 1991; Schaad *et al.*, 1997a). Cytoplasmic localisation of the replication machinery is thought to be achieved by direction of a subset of the potyviral NIa proteolytic precursor (including the amino terminal adjacent $6K_2$ hydrophobic protein) towards the endoplasmic reticulum rather than the nucleus (Schaad *et al.*, 1997a). The NIb is, in turn, postulated to be recruited to the membrane fraction via interaction with the $6K_2$-NIa complex. The TEV $6K_2$ protein is

proposed to function as an anchor to secure part or all of the TEV RNA replication apparatus to membranous sites (Schaad *et al.*, 1997b). Replication of the minus strand is initiated at the 3' poly-A tail of the plus sense genomic RNA. The presence of a poly-A tail is essential for potyvirus replication (Tacahashi and Uyeda, 1999).

1.6.4.3 Possible translation mechanism

Cellular mRNA molecules have a 5′ cap and 3′ poly-A tail; the two termini function synergistically to promote translation through protein-protein interactions. At the 5′ end the translation eukaryotic initiation factor (elF4E) which is a subunit of elF4F (containing subunits eIF4E, eIF4G and eIF4A), binds to the cap structure. EIF(iso)4E has been shown to have an important role in the regulation and initiation of translation of mRNA (Browning, 1996; McKendrick *et al.*, 1999). At the 3′ end PABP (poly A binding protein) binds to the poly-A tail. PABP then binds to elF4G resulting in a circular mRNA molecule. By bringing the 5′ and 3′ ends close to each other, translation of full length message is promoted (Gallie, 1998; Sachs *et al.*, 1997).

Although not capped the potyviral 5′ NTR of TEV has been shown to confer synergistic enhancement of translation when in combination with a poly-A tail (Carrington and Freed, 1990; Nicolaisen *et al.*, 1992; Basso *et al.*, 1994; Gallie *et al.*, 1995). There is also evidence that the 5' NTR contains an internal ribosome entry site (IRES) (Basso *et al.*, 1994; Levis & Astier-Manifacier, 1993).

The VPg of TuMV has been shown to interact with eIF(iso)4E of *Arabidopsis thaliana* (Wittmann *et al.*, 1997) as well as the eIF4E isomer of *A. thaliana* and eIF(iso)4E of *Triticum aestivum* (wheat) (Leonard *et al.*, 2000). The TuMV VPg region comprising amino acids 59 to 93 is necessary for its interaction with eIF(ios)4E and substitution of an aspartic acid residue found at position 77 abolishes the interaction (Leonard *et al.*, 2000). The VPg-eIF(iso)4E interaction has been shown to be critical in TuMV production (Leonard *et al.*, 2000) and NIa-eIF4E interaction has a positive effect on TEV genome amplification. (Schaad *et al.*, 2000).

1.6.4.4 Roles of the VPg

The 5' end of the potyviral RNA does not have a cap structure (m7GpppN, where N is any nucleotide) but is covalently linked to a virus-encoded protein (VPg) via a tyrosine residue (Murphy *et al.*, 1991, Murphy *et al.*, 1996). VPg has several suggested roles in the virus life cycle. Interaction of the VPg with the NIb polymerase (Hong *et al.*, 1995; Li *et al.*, 1997; Fellers *et al.*, 1998) suggests a role in viral RNA synthesis. VPg also performs a yet to be defined function in the nucleus (Carrington *et al.*, 1991; Hajimorad *et al.*, 1996; Restrepo *et al.*, 1990), and mutations in the VPg domain result in the inhibition of nuclear transport and debilitates viral genome amplification (Schaad *et al.*, 1996).

Additionally, VPg has been implicated in overcoming resistance in some plant cultivars:

(i) A domain of four amino acids (Cys 1928, Ser1929, Lys 1932 and Ser 1933) within the VPg protein of TVMV-S is responsible for overcoming *va* resistance in *N. tabacum* TN 86 (Nicolas *et al.*, 1997).

(ii) A single amino acid substitution (Lys105 or Arg105 to Glu105) in the VPg domain of PVY-T was shown to be responsible for overcoming resistance in *N. tabacum* VAM (Masuta *et al.*, 1999).

(iii) Specific amino acid substitutions in the $6K_2$ protein (Met5 to Val5) and the VPg (Val116 to Met116 and Leu185 to Ser185) of PVA-M permitted the virus to overcome resistance in *Nicandra physaloides* var. Black Pod (Rajamäki and Valkonen, 1999).

(iv) Chimeric pathotype P-1/P-4 PSbMV was used to demonstrate that a genomic segment which encodes the NIa and partial NIb was responsible for symptom induction in *P. sativum* (Johansen *et al.*, 1996a). It has since been shown that the VPg alone of PSbMV NY pathotype P-4 is capable of overcoming *sbm-1/sbm-1* resistance in *P. sativum* (Keller *et al.*, 1998).

1.6.4.5 Genes involved in cell to cell and long distance movement

Four viral proteins, P1, CI, HC-Pro and CP are involved in either cell-to-cell or plant-to-plant virus movement. The P1 protein has been proposed to have a function in cell-to-cell movement based on sequence similarity of the TVMV P1 with the 30K movement protein of *Tobacco mosaic virus* (TMV) (Domier *et al.*, 1987). CP is required for both cell to cell and long distance movement (Dolja *et al.*, 1994; Dolja *et al.*, 1995). The core domain of CP, which is essential for flexuous rod-shaped virion assembly, is necessary for

cell to cell movement. The N- and C-terminal domains of CP, which are exposed on the virion surface, are required for efficient long distance movement through phloem. Cell to cell movement also requires the CI protein (Riechmann *et al.*, 1992; Carrington *et al*, 1998). The HC-Pro and a particular CP amino acid triplet (DAG) are required for aphid transmission of potyviruses (Thornbury *et al.*, 1985; Berger and Pirone, 1986; Aytreya *et al.*, 1992) and the HC-Pro is needed for systemic spread in plants (Klein *et al.*, 1994; Cronin *et al.*, 1995).

1.6.4.6 Potyviral CP

The primary function of CP is to encapsidate the viral RNA. The central and C-terminal region of the CP are highly conserved within the potyviral group (Shukla *et al.*, 1988; Timmerman *et al.*, 1990). The C-terminal region of the CP also shares sequence similarity with other filamentous plant viruses, such as the potex-, carla-, clostero- and tobamoviruses (Lain *et al.*, 1988). The N- and C- terminal residues of the CP are positioned on the exterior of the virion (Allison *et al.*, 1985; Shukla *et al.*, 1988) and may be cleaved *in vivo* (Hiebert *et al.*, 1984) as well as during storage of purified virus preparations (Hiebert *et al.*, 1984; Michelin-Lansarot *et al.*, 1975). Such cleavage induces important differences in serological properties (Hiebert and McDonald, 1976), because the N- terminus constitutes the major virus specific epitopes. Mild trypsin treatment removes the N- and C- terminal segments, leaving a trypsin resistant core of about 24 kDa. All potyviruses display significant amino acid homology in the trypsin resistant core, but little sequence homology in their N- and C- terminal segments (Shukla *et al.*, 1988; Murphy *et al.*, 1995; Timmerman *et al.*, 1990).

1.6.5 Sequence variability among potyviruses

Protein sequence alignment of polyproteins from different potyviruses shows that the P1, P3 and the N-terminal region of the CP are the most variable regions among the potyviral polyproteins (Shukla *et al.*, 1991). CP sequence similarities vary between 38-71% for distinct potyviruses and 90-99% between strains of a given virus (Shukla and Ward, 1988).

Sequences from the 3'-terminal region of the genomic RNA may be used to differentiate between distinct viruses and virus strains (Shukla and Ward, 1988; Frenkel *et al.*, 1989).

Pair-wise comparisons of the conserved core region of the CP amino acid sequences also differentiated between distinct virus species and strains of a single virus (Ward and Shukla, 1991). The complete nucleotide sequence of 23 potyviruses, one ipomovirus, one rymovirus and two tritimoviruses have been determined to date (Table 1.3).

1.7 PSbMV

1.7.1 Physicochemical properties

PSbMV is a plus sense RNA virus with filamentous particles 770 nm long and 12 nm in diameter (Hampton *et al.*, 1974; Hampton and Mink, 1975). PSbMV virus preparations usually contain one sedimenting component with a sedimentation coefficient S_{20w} of 154 and a buoyant density in CsCl of 1.329 g/cm^3 (Huttinga, 1975). The $A_{260/280}$ of PSbMV is 1.14-1.18 and the absorbance of purified virus preparations at 260nm (1 mg/ml, lcm light path) is 2.5. Virions contain 5% RNA by weight and are composed of single coat protein subunits of Mr 34 kDa (Huttinga, 1975; Timmerman *et al.*, 1990).

1.7.2 Sequence information

The complete nucleic acid sequence of three PSbMV pathotypes isolate, DPD1 pathotype P-1 (Johansen *et al.*, 1991) NY pathotype P-4 (Johansen *et al.*, 1996b) and L1 pathotype P-2 (AJ252242 direct submission, 2000) have been determined. All other PSbMV sequence information available from genome databases EMBL and GenBank are for segments of the *CP* gene and *CP* and *NIb* genes.

The PSbMV DPD1 pathotype P-1 genomic RNA is 9923 nucleotides in length, excluding the polyadenylated tail (Johansen *et al.*, 1991). The RNA contains an ORF of 9618 nucleotides with the potential to encode a polyprotein with a calculated Mr of 364 kDa. The ORF is flanked by a 5' NTR sequence of 143 nucleotides and a 3' UTR of 163 nucleotides. The PSbMV NY pathotype P-4 genomic RNA is 9853 nucleotides in length, excluding the 3′-terminal poly(A) tail (Johansen *et al.*, 1996b). The sequence contained a large ORF starting at the first AUG (100-102) and terminating with a UUA stop (9697-9699). The ORF encodes a putative polyprotein of 3199 amino acids with a calculated molecular mass of 363 kDa. The PSbMV L1 pathotype P-2 genomic RNA is 9905 nucleotides in length including the 5′ NTR and poly(A) tail.

Table 1.3: Full-length sequences in the *Potyviridae.*

Potyviridae	Abbreviation	Strain/isolate	GenBank Accession no.	Year	Genome length (bp)
Potato virus Y	PVY		A08776	1993	9705
Clover yellow vein virus	ClYVV	no. 30	AB011819	1998	9584
Japanese yam mosaic virus	JYMV	Japanese yam 1	AB016500	1999	9757
	JYMV	mild	AB027007	1999	9760
Johnsongrass mosaic virus	JGMV		Z26920	1997	9779
Peanut mottle virus	PeMoV	M	AF023848	1998	9709
Bean common mosaic virus	BCMV	NL-3	U19287	1996	9612
Bean yellow mosaic virus	BYMV	S	U47033	1996	9547
	BYMV	MB4	D83749	1996	9532
Turnip mosaic virus	TuMV	Japanese	D83184	1996	9834
	TuMV		D10601, D01090	1992	9830
Sweet potato feathery mottle virus	SPFMV	S	D86371	1996	10820
Lettuce mosaic virus	LMV	O	X97704	1997	10080
	LMV	E	X97705	1997	10080
Maize dwarf mosaic virus	MDMV	Bulgarian	AJ001691	1997	9515
Pepper mottle virus	PepMoV	California	M96425	1993	9640
Tobacco vein mottling virus	TVMV		X04083	1987	9475
	TVMV	S	U38621	1997	9475
Plum pox virus	PPV	M (PS)	AJ243957	1999	9786
	PPV	SC	X81083	1994	9786
	PPV	NAT	D13751, D00424	1989	9741
	PPV	D	X16415	1989	9787
	PPV	SK 68	M2280, X56759	1993	9786
Papaya ringspot virus	PRSV	YK (Taiwan)	X97251	1997	10326
	PRSV	Hawaii	S46722	1992	10326
	PRSV	HA (Hawaii)	X67673	1998	10326
Pea seed borne mosaic virus	PSbMV	DPD1	D10930, D01152	1991	9924
	PSbMV	NY	X89997	1996	9860
	PSbMV	L1	AJ252242	2000	9905
Peanut stripe virus	PStV	blotch	U05771	1996	10056
	PStV	blotch	U34972	1997	10086
Potato virus A	PVA		Z21670	1995	9585
	PVA	Her	AJ131400	1999	9587
	PVA	Ali	AJ131401	1999	9598
	PVA	Y (U)	AJ131402	1999	9598
	PVA	TamMV	AJ131403	1999	9605
Potato virus Y	PVY	common	U09509	1997	9698
	PVY	N	D00441	1994	9704
	PVY	N (605)	X97895	1998	9701
	PVY	N	X12456	1989	9704
	PVY	Hungarian	M95491	1993	9703
Soybean mosaic virus	SMV	G2	S42280	1992	9588
Tobacco etch virus	TEV	HAT	M11458	1986	9494
	TEV		M15239	1993	9497
	TEV	Non-wilting	L38714	1996	9496
Yam mosaic virus	YMV	Ivory Coast	U42596	1996	9608
Zucchini yellow mosaic virus	ZYMV	Singapore	AF014811	1997	9603
	ZYMV	Reunion Island	L29569	1995	9642
	ZYMV	California	L31350	1995	10818
Sweet potato mild mottle ipomovirus	SPMMV		Z73124	1998	9593
Ryegrass mosaic rymovirus	RGMV	AV	AF035818	1998	9542
	RGMV	Danish	Y09854	1997	9535
Brome streak tritimovirus	BStV		Z48506	1995	9672
Wheat streak mosaic tritimovirus	WSMV	Sidney 81	AF057533	2000	9384

The nucleotide and amino acid sequence identity of PSbMV DPD1 and NY were 81% and 88% respectively (Johansen *et al.*, 1996b). The amino acid identity along the polyprotein varied: 65% (P1); 92% (HC-Pro); 82% (P3); 98% ($6K_1$); 94% (CI); 89% (6K2); 81% (VPg); 95% (NIa-Pro); 93% (NIb); 96% (CP) (Johansen *et al.*, 1996b).

The PSbMV polyprotein has a high level of amino acid identity with other potyviruses, except in the N-terminal region, which varies both in sequence and length (Johansen *et al.*, 1991). The variable region of the N-terminal region of PSbMV is between 93 and 142 amino acids longer than the corresponding regions of the other four potyviruses (Johansen *et al.*, 1991).

The P3 protein of PSbMV showed only limited sequence similarity (26-28%) with the corresponding proteins of other potyviruses (Johansen *et al.*, 1991).

The PSbMV CI protein showed 52-57% sequence similarity with the CI proteins of other potyviruses. A possible nucleotide binding motif, $(G/A)_{xxxx}GK(S/T)$ (Gorbalenya *et al.*, 1989), was also present in CI protein of PSbMV (amino acid positions 1351 to 1359) (Johansen *et al.*, 1991).

The NIb protein of PSbMV showed a high degree of sequence similarity with the corresponding proteins of other potyviruses (58% -63%). The GDD sequence motif found in many replicase enzymes (Strauss and Strauss, 1988) was also present in the PSbMV NIb protein, starting at amino acid position 2744 (Johansen *et al.*, 1991).

PSbMV CP exhibits 43%-60% amino acid sequence identity with the CP of other potyviruses (Johansen *et al.*, 1991; Timmerman *et al.*, 1990). The CP of PSbMV DPD1 pathotype P-1 (Johansen *et al.*, 1991) and another PSbMV P-1 isolate (Timmerman *et al.*, 1990) show 98% amino acid sequence similarity.

1.8 RNA virus evolution

Viruses containing RNA genomes constitute an extremely diverse group of pathogens. This high degree of variability is the result of a combination of factors including: the accumulation of mutations due to frequent errors in RNA synthesis (Domingo and Holland, 1994; Drake, 1993; Roossinck, 1997); RNA recombination (Simon and Burjarski, 1994;

Lai, 1995; White and Morris, 1995; Aranda *et al.*, 1997); genome reassortment, gene duplication, and *de novo* gene origin (Roossinck, 1997).

1.8.1 Mutation

Plant RNA viruses have a highly error prone replication mechanism, that result in numerous mutations and a quasispecies nature (Roossinck, 1997). Quasispecies are a population of viruses that share a common origin but which have distinct genomic sequences as a result of mutation, drift and the impact of selection. A single virus isolate is not a single sequence, but a swarm of mutant sequences that vary around a consensus sequence. The consensus sequence can only be obtained by direct sequencing of viral RNA.

1.8.2 Replication slippage

It has also been found that differences in length of the N-terminal portion of the CP of different potyvirus species result from repetitions that are up to 17 amino acids in length (Ward *et al.*, 1995). Such patterns of repetition suggest that replication slippage might be involved in the evolution of genes encoding these proteins. Five of nine complete potyvirus genomic sequences and 17 of 32 potyvirus CP genes were shown to have significant sequence repetitions implicating replication slippage as a mode of virus evolution (Hancock *et al.*, 1995).

1.8.3 Recombination

Recombination between virus and host genetic material evidently occurs in plant viruses as demonstrated by a luteovirus isolate with a 5′-terminal sequence derived from a chloroplast exon (Mayo and Jolly, 1991) and closteroviruses which have acquired host cellular protein-coding genes (Dolja *et al.*, 1994) which are non-essential for replication and virion production (Paremyslov *et al.*, 1998). However, recombination has rarely been observed in natural populations and has been reported for only a few viruses (Aranda *et al.*, 1997; Fraile *et al.*, 1997; Garcia-Arenal *et al.*, 1997).

Based on sequence analysis recombination has been proposed to occur among isolates of various potyviruses (Pappu *et al.*, 1994; Revers *et al.*, 1996; Bousalem *et al.*, 2000b). Comparison between phylogenies reconstructed from different short regions of the

genomes show incongruent topological positions for some PVY, BCMV, BYMV, ZYMV and YMV isolates, suggesting the occurrence of recombination events during the evolution of these potyviruses (Revers *et al.*, 1996; Bousalem *et al.*, 2000b). Complementary statistical tests have highlighted the fact that recombination events contributed to the evolution of these viruses. Unequal recombination within the genome is thought to have contributed to the evolution of the CP of *Dasheen mosaic virus* (DMV) (Pappu *et al.*, 1994).

Apart from recombination being inferred from sequence analysis, a PPV recombinant has been found in a natural population (Cervera *et al.*, 1993) and single and double recombinants have been detected when squash plants were co-bombarded with mixtures of engineered truncated constructs of ZYMV (Gal-On *et al.*, 1998).

1.9 **Phylogenetics**

The taxonomy within the Potyviridae was originally based on host range, symptomatology, cross-protection, morphology of cytoplasmic inclusions and serology (Ward and Shukla, 1991; Shukla *et al.*, 1994). This often led to conflicting or inconsistent taxonomic classification of potyviruses. Phylogenetic analysis based on amino acid sequence comparisons of the NIb and CP have helped establish new viruses as members of the family *Potyviridae,* establish new genera within the *Potyviridae* and reclassify known potyviruses (Hall *et al.*, 1998; Badge *et al.*, 1997; Drake *et al.*, 1998).

Within most virus genera different genes usually have the same phylogenies, indicating that their evolution has been linked, that they have experienced the same speciation events and have co-diverged (Gibbs *et al.*, 1997). In the case of the potyviruses phylogenetic trees based on every cistron of the YMV potyvirus genome displayed a similar topology. Phylogenetic analysis on six YMV isolates showed the same topology regardless of which gene (P1, HC-Pro, P3, NIb and partial CP region) was analysed (Aleman-Verdaguer *et al.*, 1997). Comparisons of the nucleotide sequences of potyvirus genomes showed that the degree of identity between equivalent genes of strains was greater than 96% while between distinct potyviruses the identity ranged from 42-65% suggesting that any extended sequence could be considered representative of the whole genome (Frenkel *et al.*, 1992).

Phylogenetic trees based on either 5′ NTR or P1 sequences of PVY resulted in the same clustering of the studied isolates into three groups (Tordo *et al.*, 1995). Phylogenetic studies based on the CP and 3′ UTR of PVY, however, have placed isolates into two groups (Van der Vlugt *et al*, 1993). The similar phylogenetic trees obtained for the 5′ NTR and P1 region suggests that these two adjacent regions have evolved in a coordinate manner. No correlation was observed between the CP and 3′ UTR diversities and phylogenties of 27 YMV isolates (Bousalem *et al.*, 2000b). Assessing phylogentic relationships of potyviruses with the 3′ UTR is not recommended, as there is a difference in selection pressure between the coding and non-coding regions.

1.10 Techniques for the detection of plant viruses

1.10.1 Serology

Several serological detection methods have been described for use in plant virus detection. These include precipitin tests (Hamilton and Nichols, 1978; Walkey, 1985) and variants of enzyme linked immunoassays including: ELISA (Clark and Adams, 1977); single antibody dot immunoassay (SADI) (Graddon and Randles, 1986); dot immunobinding assay (DIBA) (Hibi and Saito, 1985; Ligat *et al.*, 1991); rapid immunofilter paper assay (RIPA) (Tsuda *et al.*, 1992); and tissue-blot immunoassays (Makkouk *et al.*, 1993b).

1.10.2 Nucleic acid hybridisation

Nucleic acid hybridisation is a highly sensitive and specific procedure identifying RNA and DNA viruses and plant viroids (Maule *et al.*, 1983; Owens and Diener, 1981). Nucleic acid hybridisation has the advantage that once a diagnostic probe has been prepared to a specific virus, the technique can be used to rapidly screen a large number of crude sap samples. The limit of its sensitivity has been reported to be as low as 5-20 pg virus per spot sample (Maule *et al.*, 1983).

While there have been reports of cDNA hybridisation to successfully detect plant virus strains, the use of probes synthesised by random priming has resulted in strong hybridisation with heterologous viruses, including viruses from different groups (Koenig *et al.*, 1988). Nucleotide sequence comparisons also revealed that some parts of the genome, but not the 3' UTR, have local regions of high sequence identity and can lead to cross-hybridisation between distinct potyviruses (Frenkel *et al.*, 1992). The 3'UTR from a range

of potyviruses that were strains of a virus showed sequence identity of 80% or more, while distinct viruses showed less than 50% sequence identity (Frenkel *et al.*, 1992). Nucleic acid hybridisation with a complement of the 3' UTR of the potyvirus genome as the probe has been shown to be a relatively simple means of distinguishing between distinct potyviruses and their strains (Frenkel *et al.*, 1992).

Non-radioactive probes (biotin and Digoxigenin) are less sensitive than methods using ^{32}P labelled probes, and have been shown to cross react with mock inoculated plants giving false positives (Borja and Ponz, 1992).

1.10.3 Polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful technique used for the amplification of specific nucleic acid sequences and is capable of enrichment by a factor of 10^6-10^9, enabling the detection of a few target molecules (Saiki *et al.*, 1988). Several methods of PCR have been outlined for use in plant pathology (Henson and French, 1993; Hadidi *et al.*, 1995; Louws *et al.*, 1999).

In the case of RNA genomes, reverse transcription is also combined with PCR. Reverse transcription polymerase chain reaction (RT-PCR) from infected leaf tissues has been reported to be more sensitive than ELISA in the detection of plant viruses (Vunsh *et al.*, 1991; Borja and Ponz, 1992; Kohnen *et al.* 1992; Nolasco *et al.*, 1993; Bariana *et al.*, 1994; Rosner, 1994; Palkovics *et al.*, 1994; Matthews *et al.*, 1997).

1.10.3.1 Tissue sample preparation for RT-PCR

Plant tissues vary in the presence of inhibitory factors and the ease with which viral nucleic acid can be released (Wang *et al.*, 1993; Thomson and Dietzgen, 1995).

The approaches adopted for sample preparation of infected plant material include extraction of total plant nucleic acid, virus purification, or isolation of viral nucleic acids (Kohnen *et al.*, 1992; Borja and Ponz, 1992; Peason *et al.*, 1994). Total plant nucleic acid extraction from infected plant material with guanidinium thiocyanate (Chirgwin *et al.*, 1979; Chomczynski *et al.*, 1987) has been reported to result in the failure of subsequent PCR amplification (Kohnen *et al.*, 1992). Protocols involving crude sap extracts have been

described (Wetzel *et al.*, 1991). The simplest approach is to introduce the plant tissue directly in to the PCR reaction mixture (Berthomieu and Meyer, 1991). This method is only likely to succeed in cases where the PCR can tolerate the presence of inhibitory plant components that may be introduced. Procedural modifications to remove inhibitors from samples to be tested by PCR have been reported (Levy *et al.*, 1994; Saiz *et al.* 1994; Doyle and Doyle, 1989).

1.10.3.2 RT-PCR fidelity

Both Reverse Transcriptase and *Taq* DNA polymerase have relatively high error rates, and for *Taq* this is compounded by the multiple cycles of copying during PCR. The error rate for *Taq* ranges from 0.2-2 x 10^{-4} errors/bp (Lundberg *et al.*, 1991; Barnes, 1992). The error rates of the RNA dependant RNA polymerase of animal RNA viruses averaged about 10^{-4}, or one error per 10 kb genome (Domingo and Holland, 1994).

Errors generated during PCR can be limited by minimising the number of PCR cycles, avoiding nested PCR and optimising cycling parameters and conditions (Eckert and Kunkel, 1990). Artifactual substitution occurring PCR can be reduced by using polymerases with higher fidelity. Pwo has a higher than 10 fold increase in fidelity in DNA synthesis compared to *Taq* DNA polymerase (Annonymous, 1999a).

1.10.3.3 Internal controls for RT-PCR

The reverse transcription polymerase chain reaction (RT-PCR) is increasingly being used for the detection of RNA viruses because of its high sensitivity and specificity. For example, in plant virology new RT-PCR diagnostic methods have been recently described (Raj *et al.*, 1998; Jacobi *et al.*, 1998; Grieco and Gallitelli, 1999; Eun *et al.*, 2000). However, none of these methods describe the use of internal controls. This is important if the RT-PCR assay is to be used as a routine diagnostic assay for indexing.

Previously described internal controls for plant virus PCR and RT-PCR have targeted endogenously expressed genes. Thomson and Dietzgen (1995) used the primers of Kolchinsky *et al.*, (1991) which target the spacer regions between multiple copy ribosomal 5S RNA genes as an internal amplification control in plant virus PCR detection. Genomic DNA from each plant species produces one or more characteristic and reproducible fragments.

Primers to the 18S ribosomal gene have been used unsuccessfully as internal controls in plant virus RT-PCR (Bariana *et al.*, 1994). The primer pair targeted to the ribosomal 18S RNA in conjunction with the virus-specific primers resulted in numerous spurious bands in uninfected samples. Other internal control primers targeting malate dehydrogenase (MDH) and ribulose bisphosphate carboxylase oxygenase (RibiscoL) mRNA also produce spurious bands when multiplexed with diagnostic primers (Nassuth *et al.*, 2000).

For medical diagnostics with PCR and RT-PCR the issue of quality assurance has been addressed. Several internal control methods have been developed including: co-amplification with a second set of primers targeted to an endogenously expressed "housekeeping" gene (Denis and Lustenberger, 1995); amplification with the same set of primers targeting an exogenous internal control which has been structurally modified from the target (Pallen *et al.*, 1992; Brightwell *et al.*, 1998,); amplification with the same set of primers targeting an exogenous internal control sequence which only has homology with the target at the terminal primer recognition sites (Sachadyn and Kur, 1998); and supplementing samples with a known amount of a control sequence prior to extraction (Willhauck *et al.*, 1998).

1.11 Potyvirus group specific RT-PCR

There are many published reports of the use of RT-PCR with degenerate primers that produce specific DNA fragments from all species of a group, genus or even family of viruses, including: potyviruses (Langeveld *et al.*, 1991, Colinet and Kummert, 1993, Colinet *et al.*, 1993, Gibbs and Mackenzie, 1997, Mackenzie *et al.*, 1998); carlaviruses (Badge *et al.*, 1996); closteroviruses (Karasev *et al.*, 1994, Tian *et al.*, 1996); tospoviruses (Mumford *et al.*, 1996); geminiviruses (Deng *et al.*, 1994, Wyatt and Brown, 1996); luteoviruses (Robertson *et al.*, 1991); and carmo-, diantho- and tombusvirus groups (Morozov *et al.*, 1995).

Potyvirus group-specific RT-PCR has also been described which target conserved regions within the *CP* core domain and the *NIb* replicase proteins (Langeveld *et al.*, 1991). Two sets of degenerate primers, U335/D335 and U1000/D10000 (Table 1.4), support potyvirus-specific amplification, and do not amplify carlavirus or potexvirus RNA templates (Langeveld *et al.*, 1991). However, the U1000/D1000 primers do not amplify all

potyviruses tested (Langeveld *et al.*, 1991). The U335/D335 RT-PCR primers amplified the expected 355 bp fragment from all potyviruses tested (Langeveld *et al.*, 1991), however these primers have also been reported to amplify host nucleic acids (Pearson *et al.*, 1994).

Redundant RT-PCR primers that are specific to members of the *Potyviridae* have been reported (Gibbs and Mackenzie, 1997, Mackenzie *et al.* 1998). The degenerate *Potyviridae* specific oligonucleotide primers (PV1 and PV2, Table 1.4) amplify a 1.6-2.1 kb fragment from the 3′ end of the *Potyviridae* genome, including part of the *NIb*, *CP* and poly-A tail. These primers have been successful in detecting 18 species of the *Potyviridae* which include species from the genus *Poytyvirus*, *Rymovirus*, *Bymovirus*, *Ipomovirus* and *Macluravirus* (Gibbs and Mackenzie, 1997, Mackenzie *et al.*, 1998). Primer PV1 is targeted to the poly-A tail of the *Potyviridae* and primer PV2 is targeted to the conserved GNNSGQP region found within the *NIb* gene of all members of the *Potyviridae*.

In addition to the 1.6-2.1 kb *Potyviridae* specific amplicon generated by PV1/PV2, smaller 0.6-1 kb fragments are also produced (Gibbs and Mackenzie, 1997, Mackenzie *et al.*, 1998). The 1.6-2.1 kb fragment from 4 Potyviridae have been sequenced and their identity confirmed (Gibbs and Mackenzie, 1997). The smaller 0.6-1 kb fragments from five potyviruses have also been sequenced and identified as a region between the *NIb* and the 5′ terminal part of the *CP* gene, which is A-rich (Gibbs and Mackenzie, 1997). By modifiying the 5′ ends of the PV1 and PV2 primers, incorporating SP6 and T7 promotor sites and using deoxyinosine at sites of 3 fold degeneracy, hence creating the PV1/SP6 and PV2I/T7 primers (Appendix C), Mackenzie *et al.* (1998) have reported that only the full-length 1.6-2.1 *Potyviridae* specific fragment is obtained.

1.12 RT-PCR for PSbMV detection

Immunocapture (IC)-PCR has been described for PSbMV detection using two different primer pairs, D1/D2 and F1/F2 (Table 1.4) targeting the $6K_2$ and *NIa* genes of PSbMV (Phan *et al.*, 1997). RT-PCR primers D1/D2 amplify a 654 bp product, while RT-PCR primers F1/F2 amplify a 494 bp product (Phan *et al.*, 1997). Both the D1/D2 and F1/F2 primer pairs amplify PSbMV from infected pea embryos and testas when virions are immunocaptured with antiserum IgG 'H' (hyperimmune antisera to PSbMV antigen), but only from embryos with antisera IgG 'K' (antisera to PSbMV coat protein) (Phan *et al.*, 1997). However, primer pair F1/F2 do not amplify all PSbMV isolates tested (Phan *et al.*,

1997). IC-PCR with primers D1/D2 and F1/F2 is not 100% reproducible and approximately 30 times less sensitive than DAS-ELISA for the detection of PSbMV in seed (Phan *et al.*, 1997).

PSbMV pathotype P-1 specific RT-PCR has been reported using two primers pairs (A_{DPD1}/C_{DPD1} and B_{DPD1}/C_{DPD1}, Table 1.4) targeting the *NIa* and *NIb* genes (Kohnen *et al.*, 1992). Primers A_{DPD1} and C_{DPD1}, amplify a 1200 bp product, while primers B_{DPD1} and C_{DPD1} amplify a 730 bp product from four PSbMV pathotype P-1 isolates but not from a single PSbMV pathotype P-4 isolate (Kohnen *et al.*, 1992). Primer pair B_{DPD1}/C_{DPD1} facilitated greater PCR-product amplification. A third primer pair $E_{(DPD1)}$ /$F_{(DPD1)}$ (Table 1.4), situated within the PSbMV *CP* gene, failed to amplify of the expected 576 bp sequence from PSbMV DPD1 (Kohnen *et al.*, 1992).

PSbMV pathotype P-4 specific RT-PCR targeting the *P1/HC-Pro* genes has also been described (Kohnen *et al.*, 1995). Primer A_{NY} and B_{NY} amplified a 850 bp product from PSbMV NY pathotype P-4 but not from PSbMV DPD1 pathotype P-1 (Kohnen *et al.*, 1995). RT-PCR is 25-250 fold more sensitive than ELISA in the detection of PSbMV (Kohnen *et al.*, 1992, Kohnen *et al.*, 1995).

Significant PSbMV pathotype specific sequences have not yet been identified. Therefore, the PSbMV primers which have been termed as PSbMV pathotype specific are only PSbMV isolate specific. The PSbMV pathotype P-1 specific RT-PCR primers (Kohnen *et al.*, 1992) have only been shown to distinguish between four PSbMV pathotype P-1 isolates and one PSbMV pathotype P-4 isolate, while the PSbMV pathotype P-4 specific RT-PCR primers have only distinguished between one PSbMV pathotype P-1 isolate (DPD1) and one PSbMV pathotype P-4 isolate (NY) (Kohnen *et al.*, 1995). The F1/F2 IC-PCR primers also reveal strain specificity in PSbMV detection, which is not observed with DAS-ELISA (Phan *et al.*, 1997). Therefore, IC-PCR and RT-PCR are currently not practical alternatives to ELISA in quality control of pea seed for PSbMV.

1.13 Current detection methods for PSbMV

Screening programs based primarily on symptomatology have not proven satisfactory in detecting PSbMV infected plants both because certain pea cultivars used as indicators do not show typical symptoms of PSbMV infection and because of the reported incidence of

Table 1.4: Potyvirus specific and PSbMV primer sequences reported.

Primer	Sequence	Location within genome	Reference
U335	5'-GAATCATG(A/G)TNTGGTG (C/T)AT(A/C)GANAA(C/T)GC-3'	Conserved MVWCIENG amino acid sequence identified within the CP of nine potyviruses. Located at amino acid residues 124-131 (nucleotide positions 895-917) of TBV-lily	Langeveld *et al.*, 1991
D335	5'-GAGCTCGCNG(C/T)(C/T)TT CAT(C/T)TGN(A/G)(A/C)(A/G/T) (A/T)(G/T)NGC-3'	Conserved AHFQMKTA amino acid sequence within the CP of nine potyviruses. Located at amino acid residues 224-231 (nucleotide positions 1195-1217) of TBV-lily	Langeveld *et al.*, 1991
U1000	5'-ACIGTIGTIGA(C/T)AA(C/T) (A/T)TI(C/T)ATGG-3'	RNA-dependent RNA polymerase-related motif TVVDNTLMV within NIb	Langeveld *et al.*, 1991
D1000	5'-GTICC(A/G)TTITCIAT(A/G)CA CCAIA(C/T)CAT-3'	Conserved MVWCIENG amino acid sequence identified within the CP	Langeveld *et al.*, 1991
$A_{(DPD1)}$	5'-TGTGCATGCTTTTGATCCAT GTGAAT-3'	Located within the 5' region of the VPg-NIa region at nucleotide positions 6185-6210 of PSbMV (DPD1) pathotype P-1.	Kohnen *et al.*, 1992
$B_{(DPD1)}$	5'-CGATTATGACCGATCAATCT TTGGC-3'	Located within the 5' region of the NIa-Pro gene at nucleotide positions 6668-6692 of PSbMV (DPD1) pathotype P-1.	Kohnen *et al.*, 1992
$C_{(DPD1)}$	5'-TGTCCCGGGCAGCGGGCAAC CACT-3'	Located within the 5' region of the NIb (59K replicase) gene complementary to nucleotide positions 7379-7399 of PSbMV (DPD1) pathotype P-1.	Kohnen *et al.*, 1992
$E_{(DPD1)}$	5'-GCGGAGTGGCTATCAGC-3'	Located within the CP gene at nucleotide positions 9136-9152 of PSbMV (DPD1) pathotype P-1.	Kohnen *et al.*, 1992
$F_{(DPD1)}$	5'-CTTCTGCTGTGTGCCTC-3'	Located within the CP gene complementary to nucleotide positions 9695-9711 of PSbMV (DPD1) pathotype P-1.	Kohnen *et al.*, 1992
$A_{(NY)}$	5'-TGCGTGAAGGTTAAACAAAA CAGTAG-3'	Located within the P1 protease gene at nucleotide positions 703-728 of PSbMV (NY) pathotype P-4.	Kohnen *et al.*, 1995
$B_{(NY)}$	5'-ATTCAAGTCCTGTATTTGCC AGATGT-3'	Located within the HC-Pro gene complementary to nucleotide positions 1530-1554 of PSbMV (NY) pathotype P-4.	Kohnen *et al.*, 1995
D1	5'-GCTCTAGACTCGAGGGGAA (A/G)TC(A/G)AAAGCTAAAC-3'	Located within the VPg-NIa gene corresponded to nucleotide positions 5595-6028 of PSbMV (DPD1) pathotype P-1 and 5933-5966 PSbMV (NY) pahtotype P-4.	Phan *et al.*, 1997
D2	5'-GTCCTAGAGCTTGCGCAAT (A/T)GGATTGTA-3'	Located within the NIa-Pro gene complementary to nucleotide positions 6621-6648 of PSbMV (DPD1) pathotype P-1 and 6559-6586 of PSbMV (NY) pathotype P-4.	Phan *et al.*, 1997
F1	5'-GATTTCTTCGTTGTTTGTT-3'	Located within the $6K_2$ corresponded to nucleotide position 5916-5934 of PSbMV (DPD1).	Phan *et al.*, 1997
F2	5'-CTTGAGTGCTGGCGTGGTT-3'	Located within the VPg-NIa gene complementary to nucleotide position 6395-6413 of PSbMV (DPD1).	Phan *et al.*, 1997
PV1	5'-CACGGATCCCGGG$(T)_{17}$(A/G/C)-3'	Poly-A tail of all Potyvirdiae	Gibbs & Mackenzie, 1997
PV2	5'-GG(T/C/G)AA(C/T)AA(C/T)AG (C/T)GG(G/T/A)CA(A/G)CC-3'	Conserved GNNSGQP amino acid motif within NIb of the Potyviridae	Gibbs & Mackenzie, 1997

(N) A, C, T or G; (I) deoxyinosine. Restriction endonuclease sites are underlined.

symptomless infection in indicator plants (Maury *et al.* 1987; Hampton, 1972; Ding *et al.*, 1992; Ligat and Randles, 1993).

Gel immunodiffusion can detect PSbMV infection when infected plant tissue is diluted 1:3 in healthy plant material. However, this method is not sensitive enough for detection of low levels of PSbMV infection (Hamilton and Nichols, 1978).

Hamilton and Nichols (1978) developed an ELISA for bulk sample detection of PSbMV. PSbMV could be detected in crude homogenates of artificial mixtures of infected and healthy leaves containing 5-10% infected leaves and in crude homogenate from seedlots containing 25% infected seed. The inability to detect lower levels of PSbMV seed infection by this method could be due to the relatively high A_{405} (0.1-0.15) of healthy seed homogenates when spectrophotometrically scanning ELISA samples (Hamilton and Nichols, 1978). Purified PSbMV can be detected by ELISA at a concentration of 0.5 µg/ml (Hamilton and Nichols, 1978). Detection of PSbMV in leaf and seed tissue by ELISA is aided by the addition of cellulase or Triton X-100 in the extraction fluid at a concentration of 0.1% (Wang *et al.*, 1992).

The CP of PSbMV, analysed in mature pea seed, had a molecular weight of 33 kDa in infected embryos and a molecular weight of only 29-27 kDa in seed testas (Masmoudi *et al.*, 1994b). Antisera specific to the deleted part of the CP protein enabled detection of PSbMV in embryos only (Masmoudi *et al.*, 1994b). Antisera raised to a 26 kDa fragment of PSbMV P1 protein was shown to be specific to PSbMV pathotype P-1 isolates but not pathotype P-4 isolates (Albrechtsen & Borkhardt., 1994).

DIBA has a detection limit of 32 ng per 1 µl sample (Ligat *et al.*, 1991) and involves a crucial blocking step with healthy sap and monosaccharides to eliminate non-specific reaction due to lectins and host reacting antibodies. DIBA has advantages over ELISA in that it can be performed in a short period of time, 3-5 hours, and is economical.

Potyvirus group-specific monoclonal antibodies, recognizing conserved epitopes in the coat protein have been developed for the identification of uncharacterized potyviruses but is not useful in detecting PSbMV specific infection (Jordan, 1989).

Screening of pea germplasm for PSbMV is currently based on serological methods, such as ELISA (Maury *et al.*, 1987; Ding *et al.*, 1992; Hampton *et al.*, 1993; Masmoudi *et al.*, 1994a: Masmoudi *et al.*, 1994b) and DIBA (Ligat *et al.*, 1991).

1.14 Scope of this thesis

This thesis reports the collection and biological characterisation of PSbMV isolates from southern Australia.

This thesis also reports the molecular characterisation of a range of Australian, Pakistani and PSbMV pathotype P-1 and P-4 type isolates. RT-PCR and nucleic acid hybridisation assays were developed to detect PSbMV. The *HC-Pro* and *CI/6K$_2$/VPg* genomic regions of PSbMV were characterised by RFLP, sequence and phylogenetic analysis, allowing grouping of isolates.

This thesis further reports the development of a sensitive and specific PSbMV RT-PCR assay suitable for pea seed testing and the development of an exogenous internal RT-PCR control, which in the duplex RT-PCR does not affect the sensitivity, or specificity of the PSbMV assay. This method is suitable for pea seed batch testing and has potential as a diagnostic assay.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plants and soil

All plants were grown under glasshouse conditions at 21°C-25°C in pasteurized UC soil (Plant Research Centre, SARDI, Australia) and fertilised fortnightly with Aquasol soluble fertiliser (Hortico Pty Ltd, Australia). Table 2.1 shows the indicator and propagation plants used.

Table 2.1: Sources of indicator and propagation plants used in this study

Plant species	Seed source
Pisum sativum cv Dundale	B. Badman, Yorke Peninsula, South Australia
Vicia faba	WARI*
Phaseolus vulgaris cv Hawkesbury Wonder	Yates #
Chenopodium quinoa	WARI
Chenopodium amaranticolor	WARI
Nicotiana tabacum cv Samsun	WARI
Nicotiana clevelandii	WARI
Glycine max	WARI

* WARI; Waite Agricultural Research Institute seed collection

Yates; Arthur Yates & Co. Ltd., NSW, Australia

2.1.2 Biochemicals and miscellaneous chemicals

The biochemicals and miscellaneous chemicals used in this study are listed in Appendix A

2.1.3 Polyacrylamide and agarose gels, bacterial strains and media, and solutions and buffers

Compositions and preparation of polyacrylamide and agarose gels, bacterial strains and preparation of bacterial media, solutions and buffers are described in Appendix B.

2.1.4 Primers

All oligonucleotide primers and their sources are listed in Appendix C

2.2 Methods

2.2.1 Mechanical inoculation

Inoculum was prepared by crushing fresh or dry infected leaf tissue in 50 mM sodium borate buffer, pH 8.2. Plants were inoculated by rubbing inoculum onto all leaves of young test plants (four-leaf stage) which had been lightly dusted with carborundum powder 400 mesh.

2.2.2 Calcium chloride method of preserving virus infected tissue

Virus infected leaf tissue was collected and cut into pieces with a sterile scalpel blade. Leaf tissue was placed onto silica beads and desiccated in a vacuum chamber for 16 hrs at 4°C. Dried leaf was stored over $CaCl_2$ in a sealed McCartney bottle at 4°C for up to 6 months.

2.2.3 PSbMV purification

Approximately 150 *Pisum sativum* cv Dundale seedlings grown under continuous light and constant temperature (20-25°C) were mechanically inoculated with PSbMV 10 days post emergence. Infected plant material was harvested 3 weeks post-inoculation and used for purification of virus particles by a modification of the method of Wang *et al.* (1992). All steps were done at 4°C unless otherwise stated. Infected plant material (200 gm) was homogenised with 1 L extraction buffer (Appendix B). Homogenate was filtered through cheesecloth and clarified with 2.5% Triton® X-100 for 20 min while stirring. The sap was centrifuged at 8000 *g* for 10 min and the supernatant mixed with 100 mM NaCl and 4% (w/v) PEG_{6000} and stirred for 45 min. Following centrifugation at 8000 *g* for 10 min the pellet was resuspended in 50 mL BE buffer with stirring for 45 min. Particulate material was pelleted by low speed centrifugation at 8000 *g* for 10 min and the supernatant collected and centrifuged through a 30% sucrose cushion at 85,600 *g* for 2 hrs at 10° C (TY 60Ti Beckman, USA). The pellet was resuspended in 15 mL BE buffer and 33% w/v Cs_2SO_4 added. An isopycnic gradient was formed by high-speed centrifugation at 137,000 *g* for 16 hrs (SW 65Ti Beckman, USA). The visible virus band was collected with a syringe and 18-gauge needle. The virus was dialysed 3 times against BE buffer and pelleted by ultracentrifugation at 85,600 *g* for 2 hrs (TLA 100.3 Beckman, USA). The pellet was resuspended in 10 mM sodium borate buffer, pH 8.2.

2.2.3.1 Calculation of virus concentration

Virus concentration was determined by spectrophotometry (DU®-68 spectrophotometer Beckman, USA) using the calculation:

$$c = \frac{A_{260}}{E_{260}^{1cm,0.1\%}} \text{ x dilution factor} \quad \text{mg/mL}$$

For PSbMV $E_{260}^{1cm,0.1\%}$=2.7.

2.2.3.2 Infectivity test

Partially purified virus was tested for infectivity by mechanical inoculation onto pea seedlings and observation of the development of systemic symptoms.

2.2.4 Total nucleic acid extraction

2.2.4.1 CTAB method from leaf tissue

The CTAB method was modified from Doyle and Doyle (1989). Fresh leaf tissue (0.5 gm) was crushed in 5 volumes of CTAB buffer containing 0.2% 2-mercaptoethanol and incubated at 60°C for 30 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed, then centrifuged at 13,000 *g* (Hettich Mikrolitre centrifuge, Hettich, Germany). The aqueous phase was collected and precipitated with 0.9 vol. isopropanol in the presence of 0.3M sodium acetate, pH 5.2, and incubated at -20°C for 16 hrs. Total nucleic acid was pelleted by centrifugation at 13,000 *g* for 30 min. The pellet was washed with 70% ethanol, dried in a SpeedVac (Selby Scientific Instruments, Australia), and resuspended in 200µL sterile DEPC treated water.

2.2.4.2 CTAB method from pea germplasm

Lots of 10 seeds were crushed in an ICARDA mill (Syria) and the seed coats removed in an Aspirator (SK Engineering and Allied Works, Bahraich, India). The individual seed lots were ground into a course flour (Analysen Mühle, Janke and Kunkel IRA Labortechnik, John Morris Scientific Pty. Ltd.) and 1 gm sub-samples were incubated in 10 mL CTAB buffer containing 0.2% 2-mercaptoethanol at 60°C for 30 min in a plastic bag. Samples were briefly crushed in the bag with a roller before transfer to a tube. An equal volume of phenol:chloroform:isoamyl alcohol pH 6-7 (25:24:1) was added, samples were vortexed

and centrifuged at 10,000 *g* for 10 min. The aqueous phase was extracted with one volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 *g* for 10 min. The supernatant (1 mL) was taken and the nucleic acid was precipitated and resuspended as above.

2.2.4.3 K/SDS method

Fresh leaf tissue (0.5 gm) was crushed in a plastic bag containing 1.5 mL K/SDS solution with a roller and 150 µL of 20 % SDS added and left to stand for 15 min. One mL of slurry was transferred into a 1.5 mL eppendorf tube and centrifuged at 10,000 *g* for 10 min at room temperature. 250 µL of 5 M potassium acetate was added and mixed thoroughly, without resuspending the pellet, and incubated on ice for 30 min. Samples were gently mixed and centrifuged at 10,000 *g* for 15 min. The supernatant was collected and 0.9 vol. isopropanol added and incubated at -20°C for 16 hrs. Nucleic acids were pelleted by centrifugation at 10,000 *g* for 10 min at room temperature and washed in 700 µL 70% ethanol with gentle agitation for 1 hr. Pellets were resuspended in 150 µL sterile DEPC treated H_20.

2.2.5 Agarose gel electrophoresis

DNA was electrophoretically separated on agarose gels (Appendix B) in a submarine gel tank using 1 x TAE running buffer. Samples were loaded with 1 x gel loading buffer III and electrophoresed at 5 V/cm.

2.2.6 Formaldehyde gel electrophoresis

RNA was electrophoretically separated on formaldehyde agarose gels according to the method of Sambrook *et al.* (1989). Agarose gel were prepared in 1 x formaldehyde gel running buffer and 2.2 M formaldehyde at 60°C in a fumehood. Gels were submerged in 1 x formaldehyde gel running buffer and pre-run at 5 V/cm for 5 min prior to loading samples and then run at 3-4 V/cm.

2.2.6.1 RNA sample preparation

RNA samples in a final volume of 20 µL were incubated in 0.5 x formaldehyde gel running buffer, 2.2 M formaldehyde and 50% formamide at 65°C for 15 min then chilled on ice.

Samples were mixed with 1 x formaldehyde gel-loading buffer and loaded onto pre-run formaldehyde agarose gels.

2.2.6.2 Ethidium bromide staining

Formaldehyde gels were stained in 0.5 µg/mL EtBr in 0.1M ammonium acetate for 30-45 min. Gels were destained if required, visualized by UV excitation at 254-302 nm and photographed.

2.2.7 Dot Immunobinding Assay

2.2.7.1 Preparation of samples

2.2.7.1.1 Seed

Whole or dehusked pea seeds were ground in groups of 10 (Analysen mühle, Janke and Kunkel IRA Labortechnik, John Morris Scientific Pty. Ltd.). The coarse pea flour was incubated in 5 mL of 1 x PBS, pH 7.4 buffer at 4°C for 16 hrs. Samples were lightly macerated with a roller before the extract was transferred to a 1.5 mL eppendorf tube.

2.2.7.1.2 Leaf

Leaf samples were collected and crushed in 1:1 (w:v) PBS, pH 7.4 buffer. Sap extracts were transferred to 1.5 mL eppendorf tubes.

Both seed and sap extracts were centrifuged at 10,000 *g* for 2 minutes. One µL of the resulting supernatant was spotted in duplicate onto nitrocellulose membrane (Schleicher & Schuell, BA85/20, Germany).

2.2.7.2 Preparation of blocking buffer

2.2.7.2.1 Seed

Coarsely ground flour from seed of virus-free pea plants was added to 10 vol. 1 x PBS, pH 7.4 (w:v), shaken and left to stand for 16 hrs at 4°C. The solution was briefly macerated in a blender and particulate material removed by centrifugation at 5,000 *g* for 5 min.

2.2.7.2.2 Leaf

Leaf tissue from healthy pea was crushed in 9 vol. 1 x PSB, pH 7.4 (w:v) and centrifuged at 10,000 *g* for 5 min.

Blocking buffer was used immediately or after storage at -20°C.

2.2.7.3 Blocking, incubation and development of nitrocellulose membrane

Nitrocellulose membranes (50 cm^2) were incubated with gentle agitation for 1 hr in either 10 mL seed or leaf blocking buffer containing 0.5 M glucose and 0.5 M mannose. The type of blocking buffer used depended on the type of samples being tested; seed blocking buffer was used when testing seed and leaf blocking buffer was used when testing leaf tissue. All incubation steps were done at 25°C unless otherwise stated. Blocking buffer was replaced with 10 mL of PSbMV antibody solution (healthy blocking buffer containing 0.5 M glucose, 0.5 M mannose and 1/1000 PSbMV antibody, Waite Agricultural Research Institute antiserum collection) and incubated with gentle agitation for 1hr. PSbMV antibody solutions were prepared 1 hr before use and left to stand until required. The PSbMV antibody solution was removed and the membrane washed twice in wash buffer AP, pH 7.5 for 5 min each, then twice in wash buffer AP, pH 9.5 for 2 minutes each. The membrane was then incubated with gentle agitation for 1 hr in 10 mL BSA blocking buffer containing 0.5 M glucose, 0.5 M mannose and 1/2000 goat anti-rabbit conjugated alkaline phosphatase antibody (Appendix A). The membrane was washed as before then incubated in 10 mL substrate buffer at 37°C in the dark for 3 minutes. The reaction was stopped by transferring the membrane into stop buffer for 15 minutes.

2.2.8 Molecular cloning

2.2.8.1 Preparation of competent cells

A single colony of DH5α cells was grown in 10 mL LB for 16 hrs with shaking at 37°C. Two mL of the culture was added to 50 mL LB and grown for 2.5 hrs or until an OD at A_{600} of 0.6 was reached. Cells were pelleted by centrifugation at 4,000 *g* for 10 min at 8°C, resuspended gently in 50 mL cold 0.1 M $MgCl_2$, then again pelleted by centrifugation. Cells were resuspended in 25 mL cold 0.1 M $CaCl_2$ and incubated on ice for 25 min. Cells were pelleted by centrifugation at 4,000 *g* for 10 min at 8°C and finally resuspended in 1.6

mL cold 0.1 M $CaCl_2$ containing 15% v/v glycerol. Competent cells were stored in 200 µL aliquots at -80°C.

2.2.8.2 A-Tailing

Blunt ended PCR products generated by proof-reading Pwo DNA polymerase were A-tailed to allow cloning into the pGEM®-T vector. 5µL of purified PCR product was added to 5 U *Taq* DNA polymerase (Promega), 2.5 mM $MgCl_2$, 0.2 mM dATP in 1 x *Taq* DNA polymerase reaction buffer (Promega) and incubated at 70°C for 30 min.

2.2.8.3 Ligation

Ligations were set up in a final volume of 10 µL containing 50 ng pGEM®-T vector (Promega, Madison, WI), 3 µL A-tailed PCR product and 3 U (Weiss) T4 DNA ligase in the manufacturers rapid ligation buffer. The ligation reaction was incubated at 4°C for 16 hrs.

2.2.8.4 Transformation of competent cells (heat shock method)

50 µL of thawed competent DH5α cells was added to 2 µL of ligation reaction mixture and incubated on ice for 20 min. Cell were heat shocked at 42°C for 50 sec and immediately returned to ice for 2 min. 950 µL of SOC medium was added to the transformed cells and incubated shaking at 37°C for 1.5 hrs. Cells were pelleted by centrifugation at 4,000 *g* for 10 min and resuspended in 100 µL LB. 100 µL of cells were spread onto LB/ampicillin/IPTG/X-Gal plates and incubated at 37°C for 16 hrs.

2.2.9 Isolation of recombinant plasmid DNA

DH5α colonies containing recombinant plasmid were identified using blue/white colour selection on LB/ampicillin/IPTG/X-Gal plates. Single colonies containing recombinant plasmids were used to inoculate 10 mL LB containing 50 µg/mL ampicillin and grown with shaking at 37°C for 16 hrs. Plasmid was purified from 3 mL of overnight culture using the JETquick™ plasmid miniprep spin kit (Astral Scientific, Australia).

2.2.10 Analysis of inserts in recombinant plasmids

2.2.10.1 Restriction

Recombinant plasmids were identified by restriction endonuclease analysis. 5 µL of plasmid was restricted with 10 U *Eco*RI in the manufacturer's 1 x restriction buffer for 1hr at 37°C and analysed on a 1% agarose gel in 1 x TAE.

2.2.10.2 PCR

The plasmid preparation was diluted 1/1000 in sterile H_2O. The PCR was done in a 10 µL reaction mix containing 1 µL diluted plasmid, 0.4 µM each of the SP6 and T7 primers, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase and *Taq* DNA polymerase buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Triton® X-100). PCR was done in a GeneAmp PCR System 2400 (Perkin Elmer, USA). The reaction was incubated at 94°C for 3min followed by 20 cycles of denaturation at 94°C for 1min, annealing at 48°C for 30sec, and extension at 72°C for 30sec followed by a final extension at 74°C for 2min.

2.2.11 Primer design

All primers were designed to target genomic regions of interest with the aid of primer analysis (Oligo 4.0-s ©, Rychlik, 1992) and PCR analysing (Amplify©, Engels, 1992) software for Macintosh computers.

2.2.11.1 Calculation of primer concentration

The concentration of primers were calculated by the following methods:

$$C = \frac{A_{260}}{\text{Path length(cm)} \times E_{260}(mM^{-1}cm^{-1})} \times \text{dilution factor} \quad (mM)$$

or

$C = A_{260} \times$ weight per OD X dilution factor (µg/mL)

(Weight per OD of primers is roughly 30 µg/mL)

$$C = \frac{\mu g/mL}{MW} \quad (mM)$$

2.2.11.2 MW and E_{260} calculations

MW and the E_{260} are calculated individually for each primer sequence according to the following values

Nucleotide	MW	E_{260} ($mM^{-1}cm^{-1}$)
dA	329	15.4
dG	346	1.6
dC	306	7.3
dT	321	8.7

(Annonymous, 1999b)

2.2.12 Purification of PCR products

PCR products were either purified directly from the PCR mixture or from standard agarose gels following electrophoresis using the QIAquick PCR purification or QIAquick Gel Extraction Kits according to the manufacturer's instructions (QIAGEN, Germany).

2.2.13 Preparation of radiolabelled probes

2.2.13.1 Synthesis of RNA In Vitro

cRNA was synthsised in a 20 µL reaction volume containing 0.2-1.0 mg/mL linearized DNA template, 60 µCi α-^{32}P UTP and 10-20 U SP6 RNA polymerase, 20 U RNasin®, 40 mM Tris-HCl, pH7.9, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 0.05% Tween® 20, 10 mM DTT, 0.5 mM each ATP, CTP and GTP and 12 µM UTP. The reaction mixture was incubated at 37°C for 90 minutes. DNA template was degraded by the addition of 1 U RQ1 RNase-free DNase and incubation at 37°C for 30 minutes.

2.2.13.1.1 Removal of unincorporated nucleotides

Unincorporated nucleotides were removed from the *in vitro* synthesised RNA with the addition of 200 µL TE, 73 µL of 10M ammonium acetate and 2.5 vol. ice cold 100% ethanol and incubated on ice for 15 min. cRNA was pelleted by centrifugation at 13,000 *g* for 15 min. The pellets were washed in 70% ice cold ethanol and centrifuged at 13,000 *g* for 10 min. The wash was repeated and the pellet air dried. Pellets were resuspended in 100 µL TE containing 5 mM 2-mercaptoethanol.

2.2.13.2 5' end labelling of oligonucleotides with gamma-^{32}P ATP

End labelling was done in a reaction volume of 15 µL containing 125 pmol oligonucleotide, 25 µCi γ-^{32}P ATP, 5U T4 PNK and 1 x T4 PNK buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) and incubated at 37°C for 1 hr.

2.2.13.2.1 Purification of oligonucleotides by polyacrylamide gel fractionation

To the 5'-end labelled oligonucleotide probe reaction mix an equal volume of formamide loading dye (Appendix B) was added, heated at 80°C for 90 sec, then chilled on ice. Samples were loaded onto a 400 x 200 x 0.5 mm 20% denaturing polyacrylamide gel (Appendix B) and electrophoresed in 1 x TBE at 25 mA and 1200 V for 2.5 hrs.

2.2.13.2.2 Elution of labelled oligonucleotides from polyacrylamide gels

Wet polyacrylamide gels still attached to one glass plate were covered in GLAD® Wrap polyethylene film (GLAD Products, Australia) and exposed to autoradiographic film (Eastman KODAK Company, Rochester, N.Y., USA) for 30 sec. Autoradiographic film was developed (X-ray film developer CP1000 AGFA Gevaert, Germany) and the position of full-length oligonucleotides in the gel identified, cut out with a scalpel blade and sliced into small fragments. Gel slices were incubated in 800 µL 0.1mM EDTA for 16 hrs with agitation at 37°C. The solution containing the eluted probe was recovered and dried under vacuum in a SpeedVac (Selby Scientific Instruments, Australia). Labelled probe was re-dissolved in 200 µL SDDW, precipitated with the addition of 20 µL 3 M sodium acetate and 550 µL cold ethanol/acetone (1:1) and incubated for 16 hrs at -20°C. The labelled oligonucleotide probe was pelleted by centrifugation at 13,000 *g* for 45 min and resuspended in SDDW to equal 2000 cps/µL.

2.2.13.2.3 Removal of unincorporated radionucleotides

A sterile glass bead was placed inside a sterile 6" pasteur pipette (Chase instruments, Glens Falls, NY, USA) and ¾ filled with Sephadex® G-25 medium (Pharmacia, Sweden) in TE (Appendix B). The column was washed through with TE. The labelling reaction mix was diluted to 400 µL with TE and loaded onto the column. The column was washed through with 400 µL TE and fractions each collected in a separate eppendorf tube. This was repeated until 10 fractions were collected. Radioactivity of fractions were measured using a

hand held series 900 mini monitor (Mini Instruments Ltd., UK) and fractions corresponding to the second peak pooled.

2.2.14 Determination of radioactivity of ^{32}P labelled probes

The radioactivity of labelled probes were determined in a liquid scintillation system (LS 5000TD, Beckman, USA). Probes were added to hybridisation assay at 10^6 cpm/mL hybridisation solution.

2.2.15 Deoxyribonucleic acid from herring testes

One gm DNA from herring testes type XIV (Sigma, USA) was dissolved in 100 mL 0.4 M NaOH by stirring for 16 hrs at RT. The DNA solution was boiled for 45 min, chilled on ice and neutralised with acetic acid to pH 4.7. Particulate matter was removed by centrifugation at 5,000 *g* for 10 min, and the DNA precipitated by addition of 2 vol. 100% ethanol and incubated at -20°C for 16 hrs. DNA was pelleted by centrifugation at 8,000 *g* for 30 min, the pellet was washed in 70% ethanol and dried (SpeedVac Selby Scientific Instruments, Australia). The pellet was resuspended in 50 mL TE, DNA concentration determined using a DU®-68 spectrophotometer (Beckman, USA) and the final concentration adjusted to 10 mg/mL. This single stranded sonicated herring testes DNA was stored in 10 mL aliquots at –20°C.

2.2.16 Nucleic acid blotting protocols

2.2.16.1 Southern blotting (DNA capillary transfer)

DNA was transferred from agarose gels to nylon membranes by a modified method of Southern (1975). DNA was denatured by incubating agarose gels in 0.5 M NaOH, 1 M NaCl for 30 min, shaking gently at room temperature, and then neutralised in 1.5 M Tris-HCl, pH 7.4, 3 M NaCl for 30 min. Two 3 mm thick sponges (small pore size) were placed onto each other inside a transfer container and filled to saturation with 10 x SSC buffer. Five sheets of Whatman® 3MM chromatography paper (Whatman, England) and one sheet of Zeta-Probe® blotting membrane (BIO-RAD Laboratories, USA) were cut to the dimensions of the gel. The Zeta-Probe® membrane was soaked in SDDW for 5 min. Three sheets of pre-cut Whatman® paper were placed onto the sponges, flooded with 10 x SSC buffer, and the gel placed onto it. All bubbles were removed. A plastic template was prepared from an A4 projection transparency (Audio Visual Importers, Australia) by

cutting a hole slightly smaller than the dimensions of the gel. The template was placed over the gel. The nylon membrane was placed onto the surface of the gel, followed by two layers of pre-wet Whatman® paper, 10 cm of absorbent paper towel and a 500 gm weight. Transfer proceeded for 4-16 hrs. Following transfer the membrane was rinsed in 2 x SSC and blotted on filter paper.

2.2.16.2 Northern blotting (RNA capillary transfer)

RNA from agarose gels was transfered to nylon membranes using the same method as for DNA, however the gels were not denatured and neutralised prior to transfer.

2.2.17 Cross-linking nucleic acids to nylon mebrane

Nucleic acids were UV fixed to Zeta-Probe® blotting membrane (BIO-RAD, USA) using a GS Gene Linker™ UV Chamber (BIO-RAD,USA) with an energy setting of 125 mJoules.

2.2.18 Hybridisation with radiolabelled probes

Dot blots and capillary blots on nylon membrane (Zeta-Probe) were pre-hybridised and hybridised in 5 x SSPE, 2 x Denhardt's, 2.5% (v:v) SDS, 100 µg/mL single stranded sonicated herring sperm DNA, and 50% (v:v) deionised formamide. Pre-hybridisation was done for 1 hr at 42°C. Fresh hybridisation solution was added following pre-hybridisation. Probes were denatured by heating at 95°C for 5 min, quenching on ice and added to the hybridisation solution at 10^6 cpm/mL. Hybridisation was done for 16-24 hrs at 42°C. Following hybridization, membranes were washed once in 3 x SSC, 2.5% SDS for 15 min each at 37°C as low stringency followed by one wash in 0.5 x SSC, 2.5% SDS for 15 min each at 37°C as high stringency. Filters were blotted on filter paper, wrapped in GLAD® Wrap polyethylene film (GLAD Products, Australia) and exposed to autoradiographic film (Eastman KODAK Company, USA) in an autoradiographic cassette with two intensifying screens at -80°C. Autoradiographic film was developed as above.

2.2.19 Hybridisation with DIG labelled oligonucleotide probes

2.2.19.1 Pre-hybridisation and hybridisation

Pre-hybridisation and hybridisation of nylon membranes (Zeta-Probe®, BIO RAD, USA) was done in 5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (v/v) SDS, 2% (v:v) blocking reagent, and 50% (v:v) deionised formamide. Pre-hybridisation was done for 1 hr at 42°C.

Fresh hybridisation solution was added following pre-hybridisation containing 10 pmol/mL 5' DIG labelled VT05 oligonucleotide primer (Appendix C) used as a probe. Hybridisation was done at 42°C for 16-24 hrs.

2.2.19.2 Washes

Following hybridisation, membranes were washed twice in 3 x SSC, 2.5% SDS for 15 min each at 37°C followed by two washes in 0.5 x SSC, 2.5% SDS for 15 min each.

2.2.19.3 Detection

Membranes were briefly rinsed in DIG washing buffer (Appendix B) and incubated for 30 min in DIG blocking solution (Appendix B). Membranes were incubated in 20 mL DIG blocking solution containing 75 mU/mL anti-digoxigenin-AP-conjugated Fab fragment for 30 min. The antibody solution was removed and the membrane washed twice for 15 min each in 100 mL DIG washing buffer (Appendix B). Membranes were equilibrated for 5 min in 20 mL DIG detection buffer (Appendix B) and incubated in 1 mL DIG detection buffer containing 250 µM CSPD® for 5 min in a sealed hybridisation bag. Excess solution was removed by blotting membrane nucleic acid side up on Whatman® 3MM chromatography paper (Whatman, England), the damp membrane was sealed in a hybridisation bag and incubated for 5-15 min at 37°C. Membranes were exposed to X-ray film (Eastman KODAK Company, Rochester, N.Y., USA) for 15-25 min at room temperature and the film developed as above.

2.2.20 Sequencing

PCR products and plasmids containing cloned inserts were sequenced with appropriate primers using an automated sequencer at the Flinders University of South Australia/Flinders Medical Centre DNA Sequencing Core Facility

2.2.20.1 Sequence analysis

Sequence data was analysed using the ABI automated DNA sequence viewer EditView ABI Prism™ version 1.0 (Perkin-Elmer Corporation, USA) and edited using SeqEd™ version 1.0.3 (Applied Biosytems Inc., USA). All sequencing results were confirmed by comparing forward and reverse orientation sequencing runs.

CHAPTER 3

BIOLOGICAL CHARACTERISATION OF AUSTRALIAN PSbMV ISOLATES

3.1 Introduction

PSbMV is a quarantinable pathogen within Australia. Its presence in Australia was first reported in 1978 in imported pea germplasm from Sweden (Munro, 1978). Three pathotypes of PSbMV (P-1, P-4 and L-1) have been characterised on the basis of their ability to infect specific pea genotypes (Alconero *et al.*, 1986). A pathotype is defined as an entity of a given virus that is controlled by a specific genetic factor in a given plant species (Alconero *et al.*, 1986). However a pathotype may include a range of variants differing in virulence (Provvidenti and Alconero, 1988a). PSbMV pathotypes are serologically indistinguishable (Alconero *et al.*, 1986; Ligat *et al.*, 1991). The PSbMV pathotype L-1 has also been referred to as pathotype P-2 (Kasimor *et al.*, 1997).

Resistance to PSbMV in *P. sativum* is based on four homozygous recessive genes: *sbm*1, *sbm*2, *sbm*3 and *sbm*4 (Provvidenti, 1987; Provvidenti, 1990). Resistance to PSbMV pathotypes P-1, P-4 and P-2 is pathotype specific (Alconero *et al.*, 1986). *sbm*1, *sbm*3 and *sbm*4 are located on chromosome 6 of *P. sativum* and confer resistance to PSbMV pathotypes P-1, P-2 and P-4 respectively (Gritton and Hagedorn, 1975; Timmerman *et al.*, 1993; Provvidenti and Alconero, 1988a; Provvidenti and Alconero, 1988b) . *sbm*2 is located on chromosome 2 of *P.sativum* and confers resistance to PSbMV pathotype P-2 (Provvidenti and Alconero, 1988a). In addition to the three PSbMV pathotypes, P-1, P-4 and P-2, Khetarpal *et al.* (1990) have described two new pathotypes, Pi and Pv, which are symptomless on peas carrying the homozygous *sbm*1 gene (PI 193586) but multiply at a low level and can be detected by ELISA. Both PSbMV Pi and Pv strains multiply normally on pea genotypes carrying homozygous *sbm*2, *sbm*3 and *sbm*4 on which they produced well-marked symptoms. Two additional PSbMV pathotypes, U-1 and U-2, originating from PSbMV isolates collected in Pakistan have been proposed due to variations in infectivity observed on PI 347329 and P I 347422 lines used by Alconero *et al.* (1986) for assigning pathotype groups (Ali and Randles, 1997).

A number of PSbMV isolates have been characterised using differential pea lines with a major emphasis on symptomatology (Hampton *et al.*, 1981; Khetarpal *et al.*, 1990; Ligat *et al.*, 1991; Ali and Randles, 1997). For pea breeding purposes, isolates of PSbMV should instead be classified on the basis of genes able to control them (pathotype groups). Much work has been done on testing pea, faba bean, chickpea and lentil cultivars and plant

introductions in an effort to identify PSbMV resistance (Hampton, 1980a; Hampton, 1980b; Alconero *et al.*, 1986; Provvidenti and Alconero, 1988c; Wang *et al.*, 1993; Boulton *et al.*, 1996; Kasimor *et al.*, 1997; and Lebeda *et al.*, 1999). The resistant material identified would be a valuable resource for developing breeding programs for resistance to PSbMV. However, many of these studies have only used a single isolate of PSbMV representing only one of the three pathotypes in identifying resistance (Hampton, 1980a; Hampton, 1980b; Wang *et al.*, 1993; Boulton *et al.*, 1996; and Lebeda *et al.*, 1999). As resistance to PSbMV is pathotype specific it is important to be able to classify PSbMV isolates into pathotype groups.

It was an aim of this chapter to determine whether PSbMV is widespread in southern Australia and try to characterise isolates biologically by placing them into the three pathotype groups (P-1, P-4 and P-2) using pea differential genotypes (Alconero *et al.*, 1986).

3.2 Materials and Methods

3.2.1 Collection of Australian PSbMV isolates

Australian PSbMV isolates were collected from pea breeders' germplasm, both pea and faba bean field trial material and commercially available pea cultivars. These PSbMV isolates were collected from Western Australia, South Australia and Victoria during 1995-1998 (Table 3.1).

3.2.2 Survey of Yorke Peninsula region for legume infecting viruses

In 1995, pea crops in eight paddocks in the vicinity of Kulpara, Paskeville, Kadina and Alford on the Yorke Peninsula, South Australia were surveyed for the potyviruses PSbMV, BYMV and ClYVV.

3.2.2.1 Collection of samples.

Each of the eight paddocks was surveyed twice. The first survey was done in late June (1995), two to three weeks after emergence of the pea crop and the second survey was done in early September (1995) at the time of flowering. Leaf samples from the tips of plants were collected from single plants selected every 10 m in a linear transect across the

Table 3.1: Source of studied PSbMV isolates

PSbMV Isolate	Source [c]	Tissue	Geographic region	Year isolated
US pathotype P-1	R O Hampton [a]	virus culture	USA	1992
S6 pathotype P-4	D Cartwright [b]	virus culture	SA	1992
S4	D Cartwright [b]	virus culture	SA	1992
UK4	commercial cultivar	leaf	SA	1995
2(5)	breeder's line	seed	SA	1995
3(6)	breeder's line	seed	SA	1995
19(7)	breeder's line	seed	SA	1995
23(1)	field trial	leaf	SA - Strathalbyn	1995
26(6)	field trial	leaf	SA - Strathalbyn	1995
19(1)	field trial	leaf	SA - Strathalbyn	1995
21(1)	commercial cultivar	leaf	SA - Strathalbyn	1995
43(1)	field trial	leaf	SA - Strathalbyn	1995
VIDA	breeder's line	seed	Vic - Horsham	1996
N 20-5	breeder's line	seed	SA - Turretfield	1996
FFD	Faba bean	leaf	WA - Perth	1997
M257-7-3	breeder's line	seed	SA	1997
P515-1-1	breeder's line	seed	SA	1997
P503-4-2	breeder's line	seed	SA	1997
leafless	breeder's line	seed	SA	1998

[a] Oregon State University, Corvallis

[b] Department of Agriculture, South Australia

[c] Pea , unless otherwise indicated

paddock (Barnett, 1986). Samples were placed in 10 x 15cm plastic bags and stored on ice for transportation to the laboratory. Samples were assayed by DIBA (section 2.2.7).

3.2.3 Local lesion isolates of PSbMV

PSbMV inoculum was used to mechanically inoculate leaves of *Chenopodium quinoa* or *C. amaranticolor* (section 2.2.1). Single local lesions were isolated using a sterile 4 mm disk cutter. Individual leaf disks were placed in a 1.5 ml eppendorf tube and crushed in 200 µL of 50 mM sodium borate buffer pH 8.1. Serial passage of single local lesions on *C. quinoa* or *C. amaranticolor* was done three times for each PSbMV isolate before inoculation onto the systemic host *P. sativum* cv. Dundale.

3.2.4 Maintenance of PSbMV cultures

All PSbMV isolates were maintained by mechanical passage (section 2.2.1) to *P. sativum* cv Dundale every six to eight weeks and maintained in a glasshouse (21-24°C).

3.2.5 Source of pea differentials

The seven pea differentials, their sources and susceptibility to PSbMV pathotypes P-1, P-4 and P-2 are shown in Table 3.2

3.2.6 Biological pathotyping

Two to five seedlings of each of the seven pea differential genotypes (PI 174319, PI 193586, PI 193835, PI 193836, PI 269774, PI 347329, PI 347422) were mechanically inoculated at the four leaf stage (section 2.2.1) with each of the 15 Australian PSbMV and two PSbMV type isolates (US pathotype P-1 and S6 pathotype P-4).

3.2.7 Determination of infection on pea differentials

Infection of PSbMV isolates on the seven pea differentials was determined by three different methods.

3.2.7.1 Symptomatology

Inoculated plants were observed for the development of symptoms at two and four weeks post-inoculation and the symptoms recorded.

Table 3.2: Pea differentials and their susceptibility to PSbMV pathotypes P-1, P-4 and P-2. (Alconero *et al.*, 1987 and Provvidenti and Alconero, 1988c)

Pea differential	Pathotype P-1	Pathotype P-4	Pathotype P-2
PI 174319 [A]	S (necrosis)	S (moderate)	S (stunting)
PI 193586 [A]	R *	R	R
PI 193835 [A & F]	R [#] *	R	R
PI 193836 [F]	R [#]	S	S
PI 269774 [A]	R	S	R
PI 347329 [A & F]	S	R	R
PI 347422 [A & F]	S	R	R

R = resistant, S = susceptible

[A] = Seed held within the Department of Applied and Molecular Ecology, University of Adelaide and multiplied by M. Ali (SARDI) in 1996. J. Fletcher (Crop and Food Research, Christchurch, New Zealand) originally supplied seed in 1995.

[F] = Seed re-imported from J. Fletcher in 1997

[#] = Reported or found to contain some susceptible plants. Due to the heterozygosity of many PI seed lots, lines reported to be fully resistant may also include some susceptible plants (Provvidenti and Alconero, 1988c)

* = Seedline contained some susceptible plants (Hagedorn and Gritton, 1973)

3.2.7.2 DIBA

Individual plants were tested twice by DIBA (section 2.2.7) at 2-3 weeks post-inoculation and again one week later.

3.2.7.3 Nucleic acid hybridisation

Nucleic acid was extracted from individual plants at three weeks post-inoculation using the CTAB (section 2.2.4.1) and K/SDS (section 2.2.4.3) methods and spotted onto Zeta probe ™ nylon membrane (Bio-Rad) and probed with 5′ labelled VT02 oligonucleotide probe (2.2.13.2). The hybridisation and washing conditions were as described (section 2.2.18).

3.3 Results

3.3.1 Sources of Australian PSbMV isolates

Table 3.1 shows the sources of all Australian PSbMV isolates collected between 1995-1998. Fourteen PSbMV isolates were collected within South Australia: 2 from leaf samples of commercially available pea cultivars; 8 from pea breeders' seed; and 4 from pea field trial leaf samples. One PSbMV isolate came from Victoria from pea breeder's seed and one PSbMV isolate came from Western Australian from a faba bean leaf sample.

3.3.2 Pea crops on Yorke Peninsula Survey

The survey of the eight pea crops in the vicinity of Kulpara, Paskeville, Kadina and Alford (Figure 3.1) showed that three cultivars of *P. sativum* were grown (cvs Dundale, Early Dun and Alma). Farmers grew and maintained their own seed. Pea crops were grown in a three or five year rotation cycle with cereal crops such as wheat and barley. No colonisation of crops by aphids was noted in the 1995 growing season.

3.3.2.1 Incidences of viruses

Table 3.3 shows the results of the first and second field surveys. PSbMV infection was not detected by DIBA in the eight properties surveyed on the Yorke Peninsula in the 1995 growing season. BYMV was also not detected, however, ClYVV infection was detected in two pea crops in the first survey at a level of 0.3%.

Figure 3.1: Map of the Yorke Peninsula, South Australia, Australia.

Boxed area indicates the region where the 1995 pea surveys were done

Table 3.3: Survey of pea crops on Yorke Peninsula for PSbMV, ClYVV and BYMV

Yorke Peninsula properties	Pea cultivar grown	First Survey (June 1995)				Second Survey (September 1995)			
		no. samples	DIBA PSbMV	DIBA ClYVV	DIBA BYMV	no. samples	DIBA PSbMV	DIBA ClYVV	DIBA BYMV
Property A: David & Tony Sluggett	Early Dun	16	0	0	0	57	0	0	0
Property B: David & Tony Sluggett	Early Dun	11	0	0	0	51	0	0	0
Property C: Barry Rodda	Early Dun	7	0	0	0	51	0	0	0
Property D: Paul Rowan	Dundale	11	0	1	0	56	0	0	0
Property E: Jeff Millard	Early Dun	18	0	1	0	50	0	0	0
Property F: Jeff Ayles	Early Dun	21	0	0	0	45	0	0	0
Property G: Jeff Ayles	Early Dun	67	0	0	0	54	0	0	0
Property H: Trevor Pridham	Alma	48	0	0	0	39	0	0	0
TOTAL		199	0	2	0	403	0	0	0

3.3.3 Differential reactions of PSbMV isolates on C.quinoa

C. quinoa and *C. amaranticolor* inoculated with all PSbMV isolates developed chlorotic or necrotic local lesions on inoculated leaves (Figure 3.2a). However, two isolates, S6 and S4, also showed a systemic reaction on *C. quinoa* producing vein chlorosis and leaf distortion and chlorotic local lesions on uninoculated leaves (Figure 3.2b).

3.3.4 Variation in symptoms induced on pea differential genotypes by the Australian PSbMV isolates

Table 3.4 shows the range of symptoms induced by 15 Australian PSbMV isolates and 2 PSbMV type isolates (US pathotype P-1 and S6 pathotype P-4) on 7 pea differential genotype lines. Pea differentials PI 174319 and PI 193836 were susceptible to all PSbMV isolates tested but varied in the symptoms induced by different PSbMV isolates. The greatest range of symptom variation was observed on PI 174319, where symptoms induced by individual PSbMV isolates ranged from a mild leaf roll to whole plant necrosis.

Two of the PSbMV isolates, 2(5) and 43(1), showed symptom variability within the PI 174319 line with some plants showing whole plant necrosis and others leaf rolling.

All other PSbMV isolates expressed identical symptoms within the group of test plants for each pea differential. PI 193586 and PI 193835 were immune to all PSbMV isolates tested. All plants within the PI 269774 tested group appeared either completely symptomless or symptomatic to the tested PSbMV isolates, except PSbMV isolates 2(5), M257-7-3, 19(1) and P503-4-2, which exhibited both symptomless and symptomatic plants within the test group. PI 347329 and PI 347422 were the most variable in their response to the PSbMV isolates within test groups, showing both symptomless and symptomatic plants.

Figure 3.3 shows symptom variability among PSbMV isolates on *P. sativum* cv Dundale. Symptoms observed varied from vein clearing and mild mosaic to leaf curling, distortion and severe mosaic.

3.3.5 Comparison of symptomatology with DIBA for detecting infection

DIBA detected more PSbMV infected plants than symptomatology alone (Table 3.5). 89% of DIBA positive plants were symptomatic. All symptomatic plants were DIBA positive.

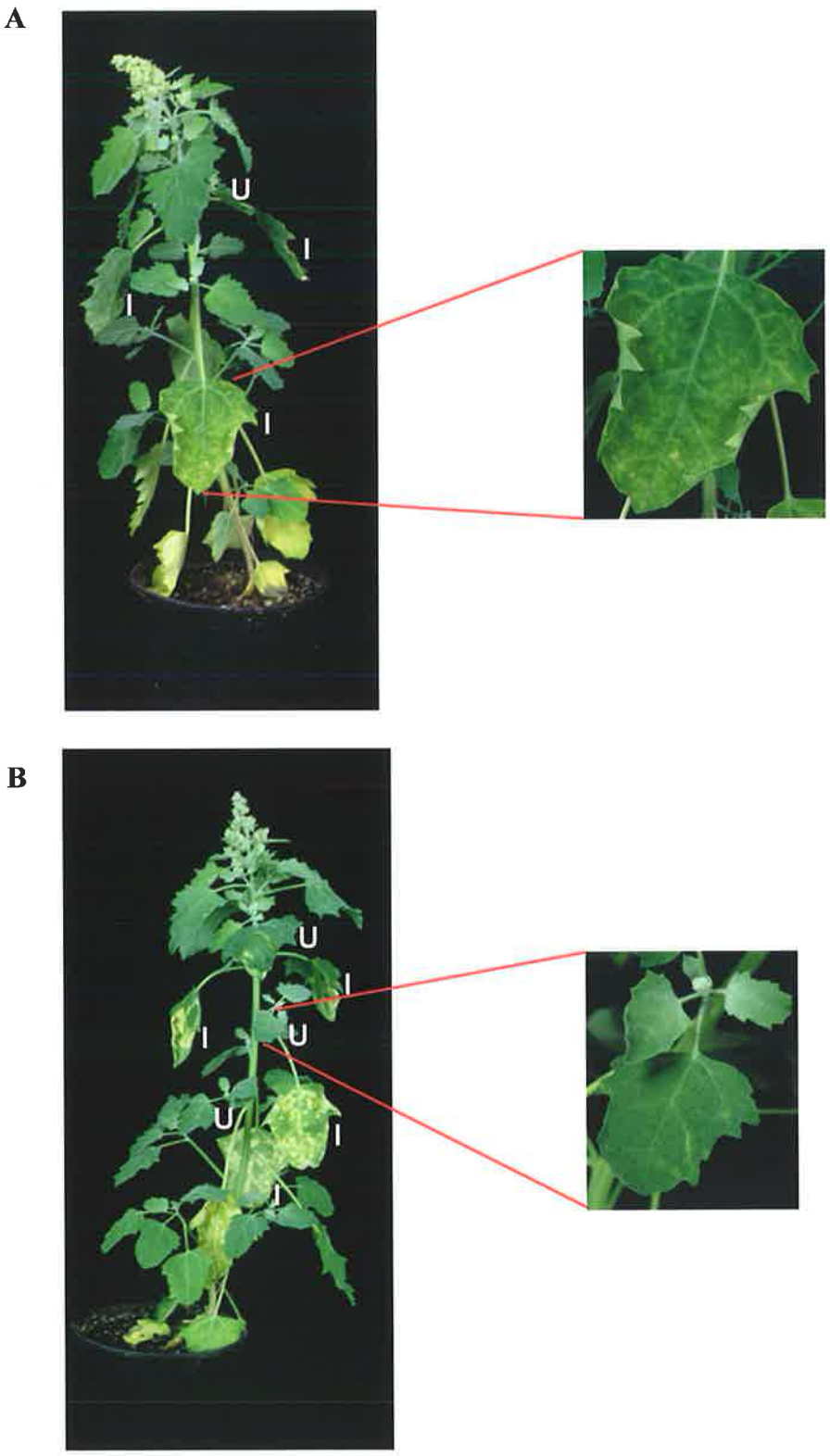


Figure 3.2: Symptoms induced by PSbMV on *C. quinoa.*

(A) *C. quinoa* inoculated with PSbMV US. (B) *C. quinoa* inoculated with PSbMV S6.

(I) Inoculated leaf; (U) Uninoculated leaf.

Table 3.4: Symptoms observed on various pea differentials inoculated with various PSbMV isolates.

Pea differential	PSbMV isolates																
	US	S6	2(5)	$N_{20\text{-}5}$	M257-7-3	3(6)	43(1)	VIDA	23(1)	19(1)	21(1)	P503-4-2	19(7)	UK4	FFD	P515-1-1	S4
PI 174319	LR M (4/4)	LR M s (5/5)	LR s (2/5) N (3/5)	LR M (5/5)	N (5/5)	LR s (2/2)	lr (1/2) N (1/2)	lr VC (5/5)	lr VC (5/5)	lr (5/5)	N (2/2)	lr m (5/5)	N (5/5)	N (4/4)	N (2/2)	LR VC (5/5)	LR (5/5)
PI 193586	- (2/2)	- (3/3)	- (3/3)	- (3/3)	- (3/3)	NA	NA	- (3/3)	- (3/3)	- (3/3)	NA	- (3/3)	- (3/3)	- (2/2)	NA	- (3/3)	- (3/3)
PI 193835	- (4/4)	- (5/5)	- (5/5)	- (5/5)	- (5/5)	- (2/2)	- (2/2)	- (5/5)	- (5/5)	- (5/5)	- (2/2)	- (5/5)	- (5/5)	- (4/4)	- (2/2)	- (5/5)	- (5/5)
PI 193836	lr VC (2/2)	lr M (2/2)	lr (2/2)	m (2/2)	lr (2/2)	LR s (2/2)	LR (2/2)	lr VC (2/2)	lr VC m (2/2)	- (1/2) lr (1/2)	lr VC (2/2)	lr m (2/2)	lr (1/1)	lr (2/2)	LR VC (2/2)	lr m (2/2)	lr VC (2/2)
PI 269774	- (4/4)	M (5/5)	- (3/5) VC (2/5)	lr m (5/5)	- (3/5) lr m (2/5)	M (2/2)	- (2/2)	M (5/5)	- (5/5)	- (2/5) m VC (3/5)	- (2/2)	- (1/5) M (4/5)	- (4/4)	M (4/4)	- (2/2)	M (5/5)	M (4/4)
PI 347329	- (2/4) lr M (2/4)	- (5/5)	- (4/5) lr (1/5)	- (3/5) M (2/5)	- (4/5) lr (1/5)	- (1/2) lr m (1/2)	- (1/2) LR (1/2)	- (5/5)	- (3/5) lr VC (1/5)	- (4/5) VC (1/5)	- (1/2) lr VC (1/2)	- (4/5) lr VC (1/5)	- (4/5) lr (1/5)	- (3/3)	- (2/2)	- (5/5)	- (4/4)
PI 347422	- (4/4)	- (2/5) m (3/5)	- (3/5) lr (2/5)	- (4/5) m (1/5)	- (1/5) lr m (4/5)	- (2/2)	- (2/2)	- (3/5) m (2/5)	- (5/5)	- (5/5)	m (2/2)	- (5/5)	- (2/5) lr (3/5)	- (2/4) lr (2/4)	- (2/2)	- (5/5)	- (4/5) lr (1/5)

LR (severe leafroll); lr (mild leafroll); M (severe mosaic); m (mild mosaic); VC (vein clearing); N (whole plant necrosis); - (symptomless); s (stunting)

The numbers in parenthesis indicated the proportion of plants within the test group displaying the observed symptoms.

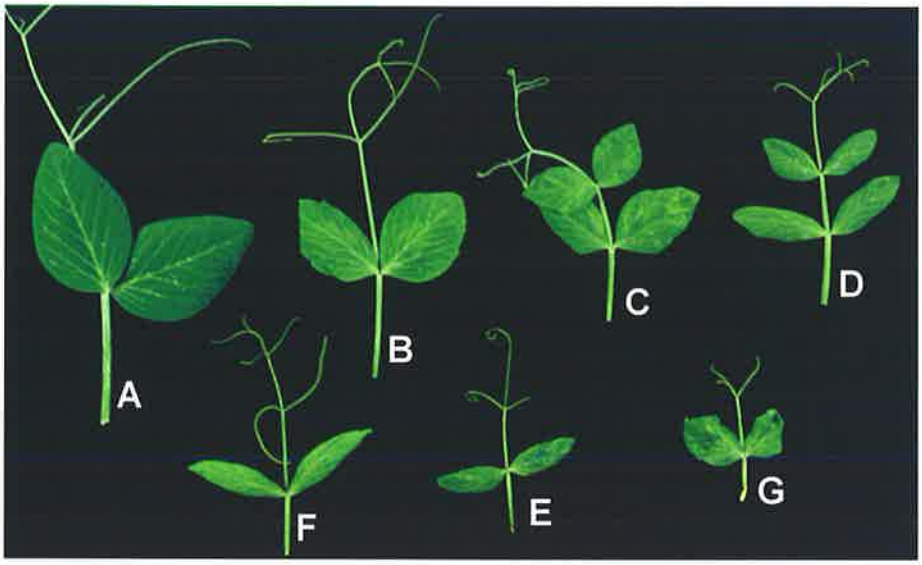


Figure 3.3: Variation in symptoms induced by a range of PSbMV isolates on *P.sativum* cv Dundale.

(A) Healthy pea; (B) PSbMV 23(1); (C) PSbMV FFD; (D) PSbMV 19(1); (E) PSbMV US; (F) PSbMV 21(1); (G) PSbMV S6

Table 3.5: Comparison of detecting PSbMV infection by symptomatology and DIBA

Pea Differentials	PSbMV isolates																
	US	S6	2(5)	N $_{20\text{-}5}$	M257-7-3	3(6)	43(1)	VIDA	23(1)	19(1)	21(1)	P503-4-2	19(7)	UK4	FFD	P515-1-1	S4
PI 174319	XXXX XXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXXX XXXX	XX	XXXXX XXXXX	XXXXX XXXXX
PI 193586	XX XX	XXX XXX	XXX XXX	XXX XXX	XXX XXX	N/A	N/A	XXX XXX	XXX XXX	XXX XXX	N/A	XXX XXX	XXX XXX	XX XX	N/A	XXX XXX	XXX XXX
PI 193835	XXXX XXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXXX XXXX	XX XX	XXXXX XXXXX	XXXXX XXXXX
PI 193836	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	X XX	XX XX	XX XX	X X	XX XX	XX XX	XX XX	XX XX
PI 269774	XXXX XXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XXXXX XXXXX	XXXX XXXX	XXXX XXXX	XX XX	XXXXX XXXXX	XXXX XXXX
PI 347329	XXXX XXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXX XXX	XX XX	XXXXX XXXXX	XXXX XXXX
PI 347422	XXXX XXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX	XX X	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XXXXX XXXXX	XXXXX XXXXX	XXXXX XXXXX	XX XX	XXXXX XXXXX	XXXXX XXXXX
Pathotype like group	P-1	P-4	?	P-4	P-4	P-4	?	P-4	P-4	P-4	P-1	P-4	P-1	P-4	P-1	P-4	P-4

X = each X represents a separate pea plant inoculated with PSbMV

= a symptomatic plant (refer to Table 3.4)

X = plant PSbMV positive by DIBA (Figure 3.4A shows examples of DIBA results obtained for some samples used in the tabulation of this table.)

N/A = not applicable as reactions of listed PSbMV isolates were not tested on indicated pea differentials.

PSbMV M257-7-3, 43(1) and FFD caused rapid whole plant necrosis on PI 174319 (Table 3.4), therefore DIBA could not be used to accurately assay these plants.

3.3.6 Comparison of DIBA with nucleic acid hybridisation for detecting infection

Figure 3.4 shows a direct comparison of PSbMV inoculated pea differentials assayed by DIBA and nucleic acid hybridisation. Table 3.6 is an interpretation of data presented in Figure 3.4. The same results were generally obtained using each of the three assay methods. A PSbMV positive was scored when two out of three assays gave a positive reaction. The level of sensitivity of DIBA and nucleic acid hybridisation with radiolabelled VT02 was similar. DIBA signal strengths varied from strong to weak (Figure 3.4). Plants showing a strong PSbMV positive signal by DIBA also showed a strong nucleic acid hybridisation signal and weak positives by DIBA were also weak by hybridisation.

PI 174319 plants inoculated with PSbMV isolates 2(5), M257-7-3, 43(1), 21(1), 19(7), UK4 and FFD gave weak positive or no signal by DIBA and hybridisation. These PSbMV isolates were observed to cause whole plant necrosis on PI 174319 (Table 3.4), therefore DIBA and nucleic acid hybridisation could not be used to accurately assay these plants. Weak hybridisation signals were observed to PSbMV isolates 2(5), 23(1), 19(1) and P503-4-2, although strong DIBA positives were observed to the same PSbMV isolates.

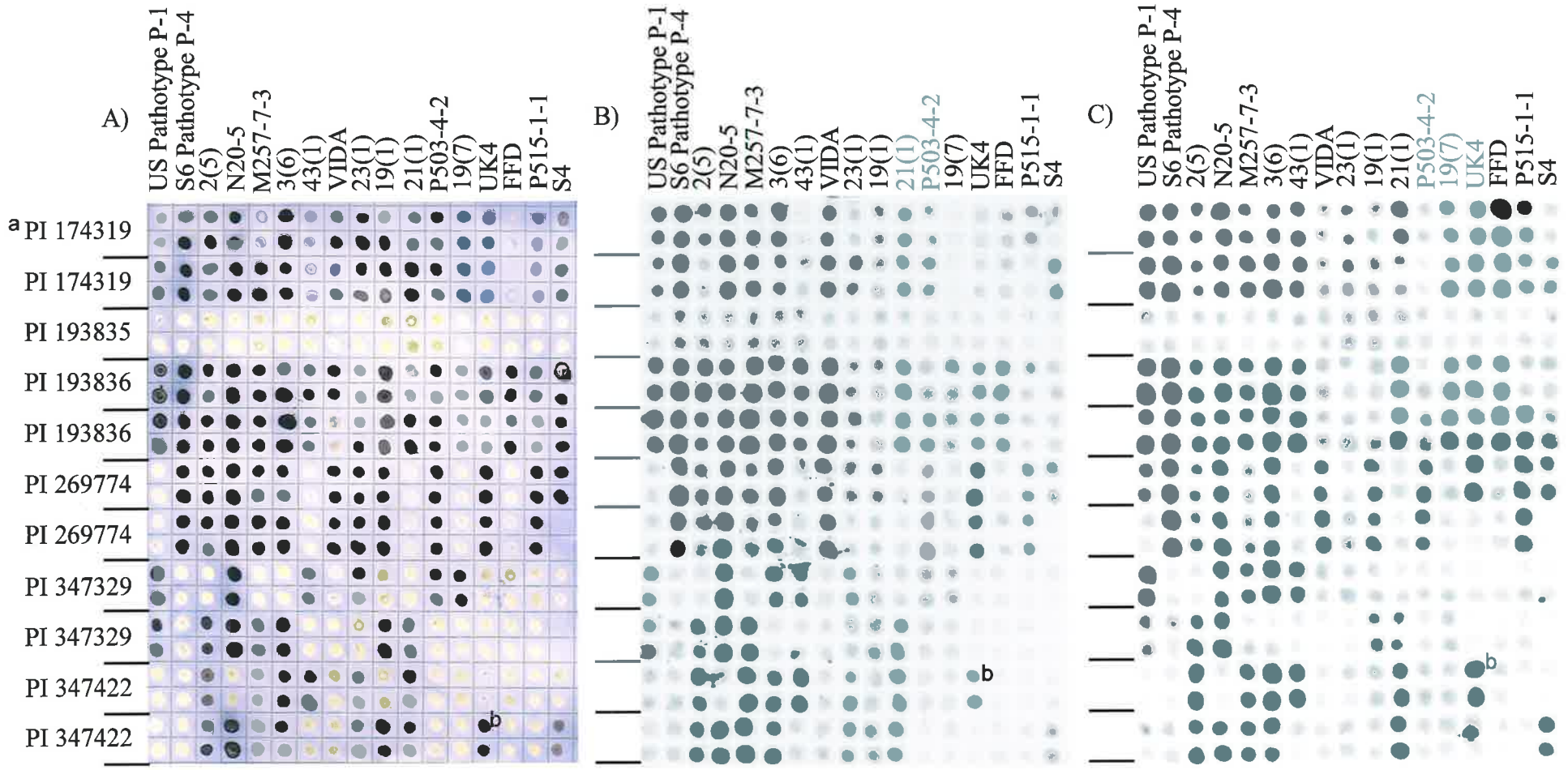
3.3.7 Placing Australian PSbMV isolates into potential biological pathotype groups

The results of challenging the seven pea differentials with 15 Australian and two PSbMV type isolates (Table 3.5) were used to place the PSbMV isolates into pathotype groups as described (Table 3.2). PI 174319, PI 193586, PI 193835 and PI 269774 were the only pea differentials out of the seven tested which reacted in the way summarised (Table 3.2) to type isolates PSbMV US (pathotype P-1) and PSbMV S-6 (pathotype P-4). Therefore, only these pea differentials were considered when placing PSbMV isolates into pathotype like groups (Table 3.5).

The PI 193836, PI 347329 and PI 347422 did not behave as predicted (Table 3.2) to the PSbMV type isolates (US pathotype P-1 and S6 pathotype P-4) and therefore were not used in establishing PSbMV pathotype grouping of tested PSbMV isolates.

Figure 3.4: Testing infectivity of PSbMV isolates on pea differentials.

All samples were tested in duplicate. (A) DIBA on pea extracts. (B) Nucleic acid hybridisation with radiolabelled VT02 oligonucleotide primer on total nucleic acid extracted by the CTAB method. (C) Nucleic acid hybridisation with radiolabelled VT02 oligonucleotide on total nucleic acid extracted by the K/SDS method. (a) Represents each pea differential plant tested. (b) Represents the same plant with a different loading order between the DIBA and hybridisation membranes. All other samples were loaded in the same order on all three membranes.



DIBA on pea sap extracts

Hybridisation with VT02 oligonucleotide probe of TNA prepared by CTAB method

Hybridisation with VT02 oligonucleotide probe of TNA prepared by K/SDS method

Table 3.6: Direct comparison of DIBA and hybridisation methods for determining PSbMV infection

Pea differentials	PSbMV isolates																
	US	S6	2(5)	N^{20-5}	M257-7-3	3(6)	43(1)	VIDA	23(1)	19(1)	21(1)	P503-4-2	19(7)	UK4	FFD	P515-1-1	S4
PI 174319	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX	XX XX XX
PI 193835	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X	X X X
PI 193836	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX
PI 269774	XX XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	X X
PI 347329	XX XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX
PI 347422	XX XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX	XX XX

X = individual pea plants inoculated with PSbMV

X = PSbMV positive plants by DIBA

X = PSbMV positive plants by nucleic acid hybridisation method (CTAB extraction)

X = PSbMV positive plants by nucleic acid hybridisation method (K/SDS extraction)

The results of DIBA and nucleic acid hybridisation are shown in Figure 3.4.

PI 193836 was completely susceptible to all PSbMV isolates including the pathotype P-1 and P-4 type isolates. PI 347329 was completely resistant to PSbMV S6, VIDA, UK4, FFD, S4 and P515-1-1 (Table 3.5). No complete susceptibility was observed in this pea line to any other tested PSbMV isolates.

PI 347422 was completely resistant to PSbMV US, P503-4-2, FFD and P515-1-1. Complete susceptibility in this pea differential was only observed to M257-7-3 and 21(1).

PSbMV 2(5) and 43(1) were not assigned potential pathotype groups as they both showed symptom variability within the PI 174319 test group and the PI 269774 test group showed a mixture of symptomless and symptomatic plants not observed for any of the other 15 PSbMV isolates.

3.4 Discussion

The limited field survey of eight pea growing properties on the Yorke Peninsula failed to detect PSbMV or BYMV in field samples in the 1995 growing season, although ClYVV was detectable in two crops at an overall level of 0.3%. However, between 1995-1998 fifteen PSbMV isolates were collected from field samples of pea and faba bean, pea breeders' germplasm and commercially available pea cultivars from South Australia, Victoria and Western Australia. This is the first report of the widespread distribution of PSbMV in South Australian pea crops and confirms that it is present in other states of Australia.

The 15 Australian PSbMV isolates are biologically distinct as observed by the range of symptoms induced on several pea differentials. PSbMV 2(5) and 43(1) showed varied reactions on PI 174319 suggesting that they may consist of a mixture of isolates representing several pathotypes. Most PSbMV isolates were observed to produce chlorotic or necrotic local lesions on *C. quinoa* and *C. amaranticolor* inoculated leaves only. PSbMV isolates S6 and S4 were also observed to produce vein yellowing, leaf distortion and chlorotic local lesions on uninoculated leaves. Systemic infection of certain PSbMV isolates, including PSbMV S6, on selected *C. quinoa* genotypes has been previously noted (Johansen *et al.*, 1996b; Andersen and Johansen, 1998).

Serologically the Australian PSbMV isolates were indistinguishable when using the PSbMV US polyclonal antiserum. Although the VT02 oligonucleotide probe did detect all Australian PSbMV isolates it did show variability in strength of hybridisation signal between PSbMV isolates. Therefore, DIBA is a reliable and rapid method of PSbMV detection in leaf tissue.

In this study biological pathotyping of PSbMV isolates using select pea differentials was not reliable because the pattern of susceptibility did not match the published reactions for PI 193836, PI 347329 and PI 347422 to the PSbMV pathotype P-1 and P-4 type isolates used (Table 3.2). Therefore the reactions on PI 174319, PI 193586, PI 193835 and PI 269774 were only considered in assigning PSbMV isolates to tentative pathotype P-1 and P-4 like groups. Using these pea differentials alone it is not possible to distinguish PSbMV pathotype P-1 from P-2 (Table 3.2). Moreover, a PSbMV pathotype P-2 type isolate was not available for this study and the possibility exists that isolates grouped in pathotype P-1 may represent P-2 pathotypes.

PI 193836 was susceptible to all 17 PSbMV isolates tested (Table 3.5). Provvidenti and Alconero (1988c) reported that PI 193836 was resistant to pathotype P-1, but susceptible to pathotypes P-4 and P-2 (Table 3.2). However, they also reported that PI 193836 contained some susceptible plants. This could explain why PI 193836 was not resistant to our PSbMV US pathotype P-1. If inadvertently some heterozygous PI 193836 susceptible plants were used for the multiplication of the seed lot then there would be a selection pressure towards the reduction of resistant genotypes/phenotypes. Dr Elizabeth Johansen (Danish Institute of Agricultural Sciences, Frederiksberg, Denmark) was also supplied with PI 193836 by John Fletcher and failed to find resistance in this line (personal communication).

PI 347329 and PI 347422 are reported to be completely resistant to pathotypes P-2 and P-4, and susceptible to pathotype P-1 (Alconero *et al.*, 1986; Provvidenti and Alconero, 1988c). In this study PI 347329 and PI 347422 only showed complete resistance to PSbMV FFD and P515-1-1, while no complete susceptibility was observed to any of the PSbMV isolates on both pea differential genotypes.

The pathotyping experiments were repeated twice. The unexpected reaction of the PSbMV isolates on PI 347432 and PI 347422 multiplied by Mushraf Ali from seed held within the Department of Applied and Molecular Ecology warranted the reimportation of these pea differentials from John Fletcher in 1997. However, the PI 347432 and PI 347422 germplasm also appeared not to be true to type. Dr E Johansen (personal communication) also observed deviations from the published reactions (Alconero *et al.*, 1986) when using the PI 347432 and PI 347422 pea differentials (supplied by J. Fletcher) to pathotype PSbMV isolates.

Provvidenti and Alconero (1988c) reported that of 40 plant introductions of *Pisum sativum* found to be resistant to PSbMV, 29 lines were resistant to all three pathotypes (P-1, P-2 and P-4). However, they also reported that in 14 lines for which resistance was shown to one or all of the pathotypes, some susceptible plants had also been found. This is believed to be due to heterogeneity in many PI seed lots. Occasional susceptible plants have been reported in the fully PSbMV resistant lines P193835 and PI 193586 (Hagedorn and Gritton, 1973). Timmerman *et al.* (1993) reported a difference in susceptibility of PI 193835 to PSbMV pathotype P-1 in seed obtained from USDA-PGRU, Pullman Washington and USDA-PGRU, maintained in the DSIR Crop Research collection, Christchurch, New Zealand. They also reported that PI 347464 was resistant to PSbMV pathotype P-1, whereas Provvidenti and Alconero (1988c) had reported this line to be susceptible to PSbMV pathotype P-1. Ali and Randles (1997) have tentatively reported two new pathotypes, U-1 and U-2, based on the deviation from expected reactions of pathotypes P-1, P-2 and P-4 on PI 347329 and PI 347422 (Alconero *et al.*, 1986). However, the study had a limited sample size of three seedlings per pea differential.

Discrepancies in reported responses of pea introduction to infections by PSbMV may be due to inadvertent changes in the germplasm accessions. It has previously been observed that genetic changes in pea accessions have occurred as a result of possible inadvertent mixtures and the elimination of PSbMV infected individuals from the gene pool (Alconero *et al.*, 1985). Alconero *et al.* (1986) have stated that accessions used to distinguish PSbMV pathotypes should be as genetically stable as possible to avoid problems in identification. It has been shown through crossing susceptible and resistance cultivars and backcrossing F1 plant to parents that resistance to the three PSbMV pathotypes is monogenically recessive (Hagedorn and Gritton, 1973; Gritton an Hagedorn 1975; Provvident and Alconero, 1988a;

Provvidenti and Alconero, 1988b). Therefore, it is important that pea differentials being used for pathotyping be tested to ensure they remain true to type and are not inadvertently changed by mixing, incorrect labelling or multiplying from small seed lots which may contain heterozygous susceptible plants creating a selection pressure for susceptible phenotypes.

Placing PSbMV isolates into pathotype groups is important for establishing the prevalence of particular pathotypes within a geographic region and, therefore in developing resistant pea cultivars suitable for cultivation in those geographic region.
However, biological pathotyping is time consuming and reliant on pea differential that are true to genotype. Therefore, testing of pea differential following each round of multiplication with known PSbMV pathotypes would be necessary to ensure the line remains true to type over time. It is important to use a large number of test plants, 10-15 seed per genotype, for testing PSbMV infection so as to detect variation/consistency in responses. Our study was unable to use a larger number of test plants due to time constrictions involved in reimportation of seedlots, multiplication of seed and testing. Hence it would be more useful to try to group PSbMV isolates by molecular techniques.

CHAPTER 4

DEVELOPING NUCLEIC ACID BASED DIAGNOSTICS FOR PSbMV

4.1 Introduction

Several serological and nucleic acid based methods have been described for plant pathogen detection (Putnam, 1995; Randles *et al.*, 1996) with a range of PCR based (Henson and French, 1993; Hadidi *et al.*, 1995; Louws *et al.*, 1999) and nucleic acid hybridisation (Hull, 1993; Randles *et al.*, 1996) assays being developed. Nucleic acid based assays have the advantage of being more specific and sensitive than the corresponding serological assay for plant virus detection (Vunsh *et al.*, 1991; Borja and Ponz, 1992; Nolasco *et al.*, 1993;Rosner, 1994; Palkovics *et al.*, 1994; Mathews *et al.*, 1997).

Although several serological based assays, such as DIBA (Ligat *et al.*, 1991) and ELISA (Stevenson and Hagedorn, 1973; Hamilton and Nichols, 1978; Maury and Khetarpal, 1997; Masmoudi *et al.*, 1994a) have been described. Few nucleic acid based assays have been described for PSbMV (Kohnen *et al.*, 1992; Kohnen *et al.*, 1995; Phan *et al.*, 1997; Ali *et al.*, 1998) but these have limitations for use as a diagnostic test for PSbMV. For example the RT-PCR (Kohnen *et al.*, 1992; Kohnen *et al.*, 1995) and IC-PCR (Phan *et al.*, 1997) were shown to be PSbMV isolate specific and the hybridisation assay (Ali *et al.*, 1998) did not test the specificity of probes to a broad range of PSbMV isolates.

This chapter describes the development of both nucleic hybridisation and RT-PCR assays for PSbMV detection and compares their sensitivities with each other and the serological PSbMV DIBA.

4.2 Methods

4.2.1 Potyvirus genome sequence comparisons

Twenty-five complete potyvirus genome sequences (Table 4.1), representing 14 species from the genus potyvirus and 1 species from the genus tritimovirus, were extracted from the GenBank database and aligned with the multiple sequence alignment program EClustALW (Thompson *et al.*, 1994) through the WebANGIS interface (http://www.angis.org.au).

Table 4.1: List of *Potyviridae* sequences used in the multiple sequence alignment

Potyviridae	**GenBank accession number**	**Reference**
Bean common mosaic virus	U19287	direct submission
Bean yellow mosaic virus	D83749	direct submission
Brome streak mosaic tritimovirus	Z48506	Gotz and Maiss, 1995
Johnsongrass mosaic virus	Z26920	Gough and Shukla, 1993
Papaya ringspot virus	X67673	direct submission
Pea seedborne mosaic virus	X89997[a] D10930/D00152[b]	[a]direct submission [b]Johansen *et al.*, 1991
Peanut stripe virus	U05771[a] U34974[b]	[a]Ganasinghe *et al.*, 1994 [b]direct submission
Pepper mottle virus	M96425	Vance *et al.*, 1992
Plum pox potyvirus	X81083[a], D13751/D00424[b] X16415[c] M92280[d]	[a]direct submission [b]Maiss *et al.*, 1989 [c]Teycheney *et al.*, 1989 [d]Palkovics *et al.*, 1993
Potato virus A	Z21670	Puurand *et al.*, 1994
Potato virus Y	A08776[a] X12456/D00441[b] M95491[c]	[a]direct submissiom [b]Robaglia *et al.*, 1989 [c]Thole *et al.*, 1993
Tobacco etch virus	M15239[a] L38714[b] M11458[c]	[a]Allison *et al.*, 1986 [b]direct submission [c]Allison *et al.*, 1985
Tobacco vein mottling virus	U38621[a] X04083[b]	[a]direct submission [b]Domier *et al.*, 1986
Turnip mosaic virus	D10927	Nicolas and Laliberte, 1992
Zucchini yellow mosaic virus	L29569	Wisler *et al.*, 1995

4.2.2 *Selection of PSbMV genome regions for PCR primer and hybridisation probe design*

Regions conserved between the PSbMV sequences, DPD1 pathotype P-1 (D10930) and NY pathotype P-4 (X89997), yet distinct from other potyvirus sequences were chosen for primer and probe design. Primers and probes were tested using Oligo® 4.01 primer analysis software (Rychlik, 1992) and Amplify 1.0 for analysing PCR experiments (Engels, 1992).

4.2.3 *Extraction of PSbMV RNA from purified virus*

PSbMV-US RNA was extracted from purified PSbMV (section 2.2.3) stored at –70°C in 50% glycerol. The glycerol stock of PSbMV was diluted to contain less than 10% (v/v) glycerol. Purified PSbMV at 1mg/mL was incubated with 1mg/mL Proteinase K (Appendix A) in Proteinase K buffer (Appendix B) for 3 hrs at 37°C. The aqueous phase was extracted once with an equal volume of 500 mM Tris saturated phenol, pH 6-7 (Appendix B) or biotechnology grade phenol saturated solution, pH 6.6-7.9 (Amresco, USA) and once with an equal volume of DEPC SDDW saturated chloroform. RNA was precipitated with 1/10 (v/v) 3 M CH_3COONa, pH 5.2 and 3vol. biotechnology grade ethanol (Amresco, USA) and incubated at -70°C for 16 hrs. RNA was pelleted by centrifugation at 13000 *g* for 45 min at 4°C. The pellet was washed with 70% ethanol prepared in DEPC SDDW and centrifuged at 13000 *g* for 30 min. The pellet was dried in a SpeedVac (Selby Scientific Instruments, Australia) and then resuspended in 30 µL DEPC SDDW (Appendix B). The concentration of RNA was determined by spectrophotometry (DU®–68 spectrophotometer Beckman, USA) and RNA integrity was determined by formaldehyde gel electrophoresis (section 2.2.6).

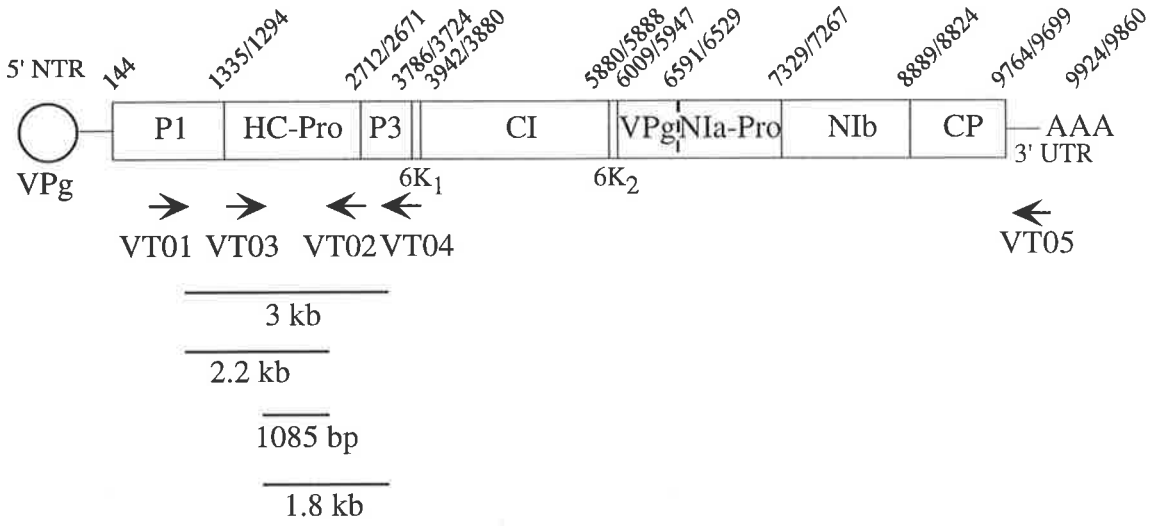
4.2.4 *Total nucleic acid extraction*

Total nucleic acid was extracted by the CTAB method (section 2.2.4.1).

4.2.5 *RT-PCR*

4.2.5.1 *Primers*

The VT04 primer (Figure 4.1) was used to prime the RT from PSbMV-US RNA and VT01/VT02, VT01/VT04, VT03/VT02 or VT03/VT04 primer pairs (Figure 4.1) were used for PCR.



VT01 (5'-ATG ACC ATG GCG CGT CCT GTG AAT C-3') position 267-291

VT02 (5'-GCA GTT GCT ACA TCC ATC ATT GTT GGC CAT-3') position 2500-2471

VT03 (5'-GTG TTG GAG GAA TCA CAC CAG AAG AAT GTG-3') position 1417-1446

VT04 (5'-CTC CAA AAC CAT GCT TCA CTC TTG A-3') position 3297-3273

VT05 (5'-GCC CTA CTG CCA CCA AAC CGA AGT CAA AGA CTC-3') position 9910-9878

Figure 4.1: Genome organisation of PSbMV and positions and sequences of RT-PCR primers and hybridisation probes.

The primer and probe positions refer to the PSbMV DPD1 sequence. The positions of gene junctions are indicated above the diagram for published PSbMV DPD1 and NY sequences respectively.

4.2.5.2 RT

PSbMV cDNA was produced by reverse transcription of 150 µg PSbMV-US RNA with 0.6 µM VT04 primer labelled at the 5′ end with γP^{32} (section 2.2.13.3), using either Retrotherm™ RT (Epicentre Technologies,USA), MMLV RT (Epicentre Technologies,USA), AMV RT (GeneWorks,Australia) or Tth polymerase (Epicentre Technologies, USA) following the manufacturer's recommendations. Radiolabelled cDNA was electrophoresed on 1% agarose gels in TAE then dried between GelAir cellophane support (Bio-Rad, USA) on a slab drier model 443 (Bio-Rad, USA). The dried agarose gel was exposed to X-ray film and developed.

4.2.5.3 PCR

Different DNA polymerases were tested on PSbMV cDNA generated from PSbMV-US RNA by RT with VT04. PCR was done with 0.5 µM of each of the VT02 and VT03 primers and either *Taq* DNA polymerase (Promega, USA), Deep Vent® (exo^-) DNA polymerase (New England BioLabs, USA), Deep Vent® (exo^+) DNA polymerase (New England BioLabs, USA), eLONGase™ enzyme mix (GIBCO BRL, Life Technologies, USA), Retrotherm™ RT (Epicentre Technologies, USA), Tth thermostable DNA polymerase (Epicentre Technologies, USA), Tth DNA polymerase (Boehringer Mannheim, Germany), or AmpliTaq DNA polymerase (Perkin Elmer, USA) according to the manufacturer's recommendations.

4.2.5.4 PCR cycling conditions

RT-PCR was done in a GeneAmp PCR System 2400 (Perkin Elmer, USA).

For the PSbMV PCR, mixtures were initially incubated at 94°C for 3min followed by 30 cycles consisting of: denaturation at 94°C for l min; annealing at 60-70°C for l min; and extension at 72°C for 1 min, with a final extension step of 72°C for 2 min. RT-PCR products were analysed by electrophoresis in a 1.5% agarose gel containing 0.5µg/ml EtBr and buffered in 1 x TAE.

4.2.6 Nucleic acid hybridization

4.2.6.1 Dot blotting

Total nucleic acid preparations were spotted onto Zeta Probe ™ nylon membrane (BIO-RAD, USA) and UV cross-linked (section 2.2.17).

4.2.6.2 Probe preparation

The VT02 (30 mer), VT03 (30 mer) and VT05 (33 mer) primers were radiolabelled for use as probes. Each primer was 5′ end-labelled with γP^{32} ATP (section 2.2.13.3) and purified from polyacrylamide (section 2.2.13.5) following fractionation (section 2.2.13.4). The VT05 5' DIG probe was purchased from Genset, Singapore.

4.2.6.3 Calculation of Tm of probes

To calculate the Tm of the oligonucleotide probes for DNA:RNA hybrids the following formula was used:

$Tm = 79.8^{\circ}C + 18.5(\log_{10}[Na^{+}]) + 0.58(\text{fraction G+C}) + 11.8(\text{fraction G+C})^2 - 0.50(\%\ \text{formamide}) - (820/l)$ (Casey and Davidson, 1977).

The Tm of the oligonucleotide probes in hybridisation mix containing 5 x SSPE and 50% formamide was 56°C (VT02), 56°C (VT03), and 64°C (VT05).

4.2.7 Hybridisation conditions for radio-labelled probes

Dot blots probed with radiolabelled VT02, VT03 or VT05 were pre-hybridised and hybridised as described in section 2.2.18.

4.2.7.1 Washing conditions

Membranes probed with radiolabelled probes were washed as described (section 2.2.18).

4.2.8 Hybridisation conditions for DIG labelled probes

Dot blots to be probed with DIG labelled VT05 were pre-hybridised and hybridised, washed and probe was detected as described in sections 2.2.19.1, 2.2.19.2, and 2.2.19.3 respectively.

4.2.9 DIBA

PSbMV US dilutions were spotted (1 µL) onto nitrocellulose membrane and air dried. The DIBA was done as described (section 2.2.7.3) with leaf blocking buffer (section 2.2.7.2.2).

4.3 Results

4.3.1 Developing RT-PCR

4.3.1.1 Testing Reverse Transcriptase efficiency

Figure 4.2 shows PSbMV-US RNA extracted from purified PSbMV. The migration of the PSbMV RNA was consistent with its previously estimated size of 9.9 kb with some smaller material possibly produced by degradation.

Figure 4.3 A shows the amount of cDNA generated from a constant amount of PSbMV-US RNA with the VT04 RT primer and four different Reverse Transcriptases. Figure 4.3 B shows the product of PCR done with cDNAs generated above, using the VT02/VT03 primer pair. AMV Reverse Transcriptase was the most efficient in generating full-length cDNA. This was the only cDNA that was amplified to the expected 1085 bp product.

4.3.1.2 Testing primer specificity

Figure 4.4 shows RT-PCR from PSbMV-US cDNA generated with VT04 RT primer and AMV Reverse Transcriptase and PCR with combinations of the PSbMV primers (VT01, VT02, VT03 and VT04). Each of the VT01/VT04, VT01/VT02, VT02/VT03 and VT03/VT04 primer pairs produced the expected sized fragment (3.0 kb, 2.2 kb, 1085 bp and 1.8 kb respectively) from the PSbMV-US cDNA. However, the VT01/VT04 and VT03/VT04 primer pairs generated additional fragments. The VT02/VT03 primer pair produced the strongest amplification signal under the PCR conditions tested.

4.3.1.3 Testing DNA polymerase efficiency

Figure 4.5 shows the effect of different DNA polymerases on the efficiency of the VT02/VT03 PCR from PSbMV-US cDNA. Of the eight different polymerases tested the Deep Vent® (exo^+) DNA polymerase failed to produce an amplification product. The Deep Vent® (exo^-) DNA Polymerase had a low efficiency of amplification and resulted in non-specific amplification. The *Taq*, Tth (Boehringer Mannheim, Germany), Pwo, AmpliTaq, Tth (Epicentre Technologies, USA) and eLONGase™ DNA polymerases all

produced a single strong amplification product of 1085 bp. The AmpliTaq, Tth (Epicentre Technologies, USA) and eLONGase™ DNA polymerases produced the strongest amplification signal under the conditions tested.

4.3.1.4 Effect of annealing temperature on PCR

Figure 4.6 shows the effect of increasing the annealing temperature by 2°C on the PCR amplification efficiency of different PSbMV isolates with the VT02/VT03 primer pair. When the PCR annealing temperature was 70°C not all the PSbMV isolates were amplified (ie. PSbMV VIDA, 43(1), S6, S4, 19(1), P503-5-2, and 23(1)) (Figure 4.6 A). When PCR for these PSbMV isolates was compared at an annealing temperature of either 70°C or 68°C, PCR either failed or was inefficient at the higher temperature but successful at the lower temperature (Figure 4.6 B).

4.3.2 Hybridisation with oligonucleotide probes

Figure 4.7 A shows the results of hybridisation with the VT02, VT03 radiolabelled probes and VT05 DIG and radiolabelled probes. Figure 4.7 B shows the results of RT with VT04 RT primer and PCR with VT02/VT03 primers for some of the nucleic acid extracts tested by hybridisation. The VT02 and VT05 probes did not detect any of the related potyviruses tested (TuMV, PeMoV, BYMV, CLYVV, LMV, BCMV, WMV-2, PMV) or TMV. All these potyvirus extracts except WMV-2, were tested by RT-PCR with *Potyviridae* specific RT-PCR primers (Mackenzie *et al.*, 1998) and shown to contain potyvirus RNA (section 5.3.2). The VT03 primer did, however hybridise to the PeMoV, BCMV, WMV-2 and TMV nucleic acid extracts. None of the probes hybridised to healthy pea nucleic acid extracts.

VT03 did not hybridise or only weakly hybridised to the nucleic acid extracts from PSbMV infected tissue. The VT02 and VT05 probes hybridised to some but not all nucleic acid extracts obtained from pea infected with different PSbMV isolates. Not all the nucleic acid extracts that tested positive with RT-PCR (Figure 4.7 B) were positive by hybridisation and some samples that were negative by RT-PCR tested positive by hybridisation.

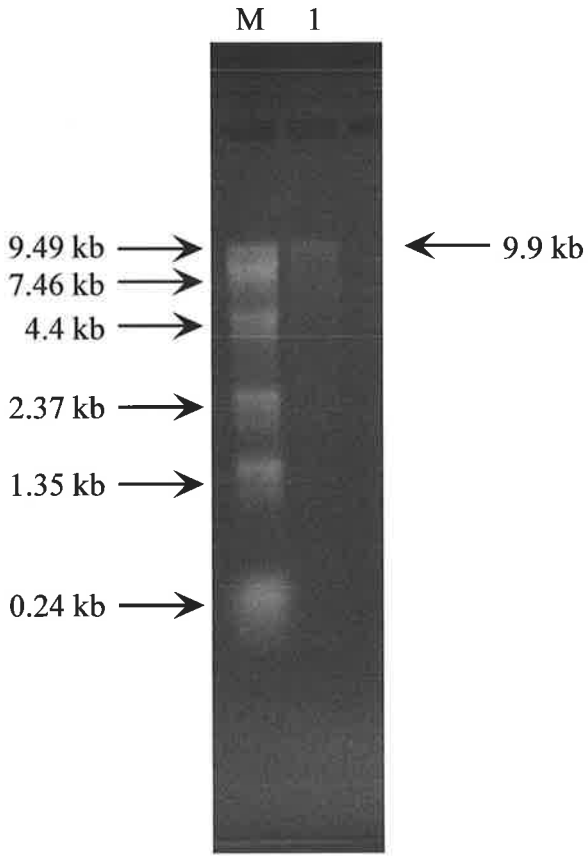


Figure 4.2: PSbMV RNA electrophoresis in formaldehyde agarose gel.

(M) 0.24-9.5 kb RNA Ladder. (1) PSbMV US RNA

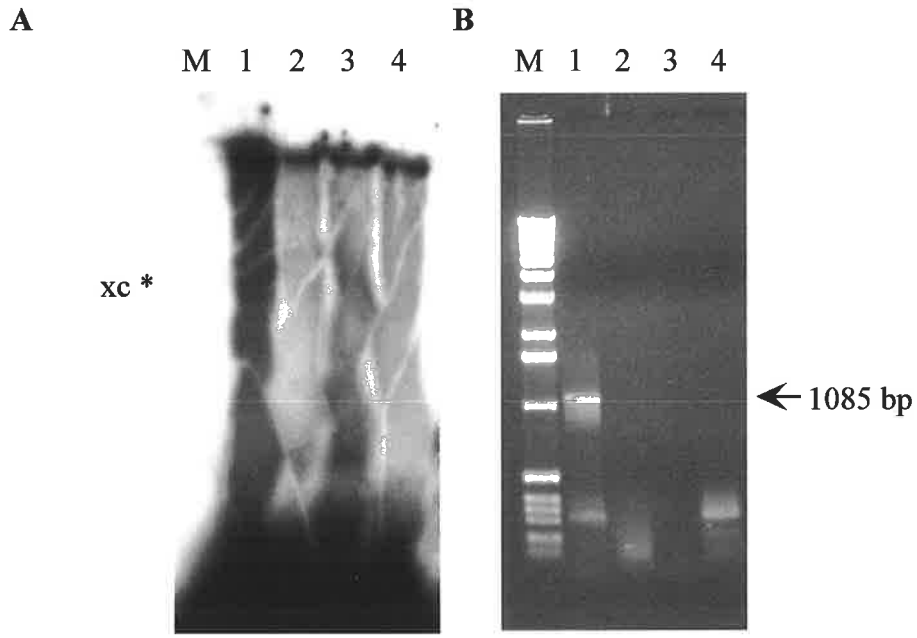


Figure 4.3: Effect of different Reverse Transcriptases on the efficiency of PSbMV amplification.

(A) Autoradiograph of radiolabelled PSbMV cDNA produced with various Reverse Transcriptases and VT04 (note: distortion due to cracking of gel during drying). cDNA transcribed with: (1) AMV RT; (2) Retrotherm RT; (3)Tth (Epicentre Technologies, USA); and (4) MMLV RT.

(B) PCR with VT02/VT03 primers on the above cDNA products.

(M) 1 kb Ladder. (xc*) position of xylene cyanol dye.

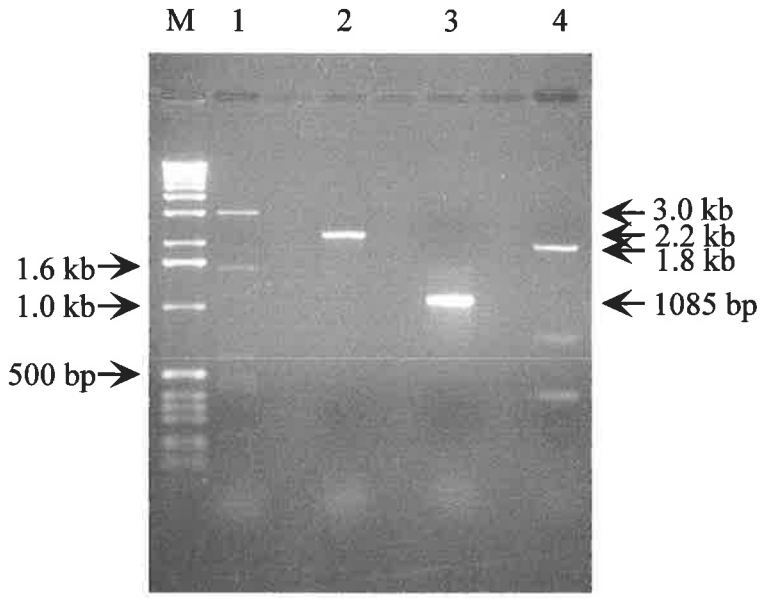


Figure 4.4: Testing combinations of PSbMV RT-PCR primers.

PCR on PSbMV cDNA generated with VT04. (1) PCR with VT01/VT04. (2) PCR with VT01/VT02. (3) PCR with VT02/VT03. (4) PCR with VT03/VT04. (M) 1 kb Ladder.

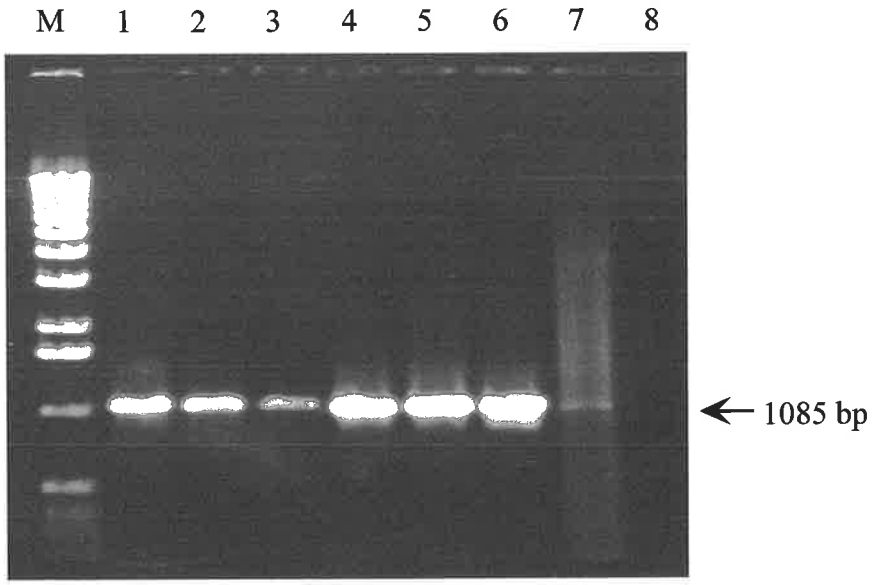


Figure 4.5: Effect of DNA polymerases on PCR.

PCR from PSbMV VT04 generated cDNA with VT02/VT03 primers and:

(1) *Taq*; (2) Tth (Boehringer Mannheim, Germany); (3) Pwo; (4) AmpliTaq;

(5) Tth (Epicentre Technologies, USA); (6) Elongase; (7) Deep Vent (exo^-); and

(8) Deep Vent (exo^+) DNA polymerases.

(M) 1 kb ladder.

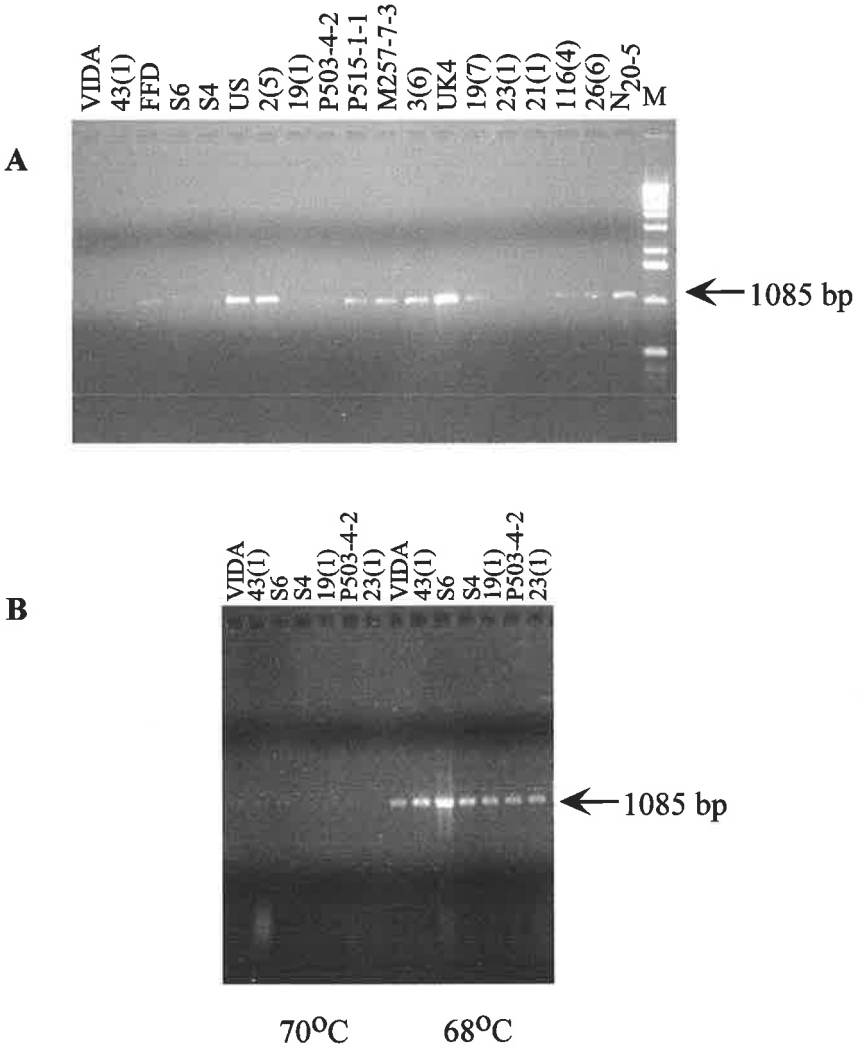
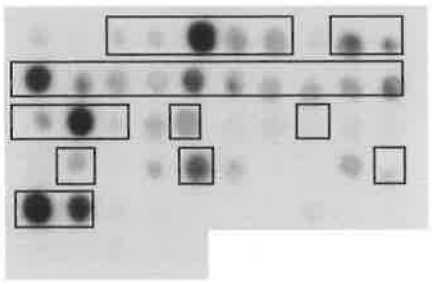


Figure 4.6: Effect of annealing temperature on RT-PCR with VT02/VT03 primers on different PSbMV isolates.

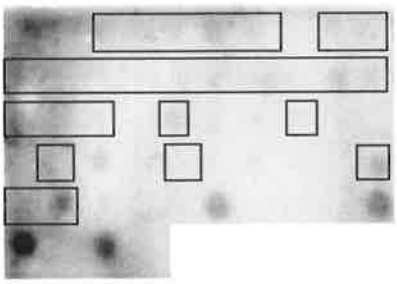
(A) PCR with annealing temperature of 70°C. (B) PCR with annealing temperature of 70°C and 68°C on identical template samples. (M) 1 kb ladder.

Figure 4.7: Hybridisation with radiolabelled and DIG-labelled oligonucleotide probes to PSbMV-RNA and comparison with RT-PCR detection of PSbMV-RNA.
(A) Autoradiographs of nylon membranes probed with radiolabelled VT02 probe, radiolabelled VT03 probe, radiolabelled VT05 probe and DIG-labelled VT05 probe. Boxed areas on autoradiograph indicate nucleic acid samples which tested positive for PSbMV by RT-PCR with VT04 RT and VT02/VT03 PCR primers. (B) RT-PCR on nucleic acid samples tested by hybridisation. Sample identities are shown on the table. Multiple PSbMV samples were tested from different sources ie fresh leaf tissue (leaf), seed from PSbMV infected mother plant (seed), dried PSbMV leaf tissue stored at 4°C on $CaCl_2$ for six months (dry), infected leaf tissue stored at 4°C for two weeks (fridge), or frozen sap from PSbMV infected plants (frozen). (*) Nucleic acid extracts which tested positive for potyvirus RNA by RT-PCR with the PV1/SP7 and PV2I/T7 primers (section 5.3.2).

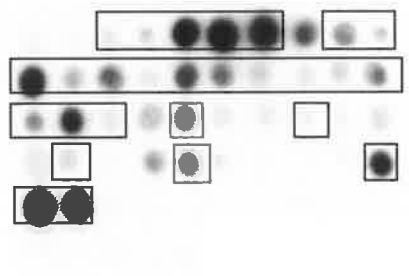
A



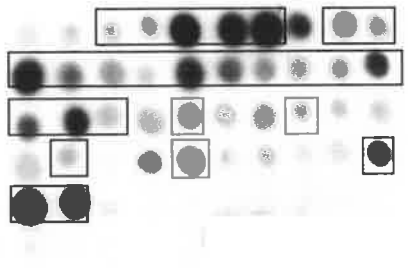
VT02 radiolabelled



VT03 radiolabelled

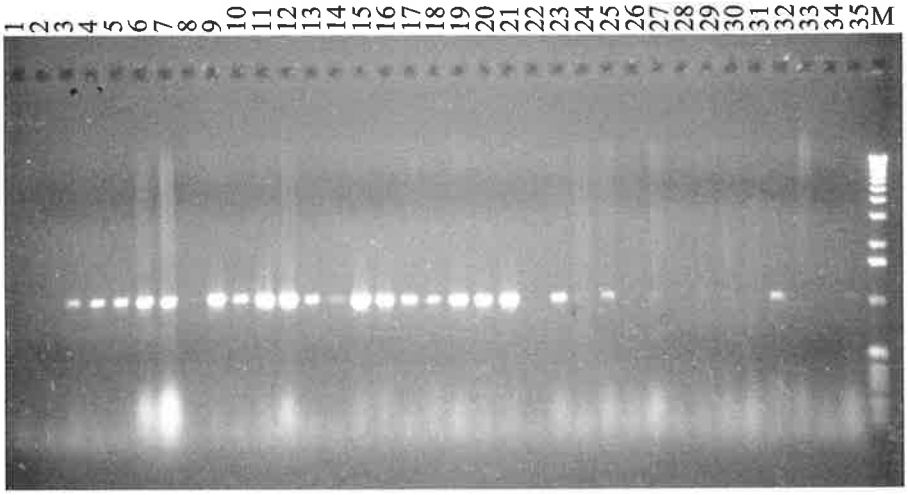


VT05 radiolabelled



VT05 DIG

B



N_{20-5} leaf 1	N_{20-5} fridge 2	VIDA leaf 3	43(1) leaf 4	FFD leaf 5	S6 leaf 6	S6 leaf 7	S6 leaf 8	S4 seed 9	S4 leaf 10
US leaf 11	US leaf 12	2(5) leaf 13	2(5) leaf 14	2(5) leaf 15	2(5) leaf 16	19(1) fridge 17	P503-4-2 fridge 18	P515-1-1 leaf 19	M257-7-3 fridge 20
3(6) leaf 21	UK4 leaf 22	19(7) leaf 23	2(5) dry 24	M257-7-3 dry 25	P503-4-2 dry 26	23(1) dry 27	N_{20-5} dry 28	116(4) dry 29	116(4) dry 30
19(1) dry 31	23(1) leaf 32	26(6) leaf 33	3(6) frozen34	21(1) frozen 35	FFD leaf 36	23(1) leaf	N_{20-5} leaf	116(4) leaf	N_{20-5} leaf
21(1) leaf	26(6) leaf	21(1) leaf	TuMV dry	TuMV leaf *	PeMV leaf *	BYMV leaf *	ClYVV leaf *	LMV leaf *	BCMV leaf *
WMV-2 leaf	PMV leaf *	TMV leaf *	Healthy leaf						

4.3.3 Sensitivity of nucleic acid and serological PSbMV detection methods

4.3.3.1 Nucleic hybridisation

Figure 4.8 shows a dilution series of PSbMV-US RNA hybridised with the VT02 and VT03 radiolabelled probes and the VT05 DIG and radiolabelled probes. VT02 detected approximately 507 pg of PSbMV-US RNA. The genome sense VT03 did not detect PSbMV-US RNA. The VT05 DIG-labelled and radiolabelled probes detected 256 pg and 126 pg PSbMV-US RNA respectively.

4.3.3.2 RT-PCR

Figure 4.9 shows RT with VT04 and a dilution series of PSbMV-US RNA and PCR with VT02/VT03. The level of sensitivity of RT-PCR was approximately 111 pg of PSbMV-US RNA in a 10 µL RT reaction of which 1 µL cDNA was added to a 10 µL PCR mixture.

4.3.3.3 DIBA

Figure 4.10 shows DIBA of a dilution series of PSbMV US. The limit of detection of DIBA was 5.4 ng/µL PSbMV.

4.4 Discussion

This chapter describes a PSbMV RT-PCR assay for detecting several PSbMV isolates from total nucleic acid extracts of infected plants. The best combination of PSbMV primers was RT with VT04, and PCR with VT02/VT03, generating a single amplicon of 1085 bp. This RT-PCR successfully amplified the expected fragment from nucleic extracts of pea leaf tissue infected with 17 different Australian PSbMV isolates, as well as the PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4 type isolates.

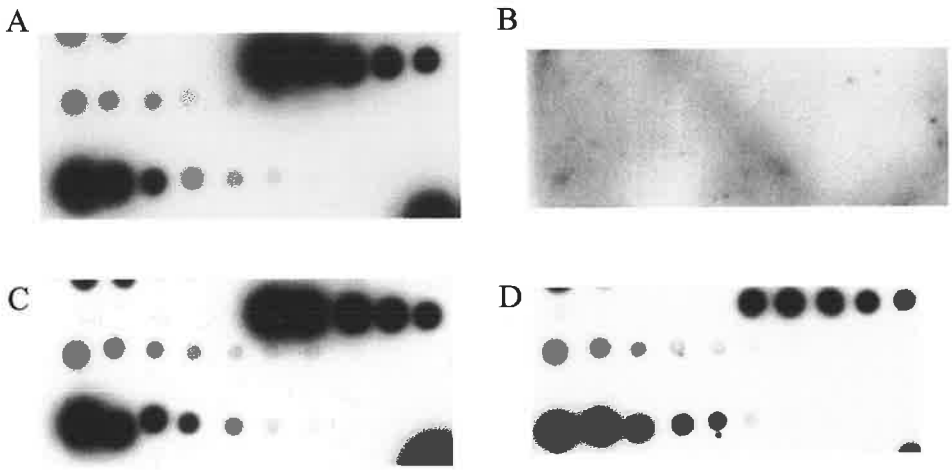
The success of the RT-PCR was mostly dependent on the RT step. RT with VT04 was dependent on the type of Reverse Transcriptase used with only AMV Reverse Transcriptase producing sufficient cDNA for successful PCR. The effect of different DNA polymerases on amplification of the PSbMV was minor with only Deep Vent® (exo^-) failing to generate a product and Deep Vent® (exo^+) giving a faint and non-specific amplification signal. Therefore, *Taq* DNA polymerase was chosen because it generated sufficient and specific amplification product and was the most cost effective DNA polymerase available. Varying the annealing temperature did affect PCR. Some PSbMV

isolates were successfully amplified at an annealing temperature of 68°C but not 70°C, while other PSbMV isolates were successfully amplified at both annealing temperatures. This suggests that there is genome sequence variability among the PSbMV isolates within either one or both VT02/VT03 primer binding sites.

Nucleic acid hybridisation with radiolabelled VT02 and VT05 oligonucleotide probes and the DIG-labelled VT05 oligonucleotide probe detected RNA in some PSbMV infected nucleic acid extracts. Not all the nucleic acid extracts that tested positive by RT-PCR were positive by hybridisation. The VT02 and VT05 probes did not hybridise non-specifically to host nucleic acid or other legume infecting potyviruses or TMV. Hybridisation signal varied between samples from different plants infected with the same PSbMV isolate indicating that the level of PSbMV varied between samples. These probes also hybridised to some PSbMV nucleic acid extracts that tested RT-PCR negative. This could be because the PSbMV template may have been partially degraded and unable to support RT-PCR, or contained inhibitors to RT-PCR. RT-PCR and hybridisation generally did not detect template extracted from dried leaf tissue suggesting that the PSbMV RNA was degraded. The VT05 probe was more sensitive, giving stronger hybridisation signals than the VT02 probe.

The radiolabelled VT03 oligonucleotide probe failed to hybridise extracts from PSbMV infected tissue but did hybridise non-specifically to PeMoV, BCMV, WMV-2 and TMV extracts. As VT03 is a sense probe it would be expected to only detect the replicative form of PSbMV. This result suggests that either the PSbMV replicative form is in low abundance compared with the PSbMV sense RNA or the extraction procedure used did not allow efficient extraction of double stranded RNA.

RT-PCR was the most sensitive method developed, approximately 10 times more sensitive than hybridisation with VT02 or VT05. The PSbMV PCR detected cDNA equivalent to the amount transcribed from 11 pg PSbMV RNA, while the VT05 and VT02 probes detected 126 pg and 507 pg PSbMV RNA respectively. RT-PCR was 20 times more sensitive than DIBA, detecting 5.4 ng/µL PSbMV which is equivalent to 250 pg/µL of PSbMV RNA.



-	-	-	-	-	260 ng	130 ng	65 ng	32ng	16 ng
8 ng	4 ng	2 ng	1 ng	507 pg	256 pg	126 pg	63 pg	31 pg	15 pg
-	-	-	-	-	-	-	-	-	-
173 ng	57 ng	19 ng	6 ng	2 ng	713 pg	237 pg	79 pg	26 pg	8 pg

Figure 4.8: Sensitivity of PSbMV hybridisation assay with radiolabelled and DIG-labelled oligonucleotide probes on PSbMV-US RNA. PSbMV-US RNA was diluted either two or four fold. The table indicates the amount of PSbMV RNA per 1 uL spot probed with: (A) radiolabelled VT02; (B) radiolabelled VT03; (C) radiolabelled VT05; (D); and DIG-labelled VT05.

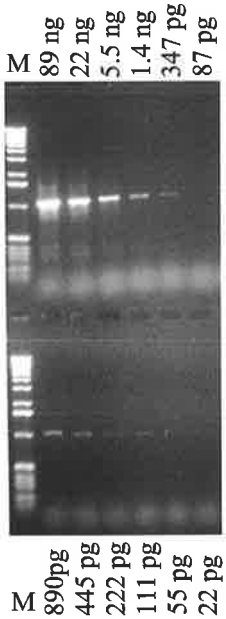
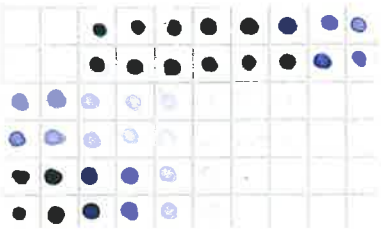


Figure 4.9: Sensitivity of RT-PCR with VT04 RT primer and VT02/VT03 PCR primers on PSbMV RNA.

PSbMV RNA was diluted either two or four fold and used as template in RT-PCR. Numbers refer to amount to PSbMV RNA added to a 10 uL RT reaction of which 1 uL was added to a 10 uL PCR.

(M) 1kb ladder



-	-	44 µg	22 µg	11µg	5.5 µg	2.7 µg	1.3 µg	687 ng	343 ng
171 ng	85 ng	42 ng	21 ng	10 ng	5.4 ng	2.7 ng	1.3 ng	671 pg	336 pg
44 µg	11 µg	2.7 µg	687 ng	172 ng	43 ng	10 ng	2.6 ng	671 pg	168 pg

Figure 4.10: Sensitivity of DIBA.

PSbMV US was diluted either two or four fold. The table indicates the amount of PSbMV per 1 uL spot. Samples were tested in duplicate.

CHAPTER 5

DETECTION OF PSbMV BY RT-PCR AND RFLP ANALYSIS

5.1 Introduction

PCR has been shown to be very useful for detecting and characterising plant pathogens and is more sensitive than direct probing or serological techniques (Hadidi *et al.*, 1995; Randles *et al.*, 1996). RT-PCR using specific primers enables small numbers of viral RNA molecules to be detected in nucleic acid mixtures. Degenerate potyvirus primers U335/D335 (Langeveld *et al.*, 1991) have been used to detect PSbMV in total nucleic acid extracts from infected pea, but also produce non specific amplification from healthy pea extracts (Pearson *et al.*, 1994). Unlike the U335/D335 primers which target a small conserved region of the CP gene, the *Potyviridae* specific primers PV1/SP6 and PV2I/T7 (Gibbs and Mackenzie, 1997; Mackenzie *et al.*, 1998) amplify a larger (1.6-2.1 kb) more variable region from the 3' end of the genome of viruses in the *Potyviridae*, including the *NIb*, entire *CP* and poly-A tail. This allows differentiation between species in the *Potyviridae* on the basis of size of the PV1/SP6 and PV2I/T7 amplicon. The PV1/SP6 and PV2I/T7 primers have not been evaluated for amplifying PSbMV, but the theoretical size of the RT-PCR amplicon would be 1674 bp. This has been inferred from nucleic acid sequence data for PSbMV DPD1 pathotype P-1 (GenBank accession number D10930).

No reliable PSbMV specific RT-PCR assay has yet been developed. RT-PCR (Kohnen *et al.*, 1992, Kohnen *et al.*, 1995) and IC-PCR (Phan *et al.*, 1997) have been described for PSbMV but these methods have limitations. They are isolate specific (Kohnen *et al.*, 1992, Kohnen *et al.*, 1995, Phan *et al.*, 1997), not always reliable, and IC-PCR is less sensitive than DAS-ELISA (Phan *et al.*, 1997). However, the PSbMV pathotype P-1 specific RT-PCR primers A_{DPD1}/C_{DPD1} (Kohnen *et al.*, 1992) and PSbMV pathotype P-4 specific primers A_{NY}/B_{NY} (Kohnen *et al.*, 1995) have been useful tools for studying: PSbMV distribution in infected pea plants; long distance movement of PSbMV DPD1 pathotype P-1, PSbMV NY pathotype P-4 and PSbMV DPD1/NY chimeras in *Chenopodium quinoa* cv. Willd (Anderson and Johansen, 1998); and the mechanism of *P. sativum* pathotype specific resistance to PSbMV (Keller *et al.*, 1998).

The only reliable method of identifying PSbMV infection has been by serology (Maury *et al.*, 1987, Ligat *et al.*, 1991, Ding *et al.*, 1992, Masmoudi *et al.*, 1994a) with biological pathotyping being used to further distinguish between PSbMV isolates (Hampton *et al.*, 1981; Alconero *et al.*, 1996; Provvidenti and Alconero, 1988). Serology is not as sensitive as RT-PCR (Kohnen *et al.*, 1992, Kohnen *et al.*, 1995) and may show cross reactivity with

related viruses or host plant antigens (Derks, 1992; Elliot *et al.*, 1996; Torrance, 1998). Furthermore, biological pathotyping is time consuming and does not allow PSbMV pathotypes to be further distinguished at the isolate level. RT-PCR based RFLPs have allowed strain discrimination for other viruses (Nemchinov and Hadidi, 1996; Yang and Mirkov 1997; Offringa *et al.*, 2000)

The aim of this chapter was to develop a specific PSbMV RT-PCR diagnostic assay. For RT-PCR to be successfully used in a diagnostic assay several criteria need to be met. These were: (1) that the assay will be specific to PSbMV and not any other legume infecting potyviruses; (2) that the RT-PCR will detect all known PSbMV isolates; and (3) that the assay will be reliable and reproducible. It was also an aim to develop a rapid and simple method for distinguishing between PSbMV isolates at the molecular level by RFLP analysis of the PSbMV RT-PCR product.

5.2 Materials and Methods

5.2.1 Total nucleic acid extraction from leaf tissue

Total nucleic acids were extracted from 0.5 gm fresh tissue using a modified CTAB method (section 2.2.4.1).

5.2.2 Virus isolates

Fourteen Pakistani PSbMV isolates (Ali and Randles, 1997) were provided by Dr A Ali, Department of Applied and Molecular Ecology, University of Adelaide, Waite Campus, Australia. 15 Australian PSbMV isolates were collected during the course of this study (Table 3.1). PSbMV type isolates US (pathotype P-1) and S6 (pathotype P-4) and virus isolates BYMV-S, PMV, TMV were provided from the Waite Campus plant virus collection. BCMV isolate 295 and PeMoV isolate 133-E were provided by Dr J Thomas, Department of Primary Industries, Indooroopilly, Queensland. ClYVV and LMV were provided by Mr D Graetz, Field Crops Pathology Unit, South Australian Research and Development Institute, South Australia, Australia. TuMV was provided by Dr J F Laliberte, INRS-Institut Armand-Frappier, Université du Québec, Québec, Canada. All virus isolates were mechanically inoculated (section 2.2.1) to the indicated host species (Table 5.1) and maintained under glasshouse conditions at 21°C.

Table 5.1: Virus isolates and host plants

Virus	Host
PSbMV	*Pisum sativum* cv. Dundale
BCMV isolate 295	*Phaseolus vulgaris* cv Hawkesbury Wonder
PeMoV isolate 133-E	*Phaseolus vulgaris* cv Hawkesbury Wonder
ClYVV	*Chenopodium quinoa*
LMV	*Chenopodium quinoa*
PMV	*Pisum sativum* cv. Dundale
BYMV-S	*Pisum sativum* cv. Dundale
TuMV	*Chenopodium quinoa*
TMV	*Nicotiana tabacum* cv Samsun

5.2.3 RT-PCR

5.2.3.1 Primers

For PSbMV RT-PCR the RT was primed with VT04 (Appendix C), targeted to a region within the *P3* gene of PSbMV corresponding to position 3297-3273 of PSbMV DPD1. The PCR was done with primers VT02 and VT03 (Appendix C) both located within the *HC-Pro* gene of PSbMV and corresponding to positions 1417-1446 and 2500-2471 of PSbMV DPD1 respectively.

Pea infecting potyviruses were tested with the *Potyviridae* specific RT-PCR primers of Mackenzie *et al.* (1998). PV1/SP6 (Appendix C) corresponding to position 9936-9919 of PSbMV DPD1 was used as both the RT and reverse PCR primers. PV2I/T7 (Appendix C) corresponding to position 8250-8266 of PSbMV DPD1 was used as the forward PCR primer.

5.2.3.2 RT-PCR conditions

RT-PCR was done in a two step reaction. RT was done in a reaction volume of 10 µL containing 1 µL CTAB extract, RT primer, 25 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.8 mM dNTPs, 1 U/µL RNase Inhibitor, and 5 U AMV RT. The RT reaction contained 0.4 µM VT04 primer for the PSbMV RT or 0.6 µM PV1/SP6 primer for the *Potyviridae* specific RT. Total nucleic acid and primer were incubated in a volume of 5 µL at 70°C for 10 min and then quenched on ice. The remainder of the RT components were added and the reaction incubated at 42°C for 40 minutes.

PCR was done in a 10 µL reaction volume containing 1 µL cDNA from the RT step, reverse and forward primers, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl, pH 9.0, 0.1% Triton® X-100, 0.8 mM dNTPs, and 1U *Taq* DNA polymerase. The PSbMV specific PCR contained 0.4 µM each of the VT02 and VT03 primers and the *Potyviridae* specific PCR contained 0.3 µM PV1/SP6 primer and 0.6 µM PV2I/T7 primer.

5.2.3.3 PCR cycling conditions

RT-PCR was done in a GeneAmp PCR System 2400 (Perkin Elmer, USA).

For the PSbMV specific PCR, mixtures were initially incubated at 94°C for 3min followed by 20 cycles consisting of: denaturation at 94°C for 30 sec; annealing at 68°C for 30 sec; and extension at 72°C for 30 sec, with a final extension step of 72°C for 2 min. RT-PCR products were analysed by electrophoresis in a 1.5% agarose gel containing 0.5µg/ml EtBr and buffered in 1 x TAE.

For the *Potyviridae* specific PCR, mixtures were initially incubated at 94°C for 3 min followed by 30 cycles consisting of: denaturation at 94°C for 30 sec; annealing at 58°C for 1 min; and extension at 72°C for 3 min, with a final extension step of 72°C for 8 min. RT-PCR products were analysed by electrophoresis in a 1% agarose gel containing 0.5 µg/mL EtBr and buffered in 1 x TAE.

5.2.4 RFLP analysis of PSbMV RT-PCR products

The 1085 bp *HC-Pro* amplicon from 15 Australian PSbMV isolates, 14 Pakistani isolates and 2 PSbMV type isolates (US pathotype P-1 and S6 pathotype P-4) were restricted with 7 unique four base pair recognition sequence restriction endonucleases (*Alu*I, *Hae*III, *Hha*I, *Rsa* I, *Sau*3AI, *Taq*αI and *Tru*91) according to the manufacturer's instructions (Appendix A). Table 5.2 shows the recognition sites of the restriction endonucleases used. RFLP products were analysed by electrophoresis in a 2.5% agarose gel containing 0.5 µg/mL EtBr and buffered in 1 x TAE. RFLP products resulting from the *Sau*3AI restriction were analysed in a 3% agarose gel.

Table 5.2: Restriction enzymes used in the RFLP analysis of the PSbMV *HC-Pro* gene

Restriction Endonuclease	Recognition sequence
*Alu*I	AG↓CT TC↑GA
*Hae*III	GG↓CC CC↑GG
*Hha*I	G CG↓C C↑GC G
*Rsa*I	GT↓AC CA↑TG
*Sau*3AI	↓GATC CTAG↑
*Taq*α1	T↓CG A A GC↑T
*Tru*91	T↓TA A A AT↑T

5.2.5 *Cloning of the 1085 bp RT-PCR amplicon from the HC-Pro of representative PSbMV isolates*

The 1085 bp PSbMV *HC-Pro* RT-PCR fragments from PSbMV US, S6, 19(1), P515-1-1, VIDA, PK6, PK7, PK10, PK13 were cloned. PCR with VT02/VT03 was done (section 5.2.3.2) using Pwo DNA polymerase instead of *Taq* DNA polymerase according to the manufacturer's recommendations. The 1085 bp amplicon was excised from agarose gels following electrophoresis and purified (section 2.2.12). Blunt end PCR fragments were A-tailed (section 2.2.8.2) and ligated into pGEM®-T vector (section 2.2.8.3) and transformed into competent DH5α cells (section 2.2.8.4). Recombinant plasmids were purified (section 2.2.9) and analysed for the presence of insert (section 2.2.10.1).

5.3 Results

5.3.1 *Specificity of the PSbMV primers*

Figure 5.1 shows the PSbMV specific RT-PCR done with RT primer VT04 and PCR primers VT02 and VT03 on total nucleic acid extracts from leaf tissue of healthy pea plants and plants infected with PSbMV US pathotype P-1, ClYVV, BYMV, PMV, PeMoV, TuMV, BCMV, LMV, and TMV. RT-PCR was also done on the above extracts which had been supplemented with 900 pg purified PSbMV-RNA (section 4.2.3) per RT reaction. No amplicon was produced from total nucleic acid extracted from healthy pea or ClYVV, BYMV, PMV, PeMoV, TuMV, BCMV, LMV or TMV infected plants. A single

1085 bp amplicon was produced from total nucleic acid extracted from PSbMV infected plants and nucleic acid samples which had been supplemented with PSbMV-RNA.

Figure 5.2 shows RT-PCR amplification of 15 Australian and 14 Pakistani PSbMV isolates with the VT04 RT primer and VT02/VT03 PCR primers. All PSbMV isolates tested produced a single amplicon of 1085 bp in size.

5.3.2 Detection of potyviruses by RT-PCR

Figure 5.3 shows the *Potyviridae* specific RT-PCR done with the PV1/SP6 and PV2I/T7 primers (Mackenzie *et. al.*, 1998) on total nucleic acid extracted from leaf tissue of healthy pea plants and plants infected with PSbMV US pathotype P-1, ClYVV, BYMV, PMV, PeMoV, TuMV, BCMV, LMV, and TMV. No amplification was observed from healthy pea nucleic acid. A distinct amplicon ranging in size from 1636-2036 bp was produced from PSbMV, ClYVV, BYMV, PMV, PeMoV, TuMV, BCMV, and LMV infected plant total nucleic acid extracts. Amplicons smaller than 1000 bp were also produced from the PSbMV, BYMV, PMV, PeMoV, BCMV and LMV infected plant nucleic acid samples. No specific product was observed for the total nucleic acid sample from the TMV infected plant.

5.3.3 RFLP analysis of 1085 bp HC-Pro generated amplicon from PSbMV

Figure 5.4 shows the different RFLP patterns generated from the 1085 bp *HC-Pro* amplicon of 31 Australian, Pakistani and type PSbMV isolates with each of seven restriction enzymes (Table 5.2). *Tru*91 generated the most RFLPs with six distinct patterns observed among the 31 PSbMV isolates tested. *Alu*I generated four distinct RFLPs, whereas *Taq*α1 generated only two distinct RFLPs. *Hae*III, *Hha*I, *Rsa*I and *Sau*3AI each generated three distinct RFLPs.

Table 5.3 shows the individual RFLP profile of each of the 31 PSbMV isolates obtained with each of the seven restriction enzymes. PSbMV isolates were grouped according to the different combinations of RFLP patterns observed from the seven restriction digests. Eight different groups were observed. Group 1 only contained one PSbMV isolate, the PSbMV US pathotype P-1 type isolate. Group 2 contained four PSbMV isolates, including the PSbMV S6 pathotype P-4 type isolate and three PSbMV Australian isolates, 3(6), $N_{20\text{-}5}$ and VIDA. Group 3 contained seventeen PSbMV isolates: eleven Australian PSbMV

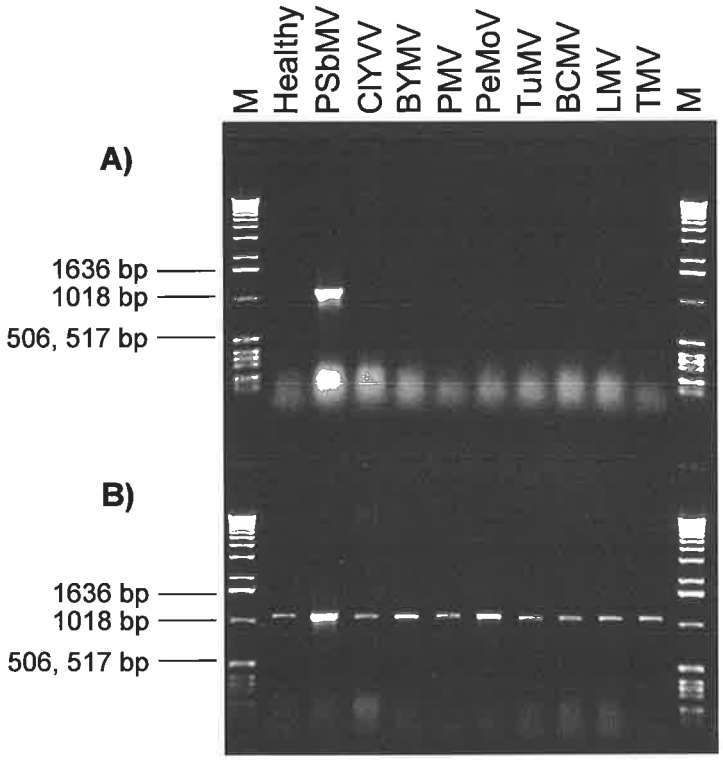


Figure 5.1: Specificity of RT-PCR primers targeted to *HC-Pro* region of PSbMV. RT with VT04 and PCR with VT02 and VT03 primers on: (A) total nucleic acids from healthy pea and PSbMV, ClYVV, BYMV, PMV, PeMoV, TuMV, BCMV, LMV and TMV infected plants; (B) the above nucleic acids which had been previously supplemented with PSbMV-RNA.

(M) kb Plus Ladder.

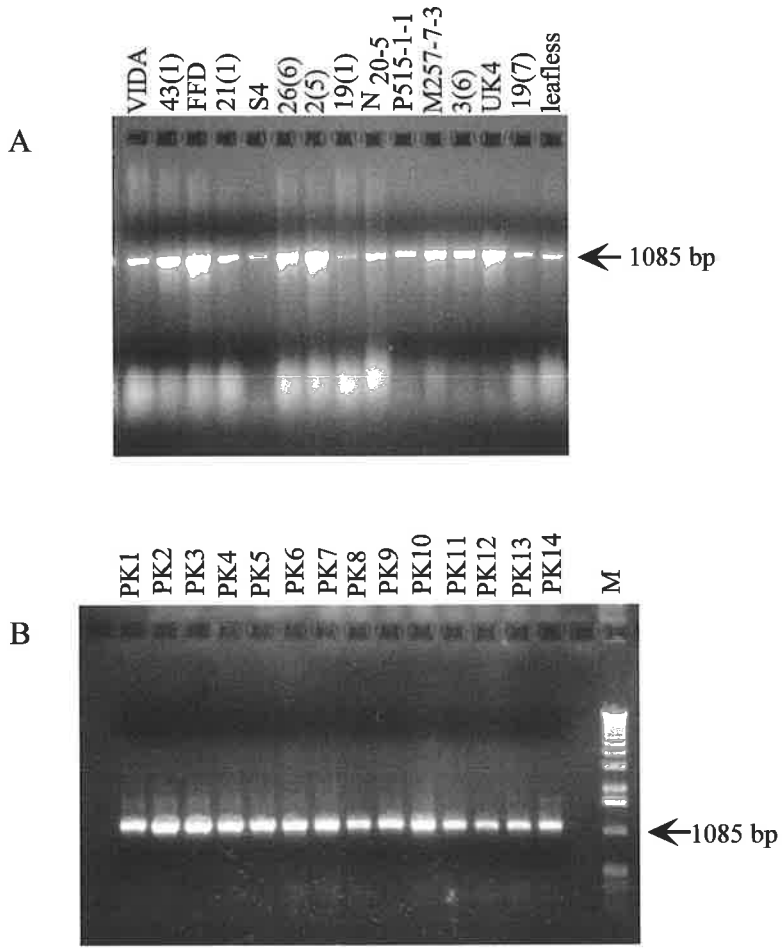


Figure 5.2: RT with VT04 and PCR with VT02/VT03 primers from TNA of 29 PSbMV isolates.

(A) Austalian PSbMV isolates.

(B) Pakistani PSbMV isolates.

(M) 1 kb Ladder.

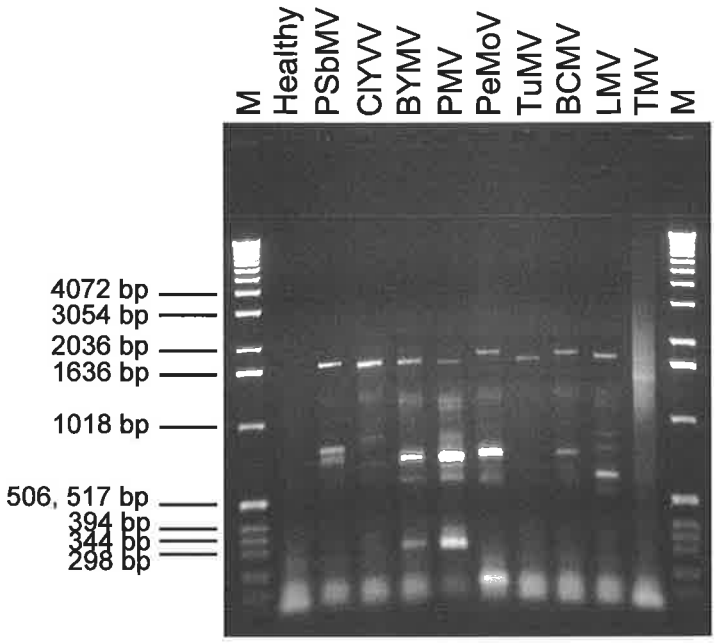


Figure 5.3: Detection of pea infecting potyviruses with *Potyviridae* specific RT-PCR primers.

RT-PCR with PV1/SP6 and PV2I/T7 primers on total nucleic acid extracted from healthy pea and virus infected plants.

(M) 1kb DNA Ladder.

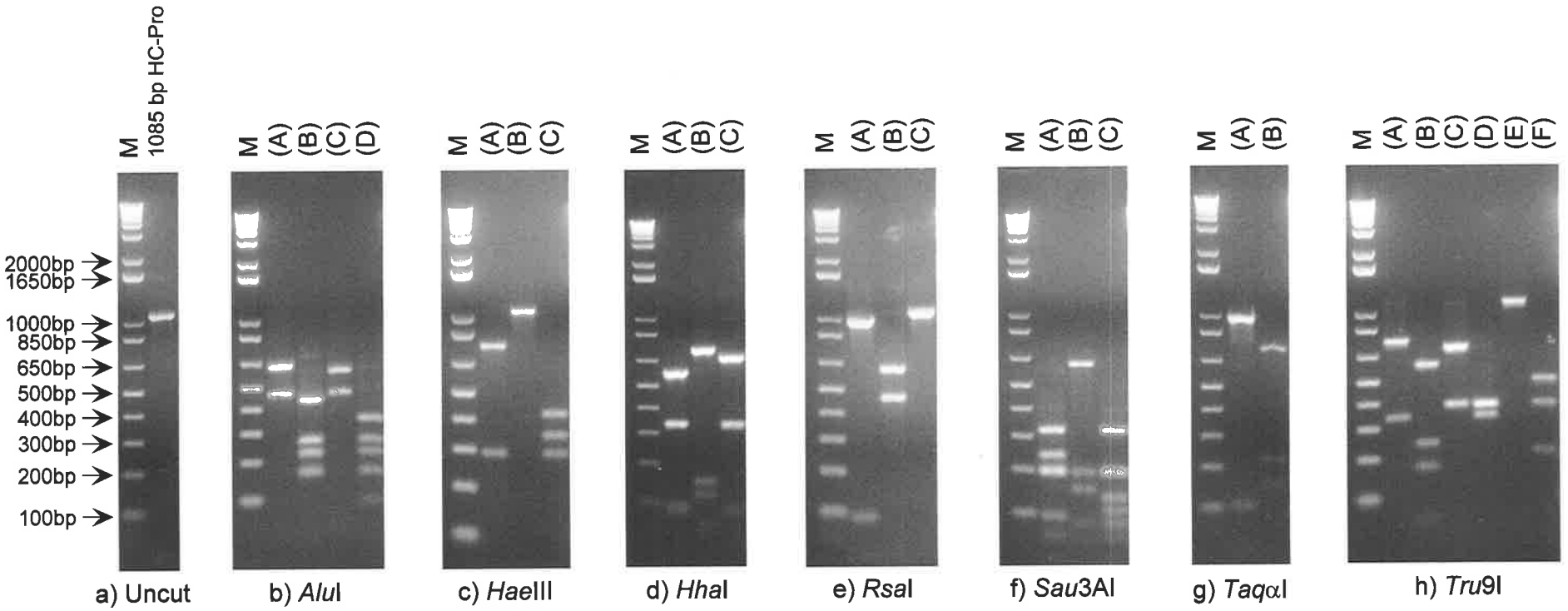


Figure 5.4: Examples of the RFLP patterns generated from the RT-PCR 1085 bp HC-Pro amplicon for the 31 PSbMV isolates.

(M) 1kb DNAPlus Ladder. (a) Shows the unrestricted RT-PCR generated amplicon from the *HC-Pro* region of PSbMV.

(b-h) Summarise the different RFLP patterns observed from 31 PSbMV isolates with each of the 7 REs.

Each of the RFLP patterns produced with a given RE are identified with an alphabetically ordered capital letter (A, B, C, etc.) which is independent of that assigned for another RE. The RFLP patterns described here are identical to those referred to in Table 5.3.

Table 5.3: RFLP analysis of *HC-Pro* regions of 31 PSbMV isolates from Australia and Pakistan

* RFLP patterns observed for each RE are identified alphabetically and directly correlate to those described in Figure 5.4.

PSbMV isolates were placed into numerical groups (1-8) based on the different combinations of RFLP patterns observed with the seven different REs. These eight groups have also been colour coded.

(X) Identifies the RFLP patterns observed in PK14 which was not placed in any group.

PSbMV Isolates		RFLP patterns with restriction endonucleases																							
		*Alu*I*				*Hae*III*			*Hha*I*			*Rsa*I*			*Sau*3AI*			*Taq*αI*		*Tru*91*					
		A	B	C	D	A	B	C	A	B	C	A	B	C	A	B	C	A	B	A	B	C	D	E	F
US pathotype P-1		1#				1			1			1			1			1		1					
S6 pathotype P-4			2				2			2			2			2			2		2					
AUSTRALIAN	3(6)		2				2			2			2			2			2		2					
	N 20-5		2				2			2			2			2			2		2					
	VIDA		2				2			2			2			2			2		2					
	S4			3				3			3	3					3	3				3				
	M257-7-3			3				3			3	3					3	3				3				
	P515-1-1			3				3			3	3					3	3				3				
	43(1)			3				3			3	3					3	3				3				
	26(6)			3				3			3	3					3	3				3				
	19(7)			3				3			3	3					3	3				3				
	2(5)			3				3			3	3					3	3				3				
	21(1)			3				3			3	3					3	3				3				
	FFD			3				3			3	3					3	3				3				
	UK4			3				3			3	3					3	3				3				
	Leafless			3				3			3	3					3	3				3				
	19(1)				4		4			4				4		4			4		4					
PAKISTANI	PK3	5				5			5			5			5			5					5			
	PK5	5				5			5			5			5			5					5			
	PK7	5				5			5			5			5			5					5			
	PK6		6				6			6				6		6			6		6					
	PK8		6				6			6				6		6			6		6					
	PK1			3				3			3	3					3	3				3				
	PK2			3				3			3	3					3	3				3				
	PK4			3				3			3	3					3	3				3				
	PK9			3				3			3	3					3	3				3				
	PK11			3				3			3	3					3	3				3				
	PK12			3				3			3	3					3	3				3				
	PK10			7				7			7	7					7	7						7		
	PK13																									
	PK14			X		X		X	X		X	X			X		X	X				X				

isolates (S4, M257-7-3, P515-1-1, 43(1), 26(6), 19(7), 2(5), 21(1), FFD, UK4 and leafless) and six Pakistani PSbMV isolates (PK1, PK2, PK4, PK9, PK11 and PK12). Group 4 only contained one PSbMV isolate the Australian 19(1). Group 5 contained three Pakistani isolates (PK3, PK5 and PK7) and was similar to Group 1, but was distinguished from it by *Tru*91. Group 6 contained two Pakistani PSbMV isolates (PK6 and PK 8) and was similar to Group 2, differing only in the *Rsa*I RFLP pattern produced. Groups 7 and 8 each contained one Pakistani PSbMV isolate (PK10 and PK 13 respectively) and were similar to Group 3, differing only in the *Tru*91 RFLP patterns produced. The Pakistani PSbMV isolate PK14 showed two distinct RFLP patterns with *Hae*III, *Hha*I and *Sau*3AI and therefore, was not placed in any of the above groups described.

5.3.4 PSbMV representative clones for each of the RFLP groups

Figure 5.5 shows the clones produced from PSbMV isolates representative of each of the eight groups described for RFLP analysis (Table 5.3). The PSbMV US, PK7, PK10 and PK13 isolates contain an additional *Eco*RI site internal to the 1085 bp *HC-Pro* RT-PCR amplicon, whereas PSbMV S6, 19(1), P515-1-1, PK6 and VIDA do not.

5.4 Discussion

The PSbMV RT-PCR developed in this chapter is specific to a 1085 bp region of the PSbMV *HC-Pro* but not to any other tested pea infecting potyvirus or TMV. This PSbMV RT-PCR appears to be ~~genus~~ species specific detecting all 31 PSbMV isolates tested. Total nucleic acid extracts tested were shown not to contain inhibitors to RT-PCR as extracts supplemented with PSbMV US RNA all produced a 1085 bp amplicon following PSbMV RT-PCR.

The PV1/SP6 and PV2I/T7 *Potyviridae* specific primers amplified a 1.6-2 kb fragment from total nucleic acid extracted from PSbMV, ClYVV, BYMV, PMV, PeMoV, TuMV, BCMV, BCMV and LMV infected plants indicating potyviral RNA had been successfully extracted by our CTAB extraction method. RT-PCR products smaller than 1 kb were also produced from PSbMV, ClYVV, BYMV, PMV, PeMoV, BCMV and LMV. These observations are consistent with those of Mackenzie *et al.*, 1998 when using the PV1/SP6 and PV2I/T7 primers to amplify the genomes of other species in the *Potyviridae*. Of the potyviruses tested this is the first report of PSbMV, ClYVV, BYMV, PMV, PeMoV, BCMV and LMV being amplified with the PV1/SP6 and PV2I/T7 primers.

RFLP analysis of the 1085 bp PSbMV specific amplicon has allowed the 31 PSbMV isolates to be partially characterised at the molecular level. Since the PSbMV specific RT-PCR does not differentiate between PSbMV isolates, RFLP analysis has been combined with RT-PCR to differentiate between isolates. This is the first report of an assay for discriminating between strains of PSbMV that does not require interpretation of symptoms on inoculated pea differential genotypes. This method should, therefore facilitate rapid identification and discrimination of strains, new field isolates, and introduction of new isolates of PSbMV into new regions. PSbMV clones of the *HC-Pro* region have also been produced for each of the RFLP patterns described and can act as standards for comparison with future PSbMV isolates identified.

By analyzing the RFLP results individually it was shown that *Tru*91 differentiated the PSbMV isolates with the largest number of groups, producing six distinct RFLP patterns, three of which were unique to the Pakistani PSbMV isolates. *Alu*I produced four RFLP patterns with one being unique to an Australian PSbMV isolate. *Hae*III, *Hha*I, *Rsa*I and *Sau*3AI each produced three distinct RFLP patterns, with one of the *Rsa*I patterns being unique to an Australian PSbMV isolate. *Taq*αI showed least discrimination among the PSbMV isolates producing only two RFLP patterns.

By analysing the combined RFLP data generated with each of the seven restriction enzymes for individual PSbMV isolates it was possible to place the 31 PSbMV isolates into eight distinct groups. Groups 2 and 4 were specific to Australian PSbMV isolates, while Groups 5, 6, 7 and 8 were specific to the Pakistani PSbMV isolates. Group 3 was the most common group found, containing 17 PSbMV isolates and was the only group representative of both Australian and Pakistani PSbMV isolates. Group 1 only contained one representative, PSbMV US pathotype P-1, which could be an indication of its geographically unique origin as the only PSbMV isolate from the United States of America. Group 1, however, is similar to the Pakistani Group 5 only differing from Group 1 in the *Tru*91 RFLP pattern. PSbMV S6 pathotype P-4 has been placed in Group 2 containing three other Australian PSbMV. The Pakistani PSbMV isolate PK14 was not placed in a group as two different RFLPs were observed with *Hae*III, *Hha*I and *Sau*3AI, probably due to a mixed PSbMV infection in the plant sampled.

Since Australian PSbMV isolates are only represented in three of the eight groups it is suggested that a limited number of PSbMV isolates have been introduced in to Australia and have subsequently become widespread. Of the three Australian PSbMV isolates in Group 2, two were isolated from South Australian breeders seed lines in 1995 and 1996 and one from Victorian breeders seed lines in 1996. Of the 11 Australian PSbMV isolates in Group 3; five were isolated from South Australian breeders seed lines between 1995-1998, two from pea field trial material in 1995, two from commercially available pea cultivars in 1995, one from a PSbMV virus culture collected in 1992, and one from faba bean field trial material from Western Australia in 1997. The one representative of Group 4 was collected from South Australian pea field trial material in 1995 and may have remained localized.

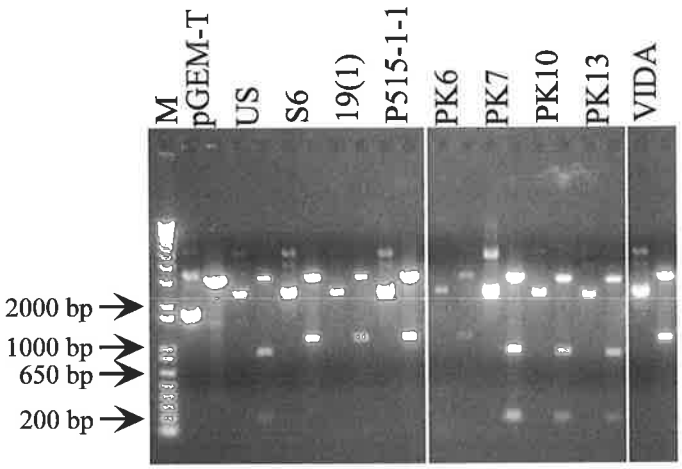


Figure 5.5: Clones of the 1085 bp RT-PCR generated *HC-Pro* region from various PSbMV isolates.

(M) 1Kb Plus ladder. (pGEM-T) non-recombinant plasmid. Each plasmid is shown first in the supercoiled form followed by restriction with *Eco*RI. Each of the PSbMV isolates cloned are representatives of the eight groups described from RFLP analysis (Table 5.2). PSbMV US (Group 1), S6 and VIDA (Group 2), P515-1-1 (Group 3), 19(1) (Group 4), PK7 (Group 5), PK6 (Group 6), PK 10 (Group 7), and PK13 (Group 8).

CHAPTER 6

DEVELOPING AN INTERNAL CONTROL FOR PSbMV RT-PCR

6.1 Introduction

The reverse transcription polymerase chain reaction (RT-PCR) is increasingly being used for the detection of RNA viruses because of its high sensitivity and specificity. New RT-PCR diagnostic methods have been recently described in the field of plant virology (Raj *et al.*, 1998; Jacobi *et al.*, 1998; Grieco and Gallitelli, 1999; Eun *et al.*, 2000). However, none of these methods describe the use of internal controls. Assays usually include a positive (with added control template) and negative (no sample added to the reaction mix) reaction. For quality assurance purposes it is important that diagnostic RT-PCR assays also have an internal standard for detecting false negative reactions. This is because negative RT-PCR results may either be real due to the absence of the target template, or false due to the failure of the RT-PCR.

For medical diagnostics with PCR and RT-PCR the issue of quality assurance has been addressed and several internal control methods have been developed (Denis and Lustenberger, 1995; Pallen *et al.*, 1992; Brightwell *et al.*, 1998; Sachadyn and Kur, 1998; Willhauck *et al.*, 1998). A disadvantage of using endogenously expressed "housekeeping" genes as internal controls is that their level of expression can vary so that amplification conditions for the control may not be suitable for detection of the pathogen. The construction of internal controls by altering the size or sequence of PCR products using cloning techniques is laborious and system specific. Other complications can arise from the formation of heteroduplexes during PCR due to sequence similarity between the target and control templates, or variable efficiency of extraction of added control sequence.

This chapter describes the development of an exogenous internal control based on TMV for use in a PSbMV diagnostic RT-PCR assay.

6.2 Materials and Methods

6.2.1 Purification of TMV

TMV U1 was partially purified from systemically infected *Nicotiana tabacum* leaves. Leaves (20 gm) were crushed in an equal volume of 0.2 M phosphate buffer (pH 7) containing 0.1% monothioglycerol, fibre was removed and the extract was clarified by heating at 60°C for 10 min and centrifugation at 10,000 *g* for 10 min, followed by a single chloroform extraction. Virus was concentrated by precipitation for 30 min with 0.5 volume

of saturated ammonium sulphate and pelleted by centrifugation at 10,000 *g* for 10 min. The pellet was resuspended in 20 mM phosphate buffer, pH 7, and dialysis against SDDW at 4°C for 16 hrs. This was repeated 2 more times. It was then clarified by centrifugation at 10,000 *g* and sedimented by ultracentrifugation at 87,000 *g* for 45 min in an Ultracentrifuge TL-100 (Beckman, USA). Pellets were resuspended in 10 mM Na borate buffer (pH 8.2) and stored at 4°C. Using a value for $E^{0.1\%}_{1cm}$ at 260 nm of 3 concentration was adjusted to 10 mg/ml,

6.2.1.1 TMV RNA extraction

TMV (1mg/ml) was incubated with 1mg/ml Proteinase K in Proteinase K buffer (Appendix B) for 3-5 hrs at 37°C. The aqueous phase was extracted once with an equal volume of phenol saturated with 100 mM Tris-HCl (pH 7) and once with an equal volume of chloroform saturated DEPC SDDW. RNA was recovered by precipitation with 3 vol. biotechnology grade ethanol (Amresco, USA) in the presence of 0.3 M sodium acetate (pH 5.2). The pellet was washed once with 70% ethanol and dried. It was resuspended in 30 µL water and the TMV-RNA concentration was determined by spectrophotometry and its size and quality was determined by agarose gel electrophoresis.

6.2.2 PSbMV RNA extraction

PSbMV RNA was extracted as above from purified PSbMV (section 2.2.3).

6.2.3 Sources of PSbMV infected seed

Pea seedlots infected with PSbMV at rates of 1-4 % were supplied by D. Graetz (Plant Pathology Unit, South Australian Research and Development Institute, Australia).

6.2.4 Extraction of total nucleic acid from pea seeds

Total nucleic acid was extracted from pea seeds (section 2.2.4.2).

6.2.5 DIBA

Both pea seed and leaf tissue were tested by DIBA (section 2.2.7).

6.2.6 TMV primers

The TMV RNA sequence (variant 1) (GenBank accession numbers V01408; J02415; Goelet *et al.*, 1982) was used to design all TMV RT-PCR primers. Figure 6.1 shows the position and sequence of all TMV primers.

6.2.7 PSbMV specific RT-PCR

This was as described in section 5.2.3

6.2.8 TMV RT-PCR

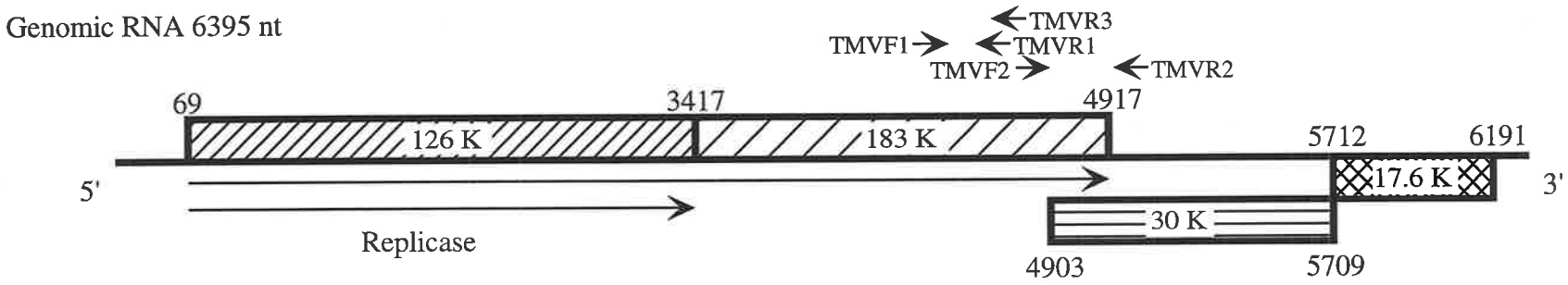
RT-PCR was done in a two step reaction. Reverse transcription was done in a 10 µL reaction volume containing 1 µL TMV-RNA or partially purified TMV, 0.4 µM TMVR1, TMVR2 or TMVR3 RT primer, 25 mM Tris HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.8 mM dNTPs, 1 U/µL RNase Inhibitor and 5 U AMV Reverse Transcriptase. Reverse primer and the TMV template were initially incubated at 70°C for 10 min and then quenched on ice. The remainder of the reagents were added and the RT done at 45°C for 40 min.

6.2.9 Conditions for combined TMV and PSbMV "duplex" RT-PCR

RT-PCR was done in a two step reaction. RT was done in a 15 µL reaction volume containing 2 µL total nucleic acid from pea seed, 0.4 µM VT04, 0.2 µM TMVR3, 10 pg TMV-RNA, 25 mM Tris HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.8 mM dNTPs, 1 U/µL RNase Inhibitor and 7.5 U AMV Reverse Transcriptase. RT was done at 45°C for 40 min. PCR was done in a 20 µL reaction volume containing 2 µL cDNA from the RT step, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% Triton® X-100, 0.8 mM dNTPs, 0.4 µM each VT02 and VT03 primers, 0.2 µM each TMVF1 and TMVR3 primers and 1U *Taq* DNA polymerase.

6.2.9.1 PCR cycling conditions

RT-PCR was done either in a PTC-225 DNA Engine Tetrad (MJ Research Inc., USA) or GeneAmp PCR System 2400 (Perkin Elmer, USA). PCR mixtures were initially incubated at 94°C for 3min followed by 20 cycles consisting of, denaturation at 94°C for 30 sec, annealing at 68°C for 30 sec and extension at 72°C for 30 sec, with a final extension step of



TMVF1 (5'-TAC CCG GCT TTG CAG ACG ATT GTG TAC CA-3') position 4023-4051

TMVF2 (5'-ATC AAA GGA GCC TTT TGC GGT GAC GAT AG-3') position 4482-4510

TMVR1 (5'-GTG ACG TCC CCG CTC TTT CTT TGA TAC CA-3') position 4414-4386

TMVR2 (5'-AAT TGT CAG GCA AGT TCC ACT CGC CCG-3') position 5152-5126

TMVR3 (5'-TTC CAA TGA ACG TCG TGA CGT C-3') position 4428-4407

Figure 6.1: TMV genome organisation (Matthews, 1992) showing positions and sequences of RT-PCR primers used for developing duplex RT-PCR

72°C for 2 min. RT-PCR products were analyzed by electrophoresis in 1.5% agarose gels buffered in 1 x TAE, pH 8, and containing 0.5 µg/mL ethidium bromide.

6.2.10 Calculation of seed infection rate for batch testing

PSbMV seed infection rate was calculated using:

$L = 100(1 - \sqrt[n]{1-G})$ (Gibbs and Gower, 1960).

Where L = percentage seed transmission, n = number of seeds in each batch, and G = decimal percentage of positive batches.

6.3 Results

6.3.1 Testing TMV RT-PCR primer combinations

To determine the best TMV primers for incorporation into a duplex TMV/PSbMV RT-PCR the TMV RT-PCR was done under the same conditions as used for the PSbMV RT-PCR. Figure 6.2 shows RT-PCR of TMV RNA with combinations of TMVR1, TMVR2, TMVF1 and TMVF2 primers. RT with TMVR1 and PCR with TMVF1/TMVR1 on TMV RNA generated a single band of predicted size, 392 bp (Figure 6.2 lane 1). RT with TMVR2 and PCR with TMVF1/TMVR1 generated the expected 392 bp fragment in addition to three other fragments, 1100 bp, 900 bp and 200 bp (Figure 6.2 lane 2). RT with TMVR2 and PCR with TMVF1/TMVR2 also generated four bands, the expected 1131 bp fragment, and smaller 800 bp, 400 bp and 200 bp fragments (Figure 6.2 lane 3). RT with TMVR2 and PCR with TMVF2/TMVR2 generated the expected 671 bp fragment in addition to a 900 bp and 400 bp fragment (Figure 6.2 lane 4).

6.3.2 Incorporating the TMV and PSbMV RT-PCR

As only the TMVF1/TMVR1 primer pair amplified a single product from TMV RNA it was tested in combination with the PSbMV RT-PCR (Figure 6.3). When total nucleic acid extracted from healthy pea was used as a template for amplification with the TMVF1/TMVR1 RT-PCR primers, a faint 200 bp product was observed (Figure 6.3 lane1). When total nucleic acid from healthy pea was used as a template for amplification with the PSbMV VT04 RT and VT02/VT03 PCR primers, no product was detected (Figure 6.3 lane 5). When PSbMV RNA was used as a template for the TMV primers a faint 400 bp product was detected (Figure 6.3 lane 2) This was also observed in the TMV/PSbMV

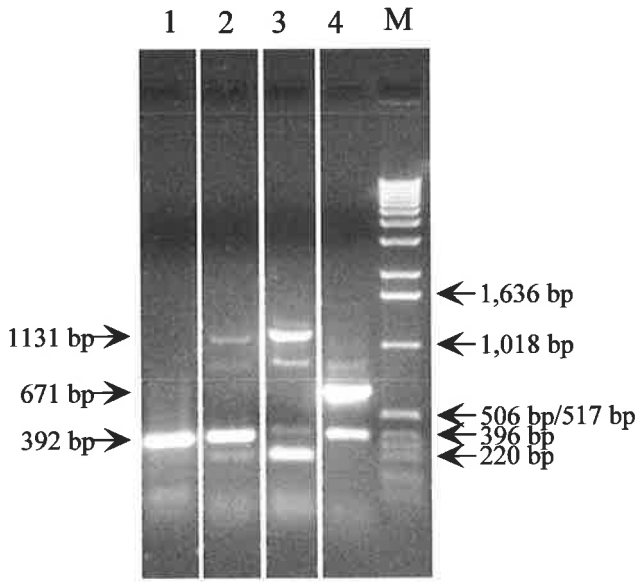


Figure 6.2: Testing different primer combinations in RT-PCR on TMV RNA.

(1) RT with TMVR1 and PCR with TMVF1/TMVR1.

(2) RT with TMVR2 and PCR with TMVF1/TMVR1.

(3) RT with TMVR2 and PCR with TMVF1/TMVR2.

(4) RT with TMVR2 and PCR with TMVF2/TMVR2.

(M) 1 Kb ladder.

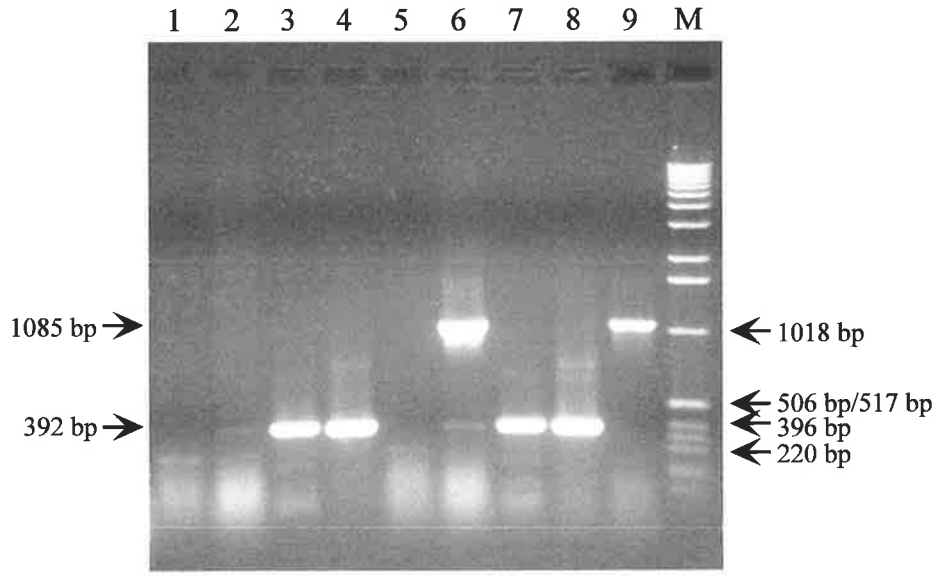


Figure 6.3: Testing duplex RT-PCR primers VT02/VT03 and TMVF1/TMVR1.

RT with TMVR1 and PCR with TMVF1/TMVR1 for:

(1) TNA from healthy pea;

(2) PSbMV US RNA;

(3) TMV RNA;

(4) Partially purified TMV.

RT with TMVR1 and VT04 and PCR with TMVF1/TMVR1 and VT02/VT03 for:

(5) TNA from healthy pea;

(6) PSbMV US RNA;

(7) TMV RNA;

(8) Partially purified TMV.

RT with VT04 and PCR with VT02/VT03 for:

(9) PSbMV US RNA.

(M) 1 kb Ladder.

duplex RT-PCR containing PSbMV RNA template only (Figure 6.3 lane 6) in which the expected 1085 bp PSbMV fragment was produced. A single 392 bp fragment was produced either when TMV RNA or partially purified TMV was used as a template with TMVF1/TMVR1 primers (Figure 6.3 lanes 3 and 4, respectively) or when TMV RNA or TMV alone was used as a template in the TMV/PSbMV duplex RT-PCR (Figure 6.3 lanes 7 and 8, respectively).

6.3.3 Modifying the TMV and PSbMV RT-PCR

As the TMVF1/TMVR1 primers were found to produce non-specific amplification from healthy pea extracts and PSbMV RNA template a new TMV reverse primer was tested (TMVR3) for incorporation into the duplex RT-PCR system. Figure 6.4 shows RT-PCR with TMV primers only, PSbMV primers only and duplex TMV/PSbMV primers with PSbMV RNA, TMV RNA or mixtures of both templates. PSbMV VT04 RT and VT02/VT03 PCR primers alone amplified the expected 1085 bp product from PSbMV RNA (Figure 6.4 lanes 1 and 2). TMVF1/TMVR3 primers alone amplified the expected 406 bp product from TMV template (Figure 6.4 lanes 3 and 4). PSbMV-RNA with all four TMV/PSbMV primers amplified the 1085 bp product alone (Figure 6.4 lane 6), and TMV-RNA template with all four TMV/PSbMV primers amplified a 406 bp product only (Figure 6.4 lane 7). In the duplex PSbMV/TMV RT-PCR both the PSbMV template RNA and the TMV control RNA were amplified without apparent interference from each other (Figure 6.4 lane 8).

6.3.4 Sensitivity of duplex PSbMV/TMV RT-PCR

Figure 6.5 shows the effect on the sensitivity of PSbMV RT-PCR when the TMV internal control RT-PCR is added. Figure 6.5 A shows that the level of detection of PSbMV by duplex RT-PCR was approximately 125 pg of PSbMV RNA per 15 µL RT reaction. When 10 pg of TMV RNA was added to the 15 µL RT reaction the level of PSbMV detection was still approximately 125 pg of PSbMV RNA per 15 µL RT reaction (Figure 6.5 B). This level of sensitivity remained approximately the same when 100 pg of TMV RNA was added to the RT reaction (Figure 6.5 C). However, when 1 ng TMV RNA was added to the RT the level of sensitivity for PSbMV detection dropped to approximately 500 pg of PSbMV RNA per 15 µl RT (Figure 6.5 D).

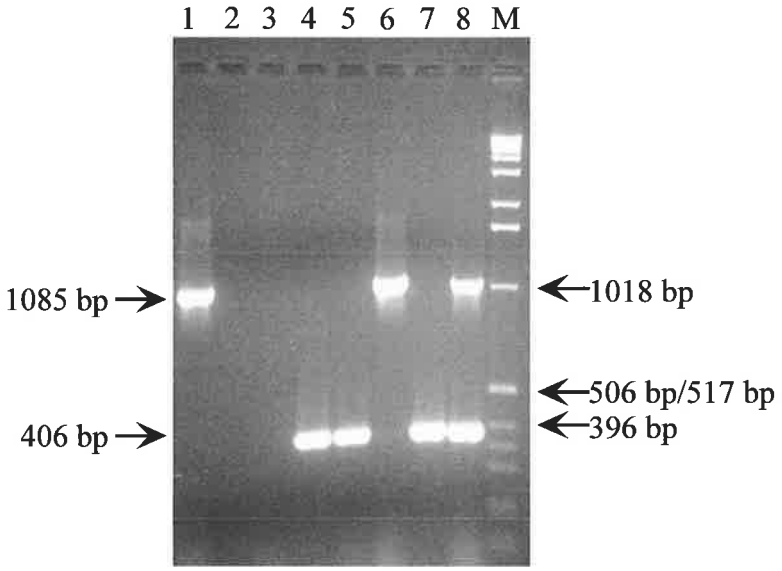


Figure 6.4: Duplex RT-PCR with TMVF1/TMVR3 and VT02/VT03.

RT with VT04 and PCR with VT02/VT03 from:

(1) PSbMV US RNA;

(2) TMV RNA.

RT with TMVR3 and PCR with TMVF1/TMVR3 from:

(3) PSbMV US RNA;

(4) TMV RNA;

(5) PSbMV US and TMV RNA mixtures.

RT with TMVR3 and VT04 and PCR with TMVF1/TMVR3 and VT02/VT03 from:

(6) PSbMV US RNA;

(7) TMV RNA;

(8) PSbMV US and TMV RNA mixtures.

(M) 1 Kb ladder.

Figure 6.5: Duplex PSbMV/TMV RT-PCR with differing concentrations of PSbMV and TMV RNA template.

(A) Duplex RT-PCR on a dilution series of PSbMV RNA template.

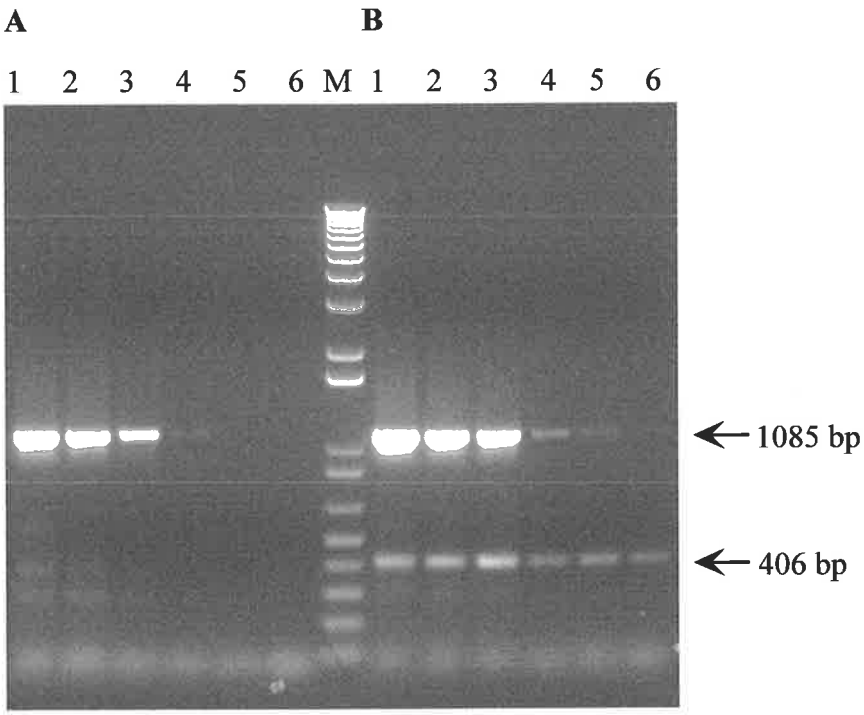
(B) Duplex RT-PCR on a dilution series of PSbMV RNA templates containing 10 pg of TMV RNA control per 15 µL RT reaction.

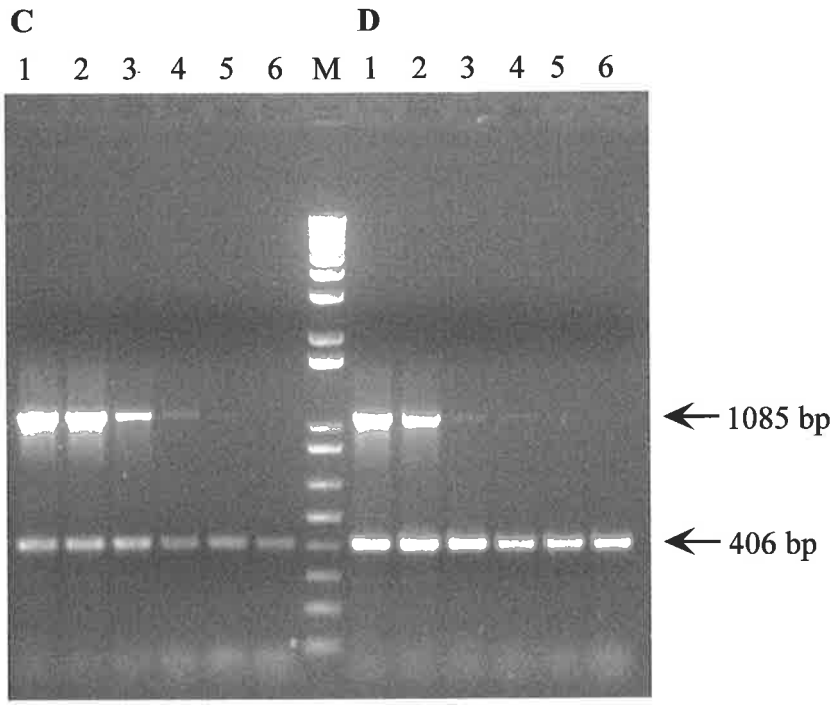
(C) Duplex RT-PCR on a dilution series of PSbMV RNA template containing 100 pg of TMV RNA control per 15 µL reaction.

(D) Duplex RT-PCR on a dilution series of PSbMV RNA template containing 1ng of TMV RNA control per 15 µL RT reaction.

Amount of PSbMV RNA per 15 µL RT reaction: (1) 1 ng; (2) 500 pg; (3) 250 pg; (4) 125 pg; (5) 63 pg; (6) 31 pg.

(M) 1kb Plus Ladder.


A
B
1 2 3 4 5 6 M 1 2 3 4 5 6
1085 bp
406 bp


C
D
1 2 3 4 5 6 M 1 2 3 4 5 6
1085 bp
406 bp

6.3.5 Using the TMV internal control in batch testing of pea seed

Figure 6.6 shows the use of the TMV internal standard in batch testing of pea seed for PSbMV. Figure 6.6 A shows that the amounts of nucleic acids extracted from 25 batches of pea seed (each consisting of 10 seeds per batch) by the extraction procedure were approximately the same for each batch. Figure 6.6 B shows the use of the TMV internal control in the testing of pea seeds for PSbMV. Each of the 25 RT-PCR reactions contained a sample from 10 pea seeds and the TMV internal control. The figure shows that 24 of the 25 duplex RT-PCR reactions contained a 406 bp TMV amplicon indicating that these RT-PCR reactions had been successful. Lane 5 lacked the 406bp TMV amplicon indicating that this RT-PCR reaction had failed. A 1085 bp PSbMV specific amplicon was present in lanes 7, 10, 14, 17, 21 (Figure 6.6 B) indicating that these samples were positive for PSbMV.

6.3.6 Direct comparison of RT-PCR and DIBA for seed testing

To determine if RT-PCR testing of pea seeds was an accurate representation of PSbMV positive samples, each batch of pea flour was divided in two and half was tested by RT-PCR while the other half was tested by DIBA.

Figure 6.7 shows the duplex RT-PCR testing of 940 pea seeds in batches consisting of 10 seeds each. Three of the 94 RT-PCRs failed (samples 61, 72 and 84) as shown by the absence of the 406 bp TMV amplicon. Thirty-nine of the successful 91 RT-PCRs were positive for PSbMV as indicated by the presence of a 1085 bp PSbMV specific amplicon. The intensity of the PSbMV positive signal varied from strong to weak.

Figure 6.8 shows the result of DIBA done on a sub-sample of pea flour from the same pea seed batches as tested by RT-PCR (Figure 6.7). Thirty PSbMV positive samples were detected by DIBA with signals varying from weak to strong. The threshold level of detection of PSbMV by DIBA was 44 ng of PSbMV per 1 µL spot.

Table 6.1 shows the correlated results obtained by RT-PCR and DIBA and calculates a seed infection rate for each method. All 30 samples that were DIBA positives also tested positive by RT-PCR, except sample 40. Nine additional samples tested positive by RT-PCR which were negative by DIBA. These positives, however, were all weak positive RT-

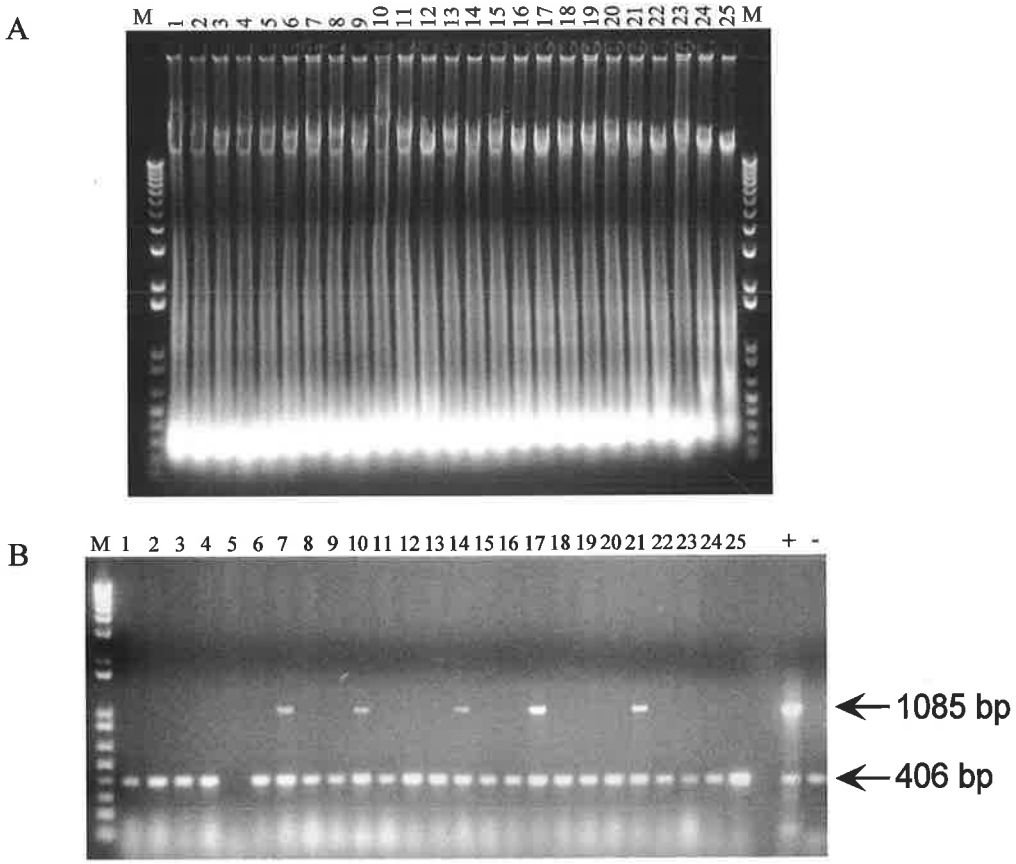


Figure 6.6: Total nucleic acid extraction from 25 batches of 10 pea seeds each and duplex PSbMV/TMV RT-PCR from those nucleic acid extracts.

(A) CTAB TNA extracts from 25 batches consisting of 10 dry pea seeds each.

(B) Duplex RT-PCR with above TNA extracts (1-25).

(+) Positive control containing PSbMV RNA and TMV RNA.

(-) Negative control containing TMV RNA only.

PSbMV positive samples are indicated by a 1085 bp amplicon and TMV positive samples are indicated by a 406 bp amplicon. Note that lane 5 lacks the 406 bp TMV specific amplicon indicating that the RT-PCR reaction had failed.

(M) 1kb Plus Ladder.

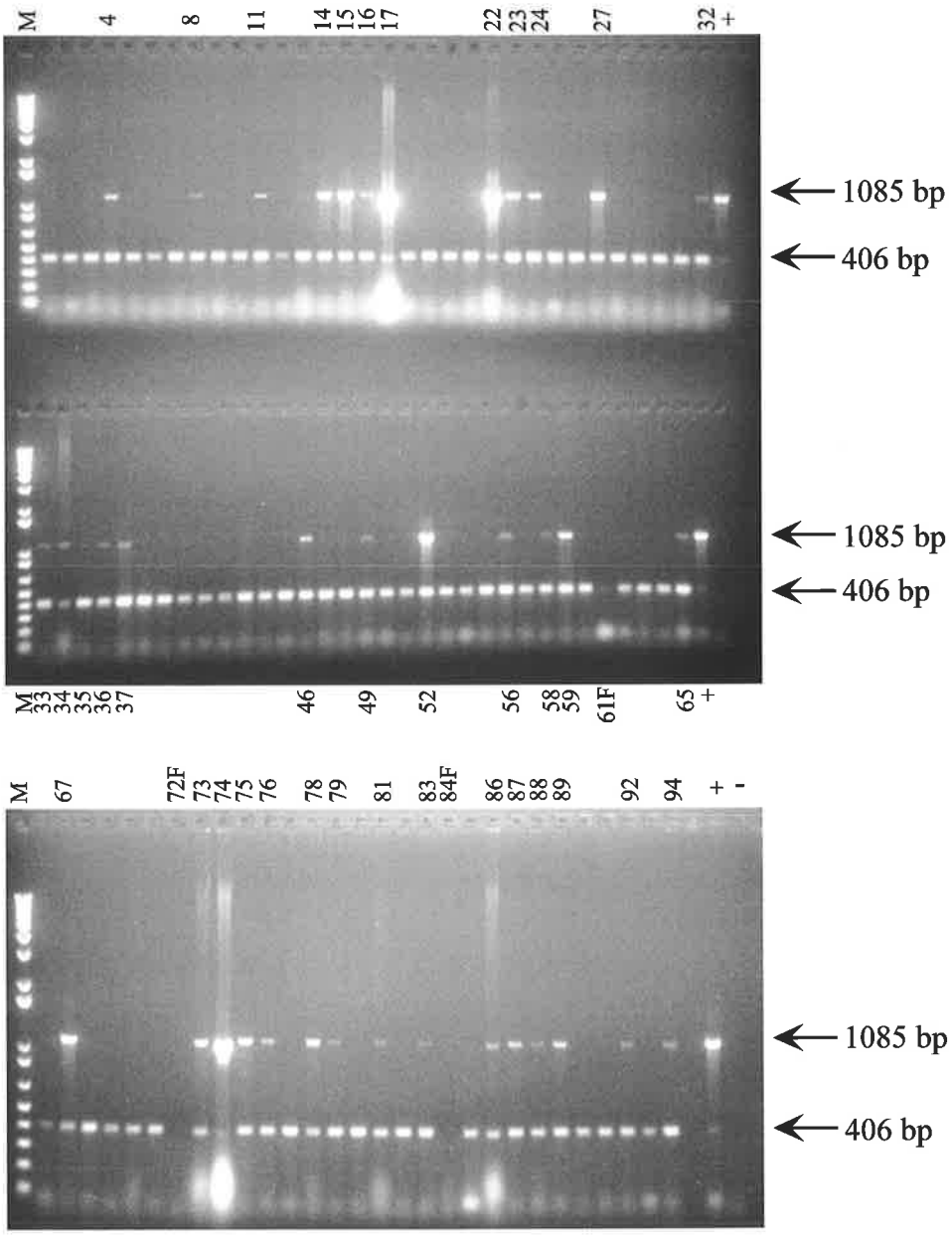


Figure 6.7: Batch testing pea seeds for PSbMV by duplex PSbMV/TMV RT-PCR.

Duplex RT-PCR was done on 94 batches of 10 pea seeds each.

PSbMV positive reactions are numbered.

(61F, 72F and 84F) are failed RT-PCR reactions.

(+) PSbMV positive RT-PCR control.

(-) RT-PCR negative control (no template).

(M) 1kb Plus Ladder.

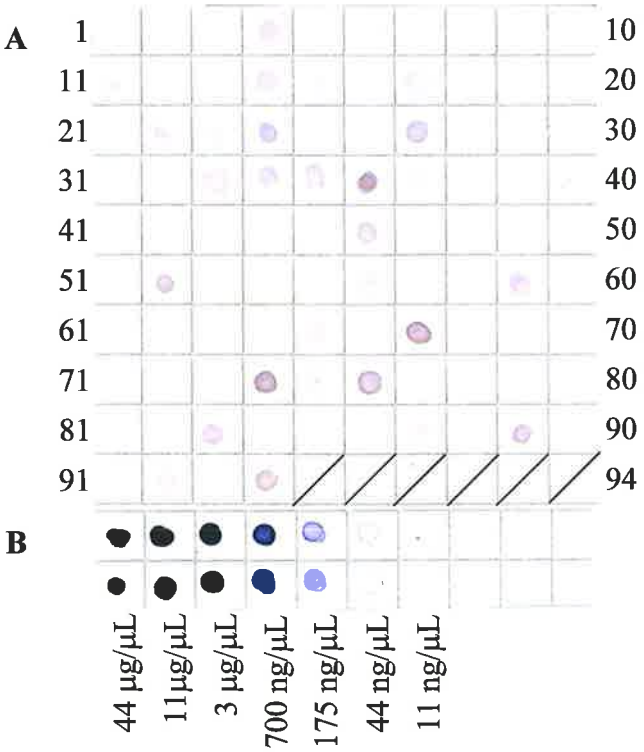


Figure 6.8: Batch testing of pea seeds for PSbMV infection by DIBA.

(A) DIBA on sub-samples of the same pea flour tested by RT-PCR (Figure 6.7). Samples were tested in duplicate on separate membranes. The results generated were identical, therefore only one membrane is shown. Numbers to left and right hand sides of the membrane are a guide to the position of samples. Each sample represents a batch of 10 pea seeds.

(B) Dilution series of purified PSbMV antigen.

Table 6.1: Comparing RT-PCR and DIBA in the detection of PSbMV from germplasm

PSbMV positive batches	Positive by RT-PCR	Positive by DIBA
4	++	++
8	+	-
11	++	+
14	+++	++
15	+++	+
16	++	-
17	++++	+
22	++++	+
23	+++	+
24	+++	++
27	+++	++
32	+	-
33	+	+
34	+	++
35	+	++
36	+	+++
37	++	+
40	-	+
46	++	++
49	+	-
52	+++	++
56	+	+
58	+	+
59	++	++
65	+	+
67	+++	+++
73	++	-
74	++++	+++
75	+++	+
76	++	+++
78	++	-
79	+	-
81	+	-
83	+	++
86	+	-
87	++	+
88	+	-
89	++	++
92	+	+
94	+	++
Total number of positives	39/91	30/94
PSbMV seed infection rate	5.4%	3.8%

Range of positive reactions from weak (+) to very strong (++++).

(-) Negative reaction observed by a particular testing method.

PCR reactions. Seed infection rates calculated from RT-PCR were 5.4% and from DIBA were 3.8%.

6.3.7 Testing pea seedling for PSbMV infection by DIBA

Two hundred individual pea seeds from the same seed lot tested by RT-PCR and DIBA were germinated and leaf tissue tested three weeks post emergence for PSbMV infection by DIBA. Figure 6.9 shows the results of the DIBA. Of the 200 seeds sown, 179 germinated and were subsequently tested. Nine seedlings tested positive giving a calculated seed infection rate of 5.0%.

6.4 Discussion

An exogenous TMV internal control for testing pea germplasm for PSbMV has been developed. Several TMV primers were designed and tested to determine those which would not give spurious products (bands not representing the expected PCR product) with templates in the duplex RT-PCR. The internal TMV RNA standard was designed to be amplified under the same conditions as the PSbMV specific RT-PCR assay but to produce a specific amplicon differing in size from the PSbMV amplicon. The TMV TMVF1/TMVR3 primers were shown to be better than other combinations for RT-PCR analysis of PSbMV RNA.

Thus, the RT-PCR of TMV-RNA with the TMVF1/TMVR3 primers produced a 406 bp amplicon from the 3' region of the 183K polymerase gene of TMV. TMV primers did not amplify PSbMV RNA and the PSbMV primers did not amplify TMV RNA. TMV RT-PCR was also successful when partially purified intact TMV was added at 1 µg per 10 µL RT as the template rather than RNA. The duplex RT-PCR assay allowed distinction between false negative reactions (failed RT-PCR) and true negative reactions (absence of pathogen template). This is important when using RT-PCR as a diagnostic method for certifying freedom from a specific seedborne pathogen.

TMV-RNA template can compete with the PSbMV-RNA template and limit the sensitivity of the test if its concentration is not accurately determined. When the concentration of the TMV-RNA template was 10 pg per 15 µL RT reaction, the sensitivity of the PSbMV assay was not affected. Under these conditions the reliable limit of PSbMV detection was 100 pg PSbMV-RNA per 15 µL RT reaction. This level of sensitivity was the same as for the

PSbMV duplex RT-PCR assay lacking TMV template. However, when the TMV-RNA control template was increased to 1 ng per 15 µL RT reaction the level of sensitivity for PSbMV was reduced to 500 pg PSbMV-RNA per 15 µL RT reaction.

Initially a larger TMV amplicon closer in size to the PSbMV amplicon was considered to be preferable because smaller PCR amplicons are expected to be amplified more efficiently than larger ones (Pallen *et al.*, 1992; Brightwell *et al.*, 1998) and may compete with the target template. Alternative TMV primers were designed to produce larger TMV amplicons, but these primers produced spurious bands in the TMV RT-PCR. It was concluded that by limiting the amount of TMV-RNA control added to the duplex RT-PCR, TMV would not be amplified in preference to PSbMV, overcoming the expected difficulties associated with the co-amplification of differently sized products. This method allows the concentration of control template to be optimised, unlike methods which use an endogenous control (Denis and Lustenberger, 1995; Nassuth *et al.*, 2000).

RT-PCR is more sensitive than DIBA as RT-PCR detected all but one of the DIBA positive reaction, however DIBA failed to detect 10 of the RT-PCR positive reactions. This therefore, resulted in a higher calculation for seed infection rates by the batch testing method based on RT-PCR results (5.4% by RT-PCR as compared with 3.8% by DIBA). These results when compared with testing of individual seedlings from the same seedlot (infection rate of 5.0%) suggest that RT-PCR yields a more accurate result. Testing by DIBA may therefore, underestimate seed infection rates due to its lower sensitivity. DIBA also has the disadvantage of not having an internal control and often producing background, making identification of weak positive reaction difficult. The possibility of detecting non-infectious PSbMV from testa was eliminated by the removal of seed coats prior to testing.

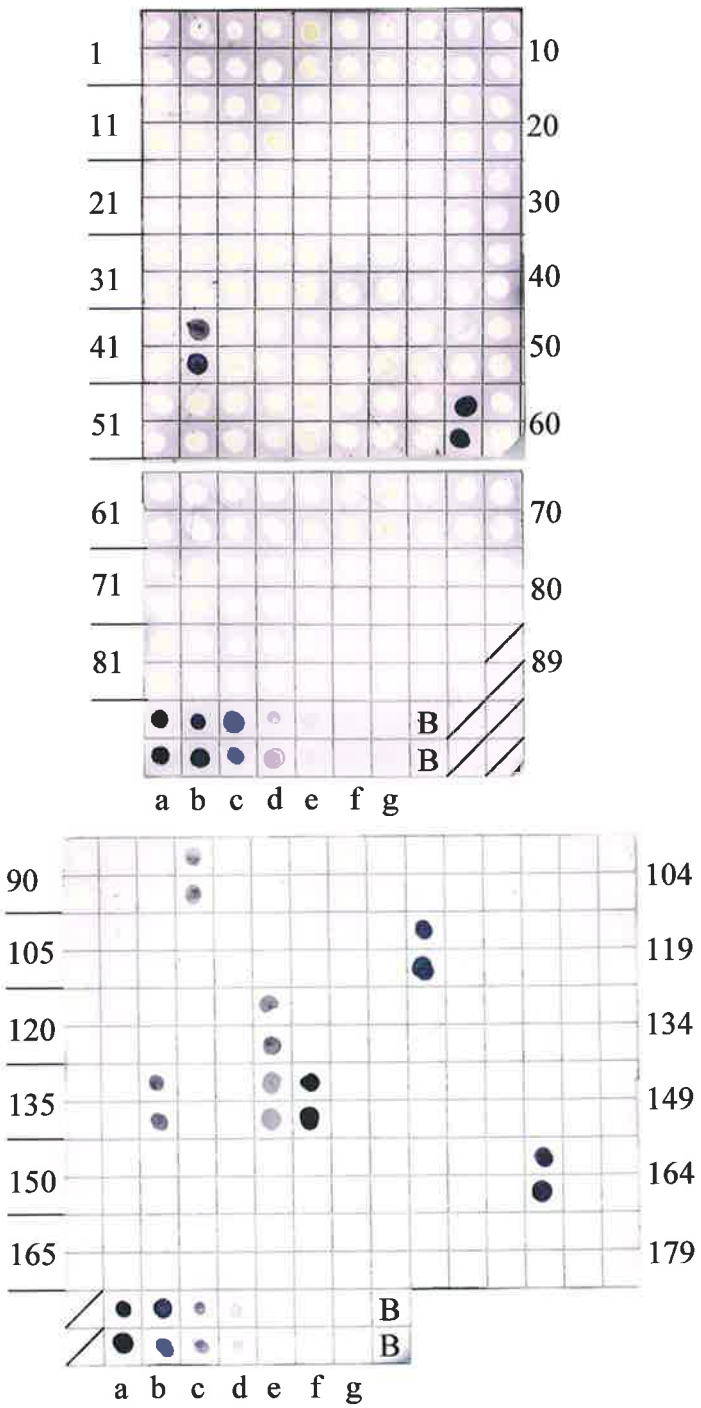


Figure 6.9: Testing pea seedlings by DIBA for PSbMV infection.

Two hundred pea seeds taken from the same seedlot as tested by RT-PCR and DIBA (Figure 6.7 and Figure 6.8) were germinated and emerged plants tested individually three weeks post-emergence by DIBA.

(a-g) PSbMV US infected pea sap diluted five fold in PBS.

(B) PBS blank.

CHAPTER 7

MOLECULAR ANALYSIS OF THE PSbMV VPg

7.1 Introduction

The VPg protein of PSbMV has been identified as the avirulence determinant of PSbMV DPD1 pathotype P-1 and has a putative function in virus replication (Keller *et al.*, 1998). This was determined by generating PSbMV chimeras between DPD1 pathotype P-1 and NY pathotype P-4 and testing their infectivity on the *sbm*-1 homozygous pea line PI 269818. The determinant region has been further narrowed down to 13 amino acids within the central part of the VPg (Borgstrom *et al.*, 1998). The potyviral VPg has also been shown to overcome resistance in certain other plant cultivars (Masuta *et al.*, 1999; Nicolas *et al.*, 1997; Rajamaki and Valkonen, 1999; Schaad *et al.*, 1997b).

The primary aim of this chapter was to characterise the VPg of PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4 and compare them with those of PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4. No PSbMV pathotype P-2 isolates were identified in the available PSbMV collection and PSbMV L1 pathotype P-2 sequence data became available after completion of this study. A second aim was to identify variable genomic regions between the PSbMV P-1 and P-4 pathotypes and develop a molecular method for differentiating between pathotypes. A third aim was to generate VPg sequence information from selected PSbMV isolates.

7.2 Materials and Methods

7.2.1 PSbMV isolates

Twenty-nine PSbMV isolates previously described and tested were used in this study: 15 Australian PSbMV isolates (Table 3.1), 12 Pakistani PSbMV isolates (Ali and Randles, 1997) and two PSbMV type isolates (PSbMV-US pathotype P-1 and PSbMV-S6 pathotype P-4).

7.2.2 RT-PCR

7.2.2.1 Primers

Primers were designed to amplify the *CI*, *NIa*, and *NIb* genes of two pathotypes of PSbMV (PSbMV DPD1 pathotype P-1; GenBank accession number D10930 and PSbMV NY pathotype P-4;EMBL accession number X89997) (section 2.2.11). Reverse transcription and PCR was done with VTNIa (5' CTA CAC ACG GCT TGC GCA AT 3') located within the PSbMV proteinase domain of the *NIa* gene, position 6649-6630 of PSbMV

DPD1, or VTNIb (5' CAC AAT ACA GCC AAC CGT CAG GTA G 3') located within the *NIb* gene, position 8064-8040 of PSbMV DPD1. PCR was also done with VTCI (5' TCG CAG GTT TAG TCA ACA GAT TGC G 3') located within the PSbMV *CI* gene, position 5674-5798 of PSbMV DPD1 or VTVPg (5' TAT CGC AGA CCC TCA TTG GAT T 3') located within the PSbMV VPg domain of the *NIa* gene, position 6453-6432 of PSbMV DPD1.

7.2.2.2 RT-PCR conditions

RT-PCR was done in a two step reaction. RT was done in a reaction volume of 10 µL containing 1 µL CTAB extract (section 2.2.4.1) or 40 ng purified PSbMV-RNA (section 4.2.3), 0.4 µM VTNIa or VTNIb primer, 25 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.8 mM dNTPs, 1 U/µL RNase Inhibitor, and 5 U AMV RT. Nucleic acid and primer were incubated in a volume of 5 µL at 70°C for 10 min and then quenched on ice. The remainder of the RT components were added and the reaction incubated at 42°C for 40 minutes.

PCR was done in a 10 µL reaction volume containing 1 µL cDNA from the RT step, 0.4 µM each forward and reverse primers, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl, pH 9.0, 0.1% Triton® X-100, 0.8 mM dNTPs, and 1U *Taq* DNA polymerase.

Alternatively, PCR fragments generated for cloning were done with the proof-reading DNA polymerase, Pwo. PCR was done in a 20 µL reaction volume containing 2 µL cDNA from the RT step, 10 mM Tris-HCl, pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.8 mM dNTPs, 0.4 µM each primer and 2.5 U Pwo DNA polymerase.

7.2.2.3 PCR cycling conditions

RT-PCR was done in a GeneAmp PCR System 2400 (Perkin Elmer, USA).

For PCR with (VTNIa and VTCI) or (VTNIb and VTCI) primer pairs mixtures were initially incubated at 94°C for 3 min followed by 30 cycles consisting of: denaturation at 94°C for 45 sec; annealing at 60°C for 1 min; and extension at 72°C for 1 min 30 sec, with a final extension step of 72°C for 8 min.

For PCR with the VTVPg and VTCI primer pair mixtures were initially incubated at 94°C for 3 min followed by 30 cycles consisting of: denaturation at 94°C for 1 min; annealing at 55°C for 1 min; and extension at 72°C for 1 min, with a final extension step of 72°C for 2 min.

RT-PCR products were analysed by electrophoresis in 1.5% agarose gels containing 0.5 µg/mL EtBr and buffered in 1 x TAE.

7.2.3 RFLP analysis

The VTCI/VTVPg generated amplicon from PSbMV were restricted with *Mfe*I (C↓AATT↑G) restriction endonucleases (New England BioLabs, USA) according to the manufacturer's instructions. RFLP products were analysed by electrophoresis in a 2% agarose gel containing 0.5 µg/mL EtBr and buffered in 1 x TAE.

7.2.4 Cloning and sequencing

PCR fragments generated with Pwo polymerase were excised from agarose gels following electrophoresis and purified (section 2.2.12). Blunt end PCR fragments were A-tailed (section 2.2.8.2) and ligated into pGEM®-T vector (section 2.2.8.3) and transformed into competent DH5α cells (section 2.2.8.4). Recombinant plasmids were purified (section 2.2.9) and analysed for the presence of insert (section 2.2.10.1). Purified plasmid DNA was sequenced (section 2.2.20) using T7 (Appendix C), SP6 (Appendix C), and VTVPgseq, (5' ATA GGG TGT CTA TCA TAA GTC AAC CC 3' located at position 6268-6243 of PSbMV DPD1) HPLC purified primers.

7.2.5 Sequence analysis

All sequence analysis was done using the WebANGIS (Australian National Genomic Information Services) interface (http://www.angis.org.au/). Protein sequences were deduced from nucleotide sequences using the "Etranslate" program. Both nucleotide and amino acid sequences were aligned using the multiple sequence alignment program "EClustALW" (Thompson *et al.*, 1994). Percentage sequence similarity was calculated from multiple sequence alignments using the "Gendoc" program. Hydrophobicity profiles were generated with "Pepwindow" (Kyte and Doolittle, 1982). Restriction endonuclease sites were predicted or confirmed from known nucleotide sequences using the "Map" program.

7.3 Results

7.3.1 Comparisons of published PSbMV pathotype P-1 and P4 VPg peptide sequences

Figure 7.1 shows the alignment of the VPg amino acid sequence for PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4. Both were 194 amino acids in length. Thirty-six amino acid differences were identified between the two pathotypes. Thirteen of these changes (amino acids 20, 23, 28, 55, 70, 105, 106, 117, 129, 130, 149, 161, and 172) were conservative changes (chemically similar residues), while 23 were non-conservative amino acid differences (amino acids 29, 35, 36, 52, 86, 96, 109, 112, 114, 115, 116, 118, 119, 139, 150, 160, 168, 173, 176, 179, 186, 187 and 190). Most of the amino acid differences were located within the central and carboxy- terminus of the VPg, with the highest proportion of changes found within the central region of the VPg (position 105 to 119).

7.3.2 RT-PCR amplification of CI, NIa and NIb of PSbMV US and S6

Figure 7.2 shows the RT-PCR amplification products from PSbMV RNA with the VTCI/VTNIa and VTCI/VTNIb primer pairs and the effects of using different DNA polymerases (*Taq* and Pwo) under identical reaction conditions. *Taq* DNA polymerase failed to amplify PSbMV US and S6 cDNA generated with VTNIb RT primer and amplified with the VTCI/VTNIa primer pair (Figure 7.2 lane 2 and 6 respectively). *Taq* DNA polymerase had a low efficiency of amplification from PSbMV US cDNA and failed to amplify PSbMV S6 cDNA generated with VTNIa RT primer and amplified with the VTCI/VTNIa primer pair (Figure 7.2 lanes 1 and 5 respectively). However, Pwo DNA polymerase, under identical reaction conditions, gave amplification products from both PSbMV US and S6 cDNA generated with VTNIa RT primer and amplified with the VTCI/VTNIa primer pair (Figure 7.2 lanes 3 and 7 respectively). Pwo also gave amplification products for PSbMV US and S6 cDNA generated with VTNIb RT primer and amplified with VTCI/VTNIb primer pair (Figure 2 lanes 4 and 8 respectively).

Reverse transcription with VTNIa and PCR with VTCI and VTNIa amplified a single 976 bp fragment of expected size from PSbMV US RNA (Figure 7.2 lane 3), and 976 bp and 350 bp fragments from PSbMV S6 RNA (Figure 7.2 lane 7). RT with VTNIb and PCR with VTCI and VTNIb amplified a 2390 bp band of expected size and smaller (≤ 500 bp)

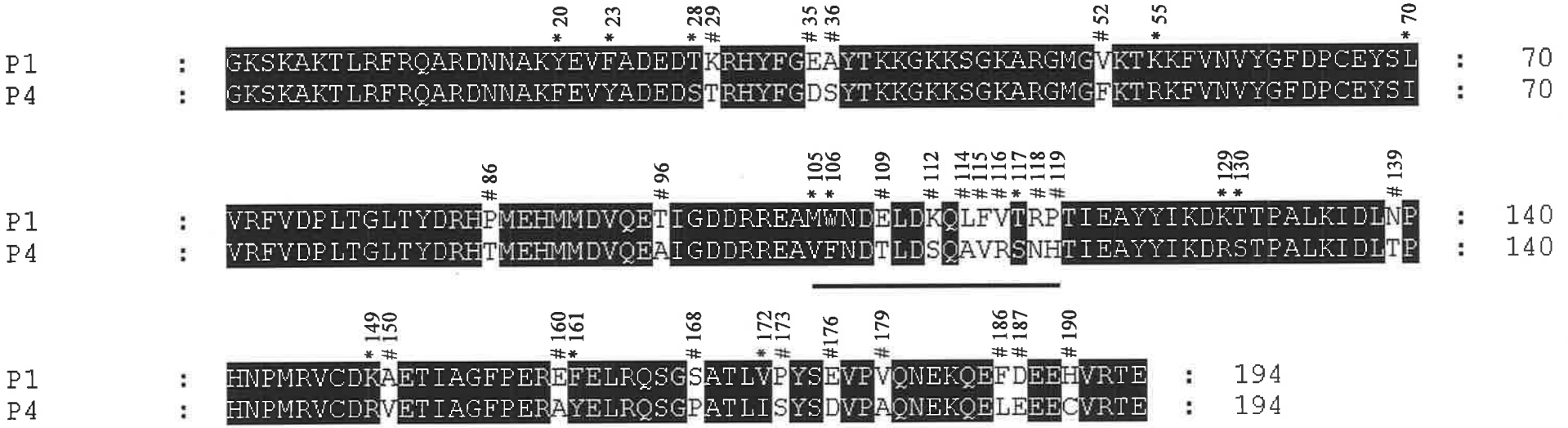


Figure 7.1: Amino acid comparison of the VPg from PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4.

* Indicates the position of conservative amino acid changes such as (D,N), (E,Q), (S,T), (K,R), (F,Y,W), (L,I,V,M).

# Indicates the position of non-conservative amino acid changes.

Underlined area indicates a highly variable region within the VPg.

(P1) PSbMV DPD1 pathotype P-1.

(P4) PSbMV NY pathotype P-4.

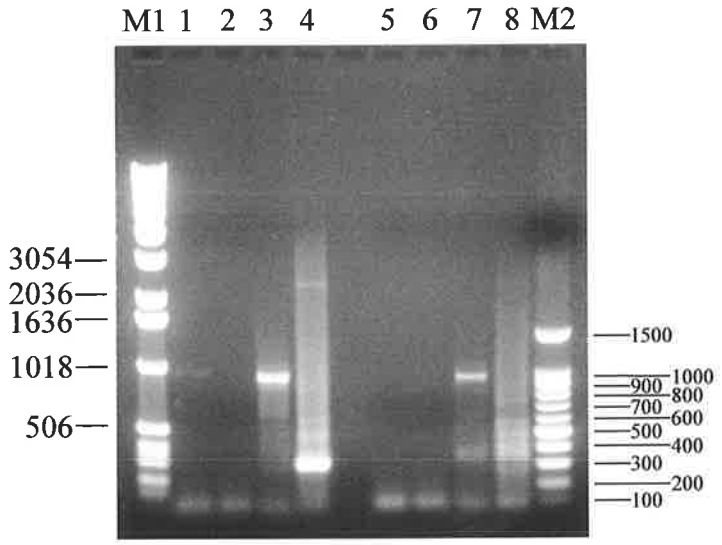


Figure 7.2: RT-PCR amplification of PSbMV *CI*, *6K*$_2$, *NIa* and *NIb* regions.

RT with VTNIa primer and PCR with VTCI/VTNIa primer pair for:

(1) PSbMV US RNA with *Taq* DNA polymerase;

(3) PSbMV US RNA with Pwo DNA polymerase;

(5) PSbMV S6 RNA with *Taq* DNA polymerase;

(7) PSbMV S6 RNA with Pwo DNA polymerase.

RT with VTNIb primer and PCR with VTCI/VTNIa primer pair for:

(2) PSbMV US RNA with *Taq* DNA polymerase;

(6) PSbMV S6 RNA with *Taq* DNA polymerase.

RT with VTNIb primer and PCR with VTCI/VTNIb primer pair for:

(4) PSbMV US RNA with Pwo DNA polymerase;

(8) PSbMV S6 RNA with Pwo DNA polymerase.

(M1) 1kb Ladder. (M2) 100 bp DNA Ladder.

non specific fragments from PSbMV US RNA (Figure 7.2 lane 4), while only non specific fragments (≤ 700 bp) were amplified from PSbMV S6 RNA (Figure 7.2 lane 8).

7.3.2.1 Cloning of the 976 bp CI/NIa RT-PCR fragment from PSbMV US and PSbMV S6

Figure 7.3 A shows the 976 bp agarose gel purified RT-PCR products generated from PSbMV US and S6 RNA with the VTNIa RT and VTCI/VTNIa PCR primers using Pwo DNA polymerase. Figure 7.3 B shows the size of inserts recovered from PSbMV US and S6 976 bp CI/NIa recombinant pGEM®-T plasmids following *Eco*RI restriction. The PSbMV US CI/NIa clone generated two fragments (850 bp and 150 bp), in addition to the vector fragment (3000 bp), indicating that there is an internal *Eco*RI restriction site in the US CI/NIa insert. The PSbMV S6 CI/NIa clone generated a single 1000 bp fragment of expected size in addition to the 3000 bp vector fragment.

7.3.2.2 Comparisons of CI/VPg peptide from PSbMV pathotype P-1 and P-4 isolates.

Figure 7.4 shows the 297 amino acid sequence alignment from the carboxy terminus of the CI, $6K_2$ and entire VPg of PSbMV DPD1 pathotype P-1, PSbMV US pathotype P-1, PSbMV NY pathotype P-4 and PSbMV S6 pathotype P-4. The VPg from all four PSbMV isolates is 194 amino acids in length. All 36 amino acid differences described between PSbMV DPD1 and PSbMV NY (Figure 7.1), with the exception of amino acid positions 28 and 150, were also found between PSbMV US and S6 (Figure 7.4). Amino acid positions 28 and 150 in both PSbMV US and S6 were identical to PSbMV NY pathotype P-4. Three additional conservative changes were identified at amino acid positions 50, 78 and 81. VPg amino acid positions 50 and 81 differed in PSbMV S6 only, whereas amino acid position 78 differed in PSbMV US only. The VPg amino acid sequences of PSbMV NY and S6 (both pathotype P-4) were identical except for amino acid positions 50 and 81 and the VPg amino acid sequences of PSbMV DPD1 and US (both pathotype P-1) were identical with the exception of amino acid positions 28, 78 and 150. There was a high level of amino acid variability within the central region of the VPg, position 105 to 119, between the pathotype P-1 (DPD1 and US) and pathotype P-4 (NY and S6) PSbMV representatives.

The 297 amino acid sequence representing the carboxy terminus of the CI, $6K_2$ and entire VPg showed 97% similarity between PSbMV US and DPD1, 98% similarity between PSbMV S6 and NY, 85% similarity between PSbMV US and NY and 84% similarity

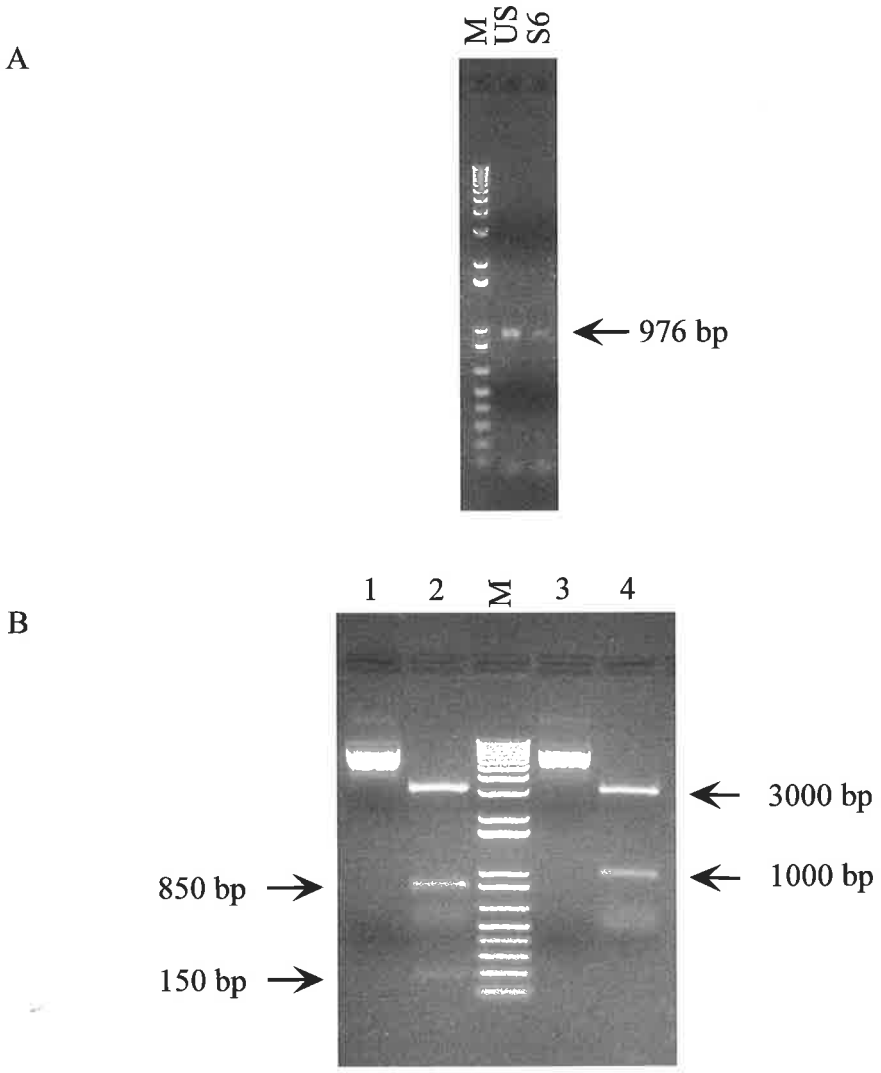


Figure 7.3: Amplification and cloning of PSbMV US and S6 976 bp *CI/NIa* amplicon.

(A) RT-PCR from purified PSbMV US and S6 RNA with VTNIa RT primer and VTCI/VTNIa PCR primer pair. (US) PSbMV US pathotype P-1. (S6) PSbMV S6 pathotype P-4.

(B) Cloning of the 976 bp *CI/NIa* fragment from PSbMV US and S6 into pGEM-T vector. (1) Unrestricted pGEM-T containing CI/NIa insert from PSbMV US. (2) pGEM-T containing CI/NIa insert from PSbMV US restricted with *Eco*RI. (3) Unrestricted pGEM-T containing CI/NIa insert from PSbMV S6. (4) pGEM-T containing CI/NIa insert from PSbMV US restricted with *Eco*RI.

(M) 1 kb Plus Ladder.

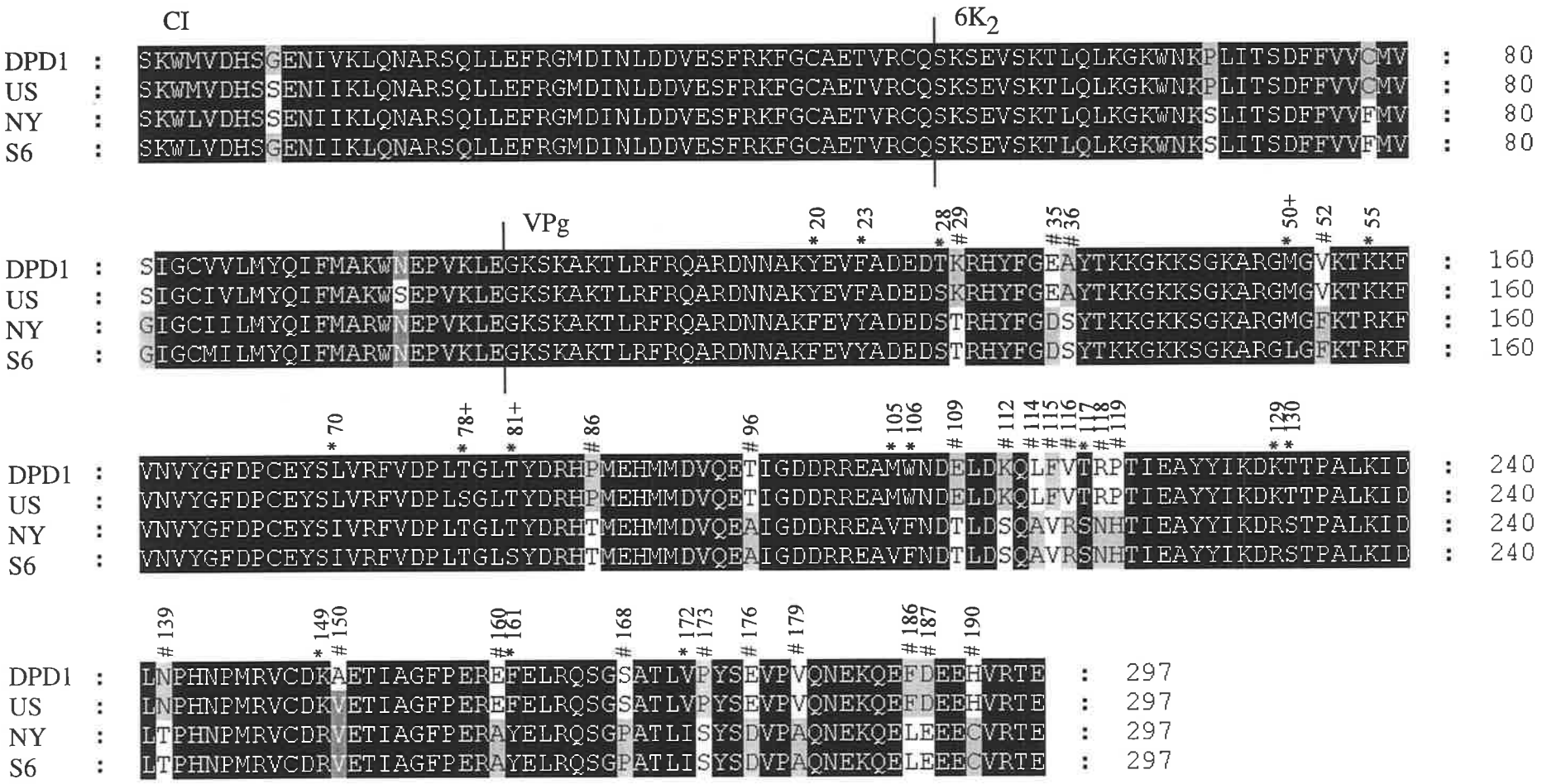


Figure 7.4: Amino acid comparison of partial CI, complete $6K_2$ and complete VPg from PSbMV pathotype P-1 (DPD1 and US) and P-4 (NY and S6) isolates. (*) Conservative amino acid changes. (#) Non-conservative amino acid changes. (+) Additional amino acid changes identified between PSbMV isolates in the VPg. Vertical lines indicate boundaries between protein products.

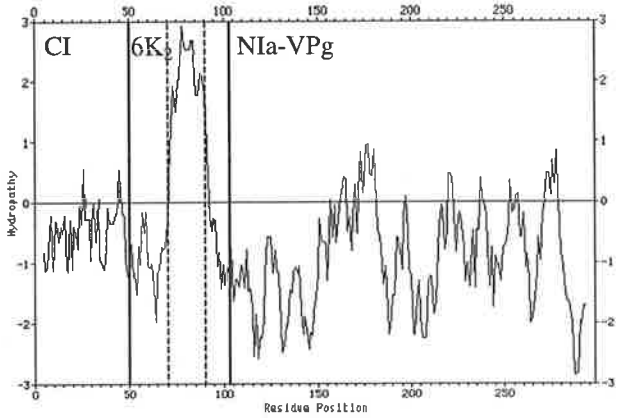
between PSbMV US and NY, and PSbMV DPD1 and NY. The 194 amino acid sequence of the VPg showed 98% similarity between PSbMV DPD1 and US and PSbMV NY and S6, 81% similarity between PSbMV DPD1 and NY, and PSbMV US and NY and 80% similar between PSbMV DPD1 and S6, and PSbMV US and S6.

Figure 7.5 shows the hybrophobicity plots of the 297 amino acids corresponding to the carboxy- terminus of the CI, entire $6K_2$ and VPg of PSbMV US and S6 compared with those for the PSbMV DPD1 and NY isolates. A region of high hybrophobicity is observed in each of the four PSbMV isolates between amino acid residue 70 to 90 corresponding to the central region of the $6K_2$ protein. Overall all the PSbMV isolates gave similar profiles.

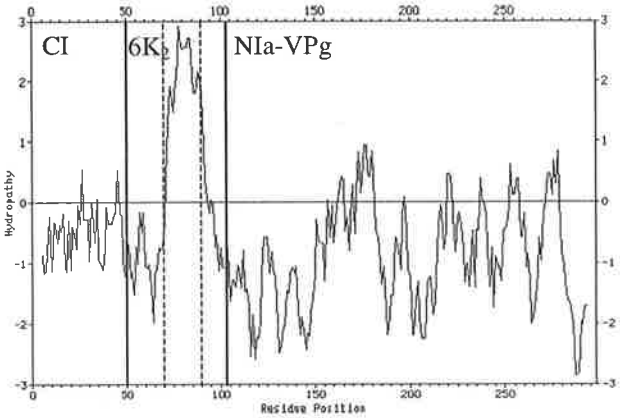
7.3.3 Targeting regions of the VPg for RFLP analysis

Figure 7.6 shows the nucleotide sequence alignment for the 891 nucleotides representing the *CI* 3′ end, *$6K_2$* and entire *VPg* coding regions from PSbMV DPD1, US, NY, and S6. Codons within the VPg corresponding to the amino acid changes identified between the PSbMV pathotype P-1 and P-4 isolates are numbered. The high proportion of amino acid changes identified within the central region of the VPg (positions 105-119) corresponded to a variable nucleotide sequence between the pathotype P-1 and P-4 representatives. However, the nucleotide sequence within this region was conserved among the PSbMV pathotype P-1, and P-4 isolates. The "Map" program was used to identify restriction sites corresponding to this variable region which would differentiate between the PSbMV P-1 and P-4 pathotypes. A *Mfe*I site corresponding to amino acids 113/114 was identified in the PSbMV pathotype P-1 (DPD1 and US) isolates but was absent in the PSbMV P-4 (NY and S6) isolates. A second *Mfe*I site was identified in the *VPg* coding region corresponding to amino acid positions 120/121/122 in PSbMV pathotype P-1 isolates (DPD1 and US) which was also absent in PSbMV pathotype P-4 isolates (NY and S6). The PSbMV pathotype P-4 isolates had a single *Mfe*I site corresponding to the VPg amino acid position 152/153/154 which was absent in the PSbMV pathotype P-1 isolates. An additional *Mfe*I site was identified outside the *VPg* coding region. This site was only present in the PSbMV US isolate and corresponded to the 3′ end of the *CI*.

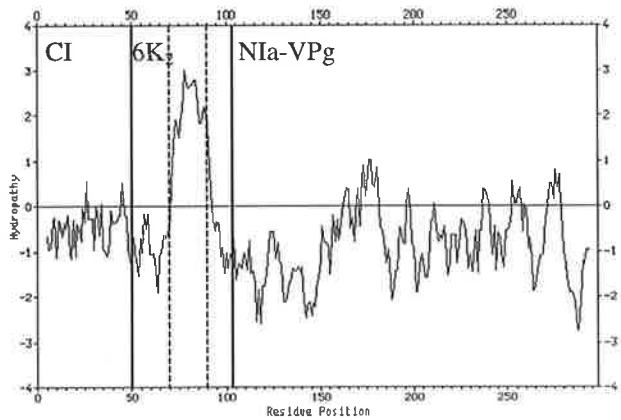
The 891 nucleotide sequence spanning the *CI*, *$6K_2$* and *VPg* of PSbMV P1 and US was 96% similar, PSbMV P4 and S6 were 98% similar, PSbMV P1 and P4, and PSbMV P1 and S6 and PSbMV US and S6 were each 79% similar, and PSbMV US and P4 were 80% similar. The 582 nucleotide *VPg* sequence of PSbMV P1 and US were 96% similar,



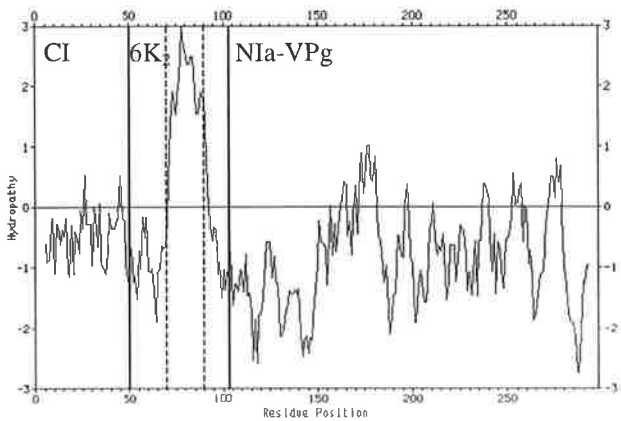
PSbMV DPD1 (pathotype P-1)



PSbMV US (pathotype P-1)



PSbMV NY (pathotype P-4)



PSbMV S6 (pathotype P-4)

Figure 7.5: Kyte-Doolittle hydrophobicity plots of PSbMV carboxy- terminus CI, entire $6K_2$, and entire NIa-VPg.

Solid vertical lines indicate boundaries between CI, $6K_2$ and NIa-VPg proteins.

Broken vertical lines show region of hydrophobicity within $6K_2$.

Figure 7.6: Nucleotide sequence alignment of PSbMV pathotype P-1 (DPD1 and US) and P-4 (NY and S6) partial *CI* 3' end, complete $6K_2$ and complete *VPg*.

MfeI sites (C↑AATT↓G).

◄——— VTVPg primer position.

(-) Boundaries between the genes with the gene indicated above the alignment.

Each codon of the VPg is indicated with a vertical line and numbers refer to the positions of amino acid changes described in Figure 7.4.


DPD1
US
NY
S6
CI
6K2
NIa-VPg
VTVPg

PSbMV P4 and S6 were 99% similar, PSbMV P1 and S6, and PSbMV US and P4 and PSbMV US and S6 were each 78% similar and PSbMV P1 and P4 were 79% similar.

7.3.4 RT-PCR of the PSbMV CI and VPg

Figure 7.7 shows the comparison of PCR with VTCI/VTNIa and VTCI/VTVPg primer pairs on dilutions of recombinant pGEM®-T plasmid containing the 976 bp CI/NIa inserts from PSbMV US and S6. Primer pair VTCI/VTVPg gave a stronger amplification signal than the VTCI/VTNIa primer pair on identical plasmid dilutions. The VTCI/VTNIa primer pair detected both the 1/100 and 1/1000 dilutions from the recombinant PSbMV US CI/NIa pGEM®-T plasmid producing a 976 bp amplicon, but only detected the 1/100 dilution of the recombinant PSbMV S6 CI/NIa pGEM®-T plasmid. Primer pair VTCI/VTVPg amplified both the 1/100 and 1/1000 PSbMV US and S6 recombinant plasmid dilutions, producing a 780 bp amplicon.

7.3.5 RFLP analysis of the 780 bp CI/VPg RT-PCR amplicon from 29 PSbMV isolates

Figure 7.8 shows the schematic representation of *Mfe*I sites and RFLP fragment sizes expected from the 780 bp VTCI/VTVPg RT-PCR amplicon from PSbMV pathotype P-1 and P-4 isolates. PSbMV pathotype P-4 isolates (NY and S6) have no *Mfe*I recognition sites within this region. PSbMV pathotype P-1 isolates (DPD1 and US) have two *Mfe*I sites at identical positions located within the variable central region of the VPg. PSbMV US has an additional *Mfe*I site within the *CI* region which is absent in PSbMV DPD1. Therefore, the restriction fragments expected in PSbMV DPD1 would be 672 bp, 22 bp and 86 bp, whereas, in PSbMV US they would be 87 bp, 585 bp, 22 bp and 86 bp.

Figure 7.9 shows the RFLP patterns generated from the 780 bp VTCI/VTVPg RT-PCR amplicon from the 29 PSbMV isolates restricted with *Mfe*I. Three distinct RFLP patterns were observed among the 29 PSbMV isolates. PSbMV US generated the predicted 585 bp and 86/87 bp fragments (the expected 22 bp fragment was not visible) and will be referred to as the "PSbMV US like pattern". PSbMV S6 contained no *Mfe*I restriction sites and will be referred to as the "PSbMV S6 like pattern". The third RFLP pattern observed was a 672 bp and 86 bp fragment (expected 22 bp fragment was not visible) as predicted from the PSbMV DPD1 sequence data and will be referred to as the "predicted PSbMV DPD1 like pattern". Among the 15 Australian PSbMV isolates two patterns were observed: the "PSbMV S6 like pattern" represented by P503-4-2, 43(1), 3(6), 19(1) and VIDA and the

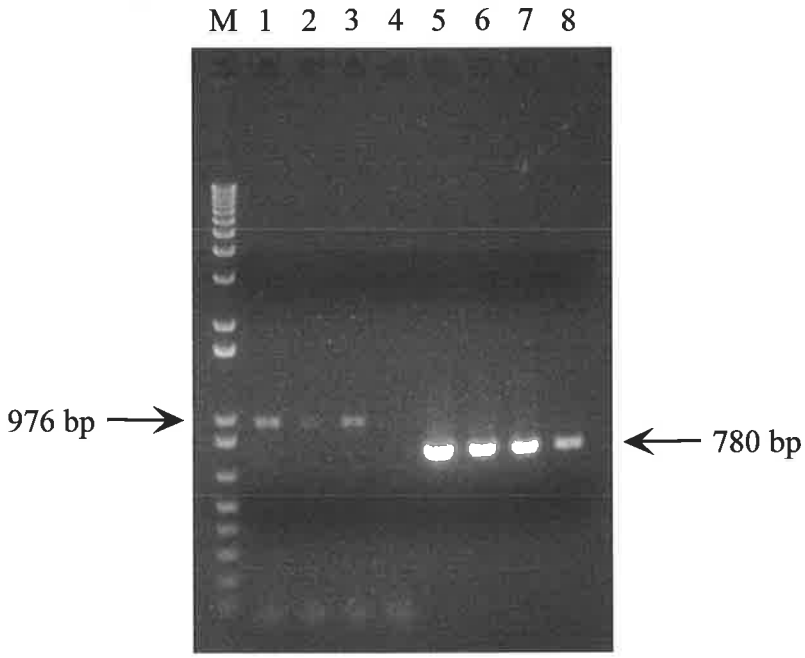


Figure 7.7: Comparison of PCR primer pairs VTCI/VTNIa and VTCI/VTVPg.

PCR with VTCI/VTNIa primer pair for:

(1) pGEM-T containing 976 bp CI/NIa insert from PSbMV US diluted 1/100;

(2) pGEM-T containing 976 bp CI/NIa insert from PSbMV US diluted 1/1000;

(3) pGEM-T containing 976 bp CI/NIa insert from PSbMV S6 diluted 1/100;

(4) pGEM-T containing 976 bp CI/NIa insert from PSbMV S6 diluted 1/1000.

PCR with VTCI/VTVPg primer pair for:

(5) pGEM-T containing 976 bp CI/NIa insert from PSbMV US diluted 1/100;

(6) pGEM-T containing 976 bp CI/NIa insert from PSbMV US diluted 1/1000;

(7) pGEM-T containing 976 bp CI/NIa insert from PSbMV S6 diluted 1/100;

(8) pGEM-T containing 976 bp CI/NIa insert from PSbMV S6 diluted 1/1000.

(M) 1 kb Plus Ladder.

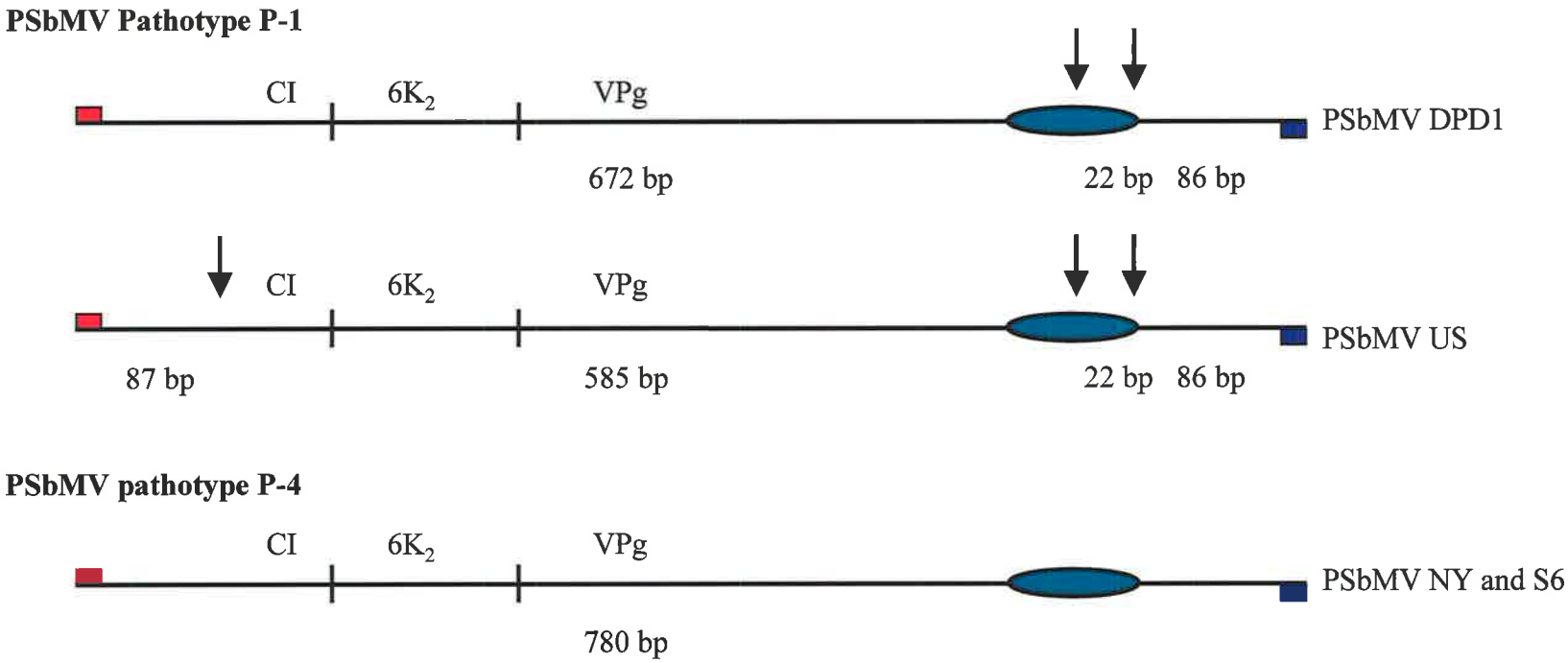


Figure 7.8: *Mfe*1 restriction sites present in the 780 bp *CI/6K$_2$/VPg* region of PSbMV pathotype P-1 (DPD1 and US) and P-4 (NY and S6) isolates. VTCI primer. VTVPg primer. *Mfe*I recognition site.

Highly variable central region of the VPg identified between PSbMV pathotype P-1 and P-4 (corresponding to amino acid position 105-119).

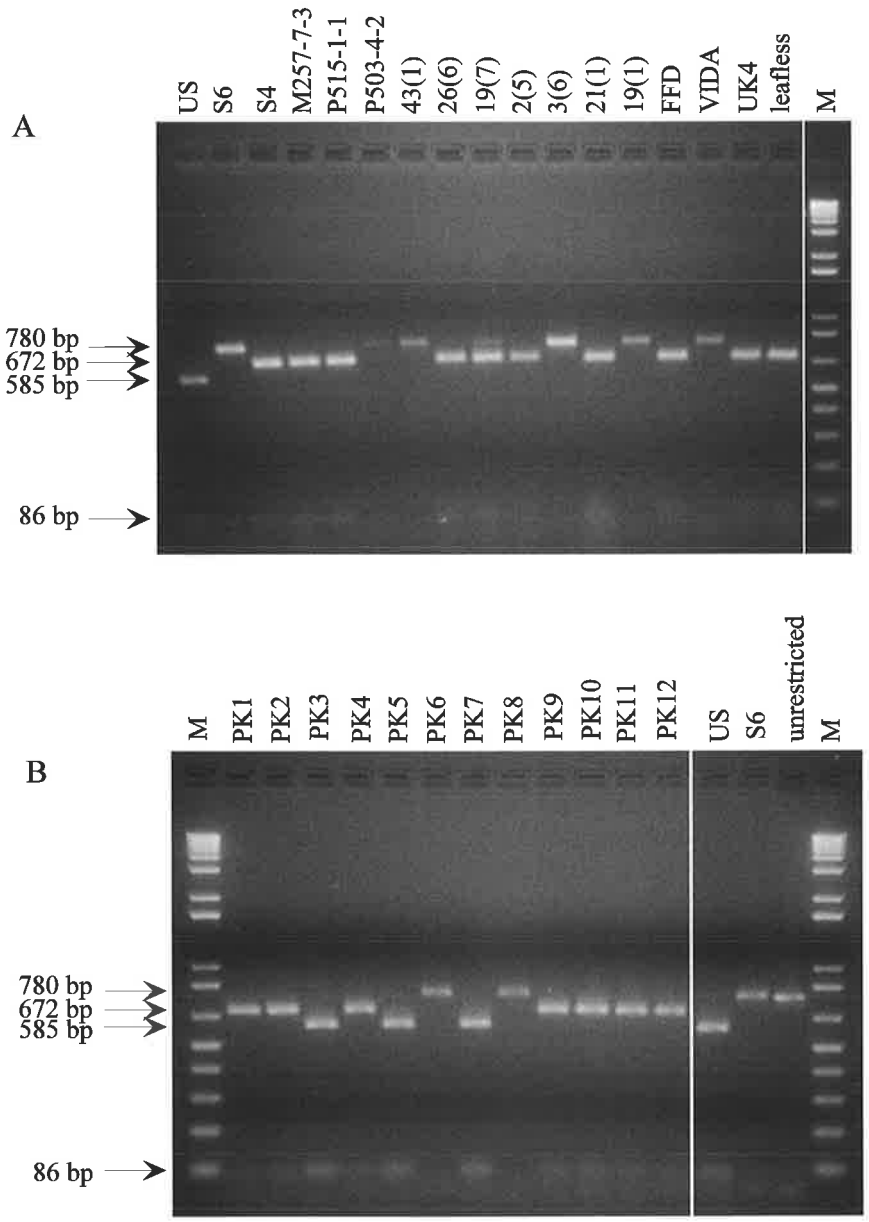


Figure 7.9: RFLP analysis of the 780 bp *CI/6K$_2$/VPg* region of 29 PSbMV isolates with *Mfe* I.

(A) Australian PSbMV isolates.

(B) Pakistani PSbMV isolates.

(M) 1 kb Plus Ladder.

"predicted PSbMV DPD1 like pattern" represented by S4, M257-7-3, P515-1-1, 26(6), 19(7), 2(5), 21(1), FFD, UK4 and leafless. Among the 12 Pakistani isolates three RFLP patterns were observed: the "PSbMV US like pattern" represented by PK3, PK5 and PK7; the "predicted PSbMV DPD1 like pattern" represented by PK1, PK2, PK4, PK9, PK10, PK11 and PK 12; and the "PSbMV S6 like pattern" represented by PK6 and PK8.

7.3.6 Sequence analysis of the 780 bp CI/VPg RT-PCR amplicon from three Australian and four Pakistani PSbMV isolates

The 780 bp RT-PCR fragment amplified with VTNIa RT and VTCI/VTVPg PCR primers were cloned from three Australian PSbMV isolates, 19(1), M257-7-3 and P515-1-1, and four Pakistani PSbMV isolates, PK4, PK6, PK7 and PK 9, representing the three different *Mfe*I RFLP patterns observed. The nucleotide and amino acid sequences from these clones were determined.

Figure 7.10 shows the alignment of the 244 amino acid sequence from the carboxy terminus of the CI, $6K_2$ and the first 141 amino acid residues of the VPg from the three Australian PSbMV isolates, four Pakistani PSbMV isolates compared with the PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4 type isolates and published PSbMV DPD1 pathotype P-1, PSbMV NY pathotype P-4 and PSbMV L1 pathotype P-2 (EMBL accession number AJ252242) sequences.

All the amino acid changes previously identified within the VPg between PSbMV pathotype P-1 (DPD1 and US) and PSbMV pathotype P-4 (NY and S6) isolates were also present in the first 141 amino acids among the three Australian and four Pakistani PSbMV isolates. No additional amino acid changes within the VPg were identified. The conservative amino acid changes identified at positions 20, 23, 55, 70, 105, 106, 117, 129, and 130 and non-conservative amino acid changes at positions 29, 35, 36, 52, 86, 96, 109, 112, 114, 115, 116, 118, 119, and 139 were identical to PSbMV DPD1 and US in the M257-7-3, P515-1-1, PK4, PK7 and PK9 PSbMV isolates and identical to PSbMV NY and S6 in the 19(1) and PK6 PSbMV isolates. The conservative amino acid change identified at position 28 of the VPg was either a threonine or serine among all the PSbMV isolates but was not pathotype specific. The additional changes identified between PSbMV DPD1, US, NY and S6 at positions 50, 78 and 81 were isolate specific and only identified in PSbMV S6, US and S6 respectively. Tyrosine and aspartic acid residues were present in all 12 PSbMV isolates at position 61 and 75 respectively.

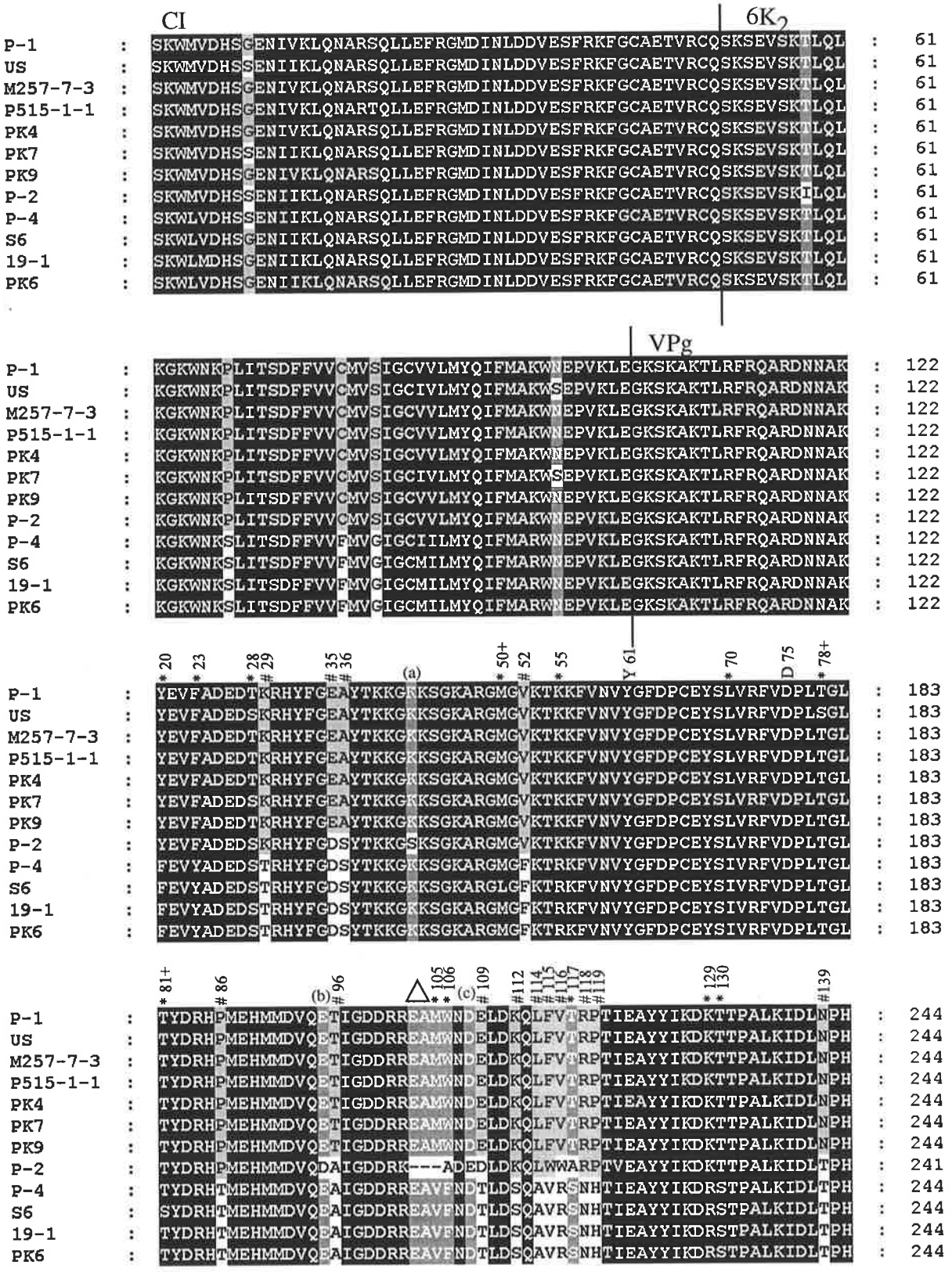
Figure 7.10: Amino acid sequence alignment of CI carboxy- terminus, entire $6K_2$ and partial VPg.

(Y61) Tyrosine residue at VPg amino acid position 61.

(D75) Aspartic acid residue at amino acid position 75.

(a, b, c) Non-conservative amino acid changes in the VPg at position 42, 95, and 108 between PSbMV L1 phototype P-2 and all other PSbMV isolates.

$\triangle$ Deletion of three amino acids in the VPg of PSbMV L1 pathotype P-2 corresponding to residue positions 103-105.


CI
6K2
VPg
P-1 : SKWMVDHSGENIVKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
US : SKWMVDHSSENIIKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
M257-7-3 : SKWMVDHSGENIVKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
P515-1-1 : SKWMVDHSGENIVKLQNARTQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
PK4 : SKWMVDHSGENIVKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
PK7 : SKWMVDHSSENIIKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
PK9 : SKWMVDHSGENIVKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
P-2 : SKWMVDHSSENIIKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKILQL : 61
P-4 : SKWLVDHSSENIIKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
S6 : SKWLVDHSGENIIKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
19-1 : SKWLMDHSGENIIKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
PK6 : SKWLVDHSGENIIKLQNARSQLLEFRGMDINLDDVESFRKFGCAETVRCQSKSEVSKTLQL : 61
P-1 : KGKWNKPLITSDFFVVCMVSIGCVVLMYQIFMAKWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
US : KGKWNKPLITSDFFVVCMVSIGCIVLMYQIFMAKWSEPVKLEGKSKAKTLRFRQARDNNAK : 122
M257-7-3 : KGKWNKPLITSDFFVVCMVSIGCVVLMYQIFMAKWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
P515-1-1 : KGKWNKPLITSDFFVVCMVSIGCVVLMYQIFMAKWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
PK4 : KGKWNKPLITSDFFVVCMVSIGCVVLMYQIFMAKWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
PK7 : KGKWNKPLITSDFFVVCMVSIGCIVLMYQIFMAKWSEPVKLEGKSKAKTLRFRQARDNNAK : 122
PK9 : KGKWNKPLITSDFFVVCMVSIGCVVLMYQIFMAKWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
P-2 : KGKWNKPLITSDFFVVCMVSIGCVVLMYQIFMAKWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
P-4 : KGKWNKSLITSDFFVVFMVGIGCIILMYQIFMARWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
S6 : KGKWNKSLITSDFFVVFMVGIGCMILMYQIFMARWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
19-1 : KGKWNKSLITSDFFVVFMVGIGCMILMYQIFMARWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
PK6 : KGKWNKSLITSDFFVVFMVGIGCMILMYQIFMARWNEPVKLEGKSKAKTLRFRQARDNNAK : 122
* 20
* 23
* 28
# 29
# 35
# 36
(a)
* 50+
# 52
* 55
Y 61
* 70
D 75
* 78+
P-1 : YEVFADEDTKRHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGL : 183
US : YEVFADEDSKRHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLSGL : 183
M257-7-3 : YEVFADEDTKRHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGL : 183
P515-1-1 : YEVFADEDTKRHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGL : 183
PK4 : YEVFADEDTKRHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGL : 183
PK7 : YEVFADEDSKRHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGL : 183
PK9 : YEVFADEDTKRHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGL : 183
P-2 : YEVFADEDSKRHYFGDSYTKKGSKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFVDPLTGL : 183
P-4 : FEVYADEDSTRHYFGDSYTKKGKKSGKARGMGFKTRKFVNVYGFDPCEYSIVRFVDPLTGL : 183
S6 : FEVYADEDSTRHYFGDSYTKKGEKSGKARGLGFKTRKFVNVYGFDPCEYSIVRFVDPLTGL : 183
19-1 : FEVYADEDSTRHYFGDSYTKKGKKSGKARGMGFKTRKFVNVYGFDPCEYSIVRFVDPLTGL : 183
PK6 : FEVYADEDSTRHYFGDSYTKKGKKSGKARGMGFKTRKFVNVYGFDPCEYSIVRFVDPLTGL : 183
* 81+
# 86
(b)
96
* 105
* 106
(c)
109
112
114
115
116
117
118
119
* 129
* 130
# 139
P-1 : TYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPH : 244
US : TYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPH : 244
M257-7-3 : TYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPH : 244
P515-1-1 : TYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPH : 244
PK4 : TYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPH : 244
PK7 : TYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPH : 244
PK9 : TYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRPTIEAYYIKDKTTPALKIDLNPH : 244
P-2 : TYDRHPMEHMMDVQDAIGDDRK---ADEDLDKQLWWARPTVEAYYIKDKTTPALKIDLTPH : 241
P-4 : TYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNHTIEAYYIKDRSTPALKIDLTPH : 244
S6 : SYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNHTIEAYYIKDRSTPALKIDLTPH : 244
19-1 : TYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNHTIEAYYIKDRSTPALKIDLTPH : 244
PK6 : TYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNHTIEAYYIKDRSTPALKIDLTPH : 244

The VPg of the PSbMV L1 pathotype P-2 isolate had a deletion of three amino acids corresponding to positions 103, 104 and 105 and was also highly variable from PSbMV pathotype P-1 and P-4 isolates within the central VPg region (amino acid position 106-119). At amino acid positions 20, 23, 29, 52, 55, 70, 86, 112, 114, 118, 119, 129, and 130 PSbMV L1 pathotype P-2 was identical to PSbMV DPD1 and US. At amino acid positions 35, 36, 96, and 139 PSbMV L1 was identical to PSbMV NY and S6. PSbMV L1 had unique amino acid changes at positions 42, 95, 106, 107, 108, 109, 115, 116 and 117.

Figure 7.11 shows the predicted *Mfe*I restriction sites present in the 780 bp VTCI/VTVPg RT-PCR amplicon of PSbMV L1 pathotype P-2, compared with those of the PSbMV pathotype P-1 and P-4 isolates. PSbMV L1 contained a single *Mfe*1 restriction site within the variable central VPg region corresponding to amino acid position 113/114 in PSbMV DPD1 and US and 110/111 in PSbMV L1 (due to a deletion of three amino acids at positions 103, 104 and 105). Therefore, in PSbMV L1 the expected RFLP fragment lengths would be 663 bp and 108 bp.

Table 7.1 shows the amino acid sequence similarity within the first 141 amino acids residues of the VPg among 12 PSbMV isolates. PSbMV DPD1 and US (pathotype P-1) and PSbMV NY and S6 (pathotype P-4) were each 98% similar. PSbMV L1 and DPD1 and PSbMV L1 and US were each 86% similar. PSbMV L1 and NY, and PSbMV L1 and S6 were 80% and 79% similar, respectively. PSbMV M257-7-3, P515-1-1, PK4, PK7 and PK9 were all 98-100% similar to PSbMV DPD1 and US, as well as each other, 81-82% similar to PSbMV NY and S6 and 86-87% similar to PSbMV L1. PSbMV 19(1) and PK6 were 98-100% similar to PSbMV NY and S6, as well as each other, 82% similar to PSbMV DPD1 and US and 80% similar to PSbMV L1

7.4 Discussion

As the *VPg* has been identified as an avirulence determinant for PSbMV DPD1 pathotype P-1 in *sbm-1/sbm-1* pea plants (Keller *et al.*, 1998) the VPg amino acid sequences of PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4 were compared. The VPg from both isolates was 194 amino acids in length with thirty-six amino acid changes (13 conservative and 23 non-conservative) being identified. High proportions of changes were observed within the central region of the VPg (amino acid position 105-119).

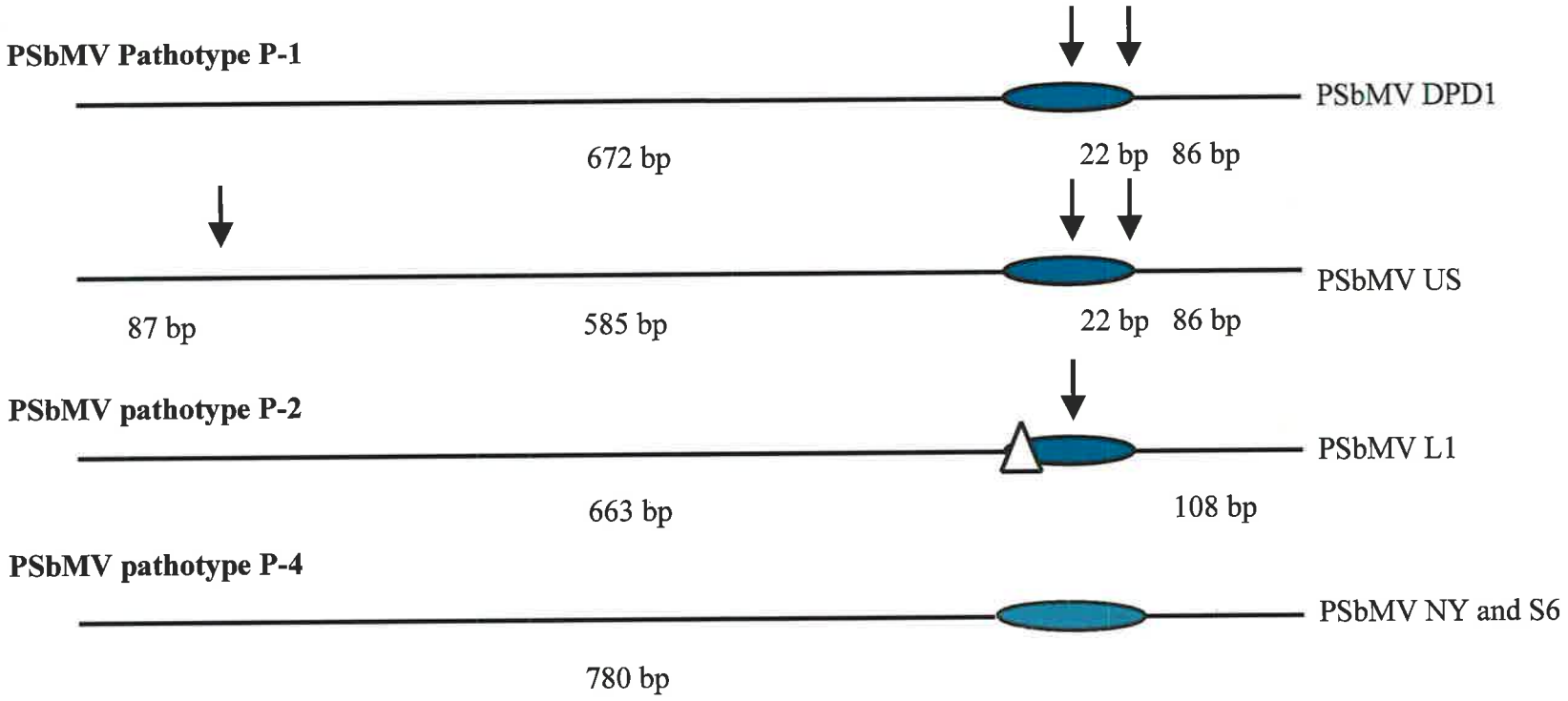


Figure 7.11: *Mfe*I recognition sites present in the 780 bp *CI/6K$_2$/VPg* region of PSbMV PSbMV pathotype P-1, P-2 and P-4 representatives.

↓ *Mfe*I recognition. △ Deletion of 9 bp in PSbMV L1 corresponding to VPg amino acid position 103-105.

Variable central region of the VPg identified between PSbMV pathotype P-1, P-2 and P-4 (corresponding to amino acid position 105-119

Table 7.1: Percentage similarity of the first 141 amino acids from the VPg of 12 PSbMV isolates

PSbMV isolates (amino acid similarity)

P-1	US	M257-7-2	P515-1-1	PK4	PK7	PK9	P-2	P-4	S6	19(1)	PK6	
	98%	100%	100%	100%	99%	100%	86%	82%	81%	82%	82%	P-1
		98%	98%	98%	99%	98%	86%	82%	81%	82%	82%	US
			100%	100%	99%	100%	86%	82%	81%	82%	82%	M257-7-3
				100%	99%	100%	86%	82%	81%	82%	82%	P515-1-1
					99%	100%	86%	82%	81%	82%	82%	PK4
						99%	87%	83%	82%	83%	83%	PK7
							86%	82%	81%	82%	82%	PK9
								80%	79%	80%	80%	P-2
									98%	100%	100%	P-4
										98%	98%	S6
											100%	19(1)
												PK6

To determine whether the amino acid sequence differences identified were pathotype or isolate specific, the *VPg* from PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4 were cloned, sequenced and compared with those of PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4. Thirty-four of the 36 amino acid changes identified between PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4 were also identified between PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4, with an additional three amino acid changes identified in PSbMV US and S6 only. Therefore, only 34 of the 39 amino acid changes identified can be described as pathotype specific changes. The highly variable central region of the VPg (position 105-119) was conserved within the PSbMV pathotype P-1 (DPD1 and US) and P-4 (NY and S6) groups and, therefore possibly could be used to distinguish between PSbMV pathotype P-1 and P4 isolates. Thirteen amino acids within the central region of the VPg have been implicated as the avirulence determinant region in PSbMV DPD1 pathotype P-1 (Borgstrom *et al.*, 1998).

A reliable RT-PCR assay was developed to amplify the variable central VPg region from total nucleic acid extracts. RT with VTNIa and PCR with the VTCI/VTVPg primer pair amplified a single 780 bp amplicon from all 29 PSbMV isolates tested without the need for further purification. This primer pair did not, however amplify the entire VPg (only amplifying the first 423 bp of the 582 bp *VPg*. Other RT-PCR primers tested that would allow the amplification of the entire VPg were not reliable or PSbMV isolate sensitive. The RT-PCR primer pair VTCI/VTNIb only amplified the expected 2300 bp product from PSbMV US RNA. Although the RT-PCR primer pair VTCI/VTNIa did amplify the expected 976 bp amplicon from PSbMV US and S6 RNA, they only amplified from a high concentration of PSbMV RNA or recombinant plasmid templates and produced additional fragments from PSbMV S6 RNA template. These primers were useful in amplifying PSbMV RNA from purified virus preparations with Pwo DNA polymerase but not for amplifying PSbMV from total nucleic extracts with *Taq* DNA polymerase.

To further examine molecular differences within the *VPg* of PSbMV, RFLP analysis targeting the variable central *VPg* region was done on the 29 PSbMV Australian, Pakistani and type isolates. RFLP analysis of the 780 bp VTCI/VTVPg amplicon with *Mfe*I produced three distinct patterns: an unrestricted PSbMV pathotype P-4 like pattern; and two PSbMV pathotype P-1 like patterns, identical to the predicted PSbMV DPD1 (672 bp, 86 bp and 22 bp) and PSbMV US (585 bp, 86/87bp and 22 bp) patterns. Among the 15 Australian PSbMV isolates only the PSbMV S6 like and predicted PSbMV DPD1 like

patterns were observed, whereas among the 12 Pakistani PSbMV isolates all three RFLP pattern types were observed.

The partial *CI/6K$_2$/VPg* from seven PSbMV isolates representing the three distinct RFLP patterns observed were cloned, sequenced and compared with the PSbMV pathotype P-1 and P-4 isolates and confirmed as being similar to PSbMV pathotype P-1 or P-4, as predicted from the RFLP analysis. At the amino acid sequence level M257-7-2, P515-1-1, PK4, PK7 and PK9 were all 98-100% similar to PSbMV pathotype P-1 isolates (DPD1 and US) and 81-83% similar to PSbMV pathotype P-4 (NY and S6) isolates, while 19(1) and PK6 are 98-100% similar to PSbMV pathotype P-4 isolates and 82% similar to PSbMV pathotype P-1 isolates.

No PSbMV pathotype P-2 type isolates were available for inclusion in the study, although full-length PSbMV L1 pathotype P-2 sequence information did become available (April, 2000) following completion of this study. The VPg of PSbMV L1 pathotype P-2 has a deletion of three amino acids, which was not observed in any of the 11 PSbMV isolates studied. The central VPg region (amino acid positions 106-119) of PSbMV L1 pathotype P-2 was also highly variable, differing from both the PSbMV pathotype P-1 and P-4 isolates. The variable central region of the VPg (amino acid positions 105-119) was conserved between the PSbMV pathotype P-1 (DPD1, US) and M257-7-3, P515-1-1, PK4, PK7, and PK9 isolates and the PSbMV pathotype P-4 (NY and S6) and 19(1) and PK6 isolates. No PSbMV pathotype P-2 like isolates were identified on this basis. Sequence data showed that the first 141 amino acids of the PSbMV L1 pathotype P-2 was 79-87% similar with the 11 other PSbMV isolates studied. PSbMV L1 pathotype P-2 was more similar to the PSbMV pathotype P-1 (DPD1 and US) isolates (86% similar) than the pathotype P-4 (NY and S6) isolates (79-80% similar).

Predicted *Mfe*I RFLP sites were identified from PSbMV L1 pathotype P-2 sequence data for the region corresponding to the 780 bp VTCI/VTVPg amplicon. One *Mfe*I site was predicted at amino acid position 110/111 of the VPg, which corresponded to the one found at position 113/114 in the PSbMV pathotype P-1 isolates. This would generate a 663 bp and 108 bp fragment, which under our conditions would be almost indistinguishable from the predicted PSbMV DPD1 like pattern (672 bp, 22 bp, 86 bp). This could, however be overcome by running restriction products on higher percentage agarose gels or polyacrylamide gels to better visualise the differences.

Hydrophobicity plots of the 297 amino acids from the carboxy- end of the CI, entire $6K_2$, and entire VPg were produced for PSbMV US pathotype P-1, PSbMV S6 pathotype P-4, PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4 and compared. All the hydrophobicity plots were similar and exhibited a region of high hydrophobicity corresponding to a stretch of approximately 20 amino acid from the central region of the $6K_2$ peptide. The $6K_2$ of TEV has been shown to be a membrane binding protein with the central 23 amino acid residues, consisting of a hydrophobic domain, required for this interaction (Schaad *et al.*, 1997a).

A tyrosine residue at position 61 and an aspartic acid at position 75 of the VPg was conserved in all 12 PSbMV isolates studied. A conserved tyrosine residue at position 1860 of the polyprotein in TVMV is responsible for the covalent attachment of the VPg to the 5' terminus of the potyviral RNA (Murphy *et al.*, 1991). Aspartic acid at residue 77 of the VPg is necessary for binding of the TuMV VPg to the translation eukaryotic initiation factor (eIF9iso)4E and shown to be conserved among eight different potyviruses (Leonard *et al.*, 2000).

CHAPTER 8

PSbMV SEQUENCE AND PHYLOGENETIC ANALYSIS

8.1 Introduction

Computer assisted comparative studies of genome sequences have greatly contributed to the understanding of the taxonomic relationships between families (Koonin and Dolja, 1993), genera (Badge *et al.*, 1997; Stenger *et al.*, 1998; Hall *et al.*, 1998) and virus species (Revers *et al.*, 1996). In addition, at the virus strain or isolate level phylogenetic analysis has allowed the correlation of sequence information with host range (Wang and Sanfacon, 2000), geographical distribution (Bousalem *et al.*, 2000a) and biological classification or genotype groups (Tordo *et al.*, 1995; Bousalem *et al.*, 2000b).

Since little PSbMV sequence information is available a primary aim for this chapter was to obtain sequence data from various PSbMV isolates within the Australian and Pakistani collection and to compare their sequences and phylogeny with PSbMV sequences available from genome databases. Furthermore, because decisive biological pathotype groups have not been established for the Australian PSbMV isolates, a second aim was to determine whether pathotypes previously described could be identified from sequence data.

8.2 Materials and Methods

8.2.1 Potyvirus isolates

Seven Australian PSbMV isolates (19(1), 43(1), VIDA, P515-1-1, 3(6), M257-7-3, and FFD; see Table 3.1 for origin), six Pakistani PSbMV isolates (PK4, PK6, PK7, PK9, PK10, PK13; Ali and Randles, 1997) and two PSbMV type isolates (US pathotype P-1 and S6 pathotype P-4) were analysed for sequence information. Sequences of PSbMV (DPD1; D10930, NY; X89997 and LI; AJ252242) and BYMV (strain MB4; D83749) were obtained from the GenBank/EMBL databases.

8.2.2 RT-PCR and direct sequencing PSbMV HC-Pro region

8.2.2.1 Primers

RT was done with VT04 (Appendix C) and PCR was done with either: the VT02/VT03 primer pair (Appendix C); or with VTHCFor (5'-ATT TCC ATC AGC ACA TCA TGT-3'), position 1602-1621 of PSbMV DPD1, and VTHCRev (5'-ACG CAT GAG CAA GGA TAC AC-3'), position 2221-2202 of PSbMV DPD1. Both primer pairs were targeted to the *HC-Pro* gene with VTHCFor/VTHCRev being internal to the VT02/VT03 primers.

8.2.2.2 RT-PCR conditions

RT-PCR was done in a two step reaction. Complementary DNA was generated from total nucleic acid with VT04 as previously described (section 5.2.3.2). PCR fragments generated for direct sequencing were amplified with the proof-reading DNA polymerase, Pwo. PCR was done in a 20 µL reaction volume containing 2 µL cDNA from the RT step, 10 mM Tris-HCl, pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.8 mM dNTPs, 0.4 µM each (VT02 and VT03) or (VTHCFor and VTHCRev) HPLC purified primer and 2.5 U Pwo DNA polymerase.

8.2.2.3 PCR cycling conditions

PCR cycling conditions with the VT02/VT03 primer pair were as previously described (section 5.2.3.3). For PCR with the VTHCFor/VTHCRev primer pair, mixtures were initially incubated at 94°C for 3 min followed by 30 cycles consisting of: denaturation at 94°C for 1 min; annealing at 50°C for 1 min; and extension at 72°C for 1 min, with a final extension step of 72°C for 2 min.

RT-PCR products were analysed by electrophoresis in 1.5% agarose gels containing 0.5 µg/mL EtBr and buffered in 1 x TAE.

8.2.2.4 Direct sequencing of PCR products

The VT02/VT03 PCR reaction was purified using the QIAquick PCR purification kit (QIAGEN, Germany) and the VTHCFor/VTHCRev PCR product was purified from agarose matrix with the QIAquick gel extraction kit (QIAGEN, Germany). Both strands of the purified RT-PCR products were directly sequenced with HPLC purified VT02, VT03, VTHCFor, or VTHCRev primers.

8.2.3 Cloning and sequencing the PSbMV CI-6K_2-VPg region

The 780 bp region representing the 3'-terminus of the CI, entire $6K_2$ and about three quarters of the VPg were amplified by RT with VTNIa and PCR with VTCI/VTVPg, then cloned and sequenced (section 7.4.2).

8.2.4 Sequence analysis

All sequence analysis was done through the WebANGIS-Australian National Genomic Information Service interface (http://www.angis.org.au/). Multiple sequence alignments of the 853 bp partial *HC-Pro* nucleotide and corresponding 284 amino acid sequences, and 732 bp partial CI, $6K_2$ and partial VPg nucleotide and corresponding 244 amino acid sequences were obtained using EClustALW (Thompson *et al.*, 1994). Pair-wise distances (without correction) and sequence similarity were calculated within a group of aligned sequences with the program Homologies.

8.2.5 Phylogenetic analysis

All phylogenetic analysis was done through the WebANGIS interface. Phylogenetic analyses were performed using the parsimony, neighbor-joining and fitch methods with BYMV defined as the outgroup. ESeqBoot was used to produce 1000 bootstrapped data sets from alignments. Parsimony analysis was done with EDnaPars or EProtPars with 1000 sets of data. Distance matrices were calculated for nucleotide sequence alignments with EDnaDist using the Kimura "2-parameter" model with a transition/transversion ratio of 2.0 and 1000 data sets. Distance matrices were calculated for amino acid sequence alignments with EProtDist using maximum likelihood estimates based on the Categories model, transition/transversion ratio of 2.0 and 1000 data sets. Phylogenies were estimated from distance matrices with ENeighbor using the Neighbor-Joining method and EFitch using the least squares formula of the Fitch-Margoliash method with 'P' value of 2.0 and 1000 replicate data sets. A majority-rule consensus tree was constructed with EConsense. Phylogenetic trees were viewed in TreeView 1.5.0.

8.3 Results

8.3.1 RT-PCR of HC-Pro region for sequencing

Figure 8.1 shows the RT-PCR amplification of a 1085 bp region of a representative group of PSbMV *HC-Pro* and internal 621 bp region for direct sequencing. PCR with primer pair VT02/VT03 on cDNA generated with VT04 produced a single 1085 bp product from the PSbMV isolates, while PCR with primer pair VTHCFor/VTHCRev from the same cDNA template produced a 621 bp fragment. Direct sequencing of these two RT-PCR products allowed the elucidation of the partial *HC-Pro* sequence from all PSbMV isolates tested.

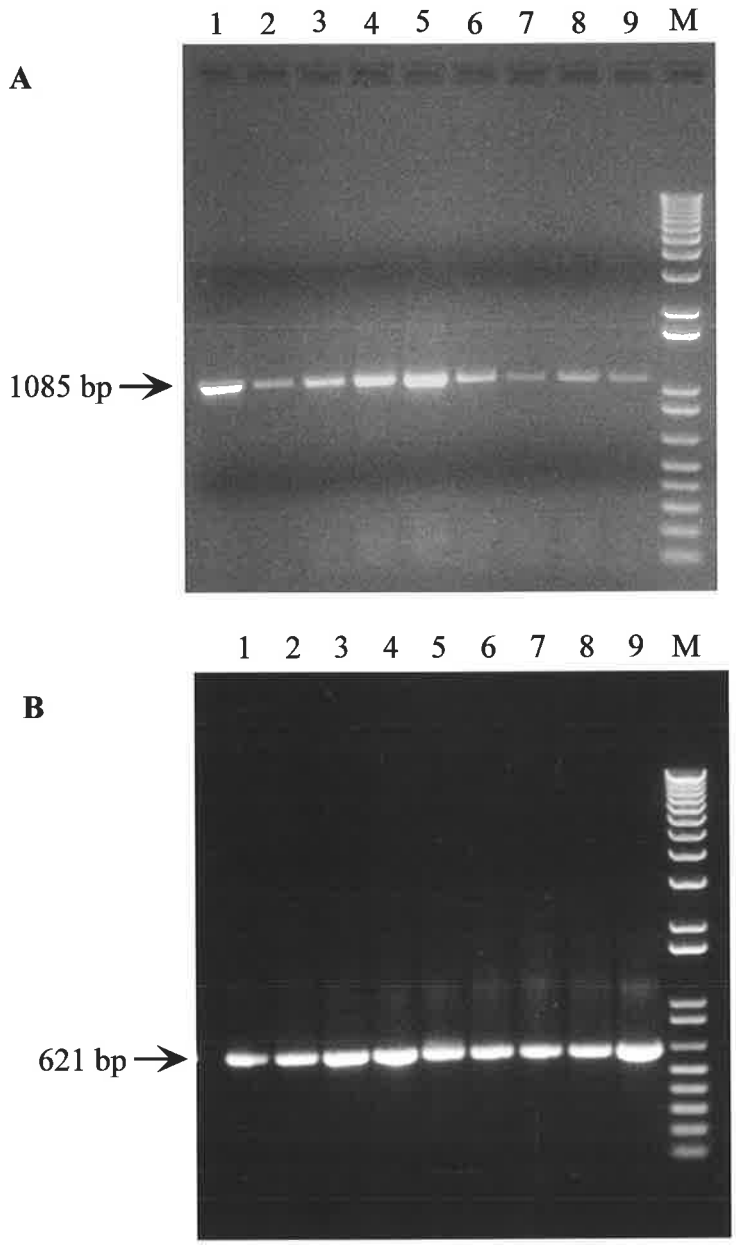


Figure 8.1: RT-PCR of *HC-Pro* from PSbMV isolates for direct sequencing. Amplification of a 1085 bp region of the *HC-Pro* from seven Australian and two PSbMV type isolates by RT-PCR for direct sequencing analysis. RT-PCR on cDNA generated from total nucleic acid with VT04 RT primer and (A) PCR of the *HC-Pro* with VT02/VT03 primer pair or (B) PCR of the internal *HC-Pro* with VTHCFor/VTHCRev primer pair. (1) PSbMV S6; (2) PSbMV 43(1); (3) PSbMV FFD; (4) PSbMV US; (5) PSbMV 3(6); (6) PSbMV VIDA; (7) PSbMV 19(1); (8) PSbMV P515-1-1; (9) PSbMV M257-7-3; (M) 1 kb Plus DNA Ladder.

8.3.2 Sequence alignments

The nucleotide and corresponding amino acid multiple sequence alignments produced from the HC-Pro and CI/6K_2/VPg regions with EClustALW are shown in Appendix D.

8.3.3 Sequence similarity

8.3.3.1 HC-Pro

Table 8.1 shows the amino acid and nucleotide sequence similarity between the HC-Pro region of 17 PSbMV and one BYMV isolate. The amino acid similarity (having the same or similar structure and/or function) was generally lower than the corresponding nucleotide sequence similarity for the comparisons between each of the PSbMV isolates and BYMV. The amino acid sequence similarity between BYMV and the 17 PSbMV isolates were in the range 37.3-39.8% and the nucleotide sequence similarities were in the range 48.2-50.1%. For the comparisons between the PSbMV isolates the amino acid sequence similarities were higher than the corresponding nucleotide sequence similarities. Among the PSbMV isolates amino acid sequence similarities ranged from 88.7-100% and nucleotide sequence similarities were in the range 79.4-99.8%.

Comparisons of the published PSbMV sequences representative of the three pathotypes showed that PSbMV L1 pathotype P-2 and PSbMV DPD1 pathotype P-1 are 94.0% and 87.5% similar at the amino acid and nucleotide sequence level respectively. PSbMV L1 pathotype P-2 and PSbMV NY pathotype P-4 are 89.8% and 79.7% similar, and PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4 are 90.5% and 79.8% similar at the amino acid and nucleotide sequence level respectively.

Comparison of the PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4 type isolates with published sequences of known pathotypes showed that PSbMV US was most similar to PSbMV DPD1 pathotype P-1 and PSbMV S6 was most similar to PSbMV NY pathotype P-4. The PSbMV US type isolate is 94.0% (amino acid) and 86.9% (nucleotide) similar with PSbMV L1 pathotype P-2; 97.5% (amino acid) and 96.8% (nucleotide) similar with PSbMV DPD1 pathotype P-1; and 90.5% (amino acid) and 79.8% (nucleotide) similar with PSbMV NY pathotype P-4. The PSbMV S6 type isolate is 88.7% (amino acid) and 79.6% (nucleotide) similar with PSbMV L1 pathotype P-2; 89.4% (amino acid) and 79.5% (nucleotide) similar with PSbMV DPD1 pathotype P-1; and 97.9% (amino acid) and 98.5% (nucleotide) similar with PSbMV NY pathotype P-4.

Table 8.1: Sequence similarity of HC-Pro region

	1	2	3	4*	5	6*	7	8*	9*	10	11	12	13	14	15	16	17	18
1. 19(1)		99.3	99.3	37.7	90.1	89.4	90.5	90.1	99.3	100.0	89.8	90.5	90.5	90.5	90.5	98.6	90.1	99.3
2. 3(6)	98.6		98.6	37.3	89.8	89.1	90.1	89.8	98.6	99.3	89.4	90.1	90.1	90.1	90.1	99.3	89.8	100.0
3. 43(1)	98.9	98.9		37.0	89.4	88.7	89.8	89.4	98.6	99.3	89.1	89.8	89.8	89.8	89.8	97.9	89.4	98.6
4. BYMV*	48.3	48.5	48.4		39.8	38.7	39.4	39.4	37.7	37.7	39.8	39.4	39.4	38.7	39.4	37.7	38.7	37.3
5. FFD	81.0	79.8	80.4	50.1		94.0	99.3	99.3	90.5	90.1	99.6	99.6	99.3	97.9	99.6	89.4	97.9	89.8
6. L1*	80.2	79.7	80.0	49.6	87.0		94.0	94.0	89.8	89.4	94.0	94.4	94.4	94.4	94.4	88.7	94.0	89.1
7. M257-7-3	80.7	79.5	80.1	49.6	99.4	87.3		99.3	90.8	90.5	98.9	99.6	99.3	97.9	99.6	89.8	97.5	90.1
8. DPD1*	80.8	79.6	80.2	49.7	99.3	87.5	99.6		90.5	90.1	98.9	99.6	99.3	97.9	99.6	89.4	97.5	89.8
9. NY*	98.2	98.7	98.6	48.5	80.1	79.7	79.7	79.8		99.3	90.1	90.8	90.8	90.8	90.8	97.9	90.5	98.6
10. P515-1-1	99.2	99.4	99.3	48.4	80.3	80.0	80.0	80.1	99.1		89.8	90.5	90.5	90.5	90.5	98.6	90.1	99.3
11. PK10	80.8	79.6	80.2	49.5	99.3	87.3	99.4	99.5	79.8	80.1		99.3	98.9	97.5	99.3	89.1	97.5	89.4
12. PK13	80.9	79.7	80.3	49.9	99.6	87.2	99.5	99.4	80.0	80.2	99.2		99.6	98.2	100.0	89.8	97.9	90.1
13. PK4	80.7	79.5	80.1	49.6	99.1	87.5	99.4	99.5	79.7	80.0	99.5	99.2		97.9	99.6	89.8	97.5	90.1
14. PK7	80.9	79.7	80.4	49.6	96.4	86.9	96.7	96.8	80.0	80.2	96.6	96.5	96.6		98.2	89.8	99.6	90.1
15. PK9	80.7	79.5	80.1	49.6	99.2	87.6	99.5	99.6	79.7	80.0	99.6	99.3	99.6	96.7		89.8	97.9	90.1
16. S6	98.4	99.8	98.7	48.7	79.7	79.6	79.4	79.5	98.5	99.2	79.5	79.6	79.4	79.6	79.4		89.4	99.3
17. US	80.9	79.7	80.4	49.4	96.4	86.9	96.7	96.8	80.0	80.2	96.6	96.5	96.6	99.8	96.7	79.6		89.8
18. VIDA	98.7	99.4	99.1	48.2	80.4	79.7	80.1	80.2	98.1	98.8	80.2	80.3	80.1	80.1	80.1	99.2	80.1	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**

Pair-wise sequence similarity within the group of aligned sequences were determined using the program Homologies with uncorrected values. Numbers above the diagonal line represent percentage amino acid sequence similarity and numbers below the diagonal represent percentage nucleotide sequence similarity. * Nucleotide sequences extracted from the GenBank/EMBL databases.

PSbMV DPD1 and US showed greater than 97% amino acid sequence similarity with PSbMV FFD, PSbMV M257-7-3, PSbMV PK10, PSbMV PK13, PSbMV PK4, PSbMV PK7 and PSbMV PK9. PSbMV NY and S6 showed greater than 97% amino acid similarity with PSbMV 19(1), PSbMV 3(6), PSbMV 43(1), PSbMV P515-1-1, and PSbMV VIDA. The amino acid sequence similarity between PSbMV L1 pathotype P-2 and the other PSbMV isolates varied from 88.7-94.4%.

8.3.3.2 CI-6K$_2$-VPg

Table 8.2 shows the amino acid and nucleotide sequence similarity between the CI/6K$_2$/VPg region of 12 PSbMV and one BYMV isolate. The amino acid similarity was lower than the corresponding nucleotide sequence similarity for the comparisons between each of the PSbMV isolates and BYMV. The amino acid sequence similarity between BYMV and the 12 PSbMV isolates were in the range 38.9-41.3% and the nucleotide sequence similarities were in the range 49.8-51.4%. For the comparisons between the PSbMV isolates, amino acid sequence similarities were higher than the corresponding nucleotide sequence similarities. Among the PSbMV isolates amino acid sequence similarities were in the range 84.4-100% and nucleotide sequence similarities were in the range 77.7-100%. Nucleotide sequence similarity of 100% was observed between the PSbMV 257-7-3, PSbMV PK4, PSbMV PK9, and PSbMV DPD1 isolates.

Comparisons of the published PSbMV sequences representative of the three pathotypes showed that PSbMV L1 pathotype P-2 and PSbMV DPD1 pathotype P-1 are 91.0% and 85.0% similar at the amino acid and nucleotide sequence level respectively. PSbMV L1 pathotype P-2 and PSbMV NY pathotype P-4 are 85.7% and 77.7% similar, and PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4 are 86.5% and 79.8% similar at the amino acid and nucleotide sequence level respectively.

Comparison of the PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4 type isolates with published sequences of known pathotypes showed that PSbMV US was most similar to PSbMV DPD1 pathotype P-1 and PSbMV S6 was most similar to PSbMV NY pathotype P-4. The PSbMV US type isolate is 91.0% (amino acid) and 83.6% (nucleotide) similar with PSbMV L1 pathotype P-2; 97.5% (amino acid) and 97.0% (nucleotide) similar with PSbMV DPD1 pathotype P-1; and 87.3% (amino acid) and 80.7% (nucleotide) similar with PSbMV NY pathotype P-4. The PSbMV S6 type isolate is 84.4% (amino acid) and

Table 8.2: Sequence identity of CI/6K$_2$/VPg region

	1	**2***	**3***	**4**	**5***	**6***	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1. 19(1)		40.9	84.8	86.5	86.5	98.8	86.1	86.5	99.6	86.5	86.5	98.8	86.1
2. BYMV*	49.9		38.9	41.3	41.3	40.9	41.3	41.3	40.9	41.3	41.3	40.1	40.9
3. L1*	77.9	50.7		91.0	91.0	85.7	90.6	91.0	85.2	91.4	91.0	84.4	91.0
4. M257-7-3	79.6	51.4	85.0		100.0	86.5	99.6	100.0	86.9	98.0	100.0	86.1	97.5
5. DPD1*	79.6	51.4	85.0	100.0		86.5	99.6	100.0	86.9	98.0	100.0	86.1	97.5
6. NY*	98.9	50.1	77.7	79.8	79.8		86.1	86.5	99.2	87.7	86.5	98.4	87.3
7. P515-1-1	79.5	51.4	84.8	99.9	99.9	79.6		99.6	86.5	97.5	99.6	85.7	97.1
8. PK4	79.6	51.4	85.0	100.0	100.0	79.8	99.9		86.9	98.0	100.0	86.1	97.5
9. PK6	99.7	49.9	77.9	79.6	79.6	99.2	79.5	79.6		86.9	86.9	99.2	86.5
10. PK7	80.5	51.1	83.7	97.1	97.1	80.9	97.0	97.1	80.5		98.0	86.1	99.6
11. PK9	79.6	51.4	85.0	100.0	100.0	79.8	99.9	100.0	79.6	97.1		86.1	97.5
12. S6	99.2	49.8	77.9	79.4	79.4	98.6	79.2	79.4	99.5	80.2	79.4		85.7
13. US	80.3	51.0	83.6	97.0	97.0	80.7	96.9	97.0	80.3	99.9	97.0	80.1	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**

Pair-wise similarity within the group of aligned sequences were determined using the program Homologies with uncorrected values. Numbers above the diagonal line represent percentage amino acid sequence similarity and numbers below the diagonal represent percentage nucleotide sequence similarity. * Nucleic acid sequences extracted from the GenBank/EMBL databases.

77.9% (nucleotide) similar with PSbMV L1 pathotype P-2; 86.1% (amino acid) and 79.4% (nucleotide) similar with PSbMV DPD1 pathotype P-1; and 98.4% (amino acid) and 98.6% (nucleotide) similar with PSbMV NY pathotype P-4.

PSbMV DPD1 and US showed greater than 97% amino acid sequence similarity with PSbMV M257-7-3, PSbMV 515-1-1, PSbMV PK4, PSbMV PK7, and PSbMV PK9. PSbMV NY and S6 showed greater than 97% amino acid sequence similarity with PSbMV 19(1) and PSbMV PK6. The amino acid sequence similarity between PSbMV L1 pathotype P-2 and the other PSbMV isolates varied from 84.4-91.4%.

8.3.4 Phylogenetic trees

8.3.4.1 HC-Pro

Figure 8.2 shows the cladograms obtained from phylogenetic analysis done on the 853 bp region of the partial *HC-Pro* nucleotide sequences by the parsimony, neighbor-joining and fitch methods. All three methods resulted in the same topologies of the PSbMV isolates within the cladograms. Three clades were produced for each of the three methods: one containing the BYMV; the second containing the PSbMV 19(1), 43(1), P515-1-1, NY pathotype P-4, VIDA, 3(6) and S6 pathotype P-4 isolates; and the third containing the PSbMV L1 pathotype P-2, US pathotype P-1, PK7, PK13, FFD, M257-7-3, DPD1 pathotype P-1, PK4, PK10 and PK 9. Within the third clade the PSbMV L1 isolate was the most distantly related to the other isolates within the group.

Figure 8.3 shows the cladograms obtained from phylogenetic analysis done on the partial HC-Pro amino acid sequences by the parsimony and neighbor-joining methods. The two methods result in slightly different topologies of the PSbMV isolates. The parsimony method resulted in four clades: one containing the BYMV isolate; the second containing the PSbMV L1 pathotype P-2 isolate; the third containing the PSbMV NY pathotype P-4, P515-1-1, 3(6), 43(1), 19(1), VIDA and S6 pathotype P-4; and the fourth containing the PSbMV PK7, US pathotype P-1, FFD, PK10, DPD1 pathotype P-1, M257-7-2, PK13, PK4, and PK9. Due to the lower bootstrap confidence values in the neighbor-joining tree (less than 50%) branches would be collapsed producing five clades. The neighbor-joining method resulted in five clades: one containing BYMV, the second containing PSbMV L1 pathotype P-2; the third containing PSbMV FFD, PK10, PK4, DPD1 pathotype P-1, PK13, PK9 and M257-7-3; the fourth containing PSbMV PK7 and US pathotype P-1; and the

Figure 8.2: Phylogenetic analysis of PSbMV isolates based on *HC-Pro* nucleotide sequences.

Phylogenetic relationships of PSbMV isolates based on multiple alignments of a 853 bp region of the *HC-Pro*. Trees were obtained by (A) parsimony, (B) neighbor-joining and (C) fitch methods. The numbers above the branches indicate the percentage of bootstrap analyses that supported the grouping at each node. BYMV was used as the outgroup.

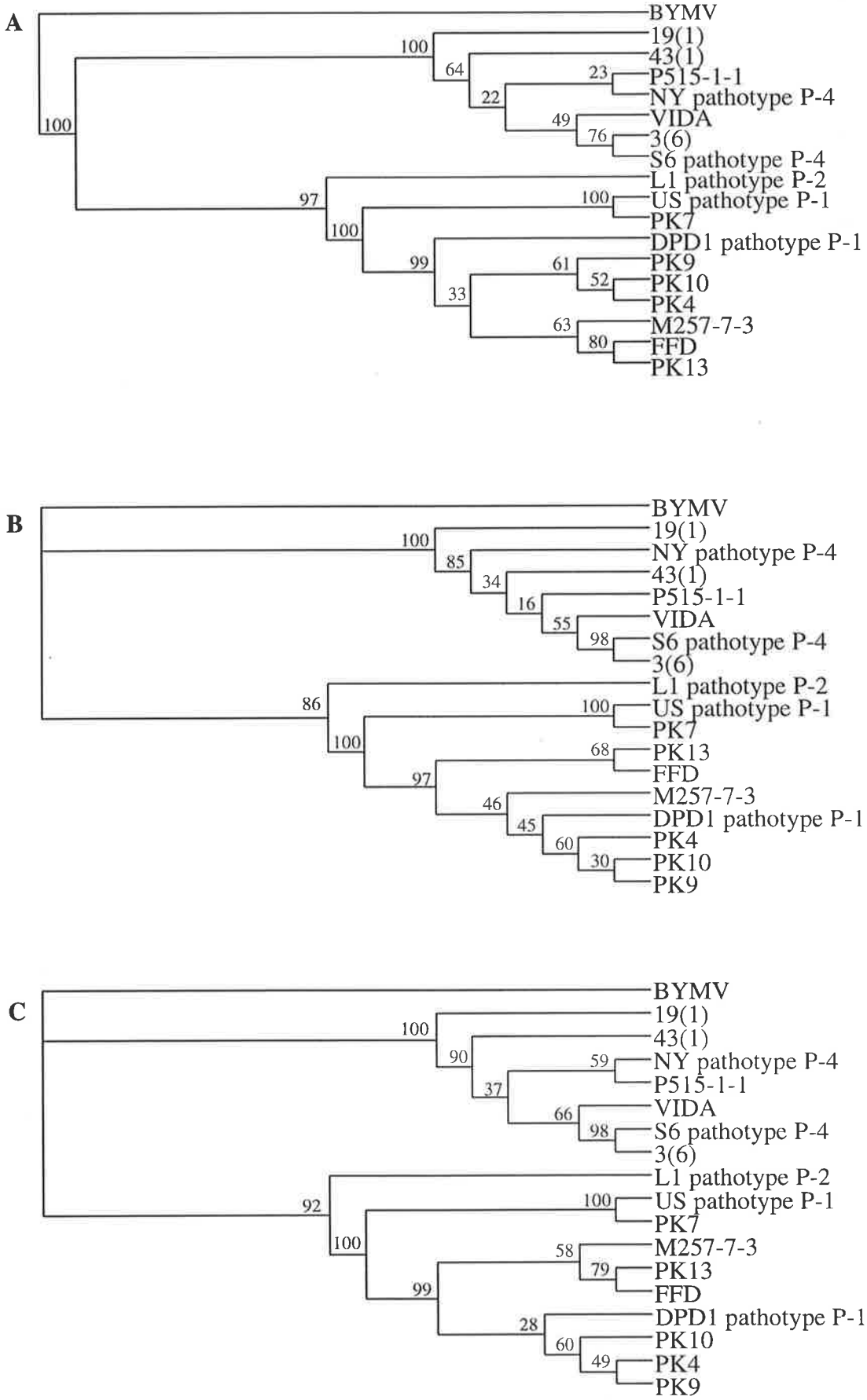
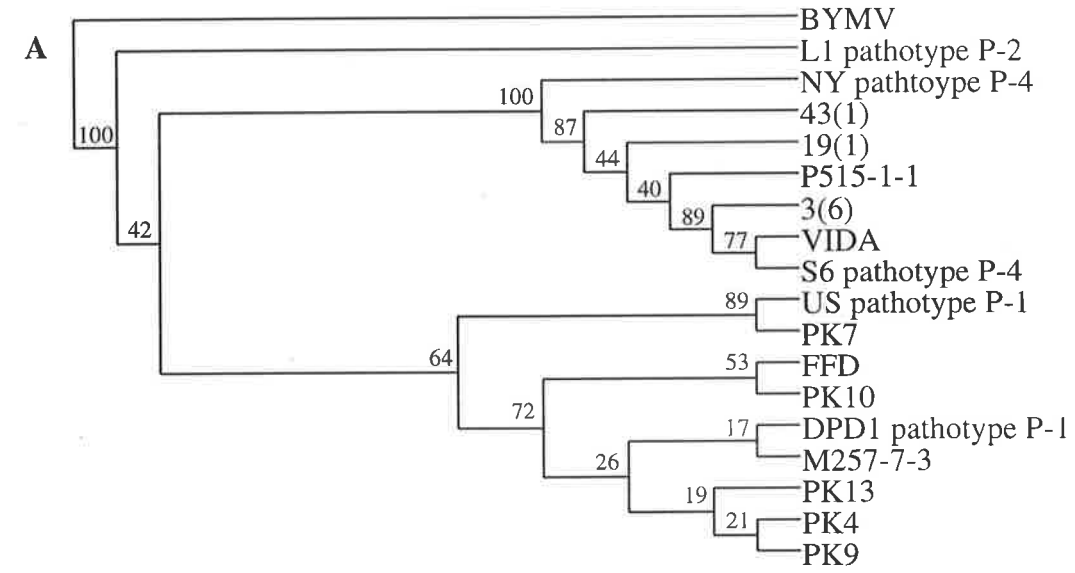
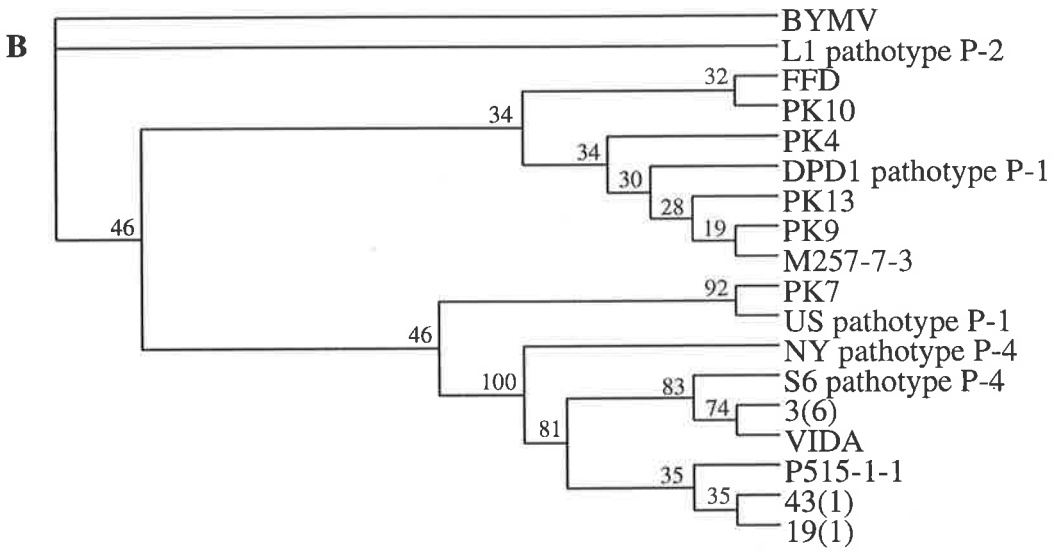

A
BYMV
19(1)
43(1)
P515-1-1
NY pathotype P-4
VIDA
3(6)
S6 pathotype P-4
L1 pathotype P-2
US pathotype P-1
PK7
DPD1 pathotype P-1
PK9
PK10
PK4
M257-7-3
FFD
PK13
100
64
23
22
49
76
100
97
100
100
99
61
52
33
63
80
B
BYMV
19(1)
NY pathotype P-4
43(1)
P515-1-1
VIDA
S6 pathotype P-4
3(6)
L1 pathotype P-2
US pathotype P-1
PK7
PK13
FFD
M257-7-3
DPD1 pathotype P-1
PK4
PK10
PK9
100
85
34
16
55
98
86
100
100
68
97
46
45
60
30
C
BYMV
19(1)
43(1)
NY pathotype P-4
P515-1-1
VIDA
S6 pathotype P-4
3(6)
L1 pathotype P-2
US pathotype P-1
PK7
M257-7-3
PK13
FFD
DPD1 pathotype P-1
PK10
PK4
PK9
100
90
59
37
66
98
92
100
100
58
79
99
28
60
49

Figure 8.3: Phylogenetic analysis of PSbMV isolates based on HC-Pro amino acid sequences.

Phylogenetic relationships of PSbMV isolates based on multiple alignments of a 284 amino acid region of the HC-Pro. Trees were obtained by (A) parsimony or (B) neighbor-joining methods. The numbers above the branches indicate the percentage of bootstrap analyses that supported the grouping at each node. BYMV was used as the outgroup.


A
BYMV
L1 pathotype P-2
NY pathtoype P-4
43(1)
19(1)
P515-1-1
3(6)
VIDA
S6 pathotype P-4
US pathotype P-1
PK7
FFD
PK10
DPD1 pathotype P-1
M257-7-3
PK13
PK4
PK9
100
87
44
40
89
77
100
42
89
64
53
72
17
26
19
21


B
BYMV
L1 pathotype P-2
FFD
PK10
PK4
DPD1 pathotype P-1
PK13
PK9
M257-7-3
PK7
US pathotype P-1
NY pathotype P-4
S6 pathotype P-4
3(6)
VIDA
P515-1-1
43(1)
19(1)
32
34
34
30
28
19
46
92
46
100
83
74
81
35
35

fifth containing PSbMV NY pathotype P-4, S6 pathotype P-4, 3(6), VIDA, P515-1-1, 43(1) and 19(1). The extra clade containing the PSbMV PK7 and US isolates observed is combined with the isolates of group three (neighbor-joining tree) in the parsimony analysis.

8.3.4.2 CI-6K$_2$-VPg

Figure 8.4 shows the cladograms obtained from phylogenetic analysis done on the 741 bp region of 3' end of the *CI*, entire 6K$_2$ and partial *VPg* nucleotide sequences by the parsimony, neighbor-joining and fitch methods. All three methods resulted in the same topologies of PSbMV isolates. Three clades were produced: one containing the BYMV; the second containing the PSbMV NY pathotype P-4, 19(1), S6 pathotype P-4, and PK6 isolates; and the third containing the PSbMV L1 pathotype P-2, US pathotype P-1, PK7, M257-7-3, P515-1-1, DPD1 pathotype P-1, PK4 and PK9.

Figure 8.5 shows the cladograms obtained from phylogenetic analysis done on the corresponding amino acid sequence from the CI/6K$_2$/VPg region by parsimony and neighbor-joining methods. The same topologies of PSbMV isolates were obtained with both methods. Three clades were produced: one containing BYMV; the second containing PSbMV NY pathotype P-4, 19(1), S6 pathotype P-4 and PK6 isolates; and the third containing PSbMV L1 pathotype P-2, US pathotype P-1, PK7, M257-3, P515-1-1, DPD1 pathotype P-1, PK4 and PK9.

8.4 Discussion

New PSbMV sequence results have been presented in this chapter. Nucleotide and amino acid sequence data was obtained for the partial *HC-Pro* (853 bp) from seven Australian, five Pakistani and two PSbMV type isolates (US and S6). Sequence data was also obtained from the *CI/6K$_2$/VPg* (732 bp) region of three Australian, four Pakistani and two PSbMV type isolates. These were compared with three other PSbMV sequences, extracted from sequence databases and representative of the three known pathotypes.

This allowed sequence and phylogenetic analysis to be done on the PSbMV isolates. The values obtained for nucleic and amino acid sequence similarities between PSbMV isolates were similar regardless of whether the HC-Pro (79.4-99.8% and 88.7-100% respectively) or CI/6K$_2$/VPg (77.7-100% and 84.4-100% respectively) regions were compared.

Figure 8.4: Phylogenetic analysis of PSbMV isolates based on *CI/6K$_2$/VPg* nucleotide sequences.

Phylogenetic relationships of PSbMV isolates based on multiple alignment of a 741 bp region of the *CI/6K$_2$/VPg*. Trees were obtained by (A) parsimony, (B) neighbor-joining and (C) fitch methods. The numbers above the branches indicate the percentage of bootstrap analyses that supported the grouping at each node. BYMV was used as the outgroup.

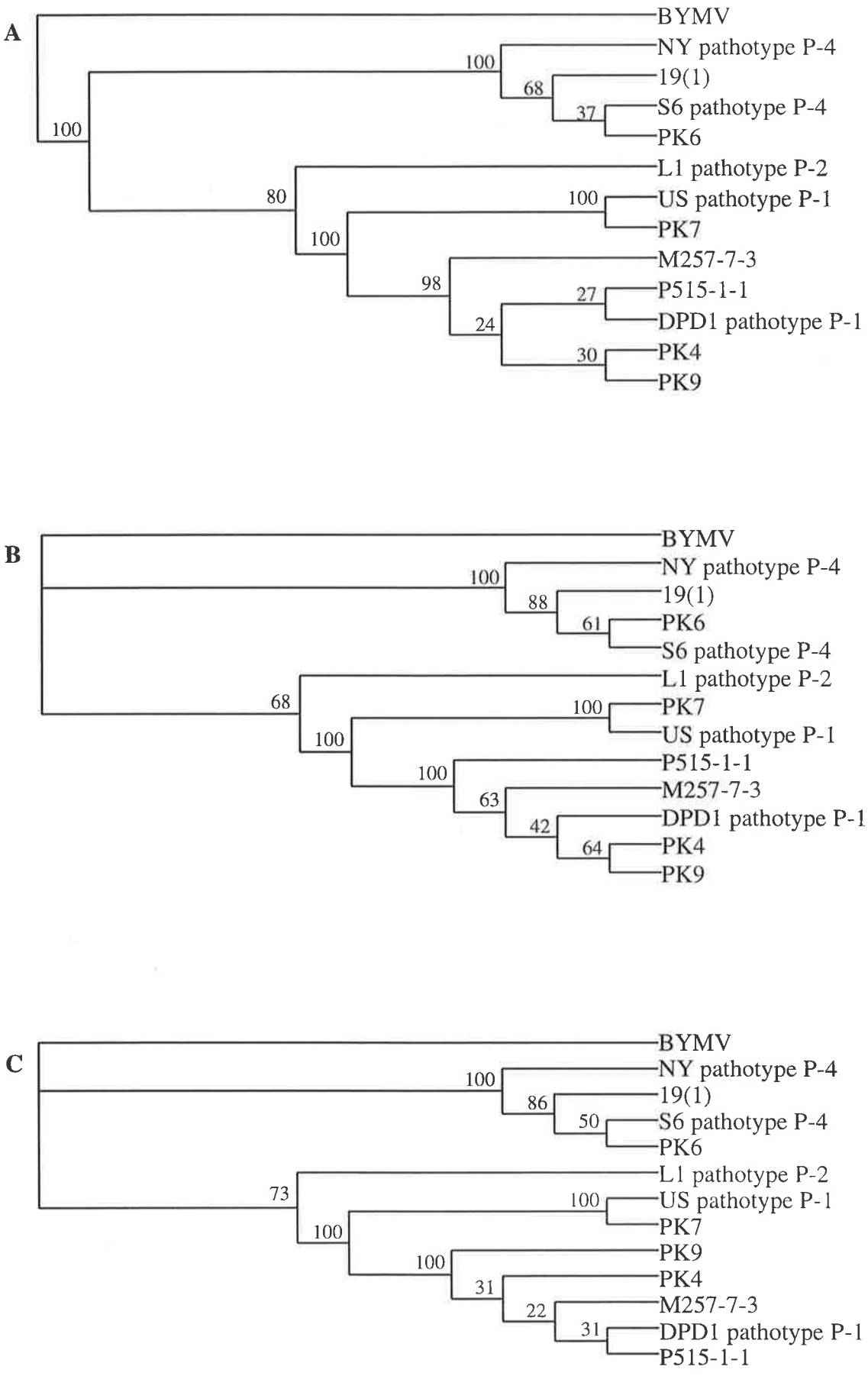
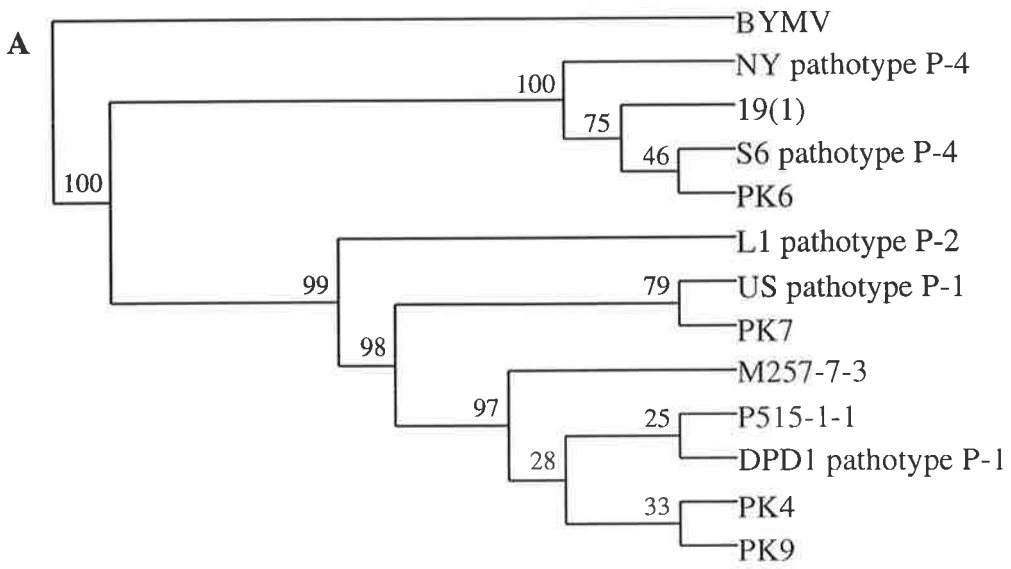
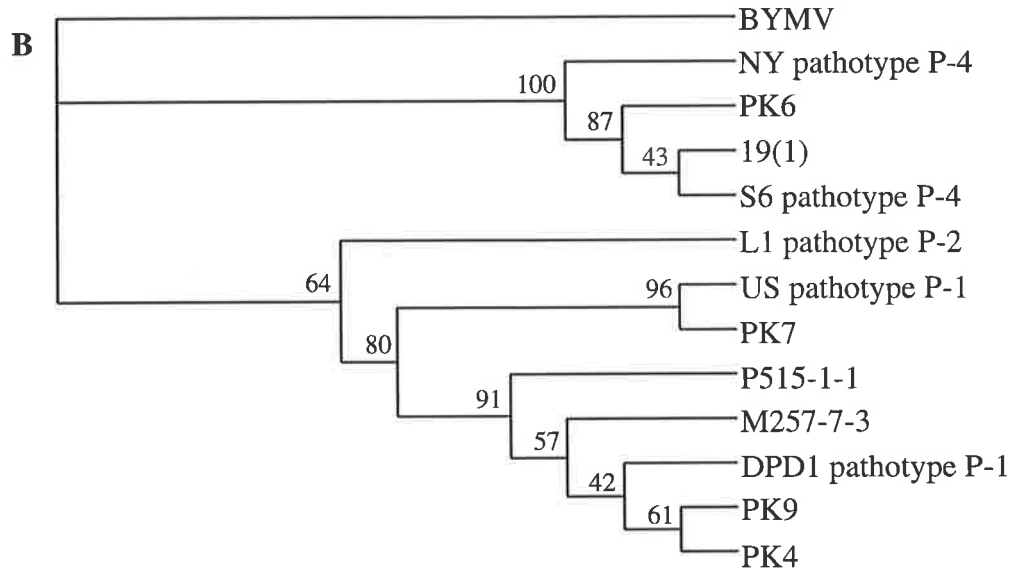

A
BYMV
NY pathotype P-4
19(1)
S6 pathotype P-4
PK6
L1 pathotype P-2
US pathotype P-1
PK7
M257-7-3
P515-1-1
DPD1 pathotype P-1
PK4
PK9
100
100
68
37
80
100
100
98
27
24
30
B
BYMV
NY pathotype P-4
19(1)
PK6
S6 pathotype P-4
L1 pathotype P-2
PK7
US pathotype P-1
P515-1-1
M257-7-3
DPD1 pathotype P-1
PK4
PK9
100
88
61
68
100
100
100
63
42
64
C
BYMV
NY pathotype P-4
19(1)
S6 pathotype P-4
PK6
L1 pathotype P-2
US pathotype P-1
PK7
PK9
PK4
M257-7-3
DPD1 pathotype P-1
P515-1-1
100
86
50
73
100
100
100
31
22
31

Figure 8.5: Phylogenetic analysis of PSbMV isolates based on CI/6K_2/VPg amino acid sequences.

Phylogenetic relationships of PSbMV isolates based on multiple alignment of a 244 amino acid region of the CI/6K_2/VPg.Trees were obtained by (A) parsimony and (B) neighbor-joining methods. The numbers above the branches indicate the percentage of bootstrap analyses that supported the grouping at each node. BYMV was used as the outgroup.


A
BYMV
NY pathotype P-4
19(1)
S6 pathotype P-4
PK6
L1 pathotype P-2
US pathotype P-1
PK7
M257-7-3
P515-1-1
DPD1 pathotype P-1
PK4
PK9
100
100
75
46
99
79
98
97
25
28
33


B
BYMV
NY pathotype P-4
PK6
19(1)
S6 pathotype P-4
L1 pathotype P-2
US pathotype P-1
PK7
P515-1-1
M257-7-3
DPD1 pathotype P-1
PK9
PK4
100
87
43
64
96
80
91
57
42
61

Comparison of BYMV with the PSbMV isolates showed approximately 40% amino acid and 50% nucleotide sequence similarity for both the HC-Pro and CI/6K$_2$/VPg regions. Previous comparisons of the nucleotide sequences of potyvirus genomes have shown that the degree of identity between equivalent genes of strains is greater than 96% while between distinct potyviruses the identity ranged from 42% to 65% suggesting that any extended genome sequence can be considered representative of the whole genome (Frenkel *et al.*, 1992).

This overall high level of sequence similarity between PSbMV isolates made distinguishing between possible pathotypes difficult. Known PSbMV pathotype P-1 isolates (DPD1 and US) showed 97.5% amino acid similarity within HC-Pro and CI/6K$_2$/VPg, while PSbMV pathotype P-4 isolates (NY and S6) showed 97.9% and 98.4% amino acid sequence similarity within HC-Pro and CI/6K$_2$/VPg respectively. No PSbMV isolate showed greater than 94.4% and 91.4% amino acid sequence similarity to PSbMV L1 pathotype P-2 within the HC-Pro and CI/6K$_2$/VPg regions respectively. Therefore, PSbMV isolates were grouped on the basis of greater than 97% similarity at the amino acid level. PSbMV isolates sequenced in both the HC-Pro and CI/6K$_2$/VPg region were always found in the same group, PSbMV pathotype P-1 and pathotype P-4 like, with the exception of PSbMV P515-1-1 which was PSbMV pathotype P-4 like based on HC-Pro sequence and PSbMV pathotype P-1 like based on CI/6K$_2$/VPg sequence data.

Phylogenetic analysis done on nucleotide and amino acid sequence data generated the same tree topologies of PSbMV isolates, regardless of the method of analysis. The neighbor-joining method resulted in a slightly different tree topology for the HC-Pro amino acid sequence data however this tree was not robust due to bootstrap values of less than 50% for major branches. When PSbMV isolates were analysed in both the HC-Pro and CI/6K$_2$/VPg region their topologies were identical except for PSbMV P515-1-1. Other studies have also observed that phylogenies generally remain the same regardless of genomic region being analysed (Tordo *et al.*, 1995; Gibbs *et al.*, 1997; Aleman-Verdaguer *et al.*, 1997; Wang and Sanfacon, 2000). The shift in the toplogy of PSbMV P515-1-1 in relation to the other PSbMV isolates could be a result of recombination. Analysis of sequence data and phylogenetic studies have indicated that recombination has occurred among other potyvirus isolates (Revers *et al.*, 1996; Bousalem *et al.*, 2000a).

The cladograms showed an absence of a correlation between sequence and geographic region, which would support the view that these seed transmitted viruses have become widely disseminated. However, PSbMV isolates did cluster into two major groups from phylogenetic analysis of both genomic regions. One group contained the PSbMV NY and S6 pathotype P-4 isolates in addition to 19(1), 43(1), P515-1-1, VIDA and 3(6) (HC-Pro analysis) or 19(1) and PK6 (CI/$6K_2$/VPg analysis). The second major group contained the PSbMV DPD1 and US pathotype P-1 isolates in addition to PSbMV L1 pathotype P-2, PK7, PK13, FFD, PK4, PK10 and PK9 (HC-Pro analysis) or PSbMV L1 pathotype P-2, PK7, P515-1, M257-7-3, PK9 and PK4 (CI/$6K_2$/VPg analysis). The PSbMV L1 pathotype P-2 isolate, however was always the most distantly related within this group.

CHAPTER 9

GENERAL DISCUSSION

9.1 Introduction

PSbMV is a potyvirus which infects pea and is seed transmitted (Maury and Khetarpal, 1992). Due to secondary spread by aphid species (Hampton and Mink, 1975), a low level of seed infection can cause significant yield losses in the field (Zimmer and Lamb, 1993). PSbMV has been reported from several countries and is believed to have spread world-wide through the international trade of infected seed (Maury and Khetarpal, 1992). PSbMV has been detected in pea germplasm collections (Hampton *et al.*, 1993). Detection of PSbMV has been by grow out experiments and visual inspection (Hampton *et al.*, 1993) or serological techniques such as ELISA (Maury *et al.*, 1987: Ding *et al.*, 1992; Hampton *et al.*, 1993; Masmoudi *et al.*, 1994a; Masmoudi *et al.*, 1994b). PSbMV isolates have been placed into three pathotype groups (Alconero *et al.*, 1986) based on their reactions on pea differential genotypes. Four recessive genes control resistance to the three PSbMV pathotypes P-1, P-4 and P-2 in pea (Provvidenti and Alconero, 1988a; Provvidenti and Alconero, 1988b). Four additional pathotypes U-1 and U-2 (Ali and Randles, 1997) and Pi and Pv (Khetarpal *et al.*, 1990) have been identified which do not fit into the grouping of PSbMV isolates on the pea differential genotypes.

The first report of PSbMV in Australia was in 1978 in seed imported from Sweden (Munro, 1978). Although PSbMV is a quarantinable pathogen in Australia occasionally new isolates have been reported (Ligat *et al.*, 1991).

9.2 Distribution of PSbMV in Australia

This thesis reports the distribution, collection and characterisation of PSbMV isolates from southern Australia. Two limited field surveys in 1995 of pea crops on the Yorke Peninsula, South Australia failed to detect PSbMV, although ClYVV was detected at an incidence of 0.3%. However, between 1995 and 1998, sixteen PSbMV isolates were collected from South Australia, Victoria and Western Australia: nine PSbMV isolates were identified in seed from South Australian and Victorian pea breeding lines; four were identified in South Australia in leaf tissue of pea field trial material; two PSbMV isolates were detected in plants from commercially available pea cultivars; one isolate was identified in a faba bean crop in Western Australia. Therefore, PSbMV is widespread in both pea seed lines and in field material in southern Australia. No quantitative survey was attempted.

9.3 Biological characterisation

Chapter 3 described the biological characterisation of PSbMV isolates on pea differential genotypes. The Australian PSbMV isolates and two PSbMV type isolates (PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4) were shown to be indistinguishable serologically yet were distinct biologically as observed by symptom variability on a range of pea differential genotypes, *P. sativum* cv Dundale and *C. quinoa*.

The Australian PSbMV isolates were placed in biologically defined groups represented by PSbMV pathotypes P-1 and P-4, based on their reactions on the four pea differential genotypes, PI 174319, PI 193586, PI 193835, and PI 269774 (Alconero *et al.*, 1986). These four pea differential genotypes do not distinguish between PSbMV pathotypes P-1 and P-2, but as no PSbMV pathotype P-2 type isolate was available for comparison in this study, isolates in the pathotype P-1 group may contain some pathotype P-2 isolates. Of the seven pea differential genotypes used in the biological pathotyping experiments, three (PI 193836, PI 347329, and PI 347422) did not react as previously described for PSbMV pathotype P-1 and P-4 type isolates. Evidence was obtained (Table 3.5) that these three pea differential genotypes were not true to type and that data from these tests were not reliable. Other researchers have also reported discrepancies from expected results with PSbMV pathotype isolates when using described pea differential genotypes (Timmerman *et al.*, 1993).

Future biological pathotyping work should test the pea differential genotypes following each round of multiplication to ensure that the genotypes remain true to type as heterogeneity has been reported in some pea differential seed lines (Hagedorn and Gritton, 1973; Alconero *et al.*, 1986; Provvidenti and Alconero, 1988c). A larger sample size (10-15 seed) should also be used to detect discrepancies in seed lines. However, in this study larger numbers of test plants for biological pathotyping were unavailable due to limited availability of seed. Biological pathotyping was found to be time consuming and unreliable in this study.

Only two PSbMV isolates, S6 and S4, were shown to become systemic in *C. quinoa* producing leaf distortion, vein yellowing and chlorotic local lesion on uninoculated leaves. Systemic spread of PSbMV on *C. quinoa* has been previously reported (Johansen *et al.*, 1996b; Andersen and Johansen, 1998).

9.4 Nucleic acid based diagnostics

Chapters 4, 5, and 7 report the development of nucleic acid based detection assays for PSbMV. Nucleic acid hybridisation with radiolabelled and DIG-labelled oligonucleotide probes VT02 and VT05 targeting the *HC-Pro* and 3'-UTR respectively showed positive signals with a range of PSbMV isolates. Both probes were PSbMV specific and did not detect a range of other legume infecting potyviruses. VT02 gave variable signal strength depending on the isolate tested. Hybridisation was ca. 10 fold less sensitive than RT-PCR for PSbMV *HC-Pro* detection and did not allow further discrimination of PSbMV isolates.

This thesis describes two RT-PCR assays for PSbMV detection which detect all PSbMV isolates tested from total nucleic acid extracts of leaf. Previously reported PSbMV RT-PCR (Kohnen *et al.*,1992; Kohnen *et al.*, 1995) and IC-PCR (Phan *et al.*, 1997) assays have been isolate specific. RT with VT04 and PCR with VT02/VT03 primers amplified a 1085 bp amplicon from the *HC-Pro* gene of all of the 31 PSbMV isolates tested and RT with VTNIa and PCR with VTCI/VTVPg primers amplified a 780 bp amplicon from the *CI/6K2/VPg* region of all of the 29 PSbMV isolates tested. This is the first report that a RT-PCR for PSbMV can detect a range of PSbMV isolates representing pathotype P-1 and P-4.

The *HC-Pro* RT-PCR did not detect a range of other legume infecting potyviruses (ClYVV, BYMV, PMV, PeMoV, TuMV, BCMV, and LMV) or TMV. This requires further testing but appears to be a PSbMV specific assay. The *HC-Pro* RT-PCR has detected PSbMV infection in dry pea seed and shown to be ca. 20 fold more sensitive than DIBA. This assay has an advantage over the reported IC-PCR for PSbMV detection from seed, which is less sensitive than ELISA and shown not to detect all isolates tested (Phan *et al.*, 1997).

Chapter 6 described the development of an exogenous internal control for use in a duplex RT-PCR with the PSbMV specific *HC-Pro* RT-PCR for detection of PSbMV from pea seed. An internal control in diagnostic RT-PCR assays is important for distinguishing a false negative reaction (failed RT-PCR) from a true negative reaction (absence of pathogen template). The internal control developed was based on a TMV RNA supplemented to the RT reaction. Conditions were optimised so that the PSbMV RT-PCR sensitivity was not compromised and non-specific products were not generated in the duplex RT-PCR. The TMVR3/TMVF1 primers amplify a 406 bp amplicon from TMV particles or TMV RNA.

By removing testas prior to testing the RT-PCR assay developed for seed testing avoided the possibility of detecting non-infectious PSbMV (Johansen *et al.*, 1994). An endogenous internal control for the RT-PCR detection of GVA, GVB and GLRaV-3 has been described, however non-specific amplification products and competition interfered with the reliability of the multiplex RT-PCR (Nassuth *et al.*, 2000). This is therefore the first reliable and specific RT-PCR internal control developed for use in a plant virus RT-PCR diagnostic assay.

The duplex RT-PCR assay has been shown to report PSbMV seed infection rates more accurately than DIBA, which under-estimated seed infection rates. The limit of detection of the PSbMV duplex RT-PCR was approximately 125 pg PSbMV RNA per 15 µL RT reaction volume which is the equivalent of cDNA generated from 17 pg PSbMV RNA in a 20 µL PCR mixture. This test would therefore be approximately 10-100 fold more sensitive than DIBA. Further work needs to be done to determine the detection threshold of RT-PCR for a single PSbMV infected seed in batches of healthy test seed. It was demonstrated that the duplex TMV/PSbMV RT-PCR could be done in a 96 well plate format, allowing the test to be automated.

9.5 Molecular Characterisation

Chapters 5 and 7 describe the molecular characterisation of a range of PSbMV isolates. RFLP analyses of both the *HC-Pro* and *CI/6K$_2$/VPg* RT-PCR amplicon have provided the first report of molecular discrimination and grouping of PSbMV isolates without the need for biological pathotyping or sequencing.

Extensive RFLP analysis of the 1085 bp *HC-Pro* amplicon was done on 31 Australian, Pakistani and PSbMV type isolates with seven restriction enzymes (*Alu*I, *Hae*III, *Hha*I, *Rsa*I, *Sau*3AI, *Taq*α1 and *Tru*91). *Tru*91 gave maximum discrimination between isolates producing six RFLP patterns, and *Taq*α1 gave least discrimination between PSbMV isolates, producing two RFLP patterns. When the RFLP results generated with each of the seven restriction enzymes for each PSbMV isolate were considered together the 31 PSbMV isolates could be placed into eight distinct groups.

The 15 Australian PSbMV isolates were represented in three of the groups, one of which was unique to a single Australian isolate. The 14 Pakistani PSbMV isolates were represented in five of the groups, four of which were unique to the Pakistani isolates.

RFLP analysis of the *HC-Pro* suggests that the Pakistani isolates are more variable than the Australian PSbMV isolates and that the Australian PSbMV isolates may have originated from few introductions. The single group represented in both the Australian and Pakistani collections contained the largest number of isolates suggesting this virus group has spread through trade of infected seed and become established in different geographic locations.

Clones of the *HC-Pro* from PSbMV isolates representing the eight groups described in this thesis have been produced for future characterisation of new PSbMV isolates.

Chapter 7 describes the characterisation of the PSbMV VPg. The VPg has been identified as the avirulence determinant in PSbMV pathotype P-1 on *sbm-1/sbm-1* pea plants (Keller *et al.*, 1998; Borgstrom *et al.*, 1998). The VPg has also been implicated as the avirulence determinant for other potyviruses on certain plant cultivars (Masuta *et al.*, 1999; Nicolas *et al.*, 1997; Rajamaki and Valkonen, 1999). The VPgs of PSbMV US pathotype P-1 and PSbMV S6 pathotype P-4 have been cloned, sequenced and compared with the VPg from PSbMV DPD1 pathotype P-1 and PSbMV NY pathotype P-4. A variable region at amino acid position 105-119 of the VPg was found to discriminate between the pathotype P-1 and P-4 isolates. This region contained an *Mfe*I site which allowed pathotype P-1 and P-4 isolates to be distinguished by RFLP analysis. RFLP analysis of the 780 bp CI/6K_2/VPg with *Mfe*I for all 29 PSbMV isolates tested produced three distinct RFLP patterns: one uncut PSbMV pathotype P-4 like pattern and two PSbMV pathotype P-1 like patterns. Sequence analysis of seven PSbMV isolates representative of these three patterns confirmed that they were either pathotype P-1 or P-4.

The variable central region identified in the VPg specific for pathotype P-1 and P-4 was also conserved among the seven sequenced PSbMV isolates. The pathotype P-1 sequence was MWNDELDKQLFVTRP and the pathotype P-4 sequence was VFNDTLDSQAVRSNH. In addition the VPg of the PSbMV L1 pathotype P-2 was shown by sequence comparisons to have a deletion of three amino acids corresponding to position 103-105, not observed in any of the other PSbMV isolates. The variable central region also differed in pathotype P-2 (-ADEDLDKQLWWARP). On this basis no PSbMV pathotype P-2 isolates were identified among the seven sequenced PSbMV isolates.

Future work could develop primers or probes targeting the variable central VPg region identified for establishing pathotype groups.

An aspartic acid residue was identified at amino acid position 75 of the VPg from 12 PSbMV isolates. Aspartic acid at residue 77 of the VPg is necessary for binding of the TuMV VPg to the translation eukaryotic initiation factor (eIF9iso)4E and shown to be conserved among eight different potyviruses (Leonard *et al.*, 2000).

9.6 Sequence and phylogenetic analysis

Chapters 8 and 9 describe new PSbMV sequence data for the *CI/6K$_2$/VPg* and *HC-Pro* regions. Published PSbMV sequence data is limited and mainly targeted to the *CP* gene. Three full-length sequences have been described for PSbMV DPD1 pathotype P-1 (GenBank D10930), PSbMV NY pathotype P-4 (EMBL X89997) and PSbMV L1 pathotype P-2 (EMBL AJ252242). Shorter sequences representing a 418 bp region of the *CP* (EMBL AF023134-AF023152: Anderson and Johansen, 1998) and other regions of the *CP* and *NIb* genes have also been reported (EMBL D10453, U16215, Z48508, Z48509 and X87938). This is the first report of PSbMV sequence data from *HC-Pro* and *CI/6K$_2$/VPg* for a range of PSbMV isolates. Sequence data are presented for a 853 bp region of the *HC-Pro* from 14 isolates and 732 bp region of the 3′ end of the *CI*, entire *6K$_2$* and partial *VPg* from 9 isolates. PSbMV isolates showed greater sequence similarity in the *HC-Pro* region than the *CI/6K$_2$/VPg* region. PSbMV isolates showed 88.7-100% similarity at the amino acid sequence level and 79.4-99.8% similarity at the nucleotide sequence level for the *HC-Pro* region and 84.4-100% similarity at the amino acid sequence level and 77.7-100% similarity at the nucleotide sequence level for the *CI/6K$_2$/VPg* region.

Chapter 8 describes the phylogenetic analysis of Australian and Pakistani PSbMV isolates. Phylogenies produced from PSbMV isolates from both the *HC-Pro* and *CI/6K$_2$/VPg* sequence data produced the same grouping for all isolates except one (PSbMV P515-1-1). This isolate may represent a recombinant, however statistical analysis (Revers *et al.*, 1996; Worobey and Holme, 1999) would be required to confirm this hypothesis. Recombination has been shown to occur among other potyvirus isolates (Revers *et al.*, 1996; Bousalem *et al.*, 2000). The PSbMV isolates were placed into two clades, which were either PSbMV pathotype P-4 like or PSbMV pathotype P-1 like. The PSbMV L1 pathotype P-2 isolate was more closely related to the PSbMV pathotype P-1 isolates.

Table 9.1 summarises the results obtained by biological pathotyping, RFLP analysis of the *CI/6K$_2$/VPg*, and phylogenetic analyses of *HC-Pro* and *CI/6K$_2$/VPg* for the Australian

PSbMV isolates. Ten of the 14 Australian PSbMV isolates tested by at least two of the methods were in agreement. Pathotype grouping of PSbMV M257-7-3, UK4, and S4 differed between the biological grouping and molecular grouping. This incongruity could be due to possible incorrect assignment to biological pathotype groups as biological pathotyping was observed to be inconsistent when done with some pea differential genotypes, and only limited tests were used for this comparison. PSbMV UK4 and S4 were only compared by two methods, biological pathotyping and RFLP analysis of the *CI/6K$_2$/VPg*. PSbMV M257-7-3 was placed into pathotype P-4 by the three molecular methods described and pathotype P-1 by the biological pathotyping method. PSbMV P515-1-1 was the only isolate that varied in its assignment of pathotype group with the molecular test analysed. However, only the VPg has been indicated as a pathotype specific determinant (Keller *et al.*, 1998; Borgstrom *et al.*, 1998) and *HC-Pro* sequence data may not be reliable in establishing pathotype groups.

The Pakistani PSbMV isolates used in these molecular studies have been placed into biological pathotype groups using pea differential genotypes (Ali and Randles, 1997). Based on their results PK4 is pathotype U-1, PK6 is pathotype P-1, PK7 is pathotype P-1 and PK9 is pathotype U-2. In this thesis PK4, PK7 and PK9 were placed into pathotype P-1 and PK6 into pathotype P-4 groups based on the RFLP analysis of the 780 bp *CI/6K$_2$/VPg*, comparison of the variable central VPg region and phylogenetic analysis of the *HC-Pro* and *CI/6K$_2$/VPg*. Therefore, the results presented in this thesis, with the exception of PK7 are not consistent with the biological pathotyping results of Ali and Randles (1997).

9.7 Conclusions

This thesis makes the following original contributions to the knowledge of PSbMV characterisation and detection:

(a) PSbMV has been detected in field trial and breeders germplasm material in South Australia, Victoria and Western Australia.

(b) Sixteen PSbMV isolates have been collected and shown to be biologically variable.

(c) Hybridisation assays have been developed which detect the Australian PSbMV isolates.

(d) A PSbMV specific RT-PCR targeting the *HC-Pro* was developed which detected all 31 biologically variable PSbMV isolates but not a range of other legume infecting potyviruses.

(e) RFLP analysis of the *HC-Pro* region of 31 PSbMV isolates has allowed isolates to be placed into eight groups.

(f) RT-PCR targeting the *CI/6K$_2$/VPg* was developed which detected all 29 PSbMV isolates tested.

(g) RFLP analysis of the 780 bp *CI/6K$_2$/VPg* region allowed PSbMV isolates to be placed into PSbMV pathotype P-1 or P-4 like groups.

(h) A method for duplex RT-PCR using an exogenous TMV internal control as a quality control measure was developed for the detection of PSbMV in pea seed.

(i) Sequence of the complete *VPg* of PSbMV pathotype P-1 and pathotype P-4 was obtained and a region identified which distinguished pathotypes P-1 and P-4 from each other and from pathotype P-2.

(j) Sequences have been obtained for a 853 bp region of the *HC-Pro* from 14 isolates and 732 bp region of the 3′ end of the *CI*, entire *6K$_2$* and partial *VPg* from 9 isolates and used for phylogenetic analysis.

Table 9.1: Placing Australian PSbMV isolates into pathotype like groups based on biological and molecular investigations.

Method of analysis	PSbMV isolates													
	US	S6	M257-7-3	3(6)	43(1)	VIDA	19(1)	21(1)	P503-4-2	19(7)	UK4	FFD	P515-1-1	S4
[a] Biological pathotyping	P-1	P-4	P-4	P-4	-	P-4	P-4	P-1	P-4	P-1	P-4	P-1	P-4	P-4
[b] RFLP VPg	P-1	P-4	P-1	P-4	P-4	P-4	P-4	P-1	P-4	P-1	P-1	P-1	P-1	P-1
[c] Phylogenetics *HC-Pro*	P-1	P-4	P-1	P-4	P-4	P-4	P-4	-	-	-	-	P-1	P-4	-
[c] Phylogenetics *CI/6K$_2$/VPg*	P-1	P-4	P-1	-	-	-	P-4	-	-	-	-	-	P-1	-

[a] Results described in Chapter 3

[b] Results described in Chapter 7

[c] Results described in Chapter 8

- not tested

APPENDICES

APPENDIX A

Biochemicals and miscellaneous chemicals

Biochemical	Source
γ-^{32}P ATP	GeneWorks, Australia
α-^{32}P UTP	GeneWorks, Australia
0.24-9.5kb RNA ladder	GIBCO BRL Life Technologies, USA
1kb DNA ladder	GIBCO BRL Life Technologies, USA
1kb DNA Plus ladder	GIBCO BRL Life Technologies, USA
100 bp DNA Ladder	Promega, USA
*Alu*I	Boehriger Mannheim, Germany
Ampicillin	Progen, Australia
AmpliTaq DNA Polymerase	Perkin Elmer, USA
AMV Reverse Transcriptase	GeneWorks, Australia
Anti-digoxigenin-AP Fab fragments	Boehriger Mannheim, Germany
CSPD®	Boehriger Mannheim, Germany
dATP	Promega, USA
dCTP	Promega, USA
Deep Vent® (exo-) DNA Polymerase	New England BioLabs, USA
Deep Vent® DNA Polymerase	New England BioLabs, USA
dGTP	Promega, USA
dTTP	Promega, USA
*Eco*RI	Promega, USA
eLONGase™ Enzyme Mix	GIBCO BRL Life Technologies, USA
Goat anti-rabbit IgG-alkaline phosphatase conjugate	Sigma,USA
Herring Testes DNA (Type XIV)	Sigma, USA
*Hae*III	Promega, USA
*Hha*1	Boehriger Mannheim, Germany
*Mbo*I	Boehriger Mannheim, Germany
*Mfe*I	New England BioLabs, USA
MMLV Reverse Transcriptase	Epicentre Technologies, USA
pGEM®-T Vector system	Promega, USA
Proteinase K	Amresco, USA
Pwo DNA Polymerase	Boehriger Mannheim, Germany

Retrotherm™ RT	Epicentre Technologies, USA
RNase Inhibitor Human	GeneWorks, Australia
RNasin®	Promega, USA
RQ1 RNase-free DNase	Promega, USA
*Rsa*1	Promega, USA
*Sau*3A1	Promega, USA
SP6 RNA Polymerase	Promega, USA
T4 PNK	GeneWorks, Australia
*Taq*αI	New England BioLabs, USA
Taq DNA Polymerase in storage buffer A	Promega, USA
*Tru*91	Boehriger Mannheim, Germany
Tth DNA Polymerase	Boehriger Mannheim, Germany
Tth DNA Polymerase	Epicentre Technologies, USA

Chemical	**Source**
2-mercaptoethanol	Riedel-deHaën, Germany
8-hydroxyquinoline	M&B Pronalys, Australia
Acetic acid	BDH AnalaR, Australia
Acetone	BDH AnalaR, Australia
Acrylamide	RIO-RAD, USA
Agar Bacteriological (Agar No.1)	Oxoid, England
Agarose 1	Amresco, USA
Ammonium acetate	UNIVAR, Australia
APS	Amresco, USA
BCIP	Sigma, USA
Blocking reagent	Boehringer Mannheim, Germany
Bis	BIO-RAD, USA
Boric acid	UNIVAR, Australia
Bromphenol blue (sodium salt)	Probing & Structure, USA
BSA (Fraction V)	Sigma, USA
$CaCl_2$ (dihydrate)	BDH AnalaR, Australia
$CaCl_2$ dried	Unilab, Australia
carborundum	Shelley's lapidary supplies, Mile End, Australia
Chloroform	BDH AnalaR, Australia

Cs_2SO_4	BDH, England
CTAB	Merck, Germany
D(+) Mannose	Sigma, USA
DEPC	Sigma,USA
D-Glucose	BDH AnalaR, Australia
DIG blocking reagent	Boehringer Mannheim, Germany
EDTA	BDH AnalaR, Australia
EtBr	Amresco, USA
Ethanol	BDH AnalaR, Australia
Ficoll (Type 400)	Pharmacia, Sweden
Formaldehyde	M&B Pronalys, Australia
Formamide	Boehringer Mannheim, Germany
Gycerol	BDH AnalaR, Australia
IPTG	Sigma, USA
Isoamyl alcohol	UNIVAR, Australia
Isopropanol	UNIVAR, Australia
KCl	BDH AnalaR, Australia
KH_2PO_4	BDH AnalaR, Australia
Maleic acid	BDH AnalaR, Australia
$MgCl_2$	UNIVAR, Australia
$MgCl_2.6H_20$	UNIVAR, Australia
$MgSO_4.7H_20$	UNIVAR, Australia
MOPS	Sigma,USA
N,N-dimethylformamide	Sigma, USA
$Na_2HPO_4.7H_20$	BDH AnalaR, Australia
Na_2SO_3	BDH AnalaR, Australia
NaCl	BDH AnalaR, Australia
NaN_3	Sigma, USA
NaOH	BDH AnalaR, Australia
NBT	Sigma, USA
N-lauroyl sarcosine	Sigma, USA
PEG_{6000}	Sigma, USA
Phenol	UNIVAR, Australia
Potassium acetate	UNILAB, Australia
Potassium acetate	UNILAB, Australia

PVP	Sigma, USA
SDS	BDH, England
Sephadex G-25	Pharmacia, Sweden
Sodium acetate	UNIVAR, Australia
Sucrose	UNIVAR, Australia
TEMED	BIO-RAD, USA
Tri sodium citrate	BDH AnalaR, Australia
Tris	Amresco, USA
Triton® X-100	Amresco, USA
Tryptone	Oxoid, England
Tween® 20	BIO-RAD, USA
Urea	BDH AnalaR, Australia
X-Gal	Amresco, USA
Xylene cyanol	LABCHEM, Australia
Yeast Extract	Oxoid, England

APPENDIX B

Polyacrylamide and agarose gels, bacterial strains and media, solutions and buffers

Polyacrylamide and agarose gels

40% Acrylamide (19:1) solution

38 g acrylamide

2 g bis – acrylamide

Make up to 100 mL with SDDW, sterilize filtration through a 0.45 µm filter (Schleicher & Schuell, Germany) and store in dark

20% denaturing polyacrylamide gel (45 mL solution)

Urea	18.9 g
40% Acrylamide solution	22.5 mL
10 X TBE	4.5 mL
10% APS	450 µL
TEMED	45 µL
SDDW	8.0 mL

Agarose gels

To 400 mL of sterile 1 X TAE add agarose to desired percentage and heat in microwave until dissolved. Add EtBr to a final concentration of 0.5 µg/mL. Allow to cool to 60°C before pouring into gel cast.

Bacterial strains and media

DH5α®

ϕ80d*lacZ*ΔM13,*rec*A1,*end*A1,*gyr*A96,*thi*-1,*hsdR*17(r_K-,m_K+),*supE*44,*rel*A1,*deoR*,Δ(*lacZYA-argF*)U169

LB medium (per L)

10 g Tryptone

5 g Yeast extract

5 g NaCl

Adjust pH to 7.0 with NaOH

Sterilise by autoclaving

LBA/ampicillin plates

Add 15 g Agar Bacteriological to 1 L of LB medium. Autoclave and allow to cool to 60°C before adding ampicillin to 100 µg/mL then pour into petri dishes. Can be stored for 1 month at 4°C in the dark.

LBA/ampicillin/X-Gal/IPTG plates

To LBA/ampicillin plate add 40 µL of X-Gal stock (20 mg/mL) and 4 µL IPTG stock (200 mg/mL) and spread over the surface. Allow to absorb for 30 min before use.

SOC medium (100 mL)

2 g Tryptone

0.5 g Yeast extract

1 mL 1M NaCl

250 µL 1M KCl

Adjust pH to 7.0 with 1N NaOH and sterilize by autoclaving. Cool to RT before adding 1 mL of 2M Mg^{2+} filter sterilised stock and 1 mL of 2M filter sterilised glucose. Sterilize the by filtration through 0.2 µM filter (Schleicher & Schuell, Germany)

2M Mg^{2+} stock (100 mL)

20.33 g $MgCl_2.6H_2O$

24.65 g $MgSO_4.7H_2O$

Sterilize by filtration

Solutions and buffers

BCIP stock

50 mg BCIP

1000 µL N,N-dimethylformamide

Store at -20°C

BE buffer

10 mM sodium borate pH 8.2

1 mM EDTA

Sterilize by autoclaving

CTAB buffer

1.4 M NaCl

0.1 M Tris HCl pH 8.0

2% CTAB

20 mM EDTA

Sterilize by autoclaving

100 X Denhardt's

2% BSA

2% PVP

2% Ficoll

Sterilize by filtration and store at –20°C

DEPC treated H_2O

0.1% (v:v) DEPC was added to H_2O and shaken vigorously. The solution was allowed to stand for 16 hrs in a fume hood and then autoclaved.

DIBA BSA Blocking Buffer

1 X PBS, pH 7.4

1% w/v BSA

DIBA Wash Buffer AP 7.5

100 mM Tris-HCl pH 7.5

100 mM NaCl

2 mM $MgCl_26H_2O$

0.05% v/v Triton® X-100

0.02% w/v NaN_3

DIBA Wash Buffer AP 9.5

100 mM Tris-HCl pH 9.5

100 mM NaCl

5 mM $MgCl_26H_2O$

0.02% w/v NaN_3

DIBA substrate buffer

10 mL wash buffer AP 9.5

44 µL NBT stock

33 µL BCIP stock

DIBA Stop Buffer

10 mM Tris-HCl pH7.5

5 mM EDTA

Sterilise by autoclaving

DIG Buffer 1 (Maleic acid buffer)

0.1 M Maleic acid

0.15 M NaCl

Adjust pH to 7.5 with NaOH

Sterilise by autoclaving

DIG Washing buffer

Maleic acid buffer with 0.3% Tween® 20 (v/v)

Do not autoclave

DIG Blocking stock solution (10 X conc)

10% Blocking reagent (w/v) in maleic acid buffer. Dissolve blocking reagent with heat.

Sterilise by autoclaving and store at 4°C.

DIG Buffer 2 (Blocking solution)

Dilute blocking stock solution 1:10 in maleic acid buffer

DIG Buffer 3 (Detection buffer)

0.1 M Tris-HCl pH 9.5

0.1 M NaCl

Sterilise by autoclaving

10mM dNTP stock

2.5 µL of 100 mM dATP

2.5 µL of 100 mM dCTP

2.5 µL of 100 mM dGTP

2.5 µL of 100 mM dTTP

90 µL sterile H_2O.

Store in 50 µL aliquots at –80°C

Extraction buffer

1 M Urea

100 mM ammonium acetate pH 7.2

20 mM EDTA

1% w/v Na_2SO_3

5 X Formaldehyde gel-running buffer

0.1 M MOPS pH 7.0

40 mM sodium acetate

5 mM EDTA

DEPC treated DDW

Filter sterilize solution through 0.2 µm filter (Schleicher & Schuell, Germany) and store in dark.

10 X Formaldehyde gel-loading buffer

50% glycerol

1 mM EDTA

0.25% bromophenol blue

0.25% xylene cyanol FF

Formamide loading dye

80% v/v formamide

10 mM EDTA

0.25% w/v bromophenol blue

0.25% w/v xylene cyanol FF

6 X Gel loading buffer III

30% glycerol in water

0.25% w/v bromphenol blue

0.25% w/v xylene cyanol FF

NBT stock (75mg/mL)

75 mg NBT

700 µL N,N-dimethylformamide

300 µL SDDW

Store at -20°C

K/SDS solution

0.5 M NaCl

100 mM sodium acetate

10 mM EDTA pH 8.0

2% soluble PVP 40

0.25% v/v 2-mercaptoethanol (add just before use)

20 X PBS Buffer

2.74 M NaCl

60 mM KCl

200 mM Na_2HPO_4

40 mM KH_2PO_4

0.4% NaN_3

Adjust pH to 6.8. Sterilise by autoclaving

Preparation of Phenol Solution

500gm of crystalline phenol was melted in a 65°C water bath and 8-hydroxyquinoline added to a final concentration of 0.1%. Once melted an equal volume of 0.5 M Tris-HCl, pH 7.0 was added and stirred for 30 min at room temperature. The phases were allowed to separate and the aqueous phase removed by aspiration. An equal volume of 0.1 M Tris-HCl, pH 7.0 was added and stirred for 30 min. The phases were allowed to separate and the aqueous phase removed. This was repeated until the pH of the phenolic phase was pH 6-7.6. The pH was measured with pH paper. Once the phenol was equilibrated the final

aqueous phase was removed and replaced with 0.1 vol. of 0.1 M Tris-HCl, pH 7.0 containing 0.2% β-mercaptoethanol. Phenol was stored in a light tight bottle at 4°C.

Phenol:Chloroform:Isoamyl Alcohol (25:24:1)

Equal parts of equilibrated phenol and chloroform:isoamyl alcohol (24:1) were added.

0.2 M Phosphate buffer pH 7.0

115.4 mL 1 M Na_2HPO_4

84.6 mL 1M NaH_2PO_4

Make upto 1L with SDDW

10 X Proteinase K buffer

100 mM Tris-HCl pH 7.8

50 mM EDTA

5% SDS

Sterilise by autoclaving

50 X TAE

2 M Tris

950 mM acetic acid

50 mM EDTA (pH 8.0)

Sterilise by autoclaving

TE Buffer

10 mM Tris-HCl pH 7.5

1 mM EDTA

Sterilise by autoclaving

10 X TBE

900 mM Tris

900 mM boric acid

20 mM EDTA (pH 8.0)

Sterilise by autoclaving

Sephadex® G-25

Add 10 g Sephadex® G-25 (Pharmacia, Sweden) to distilled sterile H_2O. Wash the swollen resin several times with SDDW. Equilibrate resin in TE pH 7.6. Sterilise by autoclaving.

50 mM Sodium borate buffer

50 mM boric acid

Adjust pH to 8.2 with 1N NaOH and sterilise by autoclaving.

20 X SSC

3 M NaCl

0.2 M Tri-sodium citrate

Sterilise by autoclaving

20 X SSPE

3.6 M NaCl

0.3 M $Na_2HPO_4.7H_2O$

20 mM EDTA

Sterilise by autoclaving

APPENDIX C

Oligonucleotides used as primers and/or probes

Name	Length (nt)	Sequence
VT01 [a]	25mer	5'-d(ATG ACC ATG GCG CGT CCT GTG AAT C)-3'
VT02 [a]	30mer	5'-d(GCA GTT GCT ACA TCC ATC ATT GTT GGC CAT)-3'
VT03 [a]	30mer	5'-d(GTG TTG GAG GAA TCA CAC CAG AAG AAT GTG)-3'
VT04 [a]	25mer	5'-d(CTC CAA AAC CAT GCT TCA CTC TTG A)-3'
VT05 [a]	33mer	5'-d(GCC CTA CTG CCA CCA AAC CGA AGT CAA AGA CTC)-3'
VTHCFor [a]	21mer	5'-d(ATT TCC ATC AGC ACA TCA TGT)-3'
VTHCRev [a]	20mer	5'-d(ACG CAT GAG CAA GGA TAC AC)-3'
VTVPg [a]	22mer	5'-d(TAT CGC AGA CCC TCA TTG GAT T)-3'
VTVPgseq [a]	26mer	5'-d(ATA GGG TGT CTA TCA TAA GTC AAC CC)-3'
VTCI [b]	25mer	5'-d(TCG CAG GTT TAG TCA ACA GAT TGC G)-3'
VTNIa [b]	20mer	5'-d(CTA CAC ACG GCT TGC GCA AT)-3'
VTNIb [b]	25mer	5'-d(CAC AAT ACA GCC AAC CGT CAG GTA G)-3'
TMVR3 [b]	22mer	5'-d(TTC CAA TGA ACG TCG TGA CGT C)-3'
TMVF1 [a]	29mer	5'-d(TAC CCG GCT TTG CAG ACG ATT GTG TAC CA)-3'
TMVR1 [a]	29mer	5'-d(GTG ACG TCC CCG CTC TTT CTT TGA TAC CA)-3'
TMVF2 [a]	29mer	5'-d(ATC AAA GGA GCC TTT TGC GGT GAC GAT AG)-3'
TMVR2 [a]	27mer	5'-d(AAT TGT CAG GCA AGT TCC ACT CGC CCG)-3'
SP6 [c]	19mer	5'-d(TAT TTA GGT GAC ACT ATA G)-3'
T7 [c]	20mer	5'-d(TAA TAC GAC TCA CTA TAG GG)-3'
PV2I/T7 [d]	38mer	5'-d(TAATACGACTCACTATAGGGIAA(C/T)AA(C/T)AG(C/T)GGICA(A/G)CC)-3'
PV1/SP6 [d]	37mer	5'-d(GATTTAGGTGACACTATAG$(T)_{17}$(A/G/C))-3'

[a] Genset, Singapore Biotech. Pty. Ltd., Singapore

[b] GIBCO BRL, Life Technologies Pty. Ltd., Mulgrave, Victoria, Australia

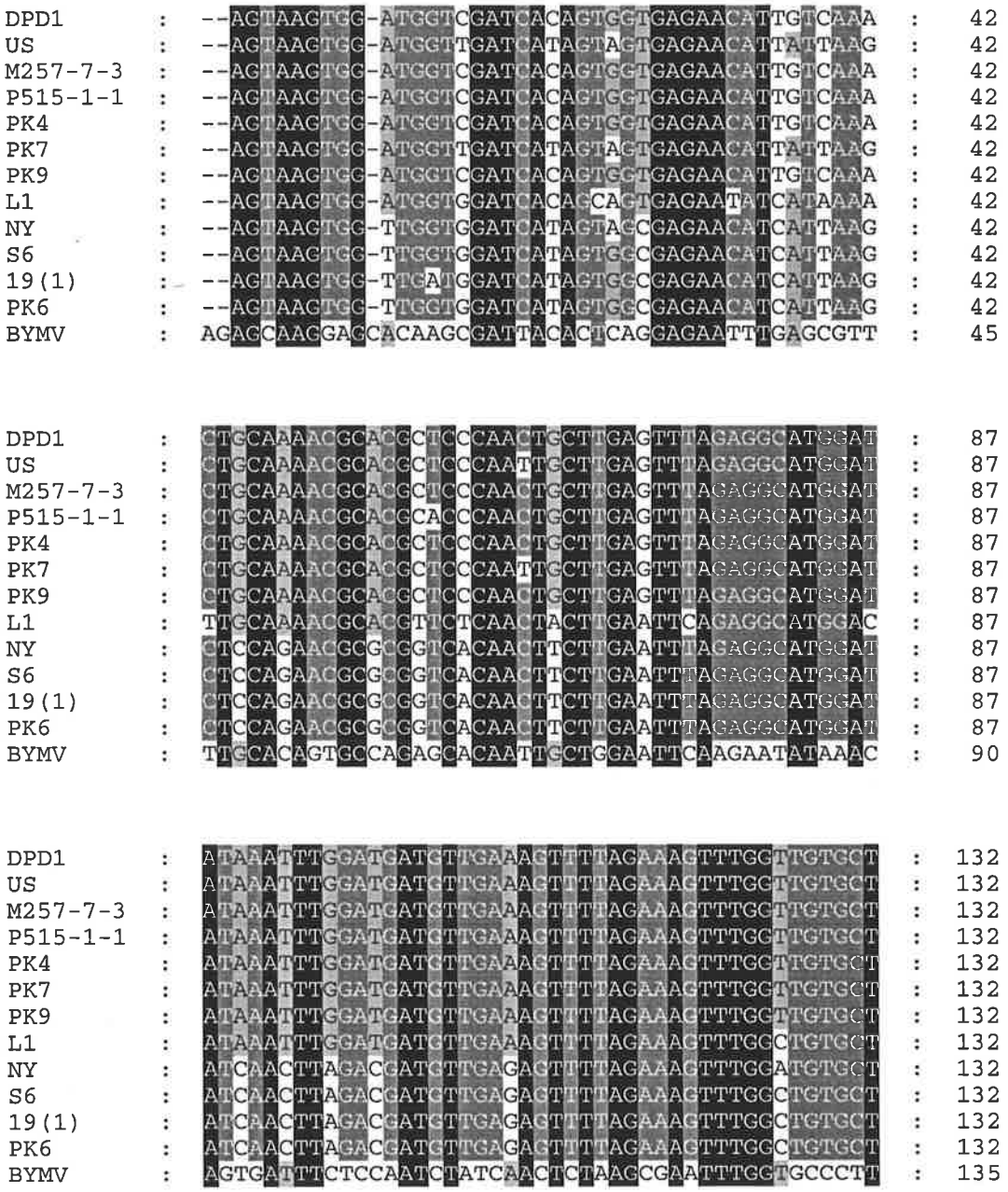
[c] Promega, Madison, WI, USA

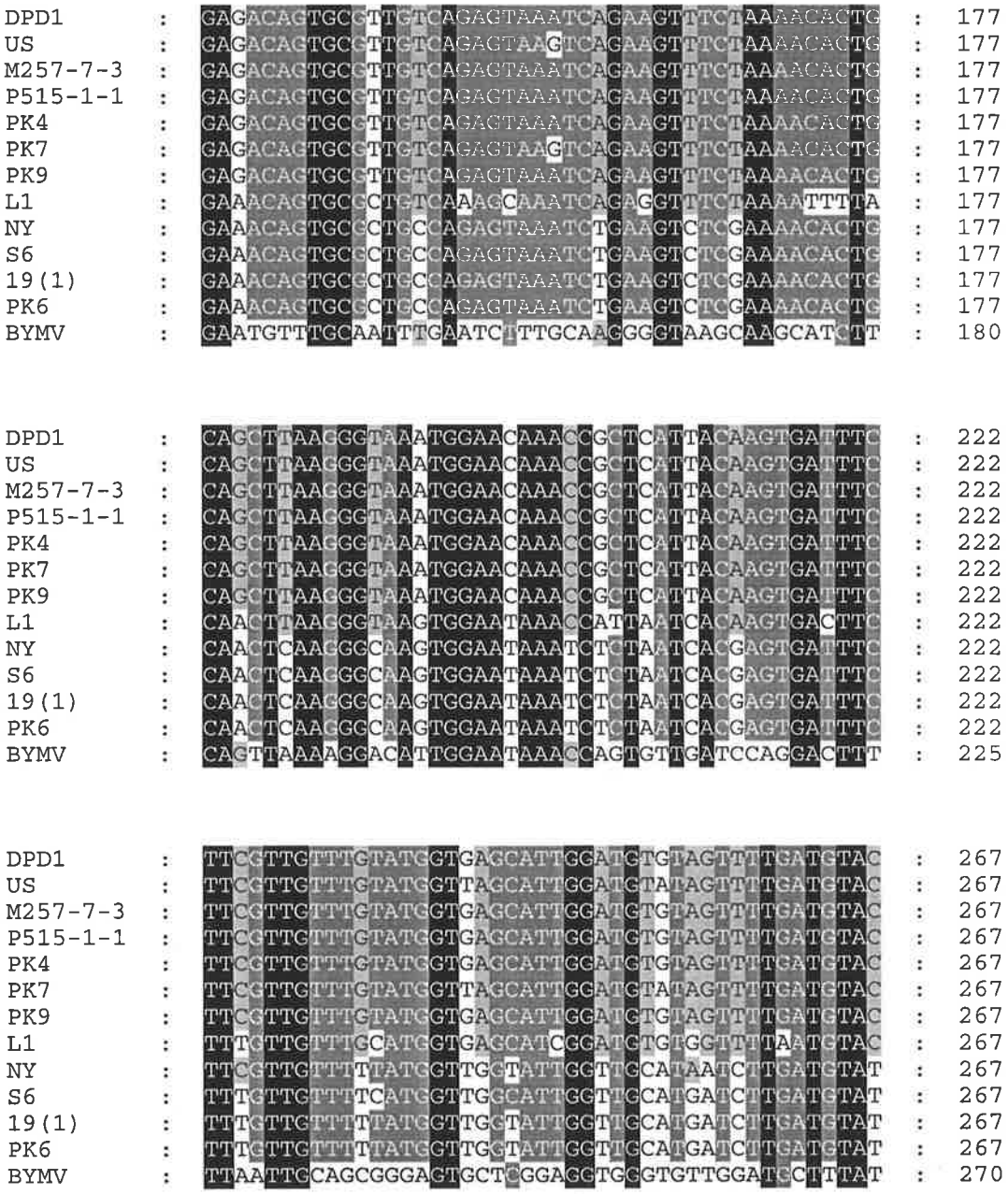
[d] Supplied by Anne Mackenzie, Research School of Biological Science, The Australian National University, Canberra, Australia.

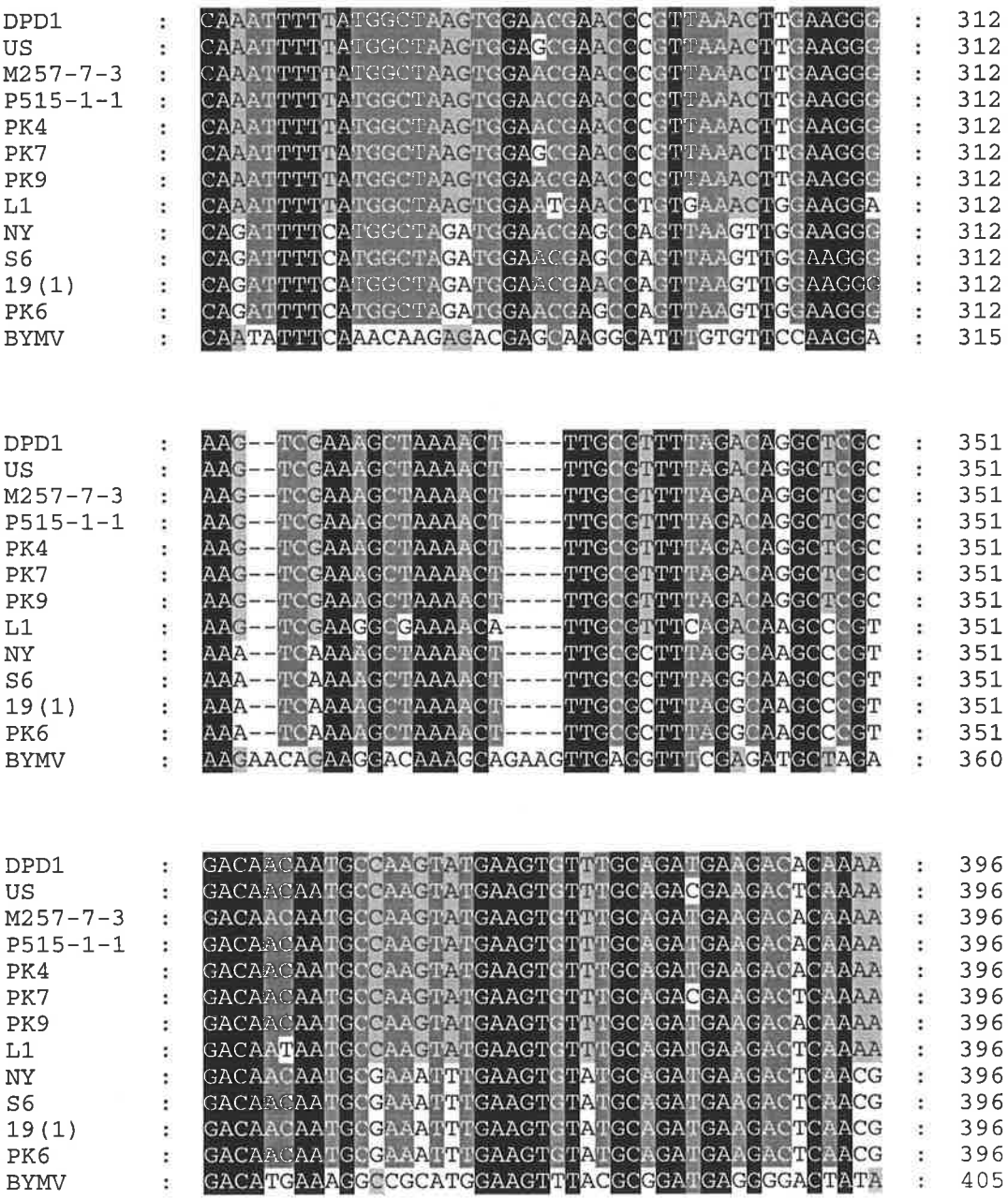
Underlined regions indicate T7 primer sequence in PV2I/T7 and SP6 primer sequence in PVI/SP6.

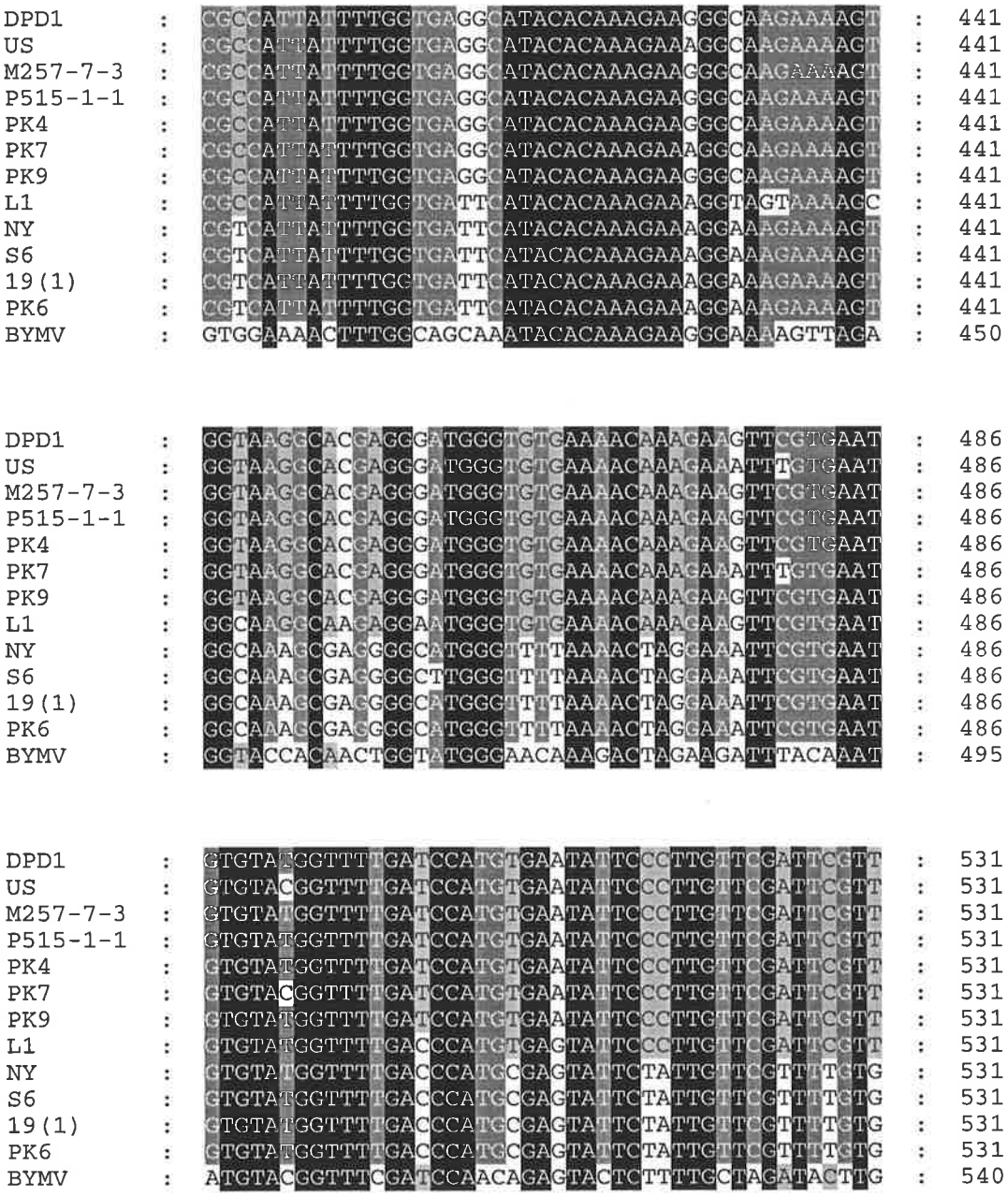
APPENDIX D

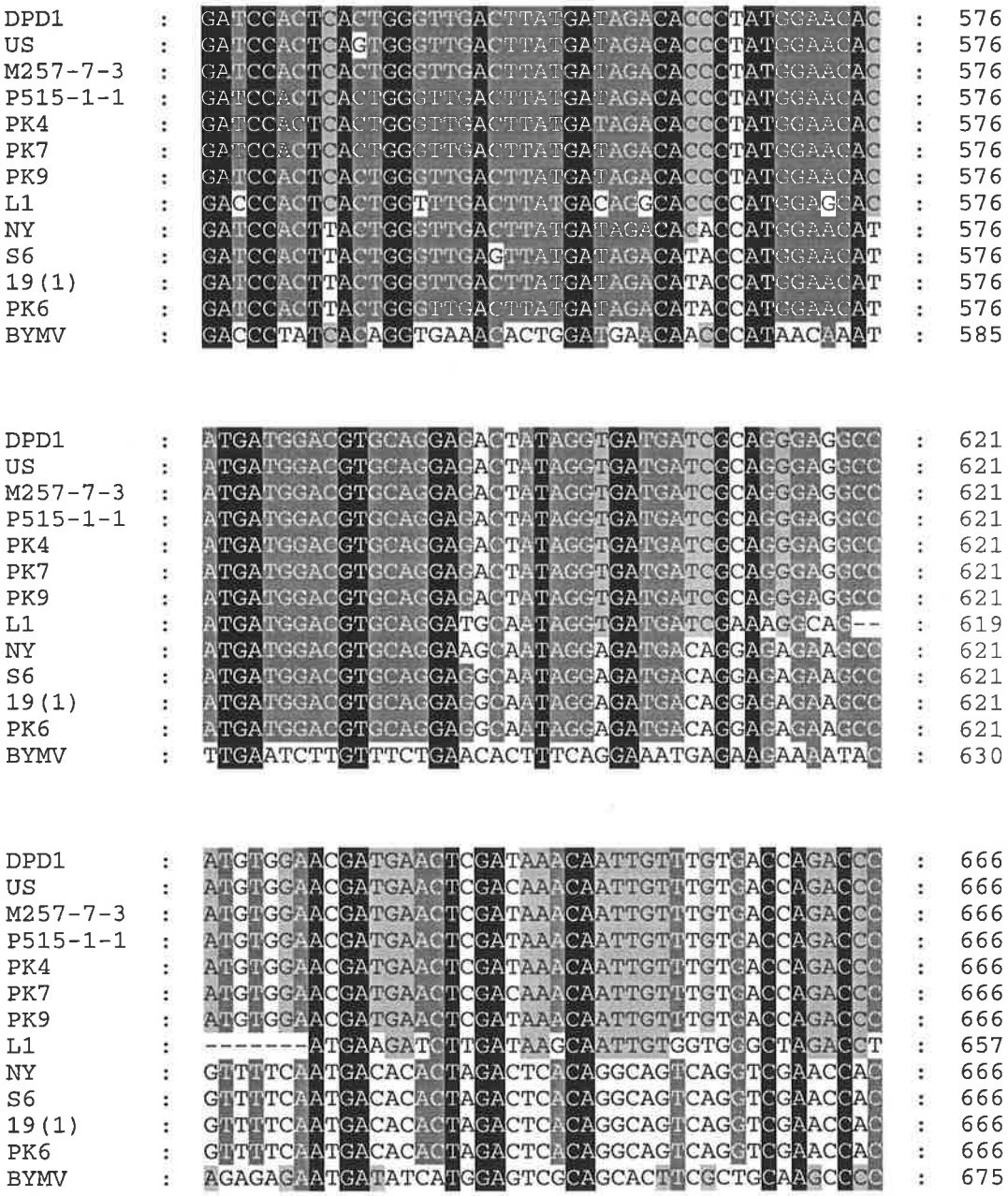
Nucleotide sequence alignment of 732 bp region of the 3′ end *CI*, entire $6K_2$ and partial *VPg* for 12 PSbMV isolates and BYMV.

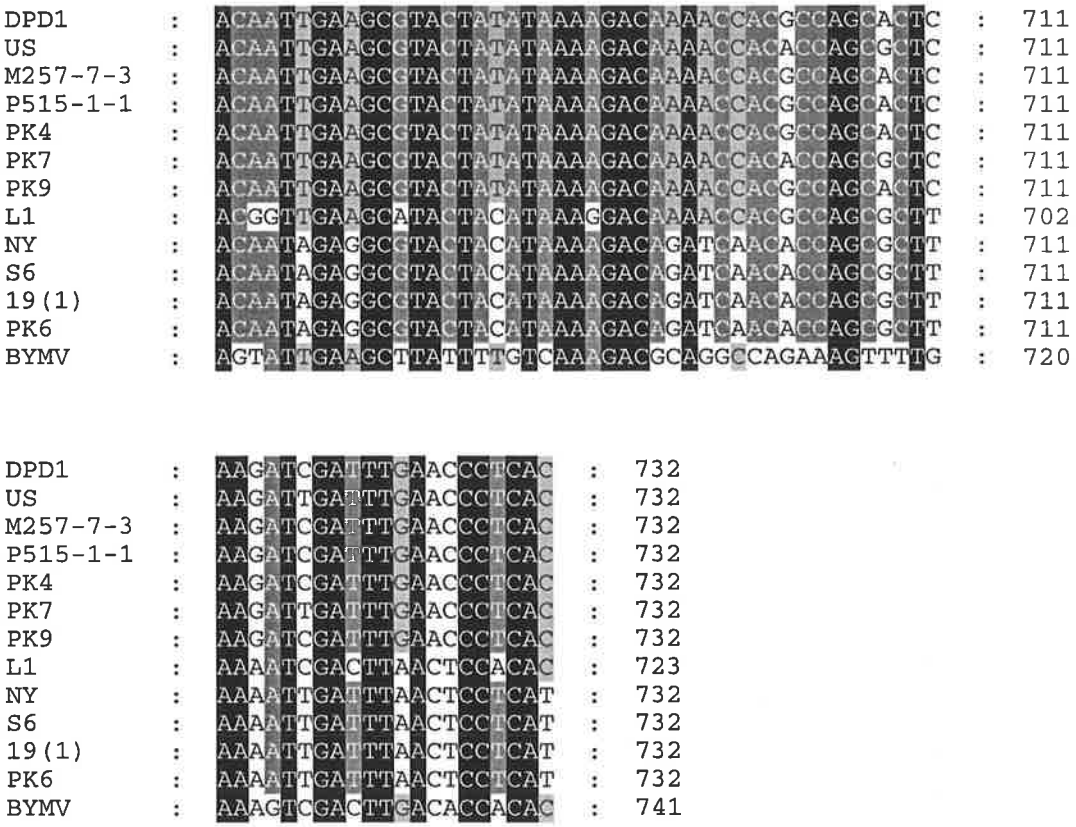



DPD1 : GAGACAGTGCGTTGTCAGAGTAAATCAGAAGTTTCTAAAACACTG : 177
US : GAGACAGTGCGTTGTCAGAGTAAGTCAGAAGTTTCTAAAACACTG : 177
M257-7-3 : GAGACAGTGCGTTGTCAGAGTAAATCAGAAGTTTCTAAAACACTG : 177
P515-1-1 : GAGACAGTGCGTTGTCAGAGTAAATCAGAAGTTTCTAAAACACTG : 177
PK4 : GAGACAGTGCGTTGTCAGAGTAAATCAGAAGTTTCTAAAACACTG : 177
PK7 : GAGACAGTGCGTTGTCAGAGTAAGTCAGAAGTTTCTAAAACACTG : 177
PK9 : GAGACAGTGCGTTGTCAGAGTAAATCAGAAGTTTCTAAAACACTG : 177
L1 : GAAACAGTGCGCTGTCAAAGCAAATCAGAGGTTTCTAAAATTTTA : 177
NY : GAAACAGTGCGCTGCCAGAGTAAATCTGAAGTCTCGAAAACACTG : 177
S6 : GAAACAGTGCGCTGCCAGAGTAAATCTGAAGTCTCGAAAACACTG : 177
19(1) : GAAACAGTGCGCTGCCAGAGTAAATCTGAAGTCTCGAAAACACTG : 177
PK6 : GAAACAGTGCGCTGCCAGAGTAAATCTGAAGTCTCGAAAACACTG : 177
BYMV : GAATGTTTGCAATTTGAATCTTTGCAAGGGGTAAGCAAGCATCTT : 180
DPD1 : CAGCTTAAGGGTAAATGGAACAAACCGCTCATTACAAGTGATTTC : 222
US : CAGCTTAAGGGTAAATGGAACAAACCGCTCATTACAAGTGATTTC : 222
M257-7-3 : CAGCTTAAGGGTAAATGGAACAAACCGCTCATTACAAGTGATTTC : 222
P515-1-1 : CAGCTTAAGGGTAAATGGAACAAACCGCTCATTACAAGTGATTTC : 222
PK4 : CAGCTTAAGGGTAAATGGAACAAACCGCTCATTACAAGTGATTTC : 222
PK7 : CAGCTTAAGGGTAAATGGAACAAACCGCTCATTACAAGTGATTTC : 222
PK9 : CAGCTTAAGGGTAAATGGAACAAACCGCTCATTACAAGTGATTTC : 222
L1 : CAACTTAAGGGTAAGTGGAATAAACCATTAATCACAAGTGACTTC : 222
NY : CAACTCAAGGGCAAGTGGAATAAATCTCTAATCACGAGTGATTTC : 222
S6 : CAACTCAAGGGCAAGTGGAATAAATCTCTAATCACGAGTGATTTC : 222
19(1) : CAACTCAAGGGCAAGTGGAATAAATCTCTAATCACGAGTGATTTC : 222
PK6 : CAACTCAAGGGCAAGTGGAATAAATCTCTAATCACGAGTGATTTC : 222
BYMV : CAGTTAAAAGGACATTGGAATAAACCAGTGTTGATCCAGGACTTT : 225
DPD1 : TTCGTTGTTTGTATGGTGAGCATTGGATGTGTAGTTTTGATGTAC : 267
US : TTCGTTGTTTGTATGGTTAGCATTGGATGTATAGTTTTGATGTAC : 267
M257-7-3 : TTCGTTGTTTGTATGGTGAGCATTGGATGTGTAGTTTTGATGTAC : 267
P515-1-1 : TTCGTTGTTTGTATGGTGAGCATTGGATGTGTAGTTTTGATGTAC : 267
PK4 : TTCGTTGTTTGTATGGTGAGCATTGGATGTGTAGTTTTGATGTAC : 267
PK7 : TTCGTTGTTTGTATGGTTAGCATTGGATGTATAGTTTTGATGTAC : 267
PK9 : TTCGTTGTTTGTATGGTGAGCATTGGATGTGTAGTTTTGATGTAC : 267
L1 : TTTGTTGTTTGCATGGTGAGCATCGGATGTGTGGTTTTAATGTAC : 267
NY : TTCGTTGTTTTTATGGTTGGTATTGGTTGCATAATCTTGATGTAT : 267
S6 : TTTGTTGTTTTCATGGTTGGCATTGGTTGCATGATCTTGATGTAT : 267
19(1) : TTTGTTGTTTTTATGGTTGGTATTGGTTGCATGATCTTGATGTAT : 267
PK6 : TTTGTTGTTTTTATGGTTGGTATTGGTTGCATGATCTTGATGTAT : 267
BYMV : TTAATTGCAGCGGGAGTGCTCGGAGGTGGGTGTTGGATCCTTTAT : 270


DPD1 : CAAATTTTTATGGCTAAGTGGAACGAACCCGTTAAACTTGAAGGG : 312
US : CAAATTTTTATGGCTAAGTGGAGCGAACCCGTTAAACTTGAAGGG : 312
M257-7-3 : CAAATTTTTATGGCTAAGTGGAACGAACCCGTTAAACTTGAAGGG : 312
P515-1-1 : CAAATTTTTATGGCTAAGTGGAACGAACCCGTTAAACTTGAAGGG : 312
PK4 : CAAATTTTTATGGCTAAGTGGAACGAACCCGTTAAACTTGAAGGG : 312
PK7 : CAAATTTTTATGGCTAAGTGGAGCGAACCCGTTAAACTTGAAGGG : 312
PK9 : CAAATTTTTATGGCTAAGTGGAACGAACCCGTTAAACTTGAAGGG : 312
L1 : CAAATTTTTATGGCTAAGTGGAATGAACCTGTGAAACTGGAAGGA : 312
NY : CAGATTTTCATGGCTAGATGGAACGAGCCAGTTAAGTTGGAAGGG : 312
S6 : CAGATTTTCATGGCTAGATGGAACGAGCCAGTTAAGTTGGAAGGG : 312
19(1) : CAGATTTTCATGGCTAGATGGAACGAACCAGTTAAGTTGGAAGGG : 312
PK6 : CAGATTTTCATGGCTAGATGGAACGAGCCAGTTAAGTTGGAAGGG : 312
BYMV : CAATATTTCAAACAAGAGACGAGCAAGGCATTTGTGTTCCAAGGA : 315
DPD1 : AAG--TCGAAAGCTAAAACT----TTGCGTTTTAGACAGGCTCGC : 351
US : AAG--TCGAAAGCTAAAACT----TTGCGTTTTAGACAGGCTCGC : 351
M257-7-3 : AAG--TCGAAAGCTAAAACT----TTGCGTTTTAGACAGGCTCGC : 351
P515-1-1 : AAG--TCGAAAGCTAAAACT----TTGCGTTTTAGACAGGCTCGC : 351
PK4 : AAG--TCGAAAGCTAAAACT----TTGCGTTTTAGACAGGCTCGC : 351
PK7 : AAG--TCGAAAGCTAAAACT----TTGCGTTTTAGACAGGCTCGC : 351
PK9 : AAG--TCGAAAGCTAAAACT----TTGCGTTTTAGACAGGCTCGC : 351
L1 : AAG--TCGAAGGCGAAAACA----TTGCGTTTCAGACAAGGCCCGT : 351
NY : AAA--TCAAAAGCTAAAACT----TTGCGCTTTAGGCAAGGCCCGT : 351
S6 : AAA--TCAAAAGCTAAAACT----TTGCGCTTTAGGCAAGGCCCGT : 351
19(1) : AAA--TCAAAAGCTAAAACT----TTGCGCTTTAGGCAAGGCCCGT : 351
PK6 : AAA--TCAAAAGCTAAAACT----TTGCGCTTTAGGCAAGGCCCGT : 351
BYMV : AAGAACACAAGGACAAAGCAGAAGTTGAGGTTTCGAGATGCTAGA : 360
DPD1 : GACAACAATGCCAAGTATGAAGTGTTTGCAGATGAAGACACAAAA : 396
US : GACAACAATGCCAAGTATGAAGTGTTTGCAGACGAAGACTCAAAA : 396
M257-7-3 : GACAACAATGCCAAGTATGAAGTGTTTGCAGATGAAGACACAAAA : 396
P515-1-1 : GACAACAATGCCAAGTATGAAGTGTTTGCAGATGAAGACACAAAA : 396
PK4 : GACAACAATGCCAAGTATGAAGTGTTTGCAGATGAAGACACAAAA : 396
PK7 : GACAACAATGCCAAGTATGAAGTGTTTGCAGACGAAGACTCAAAA : 396
PK9 : GACAACAATGCCAAGTATGAAGTGTTTGCAGATGAAGACACAAAA : 396
L1 : GACAATAATGCCAAGTATGAAGTGTTTGCAGATGAAGACTCAAAA : 396
NY : GACAACAATGCGAAATTTGAAGTGTATGCAGATGAAGACTCAACG : 396
S6 : GACAACAATGCGAAATTTGAAGTGTATGCAGATGAAGACTCAACG : 396
19(1) : GACAACAATGCGAAATTTGAAGTGTATGCAGATGAAGACTCAACG : 396
PK6 : GACAACAATGCGAAATTTGAAGTGTATGCAGATGAAGACTCAACG : 396
BYMV : GACATGAAAGGCCGCATGGAAGTTTACGCGGATGAGGCGGACTATA : 405

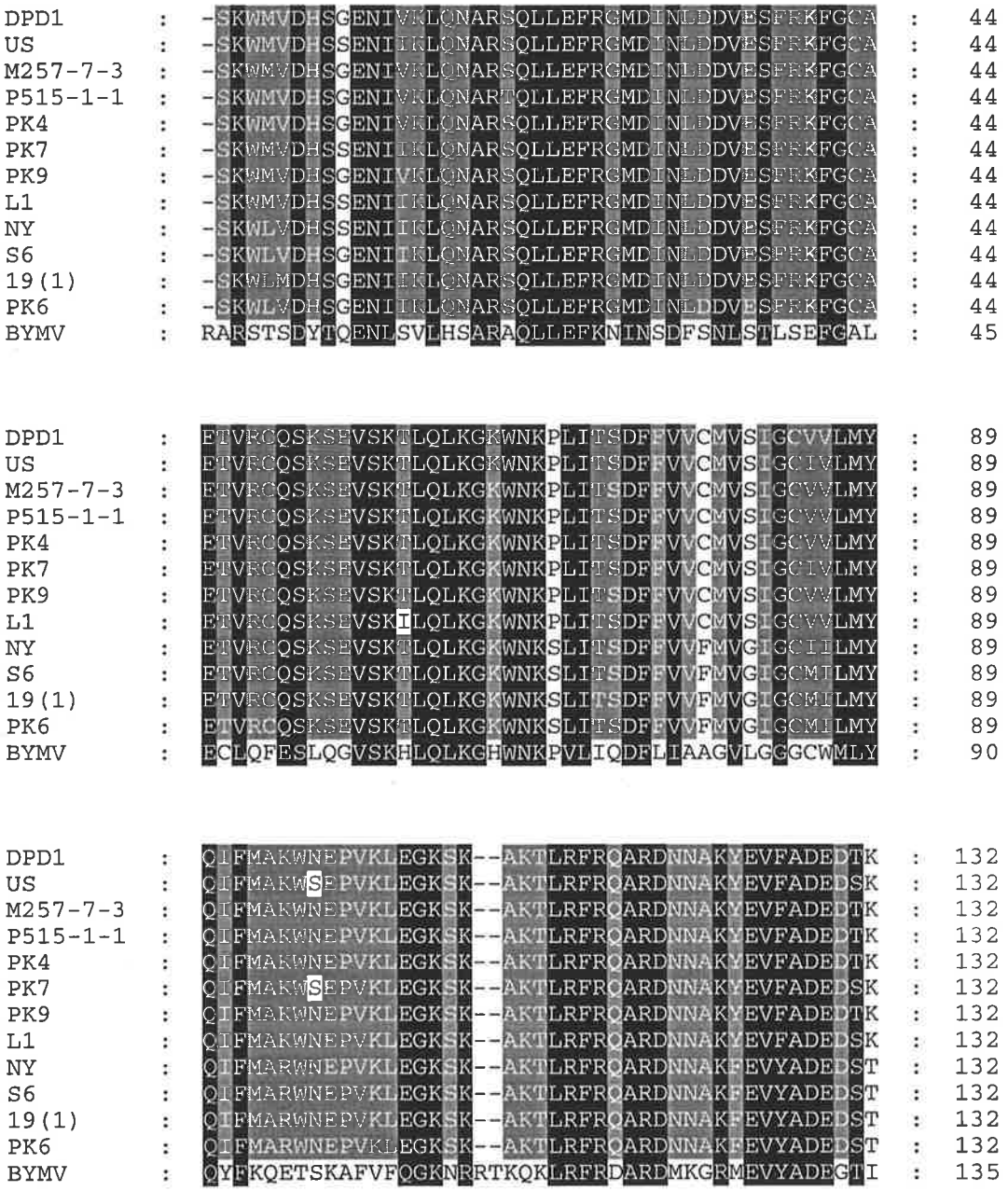

DPD1 : 441
US : 441
M257-7-3 : 441
P515-1-1 : 441
PK4 : 441
PK7 : 441
PK9 : 441
L1 : 441
NY : 441
S6 : 441
19(1) : 441
PK6 : 441
BYMV : 450
DPD1 : 486
US : 486
M257-7-3 : 486
P515-1-1 : 486
PK4 : 486
PK7 : 486
PK9 : 486
L1 : 486
NY : 486
S6 : 486
19(1) : 486
PK6 : 486
BYMV : 495
DPD1 : 531
US : 531
M257-7-3 : 531
P515-1-1 : 531
PK4 : 531
PK7 : 531
PK9 : 531
L1 : 531
NY : 531
S6 : 531
19(1) : 531
PK6 : 531
BYMV : 540

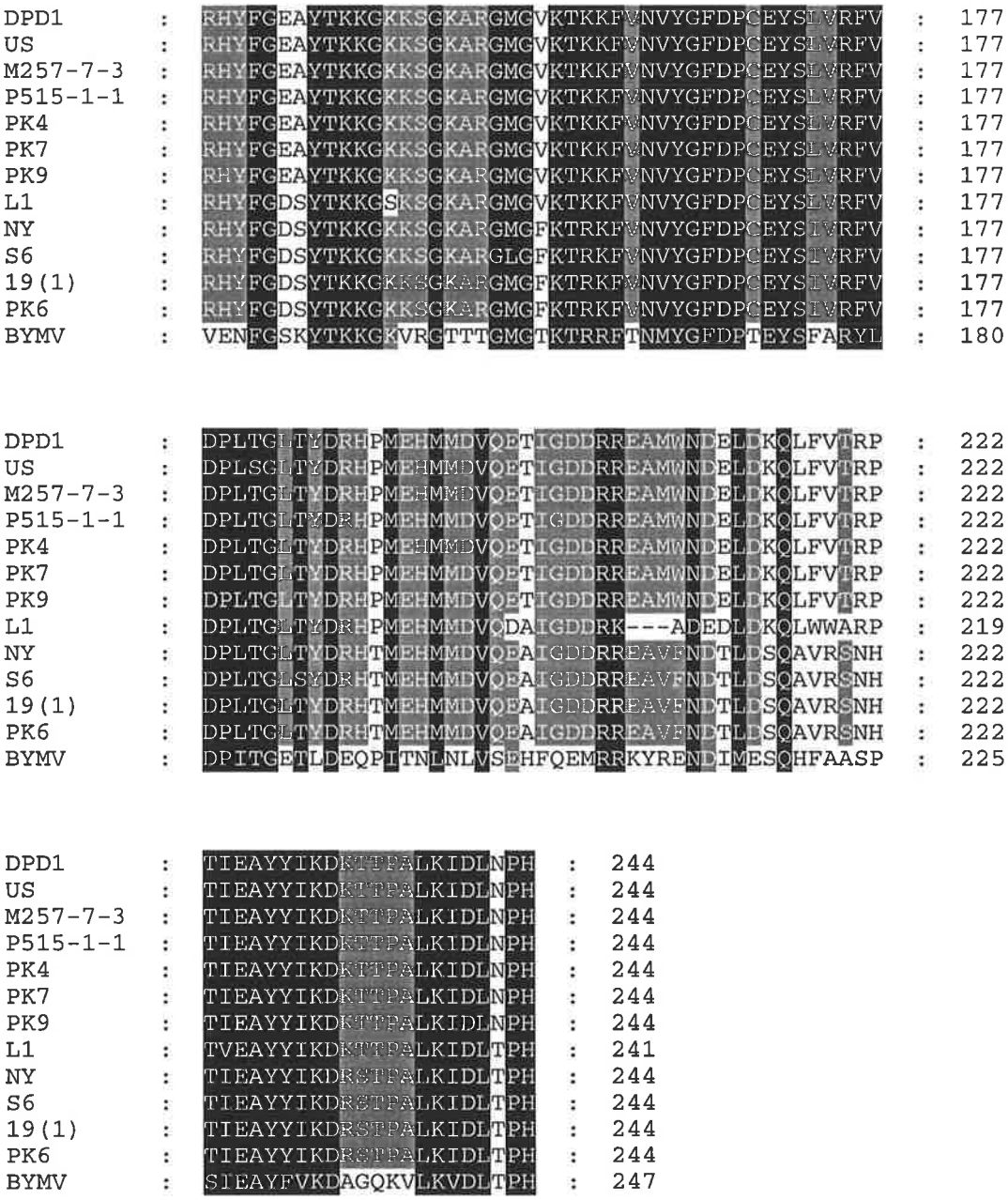

DPD1 : 576
US : 576
M257-7-3 : 576
P515-1-1 : 576
PK4 : 576
PK7 : 576
PK9 : 576
L1 : 576
NY : 576
S6 : 576
19(1) : 576
PK6 : 576
BYMV : 585
DPD1 : 621
US : 621
M257-7-3 : 621
P515-1-1 : 621
PK4 : 621
PK7 : 621
PK9 : 621
L1 : 619
NY : 621
S6 : 621
19(1) : 621
PK6 : 621
BYMV : 630
DPD1 : 666
US : 666
M257-7-3 : 666
P515-1-1 : 666
PK4 : 666
PK7 : 666
PK9 : 666
L1 : 657
NY : 666
S6 : 666
19(1) : 666
PK6 : 666
BYMV : 675


DPD1 : ACAATTGAAGCGTACTATATAAAAGACAAAACCAGGCCAGCACTC : 711
US : ACAATTGAAGCGTACTATATAAAAGACAAAACCACACCAGCGCTC : 711
M257-7-3 : ACAATTGAAGCGTACTATATAAAAGACAAAACCAGGCCAGCACTC : 711
P515-1-1 : ACAATTGAAGCGTACTATATAAAAGACAAAACCAGGCCAGCACTC : 711
PK4 : ACAATTGAAGCGTACTATATAAAAGACAAAACCAGGCCAGCACTC : 711
PK7 : ACAATTGAAGCGTACTATATAAAAGACAAAACCACACCAGCGCTC : 711
PK9 : ACAATTGAAGCGTACTATATAAAAGACAAAACCAGGCCAGCACTC : 711
L1 : ACGGTTGAAGCATACTACATAAAGGACAAAACCAGGCCAGCGCTT : 702
NY : ACAATAGAGGCGTACTACATAAAAGACAGATCAACACCAGCGCTT : 711
S6 : ACAATAGAGGCGTACTACATAAAAGACAGATCAACACCAGCGCTT : 711
19(1) : ACAATAGAGGCGTACTACATAAAAGACAGATCAACACCAGCGCTT : 711
PK6 : ACAATAGAGGCGTACTACATAAAAGACAGATCAACACCAGCGCTT : 711
BYMV : AGTATTGAAGCTTATTTTGTCAAAGACGCAGGCCAGAAAGTTTTG : 720

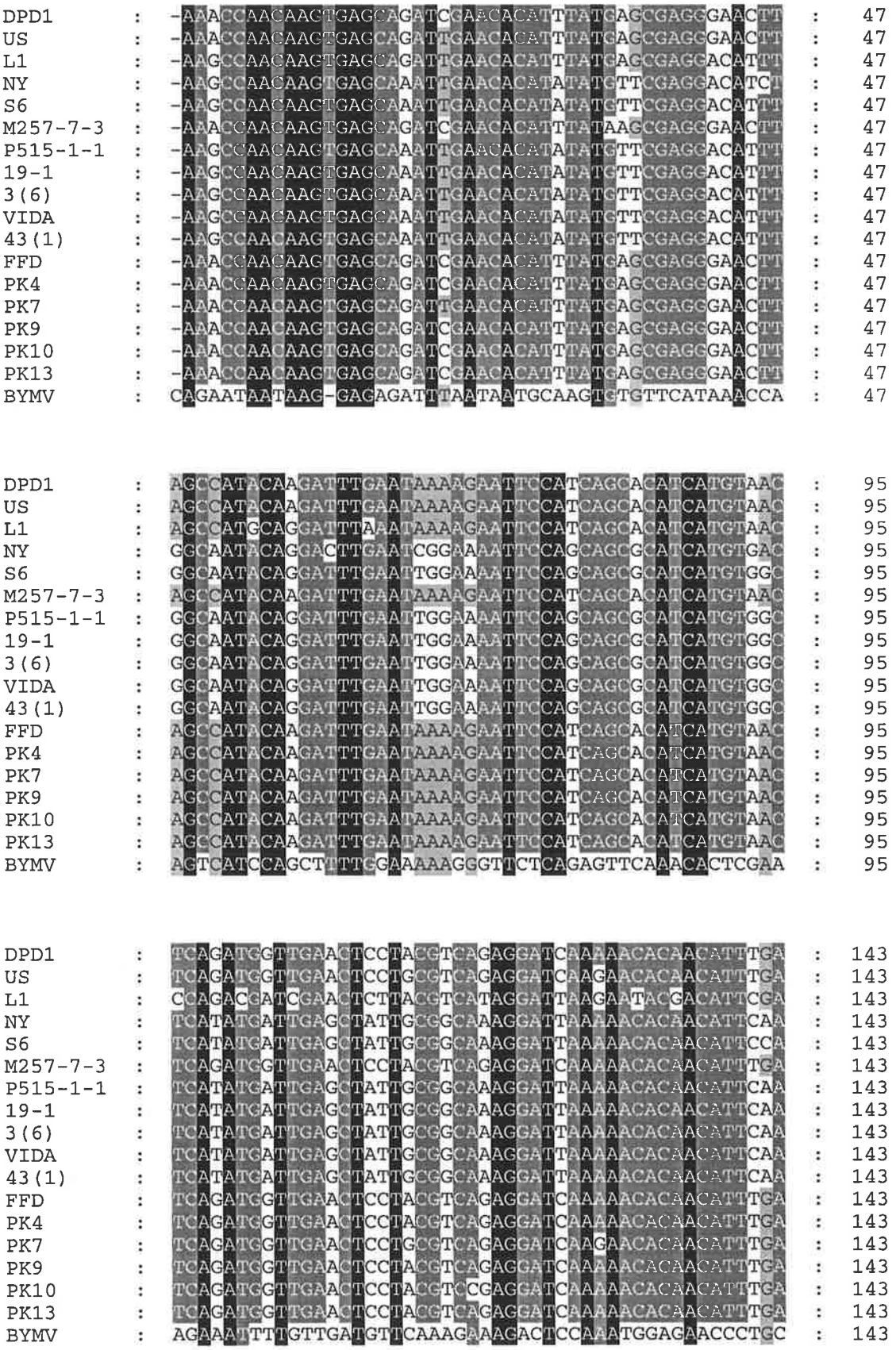
DPD1 : AAGATCGATTTGAACCCTCAC : 732
US : AAGATTGATTTGAACCCTCAC : 732
M257-7-3 : AAGATCGATTTGAACCCTCAC : 732
P515-1-1 : AAGATCGATTTGAACCCTCAC : 732
PK4 : AAGATCGATTTGAACCCTCAC : 732
PK7 : AAGATTGATTTGAACCCTCAC : 732
PK9 : AAGATCGATTTGAACCCTCAC : 732
L1 : AAAATCGACTTAACTCCACAC : 723
NY : AAAATTGATTTAACTCCTCAT : 732
S6 : AAAATTGATTTAACTCCTCAT : 732
19(1) : AAAATTGATTTAACTCCTCAT : 732
PK6 : AAAATTGATTTAACTCCTCAT : 732
BYMV : AAAGTCGACTTGACACCACAC : 741

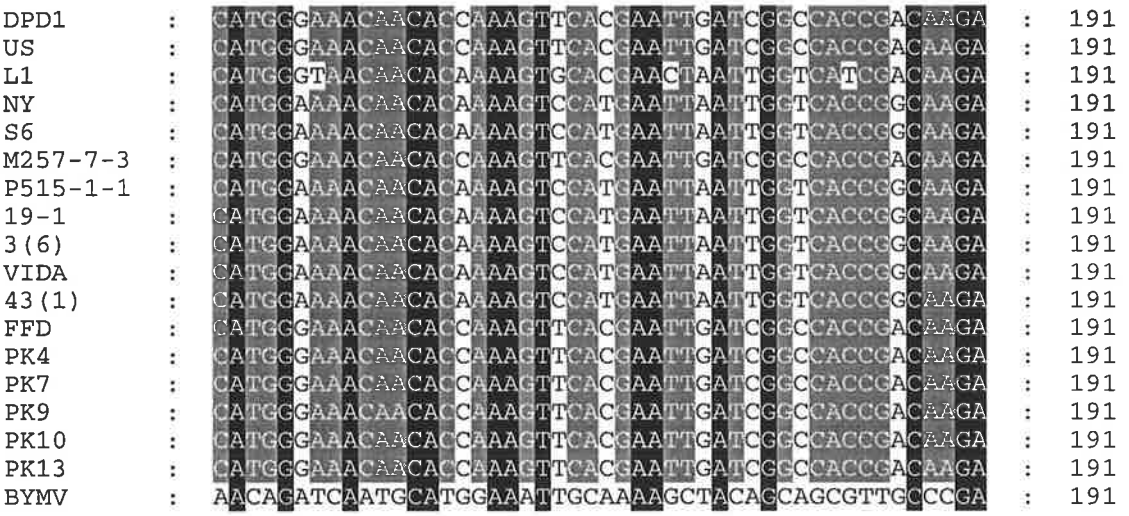
Amino acid sequence alignment of 244 amino acids from the carboxy terminus of CI, entire $6K_2$ and amino terminus VPg for 12 PSbMV isolates and BYMV.

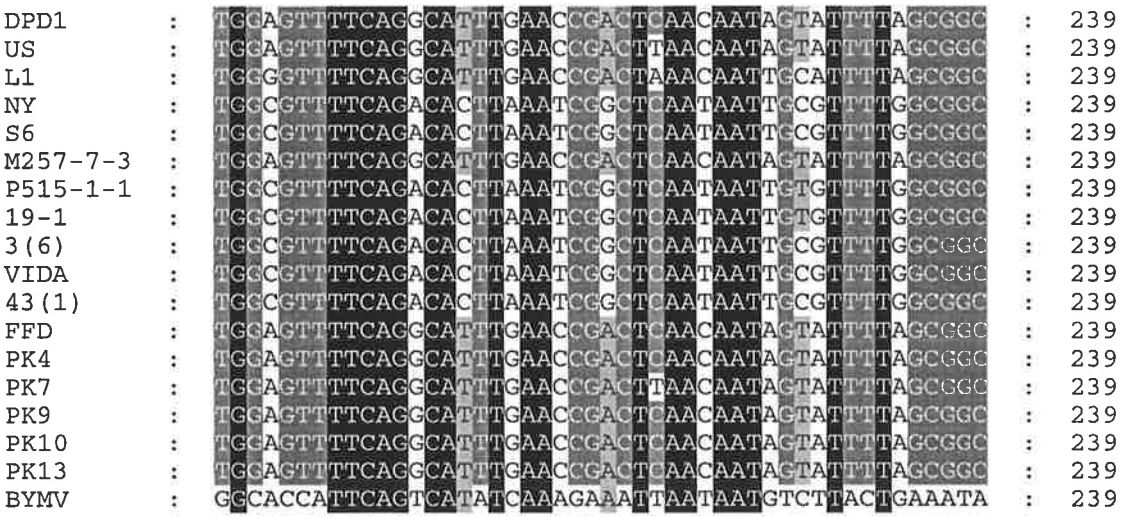


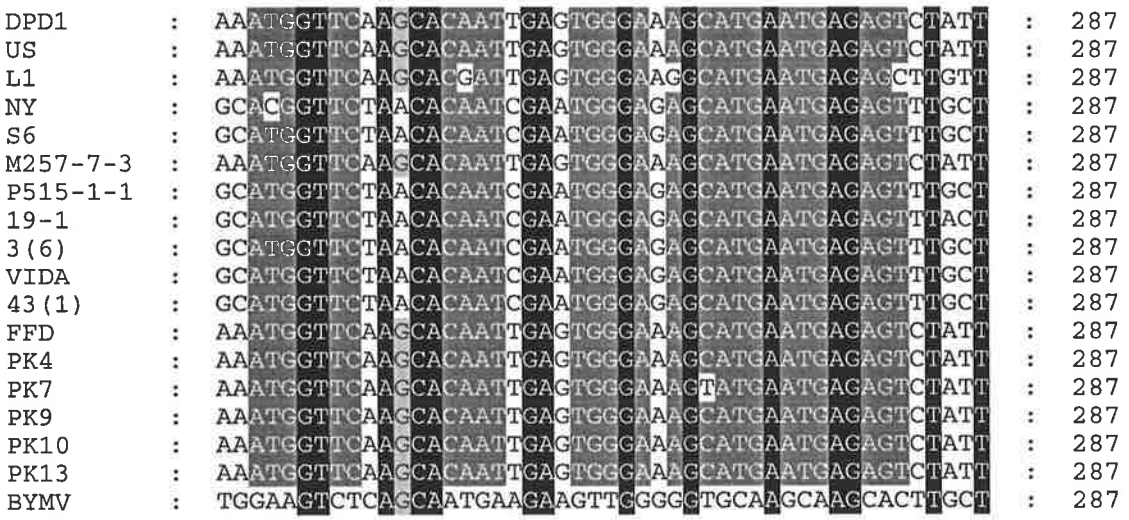

DPD1 : RHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
US : RHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
M257-7-3 : RHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
P515-1-1 : RHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
PK4 : RHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
PK7 : RHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
PK9 : RHYFGEAYTKKGKKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
L1 : RHYFGDSYTKKGSKSGKARGMGVKTKKFVNVYGFDPCEYSLVRFV : 177
NY : RHYFGDSYTKKGKKSGKARGMGFKTRKFVNVYGFDPCEYSIVRFV : 177
S6 : RHYFGDSYTKKGKKSGKARGLGFKTRKFVNVYGFDPCEYSIVRFV : 177
19(1) : RHYFGDSYTKKGKKSGKARGMGFKTRKFVNVYGFDPCEYSIVRFV : 177
PK6 : RHYFGDSYTKKGKKSGKARGMGFKTRKFVNVYGFDPCEYSIVRFV : 177
BYMV : VENFGSKYTKKGKVRGTTTGMGTKTRRFTNMYGFDPTEYSFARYL : 180
DPD1 : DPLTGLTYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRP : 222
US : DPLSGLTYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRP : 222
M257-7-3 : DPLTGLTYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRP : 222
P515-1-1 : DPLTGLTYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRP : 222
PK4 : DPLTGLTYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRP : 222
PK7 : DPLTGLTYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRP : 222
PK9 : DPLTGLTYDRHPMEHMMDVQETIGDDRREAMWNDELDKQLFVTRP : 222
L1 : DPLTGLTYDRHPMEHMMDVQDAIGDDRK---ADEDLDKQLWWARP : 219
NY : DPLTGLTYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNH : 222
S6 : DPLTGLSYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNH : 222
19(1) : DPLTGLTYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNH : 222
PK6 : DPLTGLTYDRHTMEHMMDVQEAIGDDRREAVFNDTLDSQAVRSNH : 222
BYMV : DPITGETLDEQPITNLNLVSEHFQEMRRKYRENDIMESQHFAASP : 225
DPD1 : TIEAYYIKDKTTPALKIDLNPH : 244
US : TIEAYYIKDKTTPALKIDLNPH : 244
M257-7-3 : TIEAYYIKDKTTPALKIDLNPH : 244
P515-1-1 : TIEAYYIKDKTTPALKIDLNPH : 244
PK4 : TIEAYYIKDKTTPALKIDLNPH : 244
PK7 : TIEAYYIKDKTTPALKIDLNPH : 244
PK9 : TIEAYYIKDKTTPALKIDLNPH : 244
L1 : TVEAYYIKDKTTPALKIDLTPH : 241
NY : TIEAYYIKDRSTPALKIDLTPH : 244
S6 : TIEAYYIKDRSTPALKIDLTPH : 244
19(1) : TIEAYYIKDRSTPALKIDLTPH : 244
PK6 : TIEAYYIKDRSTPALKIDLTPH : 244
BYMV : SIEAYFVKDAGQKVLKVDLTPH : 247

Nucleotide sequence alignment of 853 bp region from the *HC-Pro* for 17 PSbMV isolates and BYMV.




DPD1 : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
US : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
L1 : CATGGGTAACAACACAAAAGTGCACGAACTAATTGGTCATCGACAAGA : 191
NY : CATGGAAAACAACACAAAAGTCCATGAATTAATTGGTCACCGGCAAGA : 191
S6 : CATGGAAAACAACACAAAAGTCCATGAATTAATTGGTCACCGGCAAGA : 191
M257-7-3 : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
P515-1-1 : CATGGAAAACAACACAAAAGTCCATGAATTAATTGGTCACCGGCAAGA : 191
19-1 : CATGGAAAACAACACAAAAGTCCATGAATTAATTGGTCACCGGCAAGA : 191
3(6) : CATGGAAAACAACACAAAAGTCCATGAATTAATTGGTCACCGGCAAGA : 191
VIDA : CATGGAAAACAACACAAAAGTCCATGAATTAATTGGTCACCGGCAAGA : 191
43(1) : CATGGAAAACAACACAAAAGTCCATGAATTAATTGGTCACCGGCAAGA : 191
FFD : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
PK4 : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
PK7 : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
PK9 : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
PK10 : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
PK13 : CATGGGAAACAACACCAAAGTTCACGAATTGATCGGCCACCGACAAGA : 191
BYMV : AACAGATCAATGCATGGAAATTGCAAAAGCTACAGCAGCGTTGCCCGA : 191


DPD1 : TGGAGTTTTCAGGCATTTGAACCGACTCAACAATAGTATTTTAGCGGC : 239
US : TGGAGTTTTCAGGCATTTGAACCGACTTAACAATAGTATTTTAGCGGC : 239
L1 : TGGGGTTTTCAGGCATTTGAACCGACTAAACAATTGCATTTTAGCGGC : 239
NY : TGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGCGTTTTGGCGGC : 239
S6 : TGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGCGTTTTGGCGGC : 239
M257-7-3 : TGGAGTTTTCAGGCATTTGAACCGACTCAACAATAGTATTTTAGCGGC : 239
P515-1-1 : TGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGTGTTTTGGCGGC : 239
19-1 : TGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGTGTTTTGGCGGC : 239
3(6) : TGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGCGTTTTGGCGGC : 239
VIDA : TGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGCGTTTTGGCGGC : 239
43(1) : TGGCGTTTTCAGACACTTAAATCGGCTCAATAATTGCGTTTTGGCGGC : 239
FFD : TGGAGTTTTCAGGCATTTGAACCGACTCAACAATAGTATTTTAGCGGC : 239
PK4 : TGGAGTTTTCAGGCATTTGAACCGACTCAACAATAGTATTTTAGCGGC : 239
PK7 : TGGAGTTTTCAGGCATTTGAACCGACTTAACAATAGTATTTTAGCGGC : 239
PK9 : TGGAGTTTTCAGGCATTTGAACCGACTCAACAATAGTATTTTAGCGGC : 239
PK10 : TGGAGTTTTCAGGCATTTGAACCGACTCAACAATAGTATTTTAGCGGC : 239
PK13 : TGGAGTTTTCAGGCATTTGAACCGACTCAACAATAGTATTTTAGCGGC : 239
BYMV : GGCACCATTCAGTCATATCAAAGAAATTAATAATGTCTTACTGAAATA : 239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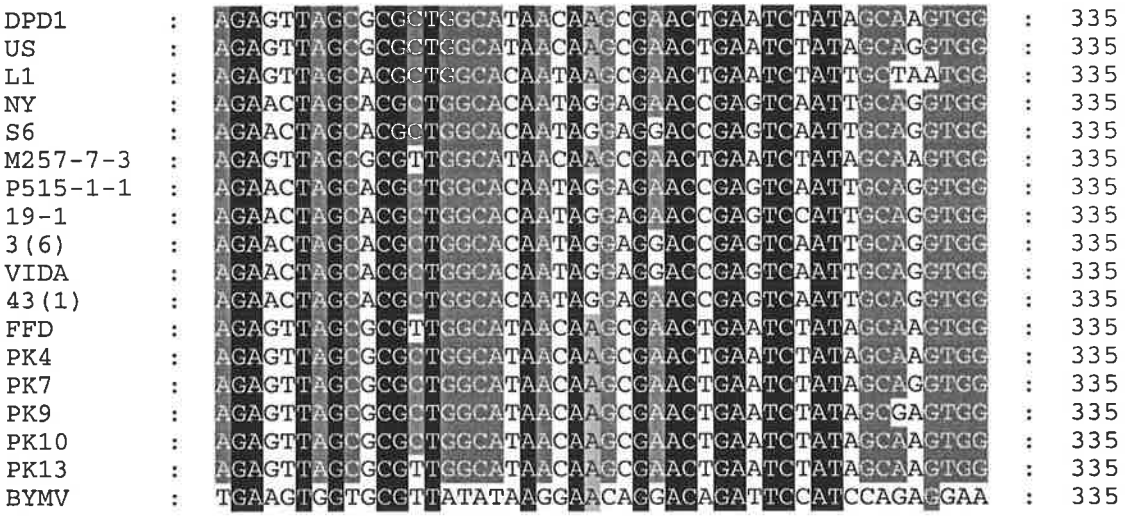
US : AAATGGTTCAAGCACAATTGAGTGGGAAAGCATGAATGAGAGTCTATT : 287
L1 : AAATGGTTCAAGCACGATTGAGTGGGAAGGCATGAATGAGAGCTTGTT : 287
NY : GCACGGTTCTAACACAATCGAATGGGAGAGCATGAATGAGAGTTTGCT : 287
S6 : GCATGGTTCTAACACAATCGAATGGGAGAGCATGAATGAGAGTTTGCT : 287
M257-7-3 : AAATGGTTCAAGCACAATTGAGTGGGAAAGCATGAATGAGAGTCTATT : 287
P515-1-1 : GCATGGTTCTAACACAATCGAATGGGAGAGCATGAATGAGAGTTTGCT : 287
19-1 : GCATGGTTCTAACACAATCGAATGGGAGAGCATGAATGAGAGTTTACT : 287
3(6) : GCATGGTTCTAACACAATCGAATGGGAGAGCATGAATGAGAGTTTGCT : 287
VIDA : GCATGGTTCTAACACAATCGAATGGGAGAGCATGAATGAGAGTTTGCT : 287
43(1) : GCATGGTTCTAACACAATCGAATGGGAGAGCATGAATGAGAGTTTGCT : 287
FFD : AAATGGTTCAAGCACAATTGAGTGGGAAAGCATGAATGAGAGTCTATT : 287
PK4 : AAATGGTTCAAGCACAATTGAGTGGGAAAGCATGAATGAGAGTCTATT : 287
PK7 : AAATGGTTCAAGCACAATTGAGTGGGAAAGTATGAATGAGAGTCTATT : 287
PK9 : AAATGGTTCAAGCACAATTGAGTGGGAAAGCATGAATGAGAGTCTATT : 287
PK10 : AAATGGTTCAAGCACAATTGAGTGGGAAAGCATGAATGAGAGTCTATT : 287
PK13 : AAATGGTTCAAGCACAATTGAGTGGGAAAGCATGAATGAGAGTCTATT : 287
BYMV : TGGAAGTCTCAGCAATGAAGAAGTTGGGGGTGCAAGCAAGCACTTGCT : 287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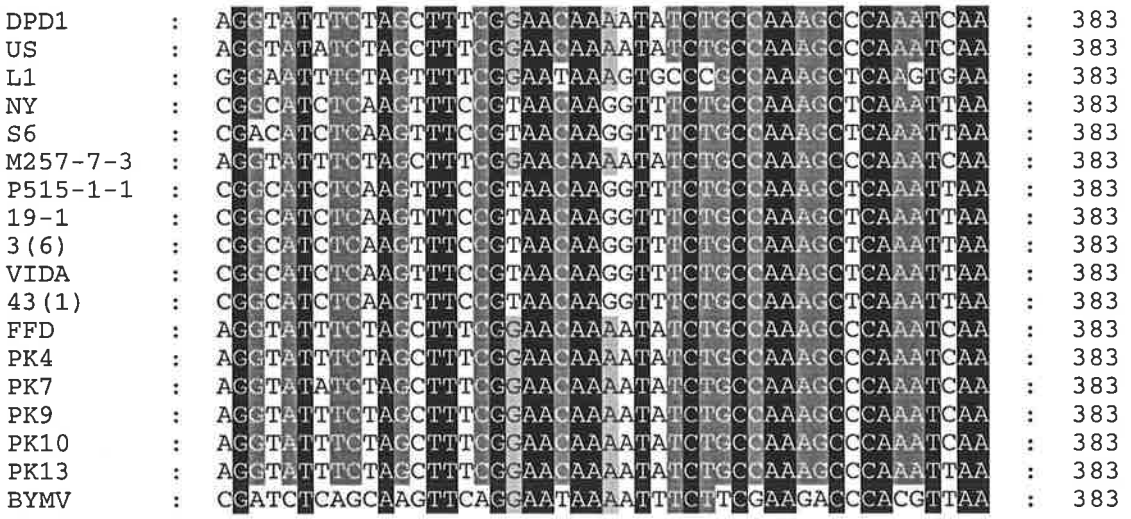
US : AGAGTTAGCGCCGCTGGCATAACAAGCCGAACTGAATCTATAGCAGGTGG : 335
L1 : AGAGTTAGCACCGCTGGCACAATAAGCCGAACTGAATCTATTGCTAATGG : 335
NY : AGAACTAGCACCGCTGGCACAATAGGAGAACCGAGTCAATTGCAGGTGG : 335
S6 : AGAACTAGCACCGCTGGCACAATAGGAGGACCGAGTCAATTGCAGGTGG : 335
M257-7-3 : AGAGTTAGCGCGTTGGCATAACAAGCCGAACTGAATCTATAGCAAGTGG : 335
P515-1-1 : AGAACTAGCACCGCTGGCACAATAGGAGAACCGAGTCAATTGCAGGTGG : 335
19-1 : AGAACTAGCACCGCTGGCACAATAGGAGAACCGAGTCCATTGCAGGTGG : 335
3(6) : AGAACTAGCACCGCTGGCACAATAGGAGGACCGAGTCAATTGCAGGTGG : 335
VIDA : AGAACTAGCACCGCTGGCACAATAGGAGGACCGAGTCAATTGCAGGTGG : 335
43(1) : AGAACTAGCACCGCTGGCACAATAGGAGAACCGAGTCAATTGCAGGTGG : 335
FFD : AGAGTTAGCGCCGTTGGCATAACAAGCCGAACTGAATCTATAGCAAGTGG : 335
PK4 : AGAGTTAGCGCCGCTGGCATAACAAGCCGAACTGAATCTATAGCAAGTGG : 335
PK7 : AGAGTTAGCGCCGCTGGCATAACAAGCCGAACTGAATCTATAGCAGGTGG : 335
PK9 : AGAGTTAGCGCCGCTGGCATAACAAGCCGAACTGAATCTATAGCGAGTGG : 335
PK10 : AGAGTTAGCGCCGCTGGCATAACAAGCCGAACTGAATCTATAGCAAGTGG : 335
PK13 : AGAGTTAGCGCCTTGGCATAACAAGCCGAACTGAATCTATAGCAAGTGG : 335
BYMV : TGAAGTGGTGCGTTATATAAGGAACAGGACAGATTCCATCCAGAGGAA : 335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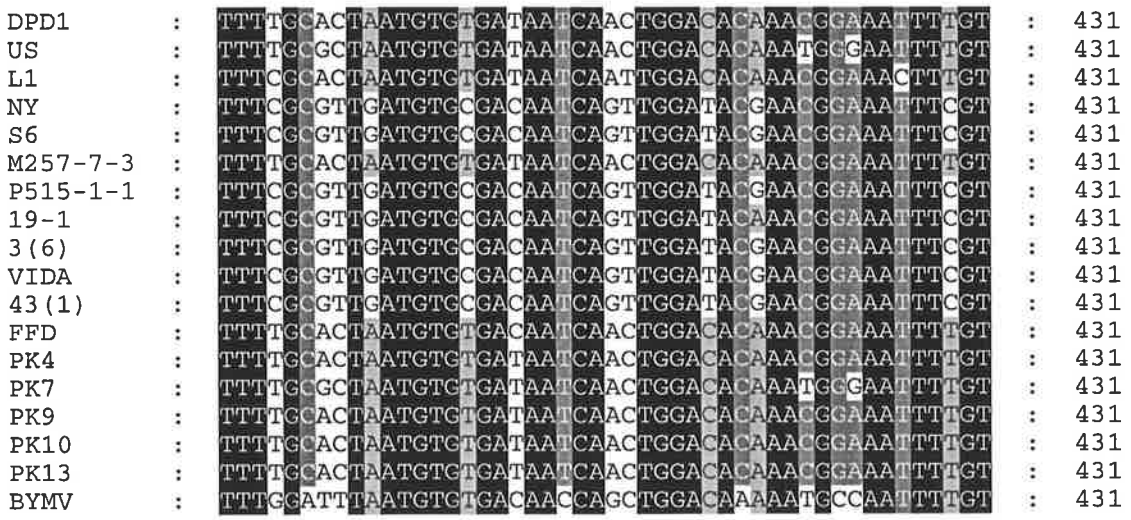
US : AGGTATATCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATCAA : 383
L1 : GGGAATTTCTACTTTTCGCAATAAAGTGCCCGCCAAAGCTCAAGTGAA : 383
NY : CGCCATCTCAACTTTCCGTAACAAGGTTTCTGCCAAAGCTCAAATTAA : 383
S6 : CGACATCTCAACTTTCCGTAACAAGGTTTCTGCCAAAGCTCAAATTAA : 383
M257-7-3 : AGGTATTTCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATCAA : 383
P515-1-1 : CGGCATCTCAACTTTCCGTAACAAGGTTTCTGCCAAAGCTCAAATTAA : 383
19-1 : CGGCATCTCAACTTTCCGTAACAAGGTTTCTGCCAAAGCTCAAATTAA : 383
3(6) : CGGCATCTCAACTTTCCGTAACAAGGTTTCTGCCAAAGCTCAAATTAA : 383
VIDA : CGGCATCTCAACTTTCCGTAACAAGGTTTCTGCCAAAGCTCAAATTAA : 383
43(1) : CGGCATCTCAACTTTCCGTAACAAGGTTTCTGCCAAAGCTCAAATTAA : 383
FFD : AGGTATTTCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATCAA : 383
PK4 : AGGTATTTCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATCAA : 383
PK7 : AGGTATATCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATCAA : 383
PK9 : AGGTATTTCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATCAA : 383
PK10 : AGGTATTTCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATCAA : 383
PK13 : AGGTATTTCTACCTTTCGCAACAAAATATCTGCCAAAGCCCAAATTAA : 383
BYMV : CGATCTCAGCAAGTTCAGGAATAAAATTTCTTCGAAGAGCCACGTTAA : 383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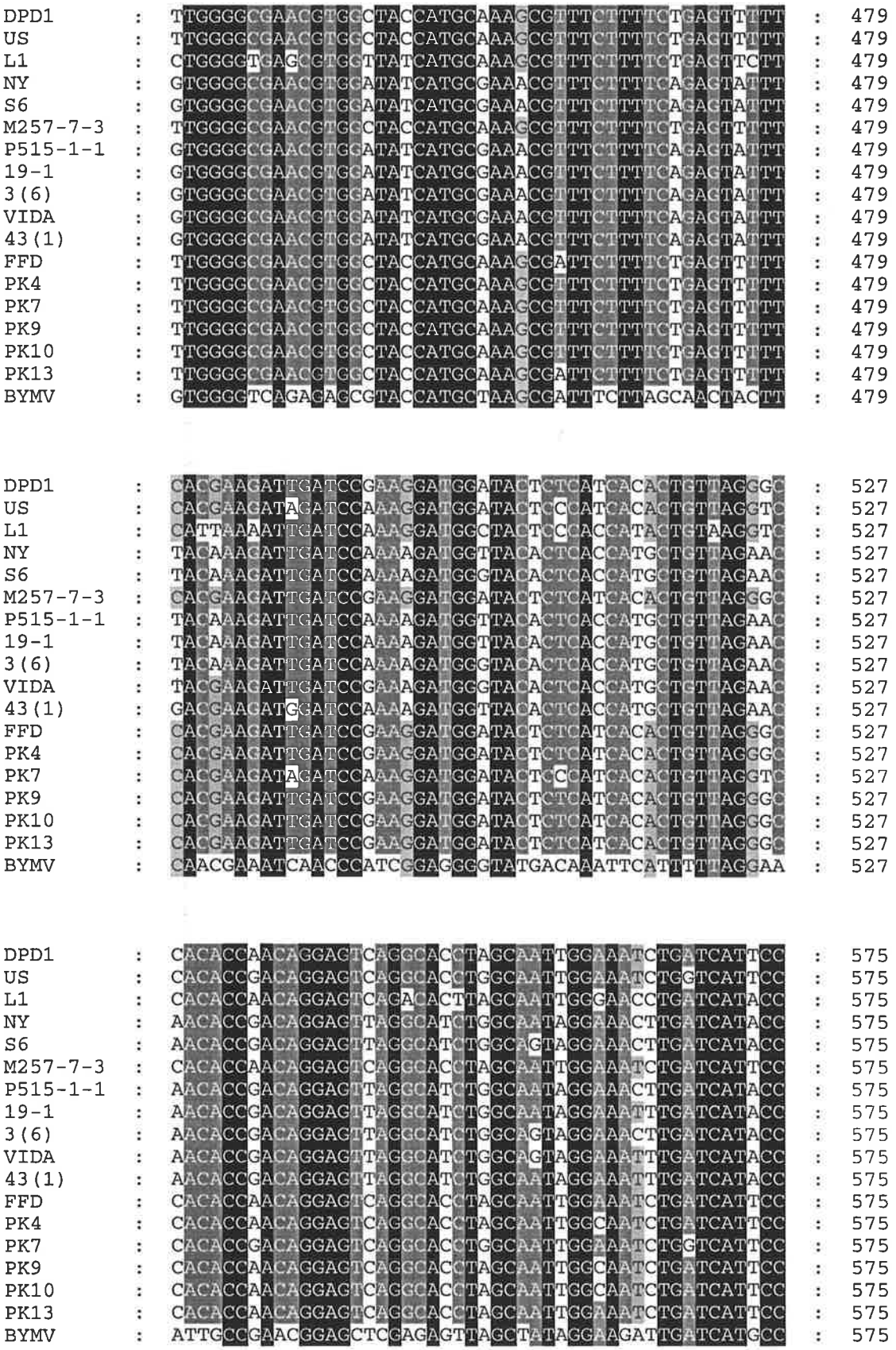
US : TTTTGCGCTAATGTGTGATAATCAACTGGACACAAATGGGAATTTTGT : 431
L1 : TTTCGCACTAATGTGTGATAATCAATTGGACACAAACGGAAACTTTGT : 431
NY : TTTCGCGTTGATGTGCGACAATCAGTTGGATACGAACGGAAATTTCGT : 431
S6 : TTTCGCGTTGATGTGCGACAATCAGTTGGATACGAACGGAAATTTCGT : 431
M257-7-3 : TTTTGCACTAATGTGTGATAATCAACTGGACACAAACGGAAATTTTGT : 431
P515-1-1 : TTTCGCGTTGATGTGCGACAATCAGTTGGATACGAACGGAAATTTCGT : 431
19-1 : TTTCGCGTTGATGTGCGACAATCAGTTGGATACAAACGGAAATTTCGT : 431
3(6) : TTTCGCGTTGATGTGCGACAATCAGTTGGATACGAACGGAAATTTCGT : 431
VIDA : TTTCGCGTTGATGTGCGACAATCAGTTGGATACGAACGGAAATTTCGT : 431
43(1) : TTTCGCGTTGATGTGCGACAATCAGTTGGATACGAACGGAAATTTCGT : 431
FFD : TTTTGCACTAATGTGTGACAATCAACTGGACACAAACGGAAATTTTGT : 431
PK4 : TTTTGCACTAATGTGTGATAATCAACTGGACACAAACGGAAATTTTGT : 431
PK7 : TTTTGCGCTAATGTGTGATAATCAACTGGACACAAATGGGAATTTTGT : 431
PK9 : TTTTGCACTAATGTGTGATAATCAACTGGACACAAACGGAAATTTTGT : 431
PK10 : TTTTGCACTAATGTGTGATAATCAACTGGACACAAACGGAAATTTTGT : 431
PK13 : TTTTGCACTAATGTGTGATAATCAACTGGACACAAACGGAAATTTTGT : 431
BYMV : TTTGGATTTAATGTGTGACAACCAGCTGGACAAAAATGCCAATTTTGT : 431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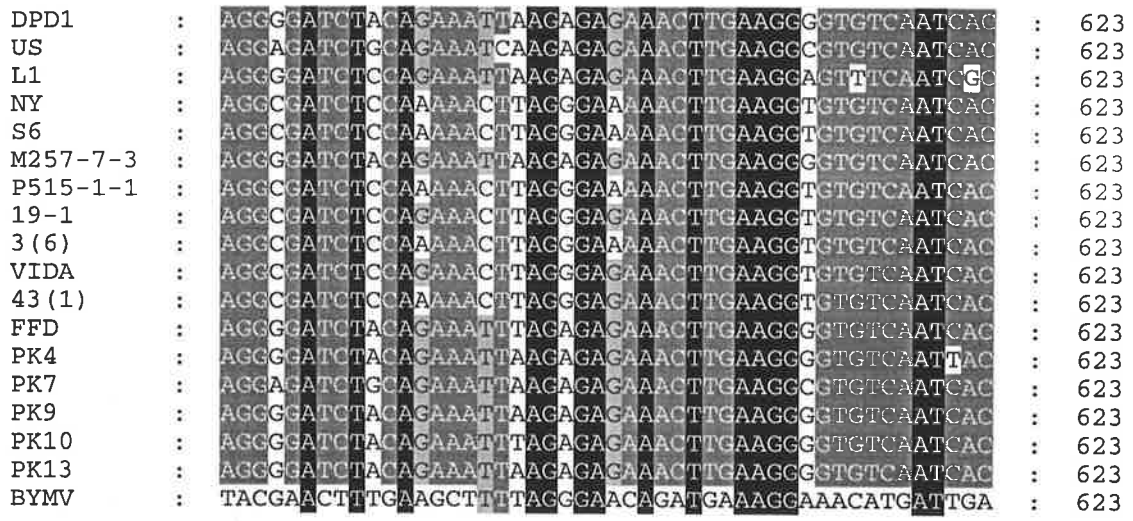
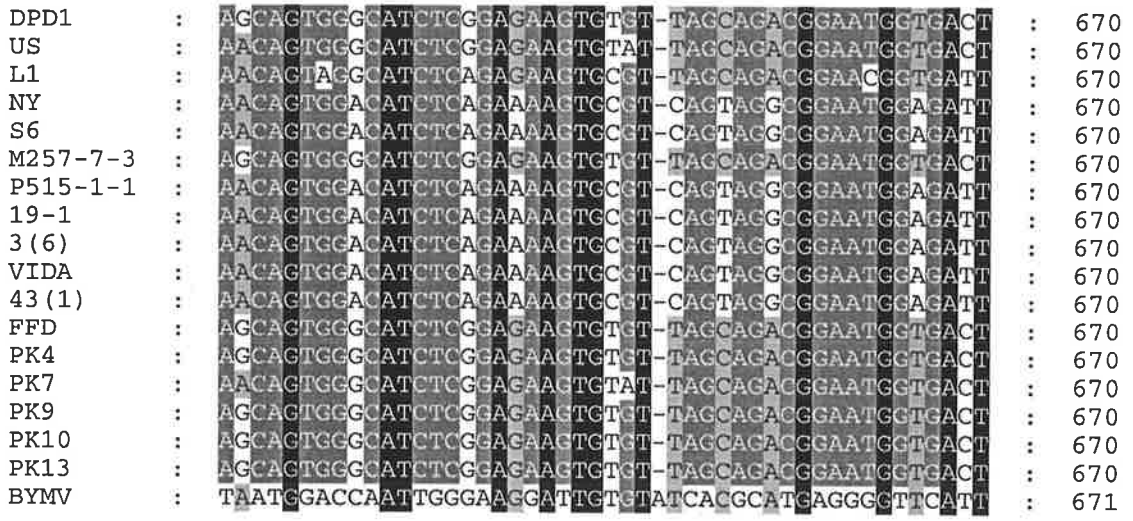
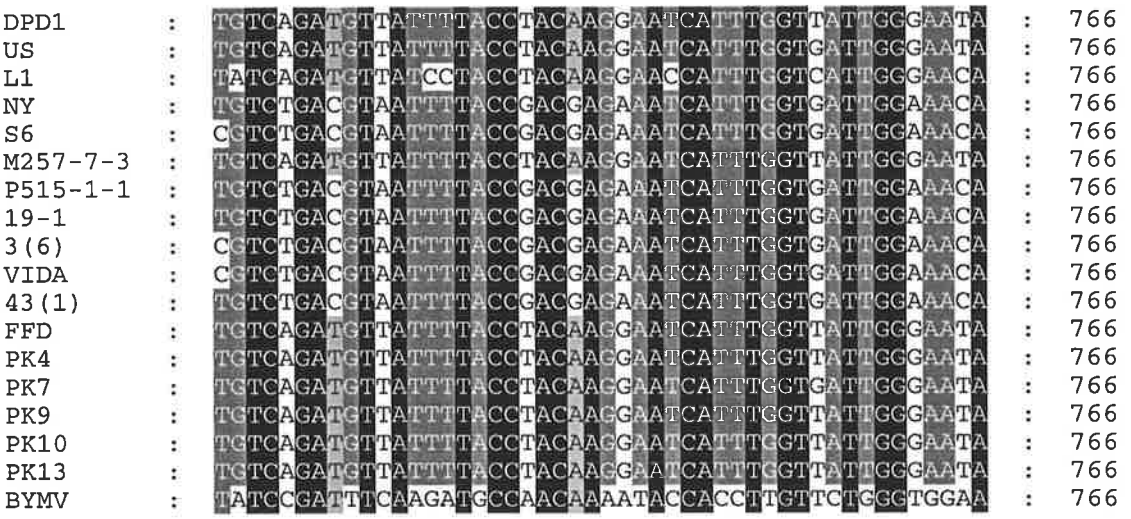
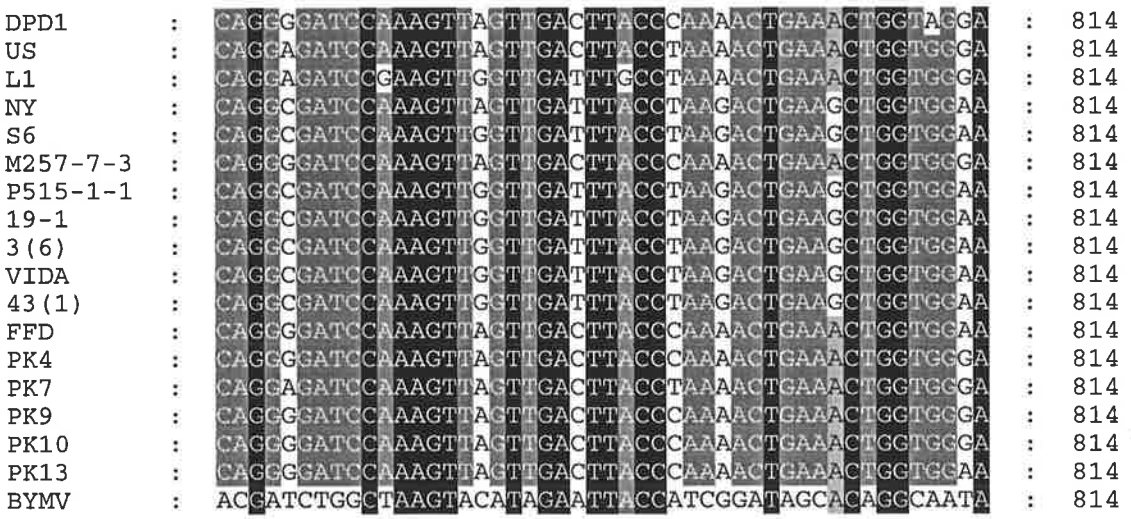
US : TTGGGGCGAACGTGGCTACCATGCAAAGCGTTTCTTTTCTGAGTTTTT : 479
L1 : CTGGGGTGAGCGTGGTTATCATGCAAAGCGTTTCTTTTCTGAGTTCTT : 479
NY : GTGGGGCGAACGTGCATATCATGCGAAACGTTTCTTTTCAGAGTATTT : 479
S6 : GTGGGGCGAACGTGGATATCATGCGAAACGTTTCTTTTCAGAGTATTT : 479
M257-7-3 : TTGGGGCGAACGTGCCTACCATGCAAAGCGTTTCTTTTCTGAGTTTTT : 479
P515-1-1 : GTGGGGCGAACGTGGATATCATGCGAAACGTTTCTTTTCAGAGTATTT : 479
19-1 : GTGGGGCGAACGTGCATATCATGCGAAACGTTTCTTTTCAGAGTATTT : 479
3(6) : GTGGGGCGAACGTGGATATCATGCGAAACGTTTCTTTTCAGAGTATTT : 479
VIDA : GTGGGGCGAACGTGCATATCATGCGAAACGTTTCTTTTCAGAGTATTT : 479
43(1) : GTGGGGCGAACGTGGATATCATGCGAAACGTTTCTTTTCAGAGTATTT : 479
FFD : TTGGGGCGAACGTGCCTACCATGCAAAGCGATTCTTTTCTGAGTTTTT : 479
PK4 : TTGGGGCGAACGTGCCTACCATGCAAAGCGTTTCTTTTCTGAGTTTTT : 479
PK7 : TTGGGGCGAACGTGCCTACCATGCAAAGCGTTTCTTTTCTGAGTTTTT : 479
PK9 : TTGGGGCGAACGTGCCTACCATGCAAAGCGTTTCTTTTCTGAGTTTTT : 479
PK10 : TTGGGGCGAACGTGCCTACCATGCAAAGCGTTTCTTTTCTGAGTTTTT : 479
PK13 : TTGGGGCGAACGTGCCTACCATGCAAAGCGATTCTTTTCTGAGTTTTT : 479
BYMV : GTGGGGTCAGAGAGCGTACCATGCTAAGCGATTTCTTAGCAACTACTT : 479
DPD1 : CACGAAGATTGATCCGAACGATGGATACTCTCATCACACTGTTAGGGC : 527
US : CACGAAGATAGATCCAAACGATGGATACTCCCATCACACTGTTAGGTC : 527
L1 : CATTAAAATTGATCCAAACGATGGCTACTCCCACCATACTGTAAGGTC : 527
NY : TACAAAGATTGATCCAAAAGATGGTTACACTCACCATGCTGTTAGAAC : 527
S6 : TACAAAGATTGATCCAAAAGATGGGTACACTCACCATGCTGTTAGAAC : 527
M257-7-3 : CACGAAGATTGATCCGAACGATGGATACTCTCATCACACTGTTAGGGC : 527
P515-1-1 : TACAAAGATTGATCCAAAAGATGGTTACACTCACCATGCTGTTAGAAC : 527
19-1 : TACAAAGATTGATCCAAAAGATGGTTACACTCACCATGCTGTTAGAAC : 527
3(6) : TACAAAGATTGATCCAAAAGATGGGTACACTCACCATGCTGTTAGAAC : 527
VIDA : TACGAAGATTGATCCGAAAGATGGGTACACTCACCATGCTGTTAGAAC : 527
43(1) : GACGAAGATGGATCCAAAAGATGGTTACACTCACCATGCTGTTAGAAC : 527
FFD : CACGAAGATTGATCCGAACGATGGATACTCTCATCACACTGTTAGGGC : 527
PK4 : CACGAAGATTGATCCGAACGATGGATACTCTCATCACACTGTTAGGGC : 527
PK7 : CACGAAGATAGATCCAAACGATGGATACTCCCATCACACTGTTAGGTC : 527
PK9 : CACGAAGATTGATCCGAACGATGGATACTCTCATCACACTGTTAGGGC : 527
PK10 : CACGAAGATTGATCCGAACGATGGATACTCTCATCACACTGTTAGGGC : 527
PK13 : CACGAAGATTGATCCGAACGATGGATACTCTCATCACACTGTTAGGGC : 527
BYMV : CAACGAAATCAACCCATCGGAGGGGTATGACAAATTCATTTTTAGGAA : 527
DPD1 : CACACCAACAGGAGTCAGGCACCTAGCAATTGGAAATCTGATCATTCC : 575
US : CACACCGACAGGAGTCAGGCACCTGGCAATTGGAAATCTGGTCATTCC : 575
L1 : CACACCAACAGGAGTCAGACACTTAGCAATTGGGAACCTGATCATACC : 575
NY : AACACCGACAGGAGTTAGGCATCTGGCAATAGGAAACTTGATCATACC : 575
S6 : AACACCGACAGGAGTTAGGCATCTGGCAGTAGGAAACTTGATCATACC : 575
M257-7-3 : CACACCAACAGGAGTCAGGCACCTAGCAATTGGAAATCTGATCATTCC : 575
P515-1-1 : AACACCGACAGGAGTTAGGCATCTGGCAATAGGAAACTTGATCATACC : 575
19-1 : AACACCGACAGGAGTTAGGCATCTGGCAATAGGAAATTTGATCATACC : 575
3(6) : AACACCGACAGGAGTTAGGCATCTGGCAGTAGGAAACTTGATCATACC : 575
VIDA : AACACCGACAGGAGTTAGGCATCTGGCAGTAGGAAATTTGATCATACC : 575
43(1) : AACACCGACAGGAGTTAGGCATCTGGCAATAGGAAATTTGATCATACC : 575
FFD : CACACCAACAGGAGTCAGGCACCTAGCAATTGGAAATCTGATCATTCC : 575
PK4 : CACACCAACAGGAGTCAGGCACCTAGCAATTGGCAATCTGATCATTCC : 575
PK7 : CACACCGACAGGAGTCAGGCACCTGGCAATTGGAAATCTGGTCATTCC : 575
PK9 : CACACCAACAGGAGTCAGGCACCTAGCAATTGGCAATCTGATCATTCC : 575
PK10 : CACACCAACAGGAGTCAGGCACCTAGCAATTGGCAATCTGATCATTCC : 575
PK13 : CACACCAACAGGAGTCAGGCACCTAGCAATTGGAAATCTGATCATTCC : 575
BYMV : ATTGCCGAACGGAGCTCGAGAGTTAGCTATAGGAAGATTGATCATGCC : 575

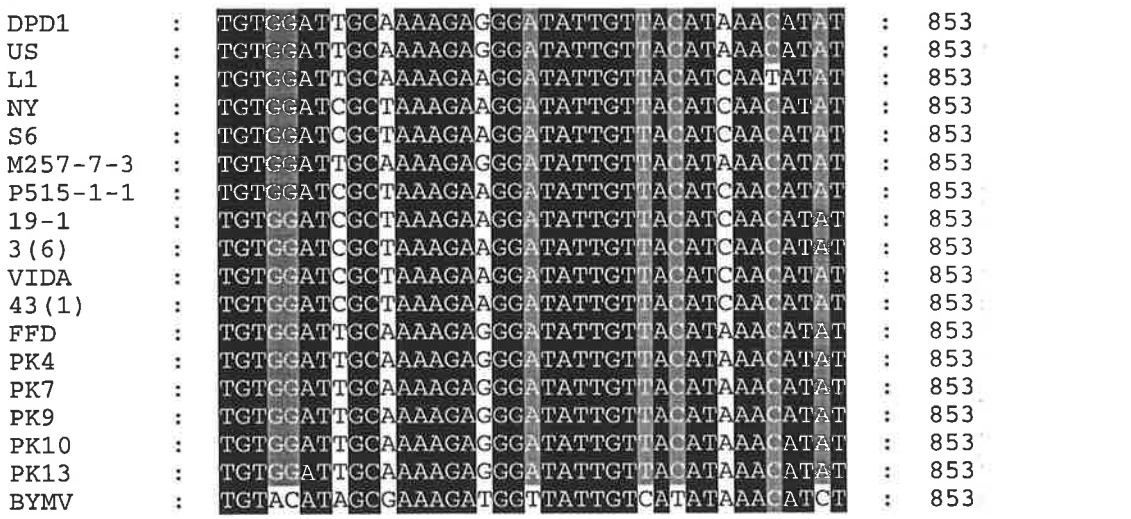

DPD1 : AGGGGATCTACAGAAATTAAGAGAGAAACTTGAAGGGGTGTCAATCAC : 623
US : AGGAGATCTGCAGAAATCAAGAGAGAAACTTGAAGGCGTGTCAATCAC : 623
L1 : AGGGGATCTCCAGAAATTAAGAGAGAAACTTGAAGGAGTTTCAATCGC : 623
NY : AGGCGATCTCCAAAAACTTAGGGAAAAACTTGAAGGTGTGTCAATCAC : 623
S6 : AGGCGATCTCCAAAAACTTAGGGAAAAACTTGAAGGTGTGTCAATCAC : 623
M257-7-3 : AGGGGATCTACAGAAATTAAGAGAGAAACTTGAAGGGGTGTCAATCAC : 623
P515-1-1 : AGGCGATCTCCAAAAACTTAGGGAAAAACTTGAAGGTGTGTCAATCAC : 623
19-1 : AGGCGATCTCCAGAAACTTAGGGAGAAACTTGAAGGTGTGTCAATCAC : 623
3(6) : AGGCGATCTCCAAAAACTTAGGGAAAAACTTGAAGGTGTGTCAATCAC : 623
VIDA : AGGCGATCTCCAGAAACTTAGGGAGAAACTTGAAGGTGTGTCAATCAC : 623
43(1) : AGGCGATCTCCAAAAACTTAGGGAGAAACTTGAAGGTGTGTCAATCAC : 623
FFD : AGGCGATCTACAGAAATTTAGAGAGAAACTTGAAGGGGTGTCAATCAC : 623
PK4 : AGGGGATCTACAGAAATTAAGAGAGAAACTTGAAGGGGTGTCAATTAC : 623
PK7 : AGGAGATCTGCAGAAATTAAGAGAGAAACTTGAAGGCGTGTCAATCAC : 623
PK9 : AGGGGATCTACAGAAATTAAGAGAGAAACTTGAAGGGGTGTCAATCAC : 623
PK10 : AGGGGATCTACAGAAATTTAGAGAGAAACTTGAAGGGGTGTCAATCAC : 623
PK13 : AGGGGATCTACAGAAATTAAGAGAGAAACTTGAAGGGGTGTCAATCAC : 623
BYMV : TACGAACTTTGAAGCTTTTAGGGAACAGATGAAAGCAAACATGATTGA : 623


DPD1 : AGCAGTGGGCATCTCGGAGAAGTGTGT-TAGCAGACGGAATGGTGACT : 670
US : AACAGTGGGCATCTCGGAGAAGTGTAT-TAGCAGACGGAATGGTGACT : 670
L1 : AACAGTAGGCATCTCAGAGAACTGCGT-TAGCAGACGGAACGGTGATT : 670
NY : AACAGTGGACATCTCAGAAAAGTGCGT-CAGTAGGCGGAATGGAGATT : 670
S6 : AACAGTGGACATCTCAGAAAAGTGCGT-CAGTAGGCGGAATGGAGATT : 670
M257-7-3 : AGCAGTGGGCATCTCGGAGAAGTGTGT-TAGCAGACGGAATGGTGACT : 670
P515-1-1 : AACAGTGGACATCTCAGAAAAGTGCGT-CAGTAGGCGGAATGGAGATT : 670
19-1 : AACAGTGGACATCTCAGAAAAGTGCGT-CACTAGGCGGAATGGAGATT : 670
3(6) : AACAGTGGACATCTCAGAAAAGTGCGT-CAGTAGGCGGAATGGAGATT : 670
VIDA : AACAGTGGACATCTCAGAAAAGTGCGT-CACTAGGCGGAATGGAGATT : 670
43(1) : AACAGTGGACATCTCAGAAAAGTGCGT-CAGTAGGCGGAATGGAGATT : 670
FFD : AGCAGTGGGCATCTCGGAGAAGTGTGT-TAGCAGACGGAATGGTGACT : 670
PK4 : AGCAGTGGGCATCTCGGAGAAGTGTGT-TAGCAGACGGAATGGTGACT : 670
PK7 : AACAGTGGGCATCTCGGAGAAGTGTAT-TAGCAGACGGAATGGTGACT : 670
PK9 : AGCAGTGGGCATCTCGGAGAAGTGTGT-TAGCAGACGGAATGGTGACT : 670
PK10 : AGCAGTGGGCATCTCGGAGAAGTGTGT-TAGCAGACGGAATGGTGACT : 670
PK13 : AGCAGTGGGCATCTCGGAGAAGTGTGT-TAGCAGACGGAATGGTGACT : 670
BYMV : TAATGGACCAATTGGGAAGGATTGTGTATCACGCATGAGGGGTTCATT : 671

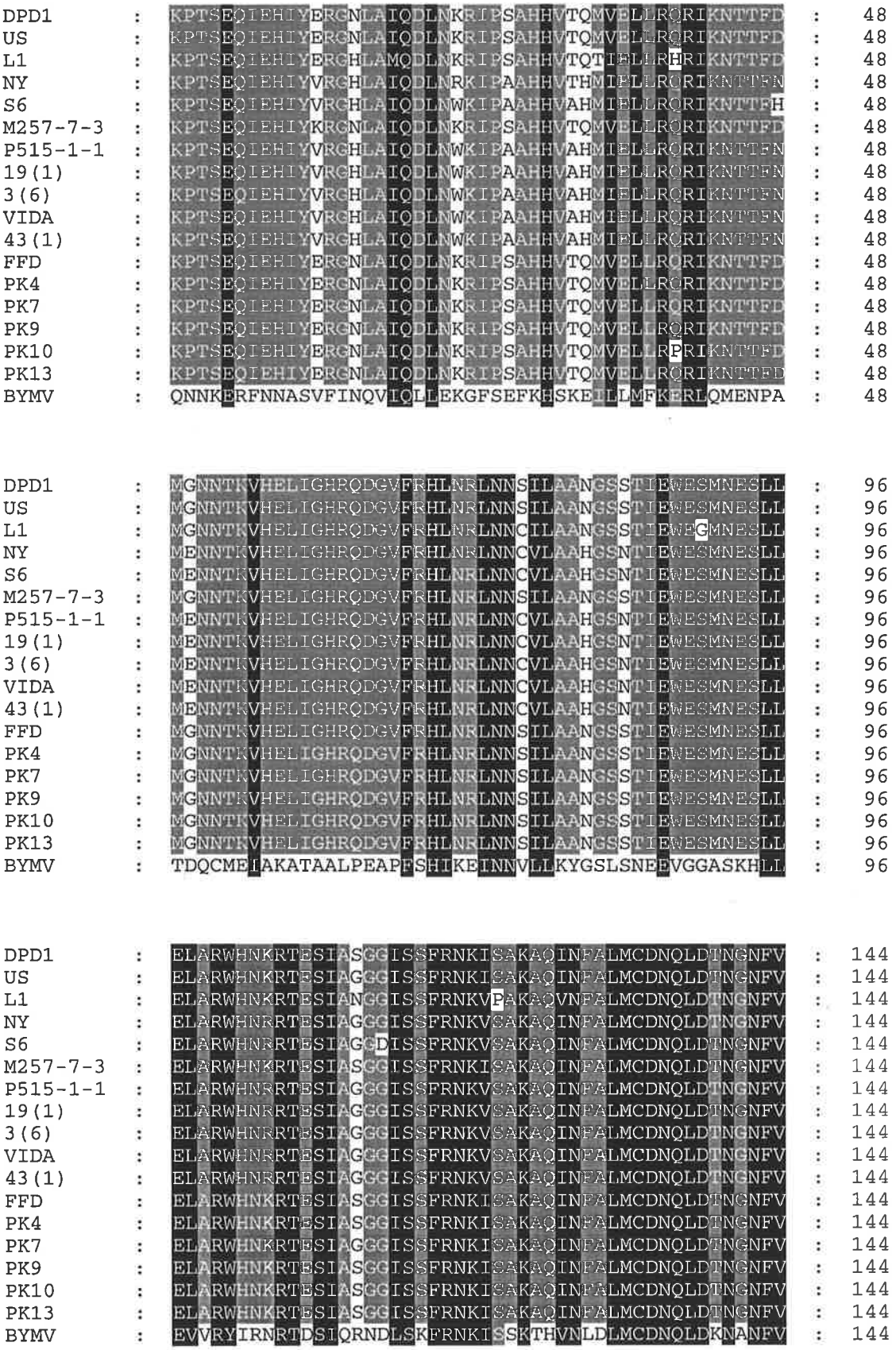

DPD1 : TTGTGTATCCTTGCTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
US : TTGTGTATCCTTGTTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
L1 : TTGTGTACCCTTGCTCGTGTGTGACATATGAGAACGGAAAACCAGTTT : 718
NY : TTGTTTATCCTTGCTCATGTGTCACACTTGAAAATGGAAAACCAGCCA : 718
S6 : TTGTTTATCCTTGCTCATGTGTAACACTTGAAAATGGAAAACCAGCCA : 718
M257-7-3 : TTGTGTATCCTTGCTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
P515-1-1 : TTGTTTATCCTTGCTCATGTGTAACACTTGAAAATGGAAAACCAGCCA : 718
19-1 : TTGTTTATCCTTGCTCATGTGTAACACTTGAAAATGGAAAACCAGCTA : 718
3(6) : TTGTTTATCCTTGCTCATGTGTAACACTTGAAAATGGAAAACCAGCCA : 718
VIDA : TTGTTTATCCTTGCTCATGTGTAACACTTGAAAATGGAAAACCAGCCA : 718
43(1) : TTGTTTATCCTTGCTCATGTGTAACACTTGAAAATGGAAAACCAGCCA : 718
FFD : TTGTGTATCCTTGCTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
PK4 : TTGTGTATCCTTGCTCATGCGTGACATNTGAGAACGGAAAACCAGTTT : 718
PK7 : TTGTGTATCCTTGTTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
PK9 : TTGTGTATCCTTGCTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
PK10 : TTGTGTATCCTTGCTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
PK13 : TTGTGTATCCTTGCTCATGCGTGACATCTGAGAACGGAAAACCAGTTT : 718
BYMV : CTGT-TACCCTTGCTGTTGCACAACTGATGATGTGGGAAATGCAGTAT : 718

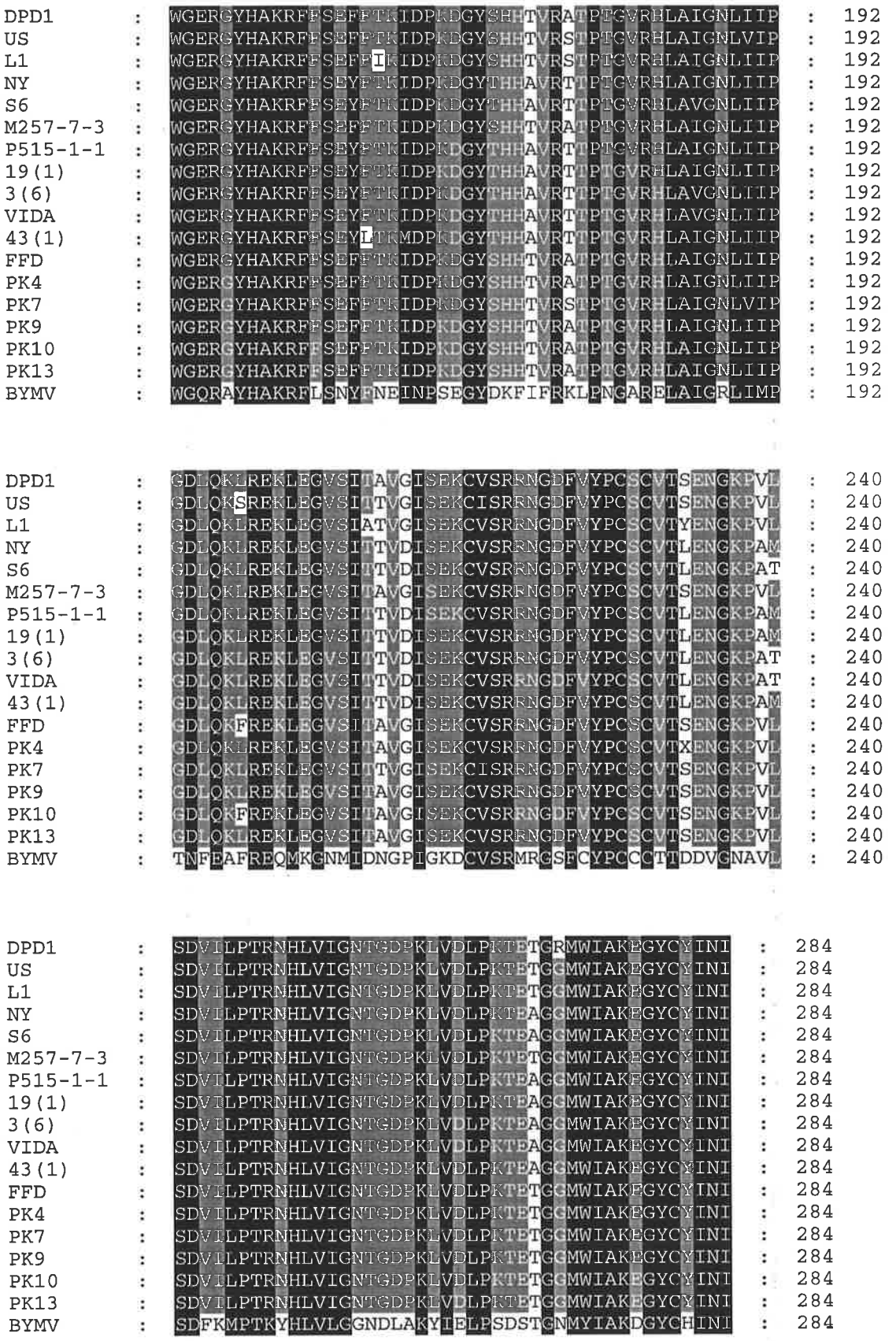

DPD1 : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTTATTGGGAATA : 766
US : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTGATTGGGAATA : 766
L1 : TATCAGATGTTATCCTACCTACAAGGAACCATTTGGTCATTGGGAACA : 766
NY : TGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAAACA : 766
S6 : CGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAAACA : 766
M257-7-3 : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTTATTGGGAATA : 766
P515-1-1 : TGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAAACA : 766
19-1 : TGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAAACA : 766
3(6) : CGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAAACA : 766
VIDA : CGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAAACA : 766
43(1) : TGTCTGACGTAATTTTACCGACGAGAAATCATTTGGTGATTGGAAACA : 766
FFD : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTTATTGGGAATA : 766
PK4 : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTTATTGGGAATA : 766
PK7 : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTGATTGGGAATA : 766
PK9 : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTTATTGGGAATA : 766
PK10 : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTTATTGGGAATA : 766
PK13 : TGTCAGATGTTATTTTACCTACAAGGAATCATTTGGTTATTGGGAATA : 766
BYMV : TATCCGATTTCAAGATGCCAACAAAATACCACCTTGTTCTGGGTGGAA : 766


DPD1 : CAGGGGATCCAAAGTTAGTTGACTTACCCAAAACTGAAACTGGTACGA : 814
US : CAGCAGATCCAAAGTTAGTTGACTTACCTAAAACTGAAACTGGTGCGA : 814
L1 : CAGCAGATCCGAAGTTGGTTGATTTGCCTAAAACTGAAACTGGTGCGA : 814
NY : CAGGCGATCCAAAGTTAGTTGATTTACCTAAGACTGAAGCTGGTGCAA : 814
S6 : CAGGCGATCCAAAGTTGGTTGATTTACCTAAGACTGAAGCTGGTGCAA : 814
M257-7-3 : CAGGGGATCCAAAGTTAGTTGACTTACCCAAAACTGAAACTGGTGCGA : 814
P515-1-1 : CAGGCGATCCAAAGTTGGTTGATTTACCTAAGACTGAAGCTGGTGCAA : 814
19-1 : CAGGCGATCCAAAGTTGGTTGATTTACCTAAGACTGAAGCTGGTGCAA : 814
3(6) : CAGGCGATCCAAAGTTGGTTGATTTACCTAAGACTGAAGCTGGTGCAA : 814
VIDA : CAGGCGATCCAAAGTTGGTTGATTTACCTAAGACTGAAGCTGGTGCAA : 814
43(1) : CAGGCGATCCAAAGTTGGTTGATTTACCTAAGACTGAAGCTGGTGCAA : 814
FFD : CAGGGGATCCAAAGTTAGTTGACTTACCCAAAACTGAAACTGGTGCAA : 814
PK4 : CAGGGGATCCAAAGTTAGTTGACTTACCCAAAACTGAAACTGGTGCGA : 814
PK7 : CAGCAGATCCAAAGTTAGTTGACTTACCTAAAACTGAAACTGGTGCGA : 814
PK9 : CAGCGGATCCAAAGTTAGTTGACTTACCCAAAACTGAAACTGGTGCGA : 814
PK10 : CAGCGGATCCAAAGTTAGTTGACTTACCCAAAACTGAAACTGGTGCGA : 814
PK13 : CAGGGGATCCAAAGTTAGTTGACTTACCCAAAACTGAAACTGGTGCAA : 814
BYMV : ACGATCTGGCTAAGTACATAGAATTACCATCGGATAGCACAGGCAATA : 814


DPD1 : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
US : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
L1 : TGTGGATTGCAAAAGAAGGATATTGTTACATCAATATAT : 853
NY : TGTGGATCGCTAAAGAAGGATATTGTTACATCAACATAT : 853
S6 : TGTGGATCGCTAAAGAAGGATATTGTTACATCAACATAT : 853
M257-7-3 : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
P515-1-1 : TGTGGATCGCTAAAGAAGGATATTGTTACATCAACATAT : 853
19-1 : TGTGGATCGCTAAAGAAGGATATTGTTACATCAACATAT : 853
3(6) : TGTGGATCGCTAAAGAAGGATATTGTTACATCAACATAT : 853
VIDA : TGTGGATCGCTAAAGAAGGATATTGTTACATCAACATAT : 853
43(1) : TGTGGATCGCTAAAGAAGGATATTGTTACATCAACATAT : 853
FFD : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
PK4 : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
PK7 : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
PK9 : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
PK10 : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
PK13 : TGTGGATTGCAAAAGAGGGATATTGTTACATAAACATAT : 853
BYMV : TGTACATAGCGAAAGATGGTTATTGTCATATAAACATCT : 853

Amino acid sequence alignment for 284 amino acids from the *HC-Pro* for 17 PSbMV isolates and BYMV.

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US : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRSTPTGVRHLAIGNLVIP : 192
L1 : WGERGYHAKRFFSEFFIKIDPKDGYSHFTVRSTPTGVRHLAIGNLIIP : 192
NY : WGERGYHAKRFFSEYFTKIDPKDGYTHFAVRTTPTGVRHLAIGNLIIP : 192
S6 : WGERGYHAKRFFSEYFTKIDPKDGYTHFAVRTTPTGVRHLAVGNLIIP : 192
M257-7-3 : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRATPTGVRHLAIGNLIIP : 192
P515-1-1 : WGERGYHAKRFFSEYFTKIDPKDGYTHFAVRTTPTGVRHLAIGNLIIP : 192
19(1) : WGERGYHAKRFFSEYFTKIDPKDGYTHFAVRTTPTGVRHLAIGNLIIP : 192
3(6) : WGERGYHAKRFFSEYFTKIDPKDGYTHFAVRTTPTGVRHLAVGNLIIP : 192
VIDA : WGERGYHAKRFFSEYFTKIDPKDGYTHFAVRTTPTGVRHLAVGNLIIP : 192
43(1) : WGERGYHAKRFFSEYLTKMDPKDGYTHFAVRTTPTGVRHLAIGNLIIP : 192
FFD : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRATPTGVRHLAIGNLIIP : 192
PK4 : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRATPTGVRHLAIGNLIIP : 192
PK7 : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRSTPTGVRHLAIGNLVIP : 192
PK9 : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRATPTGVRHLAIGNLIIP : 192
PK10 : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRATPTGVRHLAIGNLIIP : 192
PK13 : WGERGYHAKRFFSEFFTKIDPKDGYSHFTVRATPTGVRHLAIGNLIIP : 192
BYMV : WGQRAYHAKRFLSNYFNEINPSEGYDKFIFRKLPNGARELAIGRLIMP : 192
DPD1 : GDLQKLREKLEGVSITAVGISEKCVSRRNGDFVYPCSCVTSENGKPVL : 240
US : GDLQKSREKLEGVSITTVGISEKCISRRNGDFVYPCSCVTSENGKPVL : 240
L1 : GDLQKLREKLEGVSIATVGISEKCVSRRNGDFVYPCSCVTYENGKPVL : 240
NY : GDLQKLREKLECVSITTVDISEKCVSRRNGDFVYPCSCVTLENGKPAM : 240
S6 : GDLQKLREKLEGVSITTVDISEKCVSRRNGDFVYPCSCVTLENGKPAT : 240
M257-7-3 : GDLQKLREKLECVSITAVGISEKCVSRRNGDFVYPCSCVTSENGKPVL : 240
P515-1-1 : GDLQKLREKLECVSITTVDISEKCVSRRNGDFVYPCSCVTLENGKPAM : 240
19(1) : GDLQKLREKLEGVSITTVDISEKCVSRRNGDFVYPCSCVTLENGKPAM : 240
3(6) : GDLQKLREKLEGVSITTVDISEKCVSRRNGDFVYPCSCVTLENGKPAT : 240
VIDA : GDLQKLREKLEGVSITTVDISEKCVSRRNGDFVYPCSCVTLENGKPAT : 240
43(1) : GDLQKLREKLEGVSITTVDISEKCVSRRNGDFVYPCSCVTLENGKPAM : 240
FFD : GDLQKFREKLEGVSITAVGISEKCVSRRNGDFVYPCSCVTSENGKPVL : 240
PK4 : GDLQKLREKLEGVSITAVGISEKCVSRRNGDFVYPCSCVTXENGKPVL : 240
PK7 : GDLQKLREKLECVSITTVGISEKCISRRNGDFVYPCSCVTSENGKPVL : 240
PK9 : GDLQKLREKLEGVSITAVGISEKCVSRRNGDFVYPCSCVTSENGKPVL : 240
PK10 : GDLQKFREKLEGVSITAVGISEKCVSRRNGDFVYPCSCVTSENGKPVL : 240
PK13 : GDLQKLREKLEGVSITAVGISEKCVSRRNGDFVYPCSCVTSENGKPVL : 240
BYMV : TNFEAFREQMKGNMIDNGPIGKDCVSRMRGSFCYPCCGTTDDVGNAVL : 240
DPD1 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGRMWIAKEGYCYINI : 284
US : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
L1 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
NY : SDVILPTRNHLVIGNTGDPKLVDLPKTEAGGMWIAKEGYCYINI : 284
S6 : SDVILPTRNHLVIGNTGDPKLVDLPKTEAGGMWIAKEGYCYINI : 284
M257-7-3 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
P515-1-1 : SDVILPTRNHLVIGNTGDPKLVDLPKTEAGGMWIAKEGYCYINI : 284
19(1) : SDVILPTRNHLVIGNTGDPKLVDLPKTEAGGMWIAKEGYCYINI : 284
3(6) : SDVILPTRNHLVIGNTGDPKLVDLPKTEAGGMWIAKEGYCYINI : 284
VIDA : SDVILPTRNHLVIGNTGDPKLVDLPKTEAGGMWIAKEGYCYINI : 284
43(1) : SDVILPTRNHLVIGNTGDPKLVDLPKTEAGGMWIAKEGYCYINI : 284
FFD : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
PK4 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
PK7 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
PK9 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
PK10 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
PK13 : SDVILPTRNHLVIGNTGDPKLVDLPKTETGGMWIAKEGYCYINI : 284
BYMV : SDFKMPTKYHLVLGGNDLAKYIELPSDSTGNMYIAKDGYCHINI : 284

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Corrigendum

P.9, L28-29 Many potyviruses also induce cytoplasmic amorphous inclusion bodies, composed of a single protein antigenically related to the HC-Pro.

P.59, L.23 Fifteen Australian PSbMV isolates were collected during the course of this study (Table 3.1).

Table 1.3 Names of all virus species except Peanut stripe virus should be in italics.

P.64, L.20-21 This PSbMV RT-PCR appears to be species-specific detecting all 31 PSbMV isolates tested.

Comments on points raised by the examiners

Figure 4.6 - Total nucleic acid from TMV infected leaf did not product a specific amplification product in the potyvirus RT-PCR, but did produce non-specific products observed as a smear in agarose gel electrophoresis (Figure 5.3).

Figure 4.10 - Shows slight differences in the limit of detection of PSbMV by DIBA between the two and four fold dilution series. Ten nanograms of PSbMV was faintly detectable in the two fold dilution but negative in the four fold dilution. These differences could be due to errors introduced in preparing the two dilution series.

Figure 5.4 - The *Alu*I RFLP patterns (A) and (C) are slightly different with the larger fragment in (C) running faster than the larger fragment in (A) and the smaller fragment in (C) running slower than the smaller fragment in (A). These differences in *Alu*I RFLP were confirmed by checking cloned sequences of the 1085 bp HC-Pro from PSbMV isolates representative of the different RFLPs for RE recognition sites.

P.91, L10-14 - The amino acid similarities between BYMV and the PSbMV isolates were low (38.9 - 41.3%). These values represent similarity (chemically similar) and not identity (identical). Therefore, it is possible when such distantly related sequences are compared at the nucleic acid sequence level that a slightly higher similarity (in this case also identity) value may be obtained by chance in the pair-wise comparison. The nucleic acid sequence similarities were also low between BYMV and the PSbMV isolates (49.8 - 51.4%).