The Development of a Serological-Based Diagnostic Test for *Dasheen Mosaic Potyvirus* (DsMV)

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Abstract

Dasheen mosaic potyvirus (DsMV) is an important virus affecting taro. The virus has been found wherever taro is grown and infects both the edible and ornamental aroids, causing yield losses of up to 60%. The presence of DsMV, and other viruses, prevents the international movement of taro germplasm between countries. This has a significant negative impact on taro production in many countries due to the inability to access improved taro lines produced in breeding programs. To overcome this problem, sensitive and reliable virus diagnostic tests need to be developed to enable the indexing of taro germplasm. The aim of this study was to generate an antiserum against a recombinant DsMV coat protein (CP) and to develop a serological-based diagnostic test that would detect Pacific Island isolates of the virus.

The CP-coding region of 16 DsMV isolates from Papua New Guinea, Samoa, Solomon Islands, French Polynesia, New Caledonia and Vietnam were amplified, cloned and sequenced. The size of the CP-coding region ranged from 939 to 1038 nucleotides and encoded putative proteins ranged from 313 to 346 amino acids, with the molecular mass ranging from 34 to 38 kDa. Analysis of the amino acid sequences revealed the presence of several amino acid motifs typically found in potyviruses, including DAG, WCIE/DN, RQ and AFDF. When the amino acid sequences were compared with each other and the DsMV sequences on the database, the maximum variability was 21.9%. When the core region of the CP was analysed, the maximum variability dropped to 6% indicating most variability was present in the N terminus. Within seven PNG isolates of DsMV, the maximum variability was 16.9% and 3.9% over the entire CP-coding region and core region, respectively. The sequence of PNG isolate P1 was most similar to all other sequences. Phylogenetic analysis indicated that almost all isolates grouped according to their provenance. Further, the seven PNG isolates were grouped according to the region within PNG from which they were obtained.

Due to the extensive variability over the entire CP-coding region, the core region of the CP of PNG isolate P1 was cloned into a protein expression vector and expressed as a recombinant protein. The protein was purified by chromatography and SDS-PAGE and used as an antigen to generate antiserum in a rabbit. In western blots, the antiserum reacted with bands of approximately 45-47 kDa in extracts from purified DsMV and from known DsMV-infected plants from PNG; no bands were observed using healthy plant extracts. The antiserum was subsequently incorporated into an indirect ELISA. This procedure was found to be very sensitive and detected DsMV in sap diluted at least 1:1,000. Using both western blot and ELISA formats, the antiserum was able to detect a wide range of DsMV isolates including those from Australia, New Zealand, Fiji, French Polynesia, New Caledonia, Papua New Guinea, Samoa, Solomon Islands and Vanuatu. These plants were verified to be infected with DsMV by RT-PCR. In specificity tests, the antiserum was also found to react with sap from plants infected with SCMV, PRSV-P, PRSV-W, but not with PVY or CMV-infected plants.

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List of Abbreviations

µg	microgram(s)
µl	microliter(s)
bp	base pair(s)
cDNA	complementary DNA
CP	coat protein
CTAB	hexadecyltrimethylammonium bromide
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ds	double stranded
DsMV	*Dasheen mosaic potyvirus*
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
g	gravity
g/L	grams per litre
h	hour(s)
ha	hectare(s)
IAA	isoamyl alcohol
IMPACT-CN	intein mediated purification with an affinity chitin-binding tag (C- or N-terminal fusion)
IPTG	isopropyl-thiogalactoside
Kb	kilobase(s)
kDa	kilodalton(s)
LB	Luria-Bertani medium
m	metre(s)
M	molar
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar

ng	nanogram(s)
nm	nanometre(s)
OD	optical density
ORF	open reading frame
PBST	phosphate buffered saline containing Tween-20
PCR	polymerase chain reaction
pmol	picomole(s)
PVP	polyvinylpyrollidone
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
s	second(s)
ss	single stranded
TBS	tris buffered saline
TBST	TBS with tween-20
TEMED	N, N, N', N' -tetramethylethylenediamine
ton	tonne(s)
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
UV	ultra violet
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Table A. IUPAC standard codes for amino acids

RESIDUE	IUPAC LETTER CODE	
	1 LETTER	3 LETTER
Alanine	A	Ala
Cysteine	C	Cys
Aspartate	D	Asp
Glutamate	E	Glu
Phenylalanine	F	Phe
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Lysine	K	Lys
Leucine	L	Leu
Methionine	M	Met
Asparagine	N	Asn
Proline	P	Pro
Glutamine	Q	Gln
Arginine	R	Arg
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val
Tryptophan	W	Trp
Tyrosine	Y	Tyr

Statement of Original Authorship

To the best of my knowledge, the work contained in this thesis is original and has not been submitted elsewhere for the award of a higher degree, unless due reference is contained therein.

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Above all, I thank the Almighty God for the inner peace and solace during my absence from my loved ones, and for granting me a share in the ocean of knowledge.

Chapter 1 Introduction

1.1 General

Taro, *Colocasia esculenta* (L.) Schott, is an important staple crop for many people of the world, particularly in Oceania (Plucknett *et al.*, 1970). The Melanesians and Polynesians consume taro in their daily diet (Plucknett and de la Pena, 1971) while in many Pacific Island countries, taro is a small-scale commodity crop sold for income in the local markets. Further, some nations like Hawaii cultivate taro as an export crop (de la Pena, 1992). The crop also has cultural significance in the nations where it is cultivated (Barth, 1975; Connell, 1978; Morren and Hydman, 1987; Plarre, 1995) and is commonly used in ceremonies such as weddings.

The production of taro has suffered a significant decline due to numerous diseases over the past thirty years (Rodoni *et al.*, 1994). Of the pathogens associated with these diseases, viruses have caused considerable losses. Among the plant viruses affecting taro, *Dasheen mosaic potyvirus* (DsMV) occurs worldwide (Zettler, *et al.*, 1978; Shaw *et al.*, 1979; Jackson, 1980, 1982; Zettler and Hartman, 1986, 1987). Infected plants usually display a conspicuous feathery mosaic pattern although cultivars vary considerably in symptom expression. The main effect of virus infection is a reduction in corm size and quality (Hartman and Zettler, 1974; Knauss *et al.*, 1975; Wisler *et al.*, 1978; Zettler *et al.*, 1980; Chase *et al.*, 1981). In addition to taro, the virus infects several other edible aroids including *Xanthosoma* where it causes significant yield losses (Ramirez and Gamez, 1984).

There is no effective control for DsMV. The removal of infected plants (roguing) and spraying with insecticides are possible methods of control but these are likely to be ineffective and would be extremely difficult to implement in the South Pacific. There are also no taro plants that are resistant to DsMV. One possible short-term strategy to control or limit the disease is through the use of virus-indexed planting material, produced from meristem tip culture. The implementation of such a scheme requires the availability of a diagnostic test that would enable large throughput of samples, have the capability of detecting all virus isolates and

preferably be technically simple to enable use in Pacific Island laboratories. A serological test such as the enzyme-linked immunosorbent assay (ELISA) would be an ideal choice since such tests are sensitive, reliable, do not require sophisticated equipment and are suited to testing large numbers of samples. Although ELISA-based diagnostics for DsMV are commercially available, the cost of these tests precludes their regular use in Pacific Island countries. There is a real need, therefore, to produce an antiserum against Pacific isolates of DsMV which can be incorporated into an ELISA format for use in a virus-indexing scheme.

Production of antiserum against viruses has traditionally been achieved by injecting purified virions into laboratory animals. However, the use of impure virion preparations as antigen can result in cross-reactivity with plant proteins. To overcome this problem, recombinant viral coat proteins are now being used as antigen since they can be purified in large quantities and are generally free from contaminating proteins (Li *et al.*, 1998). This approach is well suited for the expression of the DsMV CP since the genomes of potyviruses have been characterised. However, because regions of the potyvirus CP-coding region are highly variable (Shukla *et al.*, 1992 ; Li *et al.*, 1998, 1999; Pappu *et al.*, 1994a), variability studies need to be instigated to determine the most appropriate CP sequence to express as antigen. At the commencement of this study, variability studies had only been done on DsMV isolates obtained from the USA (Pappu *et al.*, 1994a,b; Li *et al.*, 1998) and a single case from Taiwan (GenBank AF511485). This project aimed to analyse the variability in the coat protein-coding region of DsMV isolates from South Pacific countries, express a recombinant DsMV CP, generate antiserum and develop a serological diagnostic assay for DsMV.

1.2 The taro plant

1.2.1 Botany

Taro, *Colocasia esculenta* (L.) Schott, is a herbaceous monocot and a member of the family *Araceae*. The family has around 100 genera and approximately 1500 species, and has its origin in southeastern Asia. There are two distinct types: *Colocasia esculenta* var *esculenta* (L.) Schott, which produces one central underground corm;

and *Colocasia esculenta* var *antiquorum* (L.) Schott, having a central corm with several side cormels (Purseglove, 1988). The crop is grown primarily for food and a good source of carbohydrate and other dietary requirements.

Under optimum conditions (2500 mm rainfall per annum; wet, heavy, fertile soils; tropical climate), taro grows to a height of approximately 2 m. It produces a whorl of large leaves with long erect petioles at the apex and produces an underground starchy corm (Purseglove, 1988) (Figure 1.1). A single plant produces several suckers, which, together with the tops of the main plant (setts) are used as vegetative propagules. The crop usually matures in 8-10 months, when the leaves begin to yellow and die and there is a slight lifting of the tubers. The main crop yield averages between 15-20 ton/ha but yields as high as 37 ton/ha have been reported (Purseglove, 1988).

1.2.2 Diseases

Taro suffers from several diseases caused by viruses, fungi, bacteria, and nematodes. Taro leaf blight, caused by the fungus, *Phytophthora colocasiae*, is probably the most serious disease and results in 30-50% losses in corm yield (Jackson, 1977, 1980; Holliday, 1980). Yield losses also occur as a result of virus infections (Rodoni *et al.*, 1994; Ramirez and Gamez, 1984). There are reports of four viruses infecting taro, namely *Dasheen mosaic potyvirus* (DsMV), *Colocasia bobone disease virus* (CBDV), *Taro bacilliform badnavirus* (TaBV) and *Taro veinal chlorosis virus* (TVCV) (Brunt *et al.*, 1990; Pearson *et al.*, 1999). Apart from DsMV and TaBV, these viruses have been poorly characterised and there is confusion in the literature concerning their distribution and the symptoms associated with infection.

TaBV is thought to occur in combination with CBDV to cause "alomae" disease. This disease is considered the most destructive virus disease of taro and results in plant death (Rodoni *et al.*, 1994). In contrast, infection of taro by CBDV alone is thought to result in "bobone" disease, a similar but milder disease than alomae that causes leaf distortions and galls. The distribution of CBDV is thought to be restricted to PNG and the Solomon Islands (Gollifer *et al.*, 1977). Brunt *et al.* (1990) reported that CBDV was a possible member of the family *Rhabdoviridae* as it

a

b **c**

Figure 1.1 The taro plant. (a) Whole plant (b) taro corms with petioles (planting sett) (c) taro corm without petioles.

From Lambert (1982).

possessed morphologically characteristic bullet-shaped or bacilliform particles measuring 300-335 x 50-55 nm. A second putative rhabdovirus, TVCV, causes a very distinctive veinal chlorosis pattern in diseased taro (no galls, different symptoms) and is thought to occur in Fiji and Vanuatu (Pearson *et al.*, 1999); this virus has not been characterised.

Infection of taro by TaBV alone is thought to result in mild symptoms including mosaic and downward curling of leaves. TaBV has been classified as a possible member of the genus *Badnavirus* based on the virion size of 130 nm x 30 nm and transmission by mealybugs (Brunt *et al.*, 1990). The virus appears to be distributed throughout many taro-growing countries in the South Pacific, including PNG, Solomon Islands, Fiji, Vanuatu, Samoa and Cook Islands (Gollifer *et al.*, 1977).

Of all the viruses infecting taro, DsMV is the best characterised. This virus belongs to the family *Potyviridae* and is a member of the genus *Potyvirus*. It occurs worldwide (Zettler and Hartman, 1986, 1987; Jackson, 1980, 1982; Shaw *et al.*, 1979; Zettler *et al.*, 1978) and mainly infects crops of the family *Araceae.*

1.3 The genus *Potyvirus*

1.3.1 General

The *Potyvirus* genus derives its name from *Potato virus Y* (PVY), the type member (Harrison *et al.*, 1971; Matthews, 1982). It is grouped with 5 other genera including the *Ipomovirus, Macluravirus, Rymovirus, Tritimovirus*, and *Bymovirus* to form the family *Potyviridae*. The potyvirus genus consists of the largest membership with 179 species (88 definite and 91 possible members) (Van Regenmortel *et al.*, 2000). A large number of potyviruses are transmitted non-persistently by aphids (Matthews, 1991; Shukla *et al.*, 1994) and some are seed transmitted (mostly occur among the species of leguminous plants) (Hollings and Brunt, 1981a,b; Shukla *et al.*, 1994).

Since a suggestion by Brandes and Wetter (1959) to establish a number of groups of plant viruses based on their particle sizes and other related attributes,

addition of viruses to the group has continued to increase. For a group as large as the potyviruses, satisfactory taxonomic parameters have to be defined so that suitable criteria are employed to distinguish between viruses, and strains of viruses. To qualify for inclusion in the potyvirus genus, viruses must satisfy certain criteria including (i) have particles with characteristic modal length of 680 to 900 nm (Fenner, 1976); (ii) be able to induce typical cylindrical inclusions (ie. so called pinwheel inclusions) in the cytoplasm or nucleus of infected cells (Matthews, 1982); (iii) are transmitted non-persistently by aphids; and (iv) possess desired physicochemical properties of particles (ie. sedimentation coefficient, buoyant density, type and molecular weight of genomic nucleic acid, molecular weight of capsid protein, serological relationship) (Shukla *et al.*, 1994).

Members of the potyvirus genus occur throughout the world and have a devastating effect on the yield and quality of the crops affected (Wang *et al.*, 1978; Chang, 1983; Roperos and Magnaye, 1991; Shukla *et al.*, 1994).

1.3. 2 Particle structure and properties

Potyviruses comprise flexuous rods of about 11 to 12 nm wide with a modal length ranging from 680 to 900 nm (Fenner, 1976). Hollings and Brunt (1981a,b) reported the rods had narrow central canals, and optical diffraction assays indicate that the protein subunits are arranged in a helix with a pitch of 3.3 to 3.5 nm (Varma *et al.*, 1968). Particles contain 95% protein and 5% nucleic acid (Hollings and Brunt, 1981a,b).

The viral ssRNA genome is encapsidated by a single polypeptide of 2000 protein subunits, which are helically arranged (Hollings and Brunt, 1981a,b). The molecular weight of the subunits mostly range from 30 to 35 kDa (Hollings and Brunt, 1981a,b; Hollings *et al.*, 1976a,b; Usugi *et al.*, 1989). The capsid protein of potyviruses contains 251-332 amino acids and a protease-resistant core with molecular weight of 24 kDa.

During centrifugation, the particles usually sediment as a single component with a sedimentation coefficient of 150-160S and have buoyant densities in caesium chloride of 1.325-1.335 g cm^{-1} at 25°C (Hollings and Brunt, 1981a,b).

1.3.3 Genome organisation and function

The genome of aphid transmitted potyviruses consists of one molecule of single-stranded positive sense RNA of approximately 10 000 bases (Shukla *et al.*, 1994). The genome is 3' polyadenylated (20-160 adenosines), has a protein (VPg) of approximately 24 kDa covalently linked to the 5' terminus and functions as a single open reading frame coding for a large polyprotein (Hari *et al.*, 1979; Hari, 1981; Siaw *et al.*, 1985; Niblett *et al.*, 1988, 1991; Riechmann *et al.*, 1989, 1991).

The polyprotein of potyviruses is proteolytically cleaved into at least eight functional proteins (Hellmann *et al.*, 1983, 1986; Vance and Beachy, 1984; Yeh and Gonsalves, 1985; Dougherty and Carrington, 1988; Garcia *et al.*, 1989; Carrington *et al.*, 1989) (Figure 1.2). The proteins, VPg and CP, are present in virions while the six other proteins (P1, HC, P3, CI, NIa, NIb) occur within the cells of infected plants (Dougherty and Carrington, 1988; Rodriguez-Cerezo and Shaw, 1991). Two other putative proteins, 6K1 and 6K2, have also been identified but are yet to be detected *in vivo* (Verchot *et al.*, 1991; Riechmann *et al.*, 1992). The functions of many of the potyvirus gene products have been determined but research in this area is ongoing (Table 1.1).

1.3.4 The coat protein (CP)

The coat protein-coding region of potyviruses is located near the 3' end of the viral genome (Figure 1.2). The CP itself comprises three different regions: (i) a surface-exposed N terminus, which is heterogenous in sequence, (ii) a highly conserved core of 215-227 amino acids, and (iii) a surface-exposed C terminus of 18-20 amino acids (Shukla and Ward, 1989a). The sequence homology of the CP of potyviruses has been used to differentiate between distinct potyviruses and virus strains. Shukla and Ward (1988) proposed that isolates showing CP sequence identities of 38 to 71% and 72 to 99% be classified as either distinct viruses or potyvirus strains, respectively.

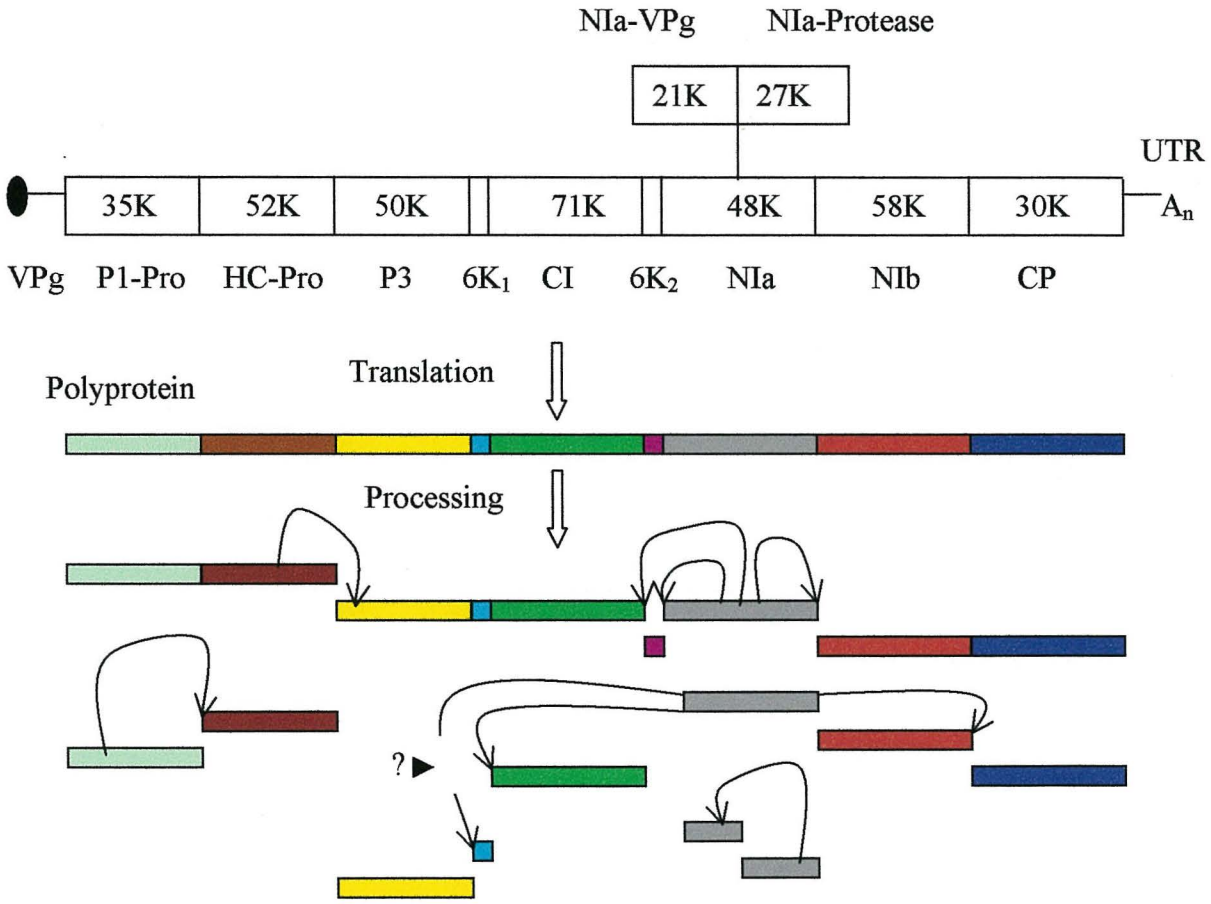

Figure 1.2: Potyvirus genome organisation and post translational cleavage patterns. In Shukla *et al.* (1994); originally from Riechmann *et al.* (1992). **Abbreviations: P1-Pro,** first protease; **HC-Pro**, helper component protease; **P3**, third protein; **6K1**, 6 kDa protein; **CI**, cytoplasmic inclusion protein; **6 K2**, second 6 kDa; **NIa**, nuclear inclusion proteins consisting of the VPg and third protease; **NIb**, RNA-dependent RNA polymerase; **CP**, coat protein; **UTR**, untranslated region; **VPg**, 5' genome-linked protein; $\mathbf{A_n}$, 3' poly (A) tail. **Note:** Changes made to Mr for some gene products are from van Regenmortel *et al.* (2000).

Table 1.1. Functions of potyvirus gene products

Protein	Function
P1	Proteinase; terminal step in polyprotein processing; cell-to-cell movement (speculation).
HC-proteinase	Aphid transmission; proteinase, polyprotein processing; cell-to-cell movement (speculation); pathogenicity; RNA replication and accumulation.
P3	Unknown; possibly genome replication.
6K1	Unknown; possibly genome replication.
CI	Genome replication; RNA helicase, unwinds dsRNA; membrane attachment; cell-to-cell movement, ATPase.
6K2	Unknown; possibly genome replication.
NIa-VPg	Genome replication; primer, host range, pathogenicity
NIa-proteinase	Proteinase; major aspects of polyprotein processing; complexed with NIb protein to initiate synthesis of +RNA
NIb	Genome replication; RNA-dependent RNA polymerase; complexed with NIa protein to initiate synthesis of +RNA.
CP	RNA encapsidation; aphid transmission; cell-to-cell movement; cross protection; symptom expression and development.

Source: Shukla *et al.* (1994), Klein *et al.* (1994), Riechmann *et al.* (1995), Maia *et al.* (1996), Pruss *et al.* (1997), Kassachau *et al.* (1997), Li *et al.* (1997), Naderi and Berger (1997), van Regenmortel *et al.* (2000).

The size estimates of the CP have been determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), amino acid analysis and protein and gene sequencing (Shukla *et al.*, 1994). Studies have shown that molecular weights determined by amino acid analysis were different to those observed in SDS-PAGE analysis (Allison *et al.*, 1985b; Shukla *et al.*, 1986). Generally, molecular weights detemined by relative mobilities of the CP in SDS-PAGE range from 28 kDa to 40 kDa, with most estimates in the 33-34 kDa range (Hiebert and McDonald, 1973; Hill *et al.*, 1973; Huttinga, 1975; Moghal and Francki, 1976; Gough and Shukla, 1981).

1.3.5 Serological diagnosis

Prior to the development of serological techniques, detection of potyviruses was based on biological diagnosis such as symptomatology, host range studies, vector transmission and cross-protection phenomenon. Generally, however, these methods are unreliable and are affected by parameters such as plant variety and environmental conditions. As such, more specific, sensitive and reliable tests have been developed based on serology of the virus particles and the coat protein (Shukla *et al.*, 1994).

1.3.5.1 Serology of the CP and virion of potyviruses

The majority of potyviruses are strongly immunogenic and antisera against the virus particles and coat protein have been produced to detect distinct members and strains (Shukla *et al.*, 1994). However, Shukla *et al.* (1992) reported some difficulties associated with serological detection of some potyviruses. These difficulties were due to inherent complexities associated with the potyvirus coat proteins and particles, and not the diagnostic technique (Shukla and Ward, 1989a,b; Ward and Shukla, 1991). Shukla *et al.* (1994) stated that the complexities resulted from three major problems, namely; (i) variable cross-reactivity of potyvirus antisera; (ii) unexpected and inconsistent paired relationship; (iii) lack of cross-reaction between some strains.

The problems led to detailed investigations into the potyvirus coat protein structure and these studies demonstrated that:

1. Distinct members, in general, posses a coat protein sequence identity of 38-71% with major differences in length and sequence of their N termini but high sequence identity in the core region (Shukla *et al.*, 1994).

2. Strains of individual viruses exhibit a sequence identity of greater than 90% and have N termini sequences that are very similar (Shukla and Ward, 1988, 1989a,b; Ward and Shukla, 1991).

3. The N and C termini of the coat protein are located on the virus particle surface, and can be removed from intact particles by mild enzyme treatment without affecting the morphology of virus particles (Hiebert *et al.*, 1984; Allison *et al.*, 1985a; Dougherty *et al.*, 1985; Shukla *et al.*, 1988).

4. The N terminus constitutes the most immunodominant region of potyvirus particles (Shukla *et al.*, 1988, 1989b).

5. Virus-specific epitopes are usually located in the N terminus, whereas cross-reacting potyvirus group-specific epitopes are contained in the highly homologous region of coat proteins (Shukla *et al.*, 1988, 1989a,b).

There are a number of serological techniques that have been used to detect potyviruses. Precipitin tests were among the earliest methods used (van Regenmortel, 1982), followed soon after by SDS-double diffusion tests (Purcifull and Batchelor, 1977; Ouchterlony and Nilsson, 1978). Both these methods, however, are reported to be insensitive (Shukla *et al.*, 1994) (Table 1.2). More recently, rapid, sensitive and specific tests including, ELISA (Clark and Adams, 1977), radioimmunoassays (Ghabrial and Shepherd, 1980) and western blotting (van Regenmortel, 1982) have been developed.

1.4 *Dasheen mosaic potyvirus* (DsMV)

1.4.1 Distribution and host range

DsMV occurs wherever taro is grown (Table 1.3). The natural host range for DsMV appears to be restricted to the *Araceae* family, with the virus infecting many aroids, both ornamental and edible (Table 1.3; Zettler and Hartman, 1986, 1987; Jackson, 1980, 1982; Shaw *et al.*, 1979). Successful mechanical infection of the virus has also been reported on plants outside this family including *Chenopodium amaranticolor,*

Table 1.2. Sensitivity of serological techniques for the detection of plant viruses

Technique	Detection range*
Liquid precipitin tests	1-10 μg virus ml^{-1}
SDS-double diffusion tests	2-20 μg virus ml^{-1}
Agglutination	5-20 ng virus ml^{-1}
Immunoelectron microscopy	1-10 ng virus ml^{-1}
Western blotting	500 pg virus
Dot-blot	0.5 pg virus
ELISA	1-10 ng virus ml^{-1}
Radioimmunosorbent assay	1-10 ng virus ml^{-1}

* Sources: Ghabrial and Shepherd, 1980; van Regenmortel, 1982; Shukla *et al.*, 1983; Berger *et al.*, 1985; Wetzel *et al.*, 1990. **In:** Shukla *et al.*, 1994.

C. quinoa, Nicotiana benthamiana, Saponaria vaccaria and *Tetragonia expansa* (Rana *et al.*, 1983; Gollifer and Brown, 1972).

1.4.2 Symptoms

Infected plants show a variety of symptoms which are often intermittent or seasonal (Zettler and Hartman, 1986). These symptoms usually include pale yellow to green patches on the leaves, characteristically as feather-like patterns along the veins, especially near the leaf surface. Occasionally, yellow and green patterns occur over the entire leaf surface, which may appear narrow, with a distorted margin (Kohler *et al.*, 1997) (Figure 1.3 a,b). It is not uncommon, however, to find symptomless infections.

A severe strain of DsMV is reported to occur in French Polynesia (Jackson, 1982). This virus causes pale green to yellow patterns on small, stunted and severely distorted leaves, some of which are reduced to strap-like structures without lobes. Plants fail to recover from infection and corms are also severely reduced in size (Kohler *et al.*, 1997) (Figure 1.3 c,d).

Table 1.3. Distribution of *Dasheen mosaic potyvirus*

Country	Host	Source
Florida, USA	*C. esculenta* and other ornamental aroids	Zettler *et al.* (1970) (First description of DsMV)
Puerto Rico	*Colocasia/Xanthosoma*	Alconero and Zettler (1971)
Netherlands	*Zentedeschia aethiopica*	Hoof (1971)
Solomon Islands	*C. esculenta*	Gollifer and Brown (1972)
Venezuela	*Colocasia/Xanthosoma*	De Brot and Ordosgoitti (1974)
Egypt	*C. antiquorum*	Mostafa *et al.* (1976)
Fiji	*C. esculenta*	Abo El-Nil *et al.* (1977)
Southwest Pacific Is.	Edible aroids	Gollifer *et al.* (1977)
Papua New Guinea	*Colocasia/Xanthosoma*	Shaw *et al.* (1979)
Japan	*Amorphophallus konjac*	Okuda *et al.* (1979)
Gilbert Islands	*C. esculenta*	Shanmuganathan (1980)
Nigeria	Cocoyam	Volin (1980)
Cameroon	*Xanthosoma* spp.	Girard *et al.* (1980)
Kiribati	Edible aroids	Shanmuganathan (1980
French Polynesia	*C. esculenta*	Jackson (1982)
California, USA	Chinese evergreen plants	Kositratana *et al.* (1983)
Costa Rica	Dasheen	Ramirez (1985)
South Africa	Dasheen	van der Meer (1985)
Queensland, Australia	*C. esculenta*	Greber and Shaw (1986)
China	Taro	Zettler *et al.* (1978)
Cuba	*Colocasia/Xanthosoma*	Quintero (1989)
Taiwan	Ornamental aroids	Liang *et al.* (1994)
Hawaii	Hawaiian taro	Hu *et al.* (1995)
New Zealand	*Zantedeschia* sp.	Matthews *et al.* (1996)

Note that the list is not exhaustive, but should indicate how widely DsMV is distributed.

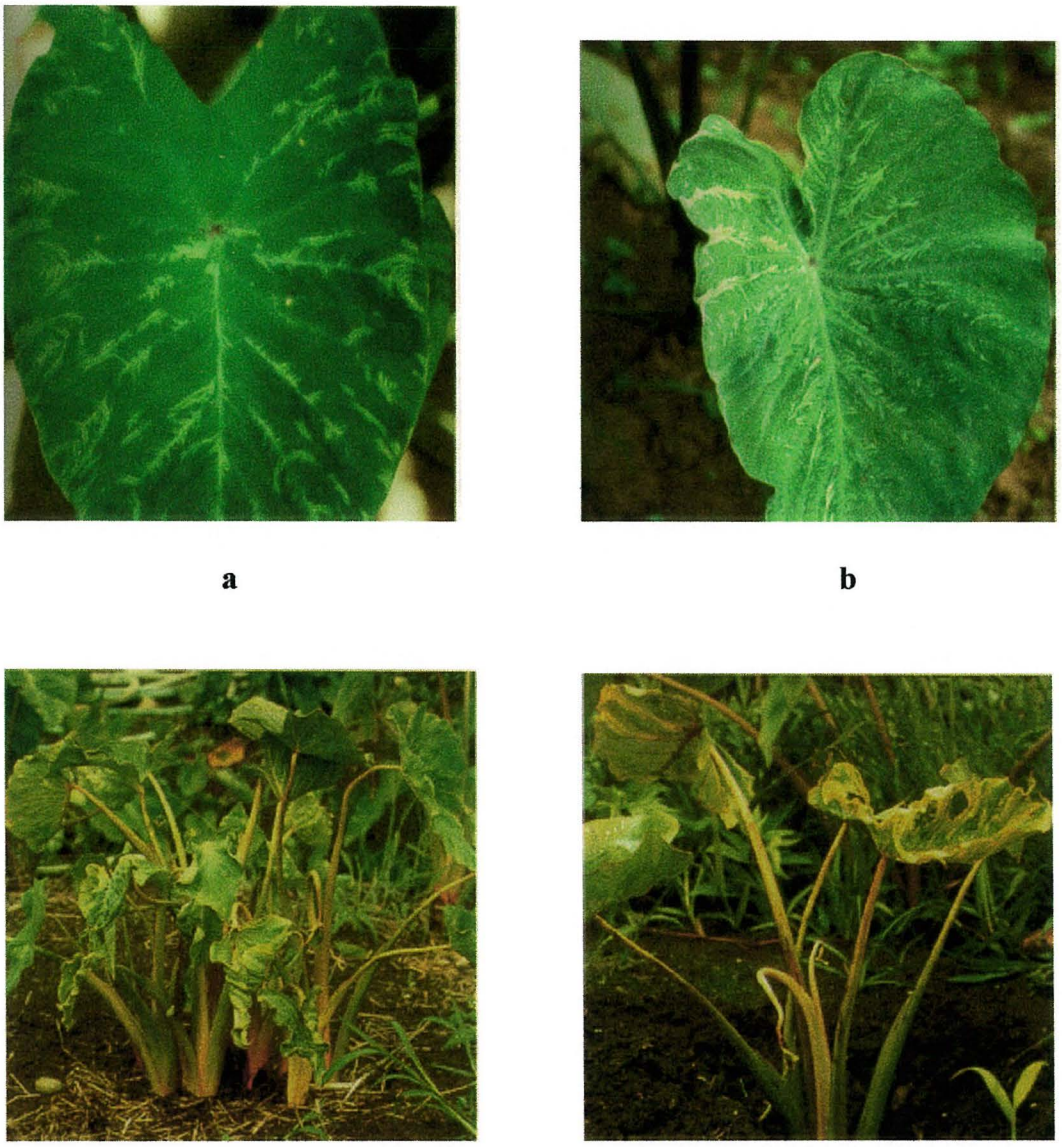

Figure 1.3. Taro plants showing symptoms of DsMV infection. Typical symptoms of DsMV (a & b), and symptoms caused by a severe strain of DsMV in French Polynesia (c & d).

Source: Kohler *et al.* (1997).

1.4.3 Morphology

DsMV has flexuous, non-enveloped particles, which are 750 nm in length and about 11 to 13 nm wide (Buddenhagen *et al.*, 1970; De Brot and Ordosgoitti, 1974; Zettler, *et al.*, 1978; Okuda *et al.*, 1979), although varying lengths of 750 to 800 nm (Gollifer and Brown, 1972) and 725 nm (Rodrigues *et al.*, 1984) have been reported.

1.4.4 Transmission

DsMV is transmitted in a non-persistent manner by aphids, *Myzus persicae* (Buddenhagen, *et al.*, 1970; Zettler *et al.*, 1970; Kenten and Woods, 1973; De Brot and Odosgoitti, 1974; van der Meer, 1985) and *Aphis craccivora* (Buddenhagen *et al.*, 1970; Zettler *et al.*, 1970). Jackson and Gollifer (1975) suggested that *A. gossypii* might also be a vector in the Solomon Islands.

Transmission of the virus through vegetative propagules used for replanting is common, (Zettler *et al.*, 1970; Gollifer *et al.*, 1977; Zettler and Hartman, 1987), but there are no reports of seed transmission.

1.4.5 Importance

There is little information on yield loss of taro caused by DsMV infection. In Costa Rica, DsMV occurs in at least 80% of all commercial plantations of taro (Ramirez, 1985) and significant yield reductions were observed in both *Xanthosoma* spp. and *Colocasia esculenta* (Ramirez and Gamez, 1984). Zettler and Hartman (1987) reported losses of up to 60% in edible aroids due to the pathogen. Preliminary studies conducted by Monge and Arias (1984) on the yield and quality of white and purple varieties of cocoyam showed that plants with DsMV symptoms yielded 25% and 51% less than healthy plants, respectively. In French Polynesia, severe symptoms caused by DsMV have been a major problem (Jackson, 1982).

Most yield studies have been conducted on ornamental plants rather than edible aroids. On all the parameters assessed in each host, significant losses were observed on those exposed to DsMV infection (Table 1.4).

Table 1.4. Yield loss on ornamental aroids due to DsMV infection

Host	Parameters assessed	Observations	Reference
Dieffenbachia picta 'Perfection'	Fresh shoot weights and leaf area	27 infected plants showed 63 and 66% reduction, respectively	Hartman and Zettler (1974)
D. maculata 'Perfection'	Cuttings	30 healthy plants: obtained an average of 18.8/plant.	Zettler *et al.* (1980)
		60 infected plants: obtained an average of 10.1 cuttings.	
		Of 564 cuttings, 98.4% were saleable. Of 606 infected cuttings, 47.3% were saleable.	
		In a similar study, healthy and infected plants produced 26.1 and 10.1 cuttings per plant, respectively.	Chase *et al.* (1981)
Philodendron selloum	Total fresh plant weights & leaf area	Of 36 diseased plants, 36% reduction was obtained.	Hartman and Zettler (1974)
	Fresh weight	diseased plants reduced by about 80%	Wisler *et al.* (1978)
P. oxycardium	Leaf number, leaf areas and vine length	infected plants had 38.6, 31.6 and 65.6% reduction, respectively.	Wisler *et al.* (1978)
Caladium hortulanum	Fresh corm weights and leaf areas	69 infected plants showed 40 and 53% reduction, respectively.	Hartman and Zetter (1974)

1.4.6 Control

There have been several approaches to control DsMV in taro. Cultural methods, such as roguing of infected plants and isolation of fields as much as possible from virus reservoirs, can delay infection and reinfection for several years (Zettler and Hartman, 1986). The establishment of taro gardens in new sites slows the build-up of vectors and may result in reduction of the disease incidence (Shaw *et al.*, 1979). However, due to a lack of economic incentives, pathogen-free planting stock is not being utilised (Zettler *et al.*, 1991). Additionally, the available land for growing taro is usually limited, hence the distance between new and existing gardens with viruliferous vectors is reduced.

Alconero (1972) tried hot water treatments to inactivate DsMV in corms of *Xanthosoma* spp. It was noted that the (i) virus was not inactivated in the corms at 65°C for 5 minutes, and (ii) germination (buds of corms) was nil with treatments for 10 minutes at that temperature. DsMV has been apparently eliminated from taro plants using tissue culture (Hartman, 1974; Zettler *et al.*, 1989) without additional heat treatment. DsMV-free plants can supposedly be obtained using this method when 0.5 mm or less of meristem-tip is used (Zettler *et al.*, 1989). The results must be treated with caution, however, since the explants were indexed for DsMV by insensitive and unreliable techniques including electron microscopy and mechanical inoculation to an indicator plant (Hartman, 1974).

Some breeding work has been undertaken to obtain taro varieties which are resistant to DsMV, but without success. Since many aroids do not breed true, seedlings exhibit a considerable amount of phenotypic variation, which is undesirable (Hartman and Zettler, 1972; Zettler and Abo El-Nil, 1979; Volin, 1980; Volin *et al.*, 1981).

1.4.7 Serological detection of DsMV

The first immunogenic studies on DsMV were by Abo El-Nil *et al.* (1977) who prepared polyclonal antiserum against purified preparations of DsMV from Florida (DMV-FL) and the Fiji Islands (DMV-FJ). The antisera were used in agar-gel double-

diffusion tests to detect DsMV in Egyptian taro (Mostafa *et al.*, 1976). Although the antisera reacted with the homologous DsMV isolates, cross-reactions were found to occur with *Tobacco etch potyvirus* and *Blackeye cowpea mosaic potyvirus*.

Rodoni and Moran (1988) were the first to use enzyme-linked immunosorbent assay (ELISA) to detect DsMV using an antiserum prepared against a Victorian isolate of DsMV, which had been maintained in *Dieffenbachia picta* and *Philodendron selloum* plants. The results from this assay were greatly influenced by the viscous nature of the araceous sap and the presence of compounds such as polyphenols. More reliable results were obtained following the addition of the sugar, mannose, and polyvinylpyrrolidone (PVP) to a Tris buffer of high ionic strength containing EDTA.

Hu *et al.* (1995) used an indirect ELISA system with anti-DsMV polyclonal antiserum and commercially available monoclonal antibody (Mab-PTY1, Agdia) to detect DsMV from different taro cultivars in Hawaii. DsMV was readily detected in leaves from plants that showed prominent symptoms. In contrast to Rodoni and Moran (1988), extracts from taro plants did not appear to interfere with the ELISA. However, an apparent uneven distribution of DsMV in taro was observed and reliable results were only obtained using pooled tissues from different leaves rather than individual leaves (Hu *et al.*, 1994, 1995).

Matthews *et al.* (1996) compared the reactions of antibodies produced against DsMV isolates from different sources and found differences in serological reactions between isolates. A serological difference between a Californian isolate from Chinese evergreen (*Aglaonema*) and two isolates from taro, Florida isolate and Fiji isolate, has been reported (Kositratana *et al.*, 1983). Sequence analysis of three DsMV isolates indicated that there was some heterogeneity at the N terminus of the CP regions but that the core was highly conserved (Li *et al.*, 1995; Pappu *et al.*, 1994a,b). Serological differences are thus attributable to the heterogenous CP region of DsMV (Li *et al.*, 1999).

Although there is a commercially available (AgDIA) DAS ELISA test system for DsMV, which uses the polyclonal antiserum for capture and detection, the cost for the test system precludes routine use in developing countries.

The preparation of antibodies against intact virions has numerous disadvantages most notably being the inability to purify sufficient quantities of "clean" virus for use as antigen. As such, the resulting antibody reacts non-specifically with host proteins. To overcome these difficulties, recombinant viral CP produced in bacteria or other hosts is being used as antigen. DsMV CP has been amplified with specific primers, cloned and expressed in *Escherichia coli* (*E. coli*) cells to yield large quantities of recombinant protein (Li *et al.*, 1998). The antigen was used to produce specific antiserum to DsMV in a rabbit. The selection of DsMV CP (*Caladium* isolate, DsMV-Ch) for expression and production of specific DsMV antisera was based on the high sequence similarity in the CP region between DsMV isolates infecting taro (Pappu *et al.*, 1993; Pappu *et al.*, 1994a,b). Li *et al.* (1998) demonstrated that the antigenic presentation by the recombinant protein lead to a good immunological response. The antiserum detected DsMV in infected taro plants at 1:10 000 dilution in both ELISA and western blots without discernible background. The antisera recognised both native viral CP in infected plants and the recombinant protein expressed in *E. coli*. However, the antiserum was not DsMV-specific and also reacted with four other potyviruses including *Zucchini yellow mosaic virus, Papaya ringspot virus type P, Papaya ringspot virus type W*, and *Watermelon mosaic virus 2*. Li *et al.* (1998) and Smith *et al.* (1995) outlined numerous advantages when using recombinant protein to produce antisera including (i) it generates large amounts of suitable antigen; (ii) it generates stable antigen since the CP of purified virions often degrades easily, (iii) the CP clones can be distributed universally since it is non-infectious and thus poses no quarantine problems.

1.4.8 DsMV CP sequence variability

The CP-coding region of four DsMV isolates has been reported. These includes two isolates from taro (DsMV-TEN and DsMV-LA) (Pappu *et al.*, 1994a,b), a *Caladium* isolate (DsMV-Ch) (Li *et al.*, 1998), and an aroid isolate from Taiwan (GenBank AF511485).

Pappu *et al.* (1994b) amplified and cloned as cDNA, the 3'-terminal 1430 nucleotides (nt) of the DsMV-TEN genome that included the CP-coding region. Sequence analysis revealed an open reading frame (ORF) encompassing 393 amino acids. Sequence similarities of 87% and 95% were found between the CPs of the TEN and LA isolates at the nucleotide and amino acid levels, respectively.

Li *et al.* (1998) cloned and sequenced the 3'-terminal 3158 nts of the *Caladium* isolate of DsMV (DsMV-Ch). The sequence included the 3' terminus of the NIa, NIb and CP-coding regions, and a 246 nt UTR (excluding the poly-A tail). The comparisons of DsMV-Ch CP with that of DsMV-LA and DsMV-TEN isolates revealed a similarity of 84-88% at the nucleotide level, and 92-94% at the amino acid level. An alignment of the amino acid sequences of the three isolates revealed N-terminal variability and a close similarity between the DsMV-Ch and DsMV-TEN isolates.

Although the CPs of potyviruses are, for the most part, highly conserved, they can differ in sequence and length at the N terminal region (Shukla *et al.*, 1994). Diversity in the CP sequences of DsMV isolates occurred predominantly at the N terminus (Pappu *et al.*, 1993, 1994a,b; Li *et al.*, 1998).

Chapter 2 Sequence variability in the DsMV CP-coding region

2.1 Introduction

DsMV is one of several plant viruses which infects taro and is considered of major importance because it causes disease in both ornamental and edible aroid crops and has a worldwide distribution (Zettler *et al.*, 1978; Shaw *et al.*, 1979; Zettler and Hartman, 1986; 1987). In some cases, yield losses of up to 60% in edible aroid crops have been reported (Zettler and Hartman, 1987). DsMV is by far the most extensively characterized virus which infects taro.

The presence of DsMV (and other viruses) currently restricts the movement of taro germplasm throughout Pacific Island countries. This has a major impact on taro production in many countries due to the inability to access agronomically elite germplasm produced in breeding programs. As such, there is a real need to develop virus diagnostic tests which can be used in indexing schemes. In addition to their importance at the international level, such tests would also have utility on a national level by enabling the indexing of tissue-cultured taro plantlets prior to their release to farmers. Ideally, the development of serological-based tests like ELISA would be advantageous since these could be easily adopted in Pacific Island laboratories which lack the infrastructure for molecular-based diagnostic tests. Although an ELISA for DsMV is commercially available, it is too expensive to be used for the large-scale testing of plants. Further, the range of DsMV isolates detected by the antiserum is unknown.

Recombinant viral coat proteins are commonly used as antigens for the production of antiserum because large quantities of highly pure protein can normally be generated. The choice of coat protein gene-coding sequence to express as a recombinant protein is especially important for potyviruses, such as DsMV, due to extensive variability that is known to occur within the CP-coding region of potyviral genome. Knowledge of the sequence variability in the CP-coding region of Pacific Island isolates of DsMV is required to allow a judicious choice of the most appropriate sequence to express as a recombinant protein, such that the resulting

antiserum will react with the widest possible range of virus isolates. The sequences of the CP-coding region of four DsMV isolates have been reported, including two isolates from taro (Pappu *et al.* 1994a;b), a *Caladium* isolate (Li *et al.*, 1998) and an aroid isolate from Taiwan (GenBank AF511485). These reports revealed a high degree of sequence variability in the CP-coding region that was mainly attributed to frequent deletions and insertions in the amino terminal region of the CP sequence (Pappu *et al.*, 1994b).

Analysis of sequence variability is also crucial to the development of long term control strategies for DsMV using pathogen derived resistance (PDR) strategies. The use of PDR in transgenic plants for virus resistance has shown great promise (Hull 1994; Beachy 1997). Coat protein-mediated resistance (CPMR) was first postulated by Sanford and Johnston (1985) and successfully demonstrated by Powell-Abel *et al.* (1986). Since then, numerous plant species have been transformed with viral CP genes (Hackland *et al.*, 1994; Luis *et al.*, 1997; Beachy 1997). These same methods can be employed to generate DsMV resistant taro. The success of CPMR, however, is greatly influenced by the sequence variability between the expressed transgene and that of the challenging virus (Tennant *et al.*, 1994). This highlights the need for extensive research into sequence variability within the DsMV CP, so that potential resistance constructs can be developed for PDR which will resist the selective pressures of virus challenge from a broad range of virus isolates.

In this study, we report an extensive investigation into the sequence variability within the CP-coding region of numerous isolates of DsMV from various countries in the South Pacific region. It was envisaged that this analysis would not only enable the selection of the most appropriate sequence to express as a recombinant antigen for antibody production but also provide important information that will subsequently facilitate the future development of DsMV-resistant transgenic crops.

2.2 Materials and methods

2.2.1 Source of plant material

Taro leaf and petiole samples displaying symptoms of DsMV infection were obtained from various South Pacific Island countries including Papua New Guinea (PNG), Samoa, Vanuatu, Solomon Islands and Fiji. The presence of DsMV was confirmed by examination of negatively stained (2% phosphotungstic acid pH 7.0) sap extracts under a transmission electron microscope (JEOL 1200 EX). Samples containing potyvirus-like virions were ground to a fine powder in liquid N_2 and stored in sterile 50 mL tubes at –80°C.

2.2.2 Extraction of total RNA

2.2.2.1 Method 1

Total RNA was extracted using the technique described by Chang *et al.* (1993). Three grams of tissue powder was added to 50 ml Falcon tubes containing 15 mL warm (65°C) extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA, 2 M NaCl, 0.5g/L spermidine, 2% *β*-mercaptoethanol, added just before use). RNA was extracted twice with an equal volume of chloroform: isoamylalcohol (IAA) (24:1) and centrifuged at 3,000 g for 10 min. A quarter volume of 10 M lithium chloride was mixed with the supernatant, transferred to Corex centrifuge tubes and precipitated overnight at 4°C. The mixture was centrifuged in a Beckman JA-20 centrifuge at 8,000 rpm for 20 min and the pellets dissolved in 500 mL SSTE buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). A single extraction was then made with an equal of volume of chloroform: IAA, followed by the addition of two volumes of 100% ethanol and RNA precipitated at –70°C for 30 min. RNA was recovered by centrifugation and pellets were finally resuspended in 50 µL diethyl pyrocarbonate (DEPC)-treated water.

2.2.2.2 Method 2

Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN). The protocol utilizes a lysis buffer containing guanidinium isothiocynate (GITC), which has powerful cell disruption and denaturation properties. All the procedures were performed at RT as per the manufacturer's instructions. RNA was eluted in a final volume of 50 µL DEPC-treated water into sterile 1.5 mL Eppendorf tubes.

2.2.2.3 Method 3

Total RNA was extracted using a protocol described by Beld *et al.* (1996). The procedure is based on the differential binding of ds- and ss-nucleic acid forms to silica particles in different lysis/binding buffers, all containing high concentrations of guanidinium thiocyanate (GuSCN). All steps were conducted at RT.

A 400 µL aliquot of lysis/binding buffer (12 g GuSCN, 10 mL 0.2 M EDTA pH 8, 100 µL β-mercaptoethanol) was added to 100 mg tissue powder in sterile 1.5 mL Eppendorf tubes. An equal volume of chloroform was added and mixed thoroughly by vortexing. The lysate was centrifuged at 10,000 g for 5 min. In new Eppendorf tubes, the supernatant was mixed with 500 µL lysis/binding buffer, 400 µL binding buffer (12 g GuSCN, 10 mL 0.35 M Tris-HCl pH 6.4, 2.2 mL 0.2 M EDTA pH 8, 0.91 g Triton X-100, 1.1 g $MgCl_2.6H_20$) and 40 µL silica particles. Tubes were incubated for 10 min at RT and then centrifuged at 8,000 g for 15 s. Pellets were washed twice with 1 mL each of washing buffer (12 g GuSCN, 10 mL 0.1 M Tris-HCl pH 6.4) and 70 % ethanol and once with acetone. Tubes were centrifuged at 8,000 rpm for 15 s between each wash. Pellets were dried (10 min at 56°C in heat block with open lid) and eluted (10 min at 56°C with closed lid) with 50 µL DEPC-treated water. After centrifugation (2 min at 8,000 g), the supernatant was transferred to sterile 1.5 mL Eppendorf tubes and immediately stored on ice.

2.2.3 Quantification of RNA concentration

RNA extracts were diluted to 1:100 in sterile water and the RNA concentration was determined by UV spectrophotometry at 260 nm according to Sambrook and Russell (2001).

2.2.4 Amplification of the DsMV CP-coding region

A 1.8 kbp fragment was amplified from each of the viral isolates either as a single fragment or as several overlapping fragments. This amplified fragment included part of the NIb-coding region, the entire CP-coding region and the 3' untranslated region of the viral genome. An oligo-dT primer, in combination with a degenerate potyvirus amplification primer (Poty-2) (Gibbs and Mackenzie, 1997) was used to amplify the entire 1.8 kbp fragment. When the entire fragment could not be amplified due to the quality of the RNA extracts, it was amplified in two separate fragments using an oligo-dT primer with a degenerate primer designed to the conserved WCIEN motif (located within the viral coat protein gene; Pappu *et al.*, 1993) and then the reverse complement of the WCIEN primer in combination with the degenerate Poty-2 primer. These primers amplified 0.7 kbp and 1.1 kbp fragments, respectively (Fig. 2.1; Table 2.1).

2.2.5 Reverse transcription and PCR

First-strand complementary DNA (cDNA) was synthesized using SuperScript II reverse transcriptase (Invitrogen). The crude extract containing 1-2 µg of total RNA was diluted to 10 µL in DEPC-treated water in a 0.5 mL sterile Eppendorf tube. A 2 µL aliquot of Oligo-dT primer (30 pmol/µL) was added and the mixture heated at 75°C for 5 min. The reaction was immediately quenched on ice for 3 min, followed by the addition of 4 µL 5x first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µL 0.1 M DTT, 1 µL 10 mM dNTP mix and 1 µL (200 U) SuperScript II reverse transcriptase . The reaction was incubated at 42°C for 60 min and the enzyme heated-inactivated at 70°C for 15 min.

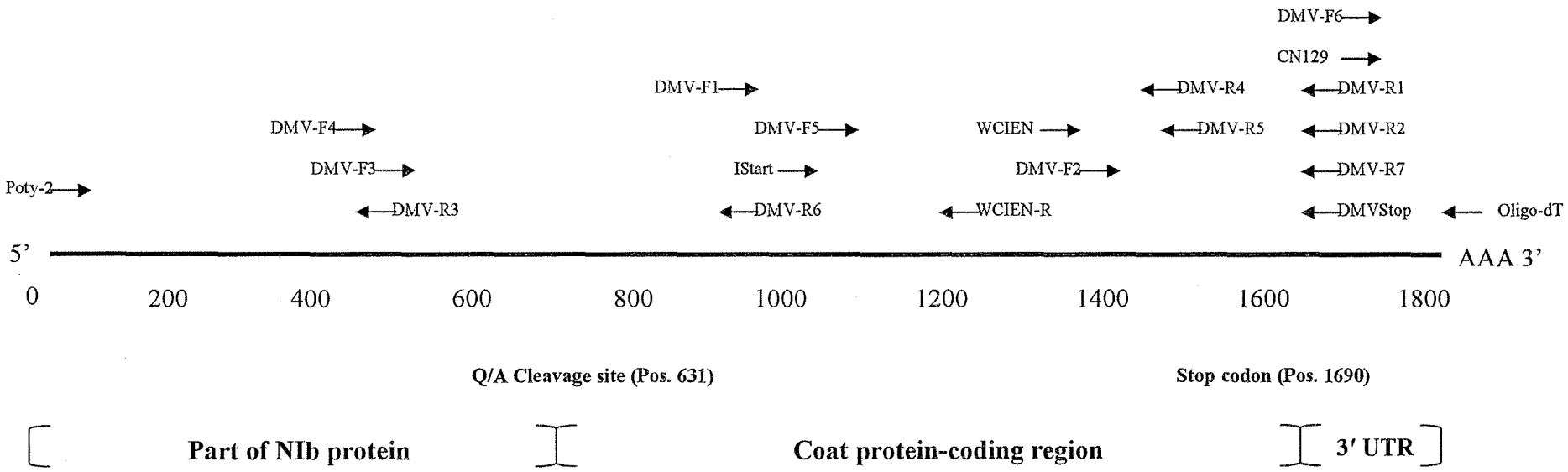

Figure 2.1: The location of primers used to amplify and sequence the DsMV CP-coding region. Initially, primers Poty-2 and Oligo-dT were used to amplify a 1.8 kbp fragment of the DsMV genome which includes part of the NIb protein, the entire CP-coding region and the 3' untranslated region of the virus. Internal primers were then used to sequence the cloned fragment. Primer sequences are listed in Table 2.1.

Table 2.1: List of primers used in the PCR amplification and sequencing.

Primer code	Sequences (5'...3')
Poty-2	GGB AAY AAY AGY GGD CAR CC
WCIEN	TGG TGY ATH GAN AAT GG
WCIEN-R	CCA TTN TCD ATR CAC CA
Oligo-dT	TTT TTT TTT TTT TTT TTT V
CN129	CAR ATG AAR GCN GCN GC
DMV-F1	CCT CCA CCG CCA CCA CCG NCG G
DMV-R1	GCA GTN TGC CTT TCA GTG TTC TCG C
DMV-F2	CCY GAY ATY AAC GGG GCT TGG G
DMV-R2	TGC CTT TCA GTG TTC TCG C
DMV-F3	CTT GAN AAN GAT GAA TTT
DMV-R3	TNTNNG TTT TCA TCT GTG
DMV-F4	GCA GCT ATG ATA GAA GCA TGG
DMV-R4	ACC NTA CCT AGG CAT
DMV-F5	AGA TGT YAA YGC TGG CAC
DMV-R5	GCT TCC CTA GCT CGA ACC GG
DMV-F6	TCW AAR ACA CCG GTB CGA GC
DMV-R6	ATG TKC CAC TDG TGC CAG
DMV-R7	ACC ARR YCY TYA CTG CGG
Istart	GGT GGT TGC TCT TCC AAC GGA AAG GGA AAA GAA ATT GTC
DMVStop	GGT GGT CTG CAG TCA CTG CGG AGA TGC CAC GCC
DMV-F7	CTW TCN AAC GTT ACC ACT AG
DMV-R8	GAW GTW GAA ACG TTA CCA TC
DMV-F8	GAR TGG GAY AGR AGY AAR GAG
DMV-R9	GTG TGA CCC CAT GCR TCT ATC
DMV-F9	TGG GGG TAA TAC AAC AAA CAC
DMV-R10	GTG ACA TCT TTC ACT ACT GG
DMV-F10	ACT ACC CCC GCA GTT ACA CC
DMV-R11	GGA GCA GAT GGA GTA ACC GTA GG
DMV-F11	CAG AAA CTC CGG TGG TGA AAG
DMV-R12	CTG GAG TTT CTG GGG GTT TTA GTG

PCR reactions (50 µL) were assembled using 3 µL of the cDNA synthesis reaction, 6 µL each of Poty-2 and Oligo-dT primers (10 pmol/µL), 1.5 µL 10 mM dNTP mix, 0.5 µL Expand DNA polymerase (Roche) (5 U/µL), 5 µL 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 50 mM $MgCl_2$), and sterile water added to bring the total volume to 50 µL. The reaction was initially heated at 92°C for 2 min followed by 40 cycles of 92°C for 30 s, 50°C for 30 s, 68°C for 3 min and finally 1 cycle of 68°C for 10 min.

2.2.6 Agarose gel electrophoresis

DNA was analysed by electrophoresis in 1% agarose gels in TBE buffer (178 mM Tris, 178 mM boric acid, 4 mM EDTA, pH 8.0) and contained 1 µL ethidium bromide (0.5 µg/mL). PCR products were mixed with a 6x agarose gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol. 0.75 mM EDTA, pH 8.0) and loaded on the gel. Appropriate DNA molecular markers were also included on the gel for size determination. DNA was subsequently electrophoresed at 100 volts and visualized under a UV transilluminator and photographed (Syngene).

2.2.7 DNA isolation from agarose gels

The appropriate sized DNA bands were excised from agarose gels under UV illumination and placed in pre-weighed sterile 1.5 mL Eppendorf tubes. The tubes were re-weighed to determine the gel volume. DNA was extracted and purified using a QIAquick Gel Extraction Kit (QIAgen) as recommended by the manufacturer.

2.2.8 Cloning

2.2.8.1 A-tailing

The enzyme mix provided with the Expand PCR kit contains a mixture of *Taq* and *Pwo* DNA polymerase. While *Taq* adds single adenosine molecules to the ends of the amplified fragments (Newton and Graham, 1994), *Pwo* has exonuclease proof-reading activity and removes the adenosine molecules. Hence to facilitate cloning, amplified fragments were A-tailed to ensure successful cloning of the fragment into a T-tailed

plasmid vector. The A-tail reaction mix (20 µL) contained 10 µL purified PCR products, 2 µL 10x PCR buffer, 1 µL 10 mM dATP, 0.5 µL Taq DNA polymerase (5 U/µL) and sterile water. The reaction mix was incubated at 72°C for 30 min.

2.2.8.2 Ligation

The A-tailed fragments were ligated to pGEMT-Easy vector (Promega) as recommended by the manufacturer. The ligation reaction (10 µL) contained 3 µL of amplified A-tailed PCR product, 1 µL pGEMT-Easy vector (50 ng), 1 µL 10x T4 DNA ligase buffer and 1 µL T4 DNA ligase (3 Weiss units/µL). In some reactions, positive and background controls were included to measure the efficacy of ligation. The reaction mixtures were incubated overnight at 4°C and the ligase was heat-inactivated at 65°C for 15 min.

2.2.8.3 Preparation of competent *E.coli* cells

The *E.coli* (JM109) cells were made electro-competent using the protocol described by Dower *et al.* (1988). A 5 mL LB broth was inoculated with a glycerol stock of JM109 cells and incubated overnight at 37°C on a shaker (225 rpm). A sterile 250 mL LB broth was subsequently inoculated with 2 mL of the overnight culture and incubated at 37°C on a shaker (225 rpm) until an OD_{600} between 0.6 and 0.8 was reached. The culture was chilled for 30 min and centrifuged at 5,000 rpm for 15 min in sterile JA-10 tubes sitting in a prechilled JA-10 rotor. Pellets were resuspended twice in sterile 1 mM HEPES; initially in 500 mL then in 250 mL and consecutively centrifuged as above. Pellets were then resuspended in 10% glycerol (sterile and chilled) in sterile Falcon tubes and centrifuged in chilled JA-17 at 5,000 rpm for 15 min. Pellets were finally resuspended in 1 mL 10% glycerol (sterile and chilled) and 40 µL aliquots of the cells were placed in sterile Eppendorf tubes, which were then quickly frozen in a dry ice/ethanol bath prior to long-term storage at –80°C.

2.2.8.4 Transformation

2.2.8.4.1 Method 1

Recombinant plasmid DNA was transformed into electro-competent JM109 cells using the protocol described by Dower *et al.* (1988). Prior to transformation, 6 µL DNA was precipitated with 0.1 volumes 3 M sodium acetate (pH 5.2), 2.5 volumes 100% ethanol and incubated at –80°C for 1 h. The suspension was centrifuged at 14,000 rpm at 4°C for 30 min. Pellet was washed with 250 µL 70% ethanol and further centrifuged at 14,000 rpm at 4°C for 15 min. The resultant pellet was finally resuspended in 6 µL sterile water of which 3 µL was used in transformation. Alternatively, 1 µL or less of the recombinant DNA was used directly from the ligation reaction without precipitation. Recombinant DNA was mixed with thawed cells and transferred to chilled cuvettes and electroporated using the standard conditions. Cells were resuscitated in 950 µL of SOC and incubated in a 37°C shaking incubator at 225 rpm for 1 h. The culture was concentrated by centrifugation and 100 µL was plated out on the selection media, supplemented with ampicillin (100 µg/mL), IPTG (0.5 mM) and X-Gal (80 µg/mL) and incubated overnight at 37°C.

2.2.8.4.2 Method 2

Recombinant DNA was transformed into *E. coli* JM109 competent cells by heat shock (Promega) as recommended by the manufacturer. A 2 µL ligation reaction was gently mixed with thawed cells in a sterile 1.5mL Eppendorf tube and incubated on ice for 20 min. Cells were heat-shocked at 42°C for 50 s in a water bath and immediately quenched on ice for 2 min. Cells were resuscitated in 950 µL SOC and incubated in a 37°C shaking incubator at 225 rpm for 1 h. The culture was concentrated by centrifugation and a 100 µL aliquot was plated out on selective media containing ampicillin (100 µg/mL), IPTG (0.5 mM) and X-Gal (80 µg/mL), and incubated overnight at 37°C.

2.2.8.5 Screening for transformants

Successful cloning in the pGEMT-Easy vector interrupts the coding sequence of β-galactosidase, which results in recombinant clones identified by a blue/white colour screening on indicator plates. In most cases, clones containing the inserts produce white colonies. Positive clones were picked with sterile tooth pick, placed in a vial containing 4 mL LB broth and incubated overnight in a 37°C shaking incubator.

2.2.8.6 Isolation of recombinant plasmid DNA

Recombinant DNA was isolated from bacterial cells using the standard plasmid miniprep procedure as described by the manufacturer (Promega). The resultant pellets were dried at RT for 30 min and finally resuspended in 50 µL sterile water containing RNase A (10 µg/mL) and incubated at 37°C for 30 min.

2.2.8.7 Confirmation of inserts

Plasmids were digested with appropriate restriction enzyme(s) to confirm the presence of the target insert. A digestion reaction (10 µL) contained 3 µL recombinant plasmid DNA, 1 µL *Eco*RI restriction enzyme (10 U/µL), 1 µL 10x buffer H (Roche) and sterile water. The reaction was incubated at 37°C for 1 h. DNA was subsequently electrophoresed through a 1 % agarose gel in TBE and visualized on a UV transilluminator (Syngene).

2.2.9 Sequencing

2.2.9.1 Preparation of template

Sequencing reaction mixtures (20 µL) contained 8 µL Big Dye terminator mix, 1 µL sequencing primer (3.2 pmol/µL) (Fig. 2.1; Table 2.1), 500 ng to 4 µg plasmid DNA and sterile water. DNA was amplified for 25 cycles at 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Following amplification, the DNA was precipitated by the addition of a 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol and incubated at RT for 15 min. The mixture was centrifuged (14,000 rpm) at RT for 20

min. The pellets were washed 70% ethanol and the DNA was dried at RT for 30 min. DNA samples were then submitted to the Australian Genomic Research Foundation (AGRF) at the University of Queensland for sequencing.

2.2.9.2 Sequence analysis

Contig analysis was carried out using SeqMan II software (DNAstar, Lasergene) and using the ABI chromatogram profiles. Sequences were submitted for BLAST analysis (Altschul *et al.*, 1997) through the NCBI database to confirm the identity of the clones. The EditSeq software (DNAstar, Lasergene) was then used to identify the coding region of the sequenced fragments and translate the region into protein sequence. Phylogenetic analysis of the compiled sequences was carried out using a compilation of computer software. Nucleic acid or protein sequences were aligned using the Clustal package described by Higgins *et al.* (1991). Phylogenetic analysis was then carried out using the Neighbor-joining tree analysis. The results were bootstrapped against 1000 replicates of the data to ensure the integrity of the results. Final graphical representations of the phylogenetic trees were visualised by the TreeView program (Page 1996). Sequence similarity tables were generated by putting the multiple sequence alignments into MegAlign program (DNAstar, Lasergene).

2.3 Results

2.3.1 RNA extraction

Three RNA extraction protocols were used to determine the most efficient and consistent method for purifying sufficient amounts of RNA of acceptable quality for amplification and cloning of the DsMV CP-coding region. Initially, a CTAB-based extraction protocol (Chang *et al.*, 1993) was trialled using three grams of starting tissue and this yielded approximately 27 µg of total RNA. An alternative procedure to purify total RNA was also examined using the RNeasy Plant Mini Kit (QIAGEN), which recommends the use of small amounts of plant material (~100 mg). Both procedures produced relatively low yields of total RNA. Further, no products were amplified from these extracts in many instances, presumably due to the presence of PCR inhibitors. Therefore, a third extraction protocol based on a modification of the

method outlined by Beld *et al.* (1996) was trialled. This procedure yielded approximately 11 μg of total RNA from 100 mg of infected tissue. Importantly, when extracts obtained using this method were used as templates for RT-PCR, products of the expected size were consistently amplified. Therefore, the third protocol was used for all subsequent RNA extractions since sufficient RNA yields from small quantities of starting material were obtained and the procedure was simple and rapid.

2.3.2 Sequence alignments

A region of the DsMV genome spanning part of the NIb-coding region, the entire CP-coding region and the 3' untranslated region was amplified from several field isolates of DsMV from various South Pacific Island countries and one isolate from Vietnam. The products were cloned and three clones from each isolate were sequenced in both directions using DsMV-specific primers. Prior to analysis, the sequences were trimmed to remove the NIb-coding sequences and the 3' UTR. The CP-coding sequence was identified by the presence of the conserved cleavage sequence VXXQ//(A/G/S/E), while the CP/3'UTR junction was identified by the stop codon for the polyprotein. The CP-coding sequences were then aligned by Clustal program, together with seven additional DsMV sequences obtained from the GenBank database. A multiple sequence alignment was generated from the amino acid sequence of the entire CP-coding region (Fig. 2.2) and a second alignment was done using only the core region of the coat protein (data not shown). The core region was identified by removing the variable portion at the N terminus of the CP upstream of the conserved KGK tripeptide.

2.3.3 Sequence analysis of the CP-coding region of DsMV

The length of the DsMV CP-coding region for the isolates sequenced in this work ranged from 939 nucleotides (PNG isolate-P16.2) to 1038 nucleotides (Samoan isolates-Sa1 and Sa2) and encoded putative proteins ranging from 313 amino acids (34 kDa) to 346 (37 kDa) amino acids, respectively. The putative amino acid sequences were analysed for conserved motifs that are common in potyviral CPs; conserved motifs were identified within the sequences although some small variations were noted. A Q//A(Gln-Ala) cleavage site was present between the NIb and CP-

Figure 2.2: Amino acid sequence alignments of the DsMV CP-coding region. Asterisks indicate identical amino acids in all isolates. Gaps inserted to optimize the alignment are indicated by dashes. Conserved potyviral motifs are indicated in bold letters.

Abbreviations and accession numbers of DsMV isolates are: Pa-P31 (Papua New Guinea isolates), Sa1 and Sa2 (Samoan isolates), SI (Solomon Island isolate), V1 (Vietnam isolate), FPa-FPc (French Polynesian isolate), NCa and NCb (New Caledonia isolates) and GenBank isolates LA (U00122), TEN (U08124), Ch (AF048981), DK3 (AJ298035), ND (AJ298034), TW (AF169832) and TW3 (AJ298036).

```
DsMV-Pa         ADDTIDAGNNDNKTKTPATGGGSNTTTPTPTAPKAPETPALKTTTPTPPAPKAPETPVVK 60
DsMV-Pb         ADDTIDAGNNDNKTKTPATGGGSNTTTPTPTAPKAPETPALKTTTPTPPALKAPETPAVK 60
DsMV-Pc         ADDTIDAGNNDNKTKTPATGGGSNTTTPTPTAPKAPETPALKTTTPTPPAPKAPETPVVK 60
DsMV-P1         ADDTVDAGKTANQTKTPATGGGNNNNTTTGGGNNNTTTGGGNNNTTTGGGNTTPNTGGGN 60
DsMV-P16        ADDTVDAGKTANQTKTPATGGGNNNNTTTDGGNNNTTTGGGNNNTTTGGGNTTPNTDGGN 60
DsMV-P16.2      ADETVDAGK----------GG----TTTPA-APKAPETPAVTPPAPKAPAVTTPTV---- 42
DsMV-P31        ADDTVDAGKTANQTKTPATGGGNNNNTTTGGGNNNTTTGGGNNNTTTGGGNTTPNTGGGN 60
DsMV-Sa1        ADDTVDAGQGENTTKTPATGGGNTNNTTTGGGNNTTMGGG--NNTTTGGGNNTTTGGGNN 58
DsMV-Sa2        ADDTVNAGQGGNTTKTPATGGGNTNNTTTGGGNNTTTGGG--NNTTTGGGNNTTTGGGNN 58
DsMV-SI         ADDTVDAGKTANQTRTPATGGGNDNNTTTGGGNNNTTTGGGNNNTTTGGGNTTPNTGGGN 60
DsMV-V1         ADETIDAGGGNNTNKTTET----------------KTPATSGGNNTTNNTT-PAAGGGN 42
DsMV-FPa        AGDTIDAGQGGNTTKTTATGGGNTNNTTTGGGNNTTTGGG--NNTTTGGGNTTPTTGGGN 58
DsMV-FPb        AGDTIDAGQGGNTTKTTATGGGNTNNTTTGGGNNTTTGGG--NNTTTGGGNTTPTTGGGN 58
DsMV-FPc        AGDTIDAGQGGNTTKTTATGGGNTNNTTTGGGNNTTTGGG--NNTTTGGGNTTPTTGGGN 58
DsMV-NCa        ADDTVDAGQ----------GGGNKSTTTPAVTPPAPKAPAVTTPTVTPPAPKAPAV---- 47
DsMV-NCb        ADDTVDAGQ----------GGGNKSTTTPAVTPPAPKAPAVTTPTVTPPAPKAPAV---- 47
DsMV-LA         ADDTVDAGNQNNTNKTTPA----------------AGGGNNTNTNTNTGNNTNTNTSTG 43
DsMV-TEN        ADDTVDAGNQNNTNKTTET----------------KTPAASGGNNTNNTPPPPA----- 39
DsMV-Ch         ADDTVDARKNNNTTKTTET----------------KTPATGGGNNTNNNTPP------- 37
DsMV-DK3        ADDTVDAGNQNNTNKTTPA----------------AGGGNNTNTNINT----------- 33
DsMV-ND         ADDTVDAGNNDNKTKTTET----------------KTPAAGGGNNTNNTPPPPA----- 39
DsMV-TW         ADDTVDAGNQNNTNKTTET----------------KTPATGGGNTTENKSTTPAAGGGN 43
DsMV-TW3        ADDTVDAGKGNNNTKTTET----------------KTPAAGNGNNTNNTPPP-A----- 38
                *  *  *

DsMV-Pa         TT----------------------PPAPPAPKAPETPVVKDVTPADTGKGKEIVRDVNAGT 99
DsMV-Pb         TT----------------------PPAPPAPKAPETPVVKDVTPADTGKGKEIVRDVNAGT 99
DsMV-Pc         TT----------------------PPAPPAPKAPETPVVKDVTPADTGKGKEIVRDVNAGT 99
DsMV-P1         NNT---NTTPTTGGGNNNNNTTP--NPPAPKTTETPVVKDVTPTDAGKGKEIVKDVNAGT 115
DsMV-P16        NNT---NTTPTTGGGNNNNNTTP--NPPAPKTTETPVVKDVTPTDAGKGKEIVKDVNAGT 115
DsMV-P16.2      -------TPPAPKAPETPAVTTPSVT--------TPVV---PPTETGKGKEIVKDVNAGT 83
DsMV-P31        NNT---NTTPTTGGGNNNNNTTP--NPPAPKTTETPVVKDVTPTDAGKGKEIVKDVNAGT 115
DsMV-Sa1        TTTGGGNNTTTGGGDTANNTTPP--APPAPKTTETPVVKDVAPTDTGKGKEIVKDVNAGT 116
DsMV-Sa2        TATGGGNTTPKTGGGNTTNTTPP--APPAPKTTETPVVKDVTPTDSGKGKEIVKDVNAGT 116
DsMV-SI         NNT---NTTPTSGGGNNNNNTTP--NPPAPKTTETPIVKDVTPTDTGKGKEIVKDVNAGT 115
DsMV-V1         NTN----NNTPPANNTTNNNPPPPPPAAPKATETSANKQVVPSTSDKGKEVVKDVNAGT 98
DsMV-FPa        TT----NTTPTTGGGNTTNNTPPPPAPPAPKTTQTPVVKDVTPTDTGKGKEIVKDVNAGT 114
DsMV-FPb        TT----NTTPTTGGGNTTNNTPPPPAPPAPKTTQTPVVKDVTPTDTGKGKEIVKDVNAGT 114
DsMV-FPc        TT----NTTPTTGGGNTTNNTPPPPAPPAPKTTQTPVVKDVTPTDTGKGKEIVKDVNAGT 114
DsMV-NCa        -------TPPAPKAPETPVVTPPTVTPSAPKATETPVVRDVSPTDTGKGKEIVKDVNAGT 99
DsMV-NCb        -------TPPAPKAPETPVVTPPTVTPSAPKATETPVVRDVSPTDTGKGKEIVKDVNAGT 99
DsMV-LA         NNT----NTNTNTNTNTTNNNPPPPPPAAPKASETPANKQVVPTTSDKGKEIVKDVNAGT 99
DsMV-TEN        --------------NNTTNNNPPPPPPTAPKATETPANTQVVPTASGKGKEVVKDVNAGT 84
DsMV-Ch         -------------VDNTTNNNPPPPPPAVTKVTEVPANKQVVPAASEKGKEVVKDVNAGT 83
DsMV-DK3        -------NTNTNTNTNTTDNTNKTPTPSAPKTSETPAVKEVTPTDTGKGKEIVKDVNAGT 85
DsMV-ND         --------------NNTTNTNPPPPPPAVPKATETPVSTQVVPAASEKGKEVVKDVNAGT 84
DsMV-TW         NTS----NTTNNTTNNTTNNILPPPPPAAPKATETSANTQVVPTASGKGKEVVKDVNAGT 99
DsMV-TW3        --------------NNTTNNTPPPPPPAAPTVTETPANKQVVPTTSDKGKEIVKDVNAGT 83
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DsMV-Pa         SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 159
DsMV-Pb         SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 159
DsMV-Pc         SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 159
DsMV-P1         SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 175
DsMV-P16        SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 175
DsMV-P16.2      SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 143
DsMV-P31        SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 175
DsMV-Sa1        SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 176
DsMV-Sa2        SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 176
DsMV-SI         SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 175
DsMV-V1         SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDISNTRATHTQFEVWYNAVK 158
DsMV-FPa        SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 174
DsMV-FPb        SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 174
DsMV-FPc        SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 174
DsMV-NCa        SGTYSVPRLNKITNKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFETWYNAVK 159
DsMV-NCb        SGTYSVPRLNKITNKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFETWYNAVK 159
DsMV-LA         SGTYSVPRLNKITNKMNLPLVKGKCILNLNHLIEHKPEQRDIFNTRATHTQFEVWYNAVK 159
DsMV-TEN        SGTYSVPRLNKITNKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 144
DsMV-Ch         SGTYSVPRLNRITNKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 143
DsMV-DK3        SGTYSVPRLNRITHKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 145
DsMV-ND         SGTYSVPRLNKITNKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 144
DsMV-TW         SGTYSVPRLNKITHKMNLPLVKGKCILNLNHLIEYKPEQRNIFNTRATHTQFEVWYNAVK 159
DsMV-TW3        SGTYSVPRLNKITNKMNLPLVKGKCILNLNHLIEYKPEQRDIFNTRATHTQFEVWYNAVK 143
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DsMV-Pa         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGSDQIEYPLKPIVENAKPTLRQ 219
DsMV-Pb         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGSDQIEYPLKPIVENAKPTLRQ 219
DsMV-Pc         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGSDQIEYPLKPIVENAKPTLRQ 219
DsMV-P1         REYELEDEQMRIVMNGFMVWCIENGTSPDINGSWVMMDGNDQIEYPLKPIVENAKPTLRQ 235
DsMV-P16        REYELEDEQMRIVMNGFMVWCIDNGTSPDINGSWVMMDGNDQIEYPLKPIVENAKPTLRQ 235
DsMV-P16.2      REYELEDEQMRIVMNGFMVWCIDNGTSPDINGSWVMMDGNDQIEYPLKPIVENAKPTLRQ 203
DsMV-P31        REYELEDEQMRIVMNGFMVWCIDNGTSPGINGSWVMMDGNDQIEYPLKPIVENAKPTLRQ 235
DsMV-Sa1        KEYELEDEQMRIVMNGFMVWCIDNGTSPDINGAWVMMDGGDQIEYPLKPIVENAKPTLRQ 236
DsMV-Sa2        KEYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 236
DsMV-SI         REYELEDEQMRIVMNGFMVWCIDNGTSPDINGSWVMMDGNDQIEYPLKPIVENAKPTLRQ 235
DsMV-V1         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 218
DsMV-FPa        KEYELDDEQMRIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 234
DsMV-FPb        KEYELDDEQMRIVMNGLIDWCIDNGTSPDINGAWVMMDGDDQIEYPLKPIVENAKPTLRQ 234
DsMV-FPc        KEYELDDEQMRIVMNGFTIWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 234
DsMV-NCa        REYDLNDDQMRIVMNGFMVWCIENGTSPDINGDWVMMDGNDQIEYPLKPIVENAKPTLRQ 219
DsMV-NCb        REYDLNDDQMRIVMNGFMVWCIENGTSPDINGDWVMMDGNDQIEYPLKPIVENAKPTLRQ 219
DsMV-LA         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 219
DsMV-TEN        REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 204
DsMV-Ch         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 203
DsMV-DK3        REYELEDDQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 205
DsMV-ND         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 204
DsMV-TW         REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 219
DsMV-TW3        REYELEDEQMHIVMNGFMVWCIDNGTSPDINGAWVMMDGNDQIEYPLKPIVENAKPTLRQ 203
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DsMV-Pa         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 279
DsMV-Pb         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 279
DsMV-Pc         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 279
DsMV-P1         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDVSLARYAFDFYEVNSKTPVRAREAVA 295
DsMV-P16        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDVSLARYAFDFYEVNSKTPVRAREAVA 295
DsMV-P16.2      IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDVSLARYAFDFYEVNSKTPVRAREAVA 263
DsMV-P31        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDVSLARYAFDFYEVNSKTPVRAREAVA 295
DsMV-Sa1        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 296
DsMV-Sa2        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 296
DsMV-SI         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDVSLARYAFDFYEVNSKTPVRAREAVA 295
DsMV-V1         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 278
DsMV-FPa        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREVVA 294
DsMV-FPb        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYALDFYEVNSKTPVRAREAVA 294
DsMV-FPc        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREVVA 294
DsMV-NCa        IMHHFSDAAEAYIEMRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 279
DsMV-NCb        IMHHFSDAAEAYIEMRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 279
DsMV-LA         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 279
DsMV-TEN        IMHHFSDAAEAYIEPRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 264
DsMV-Ch         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 263
DsMV-DK3        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 265
DsMV-ND         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 264
DsMV-TW         IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 279
DsMV-TW3        IMHHFSDAAEAYIELRNAEKPYMPRYGLIRNLRDASLARYAFDFYEVNSKTPVRAREAVA 263
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DsMV-Pa         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSPPQ 329
DsMV-Pb         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSPPQ 329
DsMV-Pc         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSPPQ 329
DsMV-P1         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPTMHTLLGVASPQ 345
DsMV-P16        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPTMHTLLGVASPQ 345
DsMV-P16.2      QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPTMHTLLGVASPQ 313
DsMV-P31        QMKAAALSNVTTRLFGLDGNVSTSSENTVRHTAKDVTPTMHTLLGVASPQ 345
DsMV-Sa1        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSPPQ 346
DsMV-Sa2        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSPPQ 346
DsMV-SI         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPTMHTLLGVAPPQ 345
DsMV-V1         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSPPQ 328
DsMV-FPa        QMKAAALSNVTTRWSGLDGNVSTSSENTERHTAKDVTPNMHTLLGVASPQ 344
DsMV-FPb        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSPPQ 344
DsMV-FPc        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVASPQ 344
DsMV-NCa        QMKAAALSNVTTRLFGLDGNVSTSSESTERHTAKDVTPNMHTLLGVSPPQ 329
DsMV-NCb        QMKAAALSNVTTRLFGLDGNVSTSSESTERHTAKDVTPNMHTLLGVSPPQ 329
DsMV-LA         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVASPQ 329
DsMV-TEN        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVAPPQ 341
DsMV-Ch         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVSSPQ 313
DsMV-DK3        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVASPQ 315
DsMV-ND         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVAPPQ 314
DsMV-TW         QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVAPPQ 329
DsMV-TW3        QMKAAALSNVTTRLFGLDGNVSTSSENTERHTAKDVTPNMHTLLGVAPPQ 313
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coding region in all the isolates sequenced, while a DAG (Asp-Ala-Gly) motif occurred near the N-terminus of all isolates except for one Samoan isolate (Sa2) which contained a NAG motif. A putative WCIEN box was also present in some isolates analysed, but most sequences had the conserved glutamic acid residue (E) conservatively replaced by an aspartic acid residue (D) (Fig. 2.2). Two other putative conserved motifs, RQ (Arg-Gln) and AFDF (Ala-Phe-Asp-Phe), were also observed in all sequences analysed except for one French Polynesian isolate which contained the motif ALDF.

Protein sequence homology tables were generated for both the entire CP-coding region (Table 2.2) and the conserved core of the CP (Table 2.3) using the MegAlign program. When the amino acid sequences of the entire CP-coding region of the isolates from this study were compared to each other and to the published sequences, the similarity levels ranged from 79.1% (P31 and NCa: P31 and NCb) to 100% (NCa and NCb; Pa and Pc). The vast majority of this variability was in the N terminus since analysis of the core region following removal of the N terminal 104 amino acids up to the KGK tripeptide revealed similarities ranging from 94% to 100%. The sequence variability within PNG DsMV isolates was also examined since seven isolates were collected from this country for comparison. At the amino acid level, similarity levels ranged from 83.1% to 100% over the entire CP-coding region and from 96.1% to 100% for the core region. Overall, the CP-coding sequence with most similarity to all others was isolate P1 from PNG.

2.3.4 Phylogenetic analysis

The alignments obtained above were used as inputs to generate phylogenetic trees based on the amino acid sequence of the entire CP-coding region (Fig. 2.3) and also the core region (data not shown). In this analysis, the bootstrap option of PAUP was used to assess the robustness of the data (1000 replications) and a *Potato virus Y* (PVY) isolate was included as an outgroup sequence for comparison. The branching pattern suggested that, in many cases, isolates grouped according to their country of origin.

Table 2.2: Amino acid sequence variability in the entire CP-coding region of numerous DsMV isolates. Figures above and below the diagonal line of squares indicate homology and divergence amongst the isolates, respectively. Abbreviations and accession numbers for the isolates are identical to those outlined in Figure 2.2.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1-DsMV-Pa		99.4	100.0	83.6	83.9	89.2	83.1	85.3	85.0	83.6	85.6	83.8	84.0	83.8	87.5	87.5	83.9	87.5	86.2	89.0	89.3	85.3	87.6
2-DsMV-Pb			99.4	83.7	83.9	89.2	83.2	85.4	85.1	83.7	85.6	83.9	84.1	83.9	87.1	87.1	84.0	87.2	86.2	89.0	88.9	85.3	87.2
3-DsMV-Pc				83.6	83.9	89.2	83.1	85.3	85.0	83.6	85.6	83.8	84.0	83.8	87.5	87.5	83.9	87.5	86.2	89.0	89.3	85.3	87.6
4-DsMV-P1					99.3	86.0	99.3	90.1	91.4	98.2	80.9	92.1	90.9	92.1	80.3	80.3	83.7	84.4	83.2	87.2	84.0	83.1	86.0
5-DsMV-P16						86.3	99.0	89.8	91.1	97.9	80.9	91.8	90.6	91.8	80.0	80.0	84.1	84.7	83.5	87.5	84.4	83.1	86.4
6-DsMV-P16.2							85.5	82.3	82.9	86.0	82.7	82.6	81.0	82.6	89.5	89.5	82.1	85.6	84.1	85.7	85.6	83.5	86.5
7-DsMV-P31								89.8	91.1	97.9	80.4	91.7	90.5	91.7	79.1	79.1	83.3	83.9	82.7	86.7	83.6	82.7	85.6
8-DsMV-Sa1									95.6	90.5	83.4	92.6	92.9	92.6	81.4	81.4	84.9	84.9	84.2	87.7	85.0	83.4	86.7
9-DsMV-Sa2										91.1	82.7	94.5	94.4	94.5	81.9	81.9	85.8	86.7	85.7	88.4	86.8	84.1	88.5
10-DsMV-SI											81.2	91.5	90.9	91.5	80.3	80.3	83.3	84.7	82.7	87.1	84.1	83.4	86.3
11-DsMV-V1												82.5	82.4	82.5	80.9	80.9	85.9	93.7	92.3	84.1	91.8	92.5	93.0
12-DsMV-FPa													97.3	98.9	79.9	79.9	84.5	85.2	83.8	87.9	84.5	83.9	86.6
13-DsMV-FPb														97.8	79.8	79.8	83.1	84.4	83.0	86.4	83.7	83.1	85.9
14-DsMV-FPc															79.9	79.9	84.5	85.2	83.8	87.9	84.5	83.9	86.6
15-DsMV-NCa																100.0	80.1	85.5	81.4	84.4	84.8	81.0	85.2
16-DsMV-NCb																	80.1	85.5	81.4	84.4	84.8	81.0	85.2
17-DsMV-LA																		91.2	89.1	94.4	88.8	87.3	91.2
18-DsMV-TEN																			93.6	88.7	96.2	95.5	95.5
19-DsMV-Ch																				86.0	94.6	91.7	93.9
20-DsMV-DK3																					85.8	87.4	88.0
21-DsMV-ND																						93.3	95.0
22-DsMV-TW																							93.0
23-DsMV-TW3																							

Table 2.3: Amino acid sequence variability in the core region of the CP-coding region from numerous DsMV isolates. Figures above and below the diagonal line of squares indicate homology and divergence amongst the isolates, respectively. Abbreviations and accession numbers for the isolates are identical to those outlined in Figure 2.2.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1-DsMV-Pa		100.0	100.0	96.5	96.9	96.9	96.1	98.5	98.8	97.3	98.5	96.1	96.5	96.1	95.3	95.3	97.7	97.7	97.7	97.7	98.1	98.1	98.5
2-DsMV-Pb			100.0	96.5	96.9	96.9	96.1	98.5	98.8	97.3	98.5	96.1	96.5	96.1	95.3	95.3	97.7	97.7	97.7	97.7	98.1	98.1	98.5
3-DsMV-Pc				96.5	96.9	96.9	96.1	98.5	98.8	97.3	98.5	96.1	96.5	96.1	95.3	95.3	97.7	97.7	97.7	97.7	98.1	98.1	98.5
4-DsMV-P1					99.6	99.6	99.1	96.9	96.9	99.3	96.5	96.5	94.9	96.5	95.3	95.3	97.3	96.5	96.5	97.3	96.9	96.9	97.3
5-DsMV-P16						100.0	99.2	97.3	97.3	99.6	96.9	96.9	95.3	96.9	94.9	94.9	97.7	96.9	96.9	97.7	97.3	97.3	97.7
6-DsMV-P16.2							99.2	97.3	97.3	99.6	96.9	96.9	95.3	96.9	94.9	94.9	97.7	96.9	96.9	97.7	97.3	97.3	97.7
7-DsMV-P31								96.4	96.5	98.9	96.1	96.1	94.4	96.1	94.0	94.0	96.9	96.1	96.1	96.9	96.5	96.5	96.9
8-DsMV-Sa1									99.2	97.7	98.1	97.3	97.7	97.3	95.7	95.7	97.3	97.3	97.3	97.3	97.7	97.7	98.1
9-DsMV-Sa2										97.7	98.8	97.3	97.3	97.3	95.7	95.7	98.1	98.1	98.1	98.1	98.5	98.5	98.8
10-DsMV-SI											97.3	96.5	95.7	96.5	95.3	95.3	97.3	97.3	96.5	97.3	97.7	97.7	98.1
11-DsMV-V1												96.1	96.1	96.1	95.3	95.3	97.7	98.5	98.5	97.7	98.8	98.8	98.5
12-DsMV-FPa													96.1	98.5	94.1	94.1	96.9	96.1	96.1	96.9	96.5	96.5	96.9
13-DsMV-FPb														96.9	94.1	94.1	95.3	95.3	95.3	95.3	95.7	95.7	96.1
14-DsMV-FPc															94.1	94.1	96.9	96.1	96.1	96.9	96.5	96.5	96.9
15-DsMV-NCa																100.0	95.3	95.6	95.3	95.3	95.7	94.9	96.1
16-DsMV-NCb																	95.3	95.6	95.3	95.3	95.7	94.9	96.1
17-DsMV-LA																		98.5	98.5	98.5	98.8	98.1	99.2
18-DsMV-TEN																			98.5	97.7	99.6	98.8	99.2
19-DsMV-Ch																				98.5	98.8	98.1	98.5
20-DsMV-DK3																					98.1	98.1	98.5
21-DsMV-ND																						99.2	99.6
22-DsMV-TW																							98.8
23-DsMV-TW3																							

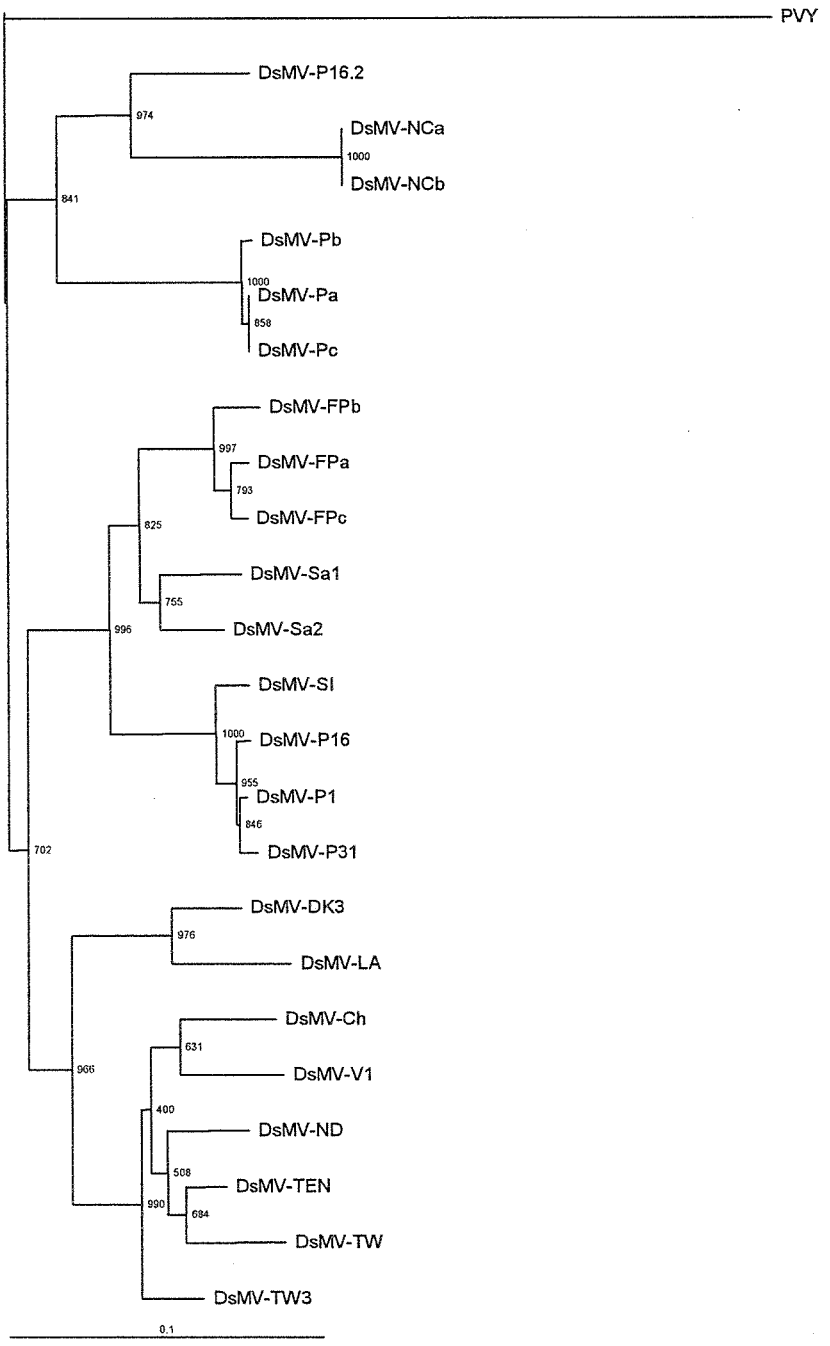

Figure 2.3: Phylogenetic tree of the amino acid sequence of the entire coat protein-coding region of DsMV isolates obtained using NEIGHBOR-JOINING analysis and using a PVY isolate as the rooted outgroup. The values at the nodes indicate the number of replicates out of 1000 trees that agreed with this grouping following bootstrap analysis. The scale bar shows the number of substitutions per base. Abbreviations and accession numbers are same as those presented in Figure 2.2.

For example, isolates from French Polynesia (FPa, FPb, FPc), New Caledonia (NCa, NCb) and Samoa (Sa1, Sa2) all formed distinct clusters. Further, isolates from PNG also generally grouped according to the regions from which they were obtained; that is, northern PNG (Lae) isolates (P1, P16, P31) and western PNG (Kiunga) isolates (Pa, Pb, Pc) forming separate clusters. One isolate (P16.2) from northern PNG grouped with the New Caledonia isolates, but sequence analysis revealed that this isolate was 32 amino acids shorter than all other northern PNG isolates. The one Solomon Islands isolate (SI) branched most closely to the northern PNG DsMV isolates, while the Vietnam isolate branched most closely to a *Caladium* isolate (Florida). However, this latter isolate was obtained from a germplasm collection in Vietnam and its origin was uncertain. A phylogenetic tree based on the core region of the CP also showed the same general trends as those outlined above, but branching was not as extensive as with the complete CP and the bootstrap values were significantly reduced. Furthermore, isolate P16.2, which previously grouped with the New Caledonian isolates, now grouped with the PNG isolates of the same origin.

2.4 Discussion

In this study, we have amplified, cloned and sequenced approximately 1.8 kbp of the 3'end of 15 South Pacific isolates of DsMV and one Vietnamese isolates. This region, which encompassed part of the NIb-coding region, the entire coat protein-coding region and the entire 3' UTR, was amplified using the generic degenerate potyvirus primers (Poty2 and Oligo-dT).

Sequence analysis of DsMV CP-coding region from all isolates revealed the presence of an open reading frame ranging from 939 to 1038 nucleotides in length, which encoded putative proteins ranging from 313 to 346 amino acids. These sizes compared favourably with those reported for DsMV isolates from the USA (Pappu *et al.*, 1994a,b; Li *et al.*, 1998) and the countries in the Asian region (Chen *et al.*, 2001), in which the sizes of the CP-coding regions ranged from 939 to 987 nucleotides and 313 to 329 amino acids. A NIa cysteine protease cleavage site (Q//A) was present at the NIb/CP junction in all isolates. Chen *et al.* (2001) recently published the complete nucleotide sequence of a Chinese DsMV isolate infecting *Zantedeschia aethiopica* and reported that the Q//A dipeptide is the preferred cleavage site between the NIb

and CP-coding regions of DsMV. Several motifs were present in the amino acid sequence of the DsMV CP-coding region which are also common in the CPs of other potyviruses. A highly conserved DAG tripeptide, which has been implicated in aphid transmission (Atreya *et al.*, 1991), was located 6 amino acids downstream of the NIb/CP cleavage site within the N-terminal region of all isolates except one Samoan isolate, Sa2), which contained a NAG motif. A WCIE/DN motif was located in the core region of the CP of all isolates; the function of this motif is unknown (Pappu *et al.*, 1993). Two additional conserved potyviral sequences, RQ and AFDF, were also present in the C-terminal region of the CP in all the isolates and are reported to be associated with the coat protein structure and assembly (Jagadish *et al.*, 1993). Pappu *et al.* (1994a) reported a tandem repeat of threonine-asparagine (TN)-rich sequences in the variable N terminus of the CP from a Florida isolate of DsMV; this TN-rich sequence was postulated to have a role in the aphid transmission of the virus. Similar stretches of TN-rich sequences were not present in DsMV isolates from the South Pacific region. Without conducting aphid transmission studies, the significance of this finding in relation to Pacific isolates is unknown.

Amino acid variability within the CP-coding region of all DsMV isolates (including those on the database) ranged from 0-21.9% for the entire CP-coding region, and 0-6% for the CP core region. PNG isolate P1 was shown to be most similar to all other isolates. Due to the small number of isolates obtained for most countries, within-country variability could only be done with the seven PNG isolates. This analysis revealed variability of up to 16.9% and 3.9% over the entire CP-coding and core regions, respectively. Previous studies have indicated that most variability in the coat protein-coding region occurs within the N terminus (Pappu *et al.*, 1994b); the results presented here further support this observation. Although it is possible that some of the sequence variations reported in this study are due to PCR error, steps were taken to minimize these artefacts including the use of proof-reading enzymes for PCR and the sequencing of multiple clones for each isolate.

Phylogenetic analysis based on the amino acid sequence of the entire CP-coding region revealed that almost all DsMV isolates from the South Pacific were grouped according to their provenance. For example, all three, two and two isolates from French Polynesia, New Caledonia and Samoa, respectively, formed distinct

clusters. Similarly, with the PNG isolates, the three isolates from northern PNG branched together as did the three isolates from western PNG.

There have been two major outcomes from this variability study. Firstly, analysis of sequence variability revealed that DsMV PNG isolate P1 was most similar to all the other Pacific Island isolates and this sequence would appear to be the most appropriate to express as a recombinant CP for antibody production. The availability of the CP-coding sequences from Pacific Island isolates of DsMV and knowledge of their variability will also enable the future development of PDR strategies to generate transgenic DsMV-resistant taro. In many cases, PDR has been shown to be virus or strain specific and the resistance of the transgenic resistant plant to infection depends on the homology between the transgene and the challenging virus (Lomonossoff, 1995). This was evident when papaya plants were transformed with the CP of a Hawaiian strain of PRSV-P and showed varying degrees of resistance to isolates of PRSV from other geographical origins (Tennant *et al.*, 1994). Due to the extensive variability observed in the entire CP-coding region of DsMV, PDR strategies based on these sequences are unlikely to provide protection against all DsMV isolates. The use of transgenes based on the more highly conserved CP core region may be more appropriate.

Chapter 3 Expression of the DsMV CP-coding region in *E. coli* and the development of a diagnostic assay

3.1 Introduction

Taro is an important staple crop of most South Pacific Island nations and other developing countries in the tropical regions of the world. The production of taro has suffered a significant decline in the past 30 years due to diseases caused by viruses and other pathogens (Rodoni *et al.*, 1994). Of the viruses that infect taro, DsMV is considered one of the most important as it occurs worldwide and infects both the ornamental and edible aroids (Zettler and Hartman, 1986, 1987; Jackson, 1980, 1982; Shaw *et al.*, 1979). In some cases, yield losses of up to 60% have been observed with the edible aroids due to DsMV infection (Zettler and Hartman, 1987). The presence of DsMV and other viruses restricts the movement of taro germplasm throughout the Pacific. Due to the significant impact of DsMV on taro, there is a need to develop reliable and sensitive diagnostic assays for the virus to enable the screening of taro germplasm.

Several molecular and serological diagnostic techniques are now being routinely used to detect plant viruses. Diagnostics based on molecular methods like polymerase chain reaction (PCR) and nucleic acid hybridization assay are highly sensitive, but are very expensive and often require trained personnel. These techniques also require sophisticated and expensive equipment, which are lacking in the laboratories of most South Pacific countries. Enzyme-linked immunosorbent assay (ELISA) is a type of serological assay that is commonly used in plant viral diagnostics (Matthews, 1991). It is cheap, simple and large number of samples can be tested simultaneously, which is appropriate for the South Pacific countries. The efficiency and reliability of ELISA depends largely on the titre and specificity of the antiserum. Traditionally, antisera have been obtained by injecting purified virions into laboratory animals. Although many antisera produced in this way have proven extremely useful, there are some instances where such antisera also cross-react with plant proteins (Smith *et al.*, 1995). This is mainly attributed to contaminating plant host proteins present in the purified virus preparations used for antiserum production. Recent developments in protein expression systems have resulted in production of high

quantities of pure recombinant protein in bacterial cell such as *E. coli* that can subsequently be used to generate protein (virus)-specific antiserum. Li *et al.* (1998) and Smith *et al.* (1995) outlined numerous advantages when using recombinant protein to produce antisera including (i) it generates large amounts of suitable antigen; (ii) it generates stable antigen (CP of purified virions often degrade easily); (iii) cloned CP can be distributed to other laboratories.

The aim of the work presented in this chapter was to express the CP of a Pacific isolate of DsMV and use the purified CP to generate a DsMV antiserum for use in diagnostics and research. The research plan to achieve this aim involved (i) expression of a conserved region of the CP as a recombinant fusion protein, (ii) production of an antiserum against the recombinant CP and (iv) testing the sensitivity and specificity of the antiserum.

3.2 Materials and methods

3.2.1 Expression of the DsMV-CP

3.2.1.1 Expression system

The IMPACT-CN protein expression and purification system (New England BioLabs) (Fig. 3.1) was used to express the DsMV-CP. This is a novel system which utilizes the inducible self-cleavage activity of a protein-splicing element (intein). The target protein is fused (N- or C-terminal fusion) to a recombinant protein element consisting of a self-cleaving intein entity and a chitin-binding protein. In the presence of thiols like DTT, the intein can undergo specific self-cleavage and releases the target protein. This results in a recombinant protein without extra non-native amino acid residues (Fig. 3.1).

3.2.1.2 Amplification of the core region of the DsMV-CP

Two primers, IStart and DMVStop, were used to amplify a 765 bp fragment of the core region of a PNG isolate of DsMV (DsMV-P1) (Table 2.1). The PCR reaction (50 µL) contained 1 µL of cloned DsMV-P1 template (1.8 kbp fragment from the 3'

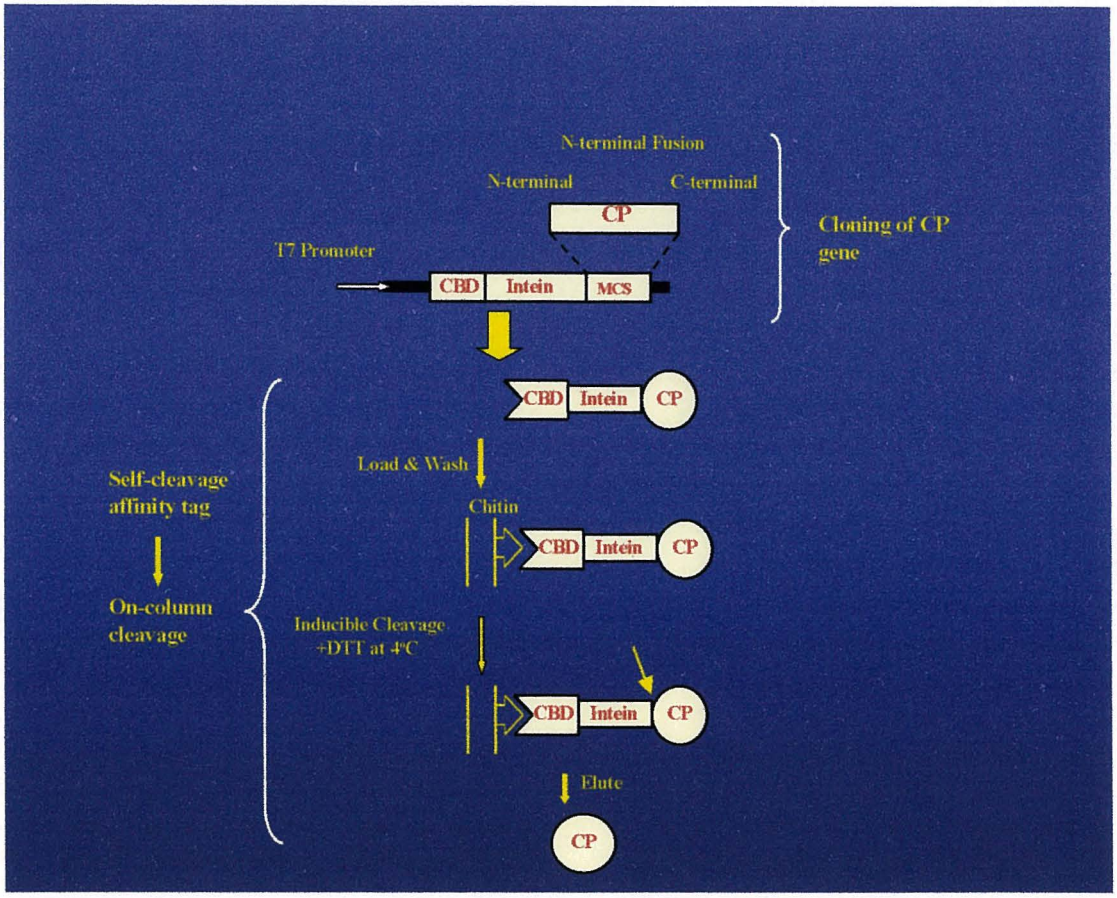

Figure 3.1: The IMPACT-CN protein expression system used to express DsMV CP. Abbreviations: **CP**, coat protein; **CBD**, chitin-binding domain; **MCS**, multiple cloning site.

terminus of the genome), 3 µL each of IStart and DMVStop primers (10 pmol/µL), 1 µL 10mM dNTPs, 0.5 µL Expand DNA polymerase (5 U/µL; Roche), 5 µL 10x Expand buffer 2 (Roche) and sterile water. The reaction mix was initially heated at 92°C for 2 min followed by 35 cycles of 92°C for 30 s, 50°C for 30 s and 68°C for 45 s, and 1 cycle of 68°C for 10 min.

3.2.1.3 Purification of DNA

Following electrophoresis through a 1% agarose gel, the fragment of the expected size (~765 bp) was excised and purified using a High Pure PCR Product Purification Kit (Roche) as described by the manufacturer.

3.2.1.4 Sticky-end ligation

The purified amplicon was initially ligated into pGEMT-Easy and transformed into *E. coli* JM109 cells (see 2.2.8.4). Putative clones were then screened by blue/white selection, extracted by mini-prep and evaluated by restriction digestion analysis. The DsMV-CP fragment was then sub-cloned into the expression vector pTYB-11 (New England Biolabs) using the restriction enzymes *Pst*I and *Sap*I, present at the termini of the cloned fragment. The ligation reaction mix (25 µL) contained 4 µL (200 ng) digested pTYB-11 vector, 12 µL (100 ng) purified insert, 2.5 µL 10x T4 ligation buffer, 2 µL T4 DNA ligase (1 U/µL) and sterile water. The mixture was incubated overnight at 4°C and the ligase heat-inactivated at 65°C for 15 min.

3.2.1.5 Transforming *E. coli* with recombinant plasmid DNA

The recombinant vector (pTYB11) was transformed into electro-competent *E.coli* (ER2566) cells using the procedures described by Dower *et al.* (1988). Procedures were same as those described earlier (section 2.2.8.4, method 1), except IPTG and X-Gal were omitted from the LB media.

3.2.1.6 Screening for transformants

Directional cloning of the CP into the multiple cloning site (MCS) of the expression vector (pTYB11) does not allow for blue/white screening so colonies had to be randomly screened for the presence of the insert. A manageable number of colonies were selected and incubated independently overnight in LB broth containing 100µg/mL ampicillin at 37°C with constant shaking (225 rpm). Recombinant plasmids were isolated using standard mini-prep procedures and screened by restriction enzyme analysis using *Pst*I and *Nco*I (*Sap*I site is lost during cloning) to confirm the presence of target inserts. The digestion reaction mix (10 µL) contained 3 µL (2 µg) plasmid DNA, 1 µL *Pst*I (10 U/µL), 1 µL *Nco*I (10 U/µL), 1 µL 10x buffer H (Roche) and sterile water. The reaction mixtures were incubated at 37°C for 60 min. DNA was electrophoresed through a 1 % agarose gel in TBE and visualized on a UV transilluminator. Several clones that contained the appropriate sized inserts were

further sequenced to verify the presence of the correct insert. Clones with correct inserts were stored in 40% glycerol at –80°C.

3.2.1.7 Induction of CP expression

3.2.1.7.1 Culture preparation

Flasks containing 200 mL LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast, 0.5% NaCl) were autoclaved and allowed to cool prior to the addition of glucose (to 0.2%) and ampicillin (100 µg/mL). The broth was inoculated with 5 mL of the overnight culture and then incubated in a 37°C shaking incubator at 225 rpm until an OD_{600} of between 0.5 and 0.6 was reached. The cultures were then induced with IPTG to initiate the production of recombinant protein. Prior to induction, 2 mL of culture was retained as an uninduced sample. In an attempt to maximise protein expression, a number of pilot experiments were conducted to optimize the concentration of IPTG, temperature and length of incubation during the induction phase.

3.2.1.7.2 Confirmation of CP expression by SDS-PAGE

Expression of DsMV-CP was confirmed by analysis on 5% stacking/12% resolving SDS-PAGE and run in Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). Gels were prepared as follows:

12% Resolving gel		**5% Stacking gel**	
Sterile water	3.3 ml	Sterile water	1.4 ml
30% acrylamide mix	4.0 ml	30% acrylamide mix	330 µL
1.5 M Tris (pH 8.8)	2.5 ml	1.0 M Tris (pH 6.8)	250 µL
10% SDS	100 µL	10% SDS	20 µL
10% ammonium persulfate	100 µL	10% ammonium persulfate	20 µL
TEMED	4 µL	TEMED	2 µL

In all cases, gels were prepared and loaded on a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad) as recommended by the manufacturer. Samples were electrophoresed at 100 volts for 45 min at 4°C. Gels were stained (0.1% Coomassie brilliant blue R-50, 40% methanol, 10% glacial acetic acid) and incubated for 30 min on a rocker. Gels were then destained with water containing 40% methanol and 10% acetic acid.

3.2.1.7.3 Cell harvest and fusion protein isolation

The 200 ml culture was aliquoted equally in 4 x 50 ml sterile Falcon tubes and centrifuged at 2,500 rpm for 10 min at RT. Pellets were resuspended in cell lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) and sonicated for 255 s, allowing 15 s interval for and between sonication. The suspension was subsequently centrifuged at 8,500 rpm for 20 min at 4°C. The supernatant was retained and the insoluble component discarded.

3.2.1.7.4 On-column cleavage of recombinant CP

One millilitre of chitin resin (2 mg/ml) was loaded into the column and equilibrated with 10 volumes of column buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA). The clarified fusion protein was slowly loaded into the chitin column and allowed to drain at a flow rate of 0.5 ml per min. The column was then washed with 10 volumes of column buffer containing 1 M NaCl at a flow rate of 1 ml per min. To induce cleavage of CP from the fusion protein, the chitin column was quickly flushed with 3 ml cleavage buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM EDTA, 50 mM DTT). All except 1 ml of the cleavage buffer was allowed to drain and the chitin column was then incubated at 4°C for 40 h.

3.2.1.7.5 Elution of recombinant protein

Ten millilitres of cleavage buffer was loaded into the chitin column and the flow-through was collected in 500 µL fractions in sterile 1.5 ml Eppendorf tubes. A UV spectrophotometer (Beckman DU-68) was used to determine the amount of protein eluted in each fraction at 280 nm. The high concentration protein fractions were analysed by SDS-PAGE to determine the protein content within the fractions.

3.2.1.7.6 Estimation of protein concentration

A commercial Bradford assay (Bio-Rad) was used to accurately determine the concentration of protein within the fractions that had high UV_{280} readings. A bovine serum albumin (BSA) (10 mg/ml) standard was used to establish a standards curve for the assay. Varying amounts of BSA (0, 4, 8, 12, 16, 20 µg) or 40 µL of the respective fractions were mixed with 1 ml of diluted assay reagent and incubated for 5 min at RT. The absorbance for the standards and samples was read at 595nm and the protein concentration of the fractions calculated by reference to the standards.

3.2.2 Production of antiserum

3.2.2.1 Immunization

All immunisations were carried out within the University of Queensland animal house. A 10 ml pre-immune serum was collected from the ear of a New Zealand white rabbit and stored as outlined below. Purified recombinant CP (150 µg in 500 µL) was emulsified with an equal volume of Freund's incomplete adjuvant and administered intramuscularly in the hind legs. Three injections were given at an interval of 3 weeks. Prior to the second and third injections, blood samples were collected to determine the titre of the antiserum.

3.2.2.2 Treatment of antiserum

Routinely collected blood and the final bleed samples were allowed to clot for 3 h at RT. Blood clots were then broken by 'ringing' the tube with a sterile spatula and incubated overnight at 4°C. The samples were centrifuge at 1,750 rpm in a swinging bucket centrifuge for 10 min. The clear aqueous phase was carefully removed, mixed with and equal volume of 100% glycerol and stored in sterile Falcon tubes at –80°C.

3.2.2.3 Titre of the antiserum

3.2.2.3.1 Electrophoretic transfer

SDS-polyacrylamide gels were prepared and 2 μg samples of the fusion protein was loaded. Bio-rad prestained protein markers were also included on the gels. Samples were electrophoresed in Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.0) at 100 volts for 45 min at 4°C. Samples were then transblotted onto nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) using a Mini-Transblot system (Bio-Rad) as recommended by the manufacturer. An overnight transfer was allowed at 30 volts at 4°C. Membranes were then cut into strips representing each lane and a reference line was drawn at the top end of the strips for correct alignment during immunoassays.

3.2.2.3.2 Immunoassays

A control strip was stained with 0.02 % Ponceau S (Sigma Diagnostics) for 2 min, washed with sterile water and then dried for 30 min at RT. Serum samples used for immunoassays included diluted (1:10) pre-immune serum and diluted (1:10, 1:100, 1:1,000, 1:10,000) final bleed serum. A corresponding number of strips were blocked with 3 % skim milk powder in TBST on a rocker for 1 h at RT. Samples were diluted in blocking reagent and incubated on respective strips for 1 h at RT. Strips were washed four times with TBST for 15 min each. Secondary antibody (anti-rabbit IgG-AP conjugate; Dako) was diluted (1:7,500) with TBST and incubated on the strips for 30 min at RT. Strips were then washed with TBST as above, followed by two brief washes with TBS. Membranes were developed by adding Western blue stabilized substrate (Promega) and washed with sterile water when sufficient colour intensity had developed.

3.2.3 Testing for DsMV with DsMV CP antiserum

Both indirect ELISA and western blot techniques were used to test antiserum response for DsMV in taro samples obtained from countries including Australia, Fiji, French Polynesia, New Caledonia, New Zealand, Papua New Guinea, Samoa, Solomon

Islands, Vanuatu and Vietnam. In all tests, purified DsMV from a Papua New Guinea isolate and sap from a healthy sample were included as positive and negative controls, respectively.

3.2.3.1 Western blot

A 100 mg tissue powder sample was thoroughly vortexed in 400 µL SDS reducing sample buffer:

1 M Tris-HCl, pH 8.0	250 µL
80% glycerol	500 µL
10% SDS	800 µL
β-mercaptoethanol	200 µL
Sterile water	2.25 ml
Bromophenol blue	1 mg

Samples were boiled at 95°C for 5 min and centrifuged at 14,000 rpm for 2 min at RT. A 2 µL (50 µg tissue) aliquot was analysed by SDS-PAGE and later transblotted using the methods described earlier. The primary antiserum (anti-DsMV recombinant CP) was diluted (1:10,000) in the blocking reagent. Procedures for hybridization, washing and colour development of the membrane were the same as previously described.

3.2.3.2 Indirect ELISA

A 100 mg sample of taro leaf tissue was homogenised in 1 ml extraction buffer in Eppendorf tubes and centrifuged at 14,000 rpm for 5 min at RT. The supernatants were transferred to new Eppendorf tubes and further centrifuged at 14,000 rpm for 2 min. A 100 µL aliquot of the clarified extracts were added onto the wells of polystyrene microtitre plates and incubated overnight at 4°C. Wells were washed 5 times with PBST. Primary antibody (polyclonal anti-DsMV recombinant CP) was diluted (1:1,000) in ECI buffer and incubated in the wells for 1 h at RT. Wells were again washed as above. A 100 µL aliquot of diluted (1:7,500) commercial secondary

antibody conjugate (anti-rabbit IgG-AP conjugate) was then incubated in the wells for 1 h at RT. A final wash was done as above. Plates were developed by adding 100 µL K-Gold substrate (ELISA Systems) to each well and incubating for 30 min at RT. The Biomek Plate Reader (Beckman) was used to determine the absorbance of the samples at 405 nm with a reference wavelength of 650 nm.

3.2.4 Testing the sensitivity and specificity of the DsMV antiserum

An indirect-ELISA was used to assay the specificity and sensitivity of the polyclonal recombinant DsMV CP antiserum. For specificity assays, isolates including *Potato Y potyvirus* (PVY), *Sugarcane mosaic potyvirus* (SCMV), *Papaya ringspot potyvirus*-P (PRSV-P), *Papaya ringspot potyvirus*-W (PRSV-W) and *Cucumber mosaic cucumovirus* (CMV) were tested. In the sensitivity assay, sap extracts from a healthy (Australian field isolate) and DsMV-infected taro samples were serially diluted 1:10, 1:50, 1:100, 1:500 and 1:1 000 in bicarbonate coating buffer and probed with the antiserum diluted to 1:1 000. Procedures for ELISA were the same as those previously outlined. For comparison, RT-PCR was used to test for DsMV in these samples at the same dilutions. Samples for RT-PCR were diluted using sterile water. For PCR, two generic primers including WCIEN and Oligo-dT were used to amplify a 765 bp fragment using the PCR conditions as previously described.

3.3 Results

3.3.1 Cloning and screening of transformants

Based on the previous sequence variability studies, a clone from the PNG isolate DsMV-P1 was chosen for expression, as this represented the most average sequence amongst all DsMV isolates. The conserved core region of DsMV-P1 was then amplified with the expression primers, IStart and DMVStop, to generate a 765 bp fragment. The fragment was initially cloned into pGEM-T Easy, digested with *Pst*I and *Sap*I and then cloned into the *Pst*I and *Sap*I sites of pTYB11 protein expression vector. The recombinant plasmid was subsequently transformed into *E. coli* strain ER2566. Since the *Sap*I site was lost during cloning, screening of transformants was achieved by digesting the plasmid with *Pst*I and *Nco*I restriction enzymes. This

resulted in a fragment size of approximately 1400 bp (Fig.3.2). The clone was then sequenced to confirm its integrity.

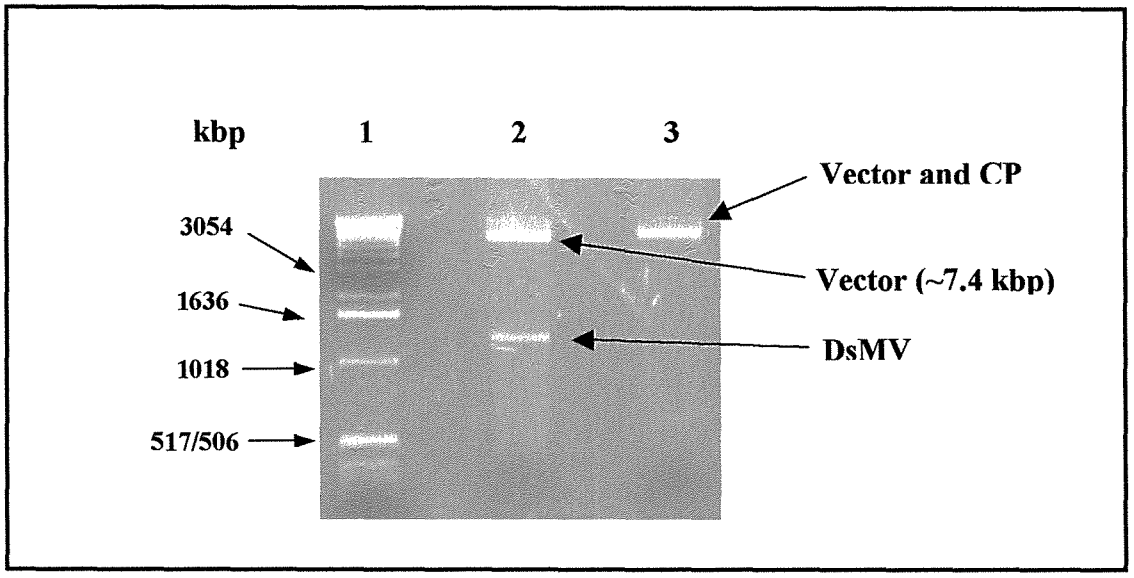

Figure 3.2: Agarose gel analysis of the DsMV CP cloned into the pTYB11 protein expression vector. Lane 1 is a DNA Molecular Marker X (Roche). Lane 2 shows plasmid digested with *Pst*I and *Nco*I (note: *Sap*I site was lost at ligation). Lane 3 shows plasmid digested with *Pst*I alone.

3.3.2 Expression of the DsMV CP in *E. coli*

The pTYB11 expression vector contains a T7 promoter, which is inducible by IPTG for basal expression of the fusion gene. The expression level of the target gene is influenced by variables including temperature of induction, duration of induction period and the concentration of inducing reagent (IPTG). Therefore, pilot experiments were conducted to optimise these variables and in the initial tests, a control plasmid (pMYB5) was included. Conditions tested included induction temperature (15°C, 23°C, 28°C and 37°C), period of incubation (1, 2 and 3 h) and the concentration of IPTG (0.3 mM and 1 mM). Protein expression levels were assessed using SDS-PAGE, with the size of the fusion protein expected to be approximately 82 kDa, comprising the DsMV CP (~28 kDa), intein and chitin-binding domain.

The effect of IPTG concentration on induction was examined first and this experiment was done using a temperature of 28°C (which is the recommended induction temperature) with the maximum induction period of 3 h. When the extracts were examined by SDS-PAGE, the use of IPTG at 0.3 mM resulted in low expression while 1 mM IPTG produced minimal expression levels that were similar to the uninduced control. Therefore, 0.3 mM IPTG was subsequently used in all future experiments to investigate the effect of temperature and induction time on expression. Based on SDS-PAGE analysis of protein extracts induced using 0.3 mM IPTG under different combinations of temperature and induction period, the conditions which resulted in the highest expression of the fusion protein were found to 23°C for 3 h (Fig. 3.3). These conditions were routinely used in subsequent protein expressions.

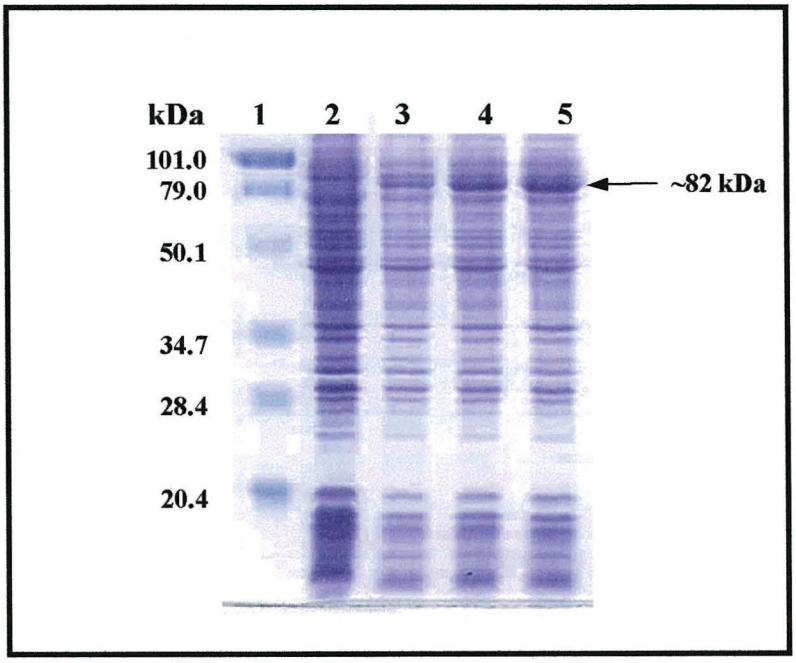

Figure 3.3: SDS-PAGE analysis showing expression of DsMV CP in *E. coli*. Lane 1 is standard prestained molecular marker (Bio-Rad). Lane 2 is uninduced sample. Lanes 3-5 are samples collected after 1, 2 and 3 h induction respectively, at 23°C. The arrow indicates the fusion protein.

3.3.3 Isolation of the recombinant fusion protein

Following the successful expression of the DsMV CP fusion protein, the recombinant protein (consisting of CBD, intein tag and DsMV CP core) was isolated from the host cells as follows (see methods and materials for details): i) pelleting of bacterial cells, ii) resuspension of pellets in cell lysis buffer, iii) sonication of cells iv) a final centrifugation to separate the soluble and insoluble protein components. A sonication trial was initially conducted to determine the optimum time at which the ER2566 bacterial host cells would be disrupted to release the recombinant protein. A 50 µl aliquot of protein was collected after each sonication period of 15 s, centrifuged and a 10 µL sample of the supernatant was used in Bradford protein assays. A total of 24 samples were obtained and read at 595 nm. The results from this experiment indicated that the highest amounts of protein were released from the bacterial cells after sonication for 255 s (Fig. 3.4). To determine the solubility of the recombinant protein, samples were taken from the supernatant and insoluble pellet obtained after the centrifugation of the sonicated extracts, and equivalent amounts of extracts were analysed by SDS-PAGE. The entire fusion protein (approx. 82 kDa) was detected both in the insoluble pellet and the supernatant, although a stronger band was present in the insoluble fraction. This suggested that significant amounts of the fusion protein still remained insoluble. Attempts to improve the yield of protein by the addition of 0.1% Triton X-100 (a non-ionic detergent) to the cell lysis buffer did not result in appreciable yield increases.

3.3.4 On-column cleavage and elution of the recombinant DsMV CP

A chitin column was used to purify and cleave the recombinant DsMV CP from the CBD and intein fusion. To determine the binding efficiency of the fusion protein onto the chitin column, the clarified fusion protein was loaded onto the chitin column and the column was washed. Following the recommended washing protocol, further washing solution was added and samples were collected and analysed by SDS-PAGE. The fusion protein was not detected in any of the fractions, indicating it had been successfully bound to the chitin column (Fig. 3.5, lane 4).

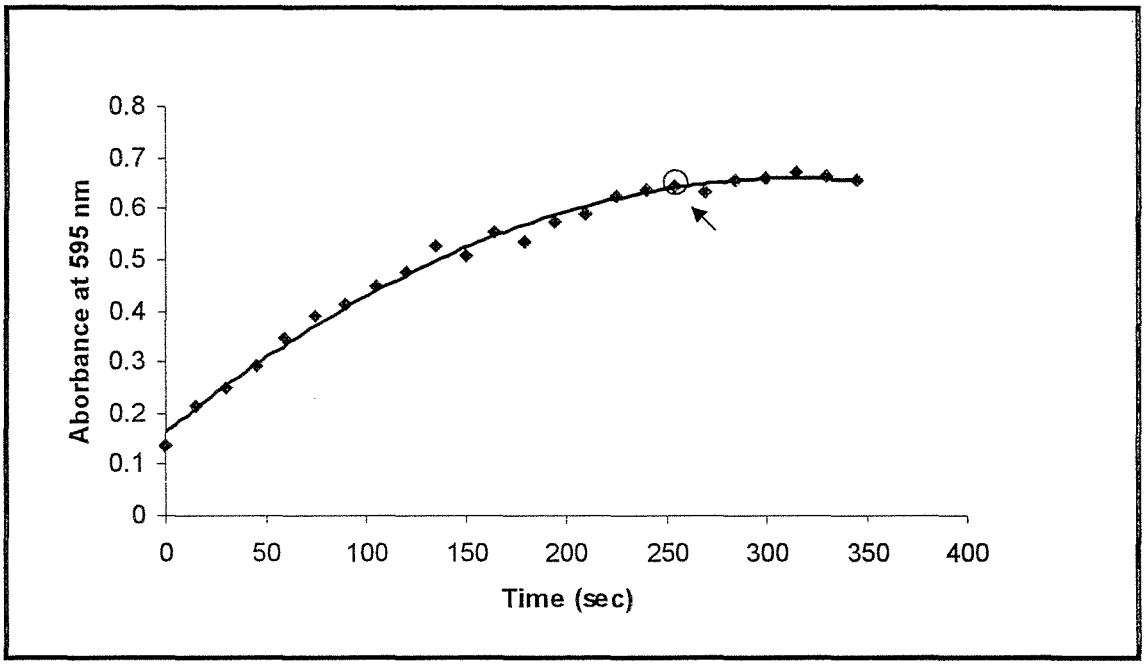

Figure 3.4: Optimum sonication for release of proteins from *E. coli*. Maximum yield was achieved after 255 s (indicated with an arrow).

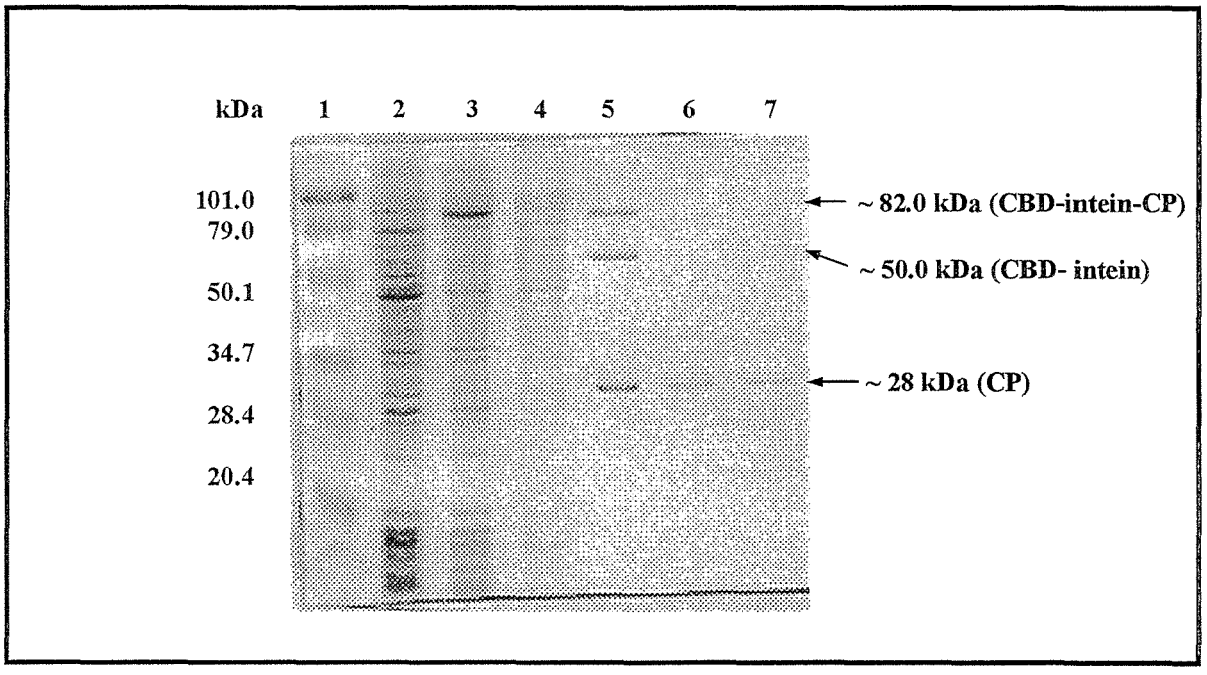

Figure 3.5: SDS-PAGE analysis of cleavage of the native protein from the fusion precursor. Lane 1 is a prestained standard protein marker (Bio-Rad). Lane 2 is uninduced sample. Lane 3 is an induced sample at 3 h. Lane 4 is a flow-through sample, collected after the clarified lysate were loaded on the column and washed. Lane 5 contains proteins bound to the column resin. Lanes 6 and 7 are samples showing successful cleavage of the recombinant DsMV CP (40 h cleavage).

To determine the optimal conditions for cleavage, the column (containing bound fusion protein) was incubated with cleavage buffer either for 16 or 40 h prior to collection of samples and analysis by SDS-PAGE. A total of 18 fractions (500 µl each) were eluted from the columns and scanned at 280 nm to determine the protein concentration. A protein elution curve was plotted (Fig. 3.6) which showed that much of the target protein was contained in the first four fractions.

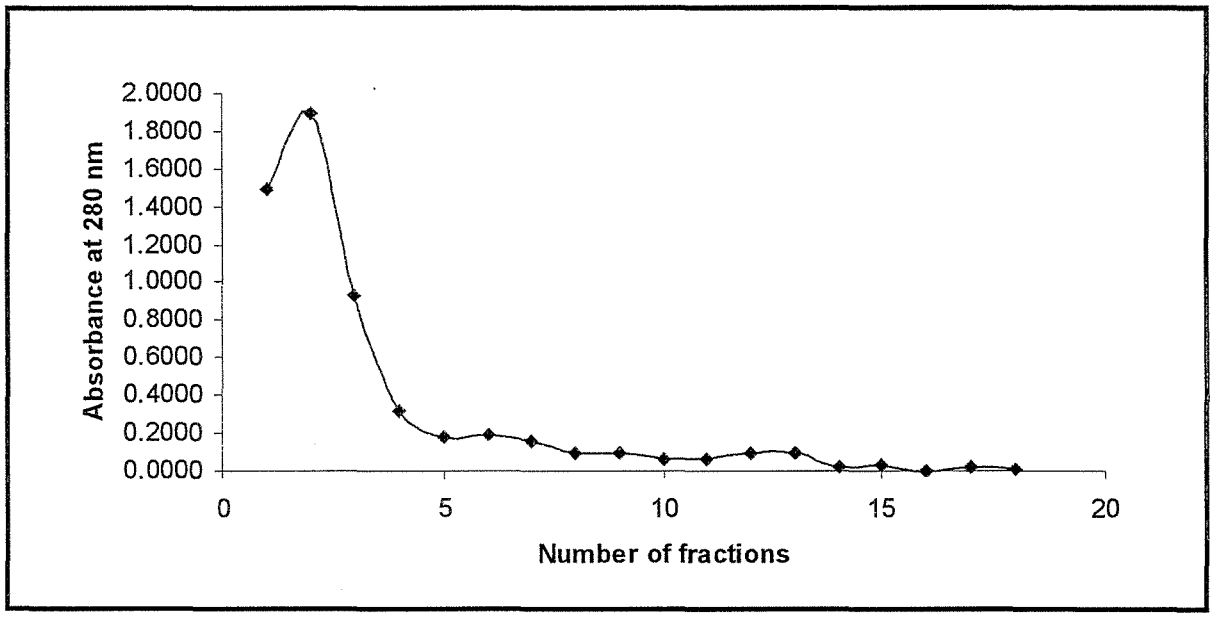

Figure 3.6: Concentration of the fusion protein in each fraction eluted from the chitin column following a 40 h cleavage. A similar trend was observed for the overnight cleavage.

Analysis of the first four eluates by SDS-PAGE showed that an incubation time of 40 h resulted in the highest yield of CP (~28 kDa) from the fusion protein (Fig. 3.5, lanes 6 and 7). However, the amount of CP eluted from the column was very low in comparison to the amount of fusion protein loaded onto the column. In an attempt to determine whether the low yields were due to inefficient cleavage of the CP or other factors, the resin from the chitin column was resuspended in denaturing buffer and analysed by SDS-PAGE. Three bands were present on the gel with molecular weights corresponding to the entire fusion protein (82 kDa), a protein comprising the chitin binding protein plus intein (~50 kDa) and the cleaved CP (~28kDa) (Fig. 3.5, lane 5). The presence of a strong band at 28 kDa indicated that cleavage of the CP had occurred on the column, which suggested that the low yields

of CP obtained in the column eluates were due to inefficient elution. Despite changing parameters such as the pH of cleavage buffer, concentration of DTT, addition of ionic detergents to the cleavage buffer and volume of elution buffer, the yield of CP eluted from the column could not be improved.

Due to the inability to purify sufficient quantities of CP from the column eluates for use as an antigen, a different approach was used to purify the CP. Following the normal 40 h on-column cleavage, the column was washed with column buffer containing 0.5% SDS to remove all bound proteins. Analysis of this eluate by SDS-PAGE revealed the presence of the three protein bands as expected (CBD-intein-CP, CBD-intein, CP). The 28 kDa CP band was subsequently excised from the gel, frozen in liquid nitrogen and ground to a fine powder, freeze-dried and this was used as antigen.

3.3.5 Titre of the DsMV CP antiserum

The purified recombinant native protein (150 μg/500 μl) was emulsified with an equal volume of Freund's incomplete adjuvant and used for immunisation. The injections were administered equally into each of the hind legs of the rabbit. The antiserum was collected 30, 60 and 90 days after the initial injection and the titre was determined by western analysis. The purified fusion protein was electrophoresed through SDS-PAGE, transblotted onto nitrocellulose membranes and probed with dilutions of the antiserum (taken from each bleed) ranging from 1:100 to 1:10,000. Identical blots were also used in immunoassays with pre-immune serum. No antigenic response was detected using the pre-immune serum (Fig 3.7, lane 3). In contrast, the antiserum obtained following injection with the DsMV CP reacted strongly with the expected sized protein of approximately 28 kDa corresponding to the recombinant DsMV CP (Fig. 3.7, lanes 4-6). The antiserum also reacted very weakly with other fragments of the recombinant fusion protein, namely the entire CBD-intein-DsMV CP fusion and the CDB-intein fragment (Fig. 3.7, lanes 4 and 5). A titre of 1:1,000 was observed 60 days after the first injection and a faint band was also evident for a dilution of up to 1:10,000. The final antiserum collected 90 days after the first injection reacted strongly at a 1:1,000 dilution and had a moderate reaction at 1:10,000 dilution (Fig. 3.7, lanes 5 & 6).

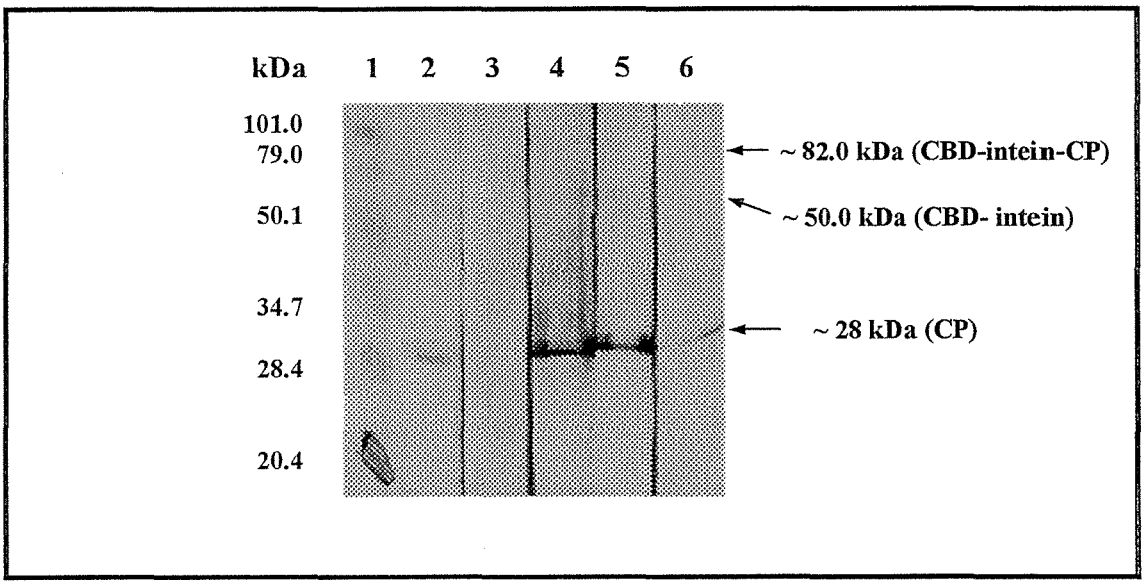

Figure 3.7: Western blot analysis to determine the titre of the DsMV CP antiserum. Lane 1 is standard prestained protein marker (Bio-Rad). Lane 2 is the fusion protein, stained with 0.02% Ponceau S (Sigma). Lanes 3 is diluted (1:10) pre-immune serum. Lanes 4-6 are probed with dilutions of the final antiserum at 1:100, 1:1,000 and 1:10,000, respectively.

3.3.7 Testing the DsMV CP antiserum in ELISA and western blotting protocols

To investigate the utility of the antiserum for the detection of DsMV in plants, the antiserum was incorporated into ELISA and western blot protocols. Based on the antiserum titre results, dilutions of 1:1,000 and 1: 10,000 were both used. In initial investigations, five known DsMV-infected taro samples from PNG and a known healthy sample (Australian field isolate) were tested. Samples for ELISA were extracted in bicarbonate coating buffer, diluted to 1:10 and a 100 µl aliquot was added to the wells. Samples for western analysis were extracted in SDS-reducing sample buffer, and 2 µl of the extracts were electrophoresed through SDS-PAGE and electroblotted to nitrocellulose membranes. In both tests, anti-rabbit IgG-AP conjugate (1:7,500) and purified PNG DsMV (1:500) were included as the secondary antibody and positive control, respectively.

In western analysis, both antiserum dilutions produced identical results although the 1:10,000 antiserum produced the least amount of background. The

antisera reacted strongly with an expected size band of ~47 kDa present in extracts from purified DsMV and the five DsMV-infected plants. No bands were observed in the extracts derived from healthy taro. Preliminary testing of the antisera in ELISA showed that both dilutions could be used to detect the presence of DsMV in plants, with strong positive reactions observed with the purified DsMV and infected extracts, compared with the healthy controls (greater than three times). However, the 1:1,000 dilution was preferred because of the stronger absorbance values. Based on these preliminary experiments, testing for DsMV was made with the antiserum diluted to 1:1,000 and 1:10,000 for ELISA and western blot analysis, respectively.

To test the ability of the antiserum to detect a wide range of DsMV isolates, extracts were prepared from nine DsMV isolates (Table 3.1), purified DsMV and a healthy taro. In Western blots, the antiserum reacted with distinct bands that corresponded to the CPs of the different DsMV isolates (Fig. 3.8, lanes 4-12). The antiserum did not react with the crude extract from a healthy taro. In all extracts except that from French Polynesia, a strong band with an approximate Mr of 47 kDa (Fig. 3.8, lanes 4-6; 8-12) was observed which corresponded to the full-length viral CP. Smaller bands were also present in some extracts. For example, a faint band with an Mr of about 30 kDa was observed in most of the extracts, while the antiserum hybridised with a band of estimated Mr of 45 kDa in extracts from the FP isolate (Fig. 3.8, lane 7) and the Papua New Guinea (PNG) isolate (Fig. 3.8, lane 9). Further, in addition to detecting the major CP band of ~47 kDa, the antiserum also reacted with a band of approximately 46 kDa in extracts from Fiji (Fig. 3.8, lane 6) and the Solomon Islands (Fig. 3.8, lane 11).

When the extracts were tested by ELISA, positive results were again observed for all geographical isolates. In all cases, the absorbance values for the isolates were more than three times those of the healthy sample (Table 3.1). To confirm the validity of the results, the extracts were also tested by RT-PCR. A band of the expected size was only amplified from each of the DsMV-infected plants and the purified DsMV confirming the plants were infected. Table 3.2 summarises the detection of DsMV using ELISA, western blot and RT-PCR analysis.

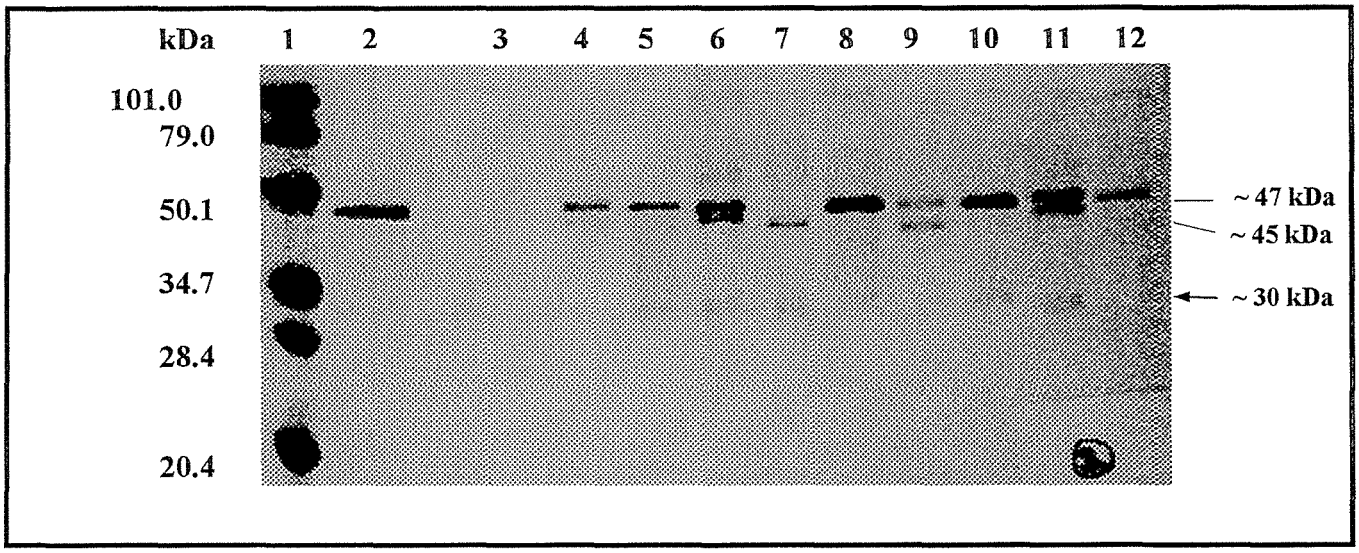

Figure 3.8: Western blot analysis of crude extracts from taro leaves tested for DsMV with the DsMV CP antiserum (1:10,000). Lane 1 is standard prestained protein marker (Bio-Rad). Lanes 2 and 3 are purified DsMV and healthy sample, respectively. Lanes 4-12 are extracts of isolates from Australia, New Zealand, Fiji, French Polynesia, New Caledonia, Papua New Guinea, Samoa, Solomon Islands and Vanuatu, respectively.

Table 3.1: ELISA of crude extracts from taro leaves tested for DsMV with the DsMV CP antiserum.[1]

Source of sample	Absorbance (405 nm)
Healthy[2]	0.120[3]
Purified DsMV	1.339
Australia	0.802
New Zealand	0.829
Fiji	0.768
French Polynesia	1.129
New Caledonia	0.948
Papua New Guinea	0.674
Samoa	0.785
Solomon Islands	0.771
Vanuatu	1.017
Vietnam	0.836

[1] Recombinant DsMV CP antiserum was diluted to 1:1,000.

[2] Sap extract of a healthy field isolate from Australia

[3] Average of duplicate readings

Table 3.2 Comparison of diagnostic assays for DsMV

Source of isolate	Method of detection		
	RT-PCR[a]	ELISA[b]	Western blot[c]
Healthy[d]	-[e]	-	-
Australia	+[f]	+	+
New Zealand	+	+	+
Fiji	+	+	+
French Polynesia	+	+	+
New Caledonia	+	+	+
Papua New Guinea	+	+	+
Samoa	+	+	+
Solomon Islands	+	+	+
Vanuatu	+	+	+
Vietnam	+	+	NT

[a] 1-2 μg of total RNA extract. [b and c] 1:1,000 and 1: 10,000 dilution of DsMV recombinant antiserum, respectively. [d] Australian field isolate. [e] Negative result. [f] Positive result. NT; Not tested

3.3.8 Testing the sensitivity and specificity of the DsMV antiserum

The sensitivity and specificity of the antiserum were tested using the ELISA and RT-PCR techniques. In the sensitivity assay, serial dilutions of sap extracts from a DsMV-infected and a healthy taro were made from 1:10 to 1:1,000 and were tested using both the ELISA and RT-PCR methods. Using both ELISA and RT-PCR, DsMV was detected at all dilutions, including the maximum dilution of 1:1,000 (Table 3.3).

Although the absorbance values for ELISA at the higher dilutions were low, they were still greater than three times than of corresponding dilutions of the healthy sample. In RT-PCR, the expected fragment size of approximately 765 bp was amplified from all extracts except the healthy control.

Table 3.3 Sensitivity and specificity assay of the DsMV antiserum using ELISA and RT-PCR.

		Dilutions of sap extracts				
Test	Healthy	1:1 000	1:500	1:100	1:50	1:10
ELISA	-[a]	++[b]	+	++	++	++
RT-PCR	-	++	++	++	++	++

		Isolates tested for specificity assay				
	Healthy	PVY	PRSV-P	PRSV-W	SCMV	CMV
ELISA	-	-[c]	+[d]	+	+	-

[a] OD less than three times the mean of healthy control or no band on agarose gel.

[b] OD greater than three times the mean of healthy control or strong band on agarose gel.

[C]Negative result [d]Positive result

In the specificity assay, crude sap extracts from four potyvirus isolates and a cucumovirus isolate were tested by ELISA. The antiserum reacted with three potyviruses including PRSV-P, PRSV-W and SCMV, but did not react with PVY and cucumber mosaic cucumovirus (CMV) (Table 3.3).

3.4 Discussion

The aim of this study was to generate an antiserum that would detect as many geographical isolates of DsMV as possible. The large amount of variability observed in the CP-coding region of South Pacific DsMV isolates (Chapter 2) precluded the expression of the entire CP-coding region as antigen. This was largely due to concerns

that the resulting antibodies might not recognize epitopes present on all virus isolates. Therefore, it was decided to express the more highly conserved CP core region for use as an antigen.

Analysis of the DsMV sequences indicated that the CP core region of PNG DsMV isolate P1 was most similar to all other isolates and hence this region was selected for protein expression. The conserved core region of the CP from this isolate was cloned into the pTYB11 protein expression vector and expressed in the *E. coli* strain ER2566, using the IMPACT-CN protein expression system. The plasmid uses a T7 RNA promoter, compatible with the inducible T7 RNA polymerase in the ER2566 cells and is well suited for high levels of expression and tight transcriptional control (Dubendorff and Studier, 1991). The majority of potyviruses are strongly immunogenic (Shukla *et al.*, 1994) and only a small amount of the recombinant DsMV CP is usually required to produce polyclonal antiserum. Although large quantities of fusion protein were expressed in this study using the IMPACT-CN system, difficulties were experienced in the elution of the DsMV CP following on-column cleavage. The reason for this is unknown but it is possible that self-assembly of the cleaved CP occurred following cleavage and that the larger protein molecules were not efficiently eluted. To overcome this problem, all proteins in the column following cleavage were eluted by washing with SDS and the CP was then purified from the gel matrix for use as an antigen. The purified protein was injected intramuscularly into a rabbit at successive intervals and the titre of the antiserum was determined by western blot analysis. The antigen was shown to be highly immunogenic, resulting in the production of antibodies which reacted strongly with the DsMV fusion protein at a dilution of 1:10,000. These results are consistent with those reported by Li *et al.* (1998) who expressed the entire DsMV CP from a *Caladium* isolate and reported that the recombinant CP was a good immunogen and yielded a high titre antiserum.

The antiserum generated in this study was able to be successfully utilized in both ELISA and western blot protocols to detect DsMV in a wide range of geographical isolates including Australia, Papua New Guinea, New Zealand, Fiji, French Polynesia, New Caledonia, Samoa, Solomon Islands and Vanuatu. In western blot analysis, the antiserum reacted with distinct bands from all isolates that

corresponded to the CP of DsMV. Three major bands ranging from 45 to 47 kDa, and several faint bands with Mr of about 30 kDa, were observed on the western blots. This is consistent with the results of other studies (Abo El-Nil *et al.*, 1977; Li *et al.*, 1999; Pappu *et al.*, 1994a) which reported that the CP of different DsMV isolates displayed considerable size variability and ranged from 30-47 kDa. Pappu *et al.* (1994a,b) reported that frequent deletions and insertions were responsible for the CP size diversity amongst DsMV isolates. Although western blot analysis was shown to be a sensitive method for detecting DsMV in infected plants, it is not suited for routine screening of large numbers of plants. As such, the focus was directed towards the development and optimization of ELISA as a diagnostic test.

Using the antiserum in an indirect-ELISA protocol, identical results were obtained to those using western blotting, with DsMV detected in a wide range of geographical isolates. The RT-PCR and indirect-ELISA were used to test the sensitivity and specificity of the antiserum. The PCR-based detection is reportedly more sensitive than serological-based tests (Randles *et al.*, 1996) and was included for comparative purposes. Sensitivity assays revealed that both RT-PCR and ELISA were able to detect DsMV in infected sap diluted to 1/1000. This result demonstrates that the DsMV CP antiserum is able to detect DsMV in sap of infected plants at low concentrations.

The specificity tests showed that the antiserum was not DsMV-specific but also reacted with sap from plants infected with other potyviruses including SCMV, PRSV-P and PRSV-W, but did not react with PVY and CMV. Li *et al.* (1998) reported similar results with an antiserum produced against the entire CP-coding region of a Caladium isolate of DsMV, with the antiserum cross-reacting with PRSV-P and PRSV-W. This is not surprising since the core region of potyviruses is highly conserved and would be expected to share epitopes in this region. In fact, when the amino acid sequence of the conserved core region of PRSV-P, PRSV-W and PVY were aligned with the sequence used as an antigen (PNG-P1), several homologous sequences of more than six amino acids were revealed in the sequences of PRSV-P and PRSV-W.

In summary, the outcome of this study has been the production of a high titre polyclonal antiserum which can be used in an ELISA to detect a wide geographical range of DsMV isolates. Such a diagnostic test can used for the routine, large scale indexing of taro plants for DsMV and, importantly, the protocol does not require sophisticated equipment and can be utilized in Pacific Island laboratories. Although the antiserum is not DsMV-specific and cross-reacts with other potyviruses, this should not pose any concerns in virus-indexing because DsMV is the only potyvirus known to infect taro.

Chapter 4 General discussion and conclusions

Taro viruses present a major constraint to the taro production throughout the Pacific. The effects are virus infection on taro plants are twofold. Firstly, virus infection causes direct losses as a result of symptoms such as chlorosis, stunting and, in some cases, plant death (Rodoni *et al.*, 1994). In addition, their presence also restricts the movement of taro germplasm through the Pacific (Zettler *et al.*, 1989). This has serious implications because many countries are unable to access agronomically elite germplasm including traditional cultivars, lines from breeding programs or plantlets held in germplasm. These strict quarantine restrictions are necessary because the taro viruses are not uniformly distributed within the Pacific region and, for most, reliable sensitive methods of detection are not yet available or are too expensive for routine use.

DsMV is amongst several plant viruses infecting taro and is considered an important virus because it infects both the ornamental and edible aroids and occurs worldwide (Zettler *et al.*, 1978; Shaw *et al.*, 1979; Zettler and Hartman, 1986, 1987). The virus is of great economic importance, causing yield losses of up to 60% in edible aroids (Zettler and Hartman, 1987). Although an ELISA for DsMV has been developed and is commercially available, the cost of the test precludes its regular use in Pacific Islands laboratories where funding is very limited. In this study, we have generated a recombinant DsMV protein comprising the core of CP-coding region, used the recombinant protein to generate antiserum and utilised the antiserum in the development of an ELISA to detect DsMV. The use of a recombinant DsMV protein as antigen was chosen primarily because (i) large quantities of pure, stable antigen can be generated thus overcoming the need to continually purify virions from infected plants. Importantly, antibodies produced against such antigens normally show little, if any, non-specific cross-reactivity with plant proteins because the antigen is purified from bacteria, and not plants.

The initial part of this study involved an investigation into the sequence variability in the CP-coding region of DsMV isolates from numerous South Pacific island countries. This was necessary to determine the most appropriate sequence to

express as a recombinant protein. At the commencement of this study, sequence information was only available for DsMV from the USA (Pappu *et al.*, 1994a,b; Li *et al.*, 1992, 1998) and a single case from Taiwan (GenBank AF511485). In these reports, characterisation was based either on DsMV isolates obtained from the ornamental aroids or isolates initially obtained from an edible aroid and maintained in a ornamental aroid species. Like other potyviruses, most variability was reported in the N terminus of the DsMV CP-coding region, which was a result of frequent deletions and insertions in this genomic region (Pappu *et al.*, 1994b).

To study the variability in the CP-coding region of DsMV, isolates were obtained from 15 countries in the South Pacific while one isolate was obtained from Vietnam. Using generic potyvirus primers, a 1.8 kbp fragment containing the entire CP-coding region was amplified, cloned and sequenced. Sequence analysis revealed that the size of the CP-coding region ranging from 939 to 1038 nucleotides which encoded putative proteins ranging from 313 to 346 amino acids, with estimated Mr's from 34 to 38 kDa. A VVLQA motif was identified at the cleavage site of the NIb and CP-coding regions which was consistent with previous reports (Pappu *et al.*, 1994a; Chen *et al.*, 2001). The amino acid sequence of the CP-coding region contained several conserved motifs which are common among the potyviruses. These included (i) a DAG tripeptide located at the N-terminal region, which is involved in aphid transmission (Atreya *et al.*, 1991), (ii) a WCIE/DN box located in the conserved core region with an unknown function (Pappu *et al.*, 1993) and (iii) AFDF and RQ sequences found at the C-terminal region of the CP which are critical for coat protein structure and assembly (Jagadish *et al.*, 1993). The maximum variability in the amino acid sequences in the complete CP-coding region between all isolates from countries in the South Pacific and Vietnam was 21.9%. When the highly variable N terminus sequence was removed and analysis done on the core region, the maximum variability dropped to 6%. The high variation observed in the entire CP-coding region is reflective of the variability in the length of the amino terminal sequences of DsMV coat proteins (Pappu *et al.* 1994a).

Phylogenetic analysis based on the complete amino acid sequence in CP-coding region revealed that most isolates grouped according to the country from which they were isolated. However, caution must be taken when interpreting these results due to the small number of isolates obtained from many countries.

The second part of this study was to express a recombinant DsMV coat protein, use the antigen to generate antiserum and incorporate the antiserum into an ELISA for serological detection of DsMV in infected taro plants. Based on the amino acid sequence variability, the sequence of PNG isolate P1 was found have most similarity to all other sequences and was deemed to be the most suitable sequence to express as a recombinant protein. However, due to the significant variability in the entire CP-coding region of all DsMV isolates, it was decided to express the more highly conserved CP-core region as antigen. Such a protein would be expected to share a greater number of epitopes with all other DsMV isolates, thus increasing the range of DsMV isolates recognised by the antiserum. The conserved core region of the CP-coding region of DsMV PNG-P1 was amplified, cloned into a protein expression vector and the recombinant protein was purified from the host *E. coli* cells. Although large amounts of fusion protein were expressed in bacterial cells, only small amounts of cleaved CP-core were eluted from the column. The reason for this was unknown, but it is likely that, following on-column cleavage, the CP reassembled and was unable to be eluted. A similar situation was reported by Smith *et al.* (1995) who were unable to elute recombinant SCMV CP from an ion-exchange chromatographic column unless a chaotropic agent (urea) was added. The assembly of potyviral CP into proviral particles has been reported in preparations containing PVY CP (McDonald *et al.*, 1976), and recombinant JGMV CP (Jagadish *et al.*, 1991). To obtain sufficient quantities of DsMV CP-core to use as antigen, columns were washed with an SDS solution following the on-column cleavage reaction, and all proteins were eluted. The CP-core was subsequently separated from other proteins by SDS-PAGE and the band was excised.

The purified recombinant DsMV CP-core was administered intramuscularly into the rabbit at successive intervals to produce antiserum. The antiserum derived from the final bleed had a titre of 1:1,000 and 1:10,000 when used in ELISA and Western blot formats, respectively. The high titres obtained in this study are not

surprising since potyviral CP is known to be a good immunogen (Shukla *et al.* 1994). The antiserum was initially tested by Western analysis using protein extracts from known DsMV-infected and healthy plants. The antiserum primarily reacted with 2-3 proteins with sizes ranging from 45-47 kDa; these bands are consistent with the size of the DsMV CP (Abo El-Nil *et al.*, 1977; Pappu *et al.* 1994b; Li *et al.*, 1998) indicating that the antiserum was reacting specifically with the DsMV CP. The antiserum was subsequently incorporated into an ELISA format using known DsMV-infected and healthy plant samples. Using an indirect ELISA, strong positive reactions were observed from the DsMV-infected samples while only faint background was observed from the healthy samples. The ELISA was also shown to be very sensitive, detecting DsMV in the highest sap dilutions of 1/1000. Further, the antiserum detected a wide range of DsMV isolates including samples from Australia, PNG, New Zealand, Fiji, French Polynesia, New Caledonia, Samoa, Solomon Islands and Vanuatu all tested positive. The DsMV status of these plants was verified using RT-PCR. Unfortunately, the antiserum was not DsMV specific and reacted with sap from plants infected with other potyviruses including SCMV, PRSV-P and PRSV-W, but did PVY and CMV. Similar cross-reactivity has also been reported using an antiserum raised against an entire intact recombinant DsMV CP (Li *et al.* 1998) and would be expected considering the relatively high conservation of sequences (and thus epitopes) in the CP-core of potyviruses. Although not DsMV specific, the use of such antiserum in virus-indexing schemes may be advantageous since other, as yet uncharacterised potyviruses, may be detected.

In conclusion, the major outcome of this research has been the development of a sensitive ELISA for the detection of a wide range of geographical isolates of DsMV. Such a test is ideally suited for Pacific Island laboratories since it is sensitive, reliable, relatively inexpensive, and does not require sophisticated equipment or highly trained personnel. Importantly, the DsMV ELISA will have utility not only in the indexing of taro prior to international movement, but can also be used on a national level to screen locally generated tissue-cultured taro cultivars prior to release to farmers. An alternative and longer term strategy to control DsMV is via genetic engineering using pathogen-derived resistance (PDR) strategies (Pappu *et al.*, 1995). The suite of CP-coding sequences generated in this study will be extremely useful in achieving future PDR against DsMV because the success of such strategies is influenced by the

sequence variability between the expressed transgene and that of the challenging virus (Tennant *et al.*, 1994). The recent report of a transformation system for taro also means that this approach is getting closer to becoming a reality (Fukino *et al.*, 2000).

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