COMPLETE SEQUENCE, IMPROVED DETECTION AND FUNCTIONAL ANALYSIS OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 1* (GLRaV-1)

Thesis submitted for the Degree of
Doctor of Philosophy
at the University of Adelaide

by

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SUMMARY

Leafroll is one of the most important virus diseases in Australia and among nine viruses currently associated with this disease, *Grapevine leafroll-associated virus 1* (GLRaV-1) is the most damaging, causing both a loss of yield and quality as well as being involved in a range of graft incompatibility problems. Leafroll disease is found wherever grapes are grown and occurs in all varieties of grapevines. The disease has been reported in Canada, Europe, New Zealand, South Africa, the United States and Australia. Currently, the most widely reported leafroll viruses worldwide are GLRaV-1 and GLRaV-3.

The specific objectives of the work described in this thesis include the:

1. Completion of the GLRaV-1 genome sequence and bioinformatic analysis of the viral open reading frames.
2. Production of an appropriate GLRaV-1 certification protocol addressing the shortcomings of the current tests for leafroll detection.
3. Intracellular localisation of the GLRaV-1 gene products via the generation of green fluorescent protein (GFP)-fusion constructs, in an attempt to further characterise the function of these proteins.

In this study the 17,647-nucleotide genome sequence of GLRaV-1 was completed and sequence data analysed. The 5'-part of the genome encodes the putative replication complex consisting of the methyltransferase (MTR), helicase (HEL) and RNA dependent RNA polymerase (RdRp) domains, with the latter domain likely to be expressed via a +1 ribosomal frameshift. GLRaV-1 contains 8 more open reading

frames (ORF) encoding in the 5' to 3' direction a small hydrophobic protein, a heat shock protein 70 (HSP70) homologue, a HSP90-like protein, the coat protein (CP), two minor copies of the CP (CPm1 and CPm2) and two other proteins of unknown function. Unlike other members of the family *Closteroviridae*, the duplication of the GLRaV-1 CP gene occurs in two ORFs. The overall organisation of the GLRaV-1 genome is similar to those of other members of the family *Closteroviridae* and it is most closely related to GLRaV-3.

The shortcomings of the current certification protocols for detection of GLRaV-1 were addressed. The current RT-PCR test was inadequate due to poor reliability and reproducibility of results, presumably due to sequence variation within the virus, targeting regions of low copy number and inhibition from excess RNA and impurities such as phenolics. The method for purifying the viral RNA has been optimised using magnetic capture hybridisation (MCH) prior to RT-PCR. This process removes any potential RT-PCR inhibitors from the sample, therefore improving reliability. This procedure also concentrates the RNA allowing addition of at least 100 fold more RNA into the reaction, therefore increasing sensitivity. The potential problem associated with viral sequence variation has been avoided by targeting primers to ORF9, a highly conserved region of the genome. This has also provided the benefit of targeting a region of the viral genome with high copy number due to the presence of ORF9 on all 3' subgenomic RNAs.

The magnetic capture RT-PCR technique has been tested on a range of different grapevine varieties from the field. 28 samples were screened for the presence of GLRaV-1 showing 9 positives using the current RT-PCR and ELISA tests. The use of

magnetic capture prior to RT-PCR allowed the detection of 16 positive samples. The MCH-RT-PCR technique provides an effective and practical screen to identify grapevine samples infected with GLRaV-1.

Research aimed at further characterising the functions of proteins encoded by GLRaV-1 has been completed. The localisation pattern of a protein with a cell can suggest possible roles for the gene in the virus life cycle. Studies involving ORF fusions with green fluorescent protein have highlighted a number of protein including ORFs 2, 3, 6 and 9, which seem to be involved in virus movement and maintenance. ORF2 appears to alter the internal structure of the cell while ORF9 is targeted to the nucleus suggesting both viral proteins are interacting with plant host factors. These results have been repeated *in planta* using confocal microscopy and transmission electron microscopy.

All the project objectives have been achieved and the research has been collated into two papers for publication in scientific journals. This work has also been presented at recent national and international conferences.

Publications

(publications arising from the work in this thesis are marked with an asterisk, *)

Book chapters

Rezaian M.A. and Little A. (2004). Grapevine viroids and viroid diseases, In Compendium of Grape Diseases. Ed. by Uyemoto J.K. APS publishing.

Little A. and Rezaian M. A. (2003). Grapevine viroids, In Viroids. Ed. by Hadidi A., Flores R., Randles J. and Semancik J. CSIRO Publishing. Australia. Pp 195-206.

Journal articles

* Little A, and Rezaian M. A. (2004). Subcellular localisation of proteins encoded by the *Grapevine leafroll-associated virus 1* reveals a vesicle inducing protein. To be submitted to *Virology*. Internal review.

* Little A, and Rezaian M. A. (2004). Improved detection *of Grapevine leafroll-associated virus 1* by magnetic capture hybridisation RT-PCR. To be submitted to *Journal of Virological Methods*. Internal review.

Little A., Fazeli C. F., and Rezaian M. A. (2001). Hypervariable genes in *Grapevine leafroll-associated virus 1*. *Virus Research* 80(1-2): 109-116.

Conference papers

* Little, A. and Rezaian, M. A. Completed sequence, improved detection and gene functional analysis of *Grapevine leafroll-associated virus 1*. Oral presentation at the 2nd Australian Virology Group meeting, Fraser Island, Australia, December 2^{nd}-6^{th} 2003.

* Little, A. and Rezaian, M. A. Completed sequence, improved detection and gene functional analysis of *Grapevine leafroll-associated virus 1*. Oral presentation at the 14th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Bari, Italy, September 12^{th}-17^{th} 2003.

Little, A., Fazeli, C. F. and Rezaian, M. A. Hypervariable genes in *Grapevine leafroll-associated virus 1*. Poster presentation at the 1st Australian Virology Group meeting, Fraser Island, Australia, December 5th-9th 2001.

Little, A., Fazeli, C. F. and Rezaian, M. A. Hypervariable genes in *Grapevine leafroll-associated virus 1*. Poster presentation at the 13th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Adelaide, Australia, March 12th-17th 2000.

STATEMENT

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan.

Alan Little

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To my family and all my friends, I thank you for your constant support and encouragement. I wish to show my appreciation to my Granda and Nan, who both recently passed away, for continually praising my academic and sporting achievements.

Most importantly, I thank my wonderful wife Claire for her love and support.

GENERAL INTRODUCTION

ONE

GRAPEVINE LEAFROLL DISEASE

1.1 INTRODUCTION

Leafroll is an important disease of grapevines, accounting for 62% of the world losses of grape production due to viruses (Walter and Martelli, 1997). The disease may cause crop loss, reduced sugar content of fruit, delayed fruit maturity, abnormal leaf coloration and rolling of leaves (Goheen, 1970, Bovey *et al.*, 1980, Bovey and Martelli, 1992). The disease was initially thought to be a deficiency in potassium (Ravaz *et al.*, 1933) until the association of viruses with leafroll was established (Goheen and Cook, 1959; Scheu, 1936; Vuittenez, 1958). Nine serologically distinct viruses are associated with leafroll disease and they are known as *Grapevine leafroll-associated virus* (GLRaV) *1-9* (Boscia *et al.*, 1995; Choueiri *et al.*, 1996; Gugerli *et al.*, 1984; Zimmerman *et al.*, 1990).

1.2 SYMPTOMS.

The symptoms associated with leafroll disease are different in red and white varieties of *Vitis vinifera* (Fig 1-1). In infected red varieties, such as Cardinal, Mission, Queen, Red Malaga, Tokay and Pinot Noir, the lower leaves redden and roll downwards. At maturity they become thick and brittle (Hoefert and Gifford, 1967). White varieties, such as Sultana, show symptoms similar to those on red varieties except that the infected leaves turn yellow instead of red before the onset of senescence (Ravaz and Verge, 1924). Symptoms can be observed from late summer onwards. No symptoms are evident in rootstocks, which can be symptomless carriers of leafroll viruses (Hewitt *et al.*, 1962).

FIGURE 1-1

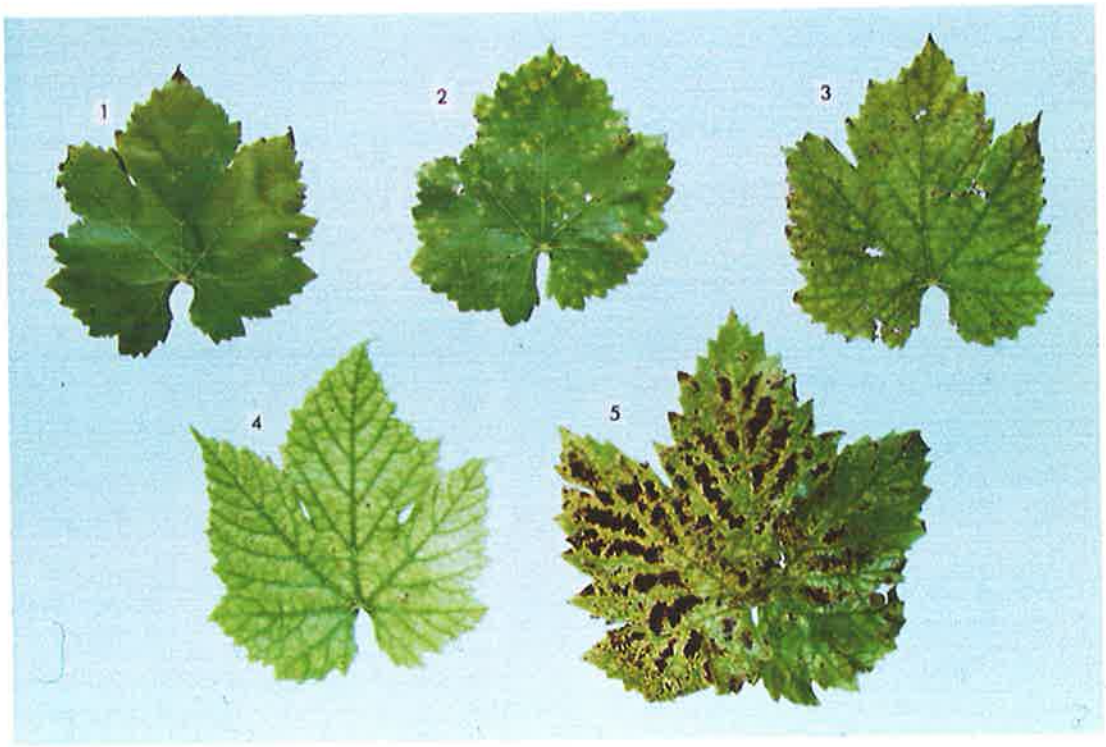
SYMPTOMS OF LEAFROLL DISEASE.

Variants of leafroll disease in Sultana (A) and Cabernet Franc (B)
(reproduced from Krake *et al.*, 1999).

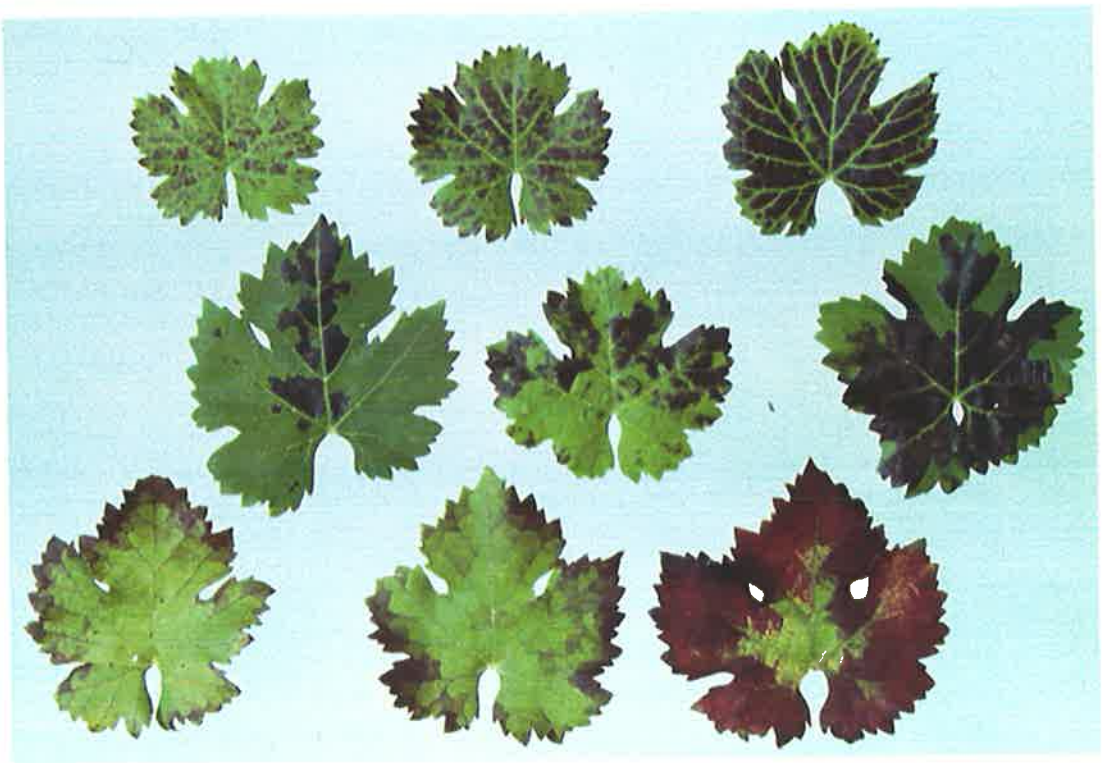
a- By early autumn, the diseased white-fruited cultivars show leaves with some degree of a green-vein pattern with or without an associated rolling of the leaves. This green-vein pattern is caused when the areas between the green main veins become yellow.

b- During late summer and early autumn the infected red- and black-fruited vines display leaves with red and reddish-purple tints and green-vein or red-vein patterns.

a



b



Leafroll virus infection induces distinctive cytological effects within the grapevine. These include the appearance of virus aggregates and vesicles in the phloem cells (Casellano *et al.*, 1985). They appear either singly or in groups, sometimes surrounded by a common membrane, possibly of mitochondrial or endoplasmic reticulum origin, and intermingled with the virus particles and other cytoplasmic components (Bar-Joseph *et al.*, 1995). These structures are referred to as *Beet yellows virus*-type (BYV-type) vesicles (Agranovsky, 1996). BYV-type vesicles are considered an important taxonomic feature of the family *Closteroviridae* and are used for diagnosis of the disease and classification of viruses within the family. It has been suggested that the finely stranded content of the BYV-type vesicles is viral RNA (Faoro and Carzaniga, 1995).

1.3 DISTRIBUTION.

Leafroll disease is found wherever grapes are grown (Goheen, 1970) and occurs in all varieties of grapevines (Bovey *et al.*, 1980). The disease has been reported in numerous regions including Canada (MacKenzie *et al.*, 1996), Europe (Gugerli *et al.*, 1984; Rosciglione and Gugerli, 1987; Tanne *et al.*, 1989), New Zealand (Peterson and Charles, 1997), South Africa (Engelbrecht and Kasdorf, 1990), the United States (Golino *et al.*, 1994; Teliz *et al.*, 1987) and Australia (see below). Currently, the most commonly reported leafroll viruses worldwide are GLRaVs 1 (GLRaV-1) and 3 (GLRaV-3) (Boscia *et al.*, 1995).

1.3.1 OCCURRENCE OF GRAPEVINE LEAFROLL IN AUSTRALIA.

Woodham *et al.* (1973) first described grapevine leafroll disease in Australia through symptomatology. The disease was probably introduced in grapevine cuttings from the U.S.A., Germany, France, South Africa, Japan and Canada after European settlement (Kerridge and Antcliff, 1996; Norrie, 1992). A virus-indexing program was established in 1974 as a part of the South Australian Vine Improvement Scheme to assess the virus content of all varieties intended for distribution to industry. This program showed that leafroll disease was present in a number of varieties of grapevines in Australia (Cirami *et al.*, 1988). No evidence of the GLRaVs occurring in these grapevines was presented. The incidence of leafroll in Australian vineyards has been increasing over the last decade. In 1997, Habili *et al.* hypothesised that the spread of leafroll may be related to a change in the transmission mode of GLRaVs. Through the use of nucleic acid-based techniques the spread of leafroll was found to be associated with GLRaV-3 (Habili *et al.*, 1995).

1.4 TRANSMISSION.

Apart from transmission via vegetative propagation and grafting GLRaV-1 and GLRaV-3 spread naturally. Experimentally, GLRaV-1 can be transmitted by the scale insects *Neopulvinaria innumerabilis* and *Parthenolecanium corni* (Fortusini *et al.*, 1997) and by the mealy bugs *Heliococcus bohemicus* and *Phenacoccus aceris* (Sforza *et al.*, 2000). The natural vector of GLRaV-2 is unknown (Zhu *et al.*, 1998), however, this virus has been transmitted by inoculation of sap from infected grapevine to *Nicotiana benthamiana* (Castellano *et al.*, 1995). GLRaV-3 can be transmitted experimentally by five species of mealy bugs (*Homoptera: Pseudococcidae*) (Rocsiglione and Gugerli, 1987) and scale insects (*Homoptera: Coccidae*) belonging to the genera *Eulecanium* and

Pulvinaria (Belli *et al.*, 1994). Vector-mediated transmission of GLRaV-5 has also been demonstrated by the mealy bug *Pseudococcus longispinus* (Golino *et al.*, 2002). However to date, none of these insect vectors have been found in the South Australian vineyards where GLRaV-3 spreads, and it is likely that other vectors may be involved (Habili *et al.*, 1995).

1.5 DETECTION AND CONTROL.

There is a requirement in the grapevine industry for the testing of grapevine material for the presence of leafroll-associated viruses. It was recommended by the general assembly of the international council for the study of virus and virus-like diseases of the grapevine that in order to preserve valuable grape clones and varieties certified selections should be tested for specific pathogens. Therefore only grape nursery stock which tests negative for the most damaging pathogens should be used. As for other countries, Australia conducts large-scale screening of planting material with the aim of eliminating Leafroll disease from the field. These tests are based on the observation of leafroll symptoms and the detection of GLRaV coat proteins or nucleic acids.

Symptomatology has traditionally been employed through biological indexing (Goheen, 1970; Woodham *et al.*, 1984). In this procedure, sensitive grapevine varieties are graft-inoculated with candidate-infected material and the development of leafroll symptoms is observed over an eighteen-month period. In addition, the infected grapevine tissue is tested for the presence of dsRNA (Habili *et al.*, 1992; Rezaian *et al.*, 1991), which is normally produced from the viral genomic RNA during its replication cycle (Valverde, 1990).

Detection of GLRaV coat protein (CP) may be achieved using specific antibodies. In the past decade, antibodies against certain GLRaV CPs have been produced (Gugerli and Ramel, 1993; Hu *et al.*, 1990a; Zimmermann *et al.*, 1990). Thus, certain GLRaVs can be detected through ELISA and Western blotting. These tests are extremely useful in speeding up the detection process. However, they may not be sensitive enough to detect GLRaVs in newly infected grapevines, and also the results for some GLRaVs may not be reproducible (Habili *et al.*, 1996).

A more sensitive technique to detect GLRaVs in infected tissues is based directly on the viral nucleic acids (Saldarelli *et al.*, 1994). A requirement for this technique is the production of nucleic acid probes to specifically target the genomic RNA of each GLRaV. However, virus purification from grapevines is hampered by the presence of phenolic compounds, which inhibit the extraction of nucleoproteins (Loomis, 1974) and are liable to interfere with the enzyme system used for RT and PCR reactions limiting the efficiency of RT-PCR (Minafra and Hadidi, 1994).

Removing infected grapevines from the vineyards and preventing the planting of already infected material by the above procedures are the means of avoiding leafroll infection in the field. However, the types of leafroll disease that spread naturally (eg. GLRaV-3) cannot be controlled until their biological vectors are eliminated or virus resistant grapevines are produced. Effective control of GLRaV-1 in Australia and worldwide will require further understanding of the basic processes involved in the replication and cell-cell movement of the viral genome.

TWO

GRAPEVINE LEAFROLL-ASSOCIATED VIRUSES

2.1 CLASSIFICATION.

GLRaVs are recognised as true members of the family *Closteroviridae* by the International Committee on Taxonomy of Viruses (ICTV) (van Regenmortel *et al.*, 2000). This classification is based on morphology, cytopathic effects and genome organisation. The family *Closteroviridae* consists of twenty-one members and seventeen tentative members (Table 2-1). Recently described molecular and biological information has prompted the revision of the taxonomic structure of the family *Closteroviridae*. In particular, mealybug transmitted species, such as GLRaV-1, have been separated from the genus *Closterovirus* (from *Kloster*, Greek for thread) and placed in the new genus *Ampelovirus* (from *Ampelos*, Greek for grapevine) (*Martelli et al.*, 2002). From this point references regarding closteroviruses refer to the family *Closteroviridae*.

Viruses previously classified in as closteroviruses, such as *Apple chlorotic leaf spot virus* (ACLSV), *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB) have been reclassified in the genus *Trichovirus* (Candresse *et al.*, 1995) with GVA and GVB reclassified again in the genus *Vitivirus* (Martelli *et al.*, 1997). The morphology of closteroviruses is similar to that of trichoviruses and vitiviruses, but trichoviruses and vitiviruses do not produce BYV-type vesicles (Agronovsky, 1995). These two groups share little sequencc homology and major differences in their genome organisation and replication strategy have been found (Candresse and Martelli, 1995).

2.2 MORPHOLOGY AND COMPOSITION.

GLRaV particles have the unique shape of closteroviruses, which are the largest known plant viruses (Dolja *et al.*, 1994; Martelli and Bar-Joseph, 1991). The thread-like structure of GLRaVs forms a flexuous filament of 1800-2200 nm long and about 12 nm in diameter (Candresse and Martelli, 1995; Gugerli *et al.*, 1984; Hu *et al.*, 1990b). Their particles sediment as a single component during sucrose or caesium sulphate gradient centrifugation. Generally, the sedimentation coefficient ($S_{20,W}$) ranges from 96 to 140, and buoyant density in caesium sulphate is 1.24-1.27 g/cm^3. Particles of GLRaVs 1, 2, 3 and 4 remain intact for 3 months when stored at 4°C or -20°C in buffers containing low salt concentrations (Hu *et al.*, 1990b). GLRaVs contain a positive sense, single-stranded genomic RNA (ssRNA), which constitutes 5-6% of the virion weight (Candresse and Martelli, 1995). The RNA is encapsidated by two types of protein subunits, the coat protein and a divergent copy of the coat protein (Agronovsky *et al.*, 1995).

Table 2-1: Members and tentative members of the family *Closteroviridae* (Martelli *et al.*, 2002)

Genus *Closterovirus*		**Genus *Ampelovirus***		**Genus *Crinivirus***	
Aphid-transmitted		**Mealy bug-transmitted**		*Lettuce infectious yellows virus (tm)*	LIYV
Beet yellows virus (type member)	BYV	*Grapevine leafroll-associated virus 3 (tm)*	GLRaV-3	*Abutilon yellows virus*	AbYV
Beet yellows stunt virus	BYSV	*Grapevine leafroll-associated virus 1*	GLRaV-1	*Cucurbit yellow stunting disorder virus*	CYSDV
Burdock yellows virus	BuYV	*Grapevine leafroll-associated virus 5*	GLRaV-5	*Lettuce chlorosis virus*	LCV
Carnation necrotic fleck virus	CNFV	*Little cherry virus 2*	LChV-2	*Sweet potato chlorotic stunt virus*	SPCSV
Carrot yellow leaf virus	CYLV	*Pineapple mealy bug wilt-associated virus 1*	PMWaV-1	*Tomato chlorosis virus*	ToCV
Citrus tristeza virus	CTV	*Pineapple mealy bug wilt-associated virus 2*	PMWaV-2	*Tomato infectious chlorosis virus*	TICV
Wheat yellow leaf virus	WYLV				
		Tentative species (Mealy bug-transmitted)		**Tentative species**	
Vector Unknown		Sugarcane mild mosaic virus	SMMV	Beet pseudo-yellows virus	BPYV
Grapevine leafroll-associated virus 2	GLRaV-2			Diodia vein chlorosis virus	DVCV
		Tentative species (Vector unknown)		Potato yellow vein virus	PYVV
Tentative species (Aphid-transmitted)		Grapevine leafroll-associated virus 4	GLRaV-4		
Clover yellows virus	CYV	Grapevine leafroll-associated virus 6	GLRaV-6	**Unassigned species**	
Dendrobium vein necrosis virus	DVNV	Grapevine leafroll-associated virus 8	GLRaV-8	Alligatorweed stunting virus	AWSV
Heracleum virus 6	HV-6	Plum bark necrosis and stem pitting-associated virus	PBNSPaV	Grapevine leafroll-associated virus 7	GLRaV-7
Festuca necrosis virus	FNV			Little cherry virus 1	LChV-1
				Megakepasma mosaic virus	MegMV
				Olive leaf yellowing-associated virus	OLYaV

2.3 GENOME ORGANISATION.

To date, there is little information available about the genome organisation of GLRaVs, therefore, the genome organisation of closteroviruses in general is reviewed.

The genomes of closteroviruses are comparable to the genomes of some animal viruses, which possess a positive-sense ssRNA genome of ca. 30 kilobases (kb) such as corona- and toroviruses (Lee *et al.*, 1991; Snijder *et al.*, 1988; Spaan *et al.*, 1988). So far, the genomic RNA of five closteroviruses has been completely sequenced with genome sizes between 10 and 20kb. These viruses are *Grapevine leafroll-associated virus 3* (GLRaV-3) (Ling *et al.*, 2004), *Beet yellows virus* (BYV) (Agranovsky *et al.*, 1991; Agranovsky *et al.*, 1994), *Citrus tristeza virus* (CTV) (Karasev *et al.*, 1995; Pappu *et al.*, 1994), *Little cherry virus* (LCV) (Jelkmann *et al.*, 1997; Keim-Konrad and Jelkmann, 1996) and *Lettuce infectious yellows virus* (LIYV) (Klaassen *et al.*, 1995). Sequence analysis of these viruses has shown that GLRaV-3, BYV, CTV and LCV have single RNA genomes, whereas, LIYV has a bipartite genome. Partial sequence information is also available (Table 2-2) for *Beet yellows stunt virus* (BYSV) (Karasev *et al.*, 1996), Pineapple mealybug wilt-associated virus-2 (Melzer *et al.*, 2001), GLRaV-1 (Fazeli *et al.*, 2000), and GLRaV-2 (Abou-Ghanem *et al.*, 1998; Zhu *et al.*, 1998).

2.3.1 THE 3'-END AND 5'-END NON-CODING REGIONS.

Nucleotide sequence analyses of the closterovirus genomes have revealed that their genomic RNAs contain non-coding regions (NCR) at the 5' or 3'-ends. These regions vary in size and sequence in different viruses (Table 2-2). The 3'-NCR in known closteroviruses lacks a poly(A) tail. Furthermore, a 60 nt stretch and potential stem-loop structures are shared among the 3'NCRs of GLRaV-2, BYSV, BYV and CTV.

Table 2-2: Summary of published genome organisations of the family *Closteroviridae*.

	BYV (Agranovsky *et al.*, 1991; Agranovsky *et al.*, 1994)	GLRaV-2 (Zhu *et al.*, 1998)	CTV (Karasev *et al.*, 1998; Pappu *et al.*, 1994)	BYSV (Karasev *et al.*, 1996)	LCV (Jelkmann *et al.*, 1997; Kelm-Konrad and Jelkmann, 1996)	LIYV (Klaassen *et al.*, 1995)		GLRaV-3 (Ling *et al.*, 1998)	GLRaV-1 (Fazeli *et al.*, 2000)	PMWaV-2 (Melzer *et al.*, 2001)
GENOME	Undivided	Undivided	Undivided	Undivided	Undivided	Bipartite		Undivided	Undivided	Undivided
SEQUENCE	Complete	Incomplete (15,000nt)	Complete	Incomplete (10,545nt)	Complete	Complete		Complete	Incomplete (12,394 nt)	Incomplete (14,861nt)
SIZE (nt)	15,480	Ca. 16,000	19,296	N/R	16,934	RNA 1 : 8118	RNA 2 : 7193	17,919	Ca. 19,500	Ca. 16,000
ORFs	9	9	12	10	9	3	7	13	10	10
5'-TERMINUS	Capped with a m^7Gpp analog (Karasev et al., 1989)	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
5'-NCR	107 nt	N/R	108 nt	N/R	76 nt	97 nt	326 nt	158 nt	N/R	N/R
ORF	1a (295 K): P-Pro, MTR, HEL	1a (incomplete): P-Pro, MTR, HEL	1a (349 K): P-Pro (duplicated), MTR, HEL	1a (Incomplete): HEL	1a (260 K): P-Pro, MTR, HEL	1a (217.3 K): P-Pro, MTR, HEL		1a (306 K): P-Pro, MTR, HEL	1a (Incomplete): HEL	1a (Incomplete): P-Pro, MTR,HEL
ORF	1b (53 K): POL	1b (52 K): POL	1b (57 K): POL	1b (52 K): POL	1b (59K): POL	1b (55.5 K): POL		1b (61K): POL	1b (60 K): POL	1b (65 K): POL
ORF			2 (33K): Unknown	2 (30 K): Unknown		3 (32 K): Unknown		2 (6 K): Unknown		
ORF	2 (6.4 K): Hydrophobic	2 (6 K): Hydrophobic	3 (6 K): Hydrophobic	3 (6 K): Hydrophobic	2 (4 K): Hydrophobic		1 (5 K): Hydrophobic	3 (5 K): Hydrophobic	2 (7 K): Hydrophobic	2 (5 K): Hydrophobic
ORF	3 (65 K): HSP70 homologue	3 (65 K): HSP70 homologue	4 (65 K): HSP70 homologue	4 (66 K): HSP70 homologue	3 (70 K): HSP70 homologue		2 (62 K): HSP70 homologue	4 (59 K): HSP70 homologue	3 (60 K): HSP70 homologue	3 (59 K): HSP70 homologue
ORF	4 (64 K): HSP90 homologue	4 (63 K): HSP90 homologue	5 (61 K): HSP90 homologue	5 (61 K): HSP90 homologue	4 (61 K): Unknown		3 (59 K): Unknown	5 (55 K): HSP90 homologue	4 (55 K): HSP90 homologue	4 (46 K): Unknown
ORF	5 (24 K): CPm	5(25 K): CPm	6 (27 K): CPm	6 (25 K): CPm	5 (46 K): CP		5 (27 K): CP	6 (35 K): CP	5 (36 K): CP	5 (34 K): CP
ORF	6 (22 K): CP	6 (22 K): CP	7 (25 K): CP	7 (24 K): CP	6 (76 K): CPm		6 (52 K): CPm	7 (53 K): CPm	6 (56 K): CPm1	6 (56 K): CPm1
ORF									7 (50 K): CPm2	
ORF	7 (20 K): Unknown	7 (19 K): Unknown	8 (18 K): Unknown	8 (18 K): Unknown	7 (21 K): Unknown		7 (26 K): Unknown	8 (21 K): Unknown	8 (26 K): Unknown	7 (20 K): Unknown
ORF	8 (21 K): Unknown	8 (24 K): Unknown	9 (13 K): Unknown	9 (22 K): Unknown	8 (27 K): Unknown			9 (20 K): Unknown	9 (24 K): Unknown	8 (22 K): Unknown
ORF			10 (20 K): Unknown					10 (20 K): Unknown		9 (6 K): Unknown
ORF			11 (23 K): RNA-binding protein					11 (4 K): Unknown		
								12 (7 K): Unknown		
3'-NCR	181 nt: a cis-element, two potential stem-loop structures	216 nt: a cis-element, a stem-loop structure	277 nt: a cis-element, several potential stem-loop structures	241 nt: a cis-element, a stem-loop structure	210 nt: a cis-element, a stem-loop structure	219 nt: no secondary structure	187 nt: no secondary structure	277 nt: no secondary structure	363 nt: several potential stem-loop structures	132 nt: several potential stem-loop structures
3'-TERMINUS	No poly(A) tract	No poly(A) tract	No poly(A) tract	No poly(A) tract	No poly(A) tract	No poly(A) tract	No poly(A) tract	No poly(A) tract	No poly(A) tract	

N/R: not reported

(Agronovsky *et al.*, 1991; Karasev *et al.*, 1996; Pappu *et al.*, 1994; Zhu *et al.*, 1998). In BYV, *in vitro* aminoacylation or adenylation of the 3'-NCR has not been successful, which suggests that there is no 3'-terminal tRNA-like structure (Agronovsky *et al.*, 1991).

Studies of secondary structure of the 5'UTR of CTV by a combination of sequence comparison and computer predictions has led to a general model with two stem loops (Lopez *et al.*, 1998). Most of the sequence variability seen in CTV isolates is accommodated in the loops and, when found in the stems, occurred in such a way that compensatory mutations maintained their general features. This conservation in secondary structure by covariation strongly supports a functional role *in vivo* for it.

2.3.2 THE 5' ORFS.

The first ORF (ORF1a) at the 5' region of closteroviruses generally codes for a polyprotein (Table 2-2) (Dolja *et al.*, 1994; Goldbach, 1990; Koonin and Dolja, 1993). This polyprotein contains papain-like protease (P-Pro), methyl-transferase (MTR) and RNA helicase (HEL) motifs. Downstream of ORF1a, ORF1b codes for an RNA-dependent RNA polymerase (POL). The MTR, HEL and POL domains are responsible for virus replication (Koonin and Dolja, 1993; Peremyslov *et al.*, 1998). These domains contain conserved sequence motifs and are similarly arranged in all known closteroviruses (Fig 4-1) (Dolja *et al.*, 1994).

In vitro translation experiments on BYV ORF1a have demonstrated an autocatalytic cleavage of the P-Pro domain (Dolja *et al.*, 1994). The predicted catalytic residues of this protease are Cys_{509} and His_{569}, which cleave between two Gly residues in BYV

(Agranovsky *et al.*, 1994). Amino acid sequences around the catalytic Cys residue are conserved in the P-Pro of positive strand RNA viruses (Gorbalenya *et al.*, 1991). These similarities have shown that BYV P-Pro is distantly related to helper component proteases (HC-Pro) of potyviruses and enhances the level of virus replication (Atreya *et al.*, 1992; Dolja *et al.*, 1993). The P-Pro domain in BYV may have similar functions, which could be important as HC-Pro is known to be a strong suppressor of post-transcriptional gene silencing (Anandalakshmi *et al.*, 1998). CTV ORF1a codes for two putative P-Pro domains that show significant similarity to each other and to the corresponding domain of BYV. This has allowed the prediction of the catalytic cysteine and histidine residues as well as the cleavage sites for both of the putative CTV proteases (Karasev *et al.*, 1995).

2.3.3 OTHER ORFS.

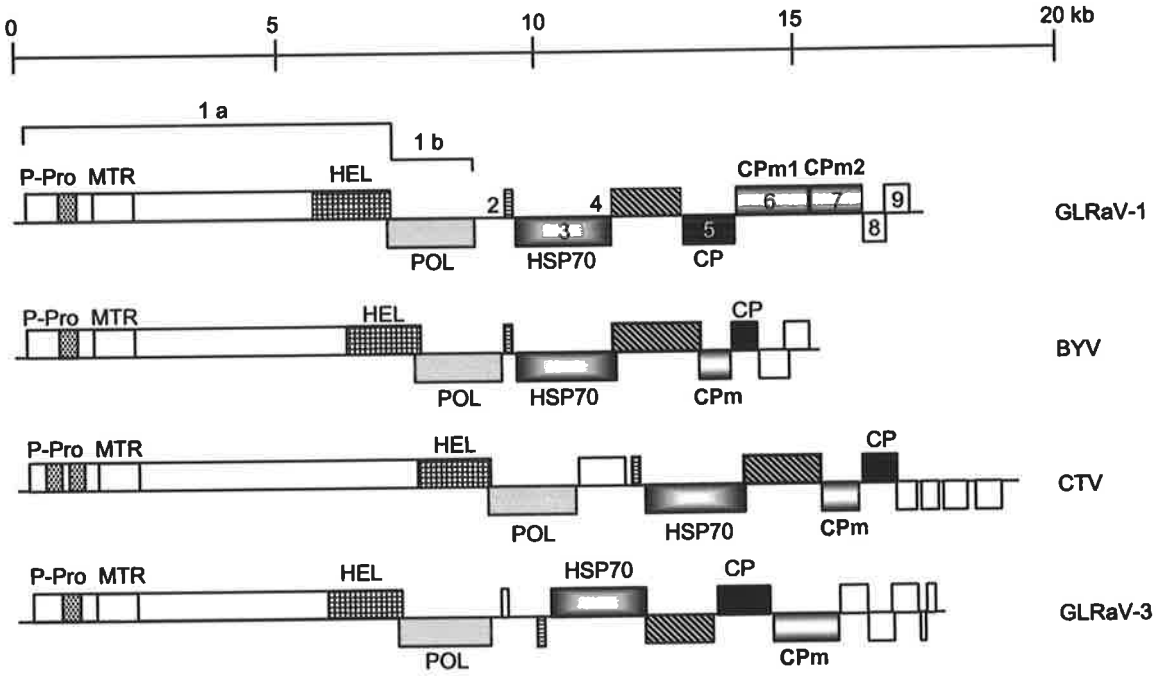
Downstream from the POL gene in CTV, a gene coding for a M_r 33,000 product (ORF2, Fig 4-1) is present which has no homologue in BYV (Pappu *et al.*, 1994). The putative product of this gene, with respect to size and location, is analogous to the M_r 32,000 product of ORF3 or LIYV RNA 1 and M_r 30,382 product of BYSV. However, there is no conservation in the amino acid sequences of these proteins.

The 3' half of the closterovirus genomic RNA has a unique gene array containing a five-gene module (Fig 4-1) (Dolja *et al.*, 1994). The first gene of this module encodes a small product with a M_r of ca. 6,000 (Table 2-2). This protein generally contains a highly hydrophobic N-terminal domain, which in the case of BYV has been shown to have membrane-binding properties. It has been suggested that this protein participates in cell-to-cell movement of the virus (Agronovsky *et al.*, 1991). The second gene codes for

FIGURE 4-1

COMPARISON OF THE GENOME ORGANISATION OF GLRaV-1 WITH THOSE OF REPRESENTATIVE SPECIES IN THE FAMILY *CLOSTEROVIRIDAE*.

Rectangles represent ORFs and homologous genes are shaded similarly. Open boxes indicate genes with no statistical similarity to other proteins in existing databases. P-Pro: papain-like protease, MTR: methyl transferase, HEL: helicase, POL: RNA-dependent RNA-polymerase, HSP70: homologue of HSP70 proteins, CP: coat protein, CPm: minor copy of coat protein.


0
5
10
15
20 kb
1 a
1 b
P-Pro
MTR
HEL
POL
2
HSP70
4
3
5
CP
CPm1
CPm2
6
7
8
9
GLRaV-1
P-Pro
MTR
HEL
POL
HSP70
CP
CPm
BYV
P-Pro
MTR
HEL
POL
HSP70
CP
CPm
CTV
P-Pro
MTR
HEL
POL
HSP70
CP
CPm
GLRaV-3

a homologue of the HSP70 family of heat shock proteins. Amino acid sequence analysis has shown that the HSP70 homologues of closteroviruses are closely related to each other, but they are distant from the cellular members of this family (Dolja *et al.*, 1994). It has been suggested that the HSP70 homologues of closteroviruses participate in cell-to-cell movement and protein-protein interactions (Karasev *et al.*, 1992). Closteroviruses are the only viruses known to encode a HSP70 homologue protein and this is a decisive factor for their classification (Pappu *et al.*, 1994). The third gene encodes a protein with M_r of 58-64,000 that has similarities to movement-related proteins and the HSP90 family of heat shock proteins in certain closteroviruses (Table 2-2). The last two genes of this module encode a minor copy of coat protein (CPm) and a coat protein (CP). The arrangement of these two genes may vary in different closteroviruses with the CPm gene located upstream of the CP gene in BYV, GLRaV-2, CTV and BYSV, and downstream of the CP gene in GLRaV-1, GLRaV-3, LCV and LIYV (Fig 4-1). The genome of GLRaV-1 is unique amongst closteroviruses as it contains two minor coat proteins (Fazeli *et al.*, 2000).

Downstream of the five gene module, there are additional 3'-terminal ORFs (Fig 4-1). The number and sizes of these ORFs vary in different closteroviruses (Table 2-2). The putative products of these ORFs do not usually show a significant similarity to other proteins in the database, and their functions are not known. Only the CTV ORF11 (Table 2-2) at the 3'-end has been shown to be an RNA-binding protein (Lopez *et al.*, 2000). It has been suggested that this protein is unique to CTV and is involved in the regulation of asymmetrical RNA accumulation (Satyanarayana *et al.*, 2002). As is characteristic of RNA viruses, wild type CTV produced more positive than negative strands, with the plus-to-minus ratios of genomic and sgRNAs estimated at 10 to 20:1

and 40 to 50:1, respectively. However, a mutant with all of the 3' genes deleted replicated efficiently, but produced plus to minus strands at a markedly decreased ratio of 1 to 2:1.

2.4 VARIATION IN CLOSTEROVIRUS GENOMES.

Sequence diversity in plant RNA viruses has been well documented both at intraspecies and interspecies levels. Closteroviruses are the most heterogeneous group among plant viruses. Sequence diversity has been clearly seen in *Citrus tristeza virus* (CTV), an aphid-transmitted closterovirus. Sequence comparison of two CTV isolates revealed approximately 89% identity throughout the ten ORFs, but only 60-70% identity throughout ORF1 (Mawassi *et al.*, 1996). The low level of sequence identity between isolates of CTV seen in ORF1 was mainly due to two 18nt deletions in one of the isolates. Clustering of variation has also been reported in GLRaV-1 ORFs 3, 6 and 7 (Little *et al.*, 2001). The sequence variation was highest in ORF7, however, there have been no deletions seen in the 28 cDNA clones sequenced. The sequence identities of these clones compared to the previously published sequence range from 97% to as low as 59.5%, therefore showing a greater range of variation than previously reported for closteroviruses.

2.5 AIMS OF THIS STUDY.

The specific objectives of the work described in this thesis include the:

1. Completion of the GLRaV-1 genome sequence and bioinformatic analysis of the viral open reading frames.
2. Production of an appropriate GLRaV-1 certification protocol addressing the shortcomings of the current tests for leafroll detection.

3. Intracellular localisation of the GLRaV-1 gene products via the generation of green fluorescent protein (GFP)-fusion constructs, in an attempt to further characterise the function of these proteins.

MATERIALS & METHODS

THREE

MATERIALS & METHODS

3.1 INTRODUCTION.

This chapter includes the general materials and methods that were used in the various experiments. Most procedures were carried out according to Sambrook and Russell (2001) or to the manufacturers' specifications. Methods, which are significantly modified from their published form, are outlined. Materials and methods specific to particular experiments are described in the corresponding chapters. A general list of solutions and buffers used is included.

3.2 MATERIALS.

3.2.1 BUFFERS AND SOLUTIONS.

Contents of the buffers and solutions are summarised in Table 3-1. All chemicals were analytical or molecular biology grade. Solutions were prepared with nanopure or deionised water and autoclaved where appropriate.

Table 3.1 - Solutions and their compositions

Solution	Composition
10× agarose gel loading dye (DNA)	78% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 10 mM EDTA
10× agarose gel loading dye (RNA)	50% (v/v) glycerol, 0.2M EDTA, 0.08% (w/v) bromophenol blue.
10× MOPS/EDTA buffer	200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH 7.0.
5× denaturing agarose gel loading buffer (RNA)	70% (v/v) deionised formamide, 10% (v/v) formaldehyde, 6% (v/v) agarose-gel loading dye (RNA), 14% (v/v) MOPS/EDTA buffer.
TAE buffer	40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA.
TBE buffer	90mM Tris.HCl, 90mM borate (pH 8.3), 2mM EDTA
STET buffer	8% (w/v) sucrose, 5% (v/v) triton X-100, 50mM EDTA (pH 8.0), 50mM Tris.HCl (pH 8.0).
dsRNA polyacrylamide gel, 6% (30ml)	25.65ml water, 6ml acrylamide solution (50%, 1:49 bis-acrylamide), 0.6ml 50x TAE, 120µl ammonium persulphate (25%), 10µl TEMED.
LB (liquid growth media)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0.
LB agar (solid growth media)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.2% (w/v) bacto-agar, pH 7.0.
Total RNA extraction buffer	50mM Tris.HCl, 100mM NaCl, 5mM EDTA, 2% (w/v) SDS, pH 8.0.
TE buffer	10mM Tris.HCl, 1mM EDTA, pH 8.0.
20× SDS	3M NaCl, 1M sodium citrate, pH 7.0.
Hybridisation buffer	0.25M Na_2HPO_4, 7% SDS (w/v), 1mM EDTA.
SSC	150mM NaCl, 15mM tri-sodium citrate, pH 7.0.
STE (10x)	500mM Tris-HCl (pH 6.85), 1M NaCl, 10mM EDTA.
dsRNA extraction buffer	0.2M Tris-HCl (pH 7.5), 0.5M NaCl, 10mM $MgCl_2$, 3% SDS, 1% β-mercaptoethanol.

3.2.2 SOURCES OF GRAPEVINES.

Leafroll infected grapevine varieties (Woodham *et al.*, 1984) were maintained at the CSIRO Plant Industry laboratory in Adelaide, South Australia. These stocks were originally obtained from the CSIRO laboratory in Merbein, Victoria. Grapevine samples with suspected leafroll disease symptoms were collected by Les Krake for the field testing of the GLRaV-1 certification protocols.

3.2.3 SYNTHETIC OLIGODEOXYRIBONUCLEOTIDES.

The oligodeoxyribonucleotides used in this study were obtained from GeneWorks (Adelaide, South Australia). Nucleotide sequences of these molecules are shown in Table 2.2.

Table 3-2: Oligodeoxyribonucleotide primers used in this study

Primer	**Description**	**Size (nt)**	**Primer sequence (5' to 3'-end)**
P1-6N	Tagged random primer	26	GCCGGAGCTCTGCAGAATTCN NNNNN
GR 5' RNA	Gene racer 5' RNA oligo	44	CGACUGGAGCACGAGGACACU GACAUGGACUGAAGGAGUAG AAA
GR 5' internal	Gene racer 5' internal oligo	23	CGACTGGAGCACGAGGACACT GA
GR 5' nested	Gene racer 5' nested oligo	26	GGACACTGACATGGACTGAAG GAGTA
1bXho1-F	ORF1b reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGGTGACGTTTT GTTGCA
1bXba1-R	ORF1b reverse primer (Xba1 site, with no stop codon)	25	CCTCTAGACCGAGTAGCCTTAC TCT
2Xho1-F	ORF2 reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGGACTTGCGGC AGTTTT
2Xba1-R	ORF2 reverse primer (Xba1 site, with no stop codon)	25	CCTCTAGACGGTTGAGCTACGT ACC
3Xho1-F	ORF3 reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGGAAGTGGGAC TTGATT
3Xba1-R	ORF3 reverse primer (Xba1 site, with no stop codon)	31	CCTCTAGATTTGGATATCTTGA AAAGTTCA
4Xho1-F	ORF4 reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGAATTCGTTGG TTTGGT
4Xba1-R	ORF4 reverse primer (Xba1 site, with no stop codon)	31	CCTCTAGAGAACCTTTTCCCAT ATTTGTAAC
5Xho1-F	ORF5 reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGGCTAGCGTTA TATCTC
5Xba1-R	ORF5 reverse primer (Xba1 site, with no stop codon)	25	CCTCTAGACACCTTAAGCTCGC TAG
6Xho1-F	ORF6 reverse primer	27	GGCTCGAGATGGCGACTGCGT

	(Xho1 site, with start codon)		TAGTGG
6Xba1-R	ORF6 reverse primer (Xba1 site, with no stop codon)	31	CCTCTAGATGATTTTTCTAATT CAGAAAAGT
7Xho1-F	ORF7 reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGGAGATCGTCG AAGCAG
7Xba1-R	ORF7 reverse primer (Xba1 site, with no stop codon)	25	CCTCTAGACAGCATCAATATCT TTT
8Xho1-F	ORF8 reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGGAATTCGCTC CAGTAT
8Xba1-R	ORF8 reverse primer (Xba1 site, with no stop codon)	28	CCTCTAGAAATAAATTTTAACG CGTCCA
9Xho1-F	ORF9 reverse primer (Xho1 site, with start codon)	27	GGCTCGAGATGGCGTCACTTA TACCTA
9Xba1-R	ORF9 reverse primer (Xba1 site, with no stop codon)	25	CCTCTAGACACCAAATTGCTA GCGA
GLRaV-1 bind	5' Biotinylated primer for magnetic capture of GLRaV-1	80	TCTATTAGCGCGTAATGGCGTT TTCTGTGATGAGAAGTATGCC ATAACCGCATTATAATATCACC GTCTCTAATTTCAAG
GFP-Rev	GFP reverse primer	39	GGACTAGTGCTTATTTGTATAG TTCATCCATGCCATGTG

3.2.4 BACTERIAL STRAINS AND PLASMID VECTORS.

Escherichia coli strain XL1-Blue (Stratagene, U.S.A) was used for all routine cloning work. *Agrobacterium tumefaciens* strain C58 was used for all agroinfiltrations of binary vectors into *N. tabacum* leaves.

The pGEM-T Easy Vector used for routine cloning of the GLRaV-1 genes was obtained from Promega (Madison, WI). A variant of the shuttle vector pART7 (Gleave, 1992), containing the full-length GFP ORF downstream of the multiple cloning site without a start codon but containing a stop codon (T. Franks, unpublished data), was used to transiently express GLRaV-1 ORF:GFP fusion proteins from a CaMV 35S promoter following biolistic bombardments. The *Not*I-digested fragments from pART7 containing

the 35S promoter, the GLRaV-1 ORF, and the Nos terminator sequence were then transferred into a binary vector pART27 (Gleave, 1992) to generate pART27:ORF:GFP allowing the transient expression of GLRaV-1 ORF:GFP fusion proteins in planta using agroinfiltration of *N. tabacum* leaves.

3.3 METHODS.

3.3.1 AGAROSE GEL ELECTROPHORESIS.

Horizontal minigel tanks (EasyCast Electrophoresis Systems, OWL Scientific Inc., Cambridge, UK) were used for electrophoresis of DNA. 0.7-2.0% (w/v) agarose gels were prepared using Type I-A low EEO agarose in TBE buffer, and contained 0.5µg/ml (w/v) ethidium bromide. Samples were adjusted to 2x DNA loading dye before loading into the wells. Gels were electrophoresed at approximately 100 volts in TBE running buffer before being visualised and photographed using a short wavelength UV transilluminator.

Electrophoresis of RNA was essentially the same as that described for DNA except gel tanks, trays, and combs were treated with 0.2M NaOH (for approximately 2 hrs) prior to use, and samples were loaded with a RNA loading dye (Table 3.1).

3.3.2 DENATURING AGAROSE GEL ELECTROPHORESIS.

The agarose gel was prepared by adding the appropriate amount of agarose to 105ml of water, autoclaving, adding 30ml 37% formaldehyde and 15ml 10× MOPS/EDTA buffer (Table 2.1), and then pouring into a NaOH pre-treated gel tray. Samples were adjusted

to 3× RNA loading dye (Table 2.1) and incubated at 65°C for 15 min before loading. Electrophoresis was carried out in 1× MOPS/EDTA buffer.

3.3.3 SILVER STAINING.

The silver staining procedure was adopted from Merril *et al.* (1981). The gel was fixed in 10% ethanol and 5% acetic acid for 45 min at room temperature and treated in a solution of 1g/l $K_2Cr_2O_7$ and 0.02% HNO_3 for 5 min. The gel was then washed three times with distilled water and impregnated with $AgNO_3$ solution (2g/l) for 30 min. The gel was rinsed with distilled water and soaked in 30g/l Na_2CO_3 and 0.05% formaldehyde for 10-15 min to develop dsRNA bands.

3.3.4 RADIOLABELLING OF DNA.

DNA probes for hybridisation analysis were synthesised by random priming (Feinberg and Vogelstein, 1983) using α-32P-dATP in a Giga-prime labelling kit (Bresatec, Adelaide, SA). The labelled DNA was denatured before hybridisation by heating at 95° for 5 min.

3.3.5 DNA AMPLIFICATION BY PCR.

Components of a PCR reaction were as follows: DNA template, oligonucleotide primers (200-500nM), 1x reaction buffer (Gibco BRL, Rockville, MD), 200μM dATP, dCTP, dGTP, and dTTP (Promega), 1.5μM MgCl2, and 0.5 units of recombinant Taq DNA polymerase (Gibco), in a 20μl or 50μl reaction vol. Thermal cycling was carried out a

PCR machine (Hybaid) and generally consisted of: 3 min at 95°C (one cycle); 45 sec at 94°C, 30 sec at 50°C, 1 min at 72°C (30 cycles); 7 min at 72°C (one cycle).

3.3.6 SINGLE TUBE RT-PCR.

Samples were synthesised with the Superscript One-Step RT-PCR Kit (Promega) in a 50µl reaction. The RT-PCR protocol consisted of 30 min at 50°C, followed by 35 cycles of 15 sec at 94°C, 30sec at 55°C, 1min at 68°C, and finally an extension time of 10min at 72°C. The PCR products were analysed on a 1% agarose/EtBr gel run in 1xTBE buffer.

3.3.7 PURIFICATION OF DNA FROM AGAROSE GEL SLICES.

Purification of specific DNA species from agarose gels after visualisation with ethidium bromide was achieved using a QIAquick Gel Extraction Kit (Qiagen, Victoria, Australia) according to the manufacturer's instructions.

3.3.8 RESTRICTION ENZYME DIGESTION OF DNA.

DNA was digested with restriction endonucleases from Roche Diagnostics (New South Wales, Australia), Promega, and New England Biolabs (Beverly, MA), using appropriate buffers supplied by the manufacturers. Generally, a ten-fold excess of the recommended amount of enzyme was utilised.

3.3.9 DNA LIGATION.

Ligation of PCR products into pGEM T-Easy was carried out according to the manufacturer's instructions. Reactions consisted of insert and vector DNA at a 3:1 ratio, 1x reaction buffer, 1 unit of T4 DNA ligase (Promega), and sterile nuclease-free water to a final vol of 10µl. After mixing, the reaction was incubated at room temperature for two hrs.

3.3.10 TRANSFORMATION OF BACTERIA WITH RECOMBINANT PLASMIDS.

Electrocompetent XL1-Blue *Escherichia coli* cells were transformed by electroporation, using a Gene-Pulser apparatus (Bio-Rad, Hercules, CA). Ligation reaction (1ul) was mixed with a 25ul aliquot of cells and transferred to an ice-cold electroporation cuvette (path length = 1mm) (Invitrogen, Groningen, The Netherlands). This was then given a single pulse in the Gene-Pulser (1.8kV, 125µFD, 200 Ohms), and immediately resuspended in 400µl of LB media. After incubation at 37°C for 1 hr to allow expression of antibiotic-resistance genes, 50 and 350µl aliquots of the transformed cells were spread on 1.2% LB agar plates with appropriate antibiotic selection (100µg/ml ampicillin, 50µg/ml kanamycin) and incubated at 37°C overnight. Alternatively, if cells were transformed with pGEM-T Easy they were grown on plates containing 100µg/ml ampicillin, 25µg/ml IPTG, and 40µg/ml X-gal, to allow blue-white selection of positive clones.

3.3.11 GROWTH OF BACTERIA IN LIQUID CULTURES.

Liquid cultures were set up by inoculating LB media with a single bacterial colony or a loopful of frozen glycerol stock. Cultures were incubated at 37°C overnight with shaking. To select for the cells of interest, appropriate antibiotics were added to the media (at the concentrations outlined in Section 3.3.11).

3.3.12 PREPARATION OF PLASMID DNA.

Preparation of plasmid DNA from 1-5ml of overnight culture was generally done using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Large-scale (20-50ml cultures) preparation of plasmid DNA was achieved using a Plasmid Midi Kit (Qiagen).

3.3.13 PREPARATION OF BACTERIAL GLYCEROL STOCKS.

Bacterial glycerol stocks were prepared by adding 1 vol of 40% or 80% sterile glycerol to overnight culture, snap-freezing in liquid nitrogen, and storing at –80°C.

3.3.14 PREPARATION OF DNA SAMPLES FOR SEQUENCING.

Preparation of DNA for sequencing was done using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, CT), according to the manufacturer's instructions. Each reaction comprised 8µl of Terminator Ready Reaction Mix, 200-400ng of double-stranded template DNA, 3.2 pmol of primer (T7 or SP6), and deionised water to a final vol of 20µl. The components were mixed, spun down in a microcentrifuge, and submitted to the following thermal cycling: 10 sec at 96°C, 5 sec at 50°C, 4 min at 60°C (25 cycles).

To precipitate the DNA, the reaction was added to 80µl of 75% isopropanol, incubated for 20 min at room temperature, and centrifuged at 11,300g for 20 min. After discarding the supernatant and adding another 250µl of 75% isopropanol, the tubes were centrifuged at 11,300g for 5 min. The samples were then drained, dried under vacuum for 15 min, and sent to the Institute of Medical and Veterinary Science (Adelaide, South Australia) for sequencing.

3.3.15 PREPARATION OF ELECTROCOMPETENT *E. coli* CELLS.

500ml of LB broth inoculated with a 5ml overnight culture of *E. coli* XL1-Blue cells were grown at 37°C with vigorous shaking to an optical density (OD600) of 0.5. Cells were chilled on ice for 10min and centrifuged for 15 min at 4°C. The cells were resuspended in 500ml of sterile ice-cold water and centrifuged again. The cells were washed and centrifuged again with 250ml sterile ice-cold water and resuspended in 10ml of sterile ice-cold 10% glycerol. The cells were transferred to a sterile 50ml tube and centrifuged again. The cells were finally resuspended in 2ml of ice-cold glycerol. Aliquots of 25µl were placed into ice-cold eppendorf tubes, snap-frozen in liquid nitrogen, and stored at –80°C.

RESULTS AND DISCUSSION

FOUR

GENOME SEQUENCE AND ANALYSIS OF GLRaV-1

4.1 INTRODUCTION.

The nucleotide sequence of the 3' 12,395-nt of the GLRaV-1 genome has been determined (Fazeli and Rezaian, 2000). The overall organisation of the GLRaV-1 genome is similar to those of other members of the family *Closteroviridae* (Dolja *et al.*, 1994) and is most closely related to GLRaV-3 (Fazeli and Rezaian, 2000). This region of the genome contains 8 open reading frames (ORF) encoding in the 5' to 3' direction a small hydrophobic protein, a heat shock protein 70 (HSP70) homologue, a HSP90-like protein, the coat protein (CP), two minor copies of the CP (CPm1 and CPm2) and two other proteins of unknown function. The aim of the work described in this chapter was to complete the sequence of the GLRaV-1 genome and to analyse the sequence data.

4.2 MATERIALS AND METHODS.

4.2.1 ISOLATION AND ANALYSIS OF GLRaV-1 dsRNA.

Viral dsRNA was extracted from green bark tissue of GLRaV-1-infected Sultana, clone B4L (Habili *et al.*, 1997; Woodham *et al.*, 1984) by the method of Rezaian *et al.*, (1991). Samples were analysed by electrophoresis in a 6% polyacrylamide gel in TAE buffer (Loening, 1967) and visualised by silver staining (Merril *et al.*, 1981).

4.2.2 SYNTHESIS OF GLRaV-1 SPECIFIC dsDNA.

First strand cDNA was synthesised using a GLRaV-1 specific primer designed to the sequence closest to the 5' end. Samples of dsRNA extracted from 5g green bark tissue of GLRaV-1 infected grapevine were combined with 1µl of 10µM specific primer in a final vol of 5µl, boiled for 5 min and cooled on ice for 2 min. Reverse transcription was carried out for 1½ hrs at 37°C in a 10µl reaction vol containing 50mM Tris-HCl (pH 8.5), 8mM $MgCl_2$, 30mM KCl, 1mM DTT, 1mM each of the four dNTPs and 20 units of AMV reverse transcriptase (Clontech, Palo Alto). Second strand DNA synthesis was carried out for 2 hrs at 16°C in a 50µl reaction by adding the second strand reaction mix containing 100mM KCl, 10mM ammonium sulphate, 5mM $MgCl_2$, 0.15mM β-NAD, 20mM Tris-HCl (pH 7.5), 0.5mg/ml BSA, 0.3mM each of the four dNTPs, 0.3 units of DNA polymerase I, 0.0125 units of RNase H and 0.06 units of *E. coli* DNA ligase (Clontech, Palo Alto). After addition of 6 units of T4 DNA polymerase, the reaction was incubated for 30min at 16°C to blunt the DNA ends. The reaction was terminated by the addition of EDTA and glycogen to final concentrations of 0.1mM and 50ng/ml respectively. After a chloroform:isoamyl alcohol (24:1) extraction, the DNA was ethanol precipitated using ultracentrifugation at 250,000g for 1 hr in a swing-out rotor. Samples were resuspended in 5µl of nuclease-free water and 2µl of 10µM Adaptor #1 from the Clontech cDNA subtraction kit (Cat #K1804-1) was added. Adaptor ligation was carried out overnight at 16°C in a final vol of 10µl containing 50mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 2mM DTT, 0.05mg/ml BSA and 40units of T4 DNA ligase (Clontech, Palo Alto). The reaction was incubated for 5min at 72°C to inactivate the enzyme. PCR was performed using the specific oligonucleotide originally used for cDNA synthesis and an oligonucleotide targeted to adaptor #1. The reaction in 25µl

contained 40mM Tris-HCl (pH 9.2), 15mM KOAc, 3.5mM $Mg(OAc)_2$, 3.75µg/ml BSA, 0.2mM of each dNTP, 1x Advantage cDNA polymerase mix (Clontech, Palo Alto) and 1ul of a 1/200 dilution of the ligation reaction product. The PCR protocol consisted of 5 min at 75°C, followed by 20 cycles of 30 sec at 94°C, 30 sec at 65°C, 4 min at 68°C, and finally an extension time of 10 min at 68°C. The PCR reaction was analysed on a 1% agarose/ethidium bromide gel run in 1x TBE buffer. A 1kb Plus DNA ladder (Gibco, BRL, Gaithersburg) was used as a size marker.

4.2.3 cDNA CLONING AND SEQUENCING.

The PCR products were run on a 1% agarose gel and products between 1000 and 4000-nt were gel extracted and ligated into a pGEM-T Easy vector (Promega, Southampton). Recombinant plasmids were digested with *Eco*RI and run on a 1% agarose gel in TBE to determine insert size. The largest positive clones were sequenced by PCR using the dideoxynucleotide chain termination method (Applied Biosystems model 377 DNA sequencer) with double stranded plasmid templates and universal sequencing primers M13 and SP6 (Stratagene, La Jolla). At least five clones were sequenced for each region of the genome.

Sequence data were analysed using the GCG package (Genetics Computer Group, USA). The non-redundant amino acid sequence database of the National Center for Biotechnology Information (NCBI) was searched using the programs BLAST and BLASTP (Altschul *et al.*, 1990). A helical wheel diagram of the 25-40 amino acid region of ORF2 was produced using the WHEEL program (Jones *et al.*, 1992).

4.2.4 5'-END SEQUENCING BY THE RACE PROCEDURE.

Clones representative of 5'-termini of the GLRaV-1 genome were obtained using a GeneRacer Kit (Invitrogen, Carlsbad) according to the manufacturer's instructions. Briefly, dsRNA extracted from 5g green bark tissue of GLRaV-1 infected grapevine was combined with 2µg of the control total RNA supplied with the kit and treated with calf intestinal alkaline phosphatase and subsequently with tobacco acid pyrophosphatase. This procedure leaves a 5'-phosphate moiety only on RNAs that contain a cap structure, thus selecting for 5'-full length RNA. The RNAs were joined to the Gene Racer 5'-RNA oligo (5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA) using RNA ligase. The ligated mRNA was reverse transcribed using Superscript II reverse transcriptase and a gene-specific primer, 5'- CAGACTCCAATTCCACAACGCTGG (reverse complement of bases 208 to 231). The initial PCR utilised GeneRacer 5'-primer (5'-CGACTGGAGCACGAGGACACTGA) and the gene-specific primer. Nested PCR, when performed, utilised GeneRacer 5'-nested primer (5'-GGACACTGACATGGACTGAAGGAGTA) and 5'-ATTAACCAGACGCACCGTCCCAGA as gene specific nested primer (reverse complement of bases 183 to 206). The clones were sequenced as outlined above.

4.3 RESULTS AND DISCUSSION.

4.3.1 COMPLETE NUCLEOTIDE SEQUENCE AND ORGANISATION OF THE GLRaV-1 GENOME.

Ten ORFs were identified within the sequence of the completed GLRaV-1 genome (Fig. 4-1) (Appendix 1). Following the convention used for *Beet yellows virus* (BYV), the type member of the genus *Closterovirus*, these ORFs were designated 1a, 1b and 2 through to 9.

Some plant viral RNAs have an end-group structure known as a 5' cap. This specialised structure is similar to the 5' cap of mammalian messenger RNAs and animal virus messenger RNAs, which have a methylated blocked 5' terminal group in the form $m^7G^5ppp^5X^{(m)}pY^{(m)}p$, where $X^{(m)}$ and $Y^{(m)}$ are two methylated bases. However, in the plant viral 5' caps the bases X and Y are not methylated (Hull, 2001). The closterovirus type member, *Beet yellows virus*, has been shown to encode a methyltransferase domain in its replicase open reading frame (Karasev *et al.*, 1989). This protein methylates the 5' guanine nucleotide using S-adenosylmethionine as the methyl donor, resulting in the 5' cap structure. The existence of a 5′ cap in GLRaV-1 RNA was postulated from the presence of a methyltransferase region in the replicase gene. This was confirmed by evidence that the 5'-terminal sequence could be obtained by 5' RACE using adaptor ligation only when the viral dsRNA was first treated with tobacco acid pyrophosphatase (TAP). This treatment hydrolyses the phosphoric acid anhydride bonds in the triphosphate bridge of the cap structure, releasing the cap nucleoside. This generates a 5'-phosphorylated terminus on the RNA molecule allowing the ligation of the adaptor oligonucleotide.

The GLRaV-1 genome contains a 32-nt 5' untranslated region (UTR) beginning with the sequence GCAAT. This sequence was present in 12 independent DNA clones from this region. The GLRaV-1 5' UTR has no significant sequence similarity to other members of the family *Closteroviridae* and is shorter in length. For example BYV and GLRaV-3 have a 5' UTR of 107-nt and 153-nt respectively (Agranovsky *et al.*, 1994, Ling, 2003). Computer assisted secondary structure predictions identified a potential stem-loop structure in the region. This structure spans 26 nucleotides between positions 2 and 27, has a stem consisting of 10 base pairs plus two mismatches, and a loop of 3 nucleotides (Fig. 4-2a). This stem-loop resembles a larger structure predicted in the 3' UTR of the GLRaV-1 genome containing 28 nucleotides at position 17600 to 17628 which includes 12 bonds, one mismatch and a stem-loop of 3 nucleotides (Fig. 4-2b) (Fazeli and Rezaian, 2000). The significance of these structures in GLRaV-1 during viral replication is unknown. In *Citrus tristeza virus* (CTV), stem-loop structures are conserved in populations with high levels of sequence variability in the 5' UTR (Lopez *et al.*, 1998). Most of the variability is accommodated in the loops and, when found in the stems, compensatory mutations maintain the base pairing. This conservation of secondary structure suggested a role *in vivo*, potentially providing sites for nucleation in RNA folding or interactions with various proteins (Witherell and Uhlenbeck, 1989).

The proposed initiation codon for ORF1a (positions 33 to 35) is the first 5' proximal AUG in the sequence and is found in a favourable context (Kozak, 1986), with a U and G at the –3 and +4 positions respectively. The 5' region of the genome contains overlapping ORFs 1a and 1b, encoding products with calculated M_r of 236K and 59K respectively. Based on homology with other members of the family *Closteroviridae,* it is predicted that the ORF1b product is expressed via a +1 ribosomal frameshift from the

ORF1a/1b transcript. The ORF1a/1b product contains the papain-like proteinase (P-Pro), methyltransferase (MTR), RNA helicase (HEL) and RNA-dependent RNA polymerase (RdRp) domains that are conserved in the 'Sindbis-like' supergroup of positive-strand RNA viruses to which the family *Closteroviridae* belongs (Koonin and Dolja, 1993).

The P-Pro domain is located at the 5'-region of ORF1a and was identified by its homology to P-Pro domains from other members of the family *Closteroviridae*. The putative catalytic cysteine and histidine residues were at positions 241 and 287 respectively, with the predicted cleavage site occurring between Gly-434 and Gly-435 (Fig. 4-3). In addition to a common proteolytic activity, the leader proteinases of closteroviruses possess specialised functions in virus RNA amplification, virus invasion and cell-cell movement (Peng *et al.*, 2001). The region of ORF1a directly downstream of the P-Pro domain was identified as an MTR domain based on homology with other members of the family *Closteroviridae* and the six conserved MTR motifs have been identified (Fig. 4-3) (Rozanov *et al.*, 1992). As previously mentioned this domain has a predicted role in the formation of the 5'cap structure.

FIGURE 4-2

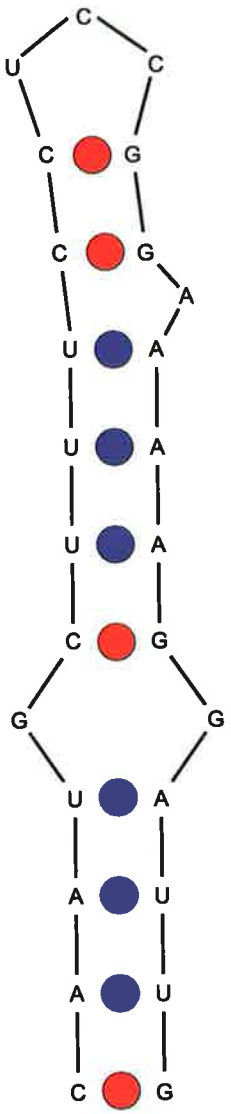
PUTATIVE STEM LOOP STRUCTURES FROM THE 5' AND 3' UTR OF GLRaV-1.

Computer assisted secondary structure predictions showed the potential for a stem-loop structure in the 5' and 3' UTRs of GLRaV-1.

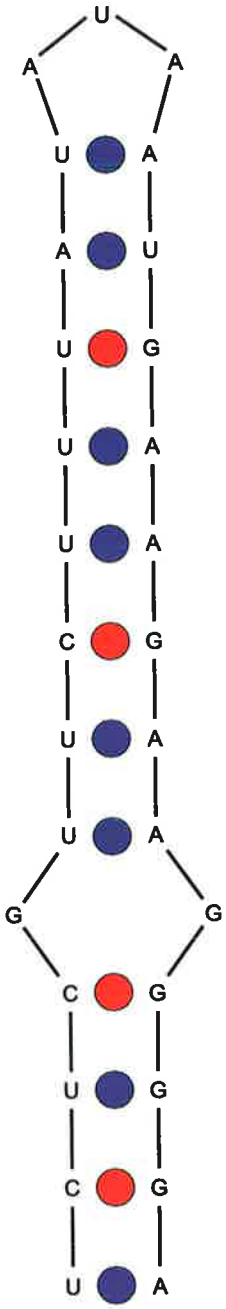
a- The 5' UTR structure contains 26 nucleotides at position 2 to 27 and includes 10 bonds, two mismatches and a loop of 3 nucleotides.

b- The 3' UTR structure contains 28 nucleotides at position 17600 to 17628 and includes 12 bonds, one mismatch and a stem-loop of 3 nucleotides (Fazeli and Rezaian, 2000).

a


C
U
C
C
G
C
G
A
U
A
U
A
U
A
C
G
G
G
U
A
A
U
A
U
C
G

b


U
A
A
U
A
A
U
G
U
A
U
A
C
G
U
A
U
A
G
G
C
G
U
G
C
G
U
A

4.3.2 PHYLOGENETIC ANALYSIS OF THE PUTATIVE REPLICASE OF GLRaV-1 CONFIRM ITS CLASSIFICATION IN THE GENUS *AMPELOVIRUS.*

Phylogenetic analysis of the complete amino acid sequences of the putative replicase consisting of the P-Pro, MTR, HEL and RdRp domains of GLRaV-1, CTV, BYV, *Little cherry virus-1* (LChV-1), *Lettuce infectious yellows virus* (LIYV), LChV-2, GLRaV-3, and the partially completed replicase sequences of GLRaV-2 and Pineapple mealybug wilt-associated virus-2 (PMWaV-2) separated them into their respective genera (Fig. 4-4). These genera were established originally from the identity of their insect vectors and the alignment of their HSP70 ORF (Martelli *et al.*, 2002). In this phylogeny GLRaV-1 is distinct from the aphid-transmissible *Closteroviruses* GLRaV-2, BYV and CTV and the whitefly transmissible *Crinivirus* LIYV. The currently unassigned LChV-1 shows stronger homology to LIYV than in the previous alignment based on the HSP70 ORF (Martelli *et al.*, 2002), strengthening the case for its assignment into the genus *Crinivirus*. However caution should still remain as LChV-1 has an *Ampelovirus*-like genome organisation. As shown in Table 4-1, GLRaV-1 shows strong homology to the other members of the mealybug transmitted ampeloviruses LChV-2, PMWaV-2 and the strongest homology to the type member of the group GLRaV-3 with 31% amino acid sequence identity and 48% sequence similarity. This strong relationship with GLRaV-3 is also seen with the HSP70, CP and CPm ORFs (Fazeli and Rezaian, 2000).

FIGURE 4-3

CONSERVED MOTIFS OF *CLOSTEROVIRIDAE* PRESENT IN THE PUTATIVE P-PRO AND MTR DOMAINS OF GLRaV-1.

The amino acid sequence alignment of GLRaV-1 with the consensus sequences of P-Pro and MTR of BYV, GLRaV-2, CTV, LChV-1, LChV-2, LIYV, PMWaV-2 and GLRaV-3 is shown. The amino acid residues that are identical or similar among the proteins are shown in upper case. The residues conserved between GLRaV-1 and other *Closteroviridae* are shown in bold. The putative catalytic cysteine and histidine residues of the P-Pro domain are marked with an asterisk (*) and an arrow (↑) is used to indicate the predicted cleavage site between two glycine residues. The conserved sequence motifs I–IV of the MTR (Rozanov *et al*, 1992) are indicated.

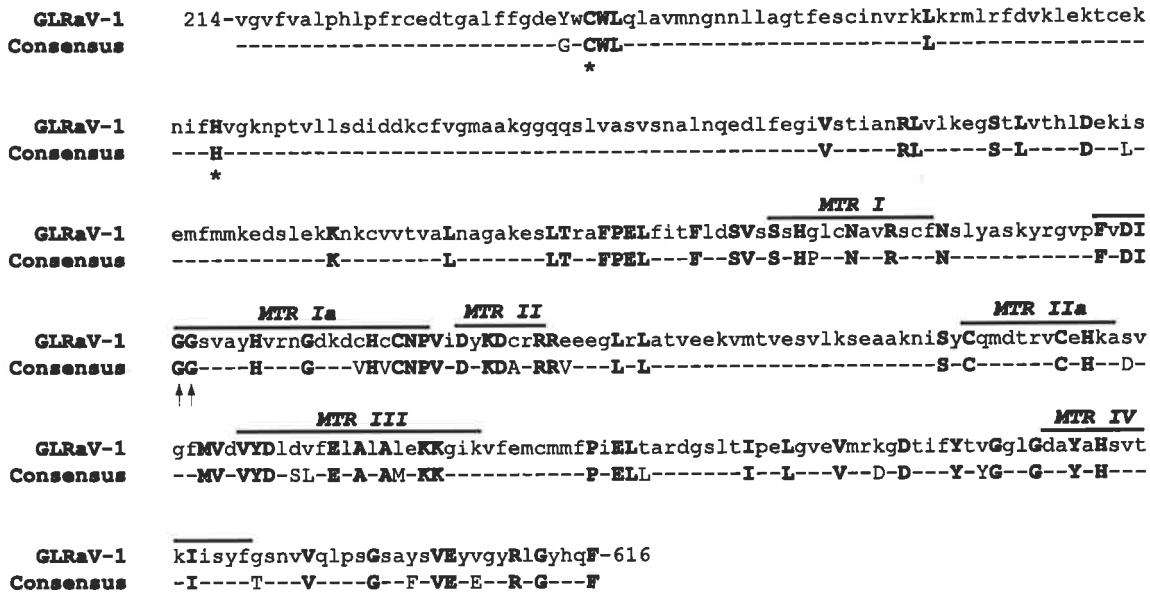

GLRaV-1 214-vgvfvalphlpfrcedtgalffgdeYwCWLqlavmngnnllagtfescinvrkLkrmlrfdvklektcek
Consensus ----------G-CWL----------L----------
*
GLRaV-1 nifHvgknptvllsdiddkcfvgmaakggqqslvasvsnalnqedlfegiVstianRLvlkegStLvthlDekis
Consensus ---H----------V-----RL-----S-L----D--L-
*
MTR I
GLRaV-1 emfmmkedslekKnkcvvtvaLnagakesLTraFPELfitFldSVsSsHglcNavRscfNslyaskyrgvpFvDI
Consensus ----------K--------L-------LT--FPEL---F--SV-S-HP--N--R---N----------F-DI
MTR Ia
MTR II
MTR IIa
GLRaV-1 GGsvayHvrnGdkdcHcCNPViDyKDcrRReeegLrLatveekvmtvesvlkseaakniSyCqmdtrvCeHkasv
Consensus GG----H---G---VHVCNPV-D-KDA-RRV---L-L----------S-C------C-H--D-
MTR III
MTR IV
GLRaV-1 gfMVdVYDldvfElAlAleKKgikvfemcmmfPiELtardgsltIpeLgveVmrkgDtifYtvGglGdaYaHsvt
Consensus --MV-VYD-SL-E-A-AM-KK----------P-ELL-------I--L---V--D-D---Y-YG--G--Y-H---
GLRaV-1 kIisyfgsnvVqlpsGsaysVEyvgyRlGyhqF-616
Consensus -I----T---V----G--F-VE-E--R-G---F

FIGURE 4-4

PHYLOGENETIC ANALYSIS OF *CLOSTEROVIRIDAE* REPLICASES.

The viruses are compared on the basis of similarity between their complete replicase domains, when available, including their P-Pro, MTR, HEL and RdRp domains.

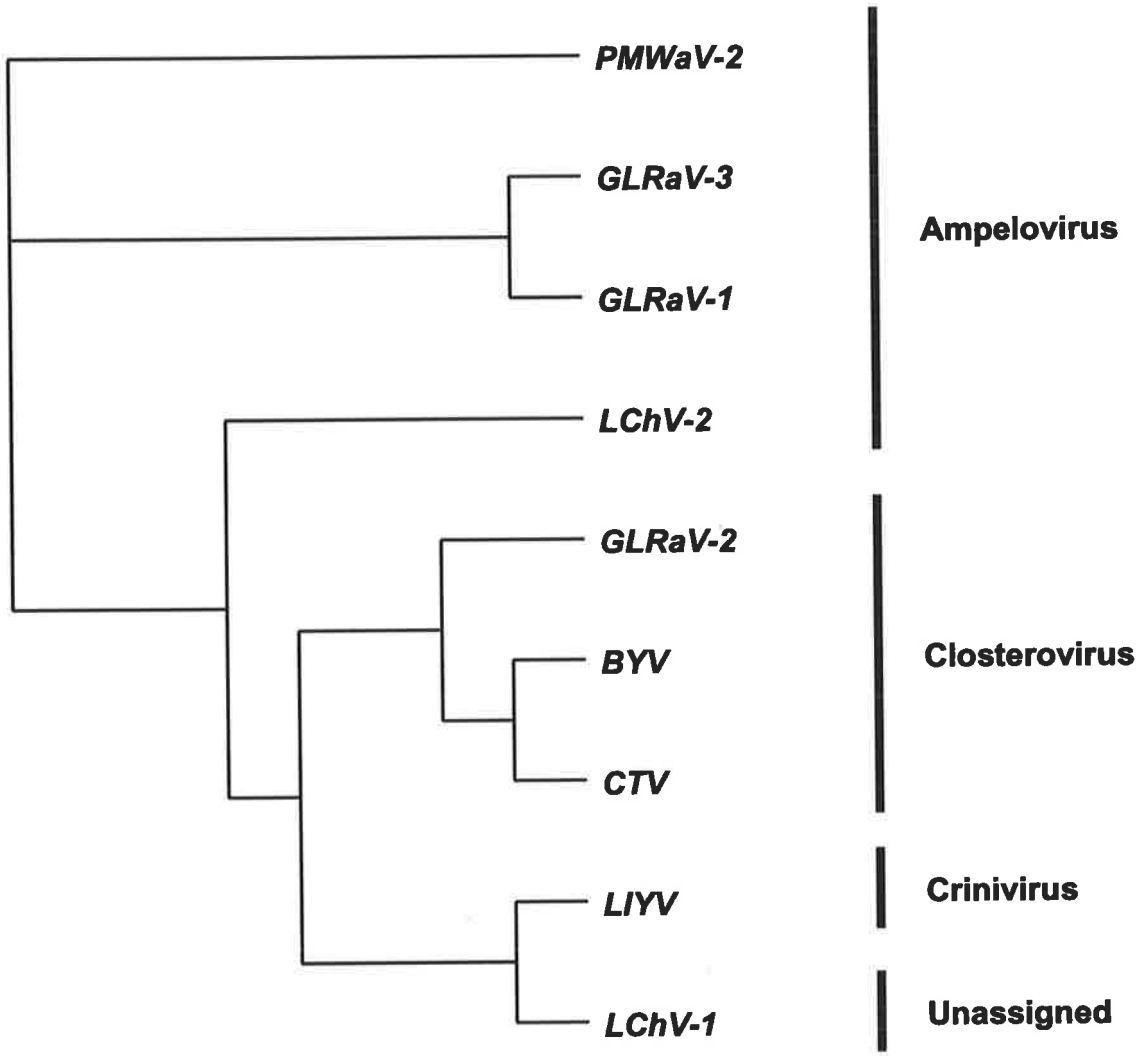

PMWaV-2
GLRaV-3
GLRaV-1
LChV-2
GLRaV-2
BYV
CTV
LIYV
LChV-1
Ampelovirus
Closterovirus
Crinivirus
Unassigned

Table 4-1: Percentage amino acid identity and similarity between GLRaV-1 and other *Closteroviridae* family replicase sequences.

	GLRaV-3	PMWaV-2	LChV-2	BYV	GLRaV-2	CTV	LIYV	LChV-1
Identity	31	18	20	15	9	13	14	14
Similarity	48	31	33	29	23	24	28	28

FIVE

GLRaV-1 CERTIFICATION PROTOCOL

5.1 INTRODUCTION.

The use of grapevine propagation material certified negative for GLRaV-1 is a common practice in viticulture. The International Council for the study of Virus and Virus-like Diseases of the Grapevine (ICVG) recommends that in order to preserve valuable grape clones and varieties, certified selections should be tested for a list of specific pathogens including GLRaV-1. Australia, conducts large scale screening of planting material with the aim of preventing GLRaV-1 distribution in the field. These tests are commonly carried out by RT-PCR that offers speed and low cost compared to biological indexing. However, shortcomings in the reproducibility of these tests have limited their reliability (GWRDC, 2002).

In an attempt to improve the consistency of GLRaV-1 detection by PCR, the level of sequence heterogeneity in the viral genome was studied (Little *et al.*, 2001). Sequence variation across the genome was found to be high, showing clustering mainly in ORFs 3, 6 and 7, corresponding to the HSP70-like protein and CPm1 and CPm2 respectively. Overall, 75 clones corresponding to ORFs 3, 6 and 7 were sequenced and 1916 nucleotide changes were recorded relative to the published sequence (Little *et al.*, 2001). None of the changes resulted in a frame shift or stop codon and there was a trend for the conservation of amino acids or change to amino acids having similar physiochemical properties. The CPm2 gene was particularly variable with a mutation seen in 60% of the

nucleotide positions in the cDNA clones sequenced. These observations suggest that GLRaV-1 may exist in the form of a heterogeneous population, possibly resulting from the lack of selective pressure and from mixing of virus strains due to viticulture practices of vegetative propagation and grafting over the centuries (Little *et al.*, 2001).

This chapter describes the development of a diagnostic method for GLRaV-1 in grapevines, which deals with the shortcomings in detection due to variability in the viral genome and the quantity of template RNA.

5.2 MATERIALS AND METHODS.

5.2.1 PREPARATION OF TOTAL RNA FOR GLRaV-1 DETECTION.

Total RNA was isolated from green bark tissue using a modification of the sodium perchlorate method (Rezaian and Krake 1987). Plant material, 1g, was ground to a powder in liquid nitrogen with a pre-cooled mortar and pestle. Tissue samples were extracted in 20ml of RNA extraction buffer [5M sodium perchlorate, 0.3M Tris-HCl (pH 8.3), 1% (w/v) SDS, 1% (v/v) b-mercaptoethanol, 8.5% (w/v) PVPP, 2% (w/v) PEG 4000] and homogenised by shaking at room temperature for 30min. The homogenate was transferred to a separation apparatus consisting of a 20ml disposable syringe plugged with silane-treated glass wool wrapped in a double layer of Miracloth. The syringe was placed in a 250ml centrifuge tube (Corning, Acton, USA) and then centrifuged at 400g for 10min at 4°C. 15ml of modified extraction buffer [5M sodium perchlorate, 0.3M Tris-HCl (pH 8.3), 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol] was added to the syringe and centrifuged as before. The combined eluate was centrifuged at 5000g for 10min at 4°C. The supernatant was removed, mixed with 2.5 vol of cold

ethanol, stored at -20°C for a minimum of 1 hr and then centrifuged at 5000g for 20min at 4°C. The resulting pellet was dried under vacuum and resuspended in 1ml of 10mM Tris-EDTA (pH 7.6). The suspension was extracted three times with an equal vol of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and once with an equal vol of chloroform:isoamyl alcohol (24:1 v/v). The upper aqueous phase was retained, mixed with 2.5 vol of cold ethanol and 0.1 vol of sodium acetate pH 5.2 and stored at -20°C overnight. The RNA precipitate was pelleted by centrifugation at 7700g for 10min at 4°C, dried under vacuum, rinsed with cold 70% ethanol, resuspended in 200ml of cold sterile water, and stored at -80°C. The concentration of the RNA was determined spectrophotometrically. Small scale total RNA extraction from 100mg green bark tissue was performed using a modified Rneasy Plant Mini kit (QIAGEN, Hilden, Germany) (MacKenzie *et al.*, 1997).

5.2.2 DESIGN AND SYNTHESIS OF BIOTIN-LABELLED CAPTURE OLIGONUCLEOTIDE.

An 80-nt capture oligonucleotide complementary to the 3'-end of the GLRaV-1 RNA genome (nucleotides 17567 to 17647, Little and Rezaian, 2004) was designed. The sequence was designed 83-nt downstream of the 3'-terminal nucleotide of ORF9, that ends at nucleotide position 17484, to avoid binding competition during subsequent RT-PCR tests. The capture oligonucleotide incorporated a biotin molecule on a twelve-atom triethylene glycol linker and was synthesised by Geneworks PTY LTD, Australia.

5.2.3 PREPARATION OF MAGNETIC PARTICLES.

Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) in 0.6ml aliquots (Promega) were completely dispersed by flicking and captured by placing the tube in a magnetic stand for approximately 30 sec. The supernatant was removed and the particles were washed three times with 300µl of 0.5xSSC. The particles were resuspended in 100µl of 0.5xSSC ready for use.

5.2.4 HYBRIDISATION OF CAPTURE OLIGONUCLEOTIDE AND VIRAL RNA.

Samples of total RNA extracted from 500mg green bark tissue were combined with 3µl of 50µM biotinylated oligonucleotide in a final vol of 500µl. The mixture was incubated at 65° for 10min, 13µl of 20xSSC was added and allowed to cool at room temperature. The entire contents of the annealing reaction was added to the washed SA-PMPs and incubated at room temperature for 10min on a spinning rotor. The SA-PMPs were collected, the supernatant removed and the particles were washed four times in 0.2x SSC and eluted twice in 250µl sterile water. The elutions were pooled for each sample and viral RNA stored at -80°.

5.2.5 SINGLE TUBE RT-PCR.

Samples were synthesised with the Superscript One-Step RT-PCR Kit (Promega) in a 50µl reaction using GLRaV-1 specific oligonucleotides pORF9F and pORF9R (GGC TCG AGA TGG CGT CAC TTA TAC CTA and CCT CTA GAC ACC AAA TTG CTA GCG A respectively), which amplify a 633-bp DNA fragment. The RT-PCR protocol consisted of 30 min at 50°, followed by 40 cycles of 15 sec at 94°, 30sec at 55°,

1min at 68°, and finally an extension time of 10min at 72°. The RT-PCR reaction was analysed on a 1% agarose/ethidium bromide gel run in 1xTBE buffer. A 1kb Plus DNA ladder (Gibco, BRL) was used as a size marker.

5.3 RESULTS AND DISCUSSION.

5.3.1 OPTIMISED TARGET REGION FOR GLRaV-1 DIAGNOSIS.

To achieve optimal detection of GLRaV-1 by RT-PCR it was necessary to target the viral RNA region with low levels of sequence variation. Using the sequence information available (Little *et al.*, 2001), we tested regions of low variability within ORFs 1b, 5 and 9 as targets (Fig. 5-1). 19 samples suspected of leafroll disease infection were collected from the field. Each sample was tested for the presence of GLRaV-1 by ELISA targeted to the coat protein (ORF5) and 7 samples gave a positive reaction. Each of the 19 samples was then tested by RT-PCR. The primers targeted to ORFs 1b, 5 and 9 (Fig 5-2) detected 4, 6, and 12 positives respectively in the 19 samples tested. An example of PCR results is shown for three of the samples in Figure 5-2b-d. The results indicated that when a PCR test was positive with a pair of primers, it was always associated with a positive result with primers targeted to regions downstream.

The lower detection levels at the 5' end of the viral genome relative to the detection levels at the 3' end is probably due to the increased copy number of GLRaV-1 ORFs at the 3' end of the genome due to the presence of sub-genomic RNAs (Fig. 5-3) (Fazeli *et al.*, 2000). GLRaV-1 encodes three sub-genomic promoters across its genome. The first sub-genomic RNA promoter is positioned upstream of ORF5, which would conform with the increase in detection using primers targeted to ORF5 compared to ORF1b. The

FIGURE 5-1

SEQUENCE VARIATION ACROSS THE GLRaV-1 GENOME.

a- Clones sequenced across the genome are shown. The sequences obtained from these clones were used for the calculation of sequence variation in Figure 5-1b.

b- The total number of nucleotide changes in all clones corresponding to a 100bp window were determined and divided by the total number of clone in that region.

c- Open reading frame organisation of GLRaV-1 and putative gene functions.

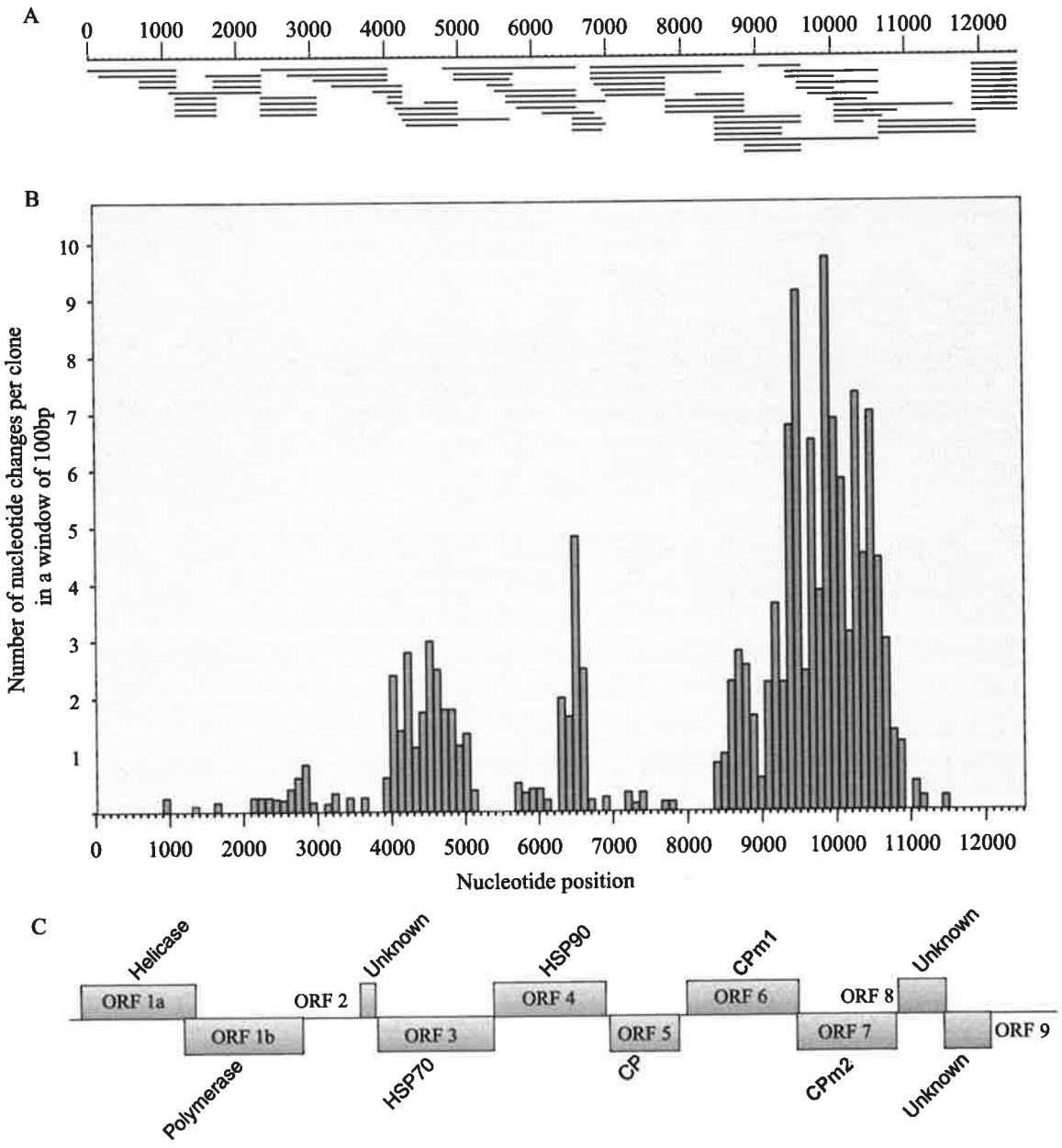

A
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
12000
B
Number of nucleotide changes per clone in a window of 100bp
1
2
3
4
5
6
7
8
9
10
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
12000
Nucleotide position
C
Helicase
ORF 1a
ORF 1b
Polymerase
ORF 2
Unknown
ORF 3
HSP70
HSP90
ORF 4
ORF 5
CP
CPm1
ORF 6
ORF 7
CPm2
ORF 8
Unknown
Unknown
ORF 9

FIGURE 5-2

REGIONS OF THE GLRaV-1 GENOME TARGETED FOR DETECTION BY RT-PCR.

a- Diagram showing the regions of the GLRaV-1 genome targeted for detection by RT-PCR.

b-d- Examples of PCR tests with three grapevine samples that gave positive results with primers from either one, two or three regions of GLRaV-1 RNA.

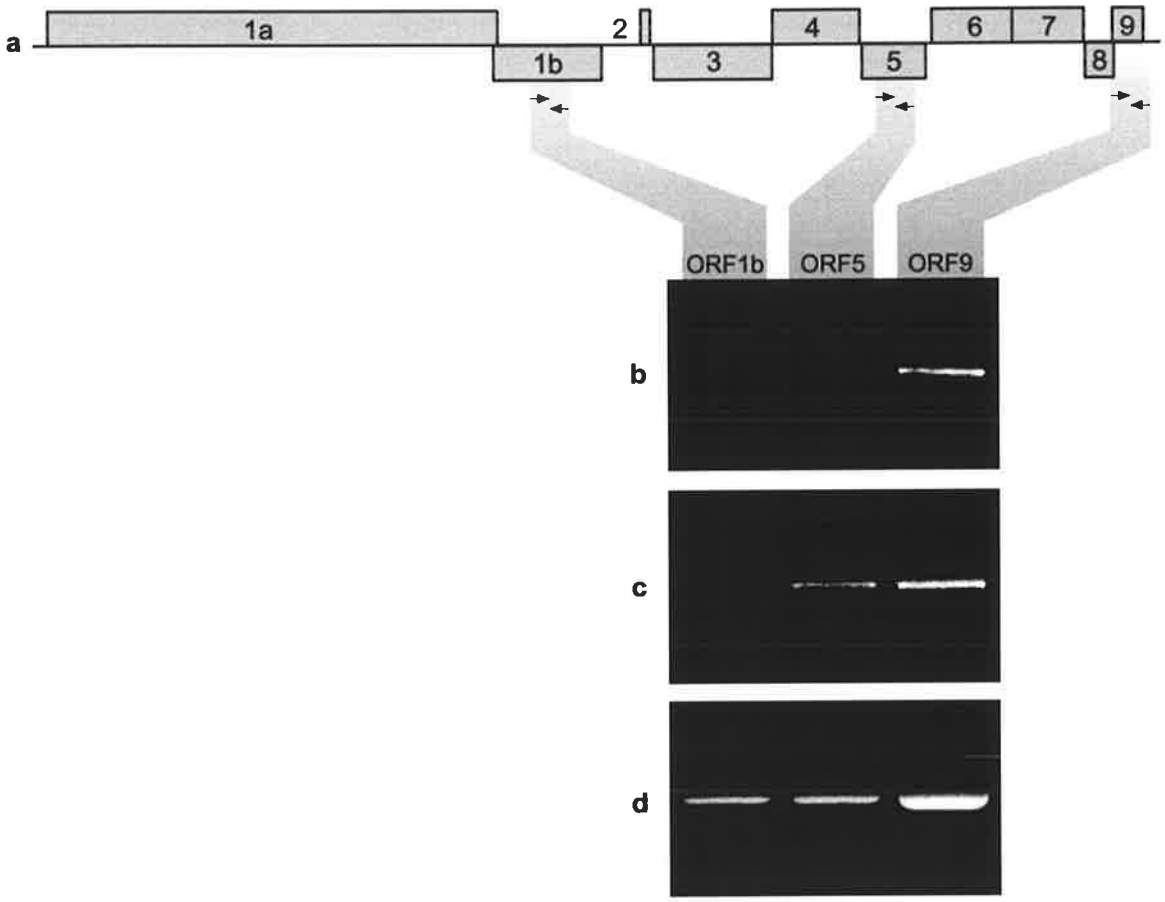
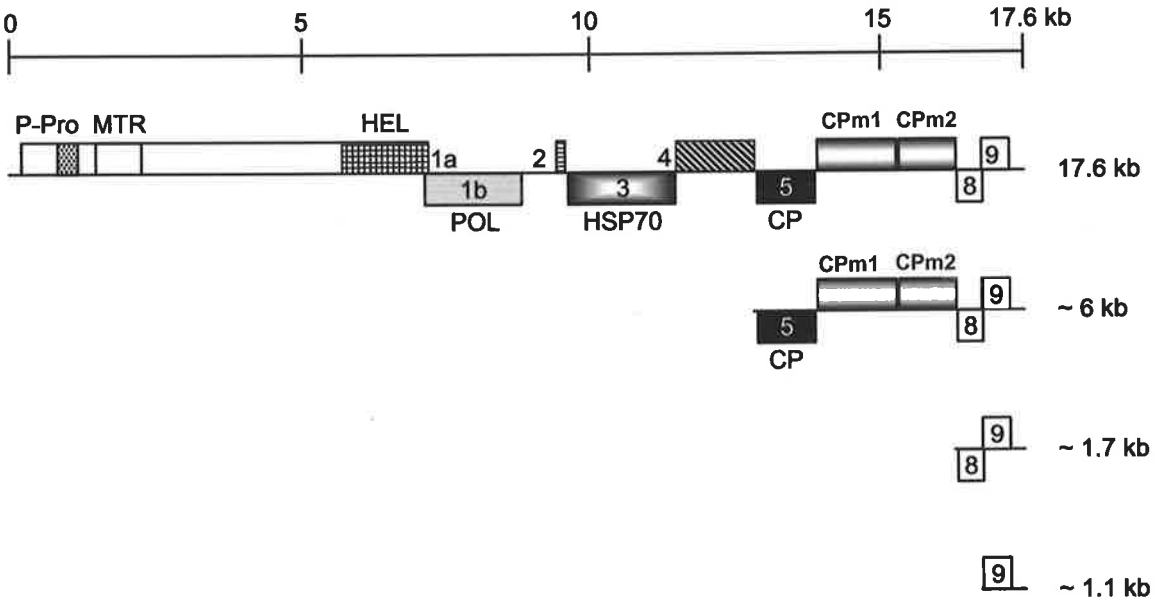

a
1a
1b
2
3
4
5
6
7
8
9
ORF1b
ORF5
ORF9
b
c
d

FIGURE 5-3

GENOME ORGANISATION OF GLRaV-1 AND SUB-GENOMIC RNAs.

Rectangles represent ORFs. P-Pro, papain-like protease; MTR, methyltransferase; HEL, helicase; POL, polymerase; HSP70, homologue of HSP70 proteins; CP, coat protein; CPm, minor copy of coat protein.


0
5
10
15
17.6 kb
P-Pro
MTR
HEL
1a
2
4
CPm1
CPm2
9
8
1b
3
5
POL
HSP70
CP
17.6 kb
CPm1
CPm2
9
8
5
CP
~ 6 kb
9
8
~ 1.7 kb
9
~ 1.1 kb

second sub-genomic promoter is positioned upstream of ORF8 and the third is upstream of ORF9. This may account for the increase in sensitivity seen when using primers targeted to ORF9 relative to ORF1b and ORF5. ORF9 was therefore considered as a suitable candidate for detection of GLRaV-1 by RT-PCR because it is present in all 3' sub-genomic RNAs (Fig. 5-3) (Fazeli *et al.*, 2000), and shows the lowest amount of variation seen for all regions across the genome (Little *et al.*, 2001).

5.3.2 ENRICHMENT OF GRLaV-1 RNA IMPROVES DETECTION.

The presence of phenolic compounds in grapevine extracts interferes with virus detection by enzymatic methods (Minafra and Hadidi, 1994). In an effort to further purify the viral RNA template, we used magnetic capture hybridisation. Initially developed for the detection of bacterial DNA from soil (Jacobsen, 1995), magnetic capture allows the separation of specific target nucleic acid from other nucleic acids and from interfering compounds. We obtained a biotinylated oligonucleotide to the 3′ untranslated region present in all GLRaV-1 genomic and sub-genomic RNA species (Fig. 5-4). Following hybridisation in solution, the RNA was captured and the non-specific RNA and other impurities were washed away. This produced a highly enriched RNA fraction after only a single round of magnetic separation that could be vacuum dried to concentrate the viral RNA without accumulation of bulky sediment.

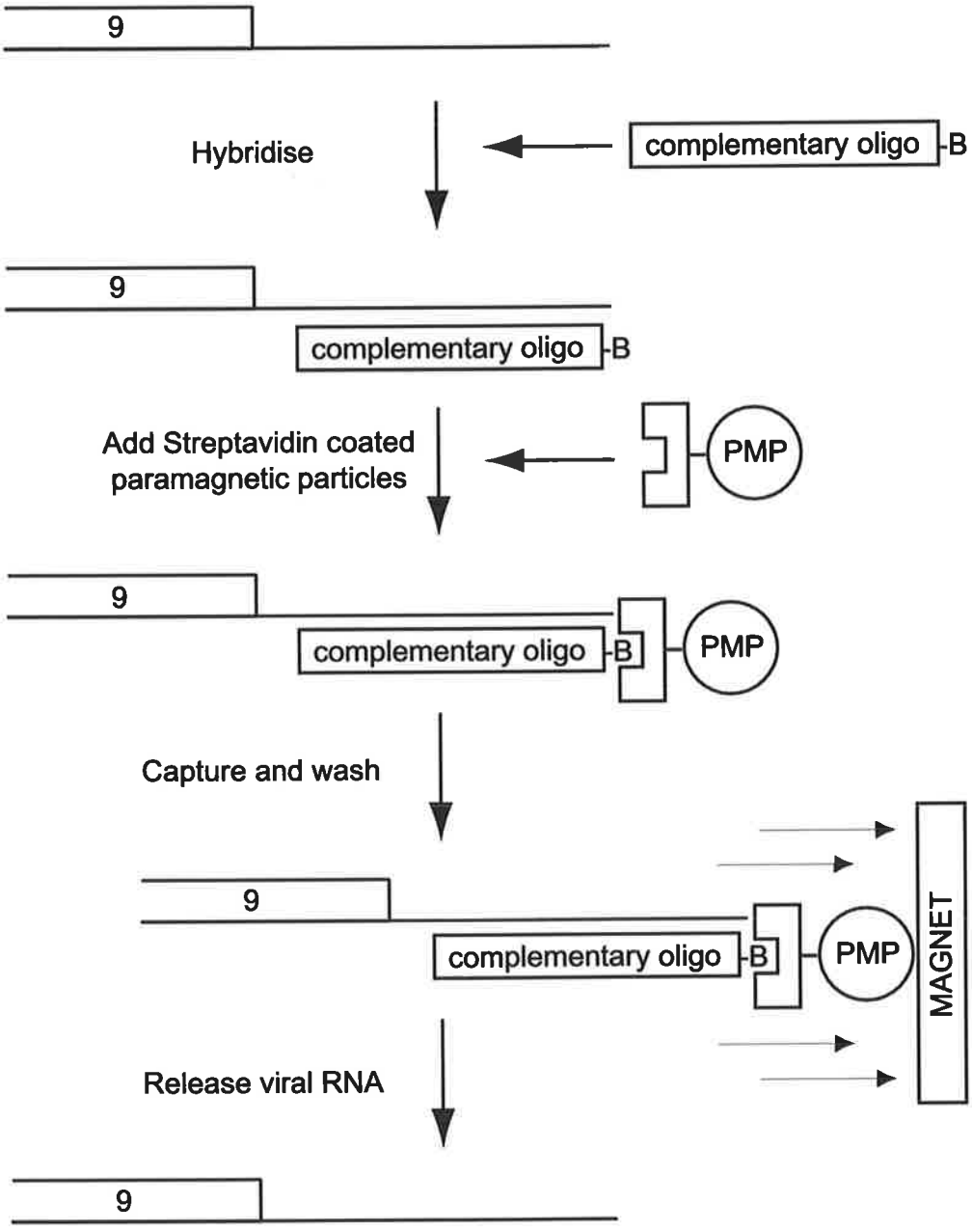
The magnetic capture technique was tested using a GLRaV-1 infected sample which produced inconclusive results when tested by RT-PCR giving a positive result using primers for ORF9 and a negative result using primers for ORF1b and ORF5. The total RNA was extracted from 1g of the grapevine sample and split into two aliquots. Half was left as total RNA and the other half was purified using magnetic capture (MC-

FIGURE 5-4

SCHEMATIC DIAGRAM OF MAGNETIC CAPTURE.

GLRaV-1 RNA isolation magnetic capture with paramagnetic particles (PMPs). The method uses a oligonucleotide labelled with Biotin (B) to hybridize at high efficiency in solution to the 3′ untranslated region present in all GLRaV-1 RNA species. The hybrids are captured and washed using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA is eluted from the solid phase by the addition of ribonuclease-free deionized water.

total RNA containing GLRaV-1 genomic and subgenomic RNAs



GLRaV-1 genomic and subgenomic RNAs

RNA). Amplification of ORF9 by RT-PCR was performed using either total RNA or MC-RNA as template (Fig. 5-5). Each reaction contained the equivalent of tissue weights ranging from 0.1mg to 100mg RT-PCR using total RNA produced a positive result using 1mg tissue, however gave a negative result reproducibly at lower and higher tissue concentrations (Fig. 5-5a, 1anes 7-10). At the lower tissue input of 0.1mg the negative reaction was presumably due to the low levels of template. Likewise, when the tissue equivalent of RNA was increased to 10mg and 100mg then a false negative occurred probably due to inhibition from impurities such as polyphenolics known to exist in grapevine extracts (Koonjul *et al.*, 1999). On the contrary, a positive result was obtained when RT-PCR was performed using magnetic captured RNA to the equivalent of 100mg tissue (Fig. 5-5b). The largest quantity used was the equivalent of adding 100-fold more viral RNA into a single RT-PCR reaction without causing an inhibition and producing a stronger signal (Fig 5-5b, lane 10). The higher viral template concentration therefore, improved the sensitivity of the certification protocol while the increased purity prevented false negatives caused by the presence of PCR inhibitors.

5.3.3 EVALUATION OF MCH-RT-PCR USING FIELD MATERIAL.

To assess the practical reproducibility of MCH-RT-PCR with field material, 28 grapevine samples were collected from the field. These samples were suspected of leafroll disease infection by the observation of leafroll symptoms. Each sample was tested for the presence of GLRaV-1 using RT-PCR targeted to ORF7 and ELISA targeted to ORF5 (Table 5-1). Nine samples tested positive by both methods. When the RT-PCR primers were changed from the variable ORF7 to the more conserved ORF9, RT-PCR resulted in an increase of the number of positive samples from 9 to 14. However, with magnetic capture RT-PCR 16 positives were detected from the sample

FIGURE 5-5

IMPROVED DETECTION OF GLRaV-1 USING MCH-RT-PCR.

a- RT-PCR without MCH; Lane 1, negative control. Lane 2, positive control using 1ng pGEM-ORF9. Lanes 3-6, healthy sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of total RNA respectively. Lanes 7-10, infected sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of total RNA respectively.

b- RT-PCR with MCH; Lane 1, negative control. Lane 2, positive control using 1ng pGEM-ORF9. Lanes 3-6, healthy sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of MC RNA respectively. Lanes 7-10, infected sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of MC RNA respectively.


a
DNA control
Healthy
Infected
- + 0.1 1 10 100 0.1 1 10 100
(mg) Tissue equivalent
633nt (ORF9)
Lane 1 2 3 4 5 6 7 8 9 10
b
DNA control
Healthy
Infected
- + 0.1 1 10 100 0.1 1 10 100
(mg) Tissue equivalent
633nt (ORF9)
Lane 1 2 3 4 5 6 7 8 9 10

group. The current certification protocol targeted to ORF7 would have therefore detected only 56% of the infected samples detected by MCH-RT-PCR as GLRaV-1 positive.

Table 5-1: Results obtained from field screen of suspected GLRaV-1 infected grapevine samples using ELISA, RT-PCR and MCH-RT-PCR.

28 samples 9 ELISA +ve	Tissue equivalent (mg)			
	0.1	1	10	100
Total RNA	7	14	11	0
MC RNA	9	15	16	16

In conclusion, the knowledge of sequence variation in GLRaV-1 and magnetic capture has been combined to devise an improved diagnostic for *Grapevine leafroll-associated virus 1*. This approach could be adapted to other grapevine viruses were detection is limited by low viral template and high levels of PCR inhibitors.

SIX

SUBCELLULAR LOCALISATION OF GLRaV-1 PROTEINS

6.1 INTRODUCTION.

GFP is a useful fluorescent tag for studying the localisation and dynamics of proteins in living cells. A number of properties make GFP a powerful reporter: it can be monitored non-invasively by external illumination, it needs no external substrates, and localisation of GFP fusions can be analysed in living cells. GFP allows the observation the subcellular localisation patterns of virus-encoded proteins. From these localisation patterns it is possible to suggest potential roles for the proteins in the virus life cycle. The aim of the work described in this chapter was to observe the intracellular localisation of the GLRaV-1 gene products via the generation of green fluorescent protein (GFP)-fusion constructs, in an attempt to further characterise the function of these proteins.

6.2 MATERIALS AND METHODS.

6.2.1 GFP FUSION CONSTRUCTS.

A variant of the shuttle vector pART7 (Gleave, 1992), containing the full-length green fluorescent protein ORF downstream of the multiple cloning site, without a start codon but containing a stop codon (T. Franks, unpublished data), was used to transiently express GLRaV-1 ORF:GFP fusion proteins from a CaMV 35S promoter. Primers were designed for ORFs 1b to 9 with the forward primer containing an *Xba*I site and a start

codon. The reverse primer contained an *Xho*I site with no stop codon allowing an in frame C-terminal fusion with the GFP ORF. The *Not*I-digested fragments from pART7 containing the cauliflower mosaic virus (CaMV)-derived 35S promoter, the GLRaV-1 ORF, and the nopaline synthase (NOS) transcription terminator sequence were then transferred into a binary vector pART27 (Gleave, 1992) to generate pART27:ORF:GFP.

6.2.2 BIOLISTIC BOMBARDMENT.

Tungsten particles (8mg) in 100µl of ethanol was vortexed for two min, spun down for about 10 sec in a microfuge, drained, washed twice with 100µl sterile water, and resuspended in 100µl sterile water. 50µl of this suspension was removed and added to 5µl of 1µg/µl plasmid preparation. While gently vortexing, 20µl of cold 1M spermidine and 50µl 2.5M CaCl2 were added drop wise and the resulting mixture incubated on ice for 10 min. The particles were spun down, drained, resuspended in cold ethanol, and placed on sterile filter holders.

Onion epidermal strips were placed on agar containing Murashige and Skoog Salt Mixture (Invitrogen, Carlsbad, California) and bombarded with settings of 620kPa helium pressure after evacuating the chamber to -90kPa. Tissue was stored in the dark for 48 hrs, and observed using a Leica stereomicroscope coupled to a fluorescence module.

6.2.3 AGROINFILTRATION.

pART27:ORF:GFP constructs were introduced into *Agrobacterium tumefaciens* (strain C58), which were subsequently introduced into *N. tabacum* leaves (Selth *et al.*, 2004). Plant tissue was sampled after five days and checked for GFP expression using a Leica stereomicroscope coupled to a fluorescence module. More detailed images were obtained with a Confocal Laser Scanning Microscope System, Bio-Rad MRC-1000UV, comprising of a Nikon Diaphot 300 inverted microscope and a Krypton/Argon laser. The excitation wavelength used for GFP analysis was 488nm.

6.3 RESULTS AND DISCUSSION.

6.3.1 SUBCELLULAR LOCALISATION OF GLRaV-1 ENCODED PROTEINS.

The subcellular localisation patterns of all of the GLRaV-1 products, except the ORF1a polyprotein, were observed using gene fusions to the green fluorescent protein (GFP). Four distinct patterns of localisation were seen when the fusion constructs were bombarded into onion epidermal cell layers (Fig. 6-1). For the majority of ORFs this has supported the putative gene functions based on alignments and in some cases allowed the assignment of previously unknown functions.

6.3.1.1 GENERAL LOCALISATION WITHIN THE CYTOPLASM.

The most common observed pattern resembled that of untargeted free GFP in that it was distributed throughout the cytoplasm and nucleus by diffusion (Fig 6-1a). The gene products in this class included the putative RNA-dependent RNA-polymerase (ORF1b, Fig 6-1b), HSP90 homologue (ORF4, Fig 6-1e), CP (ORF5, Fig 6-1f), CPm2 (ORF7,

FIGURE 6-1

TRANSIENT EXPRESSION AND VISUALIZATION OF GFP FUSED TO THE GLRaV-1 ENCODED PROTEINS IN ONION EPIDERMAL CELL LAYER.

Cells were bombarded with expression plasmids encoding the proteins indicated in each panel examined by fluorescence microscopy. Bar=100μm.

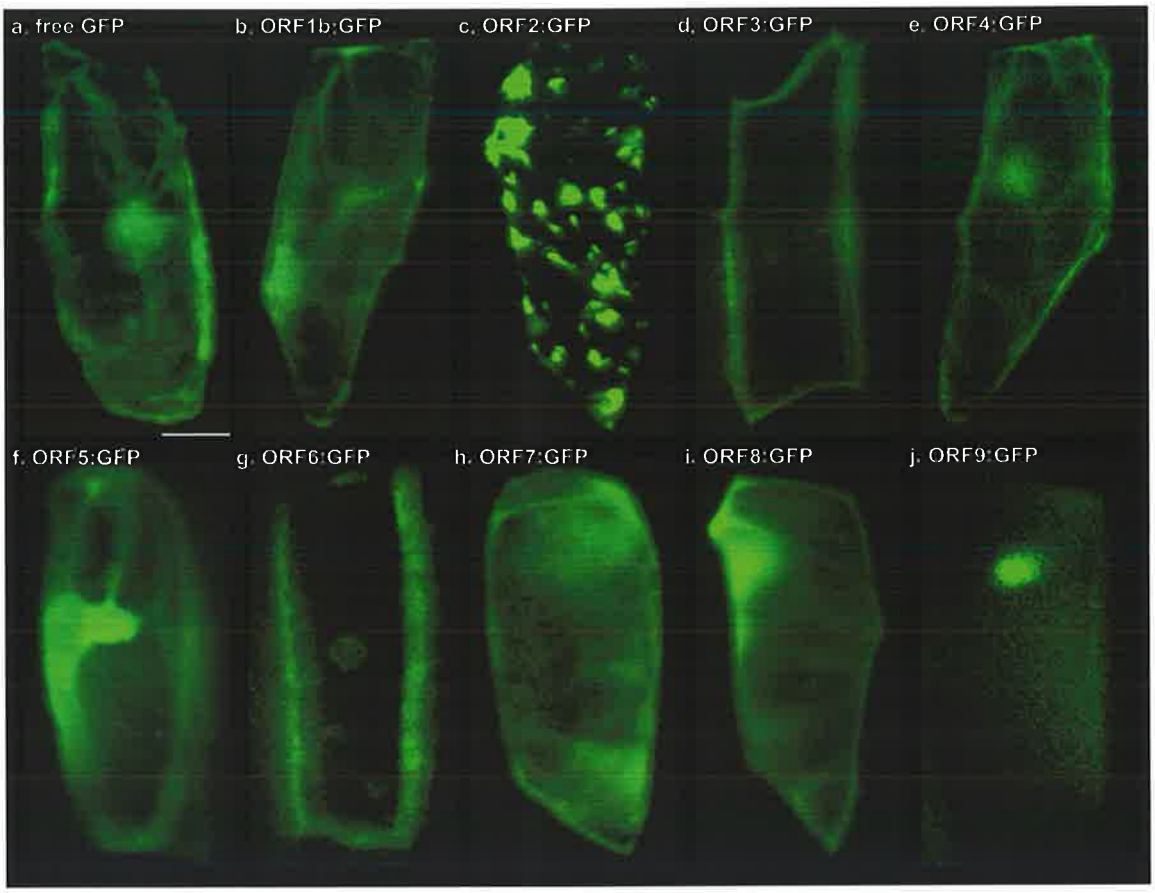

a. free GFP
b. ORF1b:GFP
c. ORF2:GFP
d. ORF3:GFP
e. ORF4:GFP
f. ORF5:GFP
g. ORF6:GFP
h. ORF7:GFP
i. ORF8:GFP
j. ORF9:GFP

Fig 6-1h) and a protein of unknown function (ORF8, Fig 6-1i). This pattern of expression would be consistent with these proteins being involved in replication and packaging of the virus that occur in the cytoplasm. The RNA dependent RNA polymerase would be expected to localise to the replication complex for transcription of the viral genome. The CP and CPm2 would also be expected to localise close to the replication complex to facilitate the formation of the virion structure following the production of single stranded copies of the viral genome. The lack of specific targeting patterns for these proteins is probably due to the absence of the membrane structures in healthy onion cells where the formation of the viral replication complex is predicted to occur in a normal virus infection process involving the coordinated expression of viral genes (Faoro and Carzaniga, 1995).

6.3.1.2 ORF3 AND 6 ARE ASSOCIATED WITH THE CELL PERIPHERY.

The HSP70 homologue (ORF3, Fig 6-1d) and the minor Coat protein #1 (ORF6, Fig 6-1g) were localised to the cell periphery, suggesting roles in cell-cell movement. This is consistent with reports that the HSP70 homologue of BYV was localised to the plasmodesmata (Medina *et al.*, 1999) and functioned as one of the viral movement proteins (Peremyslov *et al.*, 1999). The BYV HSP70 also has a role in chaperoning the assembly of the minor coat protein on the tail of the virion (Alzanhova *et al.*, 2000). Polyclonal antibodies against the BYV CP and CPm show that the CP protein covers the majority of the viral genome except for a 75nm region at the 5' end (Zinovkin *et al.*, 1999). The formation of this tail structure is a prerequisite for BYV cell-cell movement via the plasmodesmata (Alzanhova *et al.*, 2001), which is consistent with our observation of the GLRaV-1 CPm-1 localisation to the cell periphery. It is unclear why only one of the GLRaV-1 minor coat proteins are targeted to the cell wall. No

comparison can be made with other members of the family *Closteroviridae* because GLRaV-1 is the only member with two minor coat proteins. This localisation pattern was confirmed using agroinfiltration of the fusion constructs in a binary vector into *N. tabacum* and confocal laser scanning microscopy (Fig. 6-2d and e respectively). Punctate fluorescent spots and aggregates were seen at the cell periphery (arrows) and did not appear to move suggesting a possible association with plasmodesmata.

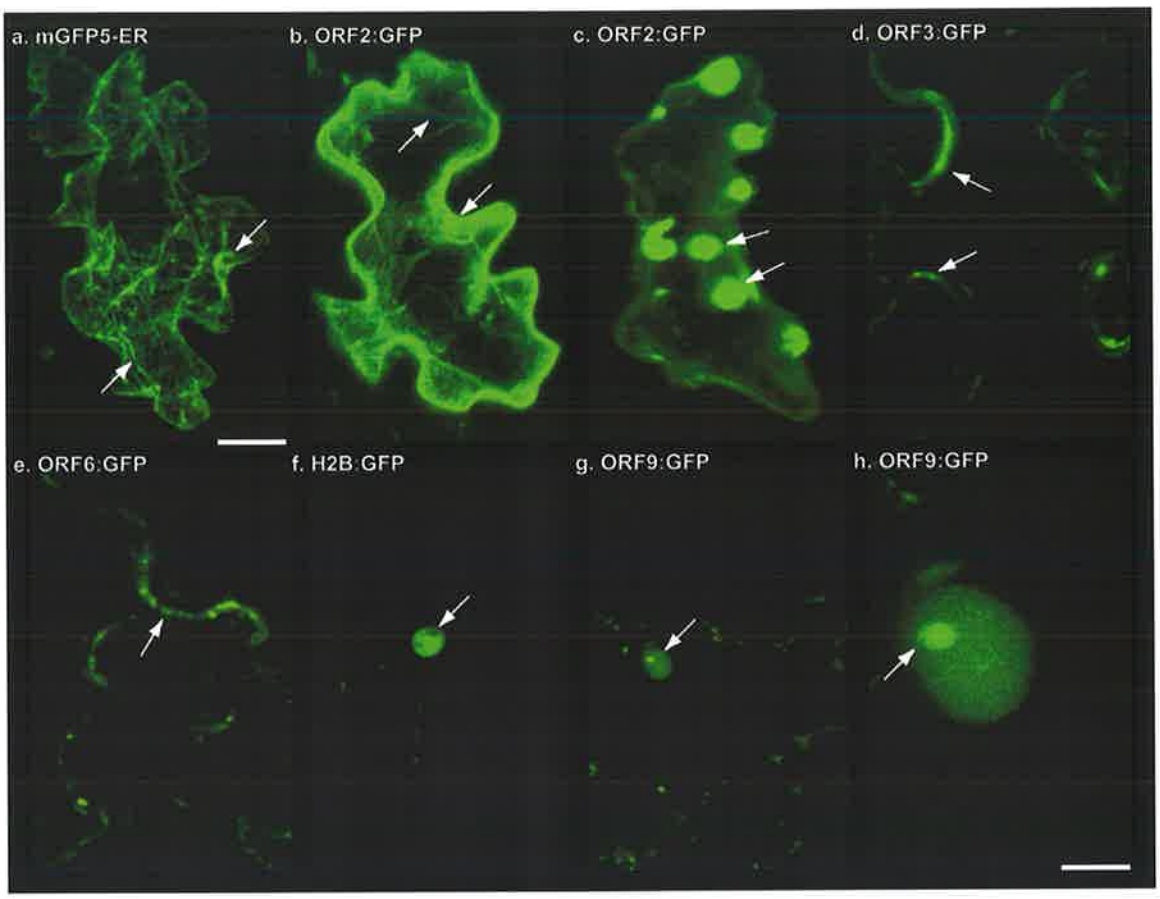
6.3.1.3 ORF9 CONGREGATES IN THE NUCLEOLUS.

The ORF9:GFP fusion appeared to localise to the nucleus of onion cells (Fig 6-1j). A potential nuclear targeting signal composing of the basic residues, RKKK, is present at positions 123 to 126. Confocal microscopy confirmed the targeting of ORF9 to the nucleus *in planta* (Fig 6-2g). A human histone 2B protein:GFP fusion (H2B:GFP), which is directly incorporated into the nucleosomes (Kanda *et al.*, 1998) was used as a control (Fig 6-2f). The ORF9 product appeared to be concentrating within the nucleolus (Fig 6-2h). Soluble proteins of M_r less than 50-60K, such as ORF9:GFP, are able to diffuse freely in and out of the nucleolus making it difficult to differentiate nuclear-targeted GFP fusions (Peters, 1986). It is generally accepted that retention of such a protein in the nucleolus involves an interaction with a nucleolar protein. This poses an interesting question as to why a protein encoded by an RNA virus would be targeting a host factor in the nucleolus. Possible cell processes involved may include host cell cycle regulation (Pederson, 1998), senescence (Olson *et al.*, 2000) or interfering with host cell defences such as RNA silencing (Carmo-Fonesca *et al.*, 2000). Non-specific RNA binding proteins that diffuse into the nucleus may also become more concentrated in the nucleolus because of the large amount of rRNA that is present (Hiscox, 2002). The M_r-23K protein encoded by the 3'-terminal gene of CTV has been shown to be an RNA

FIGURE 6-2

TRANSIENT EXPRESSION AND VISUALIZATION OF GFP FUSED TO GLRaV-1 ENCODED PROTEINS IN TOBACCO EPIDERMAL CELLS.

The leaves were agroinfiltrated with binary expression plasmids encoding the proteins indicated in each panel and examined by confocal laser scanning microscopy 5 days after infiltration. The early localisation pattern of ORF2, seen in the first 48 hours, is also shown (b). (a-g) Bar=25μm. (h) Bar=100μm.


a. mGFP5-ER
b. ORF2:GFP
c. ORF2:GFP
d. ORF3:GFP
e. ORF6:GFP
f. H2B:GFP
g. ORF9:GFP
h. ORF9:GFP

binding protein (Lopez *et al.*, 2000) and is the only non-structural within the family *Closteroviridae* for which RNA binding activity has been demonstrated. The CTV p23 protein has been reported to control asymmetric accumulation of CTV RNAs by downregulating negative-stranded RNA accumulation and indirectly increasing expression of 3' genes (Satyanarayana *et al.*, 2002). This protein is similar in size and position in the viral genome to the M_r-24K product of GLRaV-1 ORF9, but no significant sequence homology can be found between them.

6.3.1.4 ORF2 IS ASSOCIATED WITH VESICLE STRUCTURES.

The small hydrophobic protein (ORF2) gave the most striking localisation pattern showing spots of unknown identity and of various sizes (Fig 6-1c). Bioinformatic analysis of the 59 amino acid protein using the PSORT program (Nakai and Horton, 1999) suggested that it is an integral membrane protein targeted to the endoplasmic reticulum (ER) via an uncleavable N-terminal hydrophobic signal sequence (Fig 6-3a). There was no evidence of ORF2 targeting to the ER in the onion epidermal cell layer. Confocal microscopy of agroinfiltrated tobacco cells, however, gave two distinct patterns of localisation. In the first 48 hrs after infiltration, the pattern of the ORF2 encoded protein (Fig 6-2b) resembled the localisation of the ER targeted control, mgfp5-ER (Fig 6-2a) (Haseloff *et al.*, 1997), with GFP visible on the ER network and in the ER around the nucleus (arrows).). Subsequently, the pattern of ORF2 expression appeared to change (Fig 6-2c). After 48 hrs, the ER pattern was still present, but at a lower level compared to the large aggregate structures which appear to be originating from the ER (arrows). The identity and structure of these aggregates appeared uncertain and further research is required to determine their role in the viral life cycle.

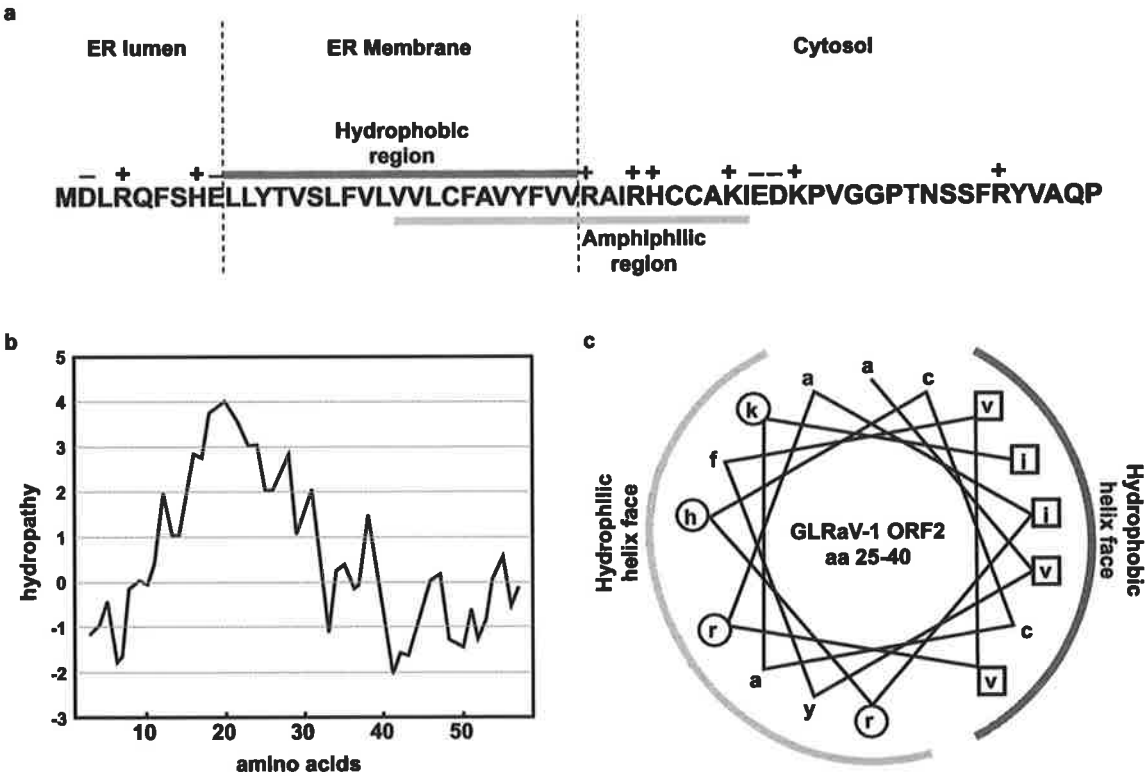
FIGURE 6-3

BIOINFORMATIC ANALYSIS OF GLRaV-1 VESICLE INDUCING PROTEIN (VIP-ER).

a- Complete amino acid sequence of VIP-ER protein (59-aa) showing the predicted hydrophobic and amphiphilic regions.

b- Hydropathy plot of VIP-ER protein using a window size of nine residues. Positive and negative hydropathy values indicate a hydrophobic and hydrophilic effects respectively.

c- Helical wheel representation of the putative cationic amphipathic α-helix formed by amino acids 25-40 of VIP-ER. Positively charged residues are circled and hydrophobic residues are boxed.


a
ER lumen
ER Membrane
Cytosol
Hydrophobic region
– + + + ++ + ––+ +
MDLRQFSHELLYTVSLFVLVVLCFAVYFVVRAIRHCCAKIEDKPVGGPTNSSFRYVAQP
Amphiphilic region
b
hydropathy
5
4
3
2
1
0
-1
-2
-3
10
20
30
40
50
amino acids
c
Hydrophilic helix face
Hydrophobic helix face
GLRaV-1 ORF2
aa 25-40
a
a
c
k
v
f
i
h
i
v
c
r
a
v
y
r

SEVEN

CYTOPATHOLOGY OF GLRaV-1 VIP-ER PROTEIN

7.1 INTRODUCTION.

The function of ORF2 could not be determined from the localisation pattern observed for the ORF2:GFP fusion protein (Chapter 6). In order to further understand the function of ORF2 the cell ultrastructural changes associated with ORF2 expression were investigated using transmission electron microscopy (TEM).

7.2 MATERIALS AND METHODS.

7.2.1 TRANSMISSION ELECTRON MICROSCOPY.

N. tabacum leaf tissue agroinfiltrated with pART27:ORF2:GFP and pART27:GFP was sampled at 3, 6, 9, 12 and 15 days post infiltration. The tissue was sectioned into 1mm squares and fixed for two to three days in 4% paraformaldehyde/1.25% glutaraldehyde (EM grade) in phosphate buffered saline (PBS) pH 7.2, with 4% sucrose. The tissue was washed twice in PBS and 4% sucrose for 10 min and post-fixed in 2% osmium tetroxide for one hr with constant mixing. The tissue was dehydrated in three changes of ethanol for twenty min each using 90, 95 and 100% ethanol sequentially. After a half hr incubation in propylene oxide, resin was infiltrated into each sample overnight at a 1:1 resin:propylene oxide ratio. This was followed by three changes of 100% resin for at least eight hrs each with constant mixing. Each sample was embedded into individual moulds and the resin polymerised at 70°C for 24 hrs. Thin sections were cut, mounted

on an EM copper grid and visualised using a PHILIPS CM100 Transmission Electron Microscope.

7.3 RESULTS AND DISCUSSION.

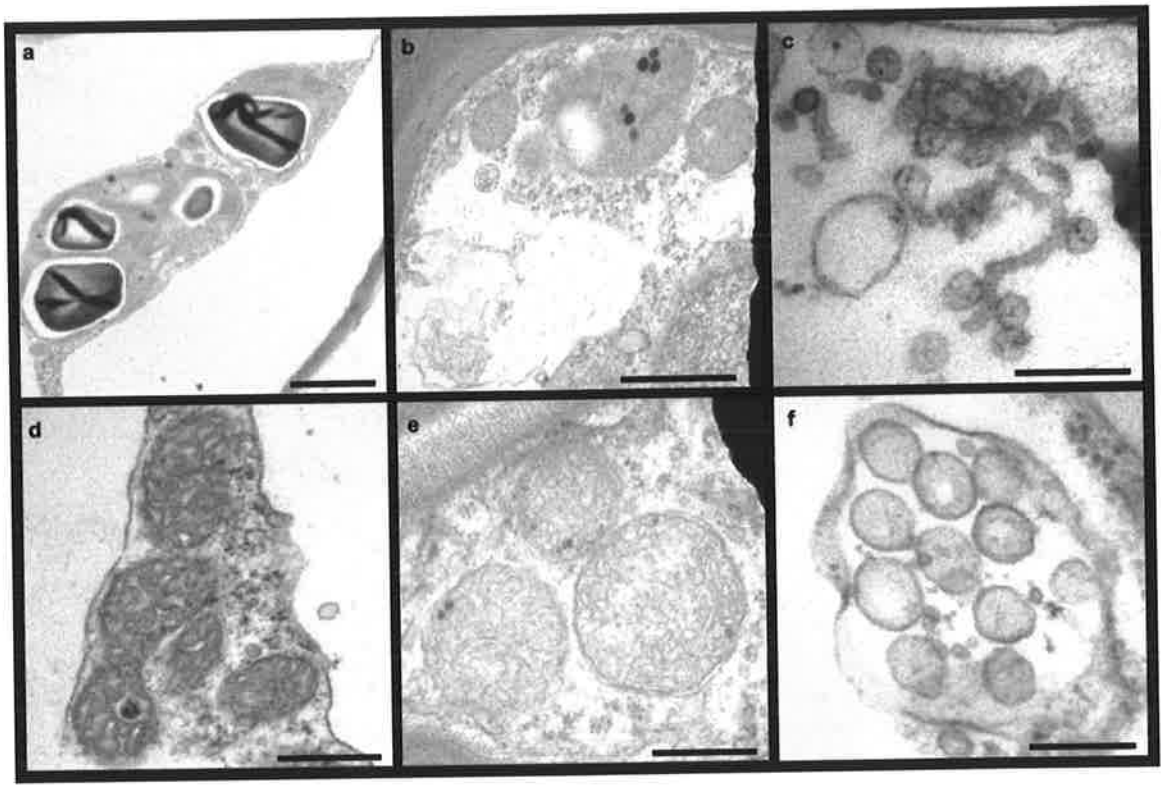
7.3.1 CELLS EXRESSING ORF2 FORM MULTIVESICULAR BODIES.

An ER targeted control, mgfp5-ER, was used as a control to observe if any changes were caused by the agroinfiltration procedure, GFP expression or processing of plant tissue for TEM. The control cells were processed in triplicate and consistently showed an organised structure (Fig 7-1a) compared to the ORF2 expressing cells (Fig 7-1b). The ER in the ORF2 expressing cells produced large numbers of vesicles of various diameters ranging from 50nm to 150nm (Fig 7-1c). Large multivesicular bodies (MVBs) were present consisting of a single membrane filled with vesicles (Fig 7-1f). These vesicles most likely originated from the ER based on the observational change of the ORF2:GFP localisation pattern from the ER to large cytoplasmic aggregates. The formation of the MVBs probably results from the collection of untargeted vesicles by the late endosome pathway. The cytopathology of most members the family *Closteroviridae* so far examined is characterised by the presence of vesiculating organelles and MVBs localised in phloem cells. GLRaV-1, 3 and 5 are thought to induce mitochondrial vesiculation (Faoro, 1997) and GLRaV-2 is believed to induce vesiculation of the endoplasmic reticulum (Castellano *et al.*, 2000). The MVBs reported in *Closteroviridae* infections consist of vesicles, 50-100nm in diameter, bound by single membranes containing viral RNA (Faoro and Carzaniga, 1995). They are hypothesised to originate from either the vesiculating mitochondria or from the endoplasmic reticulum and are thought to be released into the cytoplasm after the disruption of these

FIGURE 7-1

TRANSMISSION ELECTRON MICROSCOPY OF ORF2 EXPRESSING CELLS.

Transmission electron microscopy of tobacco epidermal cells agroinfiltrated with binary expression plasmids encoding mGFP5-ER (a, d) and VIP-ER (b, c, e and f). (a) Bar=2μm. (b) Bar=1μm. (c) Bar=250nm. (d, e) Bar=500m. (f) Bar=250m.


a
b
c
d
e
f

organelles. The involvement of the endoplasmic reticulum and tonoplast membranes in the formation of vesicles has been previously hypothesised (Faoro *et al.*, 1981), but not confirmed (Faoro *et al.*, 1991). These MVBs have been previously referred to as BYV-type vesicles, named after the *Closterovirus* type member *Beet Yellows Virus* (Agranovsky, 1995). My results indicate that the ORF2 product is the viral encoded protein responsible for the induction of the MVBs originating from the ER. I therefore refer to the ORF2 product as the Vesicle Inducing Protein originating from the Endoplasmic Reticulum (VIP-ER).

7.3.2 PUTATIVE ROLES AND MODES OF ACTION OF VIP-ER.

Amino acid sequence analysis by PSORT predicted VIP-ER to be a type 1b membrane protein with its N-terminus exposed to the ER lumen, a transmembrane region from residues 14 to 30, and its C-terminus exposed to the cytosol (Fig 6-3a). A region of hydrophobic moment was found from residues 25 to 40 (Fig 6-3b), which is predicted to form an amphiphilic α-helix containing a hydrophobic face and a hydrophilic face charged with polar residues. VIP-ER displays characteristics typical of the family of so-called membrane lytic peptides (Segrest *et al.*, 1990). Membrane lytic peptides are encoded in numerous genomes of positive-strand RNA animal viruses, such as enteroviruses (Carrasco *et al.*, 1995), and have been implicated in the proliferation or reorganisation of cellular membranes and vesicles. This is possibly a mechanism for increasing the available surface area for RNA synthesis. It has been proposed that these cytolytic peptides are able to permeate membranes either by forming multimeric membrane-integral pores or, alternatively, by lying parallel to the lipid bilayer and increasing membrane curvature (Shai, 1995).

Strong similarities can be seen between the GLRaV-1 encoded VIP-ER and the 2B protein of the enterovirus *Coxsackievirus B3*. 2B is also a small hydrophobic protein with the potential to form an amphipathic helix with a structural arrangement typical of lytic peptides (van Kuppeveld *et al.*, 1996). The 2B protein has been localised to the rough ER membrane and the outer surface of ER-derived membranous vesicles at which viral RNA replication takes place (Beinz *et al.*, 1987, 1994). Early in infection, the 2B protein forms membrane-embedded pores in the endoplasmic reticulum and subsequently disrupts its cation gradient (van Kuppeveld *et al.*, 1997). This results in the induction of vesicles from the ER and the increase in plasma membrane permeability facilitating viral replication and release respectively (Kuppeveld *et al.*, 1997). The VIP-ER protein of GLRaV-1 is predicted to have a similar mode of action. The targeting of VIP-ER to the ER with subsequent formation of ER-derived membrane structures has been observed. If VIP-ER was using the same mode of action as the 2B protein, there would be a detectable increase of cytosolic calcium levels. Our attempts to observe this expected increase in cytosolic calcium levels using calcium indicators, Indo-1 and Calcium Crimson, were unsuccessful due to technical difficulties of introducing the dyes into *N. tabacum* leaf epidermal cells. Sufficient dye accumulation could only be achieved in the guard cells with open stomata. However, increased cytosolic calcium in ORF2 expressing cells is supported by the observation that mitochondria appear to be swollen in size (Fig 7-1e) relative to those in the control cells (Fig7-1d). Mitochondria, along with the ER, play pivotal roles in regulating intracellular Ca^{2+} content. Mitochondria are endowed with multiple Ca^{2+} transport mechanisms by which they take up and release Ca^{2+} across their inner membrane. During cellular Ca^{2+} overload, mitochondria take up cytosolic Ca^{2+}, which in turn induces opening of permeability

transition pores (Kowaltowski *et al.*, 2001) allowing water and solutes to enter the matrix and cause swelling.

The role of VIP-ER in the life cycle of GLRaV-1 may not be limited to the formation of multivesicular bodies for the establishment of the viral replication complex and subsequent RNA replication, but may also have an important role in cell-cell movement. This is based on mutagenesis of a full-length construct of BYV, which showed that its small hydrophobic protein, p6, is essential for cell-cell movement (Alzanhova *et al.*, 2001). Similar investigations of GLRaV-1 movement proteins are not possible until mechanical inoculation via an infectious clone is achieved.

Viruses generally exploit plasmodesmata, which form a channel spanned by a plasma membrane and contain a thin, appressed tubule of the endoplasmic reticulum, thereby establishing cytoplasmic as well as ER continuity between neighbouring cells (Staehelin, 1997). The association of VIP-ER with the ER during infection and its impact on the morphology of the ER indicates a potential role of the endomembrane system in cell-cell spread of GLRaV-1.

Peremyslov *et al.* (2004) recently reported that the BYV p6 protein is also associated with the rough endoplasmic reticulum, but this protein was unable to induce vesicle formation. Our results indicate that the endoplasmic reticulum-derived vesicle localisation pattern develops after 48 hrs. This was possibly missed in the BYV study since p6:GFP expression was only reported up to 48 hrs. Mutagenesis of the BYV p6 protein indicated a rigid structure-function relationship with this protein (Peremyslov *et al.*, 2004) as the majority of introduced caused a loss of cell-cell movement. The

homodimerisation of the p6 protein via a disulphide bond between N-terminal cysteine residues was also shown to be essential for cell-cell movement (Peremyslov *et al.*, 2004). Like VIP-ER, some lytic peptides such as the p10 protein of Avian Reovirus S1133, contain cysteine residues in their cytoplasmic domains, often positioned proximal to their transmembrane domains (Bodelon *et al.*, 2002). Mutation of these cysteine residues results in the loss of the ability to induce vesicle formation, but not to associate with membranes. Whether VIP-ER oligomerises with or without the involvement of the cytoplasmic cysteine residues remains to be proven.

Another possible function of VIP-ER is in the assembly of the virion. Research of the coat proteins of rod shaped viruses has highlighted a central problem. Coat protein monomers can exist in a variety of conformational states based on the surrounding medium conditions. This can be clearly described with the work on the A protein of TMV (Fraenkel-Conrat and Williams, 1955). The A protein monomer can aggregate in various forms depending on pH, ionic strength and temperature. At high temperature, high pH or low ionic strength the A protein remains as a monomer. Reducing either temperature or pH and increasing ionic strength allows the stable formation of multimers until complete discs are created. These discs are incapable of forming a rod structure and require further changes of these factors to switch into the state termed the 'lockwasher'. This state can be best described as a helical form of the coat protein discs. This form allows the extension of the virion to completely cover the viral genome. An equilibrium exists between these states depending on the cellular conditions. VIP-ER could possible have a role in adjusting the balance of this equilibrium by increasing the cellular cation levels by disrupting the ER, therefore increasing the ionic strength and promoting the formation of virions. The targeting of the GLRaV-1 replication complex

to the multivesicular bodies, which are potentially part of the late endosome pathway, would provide a low pH environment. This would also promote the formation of virions. Conversely, if a complete virion was transported into a healthy cell that the cellular conditions would be such that the virion would disassemble exposing the viral genome for replication.

CONCLUDING REMARKS

EIGHT

SUMMARY AND FUTURE DIRECTIONS

8.1 SUMMARY.

The 17,647-nucleotide genome sequence of GLRaV-1 has now been completed and sequence data analysed using bioinformatics.

The shortcomings of the current tests for GLRaV-1 detection were addressed. The current RT-PCR test was inadequate due to poor reliability and reproducibility of results, presumably due to sequence variation within the virus, targeting regions of low copy number and inhibition from excess RNA and impurities such as phenolics.

The method for purifying the viral RNA has been optimised using magnetic capture hybridisation (MCH) prior to RT-PCR. This process removes any potential RT-PCR inhibitors from the sample, therefore improving reliability. This procedure also concentrates the RNA allowing addition of at least 100 fold more RNA into the reaction, therefore increasing sensitivity. The problem of RT-PCR reliability associated with viral sequence variation has been avoided by targeting primers to ORF9, a highly conserved region of the genome. This has also provided the benefit of targeting a region of the viral genome with high copy number due to the presence of ORF9 on all 3' subgenomic RNAs.

The magnetic capture RT-PCR technique has been tested on a range of different grapevine varieties from the field. 28 samples were screened for the presence of GLRaV-1 showing 9 positives using the current RT-PCR and ELISA tests. The use of magnetic capture prior to RT-PCR allowed the detection of 16 positive samples. The MCH-RT-PCR technique provides an effective and practical screen to identify grapevine samples infected with GLRaV-1.

Parallel to this research, further experiments aimed at characterising proteins encoded by GLRaV-1 have been completed. The localisation pattern of a protein with a cell can suggest possible roles for the gene in the virus life cycle. Studies involving ORF fusions with green fluorescent protein have highlighted a number of possible interesting protein functions for future research including ORFs 2, 3, 6 and 9 which seem to be involved in virus movement and maintenance. ORF2 appears to alter the internal structure of the cell while ORF9 is targeted to the nucleus suggesting both viral proteins are interacting with plant host factors. These results have been repeated *in planta* using confocal microscopy and transmission electron microscopy.

All of the project objectives have been achieved and the research has been collated into two papers for publication in scientific journals. This work has also been presented at recent national and international conferences.

8.2 FUTURE DIRECTIONS.

As the viticulture industry undergoes the current rapid expansion phase, certification of planting material has become a major issue. GLRaV-1 is the main subject of a strict screening program conducted annually by AVIA. Screening of planting material for GLRaV-1 is effective, although at present the risk remains that certain grapevine material may be carrying GLRaV-1 and be distributed unnoticed. The spread of GLRaV-1 from infected vines and natural reservoirs to the certified virus-free grapevine material still poses a substantial problem for the grapevine industry. Of major importance in designing a strategy for the control of a virus in a specific crop is an understanding of the epidemiology of that virus. Effective control of GLRaV-1 in Australia and worldwide will not be possible without a further understanding of the basic processes involved in the replication and cell-cell movement of the viral genome.

At this stage *Beet yellows virus* and *Citrus tristeza virus* are the best characterised closteroviruses with BYV research helping further characterise the function of viral open reading frames. Advances made in the research of BYV and CTV have been possible with the construction of full-length infectious clones. From this researchers were able to gain enough knowledge of the viral epidemiology and individual protein functions to design strategies to aid in the control of the viral diseases. With the completion of the GLRaV-1 genome sequence it is now feasible and essential to conduct this research with GLRaV-1. A basic project outline has been developed for future grant applications.

The aims of the proposed study are to:

1 - Prove the etiology of *Grapevine Leafroll-associated Virus 1* in Leafroll disease.

2 - Produce a viral-based protein expression system for grapevines.

3 - Determine the role of VIP-ER (ORF2).

8.2.1 PROVE THE ETIOLOGY OF GLRaV-1 IN LEAFROLL DISEASE.

The etiology of grapevine leafroll disease is yet to be proven by fulfilling Koch's postulates, hence GLRaV-1 is said to be associated with leafroll disease. To satisfy Koch's postulates the suspected disease agent must be purified from a diseased plant and must cause the same disease when introduced into an otherwise healthy plant. GLRaV-1 cannot be inoculated mechanically; therefore this cannot be achieved without a full-length infectious clone. The construction of a full-length infectious clone of GLRaV-1 would be a breakthrough, generating interest from the international community of grapevine virologists. It would be a significant outcome of this project and would benefit both the scientific field and the industry. It would also open up a broad range of research opportunities into GLRaV-1. Applications of a full length infectious construct of GLRaV-1 would be to:

- further analyse GLRaV-1 epidemiology.
- design and test possible GLRaV-1 resistance and control strategies.
- determine the effects of GLRaV-1 infection on wine quality in various grapevine cultivars, an issue debated by the wine industry.

These future applications are achievable and some have already been accomplished with full-length constructs of BYV and CTV.

8.2.1.1 APPROACH.

The 17.6 kilobase viral RNA genome must be converted into DNA and cloned into an expression vector using conventional molecular biology methods. A variety of inoculation methods will be tested for infection of plant material, including biolistic bombardment and agrobacterium transfer.

Objective #1.1 - To construct a full length cDNA clone of GLRaV-1.

Objective #1.2 - To completely sequence the full length cDNA clone.

Objective #1.3 - To insert the GFP gene into the full length clone for virus tracking during infection within the plant.

Objective #1.4 - To set up a virus infection system using various inoculation protocols.

Objective #1.5 - To prove the etiology of GLRaV-1.

8.2.2 PRODUCE A VIRAL-BASED PROTEIN EXPRESSION SYSTEM FOR GRAPEVINES.

The construction of a virus vector containing the minimal genes required for replication would provide a powerful tool for scientists wanting to express foreign proteins in grapevines for functional analysis. There are currently no viral expression vector systems designed for transient expression in grapevines. To observe the effect of a proteins expression *in planta* transgenic plants must be generated. The use of a virus expression system could significantly decrease the time and cost required for such research. Members of the family *Closteroviridae* exhibit striking similarities in genome organisation, however there are significant differences in the number and function of open reading frames encoded by these viruses. The available information concerning the functions of closterovirus proteins was inferred mainly from computer-assisted analysis

and has been investigated further with BYV and CTV using knockout mutations of each open reading frame in full-length infectious clones (Peremyslov *et al*, 1998; Satyanarayana *et al*, 1999). Performing this analysis on a full-length infectious clone of GLRaV-1 would allow the functions of each open reading frame to be characterised in more detail and provide the opportunity to compare their functions with that of BYV and CTV. Knowledge of the open reading frames essential for viral replication will also contribute to the design of virus expression vectors and resistance strategies, which would be invaluable to the grapevine industry.

8.2.2.1 APPROACH.

Open reading frame knockout mutants will be created using site-directed mutagenesis of the full-length infectious clone to reveal which products of GLRaV-1 are involved in RNA replication and which proteins are needed for cell to cell movement of the virus. With knowledge of the essential viral open reading frames a virus expression system will be constructed allowing the transient expression of foreign sequences. In the case of DNA viruses, it has generally been possible to identify nonessential regions of the genome that can be replaced by foreign sequences. However, there appear to be limitations on the size of insert which can be tolerated. In several cases as seen with viruses similar to GLRaV-1, it has been possible to substantially increase the size of the viral genome by the direct insertion of additional sequences while still retaining the ability of the viruses to multiply and spread in plants. These RNA virus-based systems appear to have the greatest potential as viral expression vectors.

Objective #2.1 - To construct a knockout mutation for each ORF in the full length GLRaV-1 clone.

Objective #2.2 - To analyse the effects of the knockout mutations on the ability of the clone to replicate the viral RNA and migrate to other cells.

Objective #2.3 - To construct a viral expression system containing only the minimal genes required for viral replication and spread.

8.2.3 DETERMINE THE ROLE OF VIP-ER (ORF2).

Research into the VIP-ER protein of GLRaV-1 has highlighted its importance in multiple areas of virus infection with potential applications in the design of resistance strategies and in the virus expression vector. The subcellular localisation patterns and TEM observations of VIP-ER have suggested a direct role in the formation of multivesicular bodies in the cell for the formation of the viral replication complex. There is an increasing interest in the origins of the ultrastructural locations of the sites of RNA replication of positive strand RNA animal and plant viruses. The genomes of positive-strand RNA viruses replicate in close association with membranes (Wimmer *et al*, 1993). Some viruses, including GLRaV-1, have been shown to induce proliferation or reorganisation of cellular membranes and vesicles (Schaad *et al*, 1997), possibly as a mechanism to increase the available surface area for RNA synthesis. The mechanisms whereby replication complexes are fixed to specific types of membranes are poorly understood, although the involvement of viral proteins as membrane anchors has been proposed (Wimmer *et al*, 1993). Understanding the ways in which viral proteins and replication complexes interact with membranes will shed considerable light on the critical virus-cell interactions that facilitate infection in plants and animals.

Secondly, the VIP-ER shows a potentially useful ability to suppress post-transcriptional gene silencing (PTGS) (Little, unpublished data). In plants, PTGS operates as a defence

strategy which prevents the expression of foreign proteins by sequence specific degradation of the encoding mRNA. This adaptive immune system is generally targeted towards plant viruses, but unfortunately the effect can also be seen when attempting to overexpress a protein in the plant. This limits the overall levels of protein that can be obtained in the plant by both transient and transgenic expression systems. As a counter-defensive strategy, many viruses have evolved proteins that suppress various steps of this mechanism allowing the efficient replication of their genomes. Co-expressing viral suppressors of PTGS in protein expression systems can alleviate the host response. This has been shown with the p19 protein encoded by *Tomato bushy stunt virus* (Voinnet *et al.*, 2003). This protein dramatically enhanced transient expression of a broad range of proteins, yielding gains exceeding 50-fold. The small size and simple structure of VIP-ER make it an ideal candidate for use in an enhanced protein expression system. We anticipate that the VIP-ER enhanced expression system will have value in industrial production as well as a research tool for the isolation and characterisation of a broad range of proteins.

8.2.3.1 APPROACH.

It will be determined whether these multivesicular bodies (MVBs) are the sites for the replication complex by observing the localisation patterns of replication proteins in the presence of VIP-ER expression. This can be done using protein fusions to green fluorescent protein and using fluorescence microscopy. Once this association has been proven it will be possible to use VIP-ER mutants, generated by oligonucleotide synthesis, to determine the regions of VIP-ER required for the formation of the MVBs and the targeting of the proteins involved in the replication complex to them. Depending

on the results obtained it may be possible to design strategies to directly inhibit the formation of the viral replication complex and viral replication.

Objective #3.A.1 - To observe if the vesicles formed by VIP-ER are required for the formation of the GLRaV-1 replication complex.

Objective #3.A.2 - To determine the regions of VIP-ER required for the formation of the vesicles and formation of the GLRaV-1 replication complex.

Objective #3.A.3 - To investigate possible options for interfering with the function of VIP-ER to inhibit viral replication.

Truncations and mutations of specific nucleotides will be done easily by designing oligonucleotides in both the sense and antisense form. These oligonucleotides will then be annealed together and end filled to create a dsDNA of the complete ORF2. These constructs will be ligated into a plasmid vector containing the GFP gene allowing expression of the VIP-ER:GFP fusion protein in planta. Particle bombardment will be used to insert the construct into the plant cells and the sub-cellular localisation of each truncation/mutation will be analysed using fluorescence microscopy. The ability of the truncation/mutation to suppress PTGS will be tested by co-bombarding a silencing construct targeted directly to GFP. This part of the project will result in the detailed characterisation of the residues necessary for the PTGS-suppressor function of VIP-ER.

Objective #3.B.1 - To confirm the ability of VIP-ER to suppress PTGS.

Objective #3.B.2 - To generate green fluorescent protein fusion constructs with truncated and/or mutated forms of VIP-ER.

Objective #3.B.3 - To analyse their sub-cellular localisation in target cells.

Objective #3.B.4 - To analyse their ability to suppress PTGS *in planta*.

This work will result in the increased understanding of GLRaV-1 and its role in leafroll disease. Knowledge of the open reading frames essential for viral replication and suppression of PTGS will also contribute to the design of enhanced virus expression vectors, which would be invaluable to the grapevine industry. The successful completion of this project will provide a strong foundation for future research of *Grapevine leafroll-associated virus 1* and alliances especially with the Australian viticulture industry. Knowledge and experience gained through this project will have a direct benefit in safeguarding the future of the grapevine industry in Australia. Recent experience has demonstrated that the Australian wine industry is likely to be confronted with new disease problems from time to time. This project will help maintaining virological skills necessary to address such issues and to maintain a leading role in the study of GLRaV-1 worldwide.

Technologies developed through the proposed study will not only contribute to our basic understanding of viral replication in plants, but also allow the development of strategies to control GLRaV-1 infections.

BIBLIOGRAPHY

Abou-Ghanem, N., Sabanadzovic, S., Minafra, A., Saldarelli, P. and Martelli, G. P. (1998). Some properties of *grapevine leafroll-associated virus 2* and molecular organisation of the 3' region of the viral genome. *J. Plant Path.* **80,** 3746.

Agranovsky, A. A. (1995). Structure and expression of RNA genomes of closteroviruses. *J. Mol. Biol.* **29,** 751-754.

Agranovsky, A. A. (1996). Principles of molecular organisation, expression and evolution of closteroviruses: Over the barriers. *Adv. in Virus Res.* **47,** 119-159.

Agranovsky, A. A., Boyko, V. P., Karasev, A. V., Lunina, N. A., Koonin, E. V. and Dolja, V. V. (1991). Nucleotide sequence of the 3'-terminal half of beet yellows closterovirus RNA genome: unique arrangement of eight virus genes. *J. Gen. Virol.* **72,** 15-23.

Agranovsky, A. A., Koonin, E. V., Boyko, V. P., Maiss, E., Frotschl, R., Lunina, N. A. and Atabekov, J. G. (1994). Beet yellows closterovirus: Complete genome structure and identification of a leader papain-like thiol protease. *Virology* **198,** 311-324.

Agranovsky, A. A., Lesemann, D. E., Maiss, E., Hull, R. and Atabekov, G. (1995). "Rattlesnake" structure of a filamentous plant RNA virus built of two capsid proteins. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 2470-2473.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Alzhanova, D. V., Hagiwara, Y., Peremyslov, V. V., Dolja, V. V. (2000). Genetic analysis of the cell-to-cell movement of beet yellows closterovirus. *Virology* **268(1),** 192-200.

Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Mallory, A. C., Smith, T. H. and Vance, V. B. (1998). A viral suppressor of gene silencing in plants. *Proc Natl Acad Sci U S A.* **95(22)**, 13079-84.

Atreya, C. D., Atreya, P. L., Thronbury, D. W. and Pirone, I. P. (1992). Site-directed mutations in the potyvirus HC-Pro gene effect helper component activity, virus accumulation, and symptom expression in infected tobacco plants. *Virology* **191,** 106-111.

Bar-Joseph, M., Lee, R. F. and Pappu, H. R. (1995). The Closteroviruses. *In* "Pathogenesis and host specificity in plant diseases. Histopathological, biochemical, genetic and molecular bases" (R. P. Singh, U. S. Singh, and K. Kohomoto, Eds.), Vol. III: Viruses and viroids, pp. 65-85. Elsevier Science Ltd.

Beinz, K., Egger, D. and Pasamontes, L. (1987). Association with polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualised by electron microscopic immunocytochemistry and autoradiography. *Virology* **160**, 220-226.

Beinz, K., Egger, D. and Pfister, T. (1994). Characteristics of poliovirus replication complex. *Arch. Virol. Suppl.* **9**, 147-157.

Belli, G., Fortusini, A., Casati, P., Belli, L., Bianco, P. A. and Prati, S. (1994). Transmission of a grapevine leafroll associated closterovirus by the scale insect *Pulvinaria vitis L. Rivista di Patologia Vagetale, S. V.* **4,** 105-108.

Boscia, D., Greif, C., Gugerli, P., Martelli, G. P., Walter, B. and Gonsalves, D. (1995). Nomenclature of grapevine leafroll-associated putative closteroviruses. *Vitis* **34,** 171-175.

Bovey, R., and Martelli, G. P. (1992). Directory of major virus and virus-like diseases of grapevines. Mediterranean Fruit Crop Improvement Council (MFCIC), and ICVG.

Bovey, R., Gartel, W., Hewitt, W. B., Martelli, G. P. and Vuittenez, A. (1980). "Virus and virus-like diseases of grapevines." Editions Payot, Lausanne.

Candresse, T. and Martelli, G. P. (1995). Closterovirus Group. *In* "Virus Taxonomy: Classification and nomenclature of viruses. Sixth report of the International Committee on Taxonomy of viruses" (F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers, Eds.), pp. 461-464. Springer-Verlag Wein New York, Austria.

Candresse, T., Namba, S. and Martelli, G. P. (1995). Trichovirus Group. *In* "Virus Taxonomy: Classification and nomenclature of viruses. Sixth report of the International Committee on Taxonomy of viruses" (F. A. Murphy. C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers, Eds.), pp. 468-470. Springer-Verlag Wein New York, Austria.

Carmo-Fonseca, M., Mendes-Soares, L. and Campos, I. (2000). To be or not to be in the nucleolus. *Nat. Cell Biol.* **2(6),** 107-112.

Carrasco, L. (1995). Modification of membrane permeability by animal viruses. *Adv. Virus Res.* **45,** 61-112.

Castellano, M. A., Abou-Ghanem, N., Choueriri, E. and Martelli, G. P. (2000). Ultrastructure of Grapevine leafroll-associated virus 2 and 7 infections. *J. Plant Pathol.* **82,** 9-15.

Castellano, M. A., Abou-Ghanem, N., Martelli, G. P., Boscia, D. and Savino, V. (1995). Cytopathology of two filamentous grapevine viruses and their intracellular identification by gold immunolabelling. *Zeitschrwt fur Pflanzenkrenkheiten und Pflanzenschutz* **102,** 23-33.

Castellano, M. A., Martelli, G. P., Savino, V. and Boscia, D. (1985). Progress in the study of the phloem-limited isometric virus-like particles associated with leafroll diseased grapevines. *Phytopath. Medit.* **24,** 165-169.

Choueiri, E., Boscia, D., Digiaro, M., Castellano, M. A. and Martelii, G. P. (1996).

Some properties of a hitherto undescribed filamentous virus of the grapevine. *Vitis* **35,** 91-93.

Cirami, R. M., Velsen, R. I. and Niejalke, I. (1988). Grapevine virus indexing in the South Australian Vine Improvement Scheme, 1974-1987. *Australian .1. Experimental Agriculture* **28,** 645-649.

Dolja, V. V., Herndon, K. L., Pirone, T. P. and Carrington, I. C. (1993). Spontaneous mutagenesis of a plant potyvirus genome after insertion of a foreign gene. *J. Virology* **67,** 5968-5975.

Dolja, V. V., Karasev, A. V. and Koonin, E. V. (1994). Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes. *Ann. Rev. Phytopathology* **32,** 261-285.

Engelbrecht, D. I. and Kasdorf, G. G. F. (1990). Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealy bug, *Planococcus Ficus. Phytophylactica* **22,** 341-346.

Faoro, F. (1997). Cytopathology of closteroviruses and trichoviruses infecting grapevines, p. 29-47. In P. L. Monette (ed.), Filamentous viruses of woody plants. Research Signpost, Trivandrum, India.

Faoro, F., and Carzaniga, R. (1995). Cytochemistry and immunocytochemistry of the inclusion bodies induced by grapevine leafroll-assoicated closteroviruses GLRaV-1 and GLRaV-3. *Riv. Pat. Veg.* **5**, 85-94.

Faoro, F., Tornaghi, R. and Belli, G. (1991). Localisation of closteroviruses on grapevine thin sections and their identification by immunogold labelling. *J. Phytopathol.* **133**, 297-306.

Faoro, F., Tornaghi, R., Fortusini, A. and Belli, G. (1981). *Riv. Pat. Veg.* **17**, 183.

Fazeli, C. F., and Rezaian, M. A. (2000). Nucleotide sequence and organization of ten open reading frames in the genome of grapevine leafroll-associated virus 1 and identification of three sub-genomic RNAs. *J. Gen. Virol.* **81(3),** 605-15.

Fazeli, C. F., Habili, N., and Rezaian, M. A. (1998). Efficient cloning of cDNA from grapevine leafroll-associated virus 4 and demonstration of probe specificity by the viral antibody. *J. Virol. Methods* **70(2),** 201-211.

Fortusini, A., Scattini, G., Prati, S., Cinquanta, S. and Belli, G. (1997). Transmission of grapevine leafroll virus 1 (GLRV-l) and grapevine virus A (GVA) by scale insects. *12th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, ICVG, Portugal.*

Fraenkel-Conrat, H. and William, R. C. (1955). Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. *Proc. Natl. Acad. Sci. U.S.A.* **41**, 690-698.

Gleave, A. P. (1992). A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20(6)**, 1203-1207.

Goheen, A. C. (1970). Grapevine leafroll. *In* "Virus diseases of small fruits and grapevines" (N. W. Frazier, Ed.). University of California, Division of Agricultural Sciences, Berkeley, California, USA.

Goheen, A. C. and Cook, I. A. (1959). Leafroll (red-leaf or rougeau) and its effects on vine growth, fruit quality and yields. *American J. Enology and Viticulture* **10,** 173-181.

Goldbach, R. (1990). Genome similarities between positive-strand RNA viruses from plants and animals. *In* "New aspects of positive-strand RNA viruses", pp. 3-12. Brinton and Heinz, American Society for Microbiology, Washington DC.

Golino, D. A., Sim, S. T., Gill, R. S. and Rowhani, A. (2002). California Mealybugs can spread grapevine leafroll disease. *California Agriculture* **56,** 196-201.

Golino, D. A., Sim, S. T., Gill, R. S. and Rowhani, A. (1994). Evidence that California Mealy bug transmit grapevine leafroll-associated viruses. *Meet. American Society of Enology and Viticulture, California.*

Gorbalenya, A. E., Koonin, E. V. and Lai, M. M. C. (1991). Putative papain-related proteases of positive-strand RNA viruses. *FEBS Lett.* **288,** 201-205.

Grape and Wine Research and Development Corporation (2002). Report of the National Vine Health Steering Committee on Proceedings of the Clean Planting Material Workshop. Adelaide, GWRDC (Executive Director D. Hall). http://www.gwrdc.com.au/WEB%20docs/Clean%20Planting%20Material%20Proceedings.pdf

Gugerli, P. and Ramel, M.-E. (1993). Grapevine leafroll associated virus II analyzed by monoclonal antibodies. *11th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, ICVG, Switzerland.*

Gugerli, P., Brugger, I. I. and Bovey, R. (1984). L'enroulment de la vigne: mise en evidence de particules virales et developpement d'une methode immunoenzymatiques pour le diagnostic rapide. *Rev. Suisse Viticult. Arboricult. Horticult.* **16,** 299-304.

Habili, N. and Rezaian, M. A. (1995). Cloning and molecular analysis of double-stranded RNA associated with grapevine leafroll disease. *Annals of Applied Biology* **127,** 95-103.

Habili, N., Ewart, A. I. W., Fazeli, C. F., Scott, N. S., Krake, L. R. and Rezaian, M. A. (1996). Virus types associated with grapevine leafroll disease in Australia. *The Australian Grape grower and Winemaker* **390a,** 25-28.

Habili, N., Fazeli C. F. and Rezaian, M. A. (1997). Identification of a cDNA clone specific to grapevine leafroll-associated virus 1, and occurrence of the virus in Australia. *Plant Pathol.* **46**, 516-522.

Habili, N., Krake, L. R., Barlass, M. and Rezaian, M. A. (1992). Evaluation of biological indexing and dsRNA analysis in grapevine virus elimination. *Annals of Applied Biology* **121,** 277-283.

Haseloff, J., Siemering, K. R., Prasher, D.C. and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. *Proc. Natl. Acad. Sci.* U. S. A. **94(6)**, 2122-2127.

Hewitt, W. B., Goheen, A. C., Raski, D. J. and Gooding, G. V. I. (1962). Studies on virus diseases of the grapevine in California. *Vitis* **3,** 57-83.

Hiscox, J. A. (2002). The nucleolus-a gateway to viral infection? *Arch. Virol.* **147(6)**, 1077-1089. Review.

Hoefert, L. L. and Gifford, E. M. (1967). Grapevine leafroll virus-History and anatomic effects. *HILGARDIA* **38,** 403-420.

Hu, I. S., Gonsalves, D. and Teliz, D. (1990b). Characterisation of clostero-like particles associated with grapevine leafroll disease. *J. Phytopathology* **128,** 1-14.

Hu, J. S., Gonsalves, D., Boscia, D. and Namba, S. (1990a). Use of monoclonal antibodies to characterise grapevine leafroll associated closteroviruses. *Phytopathology* **80,** 920-925.

Hull, R. (2001). Matthews' plant virology. Ed. by Hull, R. *Academic press.* pp 91-93.

Jacobsen, C. S. (1995). Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridization and PCR amplification assay. *Appl. Environ. Microbiol.* **61(9)**, 3347-3352.

Jelkmann, W., Fechtner, B. and Agranovsky, A. A. (1997). Complete Genome Structure and Phylogenetic Analysis Of Little Cherry Virus, a Mealy bug-Transmissible Closterovirus. *J. Gen. Virol.* **78,** 2067-2071.

Kanda, T., Sullivan, K. F. and Wahl, G. M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol.* **8(7)**, 377-85.

Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O. V., Hilt, M. E., Koonin, E. V., Niblett, C. L., Cline, K., Gumpf, D. I., Lee, R. F., Garnsey, S. M., Lewandowski, D. I. and Dawson, W. O. (1995). Complete sequence of the citrus tristeza virus RNA genome. *Virology* **208,** 511-520.

Karasev, A. V., Kashina, A. S., Gelfand, V. I. and Dolja, V. V. (1992). HSP7O-

related 65 kDa protein of beet yellows closterovirus is a microtubule-binding protein. *FEBS* **304,** 12-14.

Karasev, A. V., Nikolaeva, O. V., Mushegian, A. R., Lee, R. F. and Dawson, W. O. (1996). Organisation of the 3'-terminal half of beet yellows stunt virus genome and implications for the evolution of closteroviruses. *Virology* **221,** 199-207.

Keim-Konrad, R. and Jelkmann, W. (1996). Genome analysis of the 3'-terminal part of the little cherry disease associated dsRNA reveals a monopartite clostero-like virus. *Archives of Virology* **141,** 1437-1451.

Kerridge, G. and Antcliff, A. (1996). "Wine grape varieties of Australia." (M. Veroni, Ed.) CSIRO Australia.

Klaassen, V. A., Boeshore, M. L., Koonin, E. V., Tian, T. and Falk, B. W. (1995). Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. *Virology* **208,** 99-110.

Koonjul, P. K., Brandt, W. F., Farrant, J. M., Lindsey, G. G. (1999). Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. *Nucleic Acids Res.* **27(3),** 915-6.

Koonin, E. V., and Dolja, V. V. (1993). Evolution and taxonomy of positive strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* **28,** 374-430.

Kozak, M. (1986). Bifunctional messenger RNAs in eukaryotes. Cell **47**, 481-483.

Lee, H. I., Shieh, C. K., Gorbalenya, A. E., Koonin, E. V., la Monica, N., Tuler, I., Baghzhyan, A. and Lai, M. M. C. (1991). The complete sequence (22 kilobase) of murine coronavirus gene 1 encoding the putative proteases and RNA polymerase. *Virology* **180,** *567-582.*

Ling, K. S., Zhu, H. Y. and Gonsalves, D. (2004). Complete nucleotide and genome organisation of Grapevine leafroll-associated virus 3 the type member of the genus *Ampelovirus. J. Gen. Virol.* **85,** 2099-2102.

Little A., Fazeli C. F., and Rezaian M. A. (2001). Hypervariable genes in Grapevine Leafroll associated Virus 1. *Virus Research* **80(1-2)**, 109-116.

Loening, U. W. (1967). The fractionation of high molecular weight ribonucleic acid by polyacrylamide gel electrophoresis. *J. Biochemistry.* **102**, 251-257.

Loomis, W. D. (1974). Overcoming problems of phenolic and quinones in the isolation of plant enzymes and organelles. *In* "Methods in Enzymology" (S. Fleischer, and L. Packer, Eds.), Vol. 31, pp. *528-544.* Academic Press, New York.

Lopez, C., Ayllon, M. A., Navas-Castillo, J., Guerri, J., Moreno, P. and Flores, R. (1998). Molecular variability pf the 5' and 3'-terminal regions of *Citrus tristeza virus*

RNA. *Virology* **88(7)**, 685-691.

Lopez, C., Navas-Castillo, J., Gowda, S., Moreno, P., and Flores, R. (2000). The 23-kDa protein coded by the 3'-terminal gene of citrus tristeza virus is an RNA-binding protein. *Virology* **269(2),** 462-70.

MacKenzie, D. I., Johnson, R. C. and Warner, C. (1996). Incidence of four important viral pathogens in Canadian vineyards. *Plant Disease* **80,** *955-958.*

MacKenzie, D. J., McLean, M. A., Mukerji, S. and Green, M. (1997). Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Dis.* **81**, 222-226.

Martelli, G. P. and Bar-Joseph, M. (1991). Closterovirus Group. *In* "Classification and nomenclature of viruses" (R. I. B. Francki, C. M. Fauquet, D. L. Knudson, and F. Brown, Eds.), pp. 345-347. Springer-Verlag, Wien.

Martelli, G. P., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., Dolja, V. V., Falk, B. W., Gonsalves, D., Jelkmann, W., Karasev, A. V., Minafra, A., Namba, S., Vetten, H. J., Wisler, G. C. and Yoshikawa, N. (2002). The family *Closteroviridae* revised. *Arch. Virol.* **147(10)**, 2039-2044.

Martelli, G. P., Minafra, A. and Saldarelli, P. (1997). *Vitivirus*, a new genus of plant viruses. *Arch. Virol.* **142**, 1929-1932.

Mawassi, M., Mietkiewska, E., Gofman, R., Yang, G., and Bar-Joseph, M. (1996). Unusual sequence relationships between two isolates of citrus tristeza virus. *J. Gen. Virol.* **77(9),** 2359-64.

Medina, V., Peremyslov, V. V., Hagiwara, Y. and Dolja, V. V. (1999). Subcellular localization of the HSP70-homolog encoded by beet yellows closterovirus. *Virology.* **260(1)**, 173-181.

Melzer, M. J., Karasev, A. V., Sether, D. M. and Hu, J. S. (2001). Nucleotide sequence, genome organization and phylogenetic analysis of pineapple mealybug wilt-associated virus-2. *J. Gen. Virol.* **82(1)**, 1-7.

Merril, C. R., Goldman, D., Sedman, S. A. and Ebert, M. H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science*. **211**, 1437-1438.

Minafra, A. and Hadidi, A. (1994). Sensitive detection of grapevine virus A, B, or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. *J. Virol. Methods* **47(1-2)**, 175-187.

Nakai, K. and Horton, P. (1999). PSORT: a program for detecting the sorting signals of proteins and predicting their subcellular localization. *Trends Biochem. Sci*, **24(1)**, 34-35.

Norrie, P. A. (1992). "A study of original documents on viticulture in early New South

Wales and the role of the Macleay family 1788 to 1883." Thesis (M.Sc.)-University of Sydney, Australia.

Olson, M. O., Dundr, M. and Szebeni, A. (2000). The nucleolus: an old factory with unexpected capabilities. *Trends Cell. Biol.* **10(5)**, 189-96. Review.

Pappu, H. R., Karasev, A. V., Anderson, E. J., Pappu, S. S., Hill, M. E., Febres, V. I., Eckloff, R. M. G., McCaffery, M., Boyko, V., Gowda, S., Dolja, V. V., Koonin, E. V., Gumpf, D. I., Cline, K. C., Garnsey, S. M., Dawson, W. I., Lee, R. F. and Niblett, C. L. (1994). Nucleotide sequence and organisation of eight 3' open reading frames of the citrus tristeza closterovirus genome. *Virology* **199,** 3546.

Pederson, T. (1998). The plurifunctional nucleolus. *Nucleic Acids Res.* **26(17)**, 3871-6. Review.

Peng, C. W., Permyslov, V. V., Mushegian, A. R., Dawson, W. O. and Dolja, V. V. (2001). Functional specialization and evolution of leader proteinases in the family *Closteroviridae*. *Journal of Virology*. **75(24)**, 12153-12160.

Peremyslov, V. V., Hagiwara, Y. and Dolja, V. V. (1998). Genes acquired for replication of the 15.5-kilobase RNA genome of a plant closterovirus. *J. Virology* **72,** 5870-5876.

Peremyslov, V. V., Hagiwara, Y., and Dolja, V. V. (1999). HSP70 homolog functions in cell-to-cell movement of a plant virus. *Proc. Natl. Acad. Sci. U.S.A.* **96(26),** 14771-6.

Peremyslov, V. V., Pan, Y-W., and Dolja, V. V. (2004). Movement protein of a closterovirus is a type III integral transmembrane protein localised to the endoplasmic reticulum. *J. Virology* **78**, 3704-3709.

Peters, R. (1986). Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim Biophys Acta* **864**, 305–359.

Petersen, C. L. and Charles, J. G. (1997). Transmission of grapevine leafroll-associated closteroviruses by *Pseudococcus ion gispinus* and *P. calceolariae. Plant Pathology* **46,** 509-515.

Ravaz, L. and Verge, G. (1924). Le rougeau de la vigne. *Progr. agr. et vit.* **81,** 11-17.

Ravaz, L., Dupont, E. and Callaudeaux, R. (1933). Recherches sur le rougeau de la vigne. *Ann. Agron. n.s.* **3,** 225-231.

Regenmortel M. H. V., Fauquet C. M., Bishop D. H. L., Carstens E. B., Estes M. K., Lemon S. M., Maniloff J., Mayo M. A., McGeoch D. J., Pringle C. R. and Wickner R. B. (2000). Virus Taxonomy: The Classification and Nomenclature of Viruses. The Seventh Report of the International Committee on Taxonomy of Viruses (book). Virus Taxonomy, VIIth report of the ICTV. Academic Press, SanDiego, 1167pp.

Rezaian, M. A. and Krake, L. R. (1987). Nucleic acid extraction and virus detection in grapevine. *J. Virol. Methods* **17(3-4)**, 277-285.

Rezaian, M. A., Krake, L. R., Gunying, Q. and Hazzalin, C. A. (1991). Detection of virus-associated dsRNA from leafroll infected grapevines. *J. Virol. Methods* **31,** 325-334.

Rocsiglione, B. and Gugerli, P. (1987). Transmission of grapevine leafroll disease and an associated closterovirus to healthy grapevine by mealy bug *Pianococcus ficus* Signoret. *9th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, ICVG, Israel.*

Rozanov, M., Koonin, E. V. and Gorbalenya, A. E. (1992). Conservation of the putative methyltransferase domain: A hallmark of the 'Sinbis-like' supergroup of positive-strand RNA viruses. *J. Gen. Virol.* **73**, 2129-2134.

Saldarelli, P., Minafra, A., Martelli, G. P. and Walter, B. (1994). Detection of grapevine leafroll-associated closterovirus III by molecular hybridisation. *Plant Pathology* **43,** 91-96.

Sambrook, J. and Russell, G. W. (2001). Molecular cloning, a laboratory manual. *Cold Spring Harbour Laboratory Press*, New York.

Satyanarayana, T., Gowda, S., Ayllon, M. A., Albiach-Marti, M. R., Rabindran, S. and Dawson, W. O. (2002). The p23 protein of citrus tristeza virus controls asymmetrical RNA accumulation. *J. Virol.* **76(2)**, 473-83.

Satyanarayana, T., Gowda, S., Boyko, V. P., Albiach-Marti, M. R., Mawassi, M., Navas-Castillo, J., Karasev, A. V., Dolja, V., Hilf, M. E., Lewandowski, D. J., Moreno, P., Bar-Joseph, M., Garnsey, S. M., and Dawson, W. O. (1999). An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication. *Proc. Natl. Acad. Sci. U.S.A.* **96(13),** 7433-8.

Schaad, M. C., Jensen, P. E. and Carrington, J. C. (1997). Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. *The EMBO J.* 16(13), 4049-4059.

Scheu, G. (1936). "Mein Winzerbuch." Reichsnabrstand Verlags-G.m.b.H., Berlin.

Segrest, J. P., de Loof, H., Dohlman, J. G., Brouilette, C. G. and Anatharamaiah, G. M. (1990). Amphipathic helix motif: classes and properties. *Struct. Funct. Genet.* **8**, 103-117.

Selth, L. A., Randles, J. W., and Rezaian, M. A. (2004). Expression of the *Tomato leaf curl geminivirus* genes in host plants reveals novel viral:host interactions. *Molecular Plant Microbe Interactions* **17**, 27-33.

Sforza, R., Komar, V., and Greif, C. (2000). New scale vectors of grapevine closteroviruses. 13th Meeting of the International Council for the Study of Viruses and

Virus Diseases of the Grapevine, ICVG, Australia. Published by the University of Adelaide and CSIRO.

Shai, Y. (1995). Molecular recognition between membrane spanning polypeptides. *Trends Biol. Sci.* **20**, 460-464.

Snijder, E. I., Ecerveen, J., Spaan, W. J. M., Weiss, M. and Horzinek, M. C. (1988). Characterisation of Berne virus genomic and messenger RNAs. *J. Gen. Virol.* **69,** 2315-2144.

Spaan, W. I. M., Cavanagh, D. and Horzinek, M. C. (1988). Coronaviruses: Structure and genome expression. *J. Gen. Virol.* **69,** 2939 - 2952.

Staehelin, L. A. (1997). The plant ER: a dynamic organelle composed of a large number of discrete functional domains. *Plant J.* **11(6)**, 1151-1165.

Tanne, E., Ben-Dov, Y. and Raccah, B. (1989). Transmission of clostero-like particles associated with grapevine leafroll by mealy bugs (Pseudococcidae) in Israel. *9th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, ICVG, Israel.*

Teliz, D., Tanne, E., Gonsalves, D. and Zee, F. (1987). Field serological detection of viral antigens associated with grapevine leafroll disease. *Plant Disease* **71,** 704-709.

The International Council for the Study of Virus and Virus-like Diseases of the Grapevine (2003). Recommendation by the General Assembly of the 14th meeting of the ICVG, Locorotondo, Italy.

Valverde, R. A. (1990). Analysis of double-stranded RNA for plant virus diagnosis. *Plant Disease* **74,** 255-258.

van Kuppeveld, F. J., Hoenderop, J.G., Smeets, R. L., Willems, P. H., Dijkman, H. B., Galama, J. M. and Melchers, W. J. (1997). Coxsackievirus protein 2B modifies endoplasmic reticulum membrane and plasma membrane permeability and facilitates virus release. *EMBO J.* **16(12)**, 3519-3532.

Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* **33(5)**, 949-56.

Vuittenez, A. (1958). Transmission par greffage d'une virose du type "enroulement foliaire" commune dans las vignobles de l'est et due centre est de la France. *Compt. rend. Acad. agr.* **44,** 313-316.

Walter, B. and Martelli, G. P., (1997). Clonal and sanitary selection of the grapevine. In Sanitary Selection of the Grapevine: Protocols for Detection of Viruses and Virus-like Diseases. Ed B. Walter, 43-95. Colmar, France: Institut National De La Recherche Agronomique.

Wimmer, E., Heelen, C. U. and Cao, X. (1993). Genetics of poliovirus. *Annu. Rev. Genet.* **27**, 353-436.

Woodham, R. C., Antcliff, A. I., Krake, L. R. and Taylor, R. H. (1984). Yield differences between Sultana clones related to virus status and genetic factors. *Vitis* **23,** 73-83.

Woodham, R. C., Taylor, R. H. and Krake, L. R. (1973). Grapevine virus and virus-like diseases in Southern Australia. *Rivista Di Pathologia Vegetale* **9,** 17-22.

Zhu, H. Y., Ling, K. S., Goszczynski, D. E., McFerson, I. R. and Gonsalves, D. (1998). Nucleotide sequence and genome organisation of grapevine leafroll-associated virus-2 are similar to beet yellows virus, the closterovirus type member. *J. Gen. Virol.* **79,** 1289-1298.

Zimmermann, D., Bass, P., Legin, R. and Walter, B. (1990). Characterisation and serological detection of four closterovirus-like particles associated with leafroll disease in grapevines. *J. Phytopathology* **130,** 205-2 18.

Zinovkin, R. A., Jelkmann, W. and Agranovsky, A. A. (1999). The minor coat protein of beet yellows closterovirus encapsidates the 5' terminus of RNA in virions. *J. Gen. Virol.* **80(1)**, 269-72.

APPENDIX

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SOURCE      Grapevine leafroll-associated virus 1 (GLRaV-1)
  ORGANISM  Grapevine leafroll-associated virus 1
            Viruses; ssRNA positive-strand viruses, no DNA stage;
            Closteroviridae; Ampelovirus.
REFERENCE   1  (bases 1 to 5252)
  AUTHORS   Little,A. and Rezaian,M.A.
  TITLE     Subcellular Localisation of Proteins Encoded by the Grapevine
            Leafroll-associated Virus 1 Genome Reveals a Vesicle Inducing
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  JOURNAL   Virology (?) In press
REFERENCE   2  (bases 5253 to 17647)
  AUTHORS   Fazeli,C.F. and Rezaian,M.A.
  TITLE     Nucleotide sequence and organization of ten open reading frames in
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  JOURNAL   J. Gen. Virol. 81 (Pt 3), 605-615 (2000)
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LIST OF FIGURES