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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B. Sc. (Hons)

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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

Journal articles


Conference papers


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 supervisors, Ali Rezaian and John Randles, goes my sincere appreciation. Their guidance and support during my introduction to the scientific, industrial and political worlds of plant pathology has been exceptional. Special thanks to Ali for his constant belief in my abilities and his infamous advice to ‘focus and concentrate’.

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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.
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., 1985). 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
**Introduction**

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 *Kioster*, 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 sequence 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)

<table>
<thead>
<tr>
<th>Genus Closterovirus</th>
<th>Genus Ampelovirus</th>
<th>Genus Criniviridae</th>
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<tr>
<td><strong>Aphid-transmitted</strong></td>
<td><strong>Mealy bug-transmitted</strong></td>
<td><strong>Lettuce infectious yellows virus (tm)</strong></td>
</tr>
<tr>
<td>Beet yellows virus (type member)</td>
<td>Grapevine leafroll-associated virus 3 (tm)</td>
<td>AbYV</td>
</tr>
<tr>
<td>Beet yellows stunt virus</td>
<td>Grapevine leafroll-associated virus 1</td>
<td>GLRaV-1</td>
</tr>
<tr>
<td>Burdock yellows virus</td>
<td>Grapevine leafroll-associated virus 5</td>
<td>GLRaV-5</td>
</tr>
<tr>
<td>Carnation necrotic fleck virus</td>
<td>Little cherry virus 2</td>
<td>LChV-2</td>
</tr>
<tr>
<td>Carrot yellow leaf virus</td>
<td>Pineapple mealy bug wilt-associated virus 1</td>
<td>PMWaV-1</td>
</tr>
<tr>
<td>Citrus tristeza virus</td>
<td>Pineapple mealy bug wilt-associated virus 2</td>
<td>PMWaV-2</td>
</tr>
<tr>
<td>Wheat yellow leaf virus</td>
<td><strong>Tentative species (Mealy bug-transmitted)</strong></td>
<td>SMMV</td>
</tr>
<tr>
<td>Vector Unknown</td>
<td>Sugarcane mild mosaic virus</td>
<td></td>
</tr>
<tr>
<td>Grapevine leafroll-associated virus 2</td>
<td><strong>Tentative species (Vector unknown)</strong></td>
<td></td>
</tr>
<tr>
<td>Tentative species (Aphid-transmitted)</td>
<td>Grapevine leafroll-associated virus 4</td>
<td>GLRaV-4</td>
</tr>
<tr>
<td>Clover yellows virus</td>
<td>Grapevine leafroll-associated virus 6</td>
<td>GLRaV-6</td>
</tr>
<tr>
<td>Dendrobium vein necrosis virus</td>
<td>Grapevine leafroll-associated virus 8</td>
<td>GLRaV-8</td>
</tr>
<tr>
<td>Heracleum virus 6</td>
<td>Plum bark necrosis and stem pitting-associated virus</td>
<td>PBNSPaV</td>
</tr>
<tr>
<td>Festuca necrosis virus</td>
<td></td>
<td></td>
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<tr>
<td><strong>Tentative species</strong></td>
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<tr>
<td>Beet pseudo-yellows virus</td>
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<td></td>
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<tr>
<td>Diodia vein chlorosis virus</td>
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<td></td>
</tr>
<tr>
<td>Potato yellow vein virus</td>
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<td></td>
</tr>
<tr>
<td><strong>Unassigned species</strong></td>
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<td></td>
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<tr>
<td>Alligatorweed stunting virus</td>
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<td>Grapevine leafroll-associated virus 7</td>
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<td></td>
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<tr>
<td>Little cherry virus 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Megakepasma mosaic virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olive leaf yellowing-associated virus</td>
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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 coronaviruses 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 20 kb. 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.**

<table>
<thead>
<tr>
<th>Genome</th>
<th>BYV (Agronovskiy et al., 1991; Agronovskiy et al., 1994)</th>
<th>GLRaV-2 (Zhu et al., 1998)</th>
<th>CTV (Karassen et al., 1998; Pappo et al., 1994)</th>
<th>BYSV (Karassen et al., 1996)</th>
<th>LIVV (Karassen et al., 1995)</th>
<th>GLRaV-3 (Ling et al., 1998)</th>
<th>GLRaV-1 (Fadul et al., 2000)</th>
<th>PMVAV-2 (Melzer et al., 2001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1</td>
<td>15-480 nt</td>
<td>Ca 16,000</td>
<td>N/R</td>
<td>19-296</td>
<td>N/R</td>
<td>16,054</td>
<td>RNA 1: 8115</td>
<td>RNA 2: 7190</td>
</tr>
<tr>
<td>ORF1a</td>
<td>3</td>
<td>N/R</td>
<td>N/R</td>
<td>12</td>
<td>N/R</td>
<td>7</td>
<td>7</td>
<td>N/R</td>
</tr>
<tr>
<td>5’-NTR</td>
<td>107 nt</td>
<td>N/R</td>
<td>108 nt</td>
<td>N/R</td>
<td>70 nt</td>
<td>97 nt</td>
<td>120 nt</td>
<td>158 nt</td>
</tr>
<tr>
<td>ORF</td>
<td>1b (52 K)</td>
<td>POL</td>
<td>1b (52 K)</td>
<td>POL</td>
<td>1b (59 K)</td>
<td>POL</td>
<td>1b (55.5 K)</td>
<td>POL</td>
</tr>
<tr>
<td>ORF</td>
<td>1a (265 K)</td>
<td>P-Pro</td>
<td>1a (265 K)</td>
<td>P-Pro</td>
<td>1a (217.5 K)</td>
<td>P-Pro, MTR, HEL</td>
<td>1a (270 K)</td>
<td>P-Pro, MTR, HEL</td>
</tr>
<tr>
<td>ORF2</td>
<td>2 (64 K)</td>
<td>Hydrophobic</td>
<td>2 (64 K)</td>
<td>Hydrophobic</td>
<td>2 (40 K)</td>
<td>Hydrophobic</td>
<td>2 (40 K)</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>ORF2</td>
<td>3 (65 K)</td>
<td>HSP70 homologue</td>
<td>3 (65 K)</td>
<td>HSP70 homologue</td>
<td>2 (72 K)</td>
<td>HSP70 homologue</td>
<td>2 (72 K)</td>
<td>HSP70 homologue</td>
</tr>
<tr>
<td>ORF2</td>
<td>4 (64 K)</td>
<td>HSP90 homologue</td>
<td>4 (64 K)</td>
<td>HSP90 homologue</td>
<td>4 (61 K)</td>
<td>HSP90 homologue</td>
<td>4 (61 K)</td>
<td>HSP90 homologue</td>
</tr>
<tr>
<td>ORF2</td>
<td>5 (24 K)</td>
<td>CPM</td>
<td>5 (24 K)</td>
<td>CPM</td>
<td>5 (46 K)</td>
<td>CP</td>
<td>5 (27 K)</td>
<td>CP</td>
</tr>
<tr>
<td>ORF2</td>
<td>6 (22 K)</td>
<td>CP</td>
<td>6 (22 K)</td>
<td>CP</td>
<td>7 (24 K)</td>
<td>CP</td>
<td>6 (76 K)</td>
<td>CPM</td>
</tr>
<tr>
<td>ORF2</td>
<td>7 (20 K)</td>
<td>Unknown</td>
<td>7 (19 K)</td>
<td>Unknown</td>
<td>8 (18 K)</td>
<td>Unknown</td>
<td>8 (18 K)</td>
<td>Unknown</td>
</tr>
<tr>
<td>ORF2</td>
<td>8 (21 K)</td>
<td>Unknown</td>
<td>8 (24 K)</td>
<td>Unknown</td>
<td>9 (23 K)</td>
<td>Unknown</td>
<td>9 (22 K)</td>
<td>Unknown</td>
</tr>
<tr>
<td>ORF2</td>
<td>10 (20 K)</td>
<td>Unknown</td>
<td>11 (23 K)</td>
<td>RNA-binding protein</td>
<td>N/R</td>
<td>N/R</td>
<td>11 (23 K)</td>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>3’-NTR</td>
<td>181 nt; a cis-element, two potential stem-loop structures</td>
<td>216 nt; a cis-element, several potential stem-loop structures</td>
<td>577 nt; a cis-element, several potential stem-loop structures</td>
<td>264 nt; a cis-element, several potential stem-loop structures</td>
<td>210 nt; a cis-element, several potential stem-loop structures</td>
<td>210 nt; no secondary structure</td>
<td>187 nt; no secondary structure</td>
<td>365 nt; several potential stem-loop structures</td>
</tr>
<tr>
<td>5’-TERMINUS</td>
<td>No poly(A) tract</td>
<td>No poly(A) tract</td>
<td>No poly(A) tract</td>
<td>No poly(A) tract</td>
<td>No poly(A) tract</td>
<td>No poly(A) tract</td>
<td>No poly(A) tract</td>
<td>No poly(A) tract</td>
</tr>
</tbody>
</table>

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 Cys509 and His569, which cleave between two Gly residues in BYV
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 (Arandalakshmi 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, 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, 32,000 product of ORF3 or LIYV RNA 1 and M, 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, 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 (Agranovsky 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.
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, 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

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x agarose gel loading dye (DNA)</td>
<td>78% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 10 mM EDTA</td>
</tr>
<tr>
<td>10x agarose gel loading dye (RNA)</td>
<td>50% (v/v) glycerol, 0.2M EDTA, 0.08% (w/v) bromophenol blue.</td>
</tr>
<tr>
<td>10x MOPS/EDTA buffer</td>
<td>200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH 7.0.</td>
</tr>
<tr>
<td>5x denaturing agarose gel loading buffer (RNA)</td>
<td>70% (v/v) deionised formamide, 10% (v/v) formaldehyde, 6% (v/v) agarose-gel loading dye (RNA), 14% (v/v) MOPS/EDTA buffer.</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA.</td>
</tr>
<tr>
<td>TBE buffer</td>
<td>90mM Tris.HCl, 90mM borate (pH 8.3), 2mM EDTA.</td>
</tr>
<tr>
<td>STET buffer</td>
<td>8% (w/v) sucrose, 5% (v/v) triton X-100, 50mM EDTA (pH 8.0), 50mM Tris.HCl (pH 8.0).</td>
</tr>
<tr>
<td>dsRNA polyacrylamide gel, 6% (30ml)</td>
<td>25.65ml water, 6ml acrylamide solution (50%, 1:49 bis-acrylamide), 0.6ml 50x TAE, 120µl ammonium persulphate (25%), 10µl TEMED.</td>
</tr>
<tr>
<td>LB (liquid growth media)</td>
<td>1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0.</td>
</tr>
<tr>
<td>LB agar (solid growth media)</td>
<td>1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.2% (w/v) bacto-agar, pH 7.0.</td>
</tr>
<tr>
<td>Total RNA extraction buffer</td>
<td>50mM Tris.HCl, 100mM NaCl, 5mM EDTA, 2% (w/v) SDS, pH 8.0.</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10mM Tris.HCl, 1mM EDTA, pH 8.0.</td>
</tr>
<tr>
<td>20x SDS</td>
<td>3M NaCl, 1M sodium citrate, pH 7.0.</td>
</tr>
<tr>
<td>Hybridisation buffer</td>
<td>0.25M Na2HPO4, 7% SDS (w/v), 1mM EDTA.</td>
</tr>
<tr>
<td>SSC</td>
<td>150mM NaCl, 15mM tri-sodium citrate, pH 7.0.</td>
</tr>
<tr>
<td>STE (10x)</td>
<td>500mM Tris-HCl (pH 6.85), 1M NaCl, 10mM EDTA.</td>
</tr>
<tr>
<td>dsRNA extraction buffer</td>
<td>0.2M Tris-HCl (pH 7.5), 0.5M NaCl, 10mM MgCl2, 3% SDS, 1% β-mercaptoethanol.</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
<th>Size (nt)</th>
<th>Primer sequence (5' to 3'-end)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-6N</td>
<td>Tagged random primer</td>
<td>26</td>
<td>GCCGGAGCTCTGCGAGAATTGNNNN</td>
</tr>
<tr>
<td>GR 5'R</td>
<td>Gene racer 5' RNA oligo</td>
<td>44</td>
<td>CGACUGGAGCAGGAGGACACUGACAUGAGGAUGAA</td>
</tr>
<tr>
<td>GR 5' internal</td>
<td>Gene racer 5' internal oligo</td>
<td>23</td>
<td>CGACTGGACAGGAGGACACTGA</td>
</tr>
<tr>
<td>GR 5' nested</td>
<td>Gene racer 5' nested oligo</td>
<td>26</td>
<td>GGACACTGACATGCTGAAGAGTA</td>
</tr>
<tr>
<td>1bXho1-F</td>
<td>ORF1b reverse primer (Xho1 site, with start codon)</td>
<td>27</td>
<td>GGCTCGAGATGCTAGTGCTTTGTGCA</td>
</tr>
<tr>
<td>1bXba1-R</td>
<td>ORF1b reverse primer (Xba1 site, with no stop codon)</td>
<td>25</td>
<td>CCTCTAGACGGACTGAAGCTCTACCTGCT</td>
</tr>
<tr>
<td>2Xho1-F</td>
<td>ORF2 reverse primer (Xho1 site, with start codon)</td>
<td>27</td>
<td>GGCTCGAGATGCTAGTGCTTTGTGCA</td>
</tr>
<tr>
<td>2Xba1-R</td>
<td>ORF2 reverse primer (Xba1 site, with no stop codon)</td>
<td>25</td>
<td>CCTCTAGACGGACTGAAGCTCTACCT</td>
</tr>
<tr>
<td>3Xho1-F</td>
<td>ORF3 reverse primer (Xho1 site, with start codon)</td>
<td>27</td>
<td>GGCTCGAGATGCTAGTGCTTTGTGCA</td>
</tr>
<tr>
<td>3Xba1-R</td>
<td>ORF3 reverse primer (Xba1 site, with no stop codon)</td>
<td>31</td>
<td>CCTCTAGATTTATATCCTTGA AAGTTCA</td>
</tr>
<tr>
<td>4Xho1-F</td>
<td>ORF4 reverse primer (Xho1 site, with start codon)</td>
<td>27</td>
<td>GGCTCGAGATGCTAGTGCTTTGTGCA</td>
</tr>
<tr>
<td>4Xba1-R</td>
<td>ORF4 reverse primer (Xba1 site, with no stop codon)</td>
<td>31</td>
<td>CCTCTAGAGACCTTTTCCTCAT ATTTGTAAC</td>
</tr>
<tr>
<td>5Xho1-F</td>
<td>ORF5 reverse primer (Xho1 site, with start codon)</td>
<td>27</td>
<td>GGCTCGAGATGCTAGTGCTTTGTGCA</td>
</tr>
<tr>
<td>5Xba1-R</td>
<td>ORF5 reverse primer (Xba1 site, with no stop codon)</td>
<td>25</td>
<td>CCTCTAGACACCTTAAAGCTCCTAG</td>
</tr>
<tr>
<td>6Xho1-F</td>
<td>ORF6 reverse primer</td>
<td>27</td>
<td>GGCTCGAGATGCTAGTGCTT</td>
</tr>
</tbody>
</table>

Table 3-2: Oligodeoxyribonucleotide primers used in this study
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 NotI-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 3x RNA loading dye (Table 2.1) and incubated at 65°C for 15 min before loading. Electrophoresis was carried out in 1x 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 K₂Cr₂O₇ and 0.02% HNO₃ for 5 min. The gel was then washed three times with distilled water and impregnated with AgNO₃ solution (2g/l) for 30 min. The gel was rinsed with distilled water and soaked in 30g/l Na₂CO₃ 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, 30 sec at 55°C, 1 min at 68°C, and finally an extension time of 10 min 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 80\mu l 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 250\mu l 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 10 min 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 25\mu l 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 \textit{Closteroviridae} (Dolja \textit{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 \textit{et al}., 1997; Woodham \textit{et al}., 1984) by the method of Rezaian \textit{et al}., (1991). Samples were analysed by electrophoresis in a 6% polyacrylamide gel in TAE buffer (Loening, 1967) and visualised by silver staining (Merril \textit{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 MgCl₂, 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 MgCl₂, 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 MgCl₂, 2mM DTT, 0.05mg/ml BSA and 40 units 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
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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 1μl 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 EcoRI 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'-CGACUGGAGCACGAGGACACUGACUGACUGAAGGAGUAGAAA) using RNA ligase. The ligated mRNA was reverse transcribed using Superscript II reverse transcriptase and a gene-specific primer, 5' - CAGACTCCAATTCACAACGCTGG (reverse complement of bases 208 to 231). The initial PCR utilised GeneRacer 5'-primer (5' -CGACTGGAGCAGCAGGACACTGAG) and the gene-specific primer. Nested PCR, when performed, utilised GeneRacer 5'-nested primer (5' -GGACACTGACATGGACTGAAGGAG) and 5' -ATTAAACCAGACGCACCGTCCCAAGA 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 \( \text{m}^7 \text{G}^5 \text{ppp} \text{X}^{(m)} \text{Y}^{(m)} \text{p} \), 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, 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).
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-vgyfvalphipfrcaedtga1fgeYwCWNLqavmngnnllaqtfescinvrklormrldvklektce
| Consensus | -----------------------------G--CWL-------------------------------------L-------------------------- |
| GLRaV-1 | nifHvgknptvllsdickcfvsgmaakgqgsilvasvsnalngdfegivstianRLvikegStLwthlDeKis |
| Consensus | ----------------------------------V-----RL-----S-L-----D-L----- |
| GLRaV-1 | emfmmkedsekkncvvtvalnagakseltraFPELfitF1dSVsSsHgCSNaRsCNslyaskyrvpFvDI |
| Consensus | ----------------------------------K-------L-------LT-FPEL--F--SV-S-HF-N-R----N----------------F-DI |

| MTR 1 | MTR II | MTR II
| GLRaV-1 | GGsvayHrnnHdkdcHcCHFPVldkyKdcRReeegLrLatveekvmtvesvikseaakniSyCqmdtrvCoNkasv |
| Consensus | GG-------H---G---VHYCHFPV---D-KDA-BRV---L-L---------------S-C-------C--E--D-- |
| GLRaV-1 | givMVdVYDldvfElAlAleKKgikvfemcmfiPiElTarldgsItXpeLgyvVnrkgDtiYvGtyGdaYsHsvt |

| GLRaV-1 | kI3yfgsnvQylpsGsayseVYvgyRLGhyqF-616 |
| Consensus | --I--T--V-----G--F-VK-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.
Table 4-1: Percentage amino acid identity and similarity between GLRaV-1 and other *Closteroviridae* family replicase sequences.

<table>
<thead>
<tr>
<th></th>
<th>GLRaV-3</th>
<th>PMWaV-2</th>
<th>LChV-2</th>
<th>BYV</th>
<th>GLRaV-2</th>
<th>CTV</th>
<th>LIYV</th>
<th>LChV-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity</td>
<td>31</td>
<td>18</td>
<td>20</td>
<td>15</td>
<td>9</td>
<td>13</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Similarity</td>
<td>48</td>
<td>31</td>
<td>33</td>
<td>29</td>
<td>23</td>
<td>24</td>
<td>28</td>
<td>28</td>
</tr>
</tbody>
</table>
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) b-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
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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°,
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5min at 68°C, and finally an extension time of 10min at 72°C. 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.
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.
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.
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 an 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

Hybridise

Add Streptavidin coated paramagnetic particles

Capture and wash

Release viral RNA

GLRaV-1 genomic and subgenomic RNAs
Results and Discussion

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, lanes 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 total RNA respectively. Lanes 7-10, infected sample using 0.1mg, 1mg, 10mg and 100mg tissue equivalent of total RNA respectively.
<table>
<thead>
<tr>
<th>Lane</th>
<th>DNA control</th>
<th>Healthy (mg)</th>
<th>Infected (mg)</th>
<th>(mg) Tissue equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>0.1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1</td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td></td>
<td>10</td>
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<td>5</td>
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<td>6</td>
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<td>0.1</td>
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<td>7</td>
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<td>10</td>
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<td>8</td>
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<tr>
<td>10</td>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Diagram a:**
- 633nt (ORF9) Lane
- DNA control
- Healthy
- Infected

**Diagram b:**
- 633nt (ORF9) Lane
- DNA control
- Healthy
- Infected

**Legend:**
- 633nt: 633 nucleotides
- (ORF9): Open Reading Frame 9
- Lane: Sample number
- (mg) Tissue equivalent: Mass equivalent in milligrams
Results and Discussion

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.

<table>
<thead>
<tr>
<th>28 samples 9 ELISA +ve</th>
<th>Tissue equivalent (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Total RNA</td>
<td>7</td>
</tr>
<tr>
<td>MC RNA</td>
<td>9</td>
</tr>
</tbody>
</table>

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 XbaI site and a start
codon. The reverse primer contained an \textit{XhoI} site with no stop codon allowing an in frame C-terminal fusion with the GFP ORF. The \textit{NotI}-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 100\textmu l of ethanol was vortexed for two min, spun down for about 10 sec in a microfuge, drained, washed twice with 100\textmu l sterile water, and resuspended in 100\textmu l sterile water. 50\textmu l of this suspension was removed and added to 5\textmu l of 1\textmu g/\textmu l plasmid preparation. While gently vortexing, 20\textmu l of cold 1M spermidine and 50\textmu l 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-l 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<sub>r</sub> 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<sub>r</sub>-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.
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,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.
ER lumen | ER Membrane | Cytosol

Hydrophobic region

MDLRQFESHELTVSLFLVVLCAVYFVRARHCCAKIEDKPVGGPTNSSFRYVAQP

Amphiphilic region

(a) Schematic representation of the ER lumen, ER membrane, and cytosol with hydrophobic and amphipathic regions.

(b) Graph showing the hydropathy profile of amino acids.

(c) Circular diagram illustrating the hydrophobic helix face with amino acids 25-40 of GLRaV-1 ORF2.
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 EXPRESSING 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.
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).
Results and Discussion

Strong similarities can be seen between the GLRaV-1 encoded VIP-ER and the 2B protein of the enterovirus Coxackievirus 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
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.
Concluding remarks

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 yellow 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.
Concluding remarks

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
Concluding remarks

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.*
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Virology (?) In press

Subcellular Localisation of Proteins Encoded by the Grapevine Leafroll-associated Virus 1 Genome Reveals a Vesicle Inducing Protein


V. Little, A. and Rezaian, M.A.

V. Fazeli, C.F. and Rezaian, M.A.

Nucleotide sequence and organization of ten open reading frames in the genome of grapevine leafroll-associated virus 1 and identification of three subgenomic RNAs


V. little, A. and Rezaian, M.A.

Direct Submission


LOCUS AF195822

DEFINITION Grapevine leafroll-associated virus 1, complete genome.

ACCESSION AF195822

VERSION AF195822.1

KEYWORDS .

SOURCE Grapevine leafroll-associated virus 1 (GLRaV-1)

ORGANISM Grapevine leafroll-associated virus 1

Virus; ssRNA positive-strand viruses, no DNA stage; Closteroviridae; Ampelovirus

REFERENCE 1 (bases 1 to 5252)

AUTHORS V. Little, A. and Rezaian, M.A.

TITLE Subcellular Localisation of Proteins Encoded by the Grapevine Leafroll-associated Virus 1 Genome Reveals a Vesicle Inducing Protein

JOURNAL Virology (?) In press

REFERENCE 2 (bases 5253 to 17647)

AUTHORS V. Fazeli, C.F. and Rezaian, M.A.

TITLE Nucleotide sequence and organization of ten open reading frames in the genome of grapevine leafroll-associated virus 1 and identification of three subgenomic RNAs


REFERENCE 3 (bases 1 to 17647)

AUTHORS V. Little, A. and Rezaian, M.A.

TITLE Direct Submission


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