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Molecular characterisation of *Grapevine leafroll-associated virus 3* and implications for diagnostic testing and pathogenicity

Kar Mun Chooi

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy, The University of Auckland.
Abstract

*Grapevine leafroll-associated virus 3* (GLRaV-3) is an economically important virus that is found in all grapevine growing regions worldwide. Currently, the main strategies used to mitigate the negative impacts of GLRaV-3 on the wine industry is to prevent further introduction of GLRaV-3 by using certified planting material and reduce spread of GLRaV-3 in vineyards by roguing infected plants. Both of these approaches require a reliable and sensitive diagnostic tool. However, genetic variability within the virus population can compromise detection. Studies have shown high genetic variability in GLRaV-3 populations from different countries, but little was known about genetic variability of GLRaV-3 in New Zealand. This project examined the genetic variability within the New Zealand GLRaV-3 population to ensure that diagnostic tests would detect all known variants. In addition, knowledge of the genetic variability was used to aid the understanding of the virus biology, epidemiology, and evolution.

New Zealand GLRaV-3 variants from phylogenetic groups 1, 2, 3, 5, and 6, plus variants with more than 20% nucleotide identity to previously published GLRaV-3 variants, were identified. Sequences from these and overseas isolates were used to design and develop generic and variant-specific conventional, multiplex, and real-time RT-PCR detection assays. In addition, for the quantification of GLRaV-3 variants, ten host genes were evaluated as reference genes for real-time RT-PCR assays. GLRaV-3 variants from group 1, group 6 (specifically NZ-1), and NZ2 were graft transmitted to eight cultivars. For all cultivars, infections generally resulted in reduced growth of shoots compared to healthy controls, and for red cultivars, infections lead to typical premature leaf reddening. The molecular detection assays were used to study the spatial distribution of specific GLRaV-3 variants within a germplasm block and a commercial field plot, and within individual graft inoculated plants. The GLRaV-3 variants were found to be unevenly distributed within plants, as leaf tested from the basal position of shoots were more likely to yield reliable results, compared to the middle and apical shoot positions, and the spatial patterns were consistent with mealybug transmission within the field plots.
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List of abbreviations

AIC          Akaike information criterion
AP           Alkaline phosphatase
APET         Asymmetric PCR-ELISA
ASGV         Apple stem grooving virus
BLAST        Basic Local Alignment Search Tool
BSA          Bovine serum albumin
BYMV         Bean yellow mosaic virus
BYV          Beet yellows virus
CMV          Cucumber mosaic virus
CP           Coat protein
CPm          Minor coat protein
Ct           Cycle threshold
CTAB         Cetyl trimethylammonium bromide
CTLV         Citrus tatter leaf virus
CTV          Citrus tristeza virus
COX2         Cytochrome c oxidase
DAS-ELISA    Double antibody sandwich ELISA
DMSO         Dimethyl sulfoxide
DNA          Deoxyribonucleic acid
EF2          Elongation factor 2
ELISA        Enzyme-linked immunosorbent assay
GAPDH        Glyceraldehyde-3-phosphate dehydrogenase
HEL          Helicase
HSP70h       Heat shock protein 70 homologue
HSP90h       Heat shock protein 90 homologue
ΔG           Gibbs free energy
GFLV         Grapevine fanleaf virus
GLRaVs       Grapevine leafroll-associated viruses
GLRaV-1       Grapevine leafroll-associated virus 1
GLRaV-2       Grapevine leafroll-associated virus 2
GLRaV-3       Grapevine leafroll-associated virus 3
GLRaV-4       Grapevine leafroll-associated virus 4
GLRaV-5       Grapevine leafroll-associated virus 5
GLRaV-6       Grapevine leafroll-associated virus 6
GLRaV-7       Grapevine leafroll-associated virus 7
GLRaV-8       Grapevine leafroll-associated virus 8
GLRaV-9       Grapevine leafroll-associated virus 9
GLRaV-Car     Grapevine leafroll-associated Carnelian virus
GLRaV-De      Grapevine leafroll-associated virus De
GLRaV-Pr      Grapevine leafroll-associated virus Pr
GGS          Grafted Grapevine Standard
GVA          Grapevine virus A
GVB          Grapevine virus B
GVD          Grapevine virus D
ICTV         International Committee on Taxonomy of Viruses
IC-RT-PCR     Immuno-capture RT-PCR
IPTG         Isopropyl β-D-thiogalactopyranoside
kb           Kilobases (nucleotides)
L-Pro        Leader papain-like protease
LB           Luria bertani
LDA          Low-density array
LIYV  Lettuce infectious yellows virus
MDH  Malate dehydrogenase
MET  Methyltransferase
mRT-PCR  Multiplex RT-PCR
NAD5  NADH dehydrogenase subunit 5
NGS  Next generation sequencing
NJ  Neighbour-Joining
nt  Nucleotide
OD  Optical density
PCR–SSCP  Clonal polymerase chain reaction–single-strand conformational polymorphism
PIP2  Aquaporin PIP2.2
PMWaV-2  Pineapple mealybug wilt-associated virus 2
POLYU  Polyubiquitin-A-like
PP2a  Serine/threonine protein phosphatase-like
PPV  Plum pox virus
PVP-40  Polyvinylpyrrolidone-40
qRT-PCR  Quantitative RT-PCR (ie: Real-Time)
ORF  Open reading frame
RACE  Rapid Amplification of cDNA Ends
RdRp  RNA-dependent RNA polymerase
RNA  Ribonucleic acid
rRNA  Ribosomal RNA
RSPaV  Rupestris stem pitting-associated virus
RT-PCR  Reverse transcription polymerase chain reaction
SAND  SAND protein
sgRNA  Subgenomic RNA
SL  Stem and loop
SSCP  Single stranded conformation polymorphism
ssDNA  Single-stranded DNA
SUC11  Sucrose transporter-like
TAE  Tris-Acetate-EDTA Buffer
TAS-ELISA  Triple antibody sandwich ELISA
TATA  Transcription initiation factor subunit 7-like
TBE  Tris-Borate-EDTA-Buffer
TMV  Tobacco mosaic virus
TSS  Transcription start site
TVMV  Tobacco vein mottling virus
TYMV  Turnip yellow mosaic virus
UTR  Untranslated region
X-Gal  5-bromo-4-chloro-indolyl-β-D-galactopyranoside
Chapter 1:

Introduction

The berries from *Vitis vinifera* (grapevines) have been used as a source of fruit and beverage for thousands of years. At the start of the 1800’s, the first grapevines were brought to New Zealand by European settlers, and the first known planting in New Zealand was by Samuel Marsden in Kerikeri (Cooper, 1996; Zuur, 1987). New Zealand is well known for its production of world-class, award winning white and red wines. Within the twelve months to June 2012 the export volumes of New Zealand wine reached 178 million litres, valued at over NZD$1,176 million, which is a 7.6% increase in value from the previous year (New Zealand Winegrowers, 2012). However, grapevines are susceptible to a wide range of bacterial, fungal, and viral diseases that can reduce vine vigour and longevity, and fruit yield and quality, which in-turn can affect wine quality. There are more than 50 documented viruses infecting grapevines worldwide (Martelli et al., 2006) and in New Zealand, the most serious and most economically important of these grapevine viruses is *Grapevine leafroll-associated virus 3* (GLRaV-3), which is one of the viruses associated with grapevine leafroll disease.

1.1 Grapevine leafroll disease

Grapevine leafroll disease has been identified in viticultural areas worldwide (Martelli et al., 2006). The first descriptions of the grapevine leafroll disease are thought to have been in 1906 by Sannino in Italy, terming symptoms observed as “rossore” (Martelli et al., 2006). In Germany during 1935, Scheu also observed leafroll symptoms (Section 1.1.1) which were referred to as Rollkrankheit. These symptoms were demonstrated to be graft transmissible, resulting in the hypothesis that the disease was of viral origin (Charles et al., 2006; Martelli et al., 2006). Subsequent studies in other countries such as Australia, the United States of America (USA), France, Hungary and Switzerland observed and described grapevine diseases such as the White Emperor disease (English), Rollkrankheit (German), Blattrollkrankheit (German), and Enroulement (French) that are now accepted as synonyms for the grapevine leafroll disease (Charles et al., 2006; Martelli et al., 2006).
It has been suggested that the grapevine leafroll disease may have been in New Zealand for over 100 years (Charles et al., 2006), as premature reddening in Cabernet sauvignon vines, typical of leafroll symptoms, were observed in a report by Bragato (1902). However, the first published record of the grapevine leafroll disease in New Zealand was by McKissock (McKissock, 1964).

### 1.1.1 Symptoms

For red cultivars, in the late spring or summer, the older (lower) leaves prematurely redden as they start to develop reddish spots. These reddish spots progressively spread to cover most, if not all, of the leaf surface, while the primary and secondary vines remain green (Martelli et al., 2006). In some cases the whole leaf can become dark purple. Additionally, the leaves become brittle and the leaf margins roll downwards (Figure 1.1a). With the striking premature reddening of the leaves, the expressed phenotypic symptoms of red cultivars are easier to identify compared to those in white cultivars. For white cultivars, the leaf margins roll downwards but instead of developing reddish spots, leaves turn yellow or chlorotic (Figure 1.1b). Moreover, some white cultivars and most, if not all, rootstocks appear to be symptomless (Martelli et al., 2006; Rayapati et al., 2009). For both red and white cultivars, it has been shown that the grapevine leafroll disease can reduce the yield and delay the ripening of grapes, leading to a decrease in sugar and an increase in titratable acidity (Section 1.2).

The expression of grapevine leafroll symptoms can be affected by the *V. vinifera* cultivar, environmental conditions, scion/rootstock combination, and the virus strain/variant (for some of the grapevine leafroll causal agents) (Bertazzon et al., 2010; Golino, 1993; Martelli et al., 2006; Rowhani et al., 2000).

![Figure 1.1: Photographs of grapevine leafroll symptoms in a (a) red cultivar (Pinot noir) and (b) white cultivar (Sauvignon blanc) plant infected with *Grapevine leafroll-associated virus 3.*](image-url)
1.1.2 Viruses involved in Grapevine leafroll disease

Initial studies to identify the casual agent for the grapevine leafroll disease identified potyvirus-like, closterovirus-like, and isometric virus-like particles (Castellano et al., 1984; Namba et al., 1979; Tanne et al., 1977). Only after the successful graft transmission of closterovirus-like particles, was the association between the grapevine leafroll disease and closteroviruses confirmed (Tanne, 1988). Subsequent studies using serological and molecular testing have thus far identified 11 grapevine leafroll-associated viruses (GLRaVs) (Table 1.1), but recently a proposal has been made to combine six of these viruses into a single species (Section 1.2.1) (Martelli, 2012; Martelli et al., 2012).

In New Zealand, six GLRaVs have been identified, consisting of Grapevine leafroll-associated virus-1, -2, -3, -4, -5, and -9 (GLRaV-1, -2, -3, -4, -5, and -9) (Charles et al., 2006). GLRaV-4, -5, and -9 have each been identified just once from clones of Sauvignon blanc, and Tempranillo and Cabernet sauvignon, respectively, imported from Australia (Charles et al., 2006). GLRaV-2 was identified in a number of different cultivars imported from Bordeaux, France (Habili et al., 2002). GLRaV-1 has been detected in many cultivars in New Zealand but is always present in Chardonnay Mendoza, and one study described the spread of GLRaV-1 within a rootstock source block (Bonfiglioli et al., 2002). In New Zealand, the most economically important and most prevalent of the GLRaVs is GLRaV-3. GLRaV-3 has been extensively detected in most cultivars throughout New Zealand, except for Chardonnay Mendoza, and secondary spread is often observed (Bell et al., 2009; Charles et al., 2006; Charles et al., 2009).

1.2 Grapevine leafroll-associated virus 3

In addition to the changes to the leaf (Section 1.1.1), GLRaV-3 infection has also been shown to affect vine growth, reduce berry yield, delay berry ripening, and affect berry aspects such as the sugar accumulation, titratable acidity, and anthocyanin levels (Cabaleiro et al., 1999; Lee et al., 2009a; Lee et al., 2009b; Vega et al., 2011). Berry characteristics such as the sugar and anthocyanin levels (which are typically reduced in berries from GLRaV-3 infected vines) are likely to alter the taste and quality of the wine. For instance, studies based on spectrographic analysis and sensory evaluations have shown reduced wine quality from leafroll infected vines compared to healthy vines (Mannini et al., 1998; Over
de Linden et al., 1970; Ueno et al., 1985). Taking into consideration aspects such as possible yield loss and costs for removal and replacement of infected vines, the cost of GLRaV-3 infection to New Zealand Merlot wines alone has been estimated to be up to $85M (Nimmo-Bell, 2006).

With the high prevalence of GLRaV-3 and its economic importance to the New Zealand wine industry, studies prior to this project focused on the vectors and spread associated with GLRaV-3 in vineyards (Bell et al., 2009; Charles et al., 2010; Charles et al., 2005; Charles et al., 2009; Jordan, 1993; Petersen, 1996; Petersen et al., 1997). However, little was known about GLRaV-3 itself including which GLRaV-3 genetic variants were present in New Zealand. To help control the spread of GLRaV-3, understanding the genetic variation within the virus population is important when developing detection methods, especially the design of molecular detection methods (which is discussed later). Therefore, the focus of this project was to investigate the genetic GLRaV-3 variants present in New Zealand.

1.2.1 Current classification of GLRaVs

Based on the ninth report of the International Committee on Taxonomy of Viruses (ICTV) (King et al., 2011), the Closteroviridae family consists of three genera, Closterovirus, Ampelovirus and Crinivirus. The family was classified using features such as genome organisation, mode of transmission (aphid, mealybug, and whitefly, respectively), and conservation of amino acid sequences of relevant gene products such as the RNA-dependent RNA polymerase (RdRp), coat protein (CP), and heat shock protein 70 homologue (HSP70h). GLRaV-3 is the type species for the genus Ampelovirus (King et al., 2011; Martelli et al., 2002). This genus also includes the approved species GLRaV-1, GLRaV-5, Little cherry virus 2, Pineapple mealybug wilt-associated virus 1, Pineapple mealybug wilt-associated virus 2 (PMWaV-2), Pineapple mealybug wilt-associated virus 3, and Plum bark necrosis stem pitting-associated virus, and the putative species Fig leaf mottle-associated virus 2, GLRaV-4, GLRaV-6, GLRaV-De (variant of GLRaV-6), GLRaV-9, Grapevine leafroll-associated Carnelian virus (i.e. GLRaV-Car), GLRaV-Pr, and Sugarcane mild mosaic virus (King et al., 2011). Recently, it has been proposed that the GLRaV-5, -6, -9, -Pr, -De, and –Car viruses all represent genetically divergent variants of GLRaV-4, based on similarities in genome size and organisation, biological and epidemiological traits, and amino acid sequence
of the RdRp, CP, and HSP70h genes (Martelli, 2012; Martelli et al., 2012). In addition, the division of the *Ampelovirus* genus into two subgroups (subgroup I and II) has also been proposed (Table 1.1). This is based on the size and organisation of the genome, and phylogenetic analysis of the HSP70h, RdRp, and helicase domains of viruses (Maliogka et al., 2009; Martelli, 2012; Martelli et al., 2012).

GLRaV-2 is assigned to the genus *Closterovirus* and GLRaV-7 is currently a putative unassigned species within the family *Closteroviridae* (King et al., 2011). However, a proposal is under review by ICTV, that the putative species GLRaV-7, Little cherry virus 1, and Cordyline virus 1 comprises a fourth genus, Velarivirus, within the *Closteroviridae* family (Martelli, 2012; Martelli et al., 2012). Previously, GLRaV-8 was classified in the genus *Ampelovirus*, however the GLRaV-8 sequence has been found to be of grapevine genome origin, and has been removed from the genus (Martelli, 2012).

| Table 1.1: Eleven grapevine leafroll-associated viruses. |
|---|---|
| Genus | Species |
| Closterovirus | *Grapevine leafroll-associated virus 2* |
| **Ampelovirus** | **Subgroup I** |
| | *Grapevine leafroll-associated virus 1* |
| | *Grapevine leafroll-associated virus 3* |
| | **Subgroup II** |
| | *Grapevine leafroll-associated virus 4*<sup>b</sup> |
| | *Grapevine leafroll-associated virus 5* (GLRaV-4 strain 5) |
| | *Grapevine leafroll-associated virus 6* (GLRaV-4 strain 6) |
| | *Grapevine leafroll-associated virus 9* (GLRaV-4 strain 9) |
| | *Grapevine leafroll-associated virus De* (GLRaV-4 strain De) |
| | *Grapevine leafroll-associated virus Pr* (GLRaV-4 strain Pr) |
| | *Grapevine leafroll-associated virus Car* (GLRaV-4 strain Car) |
| Velarivirus<sup>a</sup> | *Grapevine leafroll-associated virus 7* |

<sup>a</sup> Fourth genus for the *Closteroviridae* family proposed by (Martelli, 2012; Martelli et al., 2012).

<sup>b</sup> *Grapevine leafroll-associated virus*-5, -6, -9, -Pr, -De, and –Car all represent genetically divergent variants of GLRaV-4 (Martelli, 2012; Martelli et al., 2012).

### 1.2.2 Morphology and genome organisation of GLRaV-3

GLRaV-3 virions are phloem limited, flexuous, filamentous particles, approximately 1,800 nm in length. Characteristic of the *Closteroviridae* family, the positive-sense single-stranded RNA genome is encapsidated by the CP and virion tail complex (complex includes the minor CP (CPm), HSP70h, and an approximate p60 (p55 for GLRaV-3) proteins) (Dolja et al., 2006). This accounts for 95% and 5% of the helical capsid respectively; leading to the distinctive “rattlesnake” morphology (Agranovsky et al., 1995; King et al., 2011).
The first extended GLRaV-3 genome sequence (initially thought to represent the first complete genome sequence), of the isolate NY1, was published in 2004. GLRaV-3 has a positive sense single stranded RNA genome of 17,919nt, organised into 13 open reading frames (ORFs) with 5' and 3' untranslated regions (5'UTR and 3'UTR), which corresponds to the genome organisation established for closteroviruses (Figure 1.2) (Dolja et al., 1994; Ling et al., 2004). Subsequent complete genome sequencing of other GLRaV-3 isolates, have shown the reported 17,919nt GLRaV-3 genome is incomplete, as it has a truncated 158nt 5'UTR (discussed further in Chapter 3) (Jarugula et al., 2010; Maree et al., 2008). The authentic complete GLRaV-3 genome is greater than 18,433nt (Bester et al., 2012b; Jarugula et al., 2010; Jooste et al., 2010; Maree et al., 2008).

The 13 ORFs were given the names, ORF1a and 1b, and ORF2 to 12 (Ling et al., 2004). The putative functions of the ORFs were examined and determined by comparative analysis of the amino acid sequence with other known proteins of closteroviruses (Ling et al., 2004).

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Figure 1.2: Diagrammatic representation of the *Grapevine leafroll-associated virus 3* genome and the organisation of the open reading frames (ORFs), genes, and subgenomic RNA (sgRNA). **L-Pro**, leader papain-like protease domain; **Met**, methyltransferase domain; **AlkB**, AlkB domain; **Hel**, RNA helicase domain; **RdRp**, RNA-dependent RNA polymerase; **p6**, 6kDa protein; **p5**, 5kDa protein; **HSP70h**, Heat shock protein 70 homologue; **p55**, 55kDa protein; **CP**, capsid protein; **CPm**, minor capsid protein; **p21**, 21kDa protein; **p19.6**, 19.6kDa protein; **p19.7**, 19.7kDa protein; **p4**, 4kDa protein; **p7**, 7kDa protein. Below the genome diagram is a representation of the putative (dotted lines) and experimentally confirmed (thick lines) sgRNAs for the 3'genes. The confirmed sgRNAs are based on studies by Maree et al. (2010) and Jarugula et al. (2010).

ORF1a encodes a polyprotein that contains four different domains involved in virus replication, which are the leader papain-like protease (L-Pro) (Ling et al., 2004), AlkB (Engel et al., 2008; Maree et al., 2008), methyltransferase (MET) (Ling et al.,
1998), and helicase (HEL) (Ling et al., 1998) domains. Similar to the Beet yellows virus (BYV) (type species for the genus Closterovirus), the L-Pro is predicted to cleave the ORF1a polypeptide between two glycine amino acids positioned at 371 and 372, resulting in a N'-terminal peptide of 371 amino acids and 42kDa in size (Ling et al., 2004). The protease either protects against the degradation of the RNA genome by host defences or activates the replicase for viral replication (Dolja et al., 2006). The GLRaV-3 HEL has high similarity to the Superfamily 1 helicase of positive-strand RNA viruses, sharing six conserved motifs (Ling et al., 1998).

ORF1b encodes the RdRp and has high similarity to the Supergroup 3 RdRps of the positive-strand RNA viruses, sharing eight conserved motifs (Ling et al., 1998). The +1 ribosomal frameshift mechanism is presumed to be used by GLRaV-3 to express ORF1b as an ORF1a/1b fusion protein. This is based on significant amino acid similarity to the putative frameshift point for the Lettuce infectious yellows virus (LIYV) (type species for the genus Crinivirus) (Ling et al., 1998).

ORF2 and ORF3 encode for small peptides that are approximately 6kDa (p6) and 5kDa (p5) in size, respectively. The p6 is unique to GLRaV-3, while p5 corresponds to similar hydrophobic transmembrane proteins found in other closteroviruses (Ling et al., 1998). It has been suggested that the hydrophobic p5 protein acts as a movement protein, where as a transmembrane protein it resides in the endoplasmic reticulum and is involved in virion movement from cell to cell (Dolja et al., 2006).

ORF4 encodes the HSP70h (which is unique to the Closteroviridae family), sharing eight conserved motifs (A to H) with other closteroviruses. Three of these (A to C), are functionally important as it contains the ATPase activity on its N'terminal domain (Ling et al., 1998). The HSP70h is a part of the virion tail complex, which is integral for tail assembly, virion stability, and cell-to-cell movement of closteroviruses (Alzhanova et al., 2001; Dolja et al., 2006).

ORF5 encodes for a 55kDa protein (p55). The ORF is of similar size and position in the genome to the Citrus tristeza virus (CTV) and BYV heat shock protein 90 homologue (HSP90h) gene. The p55 amino acid sequence also shares low similarity to other closteroviruses. However, the two conserved HSP90h motifs found in CTV and BYV are not found in GLRaV-3 (Ling et al., 1998). The HSP90h is a minor component of the virion tail.
ORF6 and 7 encode for the 34kDa CP and 53kDa CPm. The CP has low similarity to other closterovirus CPs, though the four amino acids (asparagine, arginine, glycine, and aspartic acid) conserved in all closterovirus CPs are also present in GLRaV-3 (Ling et al., 1997). The additional duplicated CPm is unique to the Closteroviridae family. Similar to other closteroviruses, at the C-terminal, the GLRaV-3 CPm also contains the four conserved amino acids mentioned above (Ling et al., 1998). As mentioned previously, the CP and CPm are involved in the encapsidation of the RNA.

ORF8, 9, and 10 potentially encode for 21kDa (p21), 19.6kDa (p19.6 also referred to as p20A), and 19.7kDa (p19.7 also referred to as p20B) sized proteins, respectively. The ORFs are similar in size and genome position (near the 3’ end of the genome) to the ORFs of BYV and CTV that are involved in viral silencing suppressors and systemic movement proteins (Dolja et al., 2006). Amino acid analysis of p21, p19.6, and p19.7 to other known viral suppressors of RNA silencing (VSRs) proteins indicated the p19.6 and p19.7 had regions of sequence conservation with the p21-like VSR family (Gouveia et al., 2012a). Gouveia et al. (2012a) examined the silencing suppressor activity for the p21, p19.6, and p19.7 proteins; identifying silencing suppressor activity from the p19.7 protein.

ORF11 and 12 are unique to GLRaV-3 and are predicted to encode 4kDa (p4) and 7kDa (p7) proteins, respectively. The functions for these proteins are unknown.

The 5’ and 3’UTRs of plant viruses are critical in controlling a number of different virus functions such as virus replication and virion assembly (Dreher, 1999; Gowda et al., 2003a; Jarugula et al., 2010; Satyanarayana et al., 2002; Singh et al., 1998). The reported size of the GLRaV-3 5’UTR ranges between 158 to 737nt, though as discussed above, the 158nt 5’UTR represents an incomplete sequence. At the start of this project, all sequenced GLRaV-3 3’UTRs had a uniform size of 277nt, but during this project GLRaV-3 variants with a 268nt 3’UTR were identified (Bester et al., 2012b). Typically, to regulate virus processes the UTR regions contain cis-acting control elements and potential cis-acting elements within the GLRaV-3 genome have been identified by Jarugula et al. (2010) (examined and discussed in further detail in Chapter 3).

The most common strategy for positive sense RNA viruses to express the 3’proximal ORFs involves the generation of subgenomic RNA (sgRNA)
Based on the GLRaV-3 genome, 11 putative sgRNAs are predicted to express the 11 3’proximal ORFs 2 to 12. In total, to express ORFs 3 to 12, Maree et al. (2010) and Jarugula et al. (2010) identified seven GLRaV-3 3’-coterminal sgRNAs (Figure 1.2). The expression of sgRNA for ORF2 encoding p5 (sgRNA(ORF2)) was not identified in either study, while it is likely that the sgRNA expression of ORF3 and 4 (p5 and HSP70h genes), and ORF10 to 12 (p20B, p4, and p7 genes) are translated from the same sgRNA (i.e. sgRNA(ORF3/4) and sgRNA(ORF10-12), respectively) (Jarugula et al., 2010; Maree et al., 2010).

Differential GLRaV-3 sgRNA expression levels were observed by Jarugula et al. (2010), which suggests that the expression of sgRNA is under specific regulation. Sequence analysis to identify possible cis-acting elements that control sgRNA expression was conducted, and identified that the regulation mechanism for GLRaV-3 is different to other plant viruses from the genus Closterovirus (Jarugula et al., 2010) (discussed further in Chapter 3).

1.2.3 Genetic variability of GLRaV-3

At the start of this project two well-supported GLRaV-3 variant phylogenetic groups were established. One group with high nucleotide identity to the NY1 isolate (group 1), and another group that had 91.3 to 96.2% nucleotide identity to NY1, based on sequences from ORFs 4 to 7 (Jooste et al., 2005). Additionally, sequences from Spain, France, New Zealand, and South Africa of four divergent GLRaV-3 isolates, with more than 20% nucleotide difference compared to NY1, were either reported or deposited onto the GenBank database (Angelini et al., 2006; Prosser et al., 2007). At the time, phylogenetic groupings for these four isolates could not be assigned or confirmed due to incomplete sequence information, and isolates remained classified as outlier divergent GLRaV-3 variants. Following these findings, along with this project (Chapters 2 and 3), other genetic variability studies from South Africa, Portugal, and the USA were carried out, which identified more genetic variability within the GLRaV-3 population. Based on phylogenetic analysis of the CP region, Gouveia et al. (2011) identified GLRaV-3 variants that clustered within five phylogenetic groups, while Bester et al. (2012b) established a sixth well-supported group. Therefore, based on both studies, for the remainder of the thesis, established phylogenetic groups will be referred to as groups 1 to 6. A summary of the main genetic
variation studies that have led to the establishment of the six phylogenetic groups is described below.

Following the publication of the NY1 genome sequence, a South African study by Jooste et al. (2005) identified two well-supported phylogenetic groups 1 and 2 (as described above), using single stranded conformation polymorphism (SSCP) and restriction enzyme SSCP analysis to identify possible genetic variants, that were then sequenced and analysed. Turturo et al. (2005) examined the sequence variation of 45 GLRaV-3 isolates from 14 countries. The reverse transcription polymerase chain reaction (RT-PCR) products from the RdRp, HSP70h, and CP genes were analysed using SSCP and sequence analysis. Predominantly, isolates showed SSCP profiles indicative of infection with a single GLRaV-3 variant; 90% based on the RdRp and HSP70h genes, and 85% based on the CP genes (Turturo et al., 2005). The remaining isolates showed SSCP profiles indicative of mixed infections with two or more variants (Turturo et al., 2005). Retrospective analysis of sequences deposited onto GenBank from the Turturo et al. (2005) study, revealed that all GLRaV-3 isolates identified share more than 90% nucleotide identity with each other.

In 2006 and 2007, divergent GLRaV-3 isolates Tempr (DQ314610), CB19 (EF445655 and EF445656), NZ-1 (EF508151), and WC-HSP-10 (EF103904), that differed from NY1 (group 1) by more than 20% at the nucleotide level were identified (as described above). The GLRaV-3 Tempr isolate was identified from a Spanish vineyard in a Tempranillo cultivar (Angelini et al., 2006). In 2007, the CB19 and NZ-1 isolates were identified in France and New Zealand, respectively, and sequences were directly deposited to GenBank. The CB19 isolate is a variant of the Tempr isolate, as it shares 99.3% nucleotide identity with Tempr, based on a 602nt sequence of the RdRp gene. Prosser et al. (2007) identified two GLRaV-3 variants from the table grape cultivar “Waltham Cross”, based on sequence from the HSP70h gene. The clone WC-HSP-2 had 93.2% nucleotide identity with NY1 (group 2 isolate) and clones, WC-HSP-10 and WC-HSP-28, had 72.3% nucleotide identity with NY1 (Prosser et al., 2007). The WC-HSP-10 and WC-HSP-28 clones were 98.5% identical to each other (Prosser et al., 2007), though only sequence of the WC-HSP-10 clone was submitted to GenBank.

In 2008, two complete genome sequences of a group 1 (CI-766, EU344894) and 2 (GP18, EU259806) variant from Chile and South Africa, respectively, were
published (Engel et al., 2008; Maree et al., 2008). Though, similar to NY1, the Chilean CI-766 genome sequence is now considered incomplete (Jarugula et al., 2010; Maree et al., 2008). A GLRaV-3 isolate, CI-817 (EU344894), was also described by Engel et al. (2008), which shares 94.5% nucleotide identity with group 1 variants based on the complete CP gene (representative of group 5). The description of the complete GP18 genome of 18,498nt is now considered the first report of a full length GLRaV-3 genome (Jarugula et al., 2010; Maree et al., 2008). In a survey for GLRaV-1, -2, and -3 of the Finger Lakes Vineyards in New York (USA), five GLRaV-3 variants that shared 91.5 to 98.3% nucleotide identity with NY1 were identified (based on sequence from the HSP70h gene), corresponding to groups 1 to 3 (Fuchs et al., 2009). Three GLRaV-3 genetic variants, 621 (GQ352631), 623 (GQ352632), and PL-20 (GQ352633), representative of groups 1, 2, and 3, respectively, were also identified in South Africa, and the complete genomes characterised by Jooste et al. (2010). Gouveia et al. (2011) identified 174 GLRaV-3 positive vines from 110 different varieties of grapevine, representative of mostly Portuguese cultivars. From the 174 GLRaV-3 infected vines, 74 complete CP genes were sequenced. Based on these 74 sequences and other available complete CP sequences at the time of analysis, five well-supported phylogenetic groups were identified (Gouveia et al., 2011). GLRaV-3 variants that clustered within three of the five groups corresponded to the studies by Fuchs et al. (2009) and Jooste et al. (2010) (groups 1 to 3), and variants from another group corresponded to the Chilean CI-817 variant, identified by Engel et al. (2008) (group 5) (Gouveia et al., 2011). Group 4 variants (representative sequence is the isolate Terrantez da Terceira, HQ401015) from this study did not correspond to any previously identified GLRaV-3 variants (Gouveia et al., 2011).

In 2011, two studies of GLRaV-3 variants within vineyards in the Napa Valley, California (USA) were conducted (Sharma et al., 2011; Wang et al., 2011). Wang et al. (2011) identified GLRaV-3 variants from groups 1 to 4, based on 50 sequences that were 4,711nt in length (encompasses ORF6 to 12). Sharma et al. (2011) conducted a survey for GLRaV-1 to -5, and -9, and a survey for GLRaV-3 genetic variants within 11 different vineyards. Based on sequence analysis of a 428nt region of the CP gene seven phylogenetic groups were observed (Sharma et al., 2011). Five of the seven phylogenetic groups corresponded to the previously identified groups 1 to 5 (Gouveia et al., 2011), and GLRaV-3 variants from a sixth group (denoted as the group GLRaV-3e by Sharma et al. (2011))
shared an average nucleotide identity of 91% with the NZ-1 isolate. While a single GLRaV-3 isolate, 43-15 (JF421951), did not correspond to the other established groups and only shared 79.2% nucleotide identity with NY1 (Sharma et al., 2011). Therefore, similar to GLRaV-3 isolates Tempr and WC-HSP-10, isolate 43-15 is another divergent outlier GLRaV-3 isolate.

Two complete genome sequences of South African isolates, GH11 (JQ655295) and GH30 (JQ655296) were recently published, which share approximately 68% and 91% nucleotide identity to NY1 and NZ-1, respectively (Bester et al., 2012b). Based on greater than 90% nucleotide similarities between NZ-1, GH11, GH30, and the Napa Valley GLRaV-3e variants, these variants are considered members of a sixth GLRaV-3 phylogenetic group (Bester et al., 2012b).

Prior to this project, a study investigating a link between the GLRaV-3 genetic variants described above, and the differential symptom expression had not been conducted. Since then, Habili et al. (2009) has described a possible mild biological strain of GLRaV-3, though an association to a specific GLRaV-3 variant group was not reported, and the potential mild GLRaV-3 strain was in a mixed infection with *Grapevine virus A* (GVA) and GLRaV-9. This area of interest is described further in Chapter 5.

### 1.2.3.1 Recombination

Mutation, recombination, and reassortment are the three main virus evolutionary processes that contribute to the formation of different virus variants (Domingo et al., 1997; Roossinck, 1997). The replication of RNA viruses is error-prone (an average nucleotide base misincorporation rate of $10^{-4}$ to $10^{-5}$ (Holland et al., 1998)), which can lead to the accumulation of a number of mutations, and the generation of an assortment of genetic variants (Keese et al., 1993). While the exchange of genetic material (genes) can lead to the generation of variants with enhanced fitness and adaptability to new environments (Keese et al., 1993; Roossinck, 1997; Rubio et al., 2001), recombination may also correct for deleterious mutations due to RNA virus high mutation rates (Roossinck, 1997; Rubio et al., 2001; Simon et al., 1994).

Recombination has been reported for a number of plant viruses including other members of the *Closteroviridae* family; GLRaV-1 (Alabi et al., 2011) and CTV (Rubio et al., 2001; Vives et al., 2005). At the start of this project, there was only
one report of recombination in GLRaV-3 (Turturo et al., 2005). Two recombinants were detected, AUSG5-2 (AJ748510) and IL1-1 (AJ606355), based on partial sequences of the HSP70h (546nt) and CP (484nt) genes respectively. Retrospective analysis of sequences revealed both recombination events occurred between group 1-like and group 2-like variants.

1.2.4 Transmission of GLRaV-3

GLRaV-3 can only infect *Vitis* spp. and can be transmitted and spread from vine to vine via two means, (i) grafting GLRaV-3 infected material onto another vine, or (ii) by mealybug or soft scale vector transmission. Mealybugs transmit GLRaV-3 in a semi-persistent manner (Martelli et al., 2002) and at least eight mealybug species are known vectors (Charles et al., 2006). However, a study by Cid et al. (2007) has proposed GLRaV-3 may actually be transmitted in a circulative manner, as GLRaV-3 was observed in the salivary glands of *Planococcus citri*. Worldwide, the five mealybug species most important to grapes are *P. ficus*, *P. citri*, *Pseudococcus longispinus*, *Ps. calceolariae*, and *Ps. viburni* (Charles et al., 2006). Of the five species, only *Ps. longispinus*, *Ps. calceolariae*, and *Ps. viburni* mealybugs are present in New Zealand. *Ps. longispinus* and *Ps. calceolariae* are widespread, and are the most economically important mealybugs for New Zealand (Charles et al., 2010; Charles et al., 2006; Petersen et al., 1997).

Studies have shown that the first instar mealybugs are likely to be the most important for GLRaV-3 transmission (Cabaleiro et al., 1997; Charles et al., 2006; Petersen et al., 1997). For instance, the transmission of GLRaV-3 by first and third instar *Ps. longispinus* and *Ps. calceolariae* species were compared, showing transmission was only successful when using first instar mealybugs (Petersen et al., 1997). In a study by Douglas et al. (2008), the transmission efficiency of GLRaV-3 by groups of one, five, ten, 20, or 40 first to second instar *P. ficus* and *Ps. longispinus* were examined. The transmission efficiency was similar for both mealybug species and single mealybugs successfully transmitted GLRaV-3 from LN33 rootstock plant material to virus-free Cabernet franc (Douglas et al., 2008). Therefore the natural spread of GLRaV-3 is likely the result of movement by first instar mealybugs via crawling, dispersal by wind, dispersal by traffic between blocks (e.g. humans and machinery, or a combination of these means of spread (Charles et al., 2006; Charles et al., 2009; Pietersen, 2004).
In addition to the three mealybug species, soft scale *Parthenolecanium corni* and *Pulvinaria vitis* are possible insect vectors for the spread of GLRaV-3 in New Zealand (Belli et al., 1994; Charles et al., 2006; Charles et al., 2005; Golino et al., 2002).

### 1.2.5 Spatial distribution of GLRaV-3

For the design of appropriate disease control regimes, it is important to understand the distribution of virus populations within (a) the individual host plant and (b) the plant population. Uneven distribution of different strains/variants of a virus within a host, observed for other plant viruses such as CTV, *Apple stem grooving virus* (ASGV), and *Plum pox virus* (PPV), can potentially influence the development of virus spread in field plots, and potentially affects the accuracy of diagnostic tests. The distribution and seasonal fluctuation of GLRaV-3 within the vine have been investigated using ELISA and real-time RT-PCR (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012). GLRaV-3 was observed to be unevenly distributed within the host plant, when comparing the virus levels in leaves at different positions along the cane. Typically, higher virus titre was detected from the basal position of the cane (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012). Additionally, as the season progressed, GLRaV-3 was found to move gradually with the new growth (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012). Since GLRaV-3 is phloem-limited, it has been proposed that the movement of the virus is likely to be influenced by the movements of carbohydrates in the phloem tissue (Charles et al., 2006; Cohen et al., 2004). The distribution of different GLRaV-3 genetic variants was not considered in the previously mentioned studies. However, this aspect was considered in this project and is described further in Chapter 5.

The spatial and temporal distribution of GLRaV-3 in the field has been studied widely worldwide (Cabaleiro et al., 2008; Cabaleiro et al., 2006; Charles et al., 2009; Golino et al., 2008; Habili et al., 1997; Jooste et al., 2011; Jordan, 1993; Petersen, 1996; Pietersen, 2004; Sharma et al., 2011). The graft and insect vector transmissibility of GLRaV-3 (Section 1.2.4) can contribute to the introduction and/or spread of GLRaV-3 in the field. Examples of a few GLRaV-3 epidemiological studies are summarised below.

For three years, the spread of the grapevine leafroll disease was monitored for 70 grapevine mother blocks in South Africa (Pietersen, 2004). From this study, four
distribution patterns were observed. The most common was of secondary spread within the vineyard, where from an initial infected vine, neighbouring vines along the row gradually became infected, followed by adjacent vines across the rows (Pietersen, 2004). The second most common distribution pattern observed was of infected vines predominantly at the edges of the vineyard. Generally, the number of infected vines along the vineyard edges increased towards an older GLRaV-3 infected vineyard (virus source) (Pietersen, 2004). Other distribution patterns observed included the random identification of infected vines in the vineyard, indicative of primary spread by using infected planting material, and a distribution pattern in a young vineyard that corresponded to the spatial distribution of an earlier highly infected vineyard it replaced (Pietersen, 2004).

Cabaleiro et al. (2008) studied the spread of GLRaV-3 in three vineyards from Spain. Spatial analysis of the infected vines over a varying number of years for each vineyard resulted in three different situations. In the Meaño vineyard, GLRaV-3 was monitored between 1992 and 1996, and 2003 and 2005. Similar to Pietersen (2004), over the years, infected vines were progressively detected in close proximity with each other along and across the rows. Cabaleiro et al. (2008) suggests the clustering of infecting plants is a result of vectorial field transmission likely to be from scale insects. In contrast, a random distribution of GLRaV-3 infected vines was observed in the Goián and Portomarín vineyards, which indicates virus was introduced into the vineyard through use of infected planting material (Cabaleiro et al., 2008). Though, in the last year of testing for the Goián vineyard, statistical analysis of eight rows revealed three rows with significant clustering of GLRaV-3, suggesting an emerging vectorial spread of GLRaV-3 (Cabaleiro et al., 2008). No significant clustering of GLRaV-3 was observed in the Portomarín vineyard (Cabaleiro et al., 2008).

Two studies have shown the natural spread of GLRaV-3 within New Zealand vineyards can be rapid. Jordan (1993) observed the 100% infection of vines within six years of establishing a new vineyard using disease-free Cabernet sauvignon vines. While, over five years of monitoring the GLRaV-3 infection within a block of Pinot noir vines, the level of GLRaV-3 infection increased from 12 to 92% (Petersen, 1996). More recently, over six years Charles et al. (2009) studied the spread of GLRaV-3 in newly established Chardonnay and Merlot blocks in New Zealand. The observed GLRaV-3 spread was similar to that of Jordan (1993) and
Petersen (1996), and all vines were predicted to become infected within 15 to 20 years assuming a similar rate of secondary infection (Charles et al., 2009). Generally, an increase in GLRaV-3 spread (with the formation of GLRaV-3 infected vines clusters) was observed within the blocks following the years of high mealybug (Ps. longispinus) levels and when young vines were large enough to support more mealybugs (Charles et al., 2009). In addition, GLRaV-3 spread was faster in Chardonnay vines compared to the Merlot vines, despite higher mealybug numbers in the adjacent block closest to the Merlot vines (Charles et al., 2009). Potential differences in the GLRaV-3 transmissibility in different cultivars or transmissibility of different genetic variants may account for the differential rate of spread (Charles et al., 2009).

Prior to this project, there were no published accounts of the distribution of specific GLRaV-3 variants in the field. However, along with this project (Chapter 4), the distribution of specific GLRaV-3 variants was recently examined in vineyards from South Africa and the Napa Valley (USA) (Jooste et al., 2011; Sharma et al., 2011). Generally, the different GLRaV-3 variants were unevenly distributed, and clusters of particular GLRaV-3 variants were observed, suggesting possible differences in transmission efficiencies (Jooste et al., 2011; Sharma et al., 2011). This is discussed in more detail in Chapter 4 and 6.

1.3 Detection methods for GLRaV-3

Currently, there is no “cure” for vines infected with viruses. Thus, an active approach to reduce the introduction and spread of GLRaV-3 is required. Accurate and sensitive detection of GLRaV-3 is a critical component for successful disease control (Section 1.4). Three forms of diagnostic detection methods can be used to test for GLRaV-3; biological indexing, serological-based testing, and molecular-based testing.

1.3.1 Biological indexing

Initially, detection of viral diseases was conducted using biological indexing. For the detection of GLRaV-3, which is graft-transmissible, pieces of plant material (with a bud) from test samples were grafted onto indicator plants, typically Cabernet franc (Rowhani et al., 1997). Grafted plants were then grown for two to three years and monitored for the development of symptoms. This method is labour-intensive and time-consuming, requiring two to three years before virus
infection status can be determined. In addition, testing identifies the disease and does not provide additional information about the virus(es) infecting the plant, such as which of the GLRaV(s) is present in the infected plant.

### 1.3.2 Serological testing

Serological testing is based on antibody reactions with disease agents such as viruses. Enzyme-linked immunosorbent assay (ELISA) is the most widely used method for the detection of plant viruses as it is easy, economical, fast, and large numbers of samples can be processed at one time (Narayanasamy, 1997). Since the late 1980s, ELISA has been used for the detection of GLRaV-3 infection and is now routinely used for screening (Bell et al., 2009; Cohen et al., 2004; Constable et al., 2012; Ling et al., 2000; Zee et al., 1987). In contrast to biological indexing, ELISA testing only requires two to three days of processing to determine virus infection. With the use of virus/strain specific antibodies, ELISA can also identify the different viruses and in some cases virus strains, that are in test samples.

The main drawbacks of ELISA testing include the inability to provide genetic (i.e. sequence data) information about the virus, inability to detect grapevine viruses at low titres, and the difficulty in producing antibodies for reliable detection of some viruses (Osman et al., 2008).

### 1.3.3 Molecular testing

Molecular-based assays are an alternative approach for diagnostic testing for viruses, which directly target the virus genome, i.e. specifically targeting the nucleic acid sequence that is unique to the virus. Molecular-based assays that have been used for the detection of GLRaV-3 include: conventional RT-PCR (MacKenzie et al., 1997; Martin et al., 2005b), conventional RT-PCR in conjunction with SSCP analysis and sequencing (Gouveia et al., 2011; Jooste et al., 2005; Jooste et al., 2010; Jooste et al., 2011; Turturo et al., 2005), immunocapture RT-PCR (IC-RT-PCR) (Engel et al., 2008; Nolasco et al., 1997), multiplex RT-PCR (mRT-PCR) (Fuchs et al., 2009; Gambino et al., 2006; Sharma et al., 2011), asymmetric PCR-ELISA (APET) (Gouveia et al., 2011), TaqMan real-time RT-PCR (Osman et al., 2006; Pacifico et al., 2011; Tsai et al., 2012), and low-density and oligonucleotide microarrays (Engel et al., 2010; Osman et al., 2008).
The use of conventional RT-PCR in conjunction with SSCP analysis and sequencing, has been extensively used to study the genetic variability within GLRaV-3 populations, identifying GLRaV-3 variants that represent up to five different phylogenetic groups (Gouveia et al., 2011; Jooste et al., 2005; Jooste et al., 2010; Jooste et al., 2011; Turturo et al., 2005). SSCP is an easy and inexpensive method to identify different virus variants within test samples, and identify samples with single or mixed infections. SSCP is based on the differential migration of single-stranded DNA (ssDNA) on a non-denaturing gel (refer to Chapter 2 for detailed description of SSCP protocol). Following SSCP analysis, to further examine the molecular sequence of the virus variants that generate interesting SSCP results, RT-PCR products can be cloned and sequenced. For these reasons, SSCP was also used in this project to investigate the genetic variability of GLRaV-3 in New Zealand (Chapter 2).

Conventional mRT-PCR assays (gel-based assay) allow for the simultaneous detection of multiple viruses and/or virus variants within a single test reaction, reducing reagent costs. For example, Gambino et al. (2006) developed a mRT-PCR assay that detected nine grapevine viruses, GLRaV-1, -2, and -3, *Arabis mosaic virus*, *Grapevine fanleaf virus* (GFLV), (GVA), *Grapevine virus B* (GVB), *Rupesstris stem pitting-associated virus* (RSPaV), and *Grapevine fleck virus*, in combination with a plant RNA internal control, 18S rRNA. At the start of this project, mRT-PCR assays for the detection of different GLRaV-3 variants had yet to be developed. Thus, this cost-effective form of diagnostic testing was considered in this project for the detection of the different GLRaV-3 variants present in New Zealand (Chapter 4). During the project, Sharma et al. (2011) developed two multiplex assays that detected GLRaV-3 variants from groups 1 and 3 in one assay, and variants from groups 2 and 4 in addition to a generic test for other GLRaV-3 variants in another assay (discussed further in Chapter 4).

The development of real-time RT-PCR assays have further increased the specificity, sensitivity, and efficiency of molecular-based assays compared to the gel-based conventional assays (Osman et al., 2008; Pacifico et al., 2011; Tsai et al., 2012). In addition to diagnostic detection, real-time RT-PCR assays can be used for quantification of virus targets. Quantification can be either of the absolute number of copies present or the relative expression of the virus compared to (normalised to) host genes (reference genes). The TaqMan real-time RT-PCR
has been used for the detection (Osman et al., 2006; Pacifico et al., 2011; Tsai et al., 2012) and quantification (Pacifico et al., 2011; Tsai et al., 2012) of GLRaV-3.

Molecular-based testing is fast and is generally regarded as the most sensitive form of detection currently available. PCR is estimated to be 100 to 1000 times more sensitive than ELISA (Osman et al., 2008) and is widely used by the industry and researchers (Constable et al., 2012; Gambino et al., 2006; La-Notte et al., 1997; Ling et al., 2001; Osman et al., 2007, 2008; Osman et al., 2006). However, seasonal fluctuations of virus titre and uneven virus distribution (Cohen et al., 2004; Constable et al., 2012; Osman et al., 2007; Pacifico et al., 2011; Rowhani et al., 1997) can affect the reliability of serological and molecular detection methods. Additional factors such as poor quality RNA with high amounts of reverse transcription and DNA amplification inhibitors (Henson et al., 1993; Newbury et al., 1977; Salzman et al., 1999), and genetic variability within the virus population (Constable et al., 2012; Osman et al., 2007) can also detrimentally affect molecular-based protocols. For instance, sequence variation at the primer binding site can result in a false negative result (Chapter 4). Therefore, it is important that the genetic variation within a virus population is well understood and up-to-date, as it provides valuable sequence data for the design of effective primers.

1.3.4 Other detection techniques

Possible alternative diagnostic testing approaches include using next generation sequencing (NGS) platforms and spectral reflectance analysis of the leaves. NGS platforms are not constrained by the requirement of prior sequence knowledge to design primers, and are generally used to identify new and known virus/virus strains within an individual host or multiple hosts (i.e. pooled samples) (Adams et al., 2012). Currently, NGS platforms are relatively expensive to be developed as a routine high-throughput diagnostic method. However, they can be used as an initial investigative tool to identify rapidly the potential disease causing virus in particular field plots. This can then be followed by the design of more cost-effective molecular-based assays, specific for the viruses identified in the field plots, based on the NGS sequences. For example, 454-pyrosequencing was used to diagnostically screen virally infected maize crops in Kenya (Adams et al., 2012). Samples used for sequencing were derived from a pool of 11 symptomatic leaf samples of 11 different plants from six different field plots (Adams et al., 2012). The sequencing identified two viruses, *Maize chlorotic mottle virus* and *Sugarcane
mosaic virus. From this initial diagnostic screening, the sequence data was then used to develop specific real-time PCR assays that could be used for high-throughput screening of the remainder of the untested plants in the six different field plots (Adams et al., 2012).

Naidu et al. (2009) studied the possibility of detecting GLRaV-3 in Cabernet sauvignon and Merlot vines, by analysing the changes in leaf spectral reflectance between uninfected and virus infected vines. This is based on the premise that virus infection changes the leaf pigments and biochemical components that in turn alter the leaves spectral characteristics, which can be detected using visible and/or near-infrared regions of the electromagnetic spectrum (Naidu et al., 2009). Naidu et al. (2009) compared the reflectance spectra of GLRaV-3 infected (symptomatic and non-symptomatic) and healthy leaves, between 350nm and 2500nm at 1nm increments using a portable spectrometer. Virus infections were confirmed by RT-PCR. Differences between infected and healthy were observed at the green (near 550nm), near-infrared (near 900nm) and mid-infrared (near 1600nm and 2200nm) wavelengths (Naidu et al., 2009). However, for symptomatic and non-symptomatic leaves, different wavelengths and analysis are likely to be required for accurate detection (Naidu et al., 2009). Furthermore, work to test the feasibility of this technique for white cultivars is still required (Naidu et al., 2009).

1.4 Control strategies for the grapevine leafroll disease

The current approach to reduce the negative impacts of GLRaV-3 on vineyards and wine industries, is to minimise the spread of GLRaV-3. The continual roguing of GLRaV-3 infected vines from the vineyard is important as it removes a source of GLRaV-3 that can potentially spread further in the vineyard (Charles et al., 2006; Pietersen, 2004). Furthermore, using certified virus-free plants to establish new vineyards or replacing the infected vines removed from the vineyards has been shown to be an effective method to reduce GLRaV-3 spread (Charles et al., 2006; Martelli et al., 2006; Pietersen, 2004). In an attempt to control the quality of new plant material in New Zealand, the industry implemented the New Zealand Grafted Grapevine Standard (GGS), which has strict requirements for nurseries to annually test vines used for propagation for GLRaV-3 (New Zealand Winegrowers, 2011). Therefore, it is critical that diagnostic testing protocols are accurate to ensure the correct vines are removed from the field, and that GLRaV-3 infected material are not mistakenly used as a source of rootstock or scionwood for propagation.
In addition to reducing possible sources of GLRaV-3, it is also important to contain secondary spread of leafroll infection by controlling the mealybug population within the vineyard as these are the most important insect vectors for GLRaV-3 transmission (Charles et al., 2006; Pietersen, 2004). Mealybugs can be controlled by either using insecticide sprays or biological controls in the form of mealybug predators and parasitoids (Charles et al., 2006). It is also important to control the surrounding weeds and ant population. Although ants are not GLRaV-3 vectors, ants protect mealybugs from natural predators and can lead to poor results if biological control agents are used to control mealybugs (Charles et al., 2006). Weeds are not potential GLRaV-3 hosts but can provide shelter for mealybugs and ants to hide from the weather conditions (Charles et al., 2006). Day to day vineyard hygiene protocols such as cleaning equipment and machinery, are also important in reducing the risk of viruliferous mealybugs being transported within or between blocks (Charles et al., 2006; New Zealand Winegrowers, 2010).

1.5 Objectives of this research project

The principal aim of this research was to gain an understanding of the genetic variability of GLRaV-3 in New Zealand. Prior to this project, Pong (2009) studied the GLRaV-3 genetic variability within the New Zealand population using 19 grapevine samples from six different sources. Published primers, available at the time, were used to screen material, resulting in the identification of GLRaV-3 variants with high nucleotide identity with variants from group 1 and 2. The divergent NZ-1 variant was not identified in this study. Steps to improve molecular-assays using published primers were performed; however, RT-PCR assays still could not detect GLRaV-3 from some of the ELISA or visually GLRaV-3 positive samples. This indicated that even greater genetic variability was present in New Zealand, and that the genetic variability had a detrimental effect on the accuracy and reliability of diagnostic molecular-based methods. Diagnostic methods are important to the New Zealand wine industry as it aids the control of GLRaV-3 spread.

Consequently, this project is of economic importance to New Zealand and worldwide. An improved understanding of the GLRaV-3 genetic variation in New Zealand aids (i) the development of new reliable molecular-based assays, and (ii) our understanding of the relationship between GLRaV-3 variants and their various
biological characteristics that can influence virus spread. The four key objectives for this research are:

1. To extend previous knowledge of the genetic variability of GLRaV-3 by studying a wider selection of GLRaV-3 infected samples using new protocols.

2. To sequence extended portions of the genome of different genetic variants to determine the identity of New Zealand variants and their relationship to overseas GLRaV-3 variants. In order to examine the possible implications genetic variation may have on virus functions, and to provide appropriate sequence data for the development of molecular-assays.

3. To examine the effects of genetic variation on the application of ELISA and RT-PCR, and to improve GLRaV-3 detection by developing new molecular assays based on the sequence obtained from this study and overseas.

4. To examine the effect of GLRaV-3 variation on biological features such as pathogenicity and virus movement within the vine and field plots.
Chapter 2:

The genetic diversity in New Zealand GLRaV-3 isolates

2.1 Introduction

Knowledge of the genetic diversity of a virus population can provide insights into its evolutionary history and is also of practical importance in the detection and control of virus diseases. Understanding the virus genetic variation aids the identification of new virus variants/strains, the determination of prospective evolutionary processes involved in virus evolution (such as recombination), and the identification of selective pressures on genomes and/or specific genes (Wang et al., 2011). In addition, revealing the phylogenetic relationship between isolates can also help identify phylogenetic groups that share biological characteristics (Wang et al., 2011). For example, graft incompatibilities are generally induced by the RG and PN variants of GLRaV-2, but not by the BD variants (Bertazzon et al., 2010).

Sequences from genetic diversity studies are of practical importance, as sequences are the fundamental resource used for the development of accurate and reliable molecular-based diagnostic tools (Wang et al., 2011). These diagnostic tools are critical to virus-free certification and quarantine procedures and are an important part of disease management programmes. Thus, to ensure that most, if not all, virus variants are detected with high reliability and specificity, a full understanding of the genetic variation within the virus population is required.

Until recently, little was known about the extent of the genetic diversity present in GLRaV-3 isolates found in New Zealand, with only one publicly available partial sequence, NZ-1 (EF508151), and an SSCP and sequencing based study by Pong (2009) (Chapter 1, Section 1.5). Even after attempts by Pong (2009) to improve GLRaV-3 detection by RT-PCR, some samples that were symptomatic and positive by ELISA using reagents from Bioreba could not be detected by RT-PCR using common diagnostic primers (Pong, 2009). This suggested that greater genetic diversity was potentially present within the New Zealand GLRaV-3 population.
Genetic diversity studies of plant viruses have used various molecular-based methods such as ribonuclease T1 fingerprinting (Moya et al., 1993), restriction fragment length polymorphism analysis (Corazza et al., 2012; Wang et al., 2012), and SSCP analysis (D'Urso et al., 2003; Jooste et al., 2005; Jooste et al., 2010; Papayiannis et al., 2007; Pong, 2009; Turturo et al., 2005). As mentioned in Chapter 1 Section 1.3.3, SSCP analysis is an easy, inexpensive, and fast method for identifying mutations or sequence variants within a sample, and has been used extensively in conjunction with cloning and sequencing, by researchers to investigate GLRaV-3 genetic diversity worldwide. These GLRaV-3 studies identified sequence variants that corresponded to two (Jooste et al., 2005; Pong, 2009), three (Jooste et al., 2010; Turturo et al., 2005), and five (Gouveia et al., 2011) different phylogenetic groups from groups 1 to 5 (Chapter 1). In addition, SSCP was used as an effective detection tool to determine the phylogenetic group a particular sequence variant belonged to, based only on the resulting SSCP pattern. Other GLRaV-3 genetic diversity studies based on the sequencing of PCR products and sequence analysis identified three (Fuchs et al., 2009), four (Wang et al., 2011) and seven (Sharma et al., 2011) different phylogenetic groups (Chapter 1 Section 1.2.3).

The overall objective of this chapter was to investigate the genetic diversity of GLRaV-3 in New Zealand. This was achieved by screening samples by double antibody sandwich ELISA (DAS-ELISA) and RT-PCR using newly designed primer sets to improve GLRaV-3 detection, examining a wider selection of grapevine samples, and by conducting SSCP, cloning, and sequence analyses.
2.2 Materials and methods

2.2.1 Virus source

Plant material from 55 known DAS-ELISA GLRaV-3 positive and/or visually symptomatic plants was sourced from North and South Island vineyards/collections (Table 2.1). This material included leaf and cane cuttings of different red and white cultivars.

Eight of the samples, consisting of leaf and cane material from Cabernet sauvignon, Roussanne, and Gruener Veltliner, were from plants that were still positive for GLRaV-3 by ELISA after short periods of thermotherapy (Cohen, 2007). Tests of two samples, CS341 and R2D4, by RT-PCR (Linnaeus Laboratories, Gisborne) did not detect GLRaV-3, whereas the Gruener Veltliner sample was positive by both ELISA and RT-PCR, suggesting the presence of strain variants (Cohen, 2007).

Twenty-three of the samples were from a small collection of historic grapevine accessions from the Te Kauwhata National Grapevine Repository that was established near Auckland in 2000 (referred hereafter as the Auckland collection). In an attempt to record and catalogue vines from this historic collection, each accession was allocated a number based on the apparent importation date (Dick et al., 1989b). The Auckland collection contains accessions that had been imported to New Zealand in 1900s (TK00001-TK02999) and accessions imported into New Zealand between 1960 and 1990 (TK05000-on wards); consisting of 157 accessions of 108 red varieties and 49 white varieties, refer to Appendix 1 Table A1.1 for varietal details. To enhance the likelihood of identifying different sequence variants within the New Zealand GLRaV-3 population, 20 vines from the older accessions (TK00004 to TK00213) were selected for this study (Table 2.1).
Table 2.1: ELISA and/or visual GLRaV-3 positive plant samples used for the genetic variability screen.

<table>
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<th>Cultivar</th>
<th>Source</th>
<th>Type of material</th>
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</tbody>
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<sup>a</sup> Plant samples used for further genome characterisation, refer to Chapter 3.

<sup>b</sup> Greenhouse plant samples previously heat treated.
2.2.2 Total RNA extraction

Total RNA extractions were conducted using the Sigma-Aldrich Spectrum™ Plant Total RNA Kit (St. Louis, MO, U.S.A) following the protocol provided by the manufacturer with minor modifications, as follows. Approximately 80-100mg of phloem scrapings or 6.0mm leaf discs, containing both primary and secondary veins, cut using a Harris Uni-Core™ (Ted Pella, Inc., Redding, CA, U.S.A) were sampled and stored with a 5mm stainless steel ball bearing in 2mL microcentrifuge tubes at -80°C. Samples were then homogenised using the Qiagen TissueLyser II (Qiagen, GmbH, Germany) at 30 cycles per second for 1 minute. To ensure no plant material remained at the top of the tube, samples were then centrifuged at 16,000xg for 10 seconds, this was followed by the addition of 1000µL of modified Lysis Solution buffer to each sample (Sigma-Aldrich, St. Louis, MO, USA) (containing 1% (w/v) sodium metabisulphite (Sigma-Aldrich, St. Louis, MO, USA) added just before use) and homogenisation for a further 30 seconds at 30 cycles per second. Samples were then incubated at 56°C for 5 minutes in a water bath, followed by centrifugation at 14,000xg for 5 minutes. The remainder of the total RNA extraction protocol followed the manufacturer instructions (Appendix 4 Section A4.1).

The concentration and quality of resulting total RNA extracts were measured using a Nanodrop ND-1000 spectrometer (Nanodrop technology, Wilmington, DE, USA) at the absorbance ratio of A_{260}:A_{280} and stored at -80°C.

2.2.3 Screening of plant material for GLRaV-3

To establish the GLRaV-3 infection status of plant material, samples were tested by DAS-ELISA and one-step RT-PCR.

2.2.3.1 DAS-ELISA screening

DAS-ELISA tests were conducted courtesy of D. Cohen and A. Blouin (The New Zealand Institute for Plant and Food Research Limited, Auckland, New Zealand). For extraction, approximately 100 to 200mg of leaf and petiole tissue and 200 to 300mg of leaf vein and bark tissue were used in combination with 2 to 3mL and 4 to 5mL of a grapevine extraction buffer (200mM Tris, 137mM NaCl, 0.45mM polyvinylpyrrolidone-40 (PVP-40), 1.4mM PEG, 3mM NaN₃, 2% Tween-20, pH8.2±0.2), respectively. All samples were ground in double layered plastic
extraction bags (Bioreba AG, Switzerland) using a mechanical ball-bearimg grinder and were incubated overnight at 4°C.

GLRaV-3 specific immunoglobulin G (IgG) antibodies (Bioreba AG, Reinach, Switzerland) were diluted in a coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, 3mM NaN₃, and pH9.6±0.2) at a 1 to 2000 ratio and 200μL of this mixture was used to coat each well of an ELISA microtitre plate (Nunc Maxisorp™, Germany). Plates were then incubated either overnight at 4°C or for 4 hours at 30°C. To remove excess unbound antibodies, plates were washed with deionised water three times and once with PBS-tween (0.05% Tween-20, 0.14M NaCl, 8.1mM Na₂HPO₄, 1.47mM KH₂PO₄, 2.68mM KCl, pH7.4±0.2). This was followed by the addition of 200μL of grapevine extracts, a buffer negative control, and a positive control to each well of the ELISA plate. In addition, the positive control was diluted in extraction buffer at a one to three dilution series to a factor of 1 in 729. Plates were then incubated either overnight at 4°C or for 4 hours at 30°C and then washed with deionised water three times and once with PBS-Tween.

The GLRaV-3 alkaline phosphatase (AP) conjugate antibodies (Bioreba AG, Switzerland) were diluted 1:2000 in conjugate buffer (0.45mM PVP, 2g/L bovine serum albumin (BSA), 5% PBS Tween-20, and pH7.4±0.2) before 200μL was added into each well and incubated either overnight at 4°C or for 4 hours at 30°C. To remove unbound antibody-enzyme conjugate from wells, plates were then washed with deionised water three times and once with PBS-Tween. 200μL of prepared substrate solution (1mg/ml p-nitrophenyl phosphate substrate was added to substrate buffer, 10mM diethanolamine, 3mM NaN₃, pH9.8±0.2, just before use) to each well. Plates were then incubated in the dark and the optical density (OD) was read periodically read over three to four hours at 405 nm using a Multiskan EX ELISA plate reader (Thermo-Fisher Scientific, Waltham, MA, USA).

To increase the DAS-ELISA sensitivity (reducing the effects of high background reactivity) the rate of colour development for each field sample, negative buffer and positive control was calculated (D. Cohen, Plant and Food Research, Auckland, New Zealand pers. comm.). Initially, the AP enzymatic reaction rate over a specific period of time is approximately linear (Crowther, 2009). Therefore, the reaction rate is calculated by taking the change in OD at two different time points during this linear phase (Eqn. 2.1) as the amount of enzyme conjugate present will be proportional to the rate of colour development. Field samples were
considered positive when reaction rates were greater than 0.10mOD/min, while values between 0.08 to 0.10mOD/min were considered inconclusive and the samples were re-tested.

\[
\text{Reaction rate} = \frac{(\text{OD}_2 - \text{OD}_1)}{(t_2 - t_1)} \quad \text{Eqn. 2.1}
\]

where, \(\text{OD}_2\) is the optical density at time point 2 (mOD)
\(\text{OD}_1\) is the optical density at time point 1 (mOD)
\(t_2\) is the time when the optical density reading 2 (\(\text{OD}_2\)) was taken (min)
\(t_1\) is the time when the optical density reading 1 (\(\text{OD}_1\)) was taken (min)

### 2.2.3.2 RT-PCR screening

Two sets of primers, NZ-10875F/NZ-11482R and NYSA-14109F/NYSA-14636R, (Table 2.2) were used to screen all ELISA positive and/or symptomatic samples. The primer pairs target different genomic regions and were designed based on sequences from GenBank to detect different GLRaV-3 variants. The NZ-10875F/NZ-11482R primer set targets ORF4 and NYSA-14109F/NYSA-14636R targets ORF6; generating expected PCR products sized 608bp and 528bp, respectively (Table 2.2).

#### 2.2.3.2.1 Primer design

The primer sets were designed using Primer3 (Rozen et al., 2000), to have the same RT-PCR protocol, ensuring the screening process was simple and efficient where reactions could be run in parallel. The NZ-10875F/NZ-11482R primer pair was designed based on the sequence of the GLRaV-3 NZ-1 isolate. The primer pair NYSA-14109F/NYSA-14636R was designed against three complete genomes and nine partial sequences of GLRaV-3 isolates: NY1 (AF037268), CI-766 (EU344893), GP18 (EU259806), Pet-2 (DQ680142), Pet-3 (DQ062152), Pet-1 (DQ680141), CI-817 (EU344894), Pet-4 (AY753208), SL10 (DQ911148), Dawanhong No. 2 (DQ119574), CI-664 (EU344895), and CI-765 (EU344896). The sequences were collated and aligned using ClustalX v2.0 (Larkin et al., 2007) and Geneious v5.5 (Drummond et al., 2011).

Primers were generally designed to contain a G+C content of 40 to 60%, a basic melting temperature between 53°C and 60°C, and a reduced likelihood of forming secondary structures. The basic melting temperature was calculated using the BioMath Calculator (Promega, Madison, WI, USA). To analyse the likelihood of prospective primers generating secondary structures such as hairpins, primer self-
complementarity dimers, and primer hetero-complementarity dimers, the OligoAnalyzer v3.1 program (Integrated DNA Technologies Inc., IA, USA) was used. The Gibbs Free Energy (ΔG) was utilised as an indicator of desirable or undesirable structures. Generally for hairpin structures a ΔG value of -3 kcal/mol was tolerated, while 3’end primer self- and hetero-complementarity dimers with a ΔG value of -5 kcal/mol and internal self- and hetero-complementarity dimers with a ΔG value of -6 kcal/mol were tolerated.

**Table 2.2:** Table showing the primer pairs used for the initial RT-PCR GLRaV-3 screening and SSCP analysis.

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<th>Primer Name</th>
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<td>ORF6 14617 - 14636</td>
<td></td>
</tr>
</tbody>
</table>

*The primer nucleotide positions are based on the complete GP18 (EU259806) genome sequence.*

**2.2.3.2.2 One-step RT-PCR screening protocol**

The one-step RT-PCR was carried out in 10µL reactions using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Invitrogen, Carlsbad, CA, USA) containing 100 to 400 ng of total RNA extract, 1U of the Superscript™ III RTase/Platinum® Taq mix (Invitrogen, Carlsbad, CA, USA), and a final concentration of 500 nM of forward and reverse primers. Reverse transcription was performed at 50°C for 30 minutes, followed by inactivation of the reverse transcriptase and activation of the polymerase at 94°C for 2 minutes. This was followed by 30 cycles of 94°C for 10 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 40 seconds, and a final extension at 68°C for 3 minutes.

The resultant RT-PCR products were analysed by gel electrophoresis. For each sample, 5µL of RT-PCR product was mixed with loading dye (0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 2% (w/v), Ficoll-400 (Sigma-Aldrich, St Louis, MO, USA), and 30% glycerol in 0.5xTBE buffer (45mM Tris-HCl, 45mM boric acid, and 1mM EDTA, pH8.5)) at a 5:1 ratio and analysed on a 1% agarose (Seakem® LE agarose, Cambrex, ME, USA) gel dissolved in 0.5xTBE.
buffer. Electrophoresis was then performed in a Wide Mini-Sub Cell GT electrophoresis unit (Bio-Rad Laboratories Inc, Hercules, CA, USA) using 0.5xTBE running buffer at 10V/cm for 35 to 40 minutes. Gels were stained with ethidium bromide (0.01µg/mL) for 20 minutes, and visualised under ultraviolet light at 302nm, using an ultraviolet transilluminator GelDoc 2000 (Bio-Rad Laboratories Inc, Hercules, CA, USA).

2.2.3.2.3 Specificity of GLRaV-3 detection protocols

To ensure primer specificity, oligonucleotide sequences were searched against the NCBI nucleotide database using the Basic Local Alignment Search Tool (BLAST). Additionally, both detection assays were tested against healthy grapevine samples, i.e. GLRaV-3 negative RNA, and no template controls (water).

2.2.4 SSCP analysis

SSCP is dependent on the differential mobility of ssDNA on a non-denaturing polyacrylamide gel. PCR products are denatured forming single stranded fragments and then rapidly cooled, leading to the 3-dimensional folding of single stranded fragments by intra-strand base pairing (predominantly dependent on the original nucleotide sequence) and the formation of higher-order structures. Folded ssDNAs are then run on a polyacrylamide gel, which move through the gel at different rates, according to the folded structure formed, resulting in a particular SSCP patterns (Orita et al., 1989). Thus, samples with different sequence variants generally form different SSCP patterns. SSCP banding patterns that are comprised of two or three bands generally represent samples with a single infection of one sequence variant, while banding patterns with four or more bands generally indicate samples with mixed infection of different sequence variants. These have also been referred to as “simple” and “complex” patterns respectively (Jooste et al., 2010; Turturo et al., 2005).

2.2.4.1 RT-PCR for SSCP analysis

To generate RT-PCR products for SSCP analysis, 50µL reactions were carried out using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Invitrogen, Carlsbad, CA, USA). Each reaction contained 100 to 400ng of total RNA extract, 5U of the Superscript™ III RTase/Platinum® Taq mix (Invitrogen, Carlsbad, CA, USA), and a final concentration of 2.5mM of MgSO₄ and
400nM of each forward and reverse primer. Refer to Section 2.2.3.2.2 for the RT-PCR thermocycling conditions.

To confirm successful amplification, resulting RT-PCR products were analysed on a 1% agarose gel, as described in Section 2.2.3.2.2. PCR products were gel purified before SSCP analysis, as described in Section 2.2.4.2.

**2.2.4.2 Gel extraction**

To isolate the correct sized RT-PCR product, samples were separated using crystal violet electrophoresis based on protocols by Kamath et al. (2003) and Harper (2009), excised, and purified using the Zymoclean DNA gel purification extraction kit (Zymo Research Corporation, Irvine, CA, USA). Using crystal violet electrophoresis for the purification of RT-PCR products instead of ethidium bromide, can improve downstream processes such as cloning and sequencing as the DNA is less prone to damage from exposure to ultraviolet light (Kamath et al., 2003; Rand, 1996).

Crystal violet electrophoresis was performed using 1.0 to 1.5% agarose (Seakem® LE agarose, Cambrex, ME, USA) gel dissolved in 1xTAE buffer (40mM Tris, 20mM glacial acetic acid, and 1mM EDTA; pH 7.2) with the addition of 5μg/mL of crystal violet (Sigma-Aldrich, St Louis, MO, USA) before setting the gel. For each sample, 40μL of RT-PCR product was mixed at a 5:1 ratio with crystal violet loading dye (100μg/mL crystal violet, 1mM EDTA, 2% Ficoll-400, and 30% glycerol). Electrophoresis was then preformed in a Hoefer HE33 mini-submarine electrophoresis unit (Hoefer Inc., San Francisco, CA, USA) at 80V for 60 minutes using 1xTAE running buffer. After electrophoresis, bands were visualised on a light box and the correct sized band was excised using a sterile scalpel blade. This was then weighed and purified using the Zymoclean DNA gel purification extraction kit, (Zymo Research Corporation, Irvine, CA, USA) as described by the manufacturer.

The concentration and quality of the resulting DNA extracts were measured using a Nanodrop ND-1000 spectrometer (Nanodrop technology, Wilmington, DE) at the absorbance ratio of A_{260}/A_{280} and stored at -20°C.
2.2.4.3 SSCP protocol

The SSCP protocol used was based on a combination of protocols by Rubio et al. (1996) and Ochoa et al. (2000), where 10µL of PCR product was added to 14µL of deionised formamide (Ambion, Austin, TX, USA) with 0.025% (w/v) xylene-cyanol, 0.025% (w/v) bromophenol blue, and 0.1% (w/v) Ficoll-400. To sufficiently denature the double-stranded DNA, the PCR product-loading dye mix was heated at 99°C for 10 minutes in a Techne MD-1000 dry heat block (Barloworld Scientific Ltd, Stone, Staffordshire, UK) and then, to promote the newly separated strands to fold on itself, the mix was immediately cooled on ice for 5 minutes. Samples were then separated by electrophoresis on 8 or 12% non-denaturing polyacrylamide gels in a Protean II PAGE system (Bio-Rad Laboratories Inc. Hercules, CA, USA) at 200V for three to four hours (depending on fragment length) at 4°C. Gels were stained in ethidium bromide (0.01µg/mL) for 20 minutes and visualised using an ultraviolet transilluminator GelDoc 2000 (Bio-Rad Laboratories Inc, Hercules CA, USA).

2.2.4.4 Cloning protocol

Samples generating simple and complex SSCP banding patterns (Section 2.2.4) were selected for further sequencing. Gel-purified products were cloned using the pGEM-T easy vector system (Promega, Madison, WI, USA) and DH-5α *Escherichia coli* competent cells (Invitrogen, Carlsbad, CA, USA).

The purified DNA samples were inserted into the pGEM®-T easy vector (Promega, Madison, WI, USA) using the T4 ligase and incubated overnight at 4°C. DH-5α *E. coli* competent cells (Invitrogen, Carlsbad, CA, USA) were then transformed using 5µL of the pGEM®-T easy vector ligation reactions. A 60µL aliquot of each transformation culture was plated onto two duplicate Luria Bertani (LB) agar plates (each plate contained 0.8mg ampicillin, 2mg Isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.8mg 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal)) and incubated overnight at 37°C. From these plates, 20 white colonies were transferred into falcon tubes containing 4mL of liquid LB medium (containing 0.4mg ampicillin; pH 7.0) and incubated overnight at 37°C.

To screen resultant clones for the presence of correct sized inserts, PCR and EcoRI restriction enzyme digests were conducted. The PCR clone screening was conducted using the 2xPCR Master mix Solution (i-StarTaq) (iNtRON
Biotechnology, Gyeonggi-do, South Korea) in a 10µL PCR reaction mix containing 500nM each of T7 and SP6 primer and 1µL of template plasmid DNA, following the PCR thermocycling protocol: 10 minutes at 94°C, 30 cycles of 10 seconds at 94°C, 30 seconds at 53°C, and 1kb/min at 68°C. The PCR amplification products were then visualised as described in Section 2.2.3.2.2.

2.2.4.5 Clone screening using SSCP

The correct-sized inserts in plasmids can be of the same or different sequence variant. To select for plasmids that were likely to contain interesting/different sequences, and in turn reduce the number of clones for plasmid DNA purification and sequencing, clones were further screened using the clonal polymerase chain reaction–single-strand conformational polymorphism (PCR–SSCP) approach (Zhou et al., 2008). 40µL of 0.8% Triton X-100 was added to 30µL of each clone and heated at 95°C for 10 minutes. The samples were then centrifuged for 1 minute at 12,000xg, followed by a second PCR using the 2.5U AmpliTaq® DNA Polymerase (Applied Biosystems, Foster City, CA, USA) in 25µL reaction mixtures that also contains 2µL of resultant supernatant, 2.5mM final concentration of MgCl$_2$, 400nM of the original forward and reverse primer, and 0.3mM dNTP. The PCR product from clones were then analysed using SSCP as described in Section 2.2.4.3.

Individual clone samples with different PCR-SSCP patterns indicates potential genetic variation, thus two clone samples for each different PCR-SSCP pattern was extracted using the second edition QIAprep® Miniprep kit (Qiagen, GmbH, Germany), as per the manufacturer’s instructions. To verify the insert size an EcoRI restriction enzyme (Invitrogen, Carlsbad, CA, USA) digest was utilised to release the insert from the extracted plasmid DNA and was visualised as described in Section 2.2.3.2.2. Correctly sized fragments were then sent for primer extension sequencing (Macrogen Inc., Seoul, South Korea).

2.2.5 Phylogenetic analysis of partial sequence from ORF4 and ORF6

Before phylogenetic analysis was conducted, the vector and primer sequence were removed from all sequences. The primer sequences were removed to ensure potential false homology was not introduced into the analysis. All available GLRaV-3 sequences that matched the region analysed in this study were retrieved from GenBank and aligned using Geneious v5.5 (Drummond et al., 2011) and
Multiple ORF4 and ORF6 sequence alignments were trimmed to ensure all sequences were of the same length. The nucleotide distance matrices, based on p-distance calculations, were generated in MEGA5 (Tamura et al., 2011). Since duplicate sequences do not provide additional information, unnecessarily clutters the phylogenetic trees, and increases the computation time of analysis (Hall, 2011), duplicate sequences with 100% nucleotide identity were removed from the alignment.

To ensure the best suited model was used for further phylogenetic analysis of each data set, the Akaike information criterion (AIC) and/or AIC with second order correction, and Bayesian information criterion were applied to each of the multiple sequence alignments using MEGA5 (Tamura et al., 2011) and TOPALi v2.5 programmes (Milne et al., 2004). For the ORF4 sequence data set the Tamura 3-parameter model (Tamura, 1992) was selected, while the Kimura 2-parameter model (Kimura, 1980) was selected for the ORF6 sequence data set. Phylogenetic trees were then constructed using the selected models and the Neighbour-Joining (NJ) method (Saitou et al., 1987) in MEGA5 (Tamura et al., 2011). For each analysis, bootstrap values were calculated based on $10^3$ replicates (Felsenstein, 1985).

**2.2.6 Recombination analysis**

Recombination analysis was conducted on the partial GLRaV-3 multiple sequence alignments of ORF4 and 6 using the RDP v3.44 software (Martin et al., 2010). The following recombination detection methods were used, with default settings, to detect potential recombination sites between isolates; 3Seq (Boni et al., 2007), Bootscan (Martin et al., 2005a), Chimaera (Posada et al., 2001), GENECONV (Padidam et al., 1999), MaxChi (Maynard Smith, 1992), RDP (Martin et al., 2000), and SiScan (Gibbs et al., 2000). All recombination events were confirmed by sequence and phylogenetic analysis. The nucleotide similarity between the two parents and the recombinant sequence variants was calculated using the Kimura 2-parameter model in SimPlot v3.5.1 (Lole et al., 1999) and plotted. Phylogenetic trees were constructed using sequences which flank the predicted break point(s), as described in Section 2.2.5.
2.3 Results

2.3.1 Initial RT-PCR screening for GLRaV-3

All 55 DAS-ELISA GLRaV-3 positive and/or visually symptomatic plants tested GLRaV-3 positive by RT-PCR using either the NYSA-14109F/NYSA-14636R (33) or NZ-10875F/NZ-11482R (41) primer sets, but only 19 tested positive using both primer sets (Table 2.3). Initially, nine samples tested negative using both primer sets, however retests of these samples following dilution of the total RNA in water (at a 1:10, 1:100, and 1:1000 ratio) resulted in positive outcomes (example for two samples are shown in Figure 2.1). The average absorbance $A_{260}:A_{280}$ ratio for these RNA samples with poor amplification was less than 1.60. All samples were further analysed using SSCP.

Table 2.3: Detection of GLRaV-3 in grapevine samples using the NYSA-14109F/NYSA-14636R and NZ-10875F/NZ-11482R primer sets.

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<sup>a</sup> Sample positive for GLRaV-3 using the NYSA-14109F/NYSA-14636R primer set.

<sup>b</sup> Sample positive for GLRaV-3 using the NZ-10875F/NZ-11482R primer set.
Figure 2.1: Detection of Grapevine leafroll-associated virus 3 from undiluted (lanes 1 and 5) and diluted total RNA extracts (lanes 2 – 4 and 6 – 8) using the NYSA-14109F/NYSA-14636R primer set. Lanes 1 and 5 used undiluted total RNA extracts from the TK00041 and TK00049 cane samples as RT-PCR template, respectively. Lanes 2, 3, and 4 used total RNA extracts of TK00041 as RT-PCR template that were diluted in water at a 1:10, 1:100, and 1:1000 ratio, respectively. Lanes 6, 7, and 8 used total RNA extracts of TK00049 as RT-PCR template that were diluted in water at a 1:10, 1:100, and 1:1000 ratio, respectively. Lane 9 is the positive control and lane 10 is the no template control. L = 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

2.3.2 SSCP analysis

2.3.2.1 SSCP analysis of NYSA-14109F/NYSA-14636R primers generated PCR products

The “simple” and “complex” SSCP banding patterns were used to identify samples with single and mixed infections of GLRaV-3 variants, respectively, and samples with potentially different genetic GLRaV-3 variants (Section 2.2.4). Of the 33 NYSA-14109F/NYSA-14636R RT-PCR products, 16 showed single infection banding, which comprised of three different patterns (P1, P2, and P3) (Figure 2.2a), while 17 showed mixed infection banding profiles with multiple bands per sample (Figure 2.2b).

![SSCP profiles](image)

Figure 2.2: Single stranded conformation polymorphism (SSCP) profiles generated from samples with (a) single and (b) mixed infections of Grapevine leafroll-associated virus 3 using the NYSA-14109F/NYSA-14636R primer set. (a) Three simple SSCP profiles representing samples with single infections of sequence variants that have high nucleotide identity with variants from group 1 (P1), group 2 (P2), and group 5 (P3). (b) Lanes 1 to 5 represent the five common complex SSCP profiles generated from the NYSA-14109F/NYSA-14636R primer set.

Thirteen NYSA-14109F/NYSA-14636R generated PCR products, consisting of five simple and eight complex SSCP banding profiles, were further analysed by cloning and sequencing. All clones from samples with simple band profiles had the same banding pattern as the original RT-PCR product (Figure 2.3a). In contrast, clones from samples with complex banding profiles had various simple banding profiles...
(Figure 2.3b). In most cases, the simple band profiles matched bands present in the original complex banding profile (Figure 2.3b). Sequences from the different SSCP profiles were obtained and analysed (Section 2.3.3.1).

![SSCP profiles](image1)

**Figure 2.3:** Single stranded conformation polymorphism (SSCP) profiles of seven clones from a sample with (a) single and (b) mixed infection of *Grapevine leafroll-associated virus 3* using the NYSA-14109F/NYSA-14636R primer set. Lanes O represents the original SSCP profile.

### 2.3.2.2 SSCP analysis of NZ-10875F/NZ-11482R primers generated PCR products

Thirteen out of the 41 NZ-10875F/NZ-11482R positive samples showed single infection, consisting of two different patterns (P4 and P5) (Figure 2.4a). The remaining 28 samples exhibited complex banding profiles (Figure 2.4b).

![SSCP profiles](image2)

**Figure 2.4:** Single stranded conformation polymorphism (SSCP) profiles generated from samples with (a) single and (b) mixed infections of *Grapevine leafroll-associated virus 3* using the NZ-10875F/NZ-11482R primer set. (a) Two simple SSCP profiles representing samples with single infections of sequence variants that have high nucleotide identity with NZ-1 (P4) or have low nucleotide identity with all other GLRaV-3 isolates (P5). (b) Lanes 1 to 3 represents the three common complex SSCP profiles generated from the NZ-10875F/NZ-11482R primer set.

Twelve samples that consisted of simple (5) and complex (7) SSCP banding profiles were further analysed by cloning and sequencing. Similar to the NYSA-14109F/NYSA-14636R results, clones from singly infected samples yielded clones with the same banding pattern as the original RT-PCR product (Figure 2.5a), whereas clones from the mixed infected samples yielded a range of simple banding profiles that corresponded to bands from the original complex profile (Figure 2.5b).
Figure 2.5: Single stranded conformation polymorphism (SSCP) profiles of five clones from a sample with (a) single and (b) mix infection of *Grapevine leafroll-associated virus 3* using the NZ-10875F/NZ-11482R primer set. Lanes O represents the original SSCP profile.

2.3.3 Phylogenetic analysis of SSCP partial sequence

2.3.3.1 Analysis of partial sequences from NYSA-14109F/NYSA-14636R PCR products

The P1, P2 and P3 SSCP profiles (Figure 2.2a) represent three different GLRaV-3 phylogenetic groups, their sequences (at least four clones per profile) having 98 to 99% nucleotide identity to the NY1 (group 1 variant), GP18 (group 2 variant), and CI-871 (group 5 variant) GLRaV-3 isolates, respectively. In addition, sequence from clones showing P1, P2 and P3 SSCP profiles, for example samples NZ-PG1 (P1 SSCP profile), NZ-R2D4 (P2 SSCP profile), and NZ-WCA (P3 SSCP profile), clustered in groups 1, 2, and 5 respectively (Figure 2.6).

Sequence from clones derived from complex SSCP profiles identified both mixed infections of minor sequence variants from a single phylogenetic group, and mixed infections of sequence variants from different phylogenetic groups. For instance, all clones of the TK00004 sample cluster with group 1 (Figure 2.6) with an average nucleotide difference of 0.57% between clones. Similarly, all clones from CS341 and TK00113 samples cluster in group 2 and only differed by a maximum of 0.55%. In contrast, sequences from TK00204 represented both group 1 and group 2 (Figure 2.6) with an average nucleotide difference between clones of 3.42%, while clones from WG3.17 represented GLRaV-3 phylogenetic groups 1, 2 and 3 (Figure 2.6).

In addition to identifying sequence variants that clustered with existing phylogenetic groupings, ten sequence variants identified from TK00080, TK00134, and WG3.17 did not clearly fit the current classification. These sequence variants clustered into three minor clades (a – c) (Figure 2.6), and are most closely related...
to variants from groups 3 (PL-20) and 4 (Terrantez da Terceira-2), with an overall average nucleotide identity of 97.30% and 94.91% compared to 88.39 to 93.90% with isolates from groups 1, 2, and 5 (Table 2.4). The genetic variability between the minor clades also differs, as the average nucleotide difference between clades a and b is 4.95%, clades a and c is 4.08%, and between clades b and c is 1.61%.

Table 2.4: Nucleotide identities of ten sequence variants identified from TK00080, TK00134, and WG3.17 compared to GLRaV-3 isolates from phylogenetic groups 1 to 5.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade a</td>
<td>TK00080-A</td>
<td>94.06</td>
<td>89.55</td>
<td>97.54</td>
<td>94.06</td>
</tr>
<tr>
<td></td>
<td>TK00080-C</td>
<td>94.67</td>
<td>89.55</td>
<td>97.34</td>
<td>93.85</td>
</tr>
<tr>
<td></td>
<td>Clade average</td>
<td>94.36</td>
<td>89.55</td>
<td>97.44</td>
<td>93.95</td>
</tr>
<tr>
<td>Clade b</td>
<td>TK00080-J</td>
<td>94.26</td>
<td>89.75</td>
<td>96.93</td>
<td>95.90</td>
</tr>
<tr>
<td></td>
<td>WG3.17-J</td>
<td>94.06</td>
<td>89.55</td>
<td>96.72</td>
<td>95.70</td>
</tr>
<tr>
<td></td>
<td>WG3.17-O</td>
<td>94.06</td>
<td>89.55</td>
<td>96.72</td>
<td>95.70</td>
</tr>
<tr>
<td></td>
<td>Clade average</td>
<td>94.13</td>
<td>89.62</td>
<td>96.79</td>
<td>95.77</td>
</tr>
<tr>
<td>Clade c</td>
<td>TK00080-H</td>
<td>93.44</td>
<td>88.93</td>
<td>97.75</td>
<td>94.88</td>
</tr>
<tr>
<td></td>
<td>TK00080-T</td>
<td>93.65</td>
<td>89.55</td>
<td>97.54</td>
<td>95.49</td>
</tr>
<tr>
<td></td>
<td>TK00134-A</td>
<td>92.62</td>
<td>89.34</td>
<td>98.16</td>
<td>95.49</td>
</tr>
<tr>
<td></td>
<td>WG3.17-G</td>
<td>93.24</td>
<td>88.73</td>
<td>97.54</td>
<td>94.67</td>
</tr>
<tr>
<td></td>
<td>WG3.17-L</td>
<td>93.03</td>
<td>88.52</td>
<td>97.34</td>
<td>94.47</td>
</tr>
<tr>
<td></td>
<td>Clade average</td>
<td>93.20</td>
<td>89.02</td>
<td>97.66</td>
<td>95.00</td>
</tr>
<tr>
<td>Overall average</td>
<td>93.90</td>
<td>89.39</td>
<td>97.30</td>
<td>94.91</td>
<td>92.54</td>
</tr>
</tbody>
</table>

Based on the nucleotide identity with group 1 representative NY1 (AF037268).

Based on the nucleotide identity with group 2 representative GP18 (EU259806).

Based on the nucleotide identity with group 3 representative PL-20 (GQ352633).

Based on the nucleotide identity with group 4 representative Terrantez da Terceira-2 (HQ401015).

Based on the nucleotide identity with group 5 representative CI-817 (EU344894).
Figure 2.6: Phylogenetic analysis of Grapevine leafroll-associated virus 3 isolates from this study (highlighted with red squares) and GenBank, based on a 488nt region within open reading frame 6, conducted in MEGA5 (Tamura et al, 2011). Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method was used to compute evolutionary distances. The percentages of bootstrap support (>75%) from 1,000 replicates are shown at nodes. The phylogenetic groups 1 – 5 are proposed by Gouveia et al (2011), and clades a – c consist of isolates that do not fit well with the proposed groupings, labelled with grey vertical bars. The scale represents 0.02 nucleotide substitutions per site.
2.3.3.2 Analysis of partial sequences from NZ-10875F/NZ-11482R PCR products

Based on the 564nt region of ORF4, sequences from the P4 and P5 SSCP profiles formed two separate clades, clustering separately from groups 1, 2, 3 and 5. Sequences from clones of samples with simple SSCP P4 and P5 profiles had low nucleotide identity compared to NY1 with 78.15 and 77.09%, respectively. P4 clones were 97 to 99% identical to the NZ-1 isolate (EF508151), the sequence from which the NZ-10875F/NZ-11482R primer pair were designed. Sequences with the P4 SSCP profile and the NZ-1 variant branched away from GLRaV-3 isolates, GH11 and GH30 (100% confidence), which resulted in the formation of two distinct clades within phylogenetic group 6, proposed by Bester et al. (2012b) (Figure 2.7). Sequences from the P5 SSCP profile are positioned separately from all other sequences (100% confidence) (Figure 2.7). They are most closely related to NZ-1 but with only 78.33% nucleotide identity, thus potentially forming a new seventh phylogenetic group. At least four clones per SSCP profile from at least two different plant samples were sequenced and analysed. Group 4 was not included in this analysis as there is currently no sequence data for this group in this region.

NZ-10875F/NZ-11482R primer pair did not detect variants from phylogenetic groups 1 to 5, only identifying variants with high identity to either the P4 or P5 SSCP profile sequences. Mixed infections contained either sequence variants with high identity to the P4 and/or P5 SSCP profile sequences (Figure 2.7). For example, sequence variants identified from TK00080 and WG3.9 all cluster with the P4 sequence variants and the NZ-1 isolate, with an average nucleotide difference of approximately 0.74 and 0.85% respectively, between clones that generated different SSCP profiles. Whereas the average nucleotide difference between clones with different SSCP profiles identified in TK00204 and WG3.6 was 12.73 and 15.42%, respectively, and variants clustered with both the P4 and P5 clades.
2.3.4 Recombination analysis

Based on analysis using RDP v3.44 (Martin et al., 2010) no recombination events were identified in the partial sequence alignment of ORF4, generated by the NZ-10875F/NZ-11482R primer pair. However, recombination events were revealed in
the NYSA-14109F/NYSA-14636R sequence dataset (488nt region in ORF6), with 11 of the GLRaV-3 sequence variants identified in this project containing recombination between variants from different phylogenetic groups (Table 2.5).

### Table 2.5: Recombination events identified for the NYSA-14109F/NYSA-14636R data set using RDP v3.44.

<table>
<thead>
<tr>
<th>Sequence variant</th>
<th>Break point</th>
<th>Parent isolates</th>
<th>Model</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK00080-A</td>
<td>380</td>
<td>Cl-765 (Group 1)</td>
<td>WG3.17-M (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>TK00080-C</td>
<td>358</td>
<td>Cl-765 (Group 1)</td>
<td>WG3.17-M (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>TK00080-H</td>
<td>121</td>
<td>621 (Group 1)</td>
<td>8-19 (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>TK00080-J</td>
<td>213</td>
<td>Cl-664 (Group 1)</td>
<td>LN (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>TK00080-T</td>
<td>162</td>
<td>621 (Group 1)</td>
<td>PL-20 (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td>TK00167-J</td>
<td>295</td>
<td>WG3.17-M (Group 3)</td>
<td>IS2 (Group 2)</td>
<td>MaxChi</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>TK00204-G</td>
<td>70 / 293</td>
<td>TK00004-H</td>
<td>GQ-10 (Group 2)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Group 1)</td>
<td>8-19 (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>WG3.17-G</td>
<td>121</td>
<td>621 (Group 1)</td>
<td>8-19 (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>WG3.17-L</td>
<td>121</td>
<td>621 (Group 1)</td>
<td>8-19 (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>3Seq</td>
</tr>
<tr>
<td>WG3.17-J</td>
<td>213</td>
<td>Cl-664 (Group 1)</td>
<td>LN (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
<tr>
<td>WG3.17-O</td>
<td>213</td>
<td>Cl-664 (Group 1)</td>
<td>LN (Group 3)</td>
<td>Siscan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3Seq</td>
</tr>
</tbody>
</table>

*a The parental isolates detected by the RDP v3.44 program are not the actual parent isolate but are the sequences most similar to the actual parents in the analysed dataset.*
The analysis proposed that the TK00204-G variant originated from a recombination event between a major group 1 parent (TK00004-H) and the minor group 2 parent (GQ10-12) at nucleotides 70 and 293 (Figure 2.8). Although, this was only detected by the SiScan method with a p-value of 1.12x10^{-05}, the recombination event was confirmed using SimPlot (Lole et al., 1999) (Figure 2.8b). Additionally, four recombination methods (MaxChi, Chimaera, SiScan, and 3Seq) detected a recombination break point between WG3.17-M (Group 3) and IS2 (group 2) at nucleotide 295 to produce the TK00167-J variant with relatively high confidence, p-values ranged between 1.16x10^{-05} and 3.20x10^{-09} (Table 2.5). From nucleotide 1 to 295, the TK00167-J variant has higher nucleotide identity to the group 3 variant WG3.17-M (96.61%) compared to the group 2 variant IS2 (87.80%), while the remaining sequence from nucleotide 296 to 488 has higher nucleotide identity to IS2 (98.96%) than WG3.17-M (86.01%) (Figure 2.9).

**Figure 2.8:** Nucleotide sequence comparisons between the two parent (TK00004-H and GQ10-12) and recombinant (TK00204-G) isolates by (a) sequence alignment and (b) similarity plot. (a) Sequence alignment generated in Geneious v5.5 (Drummond et al., 2011) with nucleotide disagreements highlighted in colour. The vertical black lines mark the predicted recombination break points between TK00004-H (group 1 variant) and GQ10-12 (group 2 variant) at nucleotide 70 and 293. (b) Similarity plot comparing TK00204-G (recombinant) to TK00004-H and GQ10-12 (parent), which used the Kimura 2-parameter model, window size of 20nt, step size of 10nt, and 1000 bootstraps, generated in SimPlot (Lole et al, 1999). Red arrows mark the recombination break points.
Figure 2.9: Phylogenetic analysis of the recombinant *Grapevine leafroll-associated virus 3* isolate, TK00167-J, based on the sequence (a) before (295nt) and (b) after (193nt) the predicted recombination break point between parent isolates IS2 (group 2) and WG3.17-M (group 3), conducted in MEGA5 (Tamura et al., 2011). (a and b) Recombinant and parent isolates are highlighted with a black triangle and circle, respectively. Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method was used to compute evolutionary distances. The percentages of bootstrap support (≥75%) from 1,000 replicates are shown at nodes. The phylogenetic groups 1 – 5 are proposed by Gouveia et al (2011). The scale represents 0.02 nucleotide substitutions per site.

All ten sequence variants that compose the three minor clades a, b, and c, described in Section 2.3.3.1, except the TK00134-A variant, were determined to originate from a recombination event between a group 1 and group 3 variant, although the predicted parents and position of the break point differs between the clades (Table 2.5). For example, the point of recombination for variants from clade b was predicted at nucleotide 213, whereas break points at nucleotide 121 and 162 were predicted for variants from clade c (refer to Appendix 2 for the phylogenetic trees and additional information about the sequence variants from minor clades a, b, and c).

All recombination events identified by RDP v3.44 (Martin et al., 2010) were verified by conducting further phylogenetic analysis (Figures 2.9 and Appendix 2 Figures A2.1 – A2.5) and sequence comparisons using SimPlot v3.5.1 (Lole et al., 1999) (refer to Figure 2.8b for an example of an output from SimPlot). In addition, the recombination analysis used sequence derived from a single amplified PCR fragment (i.e. fragment assembly was not conducted in this data set) and thus recombination events identified from this analysis are less likely an experimental artefact or chimera.
2.4 Discussion

Investigating the genetic variability of a virus population aids our understanding of the biology, epidemiology, and evolution of a virus. In addition, it ensures diagnostic molecular-assays remain accurate and reliable, which is important for disease management programmes. The genetic diversity of GLRaV-3 from a number of other countries has been studied (Fuchs et al., 2009; Gouveia et al., 2011; Jooste et al., 2005; Jooste et al., 2010; Pong, 2009; Sharma et al., 2011; Turturo et al., 2005; Wang et al., 2011), however at the start of this project, limited sequence data was available of New Zealand GLRaV-3 variants. To investigate the genetic diversity of New Zealand GLRaV-3 isolates, the cost-effective and simple molecular-based SSCP technique was used in combination with sequencing, successfully identifying a number of different sequence variants that belong to different phylogenetic groups.

2.4.1 Implications of RNA quality on successful RT-PCR amplification

The success of molecular-based techniques such as SSCP is dependent on the successful amplification of RT-PCR products. The quality of RNA isolated for the molecular assay is an important factor that can impede amplification. Poor RNA extracts with a high level of RT-PCR inhibitors generally repress reverse transcription and/or DNA amplification by directly interacting with the nucleic acid template or interfering and blocking the enzymatic activity of the reverse transcriptase and/or DNA polymerase (Rådström et al., 2008). Furthermore, contaminants that reduce the availability of magnesium ions required to bind to the DNA polymerase can also inhibit PCR (Rådström et al., 2008).

Woody plant materials including grapevine material are high in plant secondary metabolites such as phenolic and polysaccharide compounds (Gambino et al., 2008; Newbury et al., 1977). These compounds oxidise and bind to nucleic acids, which results in unusable RNA for downstream applications such as conventional and real-time RT-PCR (Newbury et al., 1977; Salzman et al., 1999). Furthermore, residual salts, ionic detergents, ethanol, and phenol, from the total RNA extraction process can also inhibit the successful amplification of RT-PCR products (Rådström et al., 2008; Roux, 2009).

In this project, RT-PCR inhibition was observed in the initial RT-PCR screening for GLRaV-3, with only 46 out of the 55 ELISA and/or symptomatic GLRaV-3 positive
samples, yielding RT-PCR products. Inhibition was most likely caused by high levels of contaminants, as the average spectrometric absorbance ratio $A_{260}:A_{280}$ for these nine “failed” samples was less than 1.6, which is indicative of protein contamination (Manchester, 1996). Successful amplification was only achieved following the dilution of total RNA extracts in water at a 1:10 ratio, prior to RT-PCR. Dilution of total RNA extracts is a standard approach to reduce the concentration and effects of inhibitor molecules, allowing for amplification from nucleic acid. Further work to determine the best suited total RNA extraction protocol for further experiments was conducted and is presented in Chapter 4.

2.4.2 Application of SSCP to detect GLRaV-3 sequence variants

SSCP is a simple and cost-effective tool that was successfully applied in this project to investigate the genetic diversity of GLRaV-3 in New Zealand. Two different primer sets, NYSA-14109F/NYSA-14636R and NZ-10875F/NZ-11482R, that targeted ORF 6 and 4 respectively, were used to generate RT-PCR products suitable for SSCP analysis. Distinctive SSCP profiles, P1 to P5, generated from singly infected samples, were correlated to specific phylogenetic groups 1, 2, 5, 6 (defined by Gouveia et al. (2011) and Bester et al. (2012b)), and a potentially new seventh GLRaV-3 phylogenetic group containing variants represented by the P5 SSCP profile. All distinctive SSCP profiles were confirmed by sequence analysis of at least four different clones from at least two different samples per profile. The proposed new group 7 is further analysed and discussed in Chapter 3.

The identification of a number of different GLRaV-3 variants, highlights a key feature of SSCP, as an easy tool to identify and differentiate sequence variants. This is in accordance with other studies by Jooste et al. (2005), Pong (2009), Jooste et al. (2010), and Gouveia et al. (2011). Based on an ORF5 targeting primer set, H450/C629, Jooste et al. (2005) and Pong (2009) identified two GLRaV-3 sequence variants from phylogenetic groups 1 and 2 based on distinctive SSCP profiles. A subsequent study by Jooste et al. (2010), using the same ORF5 targeting primer set, identified an additional sequence variant for a third phylogenetic group (group 3). Furthermore, Gouveia et al. (2011), assigned five distinct SSCP profiles to five phylogenetic groups, groups 1 to 5, based on sequence from ORF6 (encodes the CP).
In this study, samples with mixed infections were also identified, based on the initial RT-PCR screening and the generation of complex SSCP profiles. The NYSA-14109F/NYSA-14636R and NZ-10875F/NZ-11482R primer pairs used for the RT-PCR screening detected different GLRaV-3 groups. The NYSA-14109F/NYSA-14636R primer pair detected variants from phylogenetic groups 1, 2, 3 or 5 but not variants that formed the P4 and P5 SSCP profiles. In contrast, the NZ-10875F/NZ-11482R primer pair only detected variants that formed the P4 and P5 SSCP profiles but not variants from phylogenetic groups 1, 2, 3 or 5. Therefore, the 19 out of the 55 samples that tested positive for GLRaV-3 using both NYSA-14109F/NYSA-14636R and NZ-10875F/NZ-11482R primer sets, indicates these samples contained at least two different sequence variants. For instance, the TK00080, TK00204, and WG3.17 samples tested positive using both primer sets and all samples contained sequence variants from at least one of the five common phylogenetic groups (groups 1 to 5) plus variants similar to NZ-1 (phylogenetic group 6) and/or variants that clustered within group 7 (variants with P5 SSCP profile). In addition, similar to findings by Turturo et al. (2005) and Jooste et al. (2010), samples generating complex SSCP profiles represented samples with either mixed infections of sequence variants that clustered within the same phylogenetic group or mixed infections of sequence variants that clustered in different phylogenetic groups.

Even though no sequence variants from group 4 were identified during this study, and distinct SSCP profiles could not be assigned for groups 3 and 4, the sequence variants that were identified based on distinct SSCP profiles, had greater inter-variant variation compared to previous studies. This is apparent with the identification of sequence variants from three out of the five common phylogenetic groups (Gouveia et al, 2011) (SSCP profiles P1 to P3), and sequence variants that are more than 20% different to groups 1 to 5 (SSCP profiles P4 and P5). Furthermore, greater genetic variation within a single plant sample compared to other studies at the time, was also observed. At the time of the analysis for this current study, a genetic diversity of 6.35% between clones sequenced within a single mixed infected sample had been reported by Turturo et al. (2005), based on 546nt region of ORF4. Jooste et al. (2010) identified samples with dual infections of either variants from groups 1 and 3 or groups 1 and 2 (variants differed by <10%, based on the CP nucleotide sequence). In contrast, in this study, several mixed infected samples contained sequence variants that clustered with three
different phylogenetic groups, and variants with high identity to divergent variants from group 6 and NZ2. Some mixed infections contained variants that differed by more than 20%, based on the CP nucleotide sequence.

The differences between studies, in particular the higher GLRaV-3 genetic diversity detected between and within grapevine samples in this study, is potentially due to the type of grapevine collection(s) used. Twenty of the samples in this study originated from the old Te Kauwhata grapevine repository, some of which were imported into New Zealand in the early 1900s (Dick et al., 1989b). The long life of perennial plants such as grapevines and the spread of GLRaV-3 by mealybug vectors, in combination with the high mutation rate of RNA viruses, can lead to the extensive generation and accumulation of sequence variants within a single perennial host, resulting in a highly diverse but closely related GLRaV-3 population (Roossinck, 1997; Rubio et al., 2001). The vines from the Auckland collection were originally imported from different sources, and have been transferred from a number of different sites in New Zealand before residing in the current vineyard plot, increasing the chances for multiple virus infections via mealybug transmission.

The primer pair(s) used to generate RT-PCR products for SSCP analysis may also contribute to differences in sequence variants detected. Polymorphism(s) in the primer binding regions can prevent primer binding to the template which, in-turn, prevents amplification and identification of some sequence variants. The NYSA-14109F/NYSA-14636R primer pair was designed against GLRaV-3 isolates from groups 1, 2, and 5, while the primers used by Turturo et al. (2005), Pong (2009), Jooste et al. (2010), Gouveia et al. (2011) were all designed against the NY1 isolate from group 1. In contrast, the NZ-10875F/NZ-11482R primer pair was designed against the NZ-1 isolate and detected sequence variants generating P4 and P5 SSCP profiles, from groups 6 and 7, but did not amplify variants from groups 1 to 5. Three samples from the Pong (2009) study, MT L2-2, MT L2-3, and GV-B4, were re-tested in this study. In addition to the detection of sequence variants from groups 1 and 2, as detected by Pong (2009), sequence variants with high identity to NZ-1 were also detected in all three samples, based on SSCP profiles (generation of P4 SSCP profile) and sequence analysis. This highlights the importance of the primer pair used in molecular-based tools such as SSCP, as a key determinant to the effectiveness of the tool.
Recombination

The three main contributors to virus evolution are mutation, recombination, and reassortment (Domingo et al., 1997; Roossinck, 1997). Recombination events have been detected in a number of different plant viruses including GLRaV-1 (Alabi et al., 2011), GFLV (Sokhandan-Bashir et al., 2012), CTV (Rubio et al., 2001), and Tomato black ring virus (Le Gall et al., 1995). It has been suggested that recombination is advantageous to RNA viruses as it potentially restores/corrects the functional integrity of genes that have been altered by deleterious mutations, caused by the high mutation rates of RNA viruses (Roossinck, 1997; Rubio et al., 2001; Simon et al., 1994). In addition, the exchange of genes between related viruses in mixed infections leads to greater diversity and potential variants with greater fitness and adaptability to new environments (Roossinck, 1997; Rubio et al., 2001).

In this study, recombination events between different phylogenetic groups of GLRaV-3 were identified within ORF6 (CP), which generally resulted in recombinants that clustered poorly with the current phylogenetic groups. Similarly, recombination between GLRaV-3 variants was also reported by Turturo et al. (2005) within ORF4 (HSP70h) and 6 (CP). However, recombination was not detected in the 564nt region of ORF4 analysed in this study or the studies by Gouveia et al. (2011), Wang et al. (2011), and Sharma et al. (2011).

Recombination events may not have been detected in the ORF4 of this study as a different region from that of Turturo et al. (2005) was analysed. The region examined in this work was closer to the start of ORF, between nucleotides 10,318 to 10,881 (based on NY1 numbering), compared to a mid-ORF region between nucleotides 10,980 to 11,524 (based on NY1 numbering) examined by Turturo et al. (2005). Therefore, recombination between GLRaV-3 variants may not occur near the start of ORF4. Additionally, recombination events may have been overlooked due to limited sequence data for ORF4, particularly sequence data for the phylogenetic groups 6 and 7, formed by the P4 and P5 SSCP profile sequences.

The age of the vines used in each study may account for the varied detection of recombination in ORF6, as the probability of recombination occurring in older plants, with elevated levels of virus genetic variability (as discussed above), are
higher than younger vines with new infections. Recombinants detected from this study were all from symptomatic vines that were at least ten years old. The other studies did not report the age of the vines sampled.

In the TK00080, TK00167, and WG3.17 samples that contained recombinant sequence variants, only one of the parental isolates was identified. For instance, recombination events between a group 1-like and a group 3-like variant were detected in TK00080 clones TK00080-A, TK00080-C, TK00080-H, TK00080-J, and TK00080-T. However, of the two parental variants, only group 1 sequence variants were detected in the TK00080 sample. The failure to detect a potential parent variant could be due to recombinant variants being transmitted from another source, uneven virus distribution, low virus titre, different RT-PCR amplification efficiencies for different variants, or sequencing only a limited number of clones.

The uneven distribution of GLRaV-3 variants from different phylogenetic groups within a vine can lead to differences in the GLRaV-3 variants detected by RT-PCR and SSCP analysis, depending on the vine area sampled and tested. Uneven distribution of different strains within a host has been reported for CTV in citrus (D'Urso et al., 2000), ASGV in apple (Magome et al., 1999), and PPV in peach (Jridi et al., 2006). Studies by Monis et al. (1996) and Tsai et al. (2012) have shown GLRaV-3 is generally unevenly distributed throughout a vine. However, there are currently no published reports comparing the spatial distribution of specific GLRaV-3 variants within a vine. Spatial distribution of GLRaV-3 variants from group 1, group 6, and NZ2 within a vine was investigated and is discussed further in Chapter 5.

While the combined approach of RT-PCR, SSCP, cloning, and sequencing, has led to the detection of an increasing range of GLRaV-3 variants there is the possibility of some sequence variants being overlooked due to an unintentional bias towards higher titre sequence variants and sequence variants with superior RT-PCR efficiencies (Gouveia et al., 2011; Nolasco et al., 2009). For example, low titre sequence variants and variants that may have slight polymorphisms in the primer binding region, which reduces RT-PCR efficiency, are likely to generate less RT-PCR product compared to other sequence variants. This in-turn can result in fewer clones of this sequence variant, and reduces the probability for this particular variant to be selected for sequencing. In this study, in order to detect
less prominent variants, 20 white colonies for each sample were selected and screened for sequencing. Increasing the number of colonies to be screened and sequenced, would further improve the probability of identifying the less-represented sequence variants, however this would have been labour-intensive and more expensive. In addition, when testing samples with mixed infections, there can be added difficulties differentiating between variants with similar SSCP patterns that can lead to sequence variants being overlooked (Urzi et al., 2003).

### 2.4.4 Conclusions

This chapter describes a high-level of genetic variability within the New Zealand GLRaV-3 population. GLRaV-3 variants with high identity to isolates from groups 1, 2, 3, 5, and 6 (specifically NZ-1), as well as some variants that had low nucleotide identity to all previously known GLRaV-3 variants worldwide, were identified. Recombinant GLRaV-3 variants that do not readily fit into current phylogenetic groupings were also identified.

The work from this chapter also highlighted the advantages and limitations of using the combined approach of RT-PCR, SSCP, and sequencing to investigate virus genetic diversity. Factors such as RNA quality, primer design, and low virus titre and/or uneven distribution of particular sequence variants can influence the effectiveness of RT-PCR, and in-turn the virus variants identified by SSCP and sequencing. Regardless of these limitations, this approach was successfully used in this genetic diversity study of New Zealand GLRaV-3 isolates by identifying (i) samples with single and mixed infection based on SSCP profiles, and (ii) a high-level of GLRaV-3 genetic diversity between and within New Zealand grapevine samples. In addition, similar to other studies, SSCP analysis was effectively used as a rapid tool to identify sequence variants belonging to different phylogenetic groups, particularly in samples infected with a single variant.
Chapter 3:

Molecular characterisation and genome analysis of five New Zealand GLRaV-3 isolates

3.1 Introduction

Genetic diversity studies of a virus population aids our understanding of phylogenetic relationships, population structures, and underlying evolutionary mechanisms (Chapter 2). Analysing longer sequences can be more informative than shorter partial gene sequences (Chapter 2), improving our understanding of virus genetic variation and evolution. For instance, the genome organisation can be determined (an important taxonomic criterion), the uniformity of the genetic variation along the virus genome can be studied, and the amino acid sequences can be deduced for complete protein sequences that can help predict differences that are of possible biological importance. In addition, longer sequences aid the design and development of molecular assays.

At the start of this project, there were three complete GLRaV-3 genomes available that were from groups 1 (2) and 2 (1), and only three other sequences from China, Spain, and New Zealand that were greater than 1000nt. Since then, the majority of genetic variability studies have continued to analyse short fragments (<1000nt), except for a recent study by Wang et al. (2011), which is based on 4,711nt sequences encompassing 3’terminal ORFs 6 to 12 from 50 isolates that are representative of groups 1 (21), 2 (12), 3 (13), and 4 (4). At the time of analysis for this project, there were nine complete GLRaV-3 genomes representative of phylogenetic groups 1 (4), 2 (2), 3 (1), and 6 (2) (Bester et al., 2012b; Engel et al., 2008; Jarugula et al., 2010; Jooste et al., 2010; Ling et al., 2004; Maree et al., 2008). Thus, current sequences unevenly represent GLRaV-3 phylogenetic groups, especially group 5 (with no sequences greater than 1000nt currently available for this group) and the divergent isolates that differ from other GLRaV-3 isolates from phylogenetic groups 1 to 5 by more than 20%.

The overall objective of the work described in this chapter was to improve the molecular understanding of GLRaV-3, particularly GLRaV-3 isolates found in New Zealand. This was achieved by further characterising five New Zealand GLRaV-3
isolates that are representative of different phylogenetic groups, based on the initial SSCP and sequencing (Chapter 2), using a genome walking strategy. Furthermore, sequence data was examined to (i) determine how the New Zealand isolates fit within the current molecular understanding of GLRaV-3, (ii) provide additional sequence information to aid the design of molecular assays (Chapter 4), and, (iii) investigate the potential biological implications the genetic diversity has on aspects such as resultant protein and regulatory elements. Thus, this chapter describes the characterisation of three complete GLRaV-3 genomes that are representative of phylogenetic groups 1, 2, and 5, and partial genomes of two further isolates, NZ1-B and NZ2. In addition, sequence analyses identified several features that are of possible biological significance to the virus functionality.
3.2 Materials and methods

3.2.1 Virus source and RNA extraction

Based on the SSCP and sequence analysis (Chapter 2), five samples with single infections of GLRaV-3 isolates that clustered with different phylogenetic groups were selected for further genome characterisation. The material of the five plant samples, which comprises various cultivars, are denoted as PG1, R2D4, WCA, TK05372, and HB-PN6 were sourced from various North Island vineyards (Chapter 2 Table 2.1). These samples represent five GLRaV-3 isolates assigned the following names; NZ-PG1, NZ-R2D4, NZ-WCA, NZ1-B, and NZ2 respectively.

Total RNA was isolated from leaf and cane material using the Spectrum™ Plant RNA extraction kit as described in Chapter 2 Section 2.2.2 and the modified cetyl trimethylammonium bromide (CTAB; IUPAC: hexadecyl-trimethyl-ammonium bromide) protocol, as described by White et al. (2008) with minor modifications (Chapter 4 Section 4.2.2.3).

3.2.2 Primer design

The primers for the characterisation of isolates NZ-PG1, NZ-R2D4, and NZ-WCA were designed using the Primer3 program (Rozen et al., 2000) based on the multiple complete genome sequence alignment of NY1 (AF037268), GP18 (EU259806), and CI-766 (EU3444893) generated by Geneious v5.5 (Drummond et al., 2011) and ClustalX v2.0 (Larkin et al., 2007). Refer to Chapter 2 Section 2.2.3.2.1 for the primer design criteria. In total 18 primer sets were designed to generate amplicons sized between 855 to 1,891nt to characterise isolates NZ-PG1 and NZ-R2D4 (Figure 3.1 and Table 3.1), while 13 primer sets generating amplicons sized between 842 to 3,635nt were used to characterise NZ-WCA (Figure 3.2 and Table 3.2). To increase the reliability of the assembled sequence, resulting amplicons were expected to overlap with each other by at least 100nt.

![Diagrammatic representation of the amplicons used to characterise Grapevine leafroll-associated virus 3 (GLRaV-3) isolates NZ-PG1 and NZ-R2D4. The GLRaV-3 genome organisation diagram is based on the GP18 isolate (EU259806). Amplicon abbreviations correspond to Table 3.1 and Table 3.5.](image_url)
<table>
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<tr>
<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
<th>Position</th>
<th>Amplicon size</th>
<th>Amplicon</th>
</tr>
</thead>
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<td>1,339</td>
<td>A</td>
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<td>18,498</td>
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<td></td>
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<td>B</td>
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<td>G</td>
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<td>10,018</td>
<td>1,891</td>
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<td>1,032</td>
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<td>NYSA-5684F</td>
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<td>NYSA-1941F</td>
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<td>NYSA-580F</td>
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<td>NYSA-1547R</td>
<td>CCGGACGATAGGGAGGAAAATCTA</td>
<td>1,547</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Expected primer binding nucleotide positions are based on the complete GP18 (EU259806) genome sequence.

*b* Amplicon abbreviations are correlated to Figure 3.1.
For the NZ1-B and NZ2 isolates, primers were designed to generate four amplicons (C-NZ1 to F-NZ1) and a single amplicon (E-NZ2) against the NZ-1 sequence (EF508151) using the web-based Primer3 program (Rozen et al., 2000). A genome walking strategy was then used to further extend sequence data towards the 3' terminal end which involved the use of a combination of NZ1-B or
NZ2 sequence specific forward and non-specific reverse binding primers. The specific forward primer was based on the sequence data obtained from this study with basic melting temperatures between 50 and 55°C, while the non-specific reverse primers were previously used for the characterisation of NZ-PG1, NZ-R2D4, and NZ-WCA, had a higher basic melting temperature of 57 to 60°C (see Table 3.3 and Table 3.4 for the primer combinations used).

In addition, to obtain further sequence data from NZ1-B towards the 5’ terminal end, a similar genome walking strategy was undertaken. A specific reverse primer designed against the NZ1-B sequence (amplicon G-NZ1) with a lowered basic melting temperature was used in conjunction with a non-specific forward primer designed against a sequence from a deep sequencing analysis by Coetzee et al. (2010).

The same primer design criteria were used as described in Chapter 2 Section 2.2.3.2.1, with the exception of the aforementioned basic melting temperature criterion. In total six and five primer sets for NZ1-B (Table 3.3 and Figure 3.3) and NZ2 (Table 3.4 and Figure 3.4), respectively, were designed to generate amplicons sized from 586 to 3,096nt. Amplicons were designed to overlap with each other by at least 100nt.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Positiona</th>
<th>Amplicon size (nt)</th>
<th>Ampliconb</th>
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<td>NZ1-15625F</td>
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<td>A-NZ1</td>
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<tr>
<td>NYSA-18498R</td>
<td>GACCTAACTTATGTGCAATAA</td>
<td>18,498</td>
<td>3,096</td>
<td>A-NZ2</td>
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<tr>
<td>NZ1-13260F</td>
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<td>2,675</td>
<td>B-NZ1</td>
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<tr>
<td>NYSA-15929R</td>
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<td>3,096</td>
<td>B-NZ2</td>
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<td>NZ1-12368R</td>
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<td>1,971</td>
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<td>NZ1-9322F</td>
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<td>NZ1-10674R</td>
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<td>F-NZ2</td>
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<td>1,553</td>
<td>G-NZ1</td>
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<td>NZ1-8339R</td>
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<td>1,553</td>
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</tr>
</tbody>
</table>

Table 3.3: Primer sets used to characterise the NZ1-B GLRaV-3 isolate.

*a Expected primer binding nucleotide positions are based on the complete GP18 (EU259806) genome sequence.

b Amplicon abbreviations are correlated to Figure 3.3.
**Figure 3.3:** Diagrammatic representation of the amplicons used to characterise *Grapevine leafroll-associated virus 3* (GLRaV-3) isolate NZ1-B. The GLRaV-3 genome organisation diagram is based on the GP18 isolate (EU259806). Amplicon abbreviations correspond to Table 3.3 and Table 3.5.

**Table 3.4:** Primer sets used to characterise the NZ2 GLRaV-3 isolate.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
<th>Position</th>
<th>Amplicon size (nt)</th>
<th>Amplicon&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>NYSA-18498R</td>
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<td>B-NZ2</td>
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<td>TAAGTCGGGAAGGATGATGTG</td>
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<tr>
<td>NYSA-15929R</td>
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<td>NZ2-11321F</td>
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</table>

<sup>a</sup> Expected primer binding nucleotide positions are based on the complete GP18 (EU259806) genome sequence.

<sup>b</sup> Amplicon abbreviations are correlated to Figure 3.4.

**Figure 3.4:** Diagrammatic representation of the amplicons used to characterise *Grapevine leafroll-associated virus 3* (GLRaV-3) isolate NZ2. The GLRaV-3 genome organisation diagram is based on the GP18 isolate (EU259806). Amplicon abbreviations correspond to Table 3.4 and Table 3.5.

### 3.2.3 RT-PCR

Amplicons less than 2kb were synthesised using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Invitrogen, Carlsbad, CA, U.S.A) in a 50µL reaction mixture with a final MgSO₄ concentration of 2.5mM, 400nM of forward and reverse primer, 1U of the Superscript™ III RTase/Platinum® Taq mix (5U/µL) and 100 to 300ng of total RNA extract. Thermocycling conditions involved reverse transcription for 30 minutes at 50°C followed by 94°C for 2 minutes and 30 cycles of 94°C for 10 seconds, annealing at...
55°C for 30 seconds and extension of 1 minute per kb at 68°C, with a final extension for 3 minutes at 68°C.

For the characterisation of NZ1-B and NZ2, to successfully amplify the correct target (reducing non-specific amplification) cDNA was synthesised using the same reaction mixture described above, however the annealing temperature for the thermocycling protocol was modified to accommodate the use of specific and non-specific primer pairs. To increase the binding prospects of the non-specific reverse primer to the NZ1-B and NZ2 isolates, the annealing temperature for the first ten amplification cycles was reduced to 50°C. The remaining 20 amplification cycles were conducted at 55°C, in an attempt to reduce non-specific amplification.

Amplicons larger than 2kb were synthesised using two-step RT-PCR. To synthesise the first-strand cDNA, 100 to 300ng of total RNA extract was added to a final concentration of 200nM reverse primer, 1mM of dNTP, and made up to 10µL with distilled water. This was then incubated at 65°C for 5 minutes and immediately chilled on ice for 2 minutes. To each PCR tube 6.5µL of the following mixture was then added; 4µL of 5x First-Strand Buffer (250mM Tris-HCl, 375mM KCl, 15mM MgCl₂), 1µL of 0.1M DTT, 20U of RNaseOUT™ (40U/µl) (Invitrogen, Carlsbad, CA, USA), and 200U of Superscript™ III Reverse Transcriptase (200U/µL) (Invitrogen, Carlsbad, CA, USA). This was then incubated at 50°C for 60 minutes followed by 70°C for 5 minutes. The mix was then treated with 2U of RNase H (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C for 20 minutes.

Long-extension PCR for cDNA amplification was then conducted using the Platinum® Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA, USA). A total 50µL reaction containing 3µL of first-strand synthesis product, a final concentration of 2.5mM MgSO₄, 0.3mM dNTP, 1% dimethyl sulfoxide (DMSO) for high GC content and secondary structure (Baskaran et al., 1996), 400nM of forward and reverse primer, and 5U of Platinum® Taq High Fidelity (5U/µL) (Invitrogen, Carlsbad, CA, USA). The thermocycling PCR conditions consisted of 94°C for 2 minutes, 10 cycles of 94°C for 10 seconds, annealing at 53°C for 30 second and extension at 68 °C for 1 minute per kb, followed by 20 cycles of 94°C for 10 seconds, annealing at 57°C for 30 seconds, and extension at 68°C for 1 minute per kb plus an additional 10 seconds per cycle. The final extension was for 10 minutes at 68°C. All resultant RT-PCR products were analysed by gel electrophoresis; described in Chapter 2 Section 2.2.3.2.2.
3.2.4 Gel extraction

Correct sized amplicons were excised and purified using a crystal violet protocol and the Zymoclean DNA gel purification extraction kit (Zymo Research Corporation, Irvine, CA, USA), as described in Chapter 2 Section 2.2.4.2.

3.2.5 Cloning and sequencing protocol

Resultant purified PCR products were cloned using the pGEM®-T Easy Vector cloning system (Promega, Madison, WI, USA) and DH5α E. coli cells (Invitrogen, Carlsbad, CA, USA), refer to Chapter 2 Section 2.2.4.4. To increase reliability, at least two clones were sequenced twice for each amplicon by single direction primer extension at Macrogen Inc. (Seoul, South Korea), in both forward and reverse directions, using the universal T7 and SP6 primers. For longer fragments additional internal primers were designed to obtain the full length sequence of these amplicons (Section 3.2.7).

3.2.6 Confirmation of 5’ and 3’UTRs

To confirm that the 5’UTRs and 3’UTRs were correctly elucidated with the virus specific primers, further analysis by Rapid Amplification of cDNA Ends (RACE) was conducted.

The 5’UTR region of NZ-PG1, NZ-R2D4, and NZ-WCA GLRaV-3 isolates was verified using the 5’ RACE System for rapid amplification of cDNA ends kit v2.0 (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer. This involved the synthesis of the first-strand cDNA using the GLRaV-3 specific reverse primer NYSA-935R (Table 3.5) and nested PCR of the purified first-strand cDNA with a ‘dC’ tail using GLRaV-3 specific primers. For the nested PCR, primer pair NYSA-883R/AAP was used in the first PCR and NYSA-858R/AUAP was used in the second PCR (Table 3.5).

Nested PCR was conducted using the Platinum® Taq DNA Polymease High Fidelity kit (Invitrogen, Carlsbad, CA, USA), using the same reaction mix described in Section 3.2.3, except the PCR product from the first PCR was diluted at a ratio of 1:100 in water and used as template for the second PCR. The thermocycling PCR conditions for both rounds of PCR consisted of 94°C for 2 minutes, 10 cycles of 94°C for 10 seconds, annealing at 55°C for 30 second and extension at 68°C for 1 minute followed by 20 cycles of 94°C for 10 seconds, annealing at 57°C for 30
seconds, and extension at 68°C for 1 minute. The final extension was for 3 minutes at 68°C.

The 3' terminal region of the five selected isolates were confirmed using the Yeast Poly(A) polymerase enzyme (USB, Cleveland, OH, USA). Total RNA was polyadenylated using a 25µL reaction mix containing 1xPoly(A) Polymerase Reaction Buffer (100mM Tris-HCl, pH 7.0, 3.0mM MnCl₂, 0.1mM EDTA, 1mM DTT, 500µg/mL acetylated BSA, and 50% glycerol), 300 to 800ng of total RNA, 0.6mM ATP, and 600U of Poly(A) Polymerase. This mix was incubated at 37°C for 30 minutes and then to terminate the reaction samples were heated at 65°C for 10 minutes. Polyadenylated total RNA was then used as the template for the one-step RT-PCR protocol using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase kit (Invitrogen, Carlsbad, CA, USA) in a 50µL reaction mixture as described in Section 3.2.3. The M111 reverse primer (Jarugula et al., 2010) was used for all reactions in combination with either NYSA-17892F, NZ1-17884F, or NZ2-17870F GLRaV-3 specific forward primers (Table 3.5). Thermocycling conditions as described in Section 3.2.3.

Table 3.5: Primers used to characterise the GLRaV-3 5' and 3'UTRs.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
<th>Position</th>
<th>GLRaV-3 isolate targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' RACE AAP</td>
<td>GGCCACCGGTCGACTTAGTACGGGGGGG</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>AUAP</td>
<td>GGGCCACCGGTGGTCGACTTAGTAC</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>NYSA-935R</td>
<td>CACCCCTTTGGTGAGCTT</td>
<td>935</td>
<td>NZ-PG1, NZ-R2D4, NZ-WCA</td>
</tr>
<tr>
<td>NYSA-883R</td>
<td>GAAATCCCGACAACCTCAAGTCCC</td>
<td>883</td>
<td>NZ-PG1, NZ-R2D4, NZ-WCA</td>
</tr>
<tr>
<td>NYSA-858R</td>
<td>TGAACCTTATGGCGTACGAAAGC</td>
<td>858</td>
<td>NZ-PG1, NZ-R2D4, NZ-WCA</td>
</tr>
<tr>
<td>3'UTR M111</td>
<td>GGTCTCGAGTTTTTTTTTTTTTTTTTT</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>NYSA-17892F</td>
<td>GGGATGGTTGCTCTTGAGCCTTG</td>
<td>17,892</td>
<td>NZ-PG1, NZ-R2D4, NZ-WCA</td>
</tr>
<tr>
<td>NZ1-17884F</td>
<td>GCTGGCTAGGATGTTGAGCT</td>
<td>17,884</td>
<td>NZ1-B</td>
</tr>
<tr>
<td>NZ2-17870F</td>
<td>AGCAGCATTGTGGTTGACTA</td>
<td>17,870</td>
<td>NZ2</td>
</tr>
</tbody>
</table>

* The primer nucleotide positions are based on the complete GP18 (EU259806) genome sequence.
* Primers were included in the 5' RACE System for rapid amplification of cDNA ends kit (Invitrogen, Carlsbad, CA, USA)
* Primer sequence obtained from Jarugula et al. (2010).

All resulting PCR products from the 5' and 3' terminal reactions were analysed by gel electrophoresis, correct sized amplicons excised, purified, and cloned (Chapter 2). For each amplicon at least two clones were sequenced twice in both directions using the universal T7 and SP6 primers at Macrogen Inc. (Seoul, South Korea).
3.2.7 Sequence assembly

Prior to sequence assembly the cloned fragments were edited to remove vector sequence and primer binding sites. In addition, the virus identity of edited sequences was confirmed using BLAST, which searches the GenBank® (Benson et al., 2011) nucleotide database. Sequences were then aligned and assembled against complete GLRaV-3 NY1 and GP18 genomes using Geneious v5.5 (Drummond et al., 2011). Generally, each reaction of single direction primer extension sequencing returned 800-850nt of useable sequence data. Thus, for amplicons less than 1700nt the forward and reverse sequencing reactions overlapped and assembled into a single contiguous sequence, while the two sequencing reactions for amplicons larger than 1700nt did not overlap.

To elucidate the non-overlapping sequence and complete the sequencing of larger amplicons additional internal sequencing primers were required. Based on the alignment against NY1 and GP18, the non-overlapping area from the first sequencing reaction was identified, and primers were designed to complement sequences that were at least 100nt upstream or downstream from the region yet to be sequenced, using the Primer3 web server as described in Chapter 2 Section 2.2.3.2.1 (Table 3.6). All internal primers were synthesised by Macrogen Inc. (Seoul, South Korea) and used to sequence the remaining sections from the original sample. Resultant sequences were edited as described above and aligned to the existing multiple sequence alignment using Geneious v5.5 (Drummond et al., 2011) to produce a single contiguous sequence.

After sequence data from all amplicons were assembled into a single contiguous sequence, potential ORFs were identified using Geneious v5.5 (Drummond et al., 2011) and then compared to the existing GLRaV-3 NY1 and GP18 complete genomes. In addition, potential conserved domains such as MET and AlkB, within the assembled nucleotide sequence were identified using the National Centre for Biotechnology Conserved Domain Database (Marchler-Bauer et al., 2009).
Table 3.6: Internal primer sets used to sequence New Zealand GLRaV-3 isolates.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Position</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ-PG1 and NZ-R2D4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NYSaint-10585F</td>
<td>CGTTTGTGTCGCCAGATT</td>
<td>10,585</td>
<td>H</td>
</tr>
<tr>
<td>NYSaint-11198R</td>
<td>TGCGGCTGTGTCGTTGTT</td>
<td>11,198</td>
<td></td>
</tr>
<tr>
<td>NYSaint-8391F</td>
<td>AAGCAGACAGAAGGATTGTGTT</td>
<td>8,391</td>
<td>J</td>
</tr>
<tr>
<td>NYSaint-9213R</td>
<td>ACCCAGCTCGAGAAGGAGTGC</td>
<td>9,213</td>
<td></td>
</tr>
<tr>
<td>NYSaint-6207F</td>
<td>GCCGTCGCTACTGCTGAAAG</td>
<td>6,207</td>
<td>L</td>
</tr>
<tr>
<td>NYSaint INT-7138R</td>
<td>TCAACTGGTGTCATGCACAA</td>
<td>7,138</td>
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NZ-WCA

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<th>Amplicon</th>
</tr>
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<tbody>
<tr>
<td>WCAINT-16970F</td>
<td>ACCGATAGAGGATGCGTA</td>
<td>16,970</td>
<td>A-B</td>
</tr>
<tr>
<td>WCAINT-17734R</td>
<td>CTCGCTTCACGACACACT</td>
<td>17,734</td>
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</tr>
<tr>
<td>WCAINT-10317F</td>
<td>TCGTGTAGGGCTGCTGTT</td>
<td>10,317</td>
<td>H-I</td>
</tr>
<tr>
<td>WCAINT-11158R</td>
<td>CCAAGACTTTTGTGCTCAA</td>
<td>11,158</td>
<td></td>
</tr>
<tr>
<td>NYSaint-8391F</td>
<td>AAGCAGACAGAAGGATTGTGTT</td>
<td>8,391</td>
<td>J</td>
</tr>
<tr>
<td>WCAINT-9168R</td>
<td>CACGCTAAGAAAAAGACGACGGT</td>
<td>9,168</td>
<td></td>
</tr>
<tr>
<td>WCAINT-5198F</td>
<td>AAGCGACAAGGGCAAGGCTATT</td>
<td>5,198</td>
<td>M-K</td>
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<tr>
<td>WCAINT-7495R</td>
<td>TCCGTACCAACTCCCCTTCAAA</td>
<td>7,495</td>
<td></td>
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<td>WCAINT-5822F</td>
<td>TGGTCTCTGCTGAGGAGGAGT</td>
<td>5,822</td>
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<tr>
<td>WCAINT-6675R</td>
<td>GGATCTCTTCTTCTCACGCACAT</td>
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<td>WCAINT-2645F</td>
<td>CGATACGCTGGTGAAGG</td>
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<td>O-P</td>
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<tr>
<td>WCAINT-3668R</td>
<td>CGAGACCAGCGACATTTTC</td>
<td>3,668</td>
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NZ1-B

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<tr>
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<th>Amplicon</th>
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<tr>
<td>NZ1INT-16332F</td>
<td>GAAACAAACTCAACTGCGGTAA</td>
<td>16,332</td>
<td>A-NZ1</td>
</tr>
<tr>
<td>NZ1INT-17828R</td>
<td>CCGCTTCCACGACAGT</td>
<td>17,828</td>
<td></td>
</tr>
<tr>
<td>NZ1INT-13766F</td>
<td>TTAGCGGTGTGACGATTCTT</td>
<td>15,766</td>
<td>B-NZ1</td>
</tr>
<tr>
<td>NZ1INT-15285R</td>
<td>TCCACTCAATCAATACGACGA</td>
<td>15,285</td>
<td></td>
</tr>
<tr>
<td>NZ1INT-11095F</td>
<td>CGTGCCAGCAATTACACATC</td>
<td>11,095</td>
<td>D-NZ1</td>
</tr>
<tr>
<td>NZ1INT-11738R</td>
<td>ACCGCAGCTGACGACATC</td>
<td>11,738</td>
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NZ2

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
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<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ2INT-15742F</td>
<td>CGATGACGCGTTCTTGCAAATC</td>
<td>15,742</td>
<td>B-NZ2</td>
</tr>
<tr>
<td>NZ2INT-16761R</td>
<td>AACCTTCCACCACTCCCGAGGAC</td>
<td>16,761</td>
<td></td>
</tr>
<tr>
<td>NZ2INT-12764F</td>
<td>TTGACGGGCTACGAGGCAATTA</td>
<td>12,764</td>
<td>C-NZ2</td>
</tr>
<tr>
<td>NZ2INT-14721R</td>
<td>ACGTTTGTGCTAGCAAGGATCC</td>
<td>14,721</td>
<td></td>
</tr>
<tr>
<td>NZ2INT-13561F</td>
<td>AGACGCCATATTACACATC</td>
<td>13,561</td>
<td></td>
</tr>
<tr>
<td>NZ2INT-14284R</td>
<td>TTCTAGGACCACTTTGCCGAGA</td>
<td>14,284</td>
<td>C-NZ2</td>
</tr>
</tbody>
</table>

a Expected primer binding nucleotide positions are based on the complete GP18 (EU259806) genome sequence.
b Amplicons that correspond to the internal primers used for sequencing.

3.2.8 Phylogenetic analysis

Before phylogenetic analysis was conducted, the sequence data was prepared for analysis, as described in Chapter 2 Section 2.2.5, by generating multiple alignments of all corresponding GLRaV-3 sequences, trimming sequence alignments, and removing duplicate sequences. The MEGA5 (Tamura et al., 2011) and TOPALi v2.5 programmes (Milne et al., 2004) were used to select the
best suited model for phylogenetic analysis (Chapter 2 Section 2.2.5). For the complete and partial genome data set the Tamura-Nei model (Tamura et al., 1993) was selected, while the Kimura 2-parameter model (Kimura, 1980) was selected for the analysis of the 428nt region the ORF6 sequence data set.

Phylogenetic trees were then constructed using the selected models, the NJ method (Saitou et al., 1987) in MEGA5 (Tamura et al., 2011), and calculated bootstrap values based on $10^3$ replicates (Felsenstein, 1985). Phylogenetic trees were assumed to be rooted at the midpoint as PMWaV-2 is the closest relative to GLRaV-3, but is too distant to be reliably used as an outgroup (Tarrío et al., 2000).

### 3.2.9 Recombination and amino acid analysis

Recombination analysis was conducted on the complete and partial GLRaV-3 genome multiple sequence alignments using the RDP v3.44 software (Martin et al., 2010) and confirmed by sequence and phylogenetic analysis, as described in Chapter 2 Section 2.2.6.

### 3.2.10 RNA secondary structure analysis

To control virus functions such as virus replication and expression of sgRNAs, cis-acting control elements are commonly found within the UTRs and proceeding sequences of sgRNAs as conserved primary and/or secondary structures. To determine potential cis-acting elements for GLRaV-3 within the 5′UTR, 3′UTR, and sequence surrounding the 5′transcription start site (5′TSS) nt of seven sgRNAs, sequence and RNA secondary structures of GLRaV-3 variants, representative of each GLRaV-3 phylogenetic group, were analysed and compared. Primary or secondary structures conserved between all GLRaV-3 variants are likely cis-acting elements that are important features involved in the control of virus functions. Multiple sequence alignments of the GLRaV-3 UTRs and sequence surrounding the 5′TSS nt of seven sgRNAs were aligned using Geneious v5.5 (Drummond et al., 2011), and the RNA secondary structures of these regions were predicted using the mfold webserver (Zuker, 2003).
3.3 Results

3.3.1 Complete and partial molecular characterisation of selected New Zealand GLRaV-3 isolates

The full genome sequence was successfully elucidated for three New Zealand isolates, NZ-PG1, NZ-R2D4, and NZ-WCA, which are each comprised of 18,498nt. Approximately 64 and 41% of the NZ1-B and NZ2 genomes, respectively, were successfully assembled. Analysis of the NZ1-B sequence confirmed that this was a variant of the divergent NZ-1 strain (99.6% nucleotide identity based on 6,416nt) and extends the known sequence of this strain by 5,410nt. The NZ1-B sequence starts within ORF1a (position 6,811nt based on GP18) while the NZ2 sequence starts within ORF4 (position 10,897nt based on GP18) and both terminate at the 3'UTR, resulting in a total of 11,827nt and 7,612nt for NZ1-B and NZ2, respectively (Figure 3.5a and 3.5b). The partial sequences were deposited into the GenBank database as accessions JX220900 and JX220899, respectively.

3.3.1.1 Genome organisation

The NZ-PG1, NZ-R2D4, and NZ-WCA isolates share the same genome organisation as previously described for GLRaV-3 isolates NY1, GP18, and CI-766, which consists of the 13 ORFs and putative domains such as the MET, AlkB, and HEL within the ORF1a (Ling et al., 2004), refer to Chapter 1. In contrast, NZ1-B and NZ2 contain slight differences in their genome organisation (Figure 3.5). Similar to NZ-1 and the South African GH11 and GH30 variants, NZ1-B does not contain an ORF2, but the intergenomic region between ORF1b and ORF3 is 1,596nt, 34nt shorter than GH11 and GH30. NZ1-B, GH11, and GH30 share the same frameshift in ORF12 that leads to a premature stop codon and shortens the ORF12 polypeptide by six amino acids (Bester et al., 2012b) (Figure 3.5c).

The ORF11 of NZ2 is translated in the same frame as other GLRaV-3 isolates; however transcription is predicted to start 3nt upstream and to terminate 15nt downstream of NZ1-B, GH11, and GH30 (Figure 3.5c). Thus, the NZ2 ORF11 is 18nt longer than all previously known GLRaV-3 isolates, resulting in a six amino acid longer ORF11 polypeptide. The longer NZ2 ORF11 does not affect the positioning of ORF12, as it is predicted to start in the same position as other GLRaV-3 variants; however, as in NZ1-B GH11, and GH30, a frameshift within
ORF12 leads to a premature stop codon and a four amino acid shorter ORF12 polypeptide (Figure 3.5c). To ensure the observed variation was not due to sequencing error, eight different clones of this genomic region were sequenced and analysed.

Figure 3.5: Diagrammatic representation of the genome organisation for *Grapevine leafroll-associated virus* 3 isolates (a) NZ1-B and (b) NZ2, based on the sequence data elucidated from this study, and (c) a multiple sequence alignment of sequence from ORF10 (last 11 nt) to ORF12 (inclusive). (a and b) The blue regions represent the area analysed in the multiple sequence alignment (c). (c) The consensus sequence for groups 1 to 5 is used as the reference sequence in the alignment. All agreements with the reference sequence are displayed as dots, while disagreements are indicated with coloured letters. For each sequence the ORFs are highlighted with a green arrow. The GH11, GH30, NZ1-B, NZ2 sequences contain frameshifts (red vertical arrows) and differences in the ORF organisation (red dotted boxes). The multiple sequence alignments were generated in Geneious v5.5 (Drummond et al, 2011).

### 3.3.1.2 Pairwise comparisons of the five New Zealand isolates to other GLRaV-3 isolates

The overall nucleotide identity between NZ-PG1 and 621 (GQ352631; group 1 variant from South Africa) over the entire genome is 99.5%, while NZ-R2D4 and NZ-WCA isolates have 91.5% and 92.7% nucleotide identity respectively.
compared to 621. The NZ-R2D4 isolate is most closely related to a South African isolate from phylogenetic group 2, GP18, with 99.2% nucleotide identity based on the complete 18,498nt genome. While the NZ-WCA isolate is most closely related to a Chilean isolate from phylogenetic group 5, CI-871, with 99.2% nucleotide identity based on the complete CP gene (ORF6). A comparison of nucleotide and amino acid identities between the available ORFs of NZ-PG1, NZ-R2D4, NZ-WCA, and NY1 (AF037268) is presented in Table 3.7. The NY1 isolate was used for this analysis, as at the time, reports used NY1 as the reference sequence for genome analysis (Engel et al., 2008; Jooste et al., 2010), and it remains the reference sequence for GLRaV-3 on GenBank. The sequence variations between isolates over the entire genome are heterogeneously distributed. Regions generally showing higher nucleotide and amino acid conservation were ORF1b, ORF3, ORF4, and ORF6 with amino acid variations no greater than 4.8% compared to NY1. Lower nucleotide and amino acid conservation was observed in ORF2 and in ORFs 10, 11, and 12, where the amino acid variation for the NZ-WCA isolate compared to NY1 was 11.8%, 12.3%, 24.7%, and 13.1% respectively.

**Table 3.7:** Comparison of nucleotide and amino acid identities (%) between available open reading frames of NZ-PG1, NZ-R2D4, and NZ-WCA compared to NY1.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>5'UTR</th>
<th>ORF1a</th>
<th>1b</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ-PG1</td>
<td>100</td>
<td>96.1 / 96.2</td>
<td>99.4 / 99.8</td>
<td>98.7 / 98.1</td>
<td>96.4 / 100</td>
<td>98.8 / 98.2</td>
<td>99.2 / 98.8</td>
<td>99.3 / 99.0</td>
</tr>
<tr>
<td>NZ-R2D4</td>
<td>79.9</td>
<td>94.1 / 95.8</td>
<td>95.4 / 97.4</td>
<td>91.0 / 84.6</td>
<td>94.2 / 97.8</td>
<td>94.4 / 96.9</td>
<td>92.8 / 93.4</td>
<td>92.7 / 95.2</td>
</tr>
<tr>
<td>NZ-WCA</td>
<td>82.4</td>
<td>91.9 / 93.6</td>
<td>95.7 / 99.3</td>
<td>93.6 / 88.5</td>
<td>94.2 / 95.7</td>
<td>94.7 / 96.5</td>
<td>94.4 / 94.0</td>
<td>94.5 / 96.8</td>
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<table>
<thead>
<tr>
<th>Isolate</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<td>98.9 / 98.9</td>
<td>99.6 / 99.4</td>
<td>99.1 / 97.2</td>
<td>98.2 / 97.3</td>
<td>93.4 / 91.8</td>
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<td>1</td>
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<td>NZ-R2D4</td>
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<td>93.0 / 96.2</td>
<td>91.6 / 88.8</td>
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<td>91.9 / 89.2</td>
<td>97.3 / 93.4</td>
<td>97.5</td>
<td>2</td>
</tr>
<tr>
<td>NZ-WCA</td>
<td>92.3 / 89.7</td>
<td>94.6 / 96.8</td>
<td>95.5 / 95.5</td>
<td>90.6 / 87.2</td>
<td>87.4 / 75.7</td>
<td>90.2 / 86.9</td>
<td>96.0</td>
<td>5</td>
</tr>
</tbody>
</table>

* Based on shorter 159nt NY1 5'UTR.

Based on the partial genome sequence, NZ1-B is closely related to GH11 and GH30 with 91.5% nucleotide identity, while the NZ2 variant is most closely related to the NZ1-B, GH11, and GH30 variants with ~76% nucleotide identity. To understand the distribution of the nucleotide similarity between GLRaV-3 isolates over the partial genome, the nucleotide and amino acid sequence identities for the individual ORFs and the 3'UTR of NZ1-B and NZ2 isolates compared to NY1, GH11, and to each other, are displayed in Table 3.8. Sequence comparisons indicated that the ORF6 (CP) of NZ1-B and NZ2 had the highest sequence similarity to NY1, with 78.5% and 78.6% nucleotide and 90.1% and 91.4% amino
acid identities, respectively. Other regions of high similarity to NY1 were ORF1b and ORF4, with nucleotide and amino acid identities of at least 74.0% and 84.5% respectively. Sequence variation increased towards the 3'UTR, with nucleotide and amino acid differences of more than 34.5 and 38.0% respectively, for ORF9, ORF10, and ORF12 when compared to NY1. ORF11, which codes for the p4 (function unknown) is unique to GLRaV-3 and displayed the greatest variation. At the amino acid level NZ1-B and NZ2 are 72.3% and 86.1% different from NY1 and 16.7% and 69.4% different from GH11.

From the analysis of all GLRaV-3 isolates (based only on the available NZ2 genome region between nucleotide 10,897 and the 3'UTR), three regions consistently displayed high genetic variation for all GLRaV-3 isolates; the regions approximately between nucleotides 12,400 to 13,040 (within ORF5), 15,220 to 15,820 (within ORF7), and 17,020 to 18,190 (encompasses ORF10 to 12), based on GP18 numbering (Figure 3.6). The most variable region encompassed ORF10 to 12, with an overall average nucleotide difference of 86.4% between GLRaV-3 isolates that represent the various phylogenetic groups (NZ-PG1, NZ-R2D4, PL-20, and NZ-WCA) and divergent isolates GH11, GH30, NZ1-B, and NZ2.

| Table 3.8: Comparison of nucleotide (nt) and amino acid (aa) identities (%) between available open reading frames of NZ1-B and NZ2 with corresponding sequences from virus isolates NY1 and GH11, and between each other. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | NZ1-B versus NY1 | NZ1-B versus GH11 | NZ2 versus NY1 | NZ2 versus GH11 | NZ2 versus NZ1-B |
|                 | nt   | aa    | nt   | aa    | nt   | aa    | nt   | aa    | nt   | aa    |
| Overall a       | 70.7 | 91.5  | 70.3 | 76.3  | 76.2 | 76.2  |
| 5'UTR           | -    | -     | -    | -     | -    | -     |
| ORF1a b         | 74.4 | 87.3  | 93.8 | 98.6  | -    | -     |
| ORF1b           | 78.7 | 88.7  | 92.4 | 97.0  | -    | -     |
| ORF2            | -    | -     | -    | -     | -    | -     |
| ORF3            | 72.5 | 75.6  | 94.9 | 95.6  | -    | -     |
| ORF4 (HSP70h) c | 74.4 | 84.9  | 92.8 | 95.4  | 74.0 | 84.5  | 79.5 | 90.2  | 79.1 | 89.8  |
| ORF5 (HSP90h)   | 68.5 | 72.7  | 90.4 | 94.4  | 68.7 | 72.1  | 77.4 | 86.3  | 77.1 | 86.7  |
| ORF6 (CP)       | 78.5 | 90.1  | 92.9 | 96.5  | 78.6 | 91.4  | 82.3 | 94.9  | 81.3 | 94.6  |
| ORF7 (dCP)      | 70.5 | 77.1  | 90.7 | 93.1  | 70.9 | 77.1  | 75.2 | 81.8  | 75.1 | 82.0  |
| ORF8            | 75.4 | 77.8  | 91.8 | 95.7  | 76.0 | 78.4  | 75.6 | 81.6  | 76.7 | 83.2  |
| ORF9            | 61.6 | 55.9  | 89.7 | 88.1  | 61.8 | 59.3  | 68.5 | 66.1  | 68.9 | 65.5  |
| ORF10           | 64.3 | 62.0  | 86.7 | 90.5  | 63.3 | 63.7  | 73.0 | 78.2  | 72.8 | 78.8  |
| ORF11           | 43.6 | 27.7  | 89.1 | 83.3  | 40.9 | 13.9  | 52.7 | 30.6  | 55.4 | 38.8  |
| ORF12           | 65.5 | 61.1  | 92.7 | 88.9  | 64.8 | 61.1  | 70.9 | 72.2  | 72.1 | 75.9  |
| 3'UTR           | 79.4 | 96.5  | -    | -     | 80.9 | 87.2  | 87.9 | -     | -    | -     |

a The overall identities based on the partial genome sequence of NZ1-B (11,826nt) & NZ2 (7,613nt).
b NZ1-B identities are based on the partial ORF1a sequence (641nt).
c NZ2 identities are based on the partial ORF4 sequence (1,418nt).
Figure 3.6: Comparison between *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates at the nucleotide level over the partial genome using similarity plots comparing (a) NY1 to GP18, GH11, NZ1-B, and NZ2, and (b) NZ1-B to GH11 and NZ2. At the top of the plots is a diagrammatic representation of the GLRaV-3 genome structure from ORF4 to the 3' UTR, based on GP18. Common regions of high variation are highlighted in light blue approximately nucleotides 12,400 to 13,040, 15,220 to 15,820, and 17,020 to 18,190. Similarity plots used the Kimura 2-parameter model, window size of 350nt, step size of 10nt, and 1000 bootstraps (generated using SimPlot (Lole et al., 1999)).

### 3.3.2 Phylogenetic analysis of the five New Zealand isolates

Figure 3.7 presents the phylogenetic analysis of three New Zealand isolates compared to all complete GLRaV-3 genomes available, at the time of analysis. The NZ-PG1 and NZ-R2D4 isolates cluster with phylogenetic groups 1 and 2, respectively, while the NZ-WCA isolate branches separately from the other groups (100% confidence). Analysis of the partial genome sequences of NZ1-B and NZ2 showed that NZ1-B groups with isolates from South Africa in the proposed group 6.
by Bester et al. (2012b), while NZ2 is positioned separately from all other isolates (Figure 3.8).

**Figure 3.7:** Phylogenetic analysis of Grapevine leafroll-associated virus 3 (GLRaV-3) isolates from this study and GenBank, based on a 17,919nt region, which corresponds to nucleotide positions 580 to 18,498 (based on GP18 numbering) or 1 to 17,919 (based on NY1 numbering), conducted in MEGA5 (Tamura et al., 2011). New Zealand GLRaV-3 isolates NZ-PG1, NZ-R2D4, and NZ-WCA are highlighted with red square boxes and are in bold. Evolutionary history was inferred using the Neighbour-Joining method and the Tamura-Nei method (Tamura and Nei, 1993) was used to compute evolutionary distances. Phylogenetic groupings based on Gouveia et al. (2010) and Bester et al. (2012). The percentages of bootstrap support (≥90%) from 1,000 replicates are shown at nodes. The scale represents 0.05 nucleotide substitutions per site.

**Figure 3.8:** Phylogenetic analysis of Grapevine leafroll-associated virus 3 (GLRaV-3) isolates from this study and GenBank, based on a 7,612nt region, which corresponds to nucleotide positions 10,897 to 18,498 (based on GP18 numbering), conducted in MEGA5 (Tamura et al., 2011). New Zealand GLRaV-3 isolates NZ-PG1, NZ-R2D4, NZ-WCA, NZ1-B, and NZ2 are highlighted with red square boxes and are in bold. Evolutionary history was inferred using the Neighbour-Joining method and the Tamura-Nei method (Tamura and Nei, 1993) was used to compute evolutionary distances. Phylogenetic groupings based on Gouveia et al. (2010) and Bester et al. (2012). The percentages of bootstrap support (≥90%) from 1,000 replicates are shown at nodes. The scale represents 0.05 nucleotide substitutions per site.
A higher number of GLRaV-3 sequences, representative of a wider range of GLRaV-3 phylogenetic groups and different countries are available for a 428nt region within the CP (Figure 3.9). Consequently, this was used to further support the relationships established from the analyses described above (Figure 3.7 and 3.8) and in Chapter 2. This analysis placed most of the GLRaV-3 isolates in the previously proposed groups 1 to 5 (Gouveia et al., 2011), including NZ-PG1 into group 1 and NZ-R2D4 into group 2, as described above. NZ-WCA clustered with GLRaV-3 from group 5, which supports the previous analysis with this isolate branching separately from groups 1 and 2. This is the first known report of a complete GLRaV-3 sequence from phylogenetic group 5 as previously only partial sequences have been published.

GLRaV-3 isolates that do not fit these five groups include the isolate 43-15 (JF421951) that was referred to as GLRaV-3f by Sharma et al. (2011), NZ1-B which was positioned within the proposed group 6 with isolates from South Africa and USA, and NZ2 which was positioned independently with 99% confidence (Figure 3.9). Sequence from the outlier isolate CB-19 (EF445655) was not included in this analysis as available sequence does not span the entire 428nt analysed. Noticeably the branch lengths within group 6 are significantly longer than for the other five groups, the average genetic distance of 7.4% over all sequences within the group being considerably higher than the 0.6 to 0.9% in the other five groups.
Figure 3.9: Phylogenetic analysis of Grapevine leafroll-associated virus 3 (GLRaV-3) isolates from this study and GenBank, based on a 428nt region within open reading frame 6, conducted in MEGA5 (Tamura et al., 2011). The NZ1-B and NZ2 GLRaV-3 isolates are highlighted in bold. Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method (Kimura, 1980) was used to compute evolutionary distances. Elongated triangles represent the compressed subtrees of the phylogenetic groupings based on Gouveia et al. (2010) and Bester et al. (2012). The length of the triangle corresponds to the respective intra-group diversity and the thickness is proportional to the number of taxa. The percentages of bootstrap support (≥ 90 %) from 1,000 replicates are shown at nodes. The scale represents 0.05 nucleotide substitutions per site.
3.3.3 Analysis of UTRs

The 5′UTR of NZ-PG1, NZ-R2D4, and NZ-WCA and the 3′UTR of the five NZ isolates were confirmed using the 5′RACE System for rapid amplification of cDNA ends kit (Invitrogen, Carlsbad, CA, USA) and Yeast Poly(A) polymerase enzyme (USB, Cleveland, OH, USA), respectively. The 5′UTR and 3′UTR of the NZ-PG1, NZ-R2D4, and NZ-WCA isolates are 737nt and 277nt respectively, while the 3′UTR of NZ1-B and NZ2 are 264nt and 289nt respectively.

3.3.3.1 5′UTR analysis

The 5′UTR of NZ-PG1 and NZ-R2D4 had 99% nucleotide identity to 621 and GP18 isolates, respectively, while NZ-WCA had only 69% and 83% nucleotide identity to 621 and GP18 isolates, respectively. The NZ-PG1 isolate also contains a 65nt tandem repeat (Figure 3.10a and 3.10b), similar to that reported by Jarugula et al. (2010) between nucleotides 187 to 315 of the group 1 WA-MR and 621 isolates. In addition, to maintain the size of the 5′UTR, group 2 isolates had an additional 65nt sequence between nucleotides 502 to 566 (Jarugula et al., 2010). Both the NZ-R2D4 and NZ-WCA isolates share a similar feature, though the 65nt sequence observed in NZ-WCA has eight nucleotide differences compared to group 2 isolates such as GP18 and NZ-R2D4 (Figure 3.10a and 3.10c). Noticeably, all 5′UTRs were AU rich (68 to 70%) and high nucleotide conservation was observed within the first 46nts of the 5′UTR, with only four nucleotide differences between all GLRaV-3 analysed, in particular, the first 17nt were identical in all isolates.

To further examine possible differences in higher order structures formed by the different isolates, potential 5′UTR secondary structures, for each GLRaV-3 isolate, were predicted using the mfold web server (Zuker, 2003) and compared. Ten to 28 alternate secondary structures were predicted for each GLRaV-3 isolate and the most common type of structure predicted was of a single long complex stem and loop (SL) structure with several large interior loops and various substructural hairpins of different lengths (Figure 3.11). The complex SL structures were poorly conserved between isolates and the free energy of each structure ranged between -79.40 to -95.12kcal/mol.
Figure 3.11: Predicted secondary structures for the 5' untranslated region of Grapevine leafroll-associated virus 3 isolates (a) NZ-PG1, (b) NZ-R2D4, (c) NZ-WCA, and (d) GH11. All secondary structures were predicted using the mfold web server (Zuker, 2003).

Figure 3.10: Multiple sequence alignment of the 5' untranslated region (5'UTR) of Grapevine leafroll-associated virus 3 (GLRaV-3) isolates using (a) the complete 5'UTR sequence (73 nt) from isolates representative of phylogenetic groups 1, 2, 3, 5 and 6 respectively. All secondary structures were predicted using the mfold web server (Zuker, 2003).
3.3.3.2 3' UTR analysis

The 3’UTR of the NZ-PG1, NZ-R2D4, and NZ-WCA isolates are the same length as previously described GLRaV-3 isolates from groups 1 to 3. The NZ-PG1 and NZ-R2D4 has high nucleotide identity to other isolates from groups 1 (99%) and 2 (100%) respectively, while NZ-WCA is slightly different to groups 1 and 2 with an average nucleotide identity of 97.47 and 96.11% respectively. In contrast, the 3’UTR of the NZ1-B is 13nt shorter than the GLRaV-3 isolates from groups 1 to 3, being more similar to the South African isolates, GH11 and GH30, with an average nucleotide identity of 96.50% compared to 80.64% for groups 1 to 3. The NZ1 3’UTR is 12nt longer than other GLRaV-3 isolates characterised from groups 1 and 3 and 25nt longer than GH11, GH30, and NZ1-B. Furthermore, considerable nucleotide differences were observed, with an average nucleotide difference of 18.97% to group 1 to 3 isolates and 12.58% to GH11, GH30, and NZ1-B.

Noticeably, in addition to the relatively high sequence variation between the more divergent GLRaV-3 isolates, the sequence variation is unevenly distributed along the 3’UTR (Figure 3.12). The greatest variation was observed at the start of the 3’UTR. While the greatest similarity was observed in two regions approximately between nucleotides 18,320 to 18,382 and 18,420 to 18,498, with nucleotide identities of 90% or greater between all GLRaV-3 isolates (Figure 3.12a). Within both regions “CCR initiation box” motifs are found conserved in all GLRaV-3 isolates with a CCG motif at nucleotides 18,382 to 18,384 and two CCA motifs at nucleotides 18,427 to 18,429 and 18,434 to 18,436, based on the GP18 numbering (Figure 3.12b). The NZ1-B isolate also has a unique CCA motif at nucleotides 18,438 to 184,40 (Figure 3.12b). Furthermore, a possible 3’terminal pseudoknot structure in the 3’UTR, conserved in some members of the Crinivirus, Closterovirus, and Ampelovirus genera, including the NY1 GLRaV-3 isolate was identified by Livieratos et al. (2004). NY1 was predicted to use the sequence motif GACCUA, positioned from nucleotides 17,870 to 17,875 based on the NY1 numbering, and the complementary sequence of UAGGUC is located 38nt downstream (Livieratos et al., 2004). These predicted motifs are conserved in all GLRaV-3 isolates and are positioned in the equivalent positions, from nucleotides 18,449 to 18,454 and 18,493 to 184,98 respectively, based on the GP18 numbering (Figure 3.12b).
Figure 3.12: Comparison between Grapevine leafroll-associated virus 3 (GLRaV-3) isolates at the nucleotide level over the 3’ untranslated region (3’UTR) using (a) a similarity plot comparing phylogenetic group 1 isolates to groups 2, 3, 5, 6 and NZ2 between nucleotides 18,232 to 18,498 (based on GP18 numbering), and (b) a multiple sequence alignment of a region of the 3’UTR between nucleotides 18,353 to 18,498 (based on GP18 numbering). (a) Two regions of high conservation are highlighted in light blue approximately nucleotides 18,320 to 18,382, and 18,430 to 18,498. Similarity plots used the Kimura 2-parameter model, window size of 20nt, step size of 15nt, and 1,000 bootstraps (generated using SimPlot (Lole et al., 1999)). (b) All disagreements are highlighted with coloured letters. The “CCR initiation sites” are conserved in all isolates and NZ1-B has an additional CCA site at nucleotides 18,438 to 18,440 (pink rectangles). In addition, the sequences potentially involved in a pseudoknot secondary structure are also conserved in all GLRaV-3 isolates (green rectangles). The multiple sequence alignments were generated in Geneious v5.5 (Drummond et al., 2011).

To further examine possible differences in higher order structures formed by the different isolates, potential 3’UTR secondary structures formed by the positive and negative-sense strands, for each GLRaV-3 isolate, were predicted using the mFOLD web server (Zuker, 2003) and compared. For the positive-sense 3’UTR sequence, eight to 20 different alternate secondary structures were predicted for each GLRaV-3 isolate that included the formation of a single complex SL structure with six substructural hairpins of different lengths and complex SL structures with a combination of various simple and/or complex SL structure(s) of variable lengths.
However, one predicted secondary structure formation was similar in all GLRaV-3 isolates, consisting of a complex SL structure with four substructural hairpins that is positioned near the 3’ end. This is generally in combination with either one or two smaller SL structures of varying sizes and positions near the 5’ end (the predicted structures for three isolates NZ-PG1, NZ1-B and NZ2 are present in Figure 3.13, refer to Appendix 3 Figure A3.1 for other predicted structures). The free energy of each structure ranged between -51.75 and -57.90 kcal/mol.

There are slight size differences in the overall structure and the substructural hairpins between the different GLRaV-3 3’ complex structures (Figure 3.13). For instance, the overall size of the complex structure for the group 1 variants was 166nt and it contained four hairpins of 14, 23, 34, and 37nt in size, while the NZ1-B, GH11, and GH30 isolates had the smallest complex SL structure (161nt) and contained hairpin sizes of 14, 25, 33, and 36nt. Noticeably, the three CCR motifs are all located within the conserved SL structure and in similar positions; in the stem of two substructural hairpins (Figure 3.13 and Appendix 3 Figure A3.1). The main difference between isolates was the structure(s) formed near the 5’ end. Group 1, 2, and 5 isolates all had an 89nt SL structure 15nt from 5’ end, while two smaller SL structures were observed in the NZ1-B, GH11, and GH30 isolates including a SL structure with two or three substructural hairpins. PL-20 and NZ2 isolates form one SL structure with one hairpin, 6nt upstream from the 3’ complex structure (Figure 3.13 and Appendix 3 Figure A3.1).

![Figure 3.13: Predicted secondary structures for the 3’ untranslated region of Grapevine leafroll-associated virus 3 (GLRaV-3) isolates](Image)
For the negative-sense 3′UTR sequence, between two to 12 alternate secondary structures were predicted for each GLRaV-3 isolate. Similar to the positive-sense sequence, a range of different structures were predicted, though one complex SL structure with four substructural hairpins was common for all isolates. The structure starts at the first nucleotide of the 5′ end and is in combination with one long SL or two smaller SL structures of varying lengths, located near the 3′ end (the predicted structures for three isolates NZ-PG1, NZ1-B and NZ2 are present in Figure 3.14, refer to Appendix 3 Figure A3.2 for other predicted structures). The free energy of each structure ranged between -43.72 to -59.53 kcal/mol.

The 5′ complex structure of the 3′UTR was highly conserved throughout all GLRaV-3 isolates with only slight differences in the size of the overall structure and the substructural hairpins. For instance, the overall size of the complex structure for all group 1 GLRaV-3 isolates was 166nt and had four hairpins were 14, 23, 34, and 39nt in size, compared to the NZ1-B structure of 169nt with hairpin sizes of 10, 14, 18, and 33nt (Figure 3.14). The main difference between isolates was the structure(s) formed near the 3′ end (Figure 3.14). For example, group 1 isolates had a 101nt SL structure between nucleotide 18,225 and 18,325, while shorter SL structures (74nt) were observed in the NZ1-B and GH11 isolates 13nt away from the 3′ end. The NZ2 isolate forms the most different SL structure with three substructural hairpins that are 15, 17, and 25nt long.

![Figure 3.14: Predicted secondary structures for the 3′ untranslated region of Grapevine leafroll-associated virus 3 (GLRaV-3) isolates (a) NZ-PG1 (group 1), (b) NZ1-B (group 6), and (c) NZ2, using the negative sense sequence (i.e. complement sequence). Refer to Appendix 3 Figure A3.2 for the predicted secondary structures for GLRaV-3 isolates NZ-R2D4 (group 2), PL-20 (group 3), NZ-WCA (group 5), and GH11 (group 6). All secondary structures were predicted using the mfold web server (Zuker, 2003).](image)
3.3.4 Analysis on the 5’TSS

Using the South African isolate, GP18 (group 2 isolate), the TSSs and the corresponding leader sequence for seven GLRaV-3 subgenomic RNAs were recently elucidated by Maree et al. (2010). In addition, Jarugula et al. (2010) confirmed the 5’TSS of four sgRNA using the WA-MR isolate (group 1 isolate). Thus, to identify likely 5’TSS and leader sequences within the GLRaV-3 isolates characterised in this study and other divergent isolates, GH11 and GH30, the potential 5’TSS sites were mapped according to the sites identified by Maree et al. (2010) and Jarugula et al. (2010).

Generally, the 5'nt of each sgRNA from all five NZ isolates, GH11, and GH30 shared the same purine nucleotide predicted by Maree et al. (2010) and Jarugula et al. (2010), except for the sgRNA(ORF3/4) of NZ1-B, GH11, and GH30 and the sgRNA(ORF7) of the NZ1-B and NZ2 isolates, which had an adenine in place of the predicted guanine. In addition, the leader sequence of sgRNA(ORF6) for the GH11, GH30, NZ1-B, and NZ2 isolates is 5nt longer than other GLRaV-3 isolates. The NZ-WCA leader sequences are most similar to WA-MR and nucleotide identities to WA-MR range between 82.61 to 98.95%, whereas the NZ1-B and NZ2 isolate leader sequences showed greater variation with nucleotide identities for the NZ1-B, GH11, and GH30 isolates ranging from 47.83 to 84.21%, and NZ2 ranged between 29.73 to 86.32% (Table 3.9). In addition, sequence analysis revealed no conserved motifs or repeats between leader sequences of all predicted sgRNAs for all GLRaV-3 isolates.

Table 3.9: Comparison of nucleotide identities (%) between predicted sgRNA leader sequences of NZ-PG1, NZ-R2D4, NZ-WCA, NZ1-B and NZ2 compared to the WA-MR isolate.

<table>
<thead>
<tr>
<th>Predicted sgRNA*</th>
<th>NZ-PG1</th>
<th>NZ-R2D4</th>
<th>NZ-WCA</th>
<th>NZ1-B</th>
<th>NZ2</th>
</tr>
</thead>
<tbody>
<tr>
<td>sgRNA(ORF3/4)</td>
<td>100</td>
<td>90.63</td>
<td>93.75</td>
<td>53.13</td>
<td>n/a</td>
</tr>
<tr>
<td>sgRNA(ORF5)</td>
<td>100</td>
<td>95.08</td>
<td>95.08</td>
<td>59.02</td>
<td>63.12</td>
</tr>
<tr>
<td>sgRNA(ORF6)</td>
<td>100</td>
<td>91.67</td>
<td>93.75</td>
<td>64.58</td>
<td>54.17</td>
</tr>
<tr>
<td>sgRNA(ORF7)</td>
<td>100</td>
<td>100</td>
<td>89.19</td>
<td>48.65</td>
<td>29.73</td>
</tr>
<tr>
<td>sgRNA(ORF8)</td>
<td>100</td>
<td>86.96</td>
<td>82.61</td>
<td>47.83</td>
<td>47.83</td>
</tr>
<tr>
<td>sgRNA(ORF9)</td>
<td>100</td>
<td>92.63</td>
<td>98.95</td>
<td>84.21</td>
<td>86.32</td>
</tr>
<tr>
<td>sgRNA(ORF10-12)</td>
<td>99.20</td>
<td>89.60</td>
<td>95.20</td>
<td>52.00</td>
<td>54.40</td>
</tr>
</tbody>
</table>

*Predicted sgRNA are based on the study by Maree et al. (2010).

Secondary structures upstream of CTV 5’TSSs have been shown to influence the production of sgRNAs (Ayllón et al., 2004; Jarugula et al., 2010). Thus, to examine potential conservation of secondary structures within GLRaV-3, the
secondary structures for the sequence surrounding the 5’TSS nt of each sgRNA (25nt up- and down-stream, complement sequence) for GLRaV-3 phylogenetic groups 1 (WA-MR, NY1, and NZ-PG1), 2 (GP18 and NZ-R2D4), 3 (PL-20), 5 (NZ-WCA), 6 (GH11 and NZ1-B), and NZ2 were predicted using the mfold web server. The predicted RNA secondary structures were compared between (i) the different sgRNAs of the same GLRaV-3 variant, and (ii) the different GLRaV-3 variants for the same sgRNA. Similar to results of Jarugula et al. (2010) and Maree et al. (2010), the predicted secondary structures were poorly conserved between the different sgRNAs of the same GLRaV-3 variant, as various simple and complex SL structures were observed. On the other hand, Jarugula et al. (2010) identified some features of the predicted SL structures for sgRNA(ORF6), sgRNA(ORF8), sgRNA(ORF9), and sgRNA(10-12), were conserved between two different GLRaV-3 variants, WA-MR (group 1) and GP18 (group 2). In this study, for a number of sgRNAs similar features in the predicted SL structures were also shared between the different GLRaV-3 isolates, despite the high sequence variation between GLRaV-3 isolates analysed.

For all GLRaV-3 isolates the predicted secondary structures formed from the negative-sense sequence surrounding the TSS for sgRNA(ORF5), sgRNA(ORF8), and sgRNA(ORF10-12) (Figure 3.15a), consisted of a SL structure with the 5’TSS nt positioned inside the SL structure. sgRNA(ORF6) and sgRNA(ORF9) also generated single SL structures, though the 5’TSS nt is positioned outside the SL structure, and was conserved for all isolates with the exception of PL-20 and NZ2 sgRNA(ORF6), which formed two SL structures. Different structures were observed in some GLRaV-3 isolates for sgRNA(ORF3-4) and sgRNA(ORF7) (Figure 3.15b and 3.15c). The NZ1-B sgRNA(ORF3-4) generated a 47nt SL structure with two hairpins near the 3’end and the 5’TSS nt is located within the SL structure, while all other GLRaV-3 isolates analysed (not including NZ2 as the sequence was unavailable) generated one 14-27nt SL structure near the 3’end and the 5’TSS nt is located outside the SL structure (Figure 3.15b). For the sequence surrounding the sgRNA(ORF7), isolates from groups 1, 2, and 6 the 5’TSS nt is positioned inside a long SL structure, while PL-20 (group 3), NZ-WCA (group 5), and NZ2 isolates generated two small SL structures with the 5’TSS nt located outside the structures (Figure 3.15c).
Figure 3.15: Predicted secondary structures for the sequence surrounding the 5’transcription start site (5’TSS) (25nt up- and down-stream, complement sequence) of (a) sgRNA(10-12), (b) sgRNA(ORF3-4), and (c) sgRNA(ORF7) of Grapevine leafroll-associated virus 3 (GLRaV-3) isolates NZ-PG1 (group 1), NZ-WCA (group 5), NZ1-B (group 6), and NZ2. NZ2 sgRNA(ORF3-4) sequence was unavailable for analysis. The 5’TSS nucleotide is indicated with a red arrow. Refer to Appendix 3 Figure A3.3, A3.4, and A3.5 for the predicted secondary structures for GLRaV-3 isolates NZ-R2D4 (group 2), PL-20 (group 3), and GH11 (group 6). All secondary structures were predicted using the mfold web server (Zuker, 2003).

3.3.5 Recombination analysis

Recombination analysis using RDP v3.44 (Martin et al., 2010), based on the partial genome alignments of NZ1-B, NZ2, and corresponding GLRaV-3
sequences, revealed no recombination events between NZ1-B and NZ2 and other GLRaV-3 variants. In addition, no recombination events were identified in the New Zealand NZ-PG1, NZ-R2D4, and NZ-WCA isolates, based on analysis of the multiple sequence alignment of all the available complete genomes at the time of analysis. However, four unique recombination events were detected in NY1 (3) and CI-766 (EU344893) (1), which originate from USA and Chile respectively (Figure 3.19 and Table 3.10). Recombination analysis had not been previously performed for these two isolates, as until recently no other sequences were available for analysis.

Seven recombination methods (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq) detected three events within the NY1 sequence, which involved recombination between a WA-MR-like isolate from group 1 (major parent) and the 623-like isolate from group 2 (minor parent) (Table 3.10). The fourth recombination event was detected within the ORF1a of the CI-766 isolate, and involves the recombination between a NZ-PG1-like (major parent) and a NZ-WCA-like (minor parent) isolate, from group 1 and 5 respectively (Table 3.10). This was identified by six recombination methods (RDP, BootScan, MaxChi, Chimaera, SiScan, and 3Seq) with relatively high confidence (p-values ranged between 1.19 x10^{-09} to 1.26x10^{-30}). Similar to Chapter 2, all recombination events were confirmed using SimPlot (Lole et al., 1999) and phylogenetic analysis of the regions flanking each break point identified.

For each isolate, the position of the recombination events detected in relation to how the NY1 and CI-766 genome sequences were assembled was analysed. The NY1 genome sequence was generated using a clone library prepared from cDNA synthesised from purified dsRNA using a random primer (Ling et al., 1997; Ling et al., 1998; Ling et al., 2004). The precise regions covered by each clone are not indicated in the report, however based on the schematic diagram provided, clones 2-5 (Ling et al., 2004) and B1-1 (Ling et al., 1998) cover a similar area detected for recombination events 2 and 3, respectively. In addition, a PCR amplified fragment (Z) that was used to generate the CI-766 genome (Engel et al., 2008), which corresponds to nucleotide positions 2,719 and 3,764 of the genome matches the specified region predicted for recombination event 4 (though the recombination event is 151nt longer than the fragment). Thus, it is likely the three recombination events are experimental artefacts or chimeras. Recombination event 1 predicted
for the NY1 genome does not correspond with the clones used to generate the genome sequence. Therefore it is unlikely this event is an experimental artefact or chimera.

Figure 3.16: Diagrammatic representation of the recombination events identified in (a) NY1 and (b) CI-766. (a) Three recombination events (events 1 to 3) were identified for NY1 involving a group 1 isolate (WA-MR) as the major parent (blue) and a group 2 isolate (623) as the minor parent (purple). (b) One recombination event (event 4) was identified for CI-766 involving a group 1 isolate (NZ-PG1) as the major parent (red) and a group 5 isolate (NZ-WCA) as the minor parent (orange). Recombination events are numbered and correspond to Table 3.10, which were predicted using RDP v3.44 (Martin et al., 2010).

| Table 3.10: Recombination events identified within GLRaV-3 isolates NY1 and CI-766. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Event  | Break point (nt) | Parent isolates | Model          | p-value          |
|        | Start | End   | Major           | Minor           |                 |
| NY1    |       |       |                 |                 |                 |
| 1      | 1079  | 3247  | WA-MR (Group 1) | 623 (Group 2)   | RDP             | 1.42x10^{-25}  |
|        |       |       |                 |                 | GENECONV        | 5.28x10^{-61}  |
|        |       |       |                 |                 | BootScan        | 1.12x10^{-25}  |
|        |       |       |                 |                 | MaxChi          | 9.08x10^{-11}  |
|        |       |       |                 |                 | Chimaera        | 8.22x10^{-11}  |
|        |       |       |                 |                 | SiScan          | 1.59x10^{-12}  |
|        |       |       |                 |                 | 3Seq            | 2.42x10^{-20}  |
| 2      | 4330  | 4755  | WA-MR (Group 1) | 623 (Group 2)   | RDP             | 4.09x10^{-32}  |
|        |       |       |                 |                 | GENECONV        | 8.40x10^{-57}  |
|        |       |       |                 |                 | BootScan        | 3.35x10^{-32}  |
|        |       |       |                 |                 | MaxChi          | 8.72x10^{-11}  |
|        |       |       |                 |                 | Chimaera        | 4.60x10^{-11}  |
|        |       |       |                 |                 | SiScan          | 1.29x10^{-12}  |
|        |       |       |                 |                 | 3Seq            | 1.05x10^{-14}  |
| 3      | 17444 | 17654 | WA-MR (Group 1) | 623 (Group 2)   | RDP             | 2.07x10^{-13}  |
|        |       |       |                 |                 | GENECONV        | 4.60x10^{-9}   |
|        |       |       |                 |                 | BootScan        | 2.44x10^{-12}  |
| CI-766 |       |       |                 |                 |                 |                 |
| 4      | 2577  | 3774  | NZ-PG1 (Group 1) | NZ-WCA (Group 5) | RDP             | 8.85x10^{-29}  |
|        |       |       |                 |                 | BootScan        | 1.26x10^{-30}  |
|        |       |       |                 |                 | MaxChi          | 9.64x10^{-9}   |
|        |       |       |                 |                 | Chimaera        | 2.26x10^{-9}   |
|        |       |       |                 |                 | SiScan          | 1.19x10^{-9}   |
|        |       |       |                 |                 | 3Seq            | 6.01x10^{-9}   |

*a Based on the NY1 (AF037268) and CI-766 (EU344893) numbering.
*b The parental isolates detected by the RDP v3.44 program are not the actual parent isolate but are the sequences most similar to the actual parents in the analysed dataset.
3.3.6 Amino acid composition analysis

The amino acid composition for each ORF is fundamental to the formation of the protein structure and biological activity. Based on the sequence analysis of individual genes, the amino acid identities between GLRaV-3 isolates, NY1, NZ1-B, GH11, and NZ2, were highly variable and ranged between 13.9 to 98.6% (Section 3.3.1.2). Thus, to further understand how the amino acid substitutions observed in the divergent NZ isolates, NZ1-B and NZ2, may affect the resultant protein, multiple amino acid sequence alignments were used to identify any amino acid substitutions that are unique to individual isolates NZ1-B and NZ2, unique to group 6 isolates (GH11, GH30, and NZ1-B), and common to all four divergent isolates (GH11, GH30, NZ1-B, and NZ2) compared to other available sequences. Any unique amino acid substitutions were further classified into conservative (change in amino acid with similar physiochemical properties) and non-conservative substitutions (change in amino acid with different physiochemical properties) using NY1 as the reference sequence.

The total number of amino acid substitutions unique to each isolate and group of isolates for each ORF ranged between 9 and 129 (Table 3.11). Proteins HSP90h, CPm, and p19.6 had the greatest number of substitutions with 129, 118, and 109 respectively. NZ2 proteins consistently contained a high number of unique substitutions and also shared a high number of substitutions with isolates from group 6. For example, in the p19.7 protein NZ2 has 20 unique substitutions plus an additional 22 amino acid substitutions found only in itself and group 6 isolates. Importantly, more than half of the substitutions were conservative and are not likely to modify the protein (Table 3.11). However, all proteins except for the replicase, p5, CP, and p7, contained at least ten non-conservative substitutions and in particular, HSP90h, CPm, and p19.6 proteins contained 40 or more non-conservative substitutions (Table 3.11). The distribution of non-conservative substitutions throughout the different proteins varied, with substitutions both uniformly scattered throughout the protein and clustered in a particular region or towards either the N’ or C’ protein terminus. In addition, predicted protein secondary structures for NZ-1B and NZ2 were different compared to other GLRaV-3 isolates, particularly in regions with non-conservative substitutions.

As examples, the amino acid substitutions and predicted protein secondary structures for the CP and p19.7 proteins are described in more detail below.
Table 3.11: Amino acid substitutions within each protein that are unique to individual isolates NZ1-B and NZ2, unique to phylogenetic group 6, and unique to all divergent GLRaV-3 isolates (NZ1-B, NZ2, GH11, GH30) when compared to NY1.

<table>
<thead>
<tr>
<th></th>
<th>ORF1a (Replicase)</th>
<th>ORF1b (RdRp)</th>
<th>ORF3 (p5)</th>
<th>ORF4 (HSP70h)</th>
<th>ORF5 (HSP90h)</th>
<th>ORF6 (CP)</th>
<th>ORF7 (CPm)</th>
<th>ORF8 (p21)</th>
<th>ORF9 (p19.6)</th>
<th>ORF10 (p19.7)</th>
<th>ORF11 (p4)</th>
<th>ORF12 (p7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NZ1-B</strong></td>
<td>O&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>O&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>O&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>O&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>O&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>O&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ORF1a</td>
<td>3</td>
<td>0</td>
<td>n/a</td>
<td>18</td>
<td>3</td>
<td>n/a</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>n/a</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>ORF1b</td>
<td>8</td>
<td>4</td>
<td>n/a</td>
<td>42</td>
<td>11</td>
<td>n/a</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>n/a</td>
<td>8</td>
</tr>
<tr>
<td>ORF3</td>
<td>8</td>
<td>3</td>
<td>27</td>
<td>6</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>33</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>ORF4</td>
<td>6</td>
<td>3</td>
<td>33</td>
<td>16</td>
<td>33</td>
<td>15</td>
<td>1</td>
<td>33</td>
<td>2</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>ORF5</td>
<td>19</td>
<td>10</td>
<td>42</td>
<td>15</td>
<td>30</td>
<td>9</td>
<td>27</td>
<td>16</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ORF6</td>
<td>14</td>
<td>6</td>
<td>41</td>
<td>16</td>
<td>37</td>
<td>18</td>
<td>17</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF7</td>
<td>5</td>
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<td>20</td>
<td>11</td>
<td>16</td>
<td>5</td>
<td>22</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF8</td>
<td>2</td>
<td>0</td>
<td>20</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORF9</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Amino acid substitutions common to phylogenetic group 6 isolates NZ1-B, GH11, and GH30.
b Amino acid substitutions common to divergent isolates NZ1-B, NZ2, GH11, and GH30.
c O = Overall number of amino acid substitutions
d NC = Non-conservative amino acid substitutions
e Based on the partial ORF1a amino acid sequence (213 amino acid).
f Based on the partial ORF4 amino acid sequence (472 amino acid).
g NZ2 is six amino acids larger than all other GLRaV-3 isolates, not included in this analysis.
h Divergent isolates have smaller ORF12 amino acid sequences, analysis only included 55 amino acids.

3.3.6.1 CP amino acid composition analysis

Initially, the complete CP amino acid sequences of GH11, NZ1-B, and NZ2 were compared to NY1. There are 38 amino acid differences between these four variants, generally positioned closer to the N-terminal end (Figure 3.17). Of the 38 amino acid changes 24 are conservative and 8 out of the 14 non-conservative changes are considered neutral substitutions, i.e. a change in amino acid with different physiological properties that is less likely to affect the protein structure and/or activity (Betts et al., 2003). Five out of the six remaining non-conservative amino acid changes are located between amino acid positions 70 to 84 (Table 3.12 and Figure 3.17). In NY1 all five of these amino acids are polar and would be expected to be found on the protein surface or at active site(s), whereas in the divergent GLRaV-3 genetic variants these amino acids have been substituted by
small hydrophobic amino acids with non-reactive side chains that are rarely directly involved in protein function (Betts et al., 2003).

**Table 3.12:** Non-conservative amino acid substitutions identified in the CP of divergent isolates NZ1-B, NZ2, GH11, and GH30 compared to NY1.

<table>
<thead>
<tr>
<th>Amino acid position</th>
<th>Amino acid substitution</th>
<th>Unique to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>Glutamine → Proline</td>
<td>GH11, GH30, NZ2</td>
</tr>
<tr>
<td></td>
<td>Glutamine → Alanine</td>
<td>NZ1-B</td>
</tr>
<tr>
<td>71</td>
<td>Glutamic acid → Valine</td>
<td>Group 6</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid → Alanine</td>
<td>NZ2</td>
</tr>
<tr>
<td>75</td>
<td>Glutamine → Proline</td>
<td>All</td>
</tr>
<tr>
<td>79</td>
<td>Glutamine → Proline</td>
<td>All</td>
</tr>
<tr>
<td>84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Glutamine → Proline</td>
<td>All</td>
</tr>
<tr>
<td>206</td>
<td>Valine → Asparagine</td>
<td>NZ2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Amino acid substitution also occurs in other GLRaV-3 isolates from groups 2-5.

**Figure 3.17:** Multiple amino acid sequence alignment of the complete coat protein (open reading frame 6) of *Grapevine leafroll-associated virus 3* isolates NY1 (reference sequence) compared to divergent isolates GH11, GH30, NZ1-B, and NZ2. All amino acid substitutions are highlighted in colour, with neutral and unfavourable non-conservative substitutions indicated with blue and red squares above the NY1 sequences, respectively. All multiple sequence alignments were generated in Geneious v5.5 (Drummond et al., 2011).

When the NZ1-B and NZ2 complete CP amino acid sequences are compared to other sequences from groups 1 to 5, there are only 27 amino acid substitutions unique to the four divergent isolates (GH11, GH30, NZ1-B, and NZ2). Nine out of the 27 substitutions are non-conservative and include four substitutions located at amino acids 70, 71, 75, and 79 (Figure 3.18a). These match the non-conservative amino acid changes identified previously, based on comparisons to the NY1 isolate, and are likely to lead to changes to the protein (described above). The previous non-conservative substitution at amino acid 84 (described above)
involving a change from a glutamine to a proline, is not unique to NZ1-B, NZ2, GH11, or GH30, as it is also present in other GLRaV-3 isolates from groups 2-5.

The predicted protein secondary structure showed significant differences between the GLRaV-3 isolates from groups 1 to 5 and the four more divergent isolates, particularly at the variable 5’ region (Figure 3.18b). Most of the variability was observed between amino acids 70 and 88, which correspond to most of the non-conservative substitutions. In particular, a coil-turn structure is predicted in all groups 1 to 5 between amino acids 71 to 74, while beta-sheets or coils are present in NZ1-B, GH11, GH30, and NZ2. Additional structural differences between isolates were also observed in positions where conservative amino acid substitutions occur at the N-terminus.

![Image of secondary structure](image)

**Figure 3.18:** Comparison between *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates NY1 (reference sequence) compared to GLRaV-3 isolates from groups 2 to 6 and NZ2 for the complete coat protein sequence using (a) a multiple amino acid sequence alignment, and (b) the corresponding predicted secondary structures for each isolate. (a) All amino acid substitutions are highlighted in colour. (a and b) Neutral and unfavourable non-conservative substitutions indicated with blue and red rectangles above the NY1 sequence, respectively. All multiple sequence alignments were generated in Geneious v5.5 (Drummond et al., 2011).
3.3.6.2 p19.7 amino acid composition analysis

In total there are 63 amino acid substitutions that were unique to NZ1-B (5) and NZ2 (20), and common to group 6 (16) and to all four divergent isolates (22), based on a multiple amino acid sequence alignment that included all available sequences from groups 1 to 5 (Table 3.11). Twenty-seven of the 63 substitutions are non-conservative and are distributed throughout the protein from amino acid 24 to 167 (Figure 3.19a). Of these substitutions 14 are considered to have an increased likelihood of affecting the protein structure and/or functionality (Table 3.13), and are generally clustered in two regions between amino acids 76 to 92 and 112 to 132 (Figure 3.19a).

Table 3.13: Non-conservative amino acid substitutions identified in the p19.7 protein of divergent isolates NZ1-B, NZ2, GH11, and GH30 compared to NY1 and other isolates from phylogenetic groups 2 to 5.

<table>
<thead>
<tr>
<th>Amino acid position</th>
<th>Amino acid substitution</th>
<th>Unique to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Threonine → Leucine</td>
<td>All</td>
</tr>
<tr>
<td>33</td>
<td>Aspartic acid → Valine</td>
<td>Group 6</td>
</tr>
<tr>
<td>76</td>
<td>Phenylalanine → Serine</td>
<td>NZ2</td>
</tr>
<tr>
<td>77</td>
<td>Alanine → Asparagine</td>
<td>All</td>
</tr>
<tr>
<td>80</td>
<td>Glutamine → Alanine</td>
<td>All</td>
</tr>
<tr>
<td>90</td>
<td>Glycine → Asparagine</td>
<td>All</td>
</tr>
<tr>
<td>92</td>
<td>Leucine → Threonine</td>
<td>Group 6</td>
</tr>
<tr>
<td>112</td>
<td>Arginine → Valine</td>
<td>NZ2</td>
</tr>
<tr>
<td>122</td>
<td>Glycine → Lysine</td>
<td>All</td>
</tr>
<tr>
<td>123</td>
<td>Tyrosine → Arginine</td>
<td>NZ2</td>
</tr>
<tr>
<td>127</td>
<td>Glutamine → Leucine</td>
<td>All</td>
</tr>
<tr>
<td>132</td>
<td>Tyrosine → Asparagine</td>
<td>NZ2</td>
</tr>
<tr>
<td>157</td>
<td>Glycine → Glutamic acid</td>
<td>All</td>
</tr>
</tbody>
</table>

Similar to the CP, five substitutions involve a change from amino acids with polar characteristics that are likely to be located in enzymatic regions to hydrophobic amino acids that are generally expected buried within the protein hydrophobic core. For example, at position 24 the NY1 p19.7 protein has a polar threonine amino acid while in all four divergent isolates this is substituted with a hydrophobic leucine. In addition, the threonine and leucine amino acids also prefer to be involved in two different secondary structures, with threonine preferring to lie within beta-sheets while leucine prefers to adopt an alpha-helical conformation (Betts et al., 2003). A further six non-conservative substitutions involve a substitution from amino acids (in the NY1 isolate) with hydrophobic characteristics to polar amino acids in the divergent GLRaV-3 isolates.
Noticeably, three glycine amino acids in the NY1 isolate at positions 90, 122, and 157 are substituted for larger polar amino acids asparagine, lysine, and glutamate (glutamic acid) respectively, in the divergent GLRaV-3 isolates. These non-conservative substitutions may also have a significant impact on the protein structure and activity, as glycine is commonly located in areas, such as tight turns, that are prohibited by other amino acids (Betts et al., 2003). This is due to the amino acid possessing a hydrogen as a side chain, which provides greater conformational flexibility compared to all other amino acids (Betts et al., 2003).

Unlike the CP, the differences between predicted secondary structures for the p19.7 protein of different GLRaV-3 isolates were extensively distributed throughout the protein (Figure 3.19b). This is in accordance with the high level of amino acid substitutions identified in the protein, which were also spread throughout the protein. Noticeably, less alpha-helix structures are predicted in NZ2 with more turns and longer beta-sheets predicted, compared to all other isolates.

**Figure 3.19**: Comparison between *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates NY1 (reference sequence) and GLRaV-3 isolates from groups 2 to 6 and NZ2 for the complete p19.7 protein sequence using (a) a multiple amino acid sequence alignment, and (b) the corresponding predicted secondary structures for each isolate. (a) All amino acid substitutions are highlighted in colour. (a and b) Neutral and unfavourable non-conservative substitutions indicated with blue and red rectangles above the NY1 sequence, respectively. All multiple sequence alignments were generated in Geneious v5.5 (Drummond et al., 2011).
3.4 Discussion

Different virus isolates/strains may differ in the ability and/or efficiencies of important virus functions such as virus replication, virion assembly and movement, virulence, and transmissibility. Thus, the examination of extended sequences, deducing an increased number of complete ORFs, allows for improved understanding of the genetic variation and evolution of viruses. Additionally it aids predictions for potential differences in virus activity based on sequence variations between virus isolates/strains within genes and UTRs.

Considerable genetic variability was previously observed within New Zealand GLRaV-3 isolates based on reasonably short sequence fragments (<600nt) (Chapter 2). Thus, to extend our understanding of genetic variation from the initial sequencing, five GLRaV-3 isolates representative of five different phylogenetic clades were selected for further molecular characterisation. In particular two divergent isolates, NZ1-B and NZ2, which differed from GLRaV-3 isolates in phylogenetic groups 1 to 5 by more than 20%, were included. This lead to three complete GLRaV-3 genome sequences and partial genomes of two further isolates, which when further analysed identified a number of features that possibly influence virus functionality.

3.4.1 Comparisons between the five New Zealand isolates to other GLRaV-3 isolates from around the world

Based on the phylogenetic analysis and genome organisation it is evident NZ-PG1, NZ-R2D4, and NZ-WCA are members of GLRaV-3 phylogenetic groups 1, 2, and 5 respectively. There is high similarity to isolates found in the respective groups and the genome organisation is identical to previously described isolates GP18, 621, 623, and PL-20 from South Africa (Jooste et al., 2010; Maree et al., 2008) and WA-MR from USA (Jarugula et al., 2010).

In contrast, NZ1-B and NZ2 have substantial sequence differences and slight differences in genome organisation compared to isolates from groups 1 to 5. The NZ-1 isolate was recently proposed to be a member of the phylogenetic group 6 (Bester et al. 2012). NZ1-B is a variant of the NZ-1 (99.6% nucleotide identity) thus NZ1-B should also be classified as a group 6 isolate. Based on the results from this study, the NZ1-B isolate is also most closely related to other group 6 isolates with an overall nucleotide identity of 91.5% based on the partial genome sequence.
(11,827nt). In addition, NZ1-B shares two key genome characteristics with group 6 GLRaV-3 isolates GH11 and GH30, with no ORF2 and a six amino acid shorter ORF12 compared to isolates from groups 1 to 5. However, phylogenetic analysis from this project questions the validity of this proposed group 6 suggesting that NZ-1-like variants such as NZ1-B may represent a separate phylogenetic group.

From the genetic variability study in Chapter 2, phylogenetic analysis based on a 564nt region of ORF4 revealed two distinct clades within the phylogenetic group 6, one clade consisted of the NZ-1 and NZ-1-like isolates such as NZ1-B and the other consisted of the GH11 and GH30 isolates (100% bootstrap confidence). The average nucleotide difference between these two clades was 5.63%. This is similar to the average nucleotide differences between groups 1 to 5, which range from 4.1 to 9.6% (Chapter 2). In addition, the phylogenetic analysis based on a 428nt region of the CP revealed high (7.4%) intra-group variation between isolates from USA (21-12, 22-15, and 44-2), South Africa (GH11 and GH30), and New Zealand (NZ1-B) within the proposed group 6 (forming three potentially distinct clades), while the intra-group variation observed within each of the established phylogenetic groups 1 to 5 was only 0.6 to 0.9%. Both analyses were further supported when the longer 11,827nt partial genome sequence was also analysed, which revealed the separation of NZ1-B from the GH11 and GH30 isolates within the proposed group 6. Therefore, to ensure the phylogenetic classification is consistent for all GLRaV-3 isolates, based on the current approach for classification, the NZ1-B isolate should be considered a representative of a distinct phylogenetic group.

Key sequence-based determinants for the species demarcation in the Ampelovirus genus includes the size of the CP (based on the amino acid sequence), genome structure and organisation, and more than 25% amino acid sequence difference of relevant gene products, such as the CP, CPm, and HSP70h (King et al., 2011). The NZ2 isolate has low nucleotide identity to other GLRaV-3 isolates from groups 1 to 6 and NZ1-B (based on 7,613nt), being more than 20% different to the next most closely related GLRaV-3 variant. However, the size of the NZ2 CP is similar to other GLRaV-3 isolates (34.7kDa), and the CP, CPm, and HSP70h amino acid sequences have relatively high identity compared to other GLRaV-3 isolates with 91.4, 77.1, and 84.5% amino acid identity to NY1 respectively, and 94.9, 81.8, and 90.2% amino acid identity to GH11 respectively. In addition, the order and relative
positions of the nine ORFs are similar to previously described GLRaV-3 isolates, with only minor differences observed in ORF11 and ORF12. ORF11 overlaps ORF10 and extends further into ORF12, leading to a shorter ORF12. However, variation in the translation of ORF11 was also found by Wang et al. (2011) in GLRaV-3 variants from group 4, which requires the use of an alternative start codon (ACG). Consequently, although NZ2 is substantially different from previously described variants of GLRaV-3, based on the sequence currently available it is not sufficiently different to be considered a new species, rather it is more likely represents a new GLRaV-3 strain or phylogenetic group.

### 3.4.2 Recombination

Recombination is an important contributor to virus evolution that has been found in a number of different plant viruses including GLRaV-3 (Chapter 2). No recombination events were detected in the full genome sequences of GLRaV-3 NZ-PG1, NZ-R2D4, and NZ-WCA isolates, or the partial genome sequences of the divergent NZ1-B (11,827nt) and NZ2 (7,613nt) isolates.

In contrast, three recombination events were identified in the NY1 and one in CI-766 isolates from USA and Chile respectively, all of which involved a group 1-like major parent isolate and a group 2-like or group 5-like minor parent isolate. This is the first report of recombination occurring within both isolates. Although only recombination event 1 within the NY1 isolate is likely to be representative of an ancestral recombination event.

Limited sequence data from different phylogenetic groups and limited recombination analysis conducted in other studies may account for oversights in detecting these recombination events predicted for both GLRaV-3 isolates. The NY1 and CI-766 isolates were the first two complete genomes to be publically available, thus it was not possible for authors to identify recombination events within these isolates due to the absence of available sequence for analysis. While recombination analyses were not conducted in more recent reports (Bester et al., 2012b; Jarugula et al., 2010; Jooste et al., 2010; Maree et al., 2008) describing complete GLRaV-3 genomes. Wang et al. (2011) sequenced a 4.7kb section of GLRaV-3 (encompassing ORF6 to ORF12 and 228nt of the 3’UTR) of 50 GLRaV-3 isolates from the Napa Valley. No recombination events were detected in these 50 isolates based on analysis of these sequences using the RDP v3.15
implementing six recombination algorithms (RDP, GENECONV, Bootscan, MaxChi, Chimaera, and 3Seq) (Wang et al., 2011). Wang et al. (2011) did not include published sequences that coincided with this region into this recombination analysis and consequently, the recombination event 3 that occurs in the NY1 isolate between nucleotides 17,444 and 17,654 was overlooked (Section 3.3.5). Therefore, it is likely with more sequence data representative of greater numbers of phylogenetic groups in combination with further recombination analysis, more recombination events that may have occurred will be revealed.

3.4.3 Analysis of the potential cis-acting elements within GLRaV-3

RNA viruses contain cis-acting elements that have a number of functions that include the initiation for the synthesis of sgRNAs, regulating gene expression, virion movement, RNA stability, and induction of disease (Gowda et al., 2003b). To investigate potential cis-acting elements within the 5’UTR, 3’UTR, and 5’TSS for seven sgRNAs of GLRaV-3 isolates, sequences from this study and the other available complete genomes were analysed and compared.

3.4.3.1 5’UTR

The 5’UTR has been shown to be critical for controlling virus replication and virion assembly for many plant viruses including CTV (Gowda et al., 2003b), Brome mosaic virus (Marsh et al., 1987), Turnip yellow mosaic virus (TYMV) (Hellendoorn et al., 1997), and Papaya mosaic virus (Abouhaidar et al., 1978; Sit et al., 1994). The size of 5’UTR varies considerably within the Closteroviridae family, ranging from 105 to 737nt. For GLRaV-3, the 5’UTR sizes reported thus far range from 158 to 737nt, although subsequent studies have concluded that the 158nt 5’UTR determined for both NY1 and CI-766 represent incomplete sequences (Jarugula et al., 2010; Maree et al., 2008). This is most likely due to inaccuracies in the amplification of the 5’terminal ends (Jarugula et al., 2010). The NY1 5’UTR was determined using poly(A) tailing and an oligo (dT) primer and since the 5’UTR is AU rich, it is likely that the oligo (dT) primer non-specifically bound to other parts of the sequence (Jarugula et al., 2010). In addition, the 5’UTR of CI-766 was elucidated by using specific primers designed against the truncated 5’UTR of NY1, thus leading to the smaller sequence.
In this study, the 5′RACE kit was used to determine the complete 5′UTRs for three isolates that are members of three different phylogenetic groups (groups 1, 2, and 5). All three isolates generated a 737nt 5′UTR sequence, thus further supporting the authenticity of the extended 5′UTR. Smaller sized 5′UTRs for GLRaV-3 have also been reported for two South African isolates, PL-20 (group 3) and GH30 (group 6), with sizes of 672nt and a 642nt respectively. These shorter 5′UTRs are likely to be authentic as 5′RACE was used to accurately map the 5′end.

The genetic diversity between the 5′UTRs of CTV isolates is considerably high with intragroup nucleotide identities of more than 88% and intergroup nucleotide identities as low as 44% (Gowda et al., 2003b). Despite the high genetic diversity, for all CTV isolates, the predicted secondary structures of the positive-sense sequences are well conserved, with two simple SL structures, which are integral for virus replication and virion assembly (Gowda et al., 2003b). In contrast, although the 5′UTRs of GLRaV-3 isolates share higher nucleotide identities, the predicted secondary structures between phylogenetic groups are different. With the inclusion of divergent GLRaV-3 isolates GH11 and GH30, the overall 5′UTR intergroup nucleotide identity between GLRaV-3 isolates is approximately 67%.

Unsurprisingly, the 5′UTRs of New Zealand isolates NZ-PG1 and NZ-R2D4 have high (99%) nucleotide identity to other isolates from groups 1 and 2, respectively. While the NZ-WCA, representative of group 5, has 69% and 83% nucleotide identity compared to isolates from groups 1 and 2, respectively (based on the complete 737nt 5′UTR sequence). In addition, the intragroup nucleotide identity for phylogenetic groups 1 and 2 was approximately 99%. The predicted secondary structures for GLRaV-3 5′UTRs are long complex structures that contain numerous substructural hairpins of different lengths that are poorly conserved between GLRaV-3 isolates. Thus, GLRaV-3 likely uses a different system for virus replication and assembly (Jarugula et al., 2010), which may involve the well conserved RNA sequence at the start of the 5′UTR (discussed further in Chapter 6, Section 6.3.1). In addition, it is also possible the considerable differences in sequence and structure may signify differences in functional efficiencies between GLRaV-3 variants.
3.4.3.2 3′ UTR

The 3′UTR is integral for virus replication as it has been shown to be involved in the initiation of complementary negative strands (Dreher, 1999; Satyanarayana et al., 2002). In addition, other 3′UTR functions include controlling translation, RNA stability, cellular targeting, assembly, and inhibition of minus-strand synthesis in the later stages of virus infection (Dreher, 1999; Satyanarayana et al., 2002; Singh et al., 1998).

Recently, Jarugula et al. (2010) compared the 3′UTR from four GLRaV-3 isolates from group 1 and 2, which revealed similar sequence lengths (277nt), high sequence identity (95.7%), and folded into identical secondary structures. This is similar to CTV, as the 3′UTR nucleotide identity between CTV isolates was approximately 95% and the predicted secondary structure comprised of 10 SL structures, common to all CTV isolates (Satyanarayana et al., 2002). However, with the identification of divergent isolates such as NZ1-B and NZ2, the length and sequence variability of GLRaV-3 3′UTRs vary considerably. The lengths vary from 264nt to 289nt and the overall average nucleotide identity between isolates representative of each phylogenetic groups was approximately 89.8%. This is similar to BYV, where the 3′UTR of BYV-U and BYV-Cal isolates were 166nt and 182nt respectively, and share 89.6% nucleotide identity (Agranovsky et al., 1991; Peremyslov et al., 1998).

Potential cis-acting elements that are critical for virus replication probably have conserved primary sequence and/or secondary structures, similar to conserved replication signals found in the 3′UTR of CTV. The sequence variation along the 3′UTR of GLRaV-3 isolates examined was unevenly distributed, with the highest variation closest to ORF12 where nucleotide similarities decreased to as low as 50%, while the average nucleotide similarities between all isolates are more than 90% within two regions from nucleotides 18,320 to 18,382, and 18,430 to 18,498 (based on GP18 numbering). Thus, these areas of high conservation may represent possible cis-acting elements important for controlling GLRaV-3 replication.

CCR initiation sites/boxes are important for the initiation of minus-strand synthesis for TYMV (Singh et al., 1998) and CTV (Satyanarayana et al., 2002). In vitro experiments showed that the CCA motif present in short linear RNAs was able to
initiate transcription using the RdRps from QB bacteriophage, TYMV, and turnip crinkle virus without other promoter elements (Yoshinari et al., 2000). Many plant RNA viruses contain 3′terminal CCR motifs (Dreher, 1999), including CTV which has a 3′terminal CCA and an additional four within the 3′UTR (Satyanarayana et al., 2002). The 3′UTRs of all GLRaV-3 isolates end with a 3′terminal GTC, instead of terminating with CCR. However, within the regions of high similarity, three CCR motifs are conserved in all isolates with a CCG motif at nucleotides 18,382 to 18,384, and two CCA motifs at nucleotides 18,427 to 18,429 and nucleotides 18,434 to 18,436. The NZ1-B also contains an additional CCA motif at nucleotides 18,438 to 18,440. With multiple CCR motifs, there needs to be an additional specificity mechanism(s) to generate specific initiation by RdRps that responds to specific CCA initiation boxes. Two proposed systems are the preferential initiation from the 3′-most CCA, and use of secondary structures to control the availability of CCA potential initiation sites (Singh et al., 1998; Yoshinari et al., 2000). Mutational analysis showed that the 3′terminal CCA in CTV 3′UTR was needed to initiate replication, however exposing an internal CCA motif restored replication (Satyanarayana et al., 2002). In addition, the secondary structures formed in TYMV that make internal motifs inaccessible and others assessable to TYMV RdRp, resulted in preferential initiation from certain sites (Singh et al., 1998; Yoshinari et al., 2000). Given that a 3′terminal CCA is not present in GLRaV-3 3′UTRs it is possible the secondary structure plays a role in exposing the motif to start replication.

A similar secondary structure was predicted for the positive-sense 3′UTR of all GLRaV-3 isolates, located at the terminal 3′end, using the mFOLD web server. The predicted secondary structure is complex with four substructural hairpins and interior loops of various sizes. The CCR motifs are located in the same positions for all isolates, with CCR motifs located in the stem of two substructural hairpins and a CCA motif is located near a loop at the top of a substructural hairpin, which exposes the CCA motif making the motif more likely to be accessible to the RdRp. In addition, the secondary structures for the negative-sense 3′UTR RNA of all GLRaV-3 isolates were also predicted. A complex SL structure located at the terminal 5′end of the negative-sense RNA (i.e. the terminal 3′end of the positive strand) was similar for all isolates and consisted of four substructural hairpins and interior loops of various sizes, similarly described by Jarugula et al. (2010).
Pseudoknot structures have been shown to be involved in the RNA replication of plant viruses such as bamboo mosaic potexvirus (Cheng et al., 2002), and TYMV (Deiman et al., 1997). Because of the low stability of the structure, it has been proposed the pseudoknots may have a regulatory function of controlling replication and translation of the viral RNA genomes (Deiman et al., 2000). A 3'terminal pseudoknot structure was predicted in the NY1 isolate and other members of the Closteroviridae family using the STAR algorithm (Livieratos et al., 2004). In this study, the mfold webserver used to predict RNA structures is unable to predict pseudoknots (Eddy, 2004). Thus to predict whether the predicted pseudoknot structure is conserved in all GLRaV-3 isolates, the multiple sequence alignment of the GLRaV-3 3'UTR sequences was used to search for the sequence motif GACCUA predicted by Livieratos et al. (2004) to be involved in the pseudoknot for NY1. The predicted motif was conserved in all GLRaV-3 isolates at nucleotides 18,449 to 18,454, and the complementary sequence UAGGUC was also conserved in all isolates, located at the 3'termius (from nucleotides 18,493 to 18,498).

Despite the variability in 3'UTR size and sequence identity between GLRaV-3 isolates, a number of primary and secondary structural features are well conserved. Thus, it is probable all or some of these conserved features act as 3'cis-acting elements that play an important role for GLRaV-3 replication; either functioning independently or in combination with each other. The extensive nucleotide and structural differences between GLRaV-3 isolates, at the start of the 5'terminal of the 3'UTR, may also have variant specific control features.

3.4.3.3 5 TSSs

The production of sgRNAs is the most common strategy employed by plant viruses to express their genome within a host (Mandahar, 2006), as the sgRNA generally acts as messenger RNA for the internal and 3'proximal genes. There are two types of sgRNA, 3'co-terminal or 5'co-terminal, and of these the 3'co-terminal sgRNA is most common for plant viruses (Mandahar, 2006). Recently, studies using plant material infected with GLRaV-3 isolates GP18 (Maree et al., 2010) and WA-MR (Jarugula et al., 2010) identified, in total, seven 3'co-terminal sgRNAs that consisted of sgRNAs for p5+HSP70h (ORF3 and 4), p55 (ORF5), CP (ORF6), CPm (ORF7), p21 (ORF8), p20A (ORF9), and p20B+p4+p7 (ORF10-12).
Jarugula et al. (2010) observed that the expression of the sgRNAs were variable with high expression of sgRNA(ORF6), sgRNA(ORF8), sgRNA(ORF9), and sgRNA(ORF10-12), while sgRNA(ORF3/4), sgRNA(ORF5), and sgRNA(ORF7), were barely detectable by Northern blotting. This indicated that the production of sgRNA is most likely under independent regulation to control the timing and amounts of different proteins in relation to the virus requirements during the virus life cycle.

Generally, the *cis*-acting elements regulating expression are located upstream from the ORF (Mandahar, 2006). For instance, the sequence upstream from ORFs of *Tobacco mosaic virus* (TMV), *Citrus tatter leaf virus* (CTLV), BYV, and CTV, have been implicated in the synthesis of the respective sgRNAs (Grdzelishvili et al., 2000; Peremyslov et al., 2002; Tatineni et al., 2009). For some BYV and CTV sgRNAs, a heptanucleotide repeat is conserved in the leader sequences (sequence between the 5’terminal nucleotide of the sgRNA and the start of the ORF) (Peremyslov et al., 2002), while for TMV and CTLV octanucleotide repeats in the surrounding sequence of the sgRNA 5’terminal nucleotide are conserved (Grdzelishvili et al., 2000; Tatineni et al., 2009). In addition, the secondary structure of the upstream sequences can also play a role in controlling sgRNA synthesis as shown for TMV, CTLV, and CTV (Gowda et al., 2001; Grdzelishvili et al., 2000; Tatineni et al., 2009). To examine potential *cis*-acting elements that may control the production of GLRaV-3 sgRNAs, Jarugula et al. (2010) and Maree et al. (2010) determined the 5’terminal nucleotide for four sgRNAs (ORF6, ORF8, ORF9, and ORF10-12) and seven sgRNAs (ORF3-4, ORF5, ORF6, ORF7, ORF8, ORF9, and ORF10-12), respectively, and mapped the TSSs to the virus genome. Both studies showed that sequence or secondary structure for sequence surrounding the 5’TSS was not conserved between all sgRNAs (Jarugula et al., 2010; Maree et al., 2010). In addition, sequence and predicted structures did not match other known plant viral *cis*-acting elements (Maree et al., 2010). However, key features of the secondary structures formed by the surrounding sequences of the sgRNA(ORF6), sgRNA(ORF8), sgRNA(ORF9), and sgRNA (10-12) were conserved between isolates from groups 1 and 2 (Jarugula et al., 2010). This suggests the secondary structures may play a role in GLRaV-3 sgRNA production (Jarugula et al., 2010).
To investigate whether similar secondary structure conservation of sequences surrounding the 5’nt (25nt up- and down-stream) of each sgRNA was also maintained in the highly divergent GLRaV-3 isolates, the TSSs elucidated by Jarugula et al. (2010) and Maree et al. (2010) were mapped to sequence from this study and from overseas was used. In addition, the corresponding secondary structures were predicted using the mfold web server and compared. The predicted 5’TSS nt of each sgRNA from all five NZ isolates, GH11, and GH30 were all purine nucleotides (i.e. either an adenine or guanine) and the length of leader sequences were similar except for the leader sequence of sgRNA(ORF6) of GH11, GH30, NZ1-B, and NZ2 that had an additional 5nt.

Considerable genetic variability was observed for isolates GH11, GH30, NZ1-B, and NZ2 leader sequences, with nucleotide identities ranging from 47.6 to 84.2% for NZ1-B, GH11, and GH30 and 28.7 to 86.3% for NZ2 compared to NY1 (group 1). Despite the high genetic variability, the predicted secondary structures were relatively conserved between the different GLRaV-3 isolates. Four out of the seven predicted structures for sequence surrounding the 5’TSS (25nt up- and down-stream) were similar for all GLRaV-3 isolates and the dissimilar structures were still generally shared for at least two isolates from different phylogenetic groups. For instance, the sequence surrounding the sgRNA(ORF7) from groups 1, 2, and 6 are predicted to form a long SL structure with the 5’TSS nt positioned inside the SL structure, while PL-20 (group 3), NZ-WCA (group 5), and NZ2 isolates generated two small SL structures with the 5’TSS nt located outside the structures. Therefore, the notable conservation of predicted structures for most sgRNAs amid the high variation provides further support for the theory that the secondary structures are likely involved in the sgRNA expression strategy in GLRaV-3.

3.4.4 Amino acid analysis

The amino acid composition is fundamental to the development of the protein structure and its biological activity. GLRaV-3 isolates NZ1-B, NZ2, GH11, and GH30 showed considerable amino acid differences compared to GLRaV-3 isolates from groups 1 to 5. For example, the amino acid identities ranged between 13.9 to 98.6% when compared to NY1 (group 1 isolate). These changes to the amino acid sequence may or may not translate into changes to the protein structure.
and/or functionality. Thus in this study, to understand the degree to which amino acid changes observed only in the divergent isolates may affect the resultant protein, the primary amino acid sequences for all available ORFs were analysed by comparing the type of properties involved in each substitution. In addition, the secondary structure was predicted to evaluate possible changes to the protein structure.

The total number of amino acid substitutions for each ORF that were unique to NZ1-B and NZ2, and common to all group 6 isolates or all four divergent isolates, ranged between 9 and 129 substitutions. More than half were conservative substitutions, which are not likely to modify the protein, thus the number of non-conservative substitutions ranged between 3 and 51 substitutions. In particular, the HSP90h, CPm, and p19.6 proteins showed considerably high numbers of non-conservative substitutions with at least forty unique amino acid changes. Furthermore, the high levels of amino acid substitutions lead to significant differences in the predicted secondary protein structures for NZ-1B and NZ2 compared to other GLRaV-3 isolates, particularly in regions with non-conservative substitutions. This indicates resultant proteins from divergent isolates such as NZ1-B and NZ2 are likely to be significantly different to other GLRaV-3 isolates and potentially affect a number of virus functions.

For example, the CP has an integral role in virion assembly, replication, and movement, as it is part of the quintuple gene block and forms the helical body for the virion, encapsidating approximately 95% of the viral RNA, which protects the viral RNA during transport (Dolja et al., 2006). Comparative analysis between CPs from divergent GLRaV-3 isolates (NZ-1B, GH11, GH30, and NZ2) and NY1 lead to the identification of 38 amino acid changes, including five non-conservative amino acid changes clustered between amino acid positions 70 to 84. Four of these are unique to the divergent GLRaV-3 isolates at amino acid positions 70, 71, 75, and 79. All four changes involved polar amino acids commonly positioned on the protein surface or at active site(s) that are substituted for small hydrophobic amino acid in all divergent GLRaV-3 genetic variants, which have non-reactive side chains and are rarely directly involved in protein function (Betts et al., 2003). Changes in this region also correlated with the significant differences in predicted secondary structures. Although, experiments such as site-directed mutagenesis have not been conducted to confirm the importance of these substitutions,
differences in the immunological reactivity to the Bioreba monoclonal antibody between GLRaV-3 isolates with high identity with NY1 and the divergent NZ1-B and NZ2 isolates have been observed (Chapter 4). Since the Bioreba monoclonal antibody was prepared against the NY1 isolate, this supports the idea that the protein structures of these divergent isolates are different to NY1, which is most likely because of the four non-conservative substitutions, mentioned above, that are unique to the divergent isolates (Chapter 4).

Amino acid substitutions in the GLRaV-3 CP may lead to changes in the ability for the protein to conduct a number of different functions such as replication, movement, and/or cause different GLRaV-3 variants to have varying biological implications for the host and virus transmissibility, as discussed in Chapter 6. This has been reported in other plant viruses. For instance, amino acid sequence comparisons between Cucumber mosaic virus (CMV) mosaic-inducing and chlorosis-inducing strains revealed the amino acid at position 129 is an important determinant for chlorosis infection. All mosaic-inducing strains have proline at this position while all chlorosis-inducing strain CPs have a serine or leucine (Shintaku, 1991). In addition, predicted secondary structures were different in this position with proline disrupting the predicted beta-sheet (Shintaku et al., 1992).

The effect of the single amino acid substitution to symptom development was confirmed by two mutational analysis experiments. Shintaku et al. (1992) used site-directed mutagenesis to change the leucine at position 129 of the CP from a chlorosis-inducing strain to a proline, which resulted in Nicotiana tabacum developing green mosaic symptoms rather than chlorosis. The opposite change in phenotype was also observed, when proline was substituted for serine (Shintaku et al., 1992). Mochizuki et al. (2011) conducted a more extensive mutational study, replacing the proline with 19 different amino acids and this lead to the N. tabacum developing six types of symptoms ranging from white mosaic to systemic necrosis. Thus, experiments support residue 129 in the CP of CMV, in particular the local secondary structure surrounding amino acid 129, plays a role in the symptom development.

In another example, amino acid substitutions within the CP of various potyviruses can have an effect on aphid transmissibility of the virus (Atreya et al., 1991; Atreya et al., 1995). A three amino acid motif (aspartic acid-alanine-glycine) located near the N-terminus of the CP is conserved in aphid-transmissible potyviruses, including
Tobacco vein mottling virus (TVMV) (Atreya et al., 1991; Atreya et al., 1995). Various site-directed mutations to this triplet of amino acids and the amino acid directly adjacent to the motif in TVMV CPs have been tested, with some mutations resulting in the loss or significant reduction in aphid transmissibility. For instance, the mutation of alanine to threonine drastically reduced virus transmission by aphids (Atreya et al., 1991). In addition, sequence comparisons of potyvirus CPs indicated glycine is particularly critical for aphid transmissibility, as potyviruses that had lost aphid transmissibility generally had changes to glycine (Atreya et al., 1991). Even after the glycine was substituted with alanine, which is the most similar amino acid to glycine, aphid transmissibility was severely reduced to 7% (Atreya et al., 1995).

Recently, the p19.7 protein of GLRaV-3 has been identified as an RNA silencing suppressor (Gouveia et al., 2012a). RNA silencing suppressors are viral proteins that impede the host RNA silencing defences by targeting different stages of the silencing pathway, such as binding to the siRNAs (Lakatos et al., 2004; Senshu et al., 2009) and dsRNAs (Lakatos et al., 2006; Senshu et al., 2009), and interacting with host proteins AGO1 (Baumberger et al., 2007; Senshu et al., 2009; Zhang et al., 2006) and Dicer-like 2 and 4 (Deleris et al., 2006; Senshu et al., 2009). The activity of five GLRaV-3 p19.7 proteins, one from each of the phylogenetic groups 1 to 5, was compared (Gouveia et al., 2012b). N. benthamiana 16C plants were infiltrated with Agrobacterium tumefaciens carrying either 35S-GFP alone or with 35S-GFP and 35S-p19.7 from phylogenetic groups 1 to 5 (Gouveia et al., 2012b). Based on visual observations, northern blots, and real-time RT-PCR analysis, no significant differences in the p19.7 protein suppressor activity for groups 1, 2, and 5 was observed, however the group 3 p19.7 protein was observed to have greater activity compared to group 4 (Gouveia et al., 2012b).

Gouveia et al. (2012b) analysed the p19.7 amino acid sequences from the five groups and five non-conservative amino acid substitutions unique to groups 3 and 4 were identified. Four of these amino acid changes were similar, involving polar amino acids (in groups 1, 2, and 5) being replaced by hydrophobic amino acids (in groups 3 and 4). The substitution at amino acid 80 is of particular interest, as this is the only amino acid change that leads to amino acids with different properties in groups 3 and 4. It involves the substitution of glutamine (in groups 1, 2, and 5) to glycine, a small hydrophobic amino acid, in group 3 or glutamic acid, a polar,
negatively charged amino acid, in group 4. A difference in the secondary structure was also predicted to occur in this region (Gouveia et al., 2012b). Therefore, this analysis indicated the single amino acid substitution is a likely cause for the difference in p19.7 suppressing activity between groups 3 and 4.

Differential RNA silencing suppressor activity between strains that have been correlated to specific amino acid substitutions have also been reported in several other plant viruses (Chen et al., 2008; Siré et al., 2008; Torres-Barceló et al., 2008). For example, Siré et al. (2008) observed differences in the efficiency of the P1 silencing suppressor proteins from different phylogenetic groups of the Rice yellow mottle virus. Based on amino acid sequence and mutagenesis analysis, the cysteine and phenylalanine amino acids at positions 64 and 88, respectively, were identified as important determinants to the strength of the protein activity, as mutants that converted the cysteine to serine and the phenylalanine to tyrosine lead to significantly reduced P1 suppressor activity in Oryza sativa (Siré et al., 2008).

The amino acid identity between the GLRaV-3 group 3 and 4 p19.7 proteins (JQ763397 and JQ763396, respectively), which show significant differences in biological activity, is 87%. Since the NZ1-B and NZ2 p19.7 protein have only approximately 65% amino acid identity to the group 3 and group 4 isolates, it is likely that the considerable differences in amino acid composition will also lead to differences in protein activity. Further sequence analysis, in this study, identified 27 non-conservative amino acid substitutions that are unique to the divergent GLRaV-3 isolates. Substitutions included 14 changes that are likely candidates to alter the protein structure and/or functionality, which were generally clustered in two regions between amino acids 76 to 92 and 112 to 132. In particular, for all divergent isolates the polar glutamine positioned at amino acid 80 for groups 1, 2, and 5 was substituted with the small hydrophobic alanine, which is a similar amino acid to glycine that is present in group 3. Furthermore, the secondary structures predicted for the NZ1-B and NZ2 isolates are considerably different throughout the p19.7 protein, particularly NZ2, with longer beta-strands and less alpha-helix structures compared to all other isolates.
3.4.5 Using sequence data for the development of molecular assays

Sequence data is useful for understanding phylogenetic relationships and investigating potential biological implications, as discussed above. Additionally, sequence data is critical for the design and development of effective molecular detection assays. To ensure the assays detect most, if not all, virus variants with high reliability and specificity, an understanding of the genetic variation within the virus population is required. For instance, the sequence data described in this chapter and in Chapter 2 has improved our understanding of genetic variability within the New Zealand GLRaV-3 population, particularly with the identification of divergent isolates NZ1-B and NZ2, and this can be used to improve molecular detection (Chapter 4).

In addition, understanding the spread of genetic variation throughout the target genome can aid the design of both generic and sequence specific detection assays. Uneven genetic variation along the GLRaV-3 genome was observed between different GLRaV-3 isolates. Regions within ORF4, ORF6, and ORF8 showed the highest similarity between GLRaV-3 isolates representative of the different phylogenetic groups, and are ideal areas to target for a generic detection assay. Conversely, areas of low similarity are ideal areas for the design of variant-specific/phylogenetic group specific assays. The three regions that showed the highest genetic variability between GLRaV-3 isolates were within ORF5, ORF7, and ORF10-12. Additionally, considerable variability was observed in the 5‘UTR (Section 3.4.3.1) and Bester et al. (2012b) also identified an area of low similarity between GH11, GH30, and NY1 isolates within the ORF1a, between nucleotides 4,583 and 5,300. In this study, a similar region of low similarity between GLRaV-3 isolates from groups 1, 2, 3, and 5 was observed in approximately the same region. More sequence of the 5‘UTR and ORF1a is required to confirm if this low sequence similarity is also present in divergent isolates, NZ1-B and NZ2. But both areas are possible regions to design strain specific primers.

3.4.6 Conclusions

This chapter describes five New Zealand GLRaV-3 isolates based on the complete (NZ-PG1, NZ-R2D4, and NZ-WCA) and partial (NZ1-B and NZ2) genome sequences. NZ-PG1, NZ-R2D4, and NZ-WCA are members of the phylogenetic groups 1, 2, and 5 respectively, and NZ-WCA is the first description of a complete
GLRaV-3 genome representative of the phylogenetic group 5. NZ1-B is an isolate of the NZ-1 variant and is closely related to South African GH11 and GH30 variants, while at the nucleotide level NZ2 is more than 20% different to the next most closely related GLRaV-3 variant. The high genetic difference between NZ2 and other GLRaV-3 variants suggests NZ2 is a novel variant of GLRaV-3 and potentially represents a new phylogroup, though more sequence data is required to confirm the new grouping. Furthermore the classification of the GLRaV-3 phylogenetic groupings should be re-visited because of significantly higher genetic variability within group 6 compared to the other groups.

The high GLRaV-3 sequence variability described, highlighted a high number of amino acid substitutions that may change protein function for divergent GLRaV-3 isolates. Furthermore, analysis of regions known to contain cis-acting elements important for virus replication, assembly, and gene expression, also revealed poor conservation of possible secondary structures in the 5′UTR. Despite the high variation, primary and/or secondary structures that were conserved in GLRaV-3 isolates were identified within the 3′UTR and sequences upstream from the start of some ORFs and are likely cis-acting element candidates.
Chapter 4:
Development of molecular GLRaV-3 detection protocols improving virus detection

4.1 Introduction

The accurate and reliable detection of GLRaV-3 in nursery and field material is a critical part of grapevine improvement and disease management programmes worldwide. In 2006, the New Zealand Winegrowers established the certified GGS, with the aim of minimising the probability of infected material entering vineyards (New Zealand Winegrowers, 2011). This requires certified nurseries to annually test vines from mother blocks of rootstock and scion material, as well as testing a proportion of the new grafted vines, for GLRaV-3, thus ensuring “high-health plants” are released to vineyards (New Zealand Winegrowers, 2011). Furthermore, identifying virus infected vines in existing vineyards, which are a source of inoculum for further spread, is another important part of a disease control programme (Pietersen, 2004). This requires continual virus screening of vineyards, where identified virus infected vines are removed and replaced with nursery certified vines.

Both serological ELISA and molecular-based RT-PCR diagnostic protocols are used routinely for GLRaV-3 testing. ELISA protocols are easy to conduct with large sample numbers and can be sensitive and reliable. However, low GLRaV-3 titre and/or low antigen reactivity may compromise detection using ELISA. To date, a number of different molecular-based protocols, with high sensitivity and reliability, have been used for the detection of GLRaV-3 (Chapter 1). There are a number of factors that can lead to false negative results, reducing the reliability of molecular-based protocols, such as seasonal fluctuations of virus titre and uneven virus distribution (Cohen et al., 2004; Constable et al., 2012; Osman et al., 2007; Pacifico et al., 2011; Rowhani et al., 1997), poor quality RNA with high amounts of secondary metabolites that inhibit reverse transcription and DNA amplification (Henson et al., 1993; Newbury et al., 1977; Salzman et al., 1999), and genetic variability within the virus population (Constable et al., 2012; Osman et al., 2007). The detrimental effects of poor quality RNA extracts and considerable genetic
variability within the New Zealand GLRaV-3 population was identified and discussed in Chapters 2 and 3.

Knowledge of sequence variability is essential to ensure that RT-PCR protocols detect all variants infecting plants in a certification scheme. It is also important to determine the biological significance of these variants and the possibility of differences in vector transmission efficiency, graft transmissibility, and severity of symptom expression. In order to facilitate virus biological studies, a technique that can detect and identify multiple variants/genotypes/strains economically and with high sensitivity would be advantageous. mRT-PCR has been effectively used for the detection of multiple virus genotypes/strains infecting citrus (Roy et al., 2010), potato (Lorenzen et al., 2006), and grapevine (Sharma et al., 2011).

The overall objective of the work described in this chapter was to improve the molecular detection of GLRaV-3. This was done by addressing two areas. Firstly, to determine the most suitable total RNA extraction protocol for future experiments and large-scale screening, a comparative study of four different extraction protocols was conducted. Secondly, sequences obtained from Chapters 2, 3, and worldwide were used to develop and optimise new molecular assays for the universal and variant-specific detection of GLRaV-3. Thus, this chapter describes molecular detection assays for the generic and specific detection of all GLRaV-3 variants known to be in New Zealand, which offers the high reliability and sensitivity required for certification schemes, as well as variant-specific detection to aid biological studies of individual GLRaV-3 isolates.
4.2 Materials and methods

4.2.1 Virus source

4.2.1.1 Plant material for total RNA extraction comparisons and for the optimisation of molecular assays

Six GLRaV-3 positive and six GLRaV-3 negative samples (tested by ELISA and RT-PCR) of both leaf and cane, were used for each of the four RNA extraction protocols compared (Table 4.1).

Table 4.1: GLRaV-3 positive and negative plant material used for the comparative study of the total RNA extraction protocols.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total quantity</th>
<th>GLRaV-3 infection status</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf material</td>
<td></td>
<td></td>
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<tr>
<td>Pinot noir</td>
<td>3</td>
<td>+</td>
<td>Palliser Estate vineyard</td>
</tr>
<tr>
<td>Sauvignon blanc</td>
<td>3</td>
<td>-</td>
<td>Corbans Viticulture</td>
</tr>
<tr>
<td>Pinot gris</td>
<td>6</td>
<td>+ (3) and - (3)</td>
<td>Corbans Viticulture</td>
</tr>
<tr>
<td>Cane material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabernet sauvignon</td>
<td>6</td>
<td>+ (3) and - (3)</td>
<td>Gimblett Gravels block</td>
</tr>
<tr>
<td>Pinot noir</td>
<td>6</td>
<td>+ (3) and - (3)</td>
<td>Palliser Estate vineyard</td>
</tr>
</tbody>
</table>

*a + represents material was GLRaV-3 infected, - represents material was healthy, (3) represents that three + or – samples of this particular cultivar was used for the testing.

Plant material used for the optimisation of molecular assays was the same material as that used in the GLRaV-3 genetic variability study (Chapter 2, Section 2.2.1). In addition, root (3309 rootstock) samples from nine Chardonnay vines from Hawke’s Bay, previously DAS-ELISA tested were also used.

4.2.1.2 Plant material screened using DAS-ELISA/TAS-ELISA and mRT-PCR

Field samples from two different blocks, the Auckland collection of the Te Kauwhata National Grapevine Repository (referred hereafter as the Auckland collection) and the Hawke’s Bay Mission Estate Gimblett Gravels block (referred hereafter as the Gimblett Gravels block), were screened for GLRaV-3. From the Auckland collection, dormant cane was collected from all 157 accessions consisting of 108 red varieties and 49 white varieties (refer to Appendix 1 Table A1.1 for varietal details).

Over two years (2010 and 2011), cane, leaf, and trunk samples from Cabernet sauvignon and Syrah cultivars in the Gimblett Gravels blocks in Hawke’s Bay, were collected and tested for GLRaV-3. Leaf material was collected in late summer of both years. In the 2010 winter season, both cane and trunk material
was collected, while only cane material was collected in the winter season of 2011 (Table 4.2).

For the 2010 summer season screening of the Gimblett Gravels blocks, leaf samples were collected from a defined sub-section of the block as indicated in Figure 4.1. There were five vines per bay and a single leaf was collected from the middle cane of each vine. Leaves from the same bay were bulked and tested as one sample except for leaves from vines with apparent leafroll symptoms which were removed and tested as a separate sample. In total 271 Cabernet sauvignon and 308 Syrah leaf samples were collected, resulting in 151 and 188 tested samples, respectively. In addition, 67 leaf samples from a neighbouring Syrah block were collected, where three to six symptomatic or non-symptomatic leaves were collected from the middle cane of 19 different vines.

In the 2010 winter season, 66 trunk samples from visually GLRaV-3 positive vines consisting of 45 Cabernet sauvignon (from rows 1, 2, 11, and 12, and between bays 1 to 44) and 21 Syrah vines were collected and tested. This block had been surveyed every year and symptomatic vines are rogued, thus samples collected from symptomatic vines collected were showing symptoms for the first time.

In the 2011 summer season, 18 visually positive Cabernet sauvignon leaf samples, while in the winter season cane material from 243 vines throughout the Gimblett Gravels block were collected. Cane samples consisted of 224 Cabernet sauvignon and eight Syrah vines showing visual symptoms, and 11 non-symptomatic Cabernet sauvignon vines.

Table 4.2: Summary of the leaf and cane samples tested from the Gimblett Gravels block.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Number of samples</th>
<th>Type of material</th>
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<tbody>
<tr>
<td><strong>2010 summer season</strong></td>
<td></td>
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<tr>
<td>Cabernet sauvignon</td>
<td>271</td>
<td>Leaf</td>
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<tr>
<td>Syrah</td>
<td>375</td>
<td>Leaf</td>
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<tr>
<td><strong>2010 winter season</strong></td>
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<tr>
<td>Cabernet sauvignon</td>
<td>45</td>
<td>Cane</td>
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<tr>
<td>Syrah</td>
<td>21</td>
<td>Cane</td>
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<tr>
<td><strong>2011 summer season</strong></td>
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<tr>
<td>Cabernet sauvignon</td>
<td>18</td>
<td>Leaf</td>
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<tr>
<td><strong>2011 winter season</strong></td>
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<tr>
<td>Cabernet sauvignon</td>
<td>235</td>
<td>Cane</td>
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<td>Syrah</td>
<td>8</td>
<td>Cane</td>
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Figure 4.1: Diagrammatic representation of the Gimblett Gravels block (not to scale). The block
consists of 20 rows of Cabernet sauvignon and 20 rows of Syrah vines. This block is neighboured
by another block of older Syrah vines. For the 2010 summer season testing, the area selected for
leaf collection is marked in red.

4.2.2 RNA extraction
The quality of RNA extracts is an important aspect to consider with molecular
diagnostic assays especially for woody plant material. Poor RNA extracts with a
large amount of RT-PCR inhibitors can generate false negative results (Constable
et al., 2012; Gambino et al., 2008; Henson et al., 1993; Newbury et al., 1977;
Osman et al., 2006; Salzman et al., 1999).

This was evident in the initial

screening of GLRaV-3 positive plants (see Chapter 2), and consequently to ensure
a suitable RNA extraction protocol was used for the remainder of the project, four
total RNA extraction protocols were compared.
The RNA extraction protocols evaluated were:
(i)

the RNeasy Plant Mini Kit (Qiagen, GmbH, Germany) in conjunction with
the Mackenzie extraction buffer (MacKenzie et al., 1997)

(ii) the SpectrumTM Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA)
with the addition of 1% (w/v) meta-bisulphate into the Lysis Buffer just
before use (see Chapter 2 Section 2.2.2)

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(iii) the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) with the addition of 1% (v/v) β-mercaptoethanol into the Lysis Buffer just before use

(iv) a modified CTAB RNA extraction method described by White et al. (2008), with minor modifications

4.2.2.1 Qiagen RNeasy Plant Mini Kit

Total RNA extractions using the RNeasy Plant Mini Kit (Qiagen, GmbH, Germany) followed a protocol designed by Mackenzie et al. (1997) with minor modifications. Approximately 100mg of phloem scrapings or leaf discs were prepared for homogenisation as described in Chapter 2 Section 2.2.2. Following homogenisation 1mL of Mackenzie extraction buffer (4M guanidine isothiocyanate, 0.2M sodium acetate, 25mM EDTA pH8.0, 2.5% (w/v) PVP-40, and 1% (v/v) β-mercaptoethanol - added just before use) was added to each sample and then homogenised for a further 30 seconds at 30 cycles per second. 100µL of 20% Sarkosyl was then added to each sample, vortexed, and then incubated in a water bath for 15 minutes at 70°C with samples being vortexed every 5 minutes. To pellet the plant material and stainless steel beads, samples were centrifuged for 5 minutes at 16,000xg. The remainder of the total RNA extraction protocol followed the manufacturer instructions (Appendix 4 Section A4.2).

The concentration and quality of resulting total RNA extracts were measured as described in Section 4.2.2.5 and stored at -80°C.

4.2.2.2 Sigma-Aldrich Spectrum™ Plant Total RNA Kit

Conducted as described in Chapter 2 (Section 2.2.2) with the addition of two alternative reducing agents, (i) 1% (w/v) sodium metabisulphite, and (ii) 1% (v/v) β-mercaptoethanol, were added to the Lysis Solution Buffer (Sigma-Aldrich, St. Louis, MO, USA) just before use. All steps for the total RNA extraction using the β-mercaptoethanol reducing agent were conducted in the fume hood.

4.2.2.3 CTAB extraction method

The CTAB RNA extraction method used was based on a protocol described by White et al. (2008) with minor alterations. 100mg of phloem scrapings or leaf discs were prepared for homogenisation as described in Chapter 2 Section 2.2.2. Following homogenisation 1.2mL of pre-heated, at 65°C, RNA extraction buffer
(2% (w/v) CTAB, 2% (w/v) PVP-40, 25mM EDTA, 100mM Tris-HCl (pH8.0), 2M NaCl, 0.5g/L spermidine, and 3% β-mercaptoethanol - added just before use) was added to each sample. The tubes were then vortexed and incubated in a water bath at 65°C for 30 minutes. Throughout the incubation step, samples were periodically mixed by vortexing every 5 minutes. The remainder of the total RNA extraction protocol followed instructions by White et al. (2008) (Appendix 4 Section A4.3).

The concentration and quality of resulting total RNA extracts are measured as described in Section 4.2.2.5 and stored at -80°C.

4.2.2.4 RNA extraction for field surveys

Total RNA extractions of leaf, cane, and trunk field samples, from the Auckland collection, Gimblett Gravels block, and Hawke’s Bay Chardonnay root samples were conducted using the CTAB RNA extraction described by White et al. (2008) with minor alterations as described in Section 4.2.2.3.

Nine test samples from the first round of mRT-PCR testing for the Gimblett Gravels block were bay composites of five vines from the same bay, where two 6mm leaf discs from each vine in the bay were sampled and combined into a single tube for extraction. Leaf material from 16 vines showing possible symptoms were tested individually and all non-composite samples were sampled as described in Section 4.2.2.3.

4.2.2.5 RNA quality

The Nanodrop ND-1000 spectrometer (Nanodrop technology, Wilmington, DE, USA) was used to measure sample concentrations and verify the quality of the total RNA extracts based on the $A_{230}:A_{260}$ and $A_{260}:A_{280}$ absorbance ratio.

4.2.3 Generic conventional and real-time RT-PCR assays for the detection of GLRaV-3

4.2.3.1 Primer design

The generic forward (GEN-11112F, 5’-AACTCTTTTAAGCGGAGCTTCGTTGT-3’) and reverse (GEN-11232R, 5’-TCTTCGACTTTCGACTTAGCCAAAGGA-3’) primers, were designed in ORF4 (HSP70h) using the web-based program Primer3 (Rozen et al., 2000), to detect all of the New Zealand GLRaV-3 isolates identified from this project. The primers were designed based on the multiple sequence
alignment, using Geneious v5.5 (Drummond et al., 2011) and ClustalX v2.0 (Larkin et al., 2007), of the five main New Zealand isolates (NZ-PG1, NZ-R2D4, NZ-WCA, NZ1-B, and NZ2), partial sequence from isolates Muller-Thurgau, WC-HSP-2, WC-HSP-10 (AY424408, EF103903, and EF103904), and the complete genome sequences of GLRaV-3 isolates NY-1, GP18, and CI-766 (AF037268, EU259806, and EU344893, respectively). To ensure the primer set effectively annealed to all GLRaV-3 isolates, prospective primers were screened against the alignment ensuring minimal polymorphisms were present in the primer binding region.

The primer pair was designed to work as a conventional RT-PCR or quantitative real-time RT-PCR assay, based on the main primer criteria used for the design of quantitative real-time RT-PCR (qRT-PCR) primers (Chapter 5 Section 5.2.4.4.1). The expected amplicon size is 122nt.

**4.2.3.2 Generic conventional and real-time RT-PCR protocol**

The generic assay was optimised by testing the effects of different RT-PCR parameters including the annealing temperature (between 56 and 62°C at 1°C intervals), primer concentration, and magnesium sulphate (MgSO$_4$) concentration (ranged from 1.6 to 5mM).

The generic conventional and real-time RT-PCR protocols were carried out in 10µL reactions using either the SuperScript® III / Platinum® Taq One-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) or the SuperScript® III / Platinum® SYBR® Green One-Step qPCR Kit with ROX (Invitrogen, Carlsbad, CA, USA) respectively. Each reaction also contained 200 to 400ng of total RNA extract, 200nM of each forward and reverse primer, and a final MgSO$_4$ concentration of 4mM. Reverse transcription was conducted for 3 minutes at 55°C followed by 94°C for 2 minutes and 35 cycles of 94°C for 10 seconds, annealing and extension at 64°C for 30 seconds.

Real-time RT-PCR was performed using an ABI 7900HT real-time PCR thermocycler (Applied Biosystems, Foster City, CA, USA) and results analysed using the Sequence Detection System (SDS) software v2.3 (Applied Biosystems, Foster City, CA, USA). The resulting cDNA products from conventional RT-PCR were analysed on 2% TBE agarose gel at 10V/cm, which were then stained in ethidium bromide (0.01µg/ml) and visualised using an ultraviolet transilluminator (Bio-Rad Laboratories Inc., Hercules, CA, USA).
4.2.4 Sequence-variant specific mRT-PCR assay for the detection of GLRaV-3

In addition to the generic detection of GLRaV-3, it is also important to have sequence-variant specific assays to aid the investigation of the biological significance and implications of each isolate. Therefore, to minimise the number of single RT-PCR reactions to be conducted for each variant type, a mRT-PCR assay was developed and optimised for the simultaneous detection of GLRaV-3 isolates from groups 1 to 5, and the divergent NZ-1 and NZ2 variants.

4.2.4.1 Primer design

The mRT-PCR assay used six primer sets consisting of five virus targeting primer sets and one internal plant control. The virus variant specific primer sets target ORFs 4 to 7, generating amplicons sized between 94 to 681nt (Figure 4.2 and Table 4.3). In addition, to allow for easy discrimination between resulting bands on an agarose gel, expected amplicons differed by at least 50nt. Four of the virus targeting primer sets were designed to specifically detect variants from group 1, group 2, NZ-1, and NZ2, while the fifth primer set generically detects variants from group 1 to 5 (phylogenetic groupings based on Gouveia et al. (2011)) (Figure 4.2). Each primer set was designed using the Primer3 software (Rozen et al., 2000) based on the corresponding multiple sequence alignment to ensure amplification of all variants in each group but not non-target groups. All alignments were created using Geneious v5.5 (Drummond et al., 2011) and ClustalX v2.0 (Larkin et al., 2007).

The plant internal control for the mRT-PCR assay was designed using the NAD5 (NADH dehydrogenase subunit 5) sequence from Pinot noir (FM179380) (refer to Table 4.3). To ensure mRNA amplification, the forward primer, Vv-nad5-F, was designed across the exon-exon junction between exon a and b with five nucleotides at the primer 3′terminal end homologous to the 5′start of exon b and the remaining portion of the primer homologous to exon a (Menzel et al., 2002). The Vv-nad5-R reverse primer was designed completely within exon b. To confirm mRNA amplification, total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and then used as template.

To ensure all primers could be used in the same reaction mixture the basic melting temperature for primers was designed to range between 57 and 60°C, calculated...
using the BioMath Calculator (Promega, Madison, WI, USA). In addition, all primers were analysed for hairpins and primer dimerisation particularly primer hetero-complementarity dimers between the different variant-specific primer pairs using OligoAnalyzer 3.1 (Integrated DNA Technologies Inc., IA, USA), refer to Chapter 2 Section 2.2.3.2.1, for the criteria used. High primer specificity to GLRaV-3 was confirmed using BLAST.

**Figure 4.2:** Diagrammatic representation of the regions targeted for multiplex RT-PCR and the corresponding virtual gel (generated using Geneious v5.5 (Drummond et al., 2011)) for a sample positive for all targeted *Grapevine leafroll-associated virus 3* (GLRaV-3) variants and the plant internal control. The GLRaV-3 variants targeted for each primer set are annotated alongside the corresponding RT-PCR band.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' to 3')</th>
<th>Position*</th>
<th>Amplicon length (nt)</th>
<th>GLRaV-3 target</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY-140555F</td>
<td>CCGTACACGATAGACTGCGTTC</td>
<td>ORF6-7 14,055-14,076</td>
<td>681</td>
<td>Group 1</td>
</tr>
<tr>
<td>NY-14735R</td>
<td>CAGTGGTCTGTGCTAACCATC</td>
<td>ORF6-7 14,714-14,735</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ2-11036F</td>
<td>GGCCTGGTTTTGGAGTGAAAG</td>
<td>ORF4 11,036-11,059</td>
<td>387</td>
<td>NZ2</td>
</tr>
<tr>
<td>NZ2-11422R</td>
<td>CCCAGCTTGCGTCAGACAAC</td>
<td>ORF4 11,402-11,422</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA-15283F</td>
<td>GGATATAGTCGGTGGGACGGC</td>
<td>ORF7 15,283-15,303</td>
<td>318</td>
<td>Group 2</td>
</tr>
<tr>
<td>SA-15600R</td>
<td>GCTGGTGCTACTGGACTTTGGCG</td>
<td>ORF7 15,579-15,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vv-nad5-F</td>
<td>GCTTCTTGGGGGTTCTTGTTCG</td>
<td>n/a</td>
<td>213</td>
<td>n/a</td>
</tr>
<tr>
<td>Vv-nad5-R</td>
<td>TCCCTCCATCCAGGAATAATTG</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>qNZ1-4326F</td>
<td>CACAAAGAGGAATTTGGACGAGGA</td>
<td>ORF4-5 12,209-12,235</td>
<td>160</td>
<td>NZ1-B</td>
</tr>
<tr>
<td>qNZ1-4485R</td>
<td>AACACCTCCGACCTAGCAGCAC</td>
<td>ORF4-5 12,347-12,368</td>
<td></td>
<td></td>
</tr>
<tr>
<td>qNYSA-16117F</td>
<td>CGTCCCGAAGAGGTTCTACTCC</td>
<td>ORF7 16,117-16,137</td>
<td>94</td>
<td>Group 1-5</td>
</tr>
<tr>
<td>qNYSA-16210R</td>
<td>AGCCATTGCGTTAGCCTTCAACAC</td>
<td>ORF7 16,187-16,210</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The primer nucleotide positions are based on the complete GP18 (EU259806) genome sequence.
4.2.4.2 mRT-PCR protocol

This assay was optimised by comparing the effects of different RT-PCR parameters including the annealing temperature (between 56 and 62°C at 1°C intervals), primer concentration, MgSO₄ concentrations (ranged from 1.6 to 5mM), and various PCR additives.

A number of different chemicals can be added to RT-PCR assay, to improve RT-PCR amplification and specificity, such as betaine, BSA, DMSO, formamide, triton X-100, tween-20, and tetramethylammonium chloride. In this study, three PCR additives were compared; 2% BSA, 2% DMSO, and 2% formamide. BSA was selected as it has been shown to increase PCR efficiency and reduce the effects of PCR inhibition (Giambernardi et al., 1998; Nagai et al., 1998). DMSO and formamide are thought to reduce secondary structure formations (Baskaran et al., 1996; Varadaraj et al., 1994), which are likely in mRT-PCR assays, and formamide can also increase the stringency of molecular assays (Sarkar et al., 1990).

The optimised mRT-PCR assays were carried out in 10µL reactions using the SuperScript® III / Platinum® Taq One-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) with 100 to 400ng of total RNA extract, final MgSO₄ concentration of 2.5mM, and primer concentrations as shown in Table 4.4. Thermocycling conditions involved reverse transcription for 30 minutes at 50°C followed by 94°C for 2 minutes and 30 cycles of 94°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 42 seconds, and a final extension for 3 minutes at 68°C.

Resulting RT-PCR products were analysed on 3 to 4% TBE agarose gels at 80V and were stained in ethidium bromide (0.01µg/mL) for 20 minutes, destained, and visualised using an ultraviolet transilluminator (Bio-Rad Laboratories Inc, Hercules CA, USA).
### TABLE 4.4: Multiplex RT-PCR final primer concentrations.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY-14055F</td>
<td>450</td>
</tr>
<tr>
<td>NY-14735R</td>
<td>450</td>
</tr>
<tr>
<td>NZ2-11036F</td>
<td>400</td>
</tr>
<tr>
<td>NZ2-11422R</td>
<td>400</td>
</tr>
<tr>
<td>SA-15283F</td>
<td>350</td>
</tr>
<tr>
<td>SA-15600R</td>
<td>350</td>
</tr>
<tr>
<td>Vv-nad5-F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>300</td>
</tr>
<tr>
<td>Vv-nad5-R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>300</td>
</tr>
<tr>
<td>qNZ1-12209F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250</td>
</tr>
<tr>
<td>qNZ1-12368R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250</td>
</tr>
<tr>
<td>qNYSA-16117F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>qNYSA-16210R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primer set is also optimised for real-time RT-PCR use.

#### 4.2.5 Specificity and sensitivity of molecular assays

To ensure primer specificity oligonucleotide sequences were searched against the NCBI nucleotide database using BLAST and molecular assays were tested against healthy grapevine samples. In addition, individual RT-PCR reactions were conducted for each primer set (generic and variant specific) using total RNA from plant samples with known GLRaV-3 infections. This was carried out in 50µL reactions using the SuperScript® III / Platinum® Taq One-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) with the same final concentrations and thermocycling conditions for each respective primer sets as stated in Sections 4.2.3.2 and 4.2.4.2.

Resulting RT-PCR products were analysed by agarose gel electrophoresis and correct sized amplicons were excised, purified using the Zymoclean DNA gel purification extraction kit (Zymo Research Corporation, Irvine, CA, USA), as described in Chapter 2, Section 2.2.4.2. Purified PCR products were sequenced (Macrogen Inc, Seoul, South Korea) and compared to known/type sequences for each respective target GLRaV-3 variant.

To test the sensitivity of the molecular assays, synthetic mRNA targets were generated for all virus targeting primer sets using PCR products as template, as described by Fey et al. (2004) and Tsai et al. (2012), where PCR products encompassing the RT-PCR assay primer binding sites were used as templates for in vitro RNA transcription. Additional primers, flanking the different mRT-PCR sequences, were designed with a 5’ SP6 and T7 RNA polymerase binding site incorporated in forward and reverse primers respectively (Table 4.5). These
primers were used to generate cDNA for transcription from plasmids encompassing the RT-PCR sequences. These PCR reactions were carried out in 50µL reactions containing 10mM Tris-HCl, 50mM KCl, 300nM of each dNTP, 2.5mM MgSO$_4$, 300 – 500ng plasmid, 400nM of forward and reverse primers, 5U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA). Thermocycling conditions of 94°C for 2 minutes and 40 cycles of 94°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 68°C for 1kb per minute, and a final extension for 5 minutes at 68°C. Resulting amplicons were purified using the QIAquick PCR Purification Kit, as described by the manufacturer (Qiagen Inc., Valencia, CA, USA), eluted with 50µL RNase-free water, treated with restriction enzymes to linearise the residual plasmids, and then used as templates for positive-sense mRNA synthesis with the MAXIscript® SP6 Kit as described by the manufacturer (Ambion, Austin, TX, USA). Transcription was conducted at 37°C for 4 hours and then treated with 1U of DNase TURBO. RNA was recovered through LiCl precipitation as described by the manufacturer. The concentration and quality of mRNA were measured using the Nanodrop ND-1000 spectrometer (Nanodrop technology, Wilmington, DE, USA) at 260 and 280nm.

Table 4.5: Primer sets used for the generation of in vitro RNA transcripts.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' to 3')$^a$$^b$</th>
<th>Position$^c$ (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY1-mRNA-14011F</td>
<td>ATTAGGGACACTATAGAAACGAGAAGGTTATGGGCACAGCA</td>
<td>14,011-14,032</td>
</tr>
<tr>
<td>NY1-mRNA-14901R</td>
<td>TAATACGACTCACTATAGGGACTGGGAAACTAAGTGACC</td>
<td>14,880-14,901</td>
</tr>
<tr>
<td>NZ2-mRNA-10994F$^d$</td>
<td>ATTAGGGACACTATAGAAACGGTTTTATTACGAGTAGAGCTG</td>
<td>10,994-11,017</td>
</tr>
<tr>
<td>NZ2-mRNA-11466R$^d$</td>
<td>TAATACGACTCACTATAGGGCGGTGTGGTTCGACACTCTCCT</td>
<td>11,445-11,466</td>
</tr>
<tr>
<td>GP18-mRNA-15072F</td>
<td>ATTAGGGACACTATAGAAAGTTATGGAAGAAGCATCAGT</td>
<td>15,072-15,093</td>
</tr>
<tr>
<td>GP18-mRNA-15631R</td>
<td>TAATACGACTCACTATAGGGTTGCTCTGATGGACCTACACC</td>
<td>15,611-15,631</td>
</tr>
<tr>
<td>NZ1-mRNA-12097F</td>
<td>ATTAGGGACACTATAGAAAGCACCACGACATCCTGAAC</td>
<td>12,097-12,116</td>
</tr>
<tr>
<td>NZ1-mRNA-12457R</td>
<td>TAATACGACTCACTATAGGGCCATTACGGTCACACATACC</td>
<td>12,438-12,457</td>
</tr>
<tr>
<td>NYSA-mRNA-15813F</td>
<td>ATTAGGGACACTATAGAAAGGCAGGGAGTCACAAAACACCA</td>
<td>15,813-15,832</td>
</tr>
<tr>
<td>NYSA-mRNA-16338R</td>
<td>TAATACGACTCACTATAGGGCGGTGTGGTTCGACACTCTCCT</td>
<td>16,319-16,338</td>
</tr>
</tbody>
</table>

$^a$ Sequence in **bold** represent the SP6 RNA polymerase binding site
$^b$ Sequence in **bold and underlined** represent the T7 RNA polymerase binding site
$^c$ Binding site positions based on the genome organisation of the GP18 isolate (EU259806) excluding NY1-mRNA-14011F/NY1-mRNA-14901R, which is based on the NY1 isolate (AF037268).
$^d$ Resulting in vitro RNA transcript was used as the template for the sensitivity tests of both the generic and sequence-variant specific assays.

To calculate the number of single stranded RNA copies per µL the approximate molecular weights of each single stranded RNA (Eqn. 4.1), Avogado number, and concentrations (ng/µL) were used (Eqn. 4.2) (Fey et al., 2004). Ten-fold serial-dilutions (from $10^8$ to $10^1$ copies per µL) were made for all transcribed RNAs, for each primer set, using healthy total RNA from Cabernet sauvignon.
Molecular weight of ssRNA = (number of nucleotides x 320.5) + 159.0 \hspace{1cm} \text{Eqn. 4.1}

Number of ssRNA copies/\mu L = \left( 1 \times 10^{-9} \times 6.022 \times 10^{23} \right) / (\text{M.W.} \times n) \times \text{concentration} \hspace{1cm} \text{Eqn. 4.2}

where, M.W. is the molecular weight of the ssRNA (g/mol/nt)
n is the length of the ssRNA transcript (nt)

4.2.6 Screening of field samples for GLRaV-3

4.2.6.1 Serological detection

Field samples were serologically tested by DAS-ELISA and triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) by D. Cohen and A. Blouin (The New Zealand Institute for Plant and Food Research Limited, Auckland, New Zealand). The DAS-ELISA was performed as described in Chapter 2, Section 2.2.3.1. The TAS-ELISA used two different antibody assays. The plates were coated with polyclonal GLRaV-3 goat antibody (developed by Dr Dariusz Goszczynski (Plant Protection Research Institute, Pretoria, South Africa)) at a ratio of 1 to 1000, and to detect GLRaV-3 antigens specific rabbit anti-GLRaV-3 polyclonal antibodies (As163) developed by Ling et al. (2000) were premixed with antirabbit-AP enzyme conjugate (Sigma-Aldrich Catalogue number: S3687; St. Louis, MO, USA) each diluted at a ratio of 1 to 20,000 (Cohen et al., 2012)

4.2.6.2 Molecular detection

In total, 461 field samples, from the Auckland collection and commercial Gimblett Gravels block in Hawke’s Bay, were tested for GLRaV-3 using the hexaplex mRT-PCR protocol described in Section 4.2.4.2.
4.3 Results

4.3.1 Comparisons between total RNA extraction protocols

Four different total RNA extraction methods (modified Qiagen RNeasy Plant Mini Kit (GmbH, Germany) (MacKenzie et al., 1997), Sigma-Aldrich Spectrum™ Plant Total RNA Kit (St. Louis, MO, USA) with the addition of either 1% (v/v) of β-mercaptoethanol or 1% (w/v) sodium metabisulphite to the Lysis Buffer, and a modified CTAB protocol (White et al., 2008)) were assessed, and yielded varying qualities and quantities of total RNA (Table 4.6). For all RNA extraction methods, RNA extracted from cane material consistently produced low RNA yields, ranging between 1.93 to 12.87µg per 100mg of cane material compared to 20.87 to 71.77µg per 100mg of leaf material. In addition, for both tissue types the RNeasy kit produced the lowest amount of RNA, while the Spectrum™ kit, with the addition of either reducing agent, produced the highest amount of RNA.

Table 4.6: Total RNA quality and yield of four extraction methods from mature cane and leaf grapevine samples (n = 12).

<table>
<thead>
<tr>
<th>Method</th>
<th>Time required</th>
<th>Tissue</th>
<th>A_{260}:A_{280}</th>
<th>RNA yield (µg/100mg)a</th>
<th>RT-PCR successb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNeasy Plant Mini Kit</td>
<td>&gt;2 hrs, 1 day</td>
<td>Cane</td>
<td>1.81 ± 0.18</td>
<td>1.93 ± 0.60</td>
<td>11/12c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf</td>
<td>2.11 ± 0.04</td>
<td>20.87 ± 11.57</td>
<td>12/12</td>
</tr>
<tr>
<td>Spectrum Plant Total RNA Kit</td>
<td>&gt;2 hrs, 1 day</td>
<td>Cane</td>
<td>1.60 ± 0.14</td>
<td>9.01 ± 2.99</td>
<td>8/12c</td>
</tr>
<tr>
<td>(sodium meta-bisulphate)</td>
<td></td>
<td>Leaf</td>
<td>2.20 ± 0.01</td>
<td>71.77 ± 24.23</td>
<td>12/12</td>
</tr>
<tr>
<td>Spectrum Plant Total RNA Kit</td>
<td>&gt;2 hrs, 1 day</td>
<td>Cane</td>
<td>1.61 ± 0.27</td>
<td>12.87 ± 3.76</td>
<td>8/12c</td>
</tr>
<tr>
<td>(β-mecaptoethanol)</td>
<td></td>
<td>Leaf</td>
<td>2.14 ± 0.05</td>
<td>55.87 ± 17.66</td>
<td>12/12</td>
</tr>
<tr>
<td>CTAB</td>
<td>5 hrs, 2 days</td>
<td>Cane</td>
<td>2.14 ± 0.14</td>
<td>4.07 ± 1.84</td>
<td>12/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf</td>
<td>2.08 ± 0.03</td>
<td>32.61 ± 9.11</td>
<td>12/12</td>
</tr>
</tbody>
</table>

a Average yield is given in µg total RNA / 100mg of plant material
b Number of successful amplifications using the mRT-PCR assay / number of total samples
c Samples that did not successfully amplify product were re-tested using RNA diluted 1:10 in water and all re-tests successfully amplified product.

The purity of RNA extracts was assessed using the A_{260}:A_{280} absorbance ratio. Low A_{260}:A_{280} ratios indicate protein contamination, while a ratio between 1.8 and 2.0 indicates acceptable RNA purity (Manchester, 1996; Sambrook et al., 1989). Good quality RNA was extracted from cane and leaf material using the modified RNeasy (average of 1.81 ± 0.18 and 2.11 ± 0.04, respectively) and CTAB (average of 2.14 ± 0.14 and 2.08 ± 0.03, respectively) extraction methods. In contrast, the purity of RNA yielded by the Spectrum™ kit using either reducing agent was variable; the average A_{260}:A_{280} ratio for leaf and cane samples being greater than 2 and less than 1.61 respectively (Table 4.5). An additional measure
of quality is the $A_{230}:A_{260}$ absorbance ratio, which can indicate organic or carbohydrate contamination such as polysaccharides; ratios less than 2 indicate contamination (Nanodrop technology, Wilmington, DE, USA). For all extraction methods, contamination was evident in all cane samples ($A_{230}:A_{260}$ of less than 1.7). However, little contamination was noticeable in leaf samples, as the average $A_{230}:A_{260}$ ratio was above 2 for all methods except the modified RNeasy method (1.86 ± 0.41).

RNA quality was further analysed using the mRT-PCR assay. All RNA extracts from leaf samples consistently resulted in amplification of the plant NAD5 internal control and the correct virus variant (in GLRaV-3 positive samples) (Table 4.6). For cane samples all RNA extracts, the CTAB method produced correct sized amplicons, while varying success was achieved with the other methods. RNeasy extracted RNA resulted in 11 out of 12 successful amplifications while only eight cane samples from each Spectrum™ extraction protocol produced correct sized amplicons. RNA samples that did not generate amplicons were diluted in water (1:10) and re-tested. All re-tests resulted in successful amplification.

The cost and time required for the various extraction protocols are another important aspect to evaluate. Total RNA extractions using commercial kits, typically takes less than 2 hours to complete, while the CTAB method requires lengthy incubation and centrifugation steps resulting in a two day protocol and approximately 5 hours of hands-on work. The extended time required for extractions is particularly important to consider for commercial use. The cost to extract one sample (excluding costs for the time spent on extractions) for each method was calculated based on current pricing. The Qiagen RNeasy Plant Mini Kit (catalogue number: 74904) costs NZD$13.80 per sample, the Sigma-Aldrich Spectrum™ Plant Total RNA Kit (product number: STRN50-1KT) and sodium metabisulphate (product number: 255556-100G (Sigma-Aldrich, St. Louis, MO, USA) costs NZD$8.35 per sample, and the Sigma-Aldrich Spectrum™ Plant Total RNA Kit (product number: STRN50-1KT) and β-mercaptoethanol (provided in kit), costs NZD$7.51 per sample. In comparison, the CTAB method only costs approximately NZD$2.62 per sample.

### 4.3.2 Generic assay for all GLRaV-3 groups

#### 4.3.2.1 Optimisation of the generic assay
To ensure the generic assay, using the GEN-11112F/GEN-11232R primer set, effectively and reproducibly detected all known GLRaV-3 isolates the following parameters were assessed and optimised; primer concentration, MgSO₄ concentration, and annealing temperature. In addition, to keep the assay simple and consistent for both conventional and real-time RT-PCR protocols, all developmental optimisation tests were conducted in parallel. The resulting optimised protocol for conventional and real-time RT-PCR is described in Section 4.2.3.2.

At all primer concentrations (100, 200, 300, and 400nM), both the conventional and real-time RT-PCR successfully detected GLRaV-3; however considerable primer dimer was evident at primer concentrations higher than 300nM (Figure 4.3). In addition, at low template concentrations poor amplification was observed when using 100nM of forward and reverse primers. For instance, at 1x10⁴ template copies of amplicon per µL the average cycle threshold (Ct) value for 100nM and 200nM primer concentrations were 33.70 ± 0.44 and 19.32 ± 0.19, respectively.

Using 200nM of forward and reverse primers, the final MgSO₄ concentrations of 3, 4, and 5mM were assessed. Both conventional and real-time RT-PCR protocols successfully detected GLRaV-3 from all samples at all MgSO₄ concentrations. Optimal PCR efficiencies were observed at 4mM of MgSO₄, the average PCR efficiency being 1.86 ± 0.07 compared to 1.68 ± 0.14 and 1.53 ± 0.18 for 3mM and 5mM of MgSO₄, respectively.

**Figure 4.3:** Melting (dissociation) curve analysis of the generic real-time RT-PCR assay using a final primer concentration of (a) 400nM and (b) 200nM. (a) The arrow highlights a secondary peak, which indicates non-specific amplification such as primer dimer formation. The non-specific amplification was eliminated by reducing the final primer concentration to 200nM (b).
Conventional and real-time RT-PCR successfully amplified all GLRaV-3 positive samples at all annealing temperatures tested (60 to 65°C at 1°C intervals). In addition, to ensure that the shortened reverse transcription step of 3 minutes, generally used for real-time RT-PCR assays, did not hinder detection using conventional one-step RT-PCR, reverse transcription for 3 minutes and 30 minutes were compared. All GLRaV-3 positive samples were detected using both incubation times.

4.3.2.2 Specificity and sensitivity of the generic assay

Both the conventional one-step RT-PCR and real-time RT-PCR assays successfully amplified and detected all GLRaV-3 positive samples used previously for the genetic variability study (refer to Chapter 2 Section 2.2.1). These samples comprised of both single or mixed infections (Figure 4.4), and different grapevine varieties. Detection of GLRaV-3 was also successful from cane, leaf, and root tissues (Figure 4.5). Real-time RT-PCR Ct values ranged from 18 to 29, and no non-specific amplification was observed in healthy and water controls. The specificity of the generic assay was further confirmed by sequencing amplicons from the generic primer set, which showed 97 to 100% nucleotide identity to the corresponding GLRaV-3 isolates.

**Figure 4.4:** Detection of *Grapevine leafroll-associated virus 3* (GLRaV-3), from ten selected leaf and cane samples, using (a) the generic RT-PCR protocol and (b) the variant-specific multiplex RT-PCR protocol. (a) Arrow indicates correct sized amplicons. (a and b) Lane L, 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA); lanes 1 to 10, are different GLRaV-3 positive samples with differing GLRaV-3 variant combinations; lane 11, healthy grapevine sample (i.e. GLRaV-3 negative); lane 12, no template control.
Furthermore, both conventional and real-time assays successfully detected GLRaV-3 from all 10-fold serial dilutions of synthetic RNA (1x10^1 to 1x10^8 copies of amplicon per µL) diluted in healthy Cabernet sauvignon total RNA samples (Figure 4.6). The Ct values for the generic real-time RT-PCR assay ranged from 10 and 25.

**Figure 4.6:** Detection of synthesised *Grapevine leafroll-associated virus 3* (GLRaV-3) RNA in a 10-fold dilution series using (a) the generic real-time RT-PCR protocol, and (b) the generic conventional RT-PCR protocol. (a) Amplification plots of each 10-fold serial dilutions of synthesised GLRaV-3 RNA using healthy Cabernet sauvignon total RNA and three technical replicates, in descending order; 8 = 1x10^9, 7 = 1x10^8, 6 = 1x10^7, 5 = 1x10^6, 4 = 1x10^5, 3 = 1x10^4, 2 = 1x10^3, 1 = 1x10^1 copies of each virus amplicon per µL. (b) Agarose gel of the conventional RT-PCR with the same dilutions as (a). W = no template control, L = 1 kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

### 4.3.3 Variant specific mRT-PCR assay

#### 4.3.3.1 Optimisation of the variant specific mRT-PCR

To detect mixed infections, variant specific primers were designed and optimised for mRT-PCR. This assay was optimised by varying and comparing the effects of different RT-PCR parameters such as, primer concentration, MgSO₄ concentration...
(range from 1.6 to 5mM), the annealing temperature (range of 56 to 62°C at 1°C intervals), and the addition of PCR additives (2% BSA, 2% DMSO, and 2% formamide). The resulting optimised protocol was described in Section 4.2.4.2.

The mRT-PCR primer sets were tested as single RT-PCR reactions, using samples with known GLRaV-3 infections, to evaluate the utility of each primer set and in all cases the expected amplified fragment was generated. This was followed by testing the annealing temperatures for each primer set from 57 to 62°C at 1°C intervals. All primer sets successfully generated the expected sized amplicons for all temperatures. Subsequently, the six mRT-PCR primer sets were merged into a single RT-PCR reaction at equimolar quantities of 400nM. Since preferential amplification of smaller amplicons was observed, primer concentrations were further optimised by sequentially decreasing the primer concentrations for amplicons that were abundantly amplified, by a factor of 50nM, and increasing the concentration of NY-14055F/NY14735R to 450nM. The final primer concentrations resulting from this testing are shown in Table 4.4.

Final MgSO₄ concentrations of 1.6, 2.5, 3, 3.5, 4, 4.5, and 5mM for the 10µl mRT-PCR assay were tested. MgSO₄ concentrations higher than 4mM inhibited the amplification of the NYSA-16117F/NYSA-16210R primer pair (94nt amplicon) (Figure 4.7) and although concentrations between 1.6 to 3.5mM successfully detected all GLRaV-3 variants, the final concentration of 2.5mM produced the best results (Figure 4.7).

In addition, the annealing temperatures of 57, 58, 59, 60, 61, and 62°C were also examined. At 57°C, the group 1 and NZ2 variant-specific, and the generic groups 1 to 5 primer sets, did not successfully amplify products from GLRaV-3 RNA transcripts diluted to 1x10³ copies of each virus amplicon per µL (Figure 4.8). All remaining temperatures successfully amplified product from all samples, however 60°C yielded minimal primer dimer and the most consistent amplification of all variants between samples (Figure 4.8).
Figure 4.7: Grapevine leafroll-associated virus 3 (GLRaV-3) detection using the multiplex RT-PCR assay at different final magnesium sulphate (MgSO₄) concentrations between 1.6 to 5.0 mM. For each MgSO₄ concentration, different GLRaV-3 infections were examined. Lane 1, group 1 and NZ-1 infection; lane 2, NZ2 infection; lane 3, NZ-1 infection; lane 4, GLRaV-3 RNA transcripts of each virus amplicon diluted to 1x10⁶ and per µL using water; lane 5, GLRaV-3 RNA transcripts of each virus amplicon diluted to 1x10³ per µL using water; lanes 6, no template control. L = 1 kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

Figure 4.8: Grapevine leafroll-associated virus (GLRaV-3) detection using the multiplex RT-PCR assay at different annealing temperatures between 57 to 62°C. For each annealing temperature, leaf material with group 2, NZ-1, and NZ2 infection (lane 1), cane material with NZ-1 and NZ2 infection (lane 2), GLRaV-3 RNA transcripts diluted to 1x10⁶ (lane 3) and 1x10³ (lane 4) copies of each virus amplicon per µL using healthy Cabernet sauvignon total RNA, healthy cane samples (lane 5), and no template controls (lane 6) were examined. L = 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).
The addition of BSA reduced the inhibitory effects of secondary metabolites in undiluted samples, to some extent (Figure 4.9). However, diluting samples 1:10 in water, also effectively offset inhibition and resulted in amplification of GLRaV-3 variants (Figure 4.9). The successful use of diluted RNA is also described in Chapter 2, Section 2.3.1. The addition of DMSO and formamide did not improve the performance of the mRT-PCR assay. In most cases the correct amplicons were generated, but lowered band intensity, particularly samples with smaller amplicon size, was observed (Figure 4.9).

![Image of gel electrophoresis](image)

**Figure 4.9:** Comparing the performance of three PCR additives (2% BSA, 2% DMSO, and 2% formamide), added to the multiplex RT-PCR (mRT-PCR) assay. For each mRT-PCR condition, cane, leaf, and RNA transcripts with varying Grapevine leafroll-associated virus 3 (GLRaV-3) infections were examined. Lanes 1 – 3 consists of undiluted RNA isolated from cane material with either group 1, NZ-1, and NZ2 (lane 1 and 3) or group 2, NZ-1, and NZ2 (lane 2) mixed infection, while lanes 4 – 6 are the same RNA extracts diluted in water at a 1:10 ratio. Lanes 7 – 9 consists of RNA extracts isolated from leaf material with either group 1 and NZ-1 (lane 7) or group 3 - 5 and NZ-1 (lane 9) mixed infection or a single NZ2 infection (lane 8). Lanes 10 and 11 consists of GLRaV-3 RNA transcripts diluted to 1x10^6 (lane 10) and 1x10^3 (lane 11) copies of each virus amplicon per µL using water. Lane 12 consists of the no template control. L = 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

### 4.3.3.2 Specificity and sensitivity of variant specific mRT-PCR

All grapevine samples that tested positive from previous DAS-ELISA and single RT-PCR tests were successfully amplified and detected by the mRT-PCR assay. Samples comprised of different single or mixed infections (Figure 4.4). In addition, detection of GLRaV-3 variants was also successful from cane, leaf, and root tissues (Figure 4.10). No non-specific amplification was observed in healthy and water controls. Furthermore, the variant specific assay specificity was further confirmed, with all sequences obtained from the single RT-PCR reactions for each
primer set matching the corresponding GLRaV-3 variants with 97 to 100% nucleotide identity.

Figure 4.10: Detection of Grapevine leafroll-associated virus 3 from leaf (lanes 1 – 5), cane (lanes 6 – 10), and root samples (lanes 11 – 15), using the multiplex RT-PCR protocol. Lane L, 1kb Plus DNA (Invitrogen, Carlsbad, CA, USA); lane 16, positive control using RNA transcripts diluted in water to 1x10^6 amplicon copies per µL; lane 17, no template control.

The mRT-PCR assay shows high sensitivity, successfully detecting GLRaV-3 from all 10-fold serial dilutions of synthetic RNA diluted in healthy Cabernet sauvignon total RNA samples (1 x 10^8 to 1 x 10^1 copies of amplicon per µL) (Figure 4.11).

Figure 4.11: Detection of synthesised Grapevine leafroll-associated virus 3 (GLRaV-3) RNA in a 10-fold dilution series using the variant-specific multiplex RT-PCR protocol (mRT-PCR). Agarose gel of mRT-PCR products with the 10-fold serial dilutions of synthesised GLRaV-3 RNA using healthy Cabernet sauvignon total RNA samples (1 x 10^8 to 1 x 10^1 copies of amplicon per µL) in descending order; 8 = 1x10^8, 7 = 1x10^7, 6 = 1x10^6, 5 = 1x10^5, 4 = 1x10^4, 3 = 1x10^3, 2 = 1x10^2, 1 = 1x10^1 copies of each virus amplicon per µL. W = no template control, L = 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

4.3.4 Primer binding analysis

The primer binding sites of GEN-11112F/GEN-11233R and NYSA-16117F/NYSA-16210R were further analysed using additional sequence data from groups 3 and 4 and divergent GH11 and GH30 isolates that were not present at the time of primer design (Figures 4.12 and 4.13). For the generic primer set, GEN-11112F/GEN-11233R, isolate PL-20 from group 3 contains three polymorphisms in the forward (primer:template mismatches - G:A, C:T, and T:C) and four polymorphisms in the reverse primer binding site (primer:template mismatches - G:A, T:C, C:A, and G:A). In addition, the primer binding sites of isolates GH11
and GH30 are similar to NZ-1 with only one nucleotide difference at nucleotides 15 and 6 of the forward and reverse primer, respectively.

The multiple sequence alignment for the mRT-PCR primer set detecting groups 1 to 5, NYSA-16117F/NYSA-16210R, revealed the reverse primer of isolate WA-MR from group 1 and isolates from groups 3 and 4 all contained a single A:C mismatch at nucleotide 21 (Figure 4.13).

**Figure 4.12:** Multiple sequence alignment of different *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates within ORF4 (position 11099 to 11248, based on the GP18, EU259806) illustrating the binding site for the GLRaV-3 generic primer set, GEN-11112F/GEN-11233R, generated using Geneious v5.5 (Drummond et al., 2011).

**Figure 4.13:** Multiple sequence alignment of different *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates within ORF7 (position 16105 to 16224, based on the GP18, EU259806) illustrating the binding site for the generic GLRaV-3 groups 1 to 5 primer set, NYSA-16117F/NYSA-16210R, generated using Geneious v5.5 (Drummond et al., 2011).
4.3.5 Comparison of the performance of previously published primers and new generic and mRT-PCR assays

The reliability of two published primer sets to detect all GLRaV-3 variants, LC1/LC2 (Turturo et al., 2005) and H330/C629 (MacKenzie et al., 1997), were compared to the new generic and mRT-PCR assays, using 25 samples representing both single and mixed infections with a range of different variant combinations. Both new assays successfully detected GLRaV-3 from all samples, while only 18 out of the 25 were successfully detected using the LC1/LC2 and H330/C629 primer sets. The positive samples detected using published primers comprised single infections of group 1 variants and mixed infections of the NZ-1 and NZ2 variants with variants from group 1 to 5. All false negative samples were infected with only the NZ-1 and/or NZ2 variants (Figure 4.14).

![Figure 4.14](image)

**Figure 4.14:** Detection of *Grapevine leafroll-associated virus 3* (GLRaV-3), from ten selected leaf and cane samples, using (a) the published H330/C629 primer pair in a RT-PCR protocol and (b) the variant-specific multiplex RT-PCR (mRT-PCR) assay. (a) Arrow indicates correct sized amplicons. (a and b) Red dotted boxes highlight three samples, infected with NZ-1 and/or NZ2, that produced false negative results using the published primers but were detected using the mRT-PCR assay. Lane L, 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA); lanes 1 to 10, are different GLRaV-3 positive samples with differing GLRaV-3 variant combinations; lane 11, healthy grapevine sample (i.e. GLRaV-3 negative); lane 12, no template control.

4.3.6 Detection of GLRaV-3 infection in the Auckland collection

Of the 157 samples from the Auckland collection analysed by mRT-PCR, 13 tested negative (8.3%) while the remaining samples contained a range of single (17.8%), double (41.4%), and multiple (32.5%) GLRaV-3 sequence variants (Table 4.7 and Appendix 5 Table A5.1). The mRT-PCR results for each GLRaV-3 positive sample were plotted according to the vine position within the Auckland collection, Figure 4.15. Group 1, NZ-1, and NZ2 GLRaV-3 variants are spread
throughout the block, with no noticeable clustering pattern, however there are large spanning areas of vines infected with the same GLRaV-3 variant. Of the 13 negative samples, 11 were located in row 1 and two were located in row 2.

Table 4.7: Number of GLRaV-3 positive samples identified for each phylogenetic group from the mRT-PCR screening of the Auckland collection.

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Number of samples</th>
<th>Phylogenetic group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>Single</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>Double</td>
<td>65</td>
<td>16</td>
</tr>
<tr>
<td>Triple</td>
<td>47</td>
<td>29</td>
</tr>
<tr>
<td>Quadruple</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>144</td>
<td>63</td>
</tr>
</tbody>
</table>

a The ELISA and mRT-PCR results for each sample of the Auckland collection are in Appendix 5 Table A5.1.

b Group 3 to 5 represents the number of samples that generated mRT-PCR positive bands for the NYSA 16117F/NYSA-16210R primer set and no mRT-PCR positive bands for the NY-14055F/NY-14735R (group 1) and SA-15283F/SA-15600R (group 2) primer sets.

The incidence of GLRaV-3 group 2 variants detected in single and mixed infection was less than group 1, NZ-1, and NZ2 variants, being present in only 27 out of the 144 positive samples. Fifteen of the 144 samples tested positive using the generic group 1 to 5 primer set but not the variant specific group 1 and 2 primer sets, indicating these vines were probably infected with variants from phylogenetic groups 3, 4, or 5. Vines infected with group 2 variants and potential variants from group 3 to 5 were scattered throughout the block (Figure 4.15).
Figure 4.15: Schematic of the Auckland collection block that was ELISA and multiplex RT-PCR screened. The rows are labelled left to right and bays are numbered in ascending order from the top to the bottom of the diagram. Each “x” represents a single vine and “.” represents a missing or dead vine. Vines were infected with either a group 1 (green shading), NZ2 (blue shading), group 2 (purple shading), or NZ-1 (red shading) variant, in single or mixed infections. In addition, vines potentially infected with variants from group 3 to 5 are also marked (orange shading). These vines generated a positive amplification for the group 1 – 5 generic primer, qNYSA-16117F/qNYSA16210R, but did not generate positive amplification for the group 1 and 2 variant-specific primers.

4.3.7 Screening for GLRaV-3 infection in the commercial Gimblett Gravels block

The commercial Gimblett Gravels block in Hawke’s Bay is taking part of an ongoing programme to control GLRaV-3 spread by roguing symptomatic and/or diagnostically positive vines and re-planting with certified vines and controlling mealybugs. In this study, leaf, cane, and trunk samples from a sub-section of this block were screened for GLRaV-3 using ELISA and mRT-PCR protocols (refer to Sections 4.2.1.2 and 4.2.6).

4.3.7.1 2010 summer season testing

In the 2010 GLRaV-3 screening, samples were collected from a subsection of the Gimblett Gravels block in five-vine composites of leaves using DAS-ELISA. To test the utility and compare the new molecular-based assays with DAS-ELISA, 27 samples (19 positive and eight negative) representing 18 individual symptomatic leaves and nine composite samples were selected from the DAS-ELISA results.
and tested by mRT-PCR (Table 4.8). For positive composite samples, each vine within the composite sample was individually tested by DAS-ELISA and mRT-PCR, to identify the individual infected vine(s).

For all 27 samples, there was only one discrepancy between DAS-ELISA and mRT-PCR results. Out of the 19 positive DAS-ELISA samples, 12 were infected with a group 1 variant (nine Cabernet sauvignon and three Syrah) and seven (four Cabernet sauvignon and three Syrah) were infected with a NZ2 variant. All composite test samples had a single infected vine, except GGC1-4, which consisted of two group 1 infected vines, GG1-4-4 and GG1-4-5 (Table 4.8). The single leaf test of GG1-4-5 was negative for GLRaV-3 by DAS-ELISA.

| Table 4.8: Detection of GLRaV-3 by DAS-ELISA and mRT-PCR in leaf material from the Gimblett Gravels block. |
|---|---|---|---|---|---|
| Sample name | Reaction rates (mOD/min) | mRT-PCR results |
| Composite | Single leaf | Group 1 | NZ2 | Group 2 | NZ-1 | Group 1-5 |
| GGC1-4 | 1.26 | ✓ | ✓ |
| G1-4-1 | 0.01 | Negative |
| G1-4-2 | 0.02 | Negative |
| G1-4-3 | 0.03 | Negative |
| G1-4-4 | 3.30 | ✓ | ✓ |
| G1-4-5 | 0.05 | ✓ | ✓ |
| GGC3-7 | 0.66 | ✓ | ✓ |
| G3-7-1 | 0.80 | ✓ | ✓ |
| G3-7-2 | 0.02 | Negative |
| G3-7-3 | 0.02 | Negative |
| G3-7-4 | 0.00 | Negative |
| G3-7-5 | 0.01 | Negative |
| GGC10-13 | 1.12 | ✓ | ✓ |
| G10-13-2 | 2.96 | ✓ | ✓ |
| G10-13-3 | 0.01 | Negative |
| G10-13-4 | 0.01 | Negative |
| G10-13-5 | 0.01 | Negative |
| GGC23-5 | 0.25 | ✓ | ✓ |
| G23-5-1 | 0.01 | Negative |
| G23-5-2 | 0.01 | Negative |
| G23-5-3 | 0.51 | ✓ | ✓ |
| G23-5-4 | 0.00 | Negative |
| G23-5-5 | 0.01 | Negative |
| GGC32-6 | 0.58 | ✓ | ✓ |
| G32-6-1 | 0.02 | Negative |
| G32-6-2 | 0.01 | Negative |
| G32-6-3 | 0.01 | Negative |
| G32-6-4 | 1.46 | ✓ | ✓ |
| G32-6-5 | 0.01 | Negative |
| GGC32-7 | 2.27 | ✓ | ✓ |
| G32-7-1 | 3.29 | ✓ | ✓ |
| G32-7-2 | 0.00 | Negative |
| G32-7-3 | 0.02 | Negative |
| G32-7-4 | 0.01 | Negative |
| G32-7-5 | 0.02 | Negative |
| GGC38-6 | 1.13 | ✓ | ✓ |
| G38-6-1 | 1.95 | ✓ | ✓ |
4.3.7.2 2010 winter season testing

In the winter of 2010, trunk samples from 66 vines (45 Cabernet sauvignon and 21 Syrah) that had presumed GLRaV-3 symptoms and had been rogued, were DAS-ELISA tested. For Cabernet sauvignon 31 out of the 45 (69%) vines were confirmed to be GLRaV-3 infected compared with only four out of the 21 (19%) Syrah vines. To determine the type of GLRaV-3 infection present in these infected vines, all ELISA positive vines were analysed using the mRT-PCR assay (Table 4.9). Both single (32) and mixed (3) infections were identified. The 31 Cabernet sauvignon vines had single group 1 (23) and NZ2 (5) infections, and mixed infections of group 1 and NZ2 (2) and group 1 and NZ-1 (1). All four Syrah positives were infected with NZ2 only.
Table 4.9: Detection of GLRaV-3 by ELISA and mRT-PCR in Cabernet sauvignon and Syrah trunk samples from the Gimblett Gravels block.

<table>
<thead>
<tr>
<th>Sample name&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Reaction rates (mOD/min)</th>
<th>mRT-PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>GG1-4-4</td>
<td>9.92</td>
<td>✓</td>
</tr>
<tr>
<td>GG1-32-3</td>
<td>48.29</td>
<td>✓</td>
</tr>
<tr>
<td>GG1-32-4</td>
<td>8.94</td>
<td>✓</td>
</tr>
<tr>
<td>GG1-34-3</td>
<td>26.90</td>
<td>✓</td>
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<tr>
<td>GG1-34-4</td>
<td>41.00</td>
<td>✓</td>
</tr>
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<td>GG1-36-2</td>
<td>42.57</td>
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<td>GG1-37-4</td>
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<tr>
<td>GG2-15-3</td>
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<tr>
<td>GG2-24-1</td>
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<td>GG2-25-4</td>
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<td>GG2-26-2</td>
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</tr>
<tr>
<td>GG11-19-2</td>
<td>35.19</td>
<td>✓</td>
</tr>
<tr>
<td>GG11-20-4</td>
<td>18.73</td>
<td>✓</td>
</tr>
<tr>
<td>GG11-24-1</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>GG11-30-5</td>
<td>36.16</td>
<td>✓</td>
</tr>
<tr>
<td>GG11-33-5</td>
<td>0.85</td>
<td>✓</td>
</tr>
<tr>
<td>GG12-11-4</td>
<td>30.78</td>
<td>✓</td>
</tr>
<tr>
<td>GG12-19-1</td>
<td>36.96</td>
<td>✓</td>
</tr>
<tr>
<td>GG12-19-5</td>
<td>23.41</td>
<td>✓</td>
</tr>
<tr>
<td>GG12-23-4</td>
<td>22.64</td>
<td>✓</td>
</tr>
<tr>
<td>GG12-35-5</td>
<td>46.72</td>
<td>✓</td>
</tr>
<tr>
<td>Syrah 1</td>
<td>3.21</td>
<td>✓</td>
</tr>
<tr>
<td>Syrah 3</td>
<td>2.81</td>
<td>✓</td>
</tr>
<tr>
<td>Syrah 9</td>
<td>1.94</td>
<td>✓</td>
</tr>
<tr>
<td>Syrah 10</td>
<td>3.29</td>
<td>✓</td>
</tr>
</tbody>
</table>

<sup>a</sup>Samples with the GG prefix name denotes vine position within the block (row-bay-vine).

<sup>b</sup>Samples with the GG prefix are from Cabernet sauvignon vines.

Four of the trunk samples, GG1-4-4, GG11-12-2, GG11-13-1, and GG12-11-4, had earlier been tested as leaf samples in summer. Results confirmed the identification of group 1 infection in samples GG1-4-4, GG11-12-2, and GG12-11-4. An additional NZ2 infection was detected in vine GG11-13-1, whereas GLRaV-3 had not been detected in GG11-13-1 within a 5-vine composite sample.
containing a leaf from this vine by ELISA in summer. Vines GG10-13-1 and GG10-13-2 in the adjacent row were also infected with the NZ2 GLRaV-3 variant.

The overall 2010 mRT-PCR screening results for the Cabernet sauvignon vines (within the 2010 summer testing area) are presented in Figure 4.16, together with the vine position of the previous year’s certified replanted vines. In most cases, positive mRT-PCR vines were in close proximity to other infected vines with the same GLRaV-3 variant or in replanted vines, i.e. in close proximity to a previously infected vine.

Figure 4.16: Schematic of the selected region within the Cabernet sauvignon Gimblett Gravels block that was ELISA and multiplex RT-PCR screened in 2010. The rows are labelled left to right and bays (five vines per bay) are numbered in ascending order from the bottom to the top of the diagram. Each “x” represents a single vine. Vines were infected with either a group 1 variant (green shading) or NZ2 (blue shading). Vines replanted in 2009 are also marked (yellow shading).
4.3.7.3 2010 summer and winter season testing of the neighbouring Syrah block

In a parallel investigation, leaf and cane material from a neighbouring Syrah block was screened for GLRaV-3. At the same time as Cabernet sauvignon leaves were collected, 51 non-symptomatic and 16 symptomatic Syrah leaves were collected and tested by DAS-ELISA. From the initial results, 57 out of 67 were GLRaV-3 positive and the remaining ten samples were either borderline positive (six) or negative (four). A subset of 17 samples including all the negatives and borderline positives were also tested by mRT-PCR. All of these samples tested positive for the NZ2 variant only (Table 4.10). The ELISA borderline and negative samples were re-tested by DAS-ELISA (using the same protocol), where all samples re-tested positive except for two non-symptomatic NZ2 infected samples.

In the 2010 winter season, six dormant cane samples randomly collected from different vines within the neighbouring Syrah block were also tested by DAS-ELISA and mRT-PCR. All were positive and infected with the NZ2 variant, except one that was infected with a group 1 variant (Table 4.10).

Table 4.10: Detection of GLRaV-3 by ELISA and mRT-PCR in an old Syrah block neighbouring the main Gimblett Gravels block.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Symptomatic or Symptomless</th>
<th>Reaction rates (mOD/min)</th>
<th>mRT-PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First test</td>
<td>Second test</td>
</tr>
<tr>
<td>Leaf testing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG41-1-6b</td>
<td>Symptomatic</td>
<td>0.09</td>
<td>0.24</td>
</tr>
<tr>
<td>GG41-1-7a</td>
<td>Symptomatic</td>
<td>0.08</td>
<td>0.25</td>
</tr>
<tr>
<td>GG41-1-7f</td>
<td>Symptomless</td>
<td>0.72</td>
<td>nt</td>
</tr>
<tr>
<td>GG41-2-4a</td>
<td>Symptomless</td>
<td>0.17</td>
<td>nt</td>
</tr>
<tr>
<td>GG41-2-4b</td>
<td>Symptomless</td>
<td>0.08</td>
<td>0.15</td>
</tr>
<tr>
<td>GG41-2-4c</td>
<td>Symptomless</td>
<td>0.09</td>
<td>0.46</td>
</tr>
<tr>
<td>GG41-3-1a</td>
<td>?</td>
<td>1.14</td>
<td>0.35</td>
</tr>
<tr>
<td>GG41-3-1b</td>
<td>Symptomless</td>
<td>0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>GG41-3-1c</td>
<td>Symptomless</td>
<td>0.10</td>
<td>0.54</td>
</tr>
<tr>
<td>GG41-3-1d</td>
<td>Symptomless</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>GG41-3-2a</td>
<td>?</td>
<td>0.17</td>
<td>0.10</td>
</tr>
<tr>
<td>GG41-3-2b</td>
<td>Symptomless</td>
<td>0.04</td>
<td>2.20</td>
</tr>
<tr>
<td>GG41-3-2c</td>
<td>Symptomless</td>
<td>0.05</td>
<td>0.31</td>
</tr>
<tr>
<td>GG41-3-2d</td>
<td>Symptomless</td>
<td>0.04</td>
<td>nt</td>
</tr>
<tr>
<td>GG41-3-6a</td>
<td>Symptomless</td>
<td>0.55</td>
<td>nt</td>
</tr>
<tr>
<td>GG41-3-6b</td>
<td>Symptomless</td>
<td>0.29</td>
<td>nt</td>
</tr>
<tr>
<td>GG41-3-6c</td>
<td>Symptomless</td>
<td>0.26</td>
<td>nt</td>
</tr>
<tr>
<td>Cane testing</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>OS1</td>
<td>n/a</td>
<td>3.66</td>
<td>nt</td>
</tr>
<tr>
<td>OS2</td>
<td>n/a</td>
<td>2.74</td>
<td>nt</td>
</tr>
<tr>
<td>OS3</td>
<td>n/a</td>
<td>2.67</td>
<td>nt</td>
</tr>
<tr>
<td>OS4</td>
<td>n/a</td>
<td>3.01</td>
<td>nt</td>
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<tr>
<td>OS5</td>
<td>n/a</td>
<td>3.79</td>
<td>nt</td>
</tr>
<tr>
<td>OS6</td>
<td>n/a</td>
<td>45.13</td>
<td>nt</td>
</tr>
</tbody>
</table>

Purple and red shading indicates ELISA borderline and negative samples, respectively. 
nt = not tested.
4.3.7.4 2011 summer season testing

In the late summer of 2011, further screening of the Gimblett Gravels block was conducted. Eighteen symptomatic Cabernet sauvignon vines between rows 2 to 19 were ELISA and mRT-PCR tested. All samples were GLRaV-3 positive with single infections of either group 1 (13), NZ-1 (1), or NZ2 (4) variants (Table 4.1).

Table 4.11: Detection of GLRaV-3 by ELISA and mRT-PCR in 18 symptomatic Cabernet sauvignon vines from the Gimblett Gravels block.

<table>
<thead>
<tr>
<th>Sample number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reaction rates (mOD/min)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>mRT-PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monoclonal</td>
<td>New Assay&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GG2-25-5</td>
<td>25.95</td>
<td>26.42</td>
</tr>
<tr>
<td>GG3-6-5</td>
<td>6.01</td>
<td>24.04</td>
</tr>
<tr>
<td>GG4-25-4</td>
<td>27.06</td>
<td>25.68</td>
</tr>
<tr>
<td>GG7-35-1</td>
<td>2.50</td>
<td>23.78</td>
</tr>
<tr>
<td>GG916-3</td>
<td>30.03</td>
<td>29.98</td>
</tr>
<tr>
<td>GG9-21-3</td>
<td>27.71</td>
<td>27.37</td>
</tr>
<tr>
<td>GG9-27-2</td>
<td>27.48</td>
<td>26.65</td>
</tr>
<tr>
<td>GG10-17-2</td>
<td>4.39</td>
<td>23.75</td>
</tr>
<tr>
<td>GG11-7-2</td>
<td>3.82</td>
<td>23.95</td>
</tr>
<tr>
<td>GG11-24-2</td>
<td>6.56</td>
<td>24.48</td>
</tr>
<tr>
<td>GG13-12-2</td>
<td>26.25</td>
<td>26.70</td>
</tr>
<tr>
<td>GG13-13-1</td>
<td>27.12</td>
<td>26.44</td>
</tr>
<tr>
<td>GG14-19-4</td>
<td>29.64</td>
<td>28.78</td>
</tr>
<tr>
<td>GG14-29-4</td>
<td>27.79</td>
<td>27.63</td>
</tr>
<tr>
<td>GG15-18-5</td>
<td>25.67</td>
<td>23.24</td>
</tr>
<tr>
<td>GG15-21-2</td>
<td>26.54</td>
<td>24.86</td>
</tr>
<tr>
<td>GG16-22-2</td>
<td>26.53</td>
<td>25.65</td>
</tr>
<tr>
<td>GG19-1-5</td>
<td>27.53</td>
<td>26.91</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples with the GG prefix name denotes vine position within the block (row-bay-vine).
<sup>b</sup> Blue shading indicates samples with high reactivity ratios.
<sup>c</sup> New assay (NA) used polyclonal antibodies in a modified TAS-ELISA format.
<sup>d</sup> Ratio between the reaction rate of the new assay to reaction rate of monoclonal antibodies.

4.3.7.5 2011 winter season testing

In the 2011 winter season, cane samples from 243 vines throughout the Gimblett Gravels block were ELISA tested, resulting in detection of 228 GLRaV-3 positive vines, including five of the 11 non-symptomatic vines. A subset of 159 vines, consisting of Cabernet sauvignon (158) and Syrah (1) vines, was screened by mRT-PCR. This subset included all vines within the area used for the 2010 testing plus a selection of vines from the remaining middle and top sections of the block, and included 149 symptomatic and ten non-symptomatic vines. All 149 symptomatic vines and three non-symptomatic samples tested GLRaV-3 positive by mRT-PCR, consisting of single (98%) and double (2%) infections (Table 4.12).
However, only 149 out of the 152 mRT-PCR positive samples were ELISA positive. ELISA negatives comprised of one double infection of a group 1 variant and NZ2, and two single infections of NZ2.

Table 4.12: Number of GLRaV-3 positive samples identified for each phylogenetic group from the mRT-PCR testing of the Gimblett Gravels block.

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Number of samples</th>
<th>Phylogenetic group</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
</tr>
<tr>
<td>Single</td>
<td>149</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Double</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Triple</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Quadruple</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>152</td>
<td>64</td>
<td>0</td>
</tr>
</tbody>
</table>

a The ELISA and mRT-PCR results for each sample of the Gimblett Gravels block are in Appendix 5 Table A5.2.

b Group 3 to 5 represents the number of samples that generated mRT-PCR positive bands for the NYSA-16117F/NYSA-16210R primer set and no mRT-PCR positive bands for the NY-14055F/NY-14735R (group 1) and SA-15283F/SA-15600R (group 2) primer sets.

The mRT-PCR results for each GLRaV-3 positive sample were plotted according to the vine position within the commercial Gimblett Gravels block (Figure 4.17). Most of the infected vines were isolated plants scattered throughout the block; however, NZ2 variants and, to a lesser extent group 1 variants, also occurred in clusters.

Figure 4.18 compares the results from the two Cabernet sauvignon plot studies for the two years of screening. In many cases, positive vines from the second year of testing were adjacent to either two year old replanted vines or previously infected vines with the same GLRaV-3 variant. Furthermore, three of the two year old replanted vines became re-infected with GLRaV-3, either with a group 1 (2) or NZ2 (1) variant.
Figure 4.17: Schematic of the Grapevine leafroll-associated virus 3 (GLRaV-3) infection status from bays 1 to 44 and rows 1 to 20 of the Gimblett Gravels block. Vines were infected with either a group 1 variant (green shading), NZ-1 (red shading), NZ2 (blue shading), or mixed infection of group 1 and NZ2 (orange shading). Vines that tested GLRaV-3 negative are also marked (brown shading). The NZ2 clusters are marked with red dotted circles.
4.3.8 Comparisons between ELISA reaction rates and mRT-PCR

A large range of DAS-ELISA reaction rates were observed within each of the two locations surveyed. Reaction rates from DAS-ELISA positives samples using the Bioreba antiserum, from the Auckland collection and the second year screening of the Gimblett Gravels block, ranged between 0.14 to 57.67mOD/min and 0.15 to 24.45mOD/min, respectively (Appendix 5 Table A5.1 and Table A5.2). In contrast, infected material tested using polyclonal antibodies against GLRaV-3 from Goszczynski et al. (1995) in a TAS-ELISA format, resulted in increased reaction
rates of previously low Bioreba positive samples and reduced the range of reaction rates from the two locations to 0.64 to 33.43mOD/min and 0.14 to 10.96mOD/min, respectively (Appendix 5 Table A5.1 and Table A5.2). The reaction rate interquartile ranges for the monoclonal and polyclonal antiserum assays were calculated for both the Auckland collection and the Gimblett Gravels block (Figure 4.19), where the polyclonal interquartile range (4.76 and 2.55) was considerably lower than the monoclonal interquartile range (38.30 and 8.36). Therefore, more consistent reaction rates were obtained from all samples when polyclonal antibodies were used, however the polyclonal antibodies also increased the background for non-infected samples.

![Figure 4.19](image-url)

**Figure 4.19:** Plot showing the interquartile range (75 to 25%) for the monoclonal (blue bar) and polyclonal (red bar) antibody reaction rates from the Auckland collection and Gimblett Gravels block.

The high variation in Bioreba reaction rates and observable differences in GLRaV-3 reaction rates using different antisera suggests the different GLRaV-3 variants have different immunoreactivity (avidity). To investigate this further, for each location the ELISA and mRT-PCR results were compared. In most cases, grapevines infected with GLRaV-3 variants from groups 1 to 5 had high ELISA reaction rates, while samples with low reaction rates were infected with the NZ-1 and/or NZ2 variants of GLRaV-3. From the Auckland collection, all 96 samples with high ELISA reaction rates (7.68 and 57.67mOD/min) using the Bioreba monoclonal antibodies were infected with variants from at least one of the phylogenetic groups 1 to 5, either as single infections or in mixed infections with variants NZ-1 and NZ2. In contrast, 47 samples with low ELISA reaction rates (0.14 to 7.60mOD/min) were only infected with the NZ-1 and/or NZ2 variants, except four samples that were also infected with variants from group 1, 3, 4, or 5 (reaction rates were 0.14, 0.32, 1.00, and 4.86mOD/min).
From the 2011 winter testing of the Gimblett Gravels block, all 59 samples with high Bioreba reaction rates (3.79 - 24.45 mOD/min), were infected with group 1 GLRaV-3 variants, except one sample (GG1-35-3) was infected with NZ2 (24.45 mOD/min). Most of the 87 samples with low Bioreba reaction rates (0.09 - 2.83 mOD/min) were infected with either NZ-1 or NZ2, except five samples only infected with group 1 variants (0.09, 0.12, 0.15, 0.18, and 2.36 mOD/min).

To demonstrate differences in immunoreactivity between GLRaV-3 variants, the ratio of polyclonal to monoclonal reaction rates was calculated for all ELISA positive samples (Figure 4.20 and Figure 4.21). A high ratio indicates low immunoreactivity to the Bioreba monoclonal antibody. From the Auckland collection, 98 samples had a ratio from 0.51 to 3.06 and all were either infected with only variants from phylogenetic groups 1 to 5 or mixed infected with variants from groups 1 to 5 and NZ-1 and NZ2 variants (Figure 4.20). All samples with higher reactivity ratios (between 3.79 to 14.83) were infected with only NZ-1 and/or NZ2, with the exception of one sample TK00110, which had a ratio of 4.57 and was infected with group 1, NZ-1, and NZ2 variants (Figure 4.20).

**Figure 4.20:** Plot showing the reaction rates (mOD/min) and reactivity ratios for samples tested by ELISA from the Auckland collection using two combinations of antibodies. Samples were tested using the Bioreba monoclonal antibodies (blue bars) and polyclonal antibodies (red bars). In addition, the reactivity ratio, which is the ratio between the reaction rate of polyclonal antibodies to reaction rate of Bioreba antibodies was calculated (green triangles). Samples are ordered in ascending order of the reactivity ratios. The dotted black vertical line partitions low reactivity ratios that generally indicates infection of *Grapevine leafroll-associated virus 3* (GLRaV-3) variants from phylogenetic groups 1 to 5 or mixed infected with NZ-1 and/or NZ2, and high reactivity ratios that generally indicates GLRaV-3 infection of NZ-1 and/or NZ2. The red triangle highlights the atypical TK00110 sample that is infected with group 1, NZ-1, and NZ2 variants but has a high reactivity ratio (4.57).
From the Gimblett Gravels block, all samples infected with a group 1 variant, in single or mixed infection, had a reactivity ratio less than 2.00 (ranging between 0.32 and 1.99), with the exception of one sample (GG2-15-5) with a ratio of 3.44 (Figure 4.21). Samples with NZ-1 or NZ2 infection had reactivity ratios above 2.00 (ranging between 2.02 and 12.12) except one sample had a ratio of 0.45 (GG1-35-3) and was infected with NZ2 (Figure 4.21).

Figure 4.21: Plot showing the reaction rates (mOD/min) and reactivity ratios for samples tested by ELISA from the Gimblett Gravels block using two combinations of antibodies. Samples were tested using the Bioreba monoclonal antibodies (blue bars) and polyclonal antibodies (red bars). In addition, the reactivity ratio, which is the ratio between the reaction rate of polyclonal antibodies to reaction rate of Bioreba antibodies was calculated (green triangles). Samples are ordered in ascending order of the reactivity ratios. The dotted black vertical line partitions low reactivity ratios that generally indicates Grapevine leafroll-associated virus 3 (GLRaV-3) infection of variants from phylogenetic group 1 or mixed infected with NZ-1 and/or NZ2, and high reactivity ratios that generally indicates GLRaV-3 infection of NZ-1 or NZ2. The red triangles highlights the two atypical samples, GG1-35-3 and GG2-15-5, that are infected with NZ2 and a group 1 variant respectively, but have corresponding reactivity ratios (0.45 and 3.44 respectively) that do not follow the general trend.

In an additional analysis, nine root samples from Chardonnay vines from Hawke’s Bay (used for molecular assay optimisation tests) were ELISA tested using monoclonal and polyclonal antibodies. Samples with reactivity ratios less than 2.00 had mixed infections of group 1, NZ-1, and NZ2 variants, while samples with ratios greater than 2.00 were infected with either a single NZ-1 variant or co-infected with NZ-1 and NZ2 (Table 4.13 and Figure 4.22).
Table 4.13: Detection of GLRaV-3 by ELISA and mRT-PCR testing in nine root samples from Chardonnay vines in Hawke’s Bay.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Reaction rates (mOD/min)</th>
<th>mRT-PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monoclonal</td>
<td>New Assay</td>
</tr>
<tr>
<td>1</td>
<td>14.01</td>
<td>14.14</td>
</tr>
<tr>
<td>2</td>
<td>8.00</td>
<td>13.14</td>
</tr>
<tr>
<td>3</td>
<td>6.30</td>
<td>10.53</td>
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<tr>
<td>4</td>
<td>1.35</td>
<td>12.39</td>
</tr>
<tr>
<td>5</td>
<td>1.05</td>
<td>9.85</td>
</tr>
<tr>
<td>6</td>
<td>0.63</td>
<td>10.00</td>
</tr>
<tr>
<td>7</td>
<td>0.41</td>
<td>6.56</td>
</tr>
<tr>
<td>8</td>
<td>0.06</td>
<td>1.62</td>
</tr>
<tr>
<td>9</td>
<td>0.07</td>
<td>1.92</td>
</tr>
</tbody>
</table>

a Blue shading indicates samples with high reactivity ratios.
b Red shading indicates ELISA negative samples.
c New assay (NA) uses polyclonal antibodies in a modified TAS-ELISA format.
d Ratio between the reaction rate of the new assay to reaction rate of monoclonal antibodies.

Figure 4.22: Plot showing the reaction rates (mOD/min) and reactivity ratios for samples tested by ELISA from the nine 3309 rootstock root samples using two combinations of antibodies. Samples were tested using the Bioreba monoclonal antibodies (blue bars) and polyclonal antibodies (red bars). In addition, the reactivity ratio, which is the ratio between the reaction rate of polyclonal antibodies to reaction rate of Bioreba antibodies was calculated (green triangles). Samples are ordered in ascending order of the reactivity ratios. The dotted black vertical line partitions low reactivity ratios that generally indicates mixed infections of Grapevine leafroll-associated virus 3 (GLRaV-3) variants from phylogenetic group 1, NZ-1, and NZ2, and high reactivity ratios that generally indicates GLRaV-3 infection of NZ-1 and/or NZ2.
4.4 Discussion

Factors that can lead to false negative RT-PCR results for GLRaV-3 include high amounts of RT-PCR inhibiting secondary metabolites in total RNA extracts (Chapter 2) and sequence variation in the New Zealand virus population (Chapter 3). In this chapter, both total RNA extraction and molecular detection protocols were examined in order to improve the accuracy and reliability of molecular based techniques. This involved the comparison of four total RNA extraction methods, and the development of new generic conventional RT-PCR and real-time RT-PCR GLRaV-3 assays, as well as an mRT-PCR GLRaV-3 variant specific assay. Assays were designed using sequence data obtained during this study and available from other regions of the world. These assays detected GLRaV-3 readily and reproducibly regardless of the plant tissue type or variety of grapevine.

4.4.1 Plant RNA extractions

The isolation of high quality RNA is a critical factor, fundamental to the success of molecular assays. This is particularly difficult to achieve when extracting from tissues of woody and perennial plants, such as grapevine, due to the high concentrations of RT-PCR inhibitory polysaccharides and phenolic compounds (Carra et al., 2007; Gambino et al., 2008; Henson et al., 1993; MacKenzie et al., 1997; Nassuth et al., 2000; Newbury et al., 1977; Osman et al., 2006; Salzman et al., 1999). A number of different extraction methods, using different detergents, denaturing and reducing agents, and denaturing organic solvents, have been developed to minimise the presence of inhibitory compounds in RNA extracts (Gambino et al., 2008).

Variable success in isolating high quality RNA from cane and leaf material was observed. All extractions from leaves successfully generated RT-PCR products but all four extraction methods showed indications of polysaccharide contamination ($A_{230}:A_{260}$ of less than 1.7) in cane RNA extracts, with several samples that did not successfully generate mRT-PCR amplification without prior dilution. Similarly, Constable et al. (2012) reported difficulties using mature cane material, where an increased number of false-negative results were observed as canes matured and lignified. This is most likely due to an increased presence of inhibitory compounds in woody tissue (Constable et al., 2012). For instance, lignin is a phenolic compound within the plant cell wall that provides support and is most
abundant in vascular and support structures of woody tissue, such as the cane tissue of grapevines (Bruce et al., 1989).

Of the methods tested in this project, the modified RNeasy and CTAB methods yielded better quality RNA extracts compared to the Spectrum™ Plant RNA extraction kit. The RNA extracts were successfully used in downstream applications such as mRT-PCR, with only one RNeasy RNA extract requiring post-extraction modification (dilution of RNA). Nassuth et al. (2000) experienced problems with the modified RNeasy method, where RNA extracts from grapevine varieties SO4 and Riparia, and from older leaf tissue could not be used for RT-PCR. This problem was not encountered with the varieties and mature leaf material (harvest in the mid-summer season) used during the current study. All 24 RNeasy extracted RNA samples being successfully amplified, although one extract required dilution.

The Spectrum™ Plant RNA extraction kit (Sigma-Aldrich, St. Louis, MO, USA) consistently yielded the highest RNA concentrations, though it did not perform as well as the other methods in this study. Reducing agents are additives that can help improve RNA extraction methods, reducing the effects of RNase enzymes. Two alternatives, sodium metabisulphite and β-mercaptoethanol, were compared in conjunction with Spectrum™ kit. The sodium metabisulphite was found to be a viable alternative to β-mercaptoethanol provided in the Spectrum™ Kit (Sigma-Aldrich, St. Louis, MO, USA), with the added advantage that sodium metabisulphite extractions do not require the use of a fume hood. Increasing the concentration of this reducing agent could further help improve RNA extractions using the Spectrum™ kit and from cane material; only 1% reducing agent was used in both commercial kits as recommended by the manufacturer, while the modified CTAB method used 3% β-mercaptoethanol. Additional modifications that can improve RNA isolation include conducting an initial chloroform:isoamyl alcohol extraction step before proceeding to the commercial kit steps (Gambino et al., 2006; Gambino et al., 2008), using only 50 to 80 mg of plant material (Kalinowska et al., 2012), and eluting RNA with a larger volume of water/elution buffer (Constable et al., 2012).

Immunocapture is another method that selectively isolates viral RNA for testing. This uses virus specific antibodies bound to the surface of microtitre plate wells or
PCR tubes to trap virus particles that are then used as template for RT-PCR assays (Engel et al., 2008; Nolasco et al., 1997). A less specific method is the binding of virions (and other cell components) on charged/treated nylon and nitrocellulose membranes (Dovas et al., 2003; La-Notte et al., 1997; Olmos et al., 1996; Osman et al., 2006).

Based on the results of this study, the modified CTAB method was used for the remainder of the project as it was most cost effective (less than $3.00 per sample) and yielded RNA of relatively high quality and integrity. This was particularly critical given that over 800 field and experimental samples were to be screened using the mRT-PCR and/or real-time RT-PCR assays. Other studies have also effectively used CTAB-based RNA extraction protocols for woody species (Bester et al., 2012b; Carra et al., 2007; Gambino et al., 2008; Hu et al., 2002; Jooste et al., 2010; White et al., 2008). However, this extraction method is time and labour intensive compared to commercial kits, and is harder to automate. Therefore, it would be less suitable for high-throughput diagnostic screening that requires rapid extraction of a large number of samples per day. In a recent study, Osman et al. (2012), compared automated systems that extracted up to 96 samples at a time, and effectively extracted 96 samples in less than an hour. This type of system was not available for this project and so the CTAB method was selected.

4.4.2 Molecular assays

4.4.2.1 Generic assay

The reliability of molecular techniques is sequence dependent, and genetic variability in a virus population can result in reduced reliability. A number of molecular techniques have been developed for the generic detection of GLRaV-3 isolates, including the development of conventional RT-PCR and a gel-free TaqMan real-time RT-PCR assay using primer sets designed in ORF4 and 5 (Jooste et al., 2010; Osman et al., 2006; Turturo et al., 2005). However, in recent years our knowledge of the genetic variability between GLRaV-3 isolates has greatly increased with the rising number of samples sequenced (Chapter 2 and 3). Phylogenetic analysis has demonstrated that most of these sequences cluster into five groups; however, a number of sequences remain as outliers (Angelini et al., 2006; Prosser et al., 2007; Sharma et al., 2011) including NZ variants NZ1-B and NZ2 (refer to Chapter 3). Therefore, in order to accommodate the increased
GLRaV-3 genetic variability, sequence information from this project and from GenBank, with nucleotide differences of more than 20% were included in primer design.

Common fluorescence chemistry that has been used to successfully detect GLRaV-3 by real-time RT-PCR includes SYBR Green (Bell et al., 2009) and TaqMan® (Osman et al., 2007, 2008; Osman et al., 2006; Pacifico et al., 2011; Tsai et al., 2012) assays. SYBR Green is a cyanine dye that binds to the minor groove of any double-stranded DNA (non-sequence specific) and once bound, the dye emits a fluorescent signal that is more than 1000 times greater than unbound dye (Houghton et al., 2006). The TaqMan probe-based assay uses the Taq polymerase ability to hydrolyse DNA. A probe that is complimentary to a region between the forward and reverse primer is designed and contains a fluorescent reporter and quencher dye and the 5′ and 3′ ends of the probe, respectively (Houghton et al., 2006). The intact probe does not emit a fluorescent signal. During the annealing stage of the PCR reaction, the probe will bind to the sequence, and then be cleaved by the Taq polymerase during primer extension (Houghton et al., 2006). The separation of the two dyes results in the report dye emitting a fluorescent signal (Houghton et al., 2006).

The advantages of the SYBR Green based real-time RT-PCR include the reduced complexity and relatively lower cost of designing and manufacturing additional probes. The development and optimisation of the generic conventional and real-time protocols in this study was conducted in parallel, which has resulted in a generic protocol that is easily inter-changeable between the two assays without losing specificity or sensitivity, as both assays detected 10 transcribed RNA copies per µL. Furthermore, fewer false negative results are expected using this non-probe based assay compared with the probe-based TaqMan assay because of its added constraint of potential polymorphisms within the probe target sequence (Beuve et al., 2007; Papin et al., 2004; Varga et al., 2005).

Conversely, due to the non-sequence specific binding nature of the SYBR Green dye, all non-specific amplification such as PCR primer dimers results in a fluorescent signal. This drawback was addressed in the development of the generic GLRaV-3 assay in this project, with the protocol undergoing a series of steps to reduce the likelihood of non-specific amplification. These steps included the design of primers with reduced likelihood of forming secondary structures, and
the optimisation of important PCR parameters such as primer and magnesium concentration. Melting curve analysis and gel electrophoresis were used to detect non-specific amplification, and Ct values and PCR efficiencies to ensure high assay effectiveness.

4.4.2.2 Sequence-variant specific assay

With the increasing number of different GLRaV-3 sequence variants being identified our understanding of the biological properties of these variants and their potential impact on grapevines is of increasing importance. Therefore, there is a need for a protocol that can detect and discriminate the range of variants easily and cost effectively. Conventional RT-PCR in conjunction with the SSCP technique has thus far been the main method used to easily identify different GLRaV-3 variants and to identify mixed infections (Chapter 2). The initial SSCP analysis designed by Turturo et al. (2005) identified sequence variants in mixtures from groups 1 and 2, analyses by Jooste et al. (2010) and Gouveia et al. (2011) identified GLRaV-3 variants from groups 1 to 3 and groups 1 to 5, respectively, and analysis from this project identified GLRaV-3 variants from groups 1, 2, 3, 5, and divergent variants NZ-1 and NZ2 (Chapter 2). The main drawbacks of this method are the reduced specificity when testing mixed infections because of difficulties differentiating between isolates with similar SSCP patterns (Urzi et al., 2003), and possible bias against less-represented virus isolates, depending on the number of clones selected for sequencing or PCR amplification (Gouveia et al., 2011; Nolasco et al., 2009). In addition, the analysis requires prior knowledge of the expected SSCP patterns for each viral group (Emeny et al., 1999).

Other sequence variant-specific protocols include an APET assay developed to detect GLRaV-3 isolates from groups 1 to 5 within the Portuguese population, detecting isolates with approximately 6.3% average genetic difference (Gouveia et al., 2011). Sharma et al. (2011) designed two triplex assays where the first triplex specifically detects variants from groups 1 and 3 using 18S rRNA as an internal control, and the second triplex specifically detects isolates from groups 2 and 4 in addition to the generic detection of other GLRaV-3 variants. Samples that only showed positive amplification for the generic primer set, from the second triplex assay, required additional RT-PCR amplification and sequencing reactions to identify two further variant groups denoted as GLRaV-3e and -3f (Sharma et al., 2011). From this study, using the two triplex assays and additional RT-PCR and
sequencing reactions, the overall mean nucleotide difference between the main isolates, from each GLRaV-3 phylogenetic group (NY1, AF037268; GP18, EU259806; 9-221, HQ130332; 7-110, HQ130309; 7-1006, JF421962; 43-15, JF421951; CI817, EU344894) detected was approximately 20.5% based on a 428nt of the CP region (Sharma et al., 2011).

In comparison, this current study describes the development of a hexaplex RT-PCR assay. Within a single 10µL RT-PCR reaction, GLRaV-3 variants from groups 1 to 5 and outliers NZ-1 and NZ2 were simultaneously detected using the plant NAD5 gene as an internal control. Thus, the proposed mRT-PCR assay identifies variants from more phylogenetic groups within a single one-step RT-PCR reaction, where the overall mean nucleotide difference between the main isolates, from each phylogenetic GLRaV-3 group, (NY1, GP18, 9-221, 7-110, CI817, NZ-1, and NZ2) detected is approximately 20.8% based on a 428nt of the CP region. Furthermore, the inclusion of an internal control reduces the likelihood of false negative results and by targeting the NAD5 gene across a splice junction, plant genomic contamination of RNA samples is less of a concern compared with using an 18S rRNA control (Du et al., 2006; Menzel et al., 2002). The mRT-PCR assay has high specificity, correctly identifying all previous GLRaV-3 positive isolates from the initial genetic variability study (refer to Chapter 2), and high sensitivity, as transcribed RNA as low as 10 RNA copies per µL was successfully detected. Moreover the mRT-PCR assay successfully detected GLRaV-3 from three ELISA GLRaV-3 negative field samples.

4.4.3 Comparison between published LC1/LC2 and H330/C629 primers and new generic and mRT-PCR assays

Both the generic and sequence-variant specific assays readily detected 100% of the samples with single and/or mixed infection of New Zealand isolates. However, both LC1/LC2 and H330/C629 primer sets only gave a positive result for 72% of the infected samples; all samples not detected consisted of NZ-1 and/or NZ2 infection. This is comparable with the findings of Bester et al. (2012b), where published primers, including the LC1/LC2 primer set, were unable to detect the South African GH11 and GH30 isolates that have high similarity to NZ-1.

The LC1/LC2 and H330/C629 primer pairs have been used in a number of GLRaV-3 surveys (Engel et al., 2008; Fiore et al., 2008; Fiore et al., 2011; Fuchs et al., 2009; Jooste et al., 2005; Jooste et al., 2010; Martin et al., 2005b; Turturo et
al., 2005) and comparative studies between RT-PCR and other molecular assays (Engel et al., 2010; Osman et al., 2007, 2008). Osman et al. (2007, 2008) and Engel et al. (2010) compared the reliability of RT-PCR, TaqMan real-time PCR, low-density array (LDA), and microarray assays, for the detection of grapevine viruses; including GLRaV-3 detection using the LC1/LC2 RT-PCR primer pair. In all studies the conventional RT-PCR protocol using LC1/LC2 detected GLRaV-3 with the lowest detection rates compared to the other molecular assays. Better detectability can be a result of increased assay sensitivity that identifies low titre virus and/or detection of previously unknown virus isolates.

The TaqMan real-time PCR and LDA assays used a primer pair (56F/285R) and probe (181p) that were designed against the NY1 isolate (Osman et al., 2007; Osman et al., 2006). The primer binding sites had 100% homology to other GLRaV-3 sequences, available at the time, from GenBank and sequences obtained in the Osman et al. (2006, 2007) study using the LC1/LC2 primer pair. This analysis would not have included NZ-1 and NZ2 sequences. In addition, Pong (2009) used a number of different published primers, including 56F/285R, to screen grapevine material from selected New Zealand vineyards, which included samples (GV-B4, MT L2-2, and MT L2-3) used in this project (refer to Chapter 2). The GV-B4 sample tested positive for a dual infection of group 2 and NZ-1 variants, while samples MT L2-2 and MT L2-3 tested positive for group 1 and NZ-1. Pong (2009) did not detect group 2 and NZ-1 variants from these samples, only positively detecting the group 1 variant from the MT L2-2 and MT L2-3 samples. Therefore, it is likely that the 56F/285R primer set improved the detection of low titre GLRaV-3.

4.4.4 Primer binding analysis

In this present study, sequence information for groups 3, 4, and outlier isolate 43-15, were not available at the time of primer design. Therefore to predict PCR success, the primer binding sites of these additional GLRaV-3 isolates, where possible, were examined for potential mismatches that could affect detection. In particular, primer-template mismatches at or near the 3’terminal end are more likely to affect PCR success and efficiency (Kwok et al., 1990; Stadhouders et al., 2010). Even though the generic forward and reverse primer binding sites, GEN-11112F/GEN-11233R, of the PL-20 isolate from group 3 contained a number of polymorphisms, only one primer-template mismatch occurs within the last five
nucleotides of the each 3' terminal primer ends. Both mismatches occur three nucleotides from the 3' terminal, comprising of a T:C and G:A (primer:template) mismatch for the forward and reverse primer respectively. These mismatches are considered as acceptable for Taq/Moloney murine leukemia virus based real-time RT-PCR (Stadhouders et al., 2010). The generic primer binding sites of the GH11 and GH30 isolates are similar to NZ-1, with all polymorphisms within the binding site positioned in the centre, more than eight nucleotides away from the 3' terminal primer ends. Thus, the primer set should effectively detect both isolates.

The mRT-PCR primer set NYSA-16117F/NYSA-16210R, detecting groups 1 to 5, revealed isolate WA-MR from group 1 and isolates from groups 3 and 4 all contain a single A:C mismatch at nucleotide 21 of the reverse primer, four nucleotides from the 3' terminal. This mismatch is also considered acceptable (Stadhouders et al., 2010). In addition, samples from the Auckland collection that contained isolates from groups with the aforementioned primer:template mismatches, were screened using both primer sets and were successfully detected.

Sequence data available for outlier variants such as Tempr, CB-19, and 43-15, do not coincide with the region used to design generic primers, GEN-11112F/GEN-11233R. Moreover, there is little or no overlap between available sequences for outlier isolates, highlighting the lack of comparable sequence data, particularly between highly divergent GLRaV-3 isolates. This impedes effective sequence comparisons for primer design and in silico primer analysis. Therefore, the following precautions were taken to increase the likelihood of detecting divergent isolates: (i) primers GEN-11112F/GEN-11233R were designed and tested against a highly divergent sample set, (ii) primers were designed with minimal primer-template mismatch, particularly within the last five nucleotides of the 3'terminal end, and the forward primer terminates with a thymidine residue, increasing degeneracy and therefore the likelihood of primer extension (Apte et al., 2009; Kwok et al., 1990), and (iii) a non-probe based assay was used.

### 4.4.5 Application of mRT-PCR in pilot field surveys

The hexaplex RT-PCR assay was successfully applied to screen field samples. In this study, 74% of the Auckland collection and 1.9% of the commercial vineyard samples contained multiple GLRaV-3 sequence variants. Mixed infections of different plant viruses and different strains of the same virus are a common
occurrence (Roossinck, 2005; Syller, 2012). GLRaV-3 has been found to co-infect grapevines in combination with other grapevine viruses including GLRaV-1, GLRaV-2, GVA, GVB, and GFLV (Engel et al., 2010; Fuchs et al., 2009; Gambino et al., 2006; Nassuth et al., 2000; Osman et al., 2008; Sharma et al., 2011) and in combinations of GLRaV-3 variants from different phylogenetic groups, refer to Chapter 2 (Gouveia et al., 2011; Jooste et al., 2005; Jooste et al., 2010; Jooste et al., 2011; Sharma et al., 2011; Turturo et al., 2005; Wang et al., 2011). A much higher percentage of the Auckland collection samples contained mixed infections compared with the commercial Gimblett Gravels block and other published reports (Gouveia et al., 2011; Jooste et al., 2011; Sharma et al., 2011). This might be expected as it consists of older virally-infected plants that have been transferred from a number of different sites in New Zealand. In addition, the Auckland collection is a repository of virally infected material for research purposes, thus no disease control measures are undertaken in this block. The presence of multiple virus sources in a single site also increases the chances for multiple virus infections via mealybug transmission. In contrast, the Gimblett Gravels block is part of an on-going programme to control GLRaV-3 spread via annual roguing of plants with GLRaV-3 visual symptoms. This reduces the number of virus sources and in-turn reduces the chance of multiple virus infections via mealybug transmission. Other published studies may have detected a reduced number of mixed infections because the primer sets are unlikely to have reliably detected divergent isolates such as NZ-1 and NZ2.

The initial detection of NZ-1 and NZ2 (refer to Chapter 2) was not an isolated occurrence. Both occur frequently within the New Zealand GLRaV-3 population and have been detected in both the commercial vineyard and older germplasm collections, individually and in mixed infection with other GLRaV-3 variants. In particular, NZ2 was detected in more than half the samples from both collections; 93 out of 144 (64.6%) in the Auckland collection and 81 out of 152 (53.3%) from the Gimblett Gravels block. In an attempt to further understand the potential significance of NZ2, the mRT-PCR data were used to map the positions of each GLRaV-3 variant within the Auckland collection and the commercial Gimblett Gravels block, providing a convenient means to investigate the epidemic situation of GLRaV-3 variants. The spatial distribution of GLRaV-3 variants within the Auckland collection had long spanning stretches of infected vines with the same GLRaV-3 variant infection. This is likely due to mealybug transmission within the
current Auckland collection block (as mentioned above) and/or mealybug transmission that occurred before being located to Auckland.

As mentioned above, the Gimblett Gravels block is under an on-going roguing programme to control the GLRaV-3 spread. Thus, potential sources of new infections include (i) planting infected nursery stocks, (ii) mealybug transmission from nearby non-symptomatic plants or symptomatic plants before roguing, or possibly from neighbouring blocks/vineyards, and (iii) mealybug transmission from residual roots from rogued vines.

Where diseased nursery stock has been used for planting, it is possible that current diagnostic tests used to certify nursery stocks may have failed to detect some GLRaV-3 variants, particularly NZ-1 or NZ2, resulting in virus introduction into the block. Misdiagnosis can be because of poor primer specificity to divergent genetic variants, for example primer sets LC1/LC2 and H330/C629 do not detect NZ-1 or NZ2, or virus titre below the limits of detection in new or recent infections within the nursery block (Constable et al., 2012; Pietersen, 2004). However, misdiagnosis does not explain the high numbers of both group 1 and NZ2 variants, or why on several occasions GLRaV-3 positive plants were detected in close proximity to one another.

GLRaV-3 infection between adjacent vines along a row is the most common spatial patterning observed in epidemiological studies worldwide (Cabaleiro et al., 2008; Charles et al., 2009; Jooste et al., 2011; Pietersen, 2004). As described in Chapter 1, GLRaV-3 typically spreads within the vineyard from single infected vines that act as an inoculum source for spread to adjacent vines along the rows, and then to neighbouring vines across rows, leading to “GLRaV-3 clusters” (Cabaleiro et al., 2008; Pietersen, 2004). This secondary spread could be via root graft unions (Cabaleiro et al., 2008; Pietersen, 2004), where roots from neighbouring vines graft together, sharing vascular tissue thus allowing pathogen transmission (Cabaleiro et al., 2008; Epstein, 1978). Although, this method of transmission has been suggested for Apple mosaic virus and PPV, it has yet to be reported for GLRaV-3 and would only be a minor means of spread (Cabaleiro et al., 2008).

GLRaV-3 transmission is more likely by means of insect vectors (Pietersen, 2004). Mealybugs can move through a block by their own mobility, Charles et al. (2009)
frequently observed mealybug crawlers moving between vines along the trellis support wires in New Zealand vineyards, or by passive assistance from vineyard workers, machinery, animals, and wind (Charles et al., 2009; Pietersen, 2004). Airborne dispersal from neighbouring blocks or vineyards is also possible, leading to either a GLRaV-3 infection gradient on block edges (Pietersen, 2004) or random emergence of infected vines within the block (Charles et al., 2009). To date, most spatial and temporal analyses of GLRaV-3 have not examined the spread of specific genetic variants, apart from two recent studies that investigated the distribution of GLRaVs including GLRaV-3 variants from groups 1 to 6 within vineyards in the Napa Valley, USA (Sharma et al., 2011) and the distribution of GLRaV-3 genetic variants from groups 1 to 3 within mother blocks in South Africa (Jooste et al., 2011).

Sharma et al. (2011) tested symptomatic plants for six GLRaV species (GLRaV-1 to -5, and -9) and seven GLRaV-3 genetic variants, from 11 different vineyards that consisted of a total of 36 blocks and the following cultivars: Cabernet sauvignon, Cabernet franc, Chardonnay, Merlot, Malbec, and Pinot noir. GLRaV-1, -2, -3, -4, and -9 were identified from 216 samples, and approximately 81% of these positive GLRaV samples were infected with GLRaV-3. To investigate the spread of GLRaV-3 genetic variants, an additional 468 samples were screened for GLRaV-3 variants using two triplex assays, RT-PCR, and sequencing, as described above (Sharma et al., 2011). Of these samples, 65% were GLRaV-3 positive predominantly infected with variants from groups 1 to 3, and of the 65% positives, 21% were mixed infections of mostly dual infection, except for one triple infection of groups 1 to 3. The relatively high numbers of mixed infections in vineyards was hypothesised to be due to either primary spread with re-plants infected with mixed infections or the more likely scenario of secondary spread with multiple introductions via mealybug transmission (Sharma et al., 2011). Based on Hot-Spot analysis, the distribution of GLRaV-3 variants within the Napa Valley appeared uneven, with more groups 1 and 2 variants located in the North and Central vineyards respectively, and group 3 numbers were generally high throughout all vineyards (Sharma et al., 2011). In addition, analysis based on the number and the distribution patterns of specific variants within individual vineyards, showed evidence of vector transmission within and between vineyard blocks (Sharma et al., 2011).
In the South African study, SSCP analysis was used to identify different GLRaV-3 variants and the predominant GLRaV-3 variant identified from 14 mother blocks was from group 2 (Jooste et al., 2011). In addition, based on visual symptoms observed from 2001 to 2005 within a Cabernet sauvignon block, three GLRaV-3 clusters were further examined by SSCP analysis (Jooste et al., 2011). Each cluster started from a single visually positive vine observed in 2001, where mealybug virus transmission to numerous neighbouring vines resulted in the gradual appearance of symptomatic positive vines in following years. The three clusters contained either a single infection of a group 2 or 3 variant or a mixed infection of both group 2 and 3 variants (Jooste et al., 2011). In all three clusters, the GLRaV-3 variant(s) in the centre of each cluster were also identified in surrounding vines within and/or across rows. However, in two clusters neighbouring vines had GLRaV-3 variants not present in the centre vine (Jooste et al., 2011).

Possible differences in the rate of symptom expression between clusters were also observed (Jooste et al., 2011). Symptoms in neighbouring vines of the group 2 cluster were observed after only a year, while the other clusters took three to four years for symptoms to appear. It was suggested that the genetic variants may have differential symptom expression rates and/or differential virus-vector relationship, with group 2 variants having a more efficient transmission rate (Jooste et al., 2011). However, the mealybug population and their role in transmitting GLRaV-3 variants were not monitored (Jooste et al., 2011).

Similar to both studies, the spatial distribution of the GLRaV-3 genetic variants from the Gimblett Gravels block indicated mealybug transmission of group 1, NZ-1, and NZ2 variants within and across the rows. Where infected vines in close proximity to one other had the same GLRaV-3 variant infection was often found. In particular, NZ2 and, to a lesser extent, group 1 variants formed disease clusters, similar to Jooste et al. (2011).

In an attempt to control this secondary spread within a block, roguing and replacing infected vines with certified virus-free vines is frequently carried out in New Zealand and worldwide (Bell et al., 2009; Pietersen, 2004). However, as observed in this project, it is also common for new infections to reappear 12 to 18 months after vine removal and replanting (Bell et al., 2009; Pietersen, 2004). For example, testing of the selected region within the Cabernet sauvignon block during
the 2010/2011 summer and winter seasons, frequently detected group 1 and NZ2 variants in vines neighbouring the replanted vines (i.e. previously infected vines). In addition, in 2011 symptomatic vines often neighboured two-year old replanted vines or 2010 positive vines, and were infected with the same GLRaV-3 variant. On two occasions, two-year-old replanted vines were re-infected with a group 1 variant. Potential sources of the new infections include viruliferous mealybugs moving/transmitting to neighbouring vines before removal of infected vines, or viruliferous mealybugs left behind on remnant infected roots moving to neighbouring vines or the roots of replanted vines (Pietersen, 2004).

Bell et al. (2009) found that even 12 months after vine removal, GLRaV-3 was still detectable by DAS-ELISA and real-time RT-PCR in remnant roots and in *Ps. calceolariae* mealybugs found on remnant roots. In addition, 12 days after planting, *Ps. calceolariae* were found on roots of replant young bare-rooted vines (Bell et al., 2009). In this project, the roots of the Cabernet sauvignon vines were not tested, however in 2010, 35 samples from the trunks tested positive for group 1 (26), NZ-1 (1), and NZ2 (11), which suggests variants are also present in the roots. In addition, group 1, NZ-1, and NZ2 were all detected in nine herbicide treated 3309 root samples from Chardonnay vines. Thus, the group 1, NZ-1, and NZ2 variants can survive in remnant roots and in-turn could have been an additional source of inoculum for mealybug transmission in the Gimblett Gravels block.

During the 2011 winter season more group 1 (44.1%) and NZ2 (53.3%) variants were distributed throughout the block than NZ-1 (4.6%). This large difference in numbers between different variants could be a result of neighbouring blocks containing only group 1 and NZ2 infected vines, providing a source of inoculum for transmission, and/or enhanced mealybug transmission of group 1 and NZ2 variants within the block, compared to NZ-1 variants. In 2010 and 2011, both group 1 and NZ2 infected vines were identified in the neighbouring Syrah blocks. Thus, aerial dispersal of viruliferous mealybugs from these blocks possibly accounts for the apparent random appearances of some isolated group 1 and NZ2 vines, which do not neighbour other infected vines, similar to suggestions by Charles et al. (2009). However, only a small number of vines from the neighbouring blocks were screened and vines with NZ-1 may have been missed.
Based on the genetic diversity, spatial, and temporal analysis of GLRaV-3 worldwide, many publications have hypothesised that the different GLRaV-3 genetic variants may have different virulence and/or vector transmissibility (Charles et al., 2009; Gouveia et al., 2012b; Gouveia et al., 2011; Habili et al., 1997; Jooste et al., 2011; Sharma et al., 2011). Habili et al. (2009) reported the identification of two biologically different GLRaV-3 strains in Crimson Seedless table grapes; GLRaV-3-s that results in a severe phenotype and GLRaV-3-m that results in a mild phenotype and produces larger, crisper, and heavier berries compared to virus-free vines. Thus, differential virulence and/or transmission, may explain the higher number of NZ2 variant clusters observed in the Gimblett Gravels block. If the visual symptoms of Cabernet sauvignon vines infected with a group 1 variant are expressed earlier than NZ2 infected vines, this would lead to the identification and earlier removal of group 1 variant vines, and over several seasons, the overlooked NZ2 infected vines are sources of inoculum for mealybug transmission to adjacent plants, which generate the observed clusters. Alternatively, if NZ2 had a greater transmission rate than other variants, this would increase the likelihood of successful transmission into more neighbouring vines, which would also generate the observed larger NZ2 clusters. However, the relationship between host, virus, and vector remains poorly understood.

With other closteroviruses, phylogenetically different variants with different virus virulence have been reported (Bertazzon et al., 2010; Huang et al., 2005; Nolasco et al., 2009; Raccah et al., 1980; Rowhani et al., 2000). For example, vines infected with the GLRaV-2 RG variant are consistently asymptomatic (Bertazzon et al., 2010; Rowhani et al., 2000), while GLRaV-2 PN3 variants, in single or mixed infections, lead to symptom expression (Bertazzon et al., 2010). In addition, GLRaV-2 variants from groups RG and PN generally induce graft incompatibilities, while symptoms related to graft incompatibility are not observed in vines infected with the GLRaV-2 BD variant (Bertazzon et al., 2010).

Moreover, studies have shown different CTV genetic strains can lead to different symptom expression and differences in their aphid transmissibility (Huang et al., 2005; Raccah et al., 1980). CTV mild strains do not produce noticeable symptoms while other strains have a damaging effect, resulting in stem pitting syndrome which lowers yield and reduces fruit quality, or results in “quick decline” or tristeza syndrome which can cause death to trees shortly after infection (Nolasco et al.,
Raccah et al. (1980) observed citrus trees infected with a mixture of genotypes, which also had mixed ability for aphid transmission and Huang et al. (2005) determined that brown citrus aphids transmitted the CTV T35 variant more efficiently than the T30 variant in plants infected with both genotypes.

4.4.6 Comparisons between ELISA and mRT-PCR detection using field samples

Thus far, comparative ELISA and RT-PCR studies for the detection of grapevine viruses, including GLRaV-3, have shown the reliability and sensitivity of both detection methods is dependent on the time of sampling, type and age of tissue, and the environment influencing virus titre (Constable et al., 2012; Fiore et al., 2009; Ling et al., 2001). For instance, studies have shown RT-PCR is more reliable than ELISA when testing leaf and cane material from hot and cold climate regions of Australia (Constable et al., 2012), and the opening buds and tips/unfolded leaves in Chile (Fiore et al., 2009). In contrast, Ling et al. (2001) and Fiore et al. (2009) found ELISA and RT-PCR had equal reliability detecting grapevine viruses in mature cane material, though dependent on the time of sampling (Fiore et al., 2009), and ELISA was more reliable than RT-PCR when testing leaf petioles, completely expanded leaves, and green phloem tissues (Fiore et al., 2009).

Throughout this project, ELISA and RT-PCR protocols have been used to screen cane, leaf, and root samples. In most tests, ELISA and RT-PCR protocols gave consistent results. There were only two occasions where a small number of GLRaV-3 positive samples were detected by mRT-PCR but not by ELISA. Firstly, the 17 ELISA negative or borderline ELISA positive Syrah leaf samples from the Gimblett Gravels block, all tested mRT-PCR positive for NZ2. Only after an ELISA re-test did all but two samples test positive by ELISA. The two leaves that remained ELISA negative were symptomless. Difficulty in detecting GLRaV-3 infection in symptomless leaves is presumably due to lower virus titres. Ling et al. (2001) and Chen et al. (2003) have also reported the inconsistent detection of GLRaV-3 in non-symptomatic leaves using either method, though RT-PCR protocols showed higher sensitivity. For example, Ling et al. (2001) found that at the onset of grape ripening, all symptomatic leaves had 100% detection using both methods, while less than 60% and 30% of non-symptomatic leaves, at the same stage, detected positive using nested IC-RT-PCR and DAS-ELISA respectively.
Also, in a study by Constable et al. (2012), to improve the likelihood of virus detection, symptomatic leaves were preferentially selected and sampled rather than non-symptomatic leaves.

The second instance of inconsistent results between ELISA and molecular-based testing occurred when screening mature Cabernet sauvignon cane material from the Gimblett Gravels block. Three mRT-PCR positive samples consisting of one double infection of a group 1 variant and NZ2 and two single infections of NZ2, tested negative by ELISA. This suggests the current mRT-PCR protocol had higher sensitivity than the ELISA protocol. It is also likely that virus titre in these vines were low, as the samples are from a block where symptomatic vines are removed annually. Thus, positive vines are likely to have been recently infected, i.e. in the first year of infection. Studies by Cohen et al. (2004) and Constable et al. (2012) also comment on the irregular detectability of grapevine viruses in vines with recent infections caused by low virus titre, as virus titre have yet to reach the levels for detectability. Other potential reasons for differences in reliability and sensitivity between the two different methods include the uneven distribution of viruses within the vines and the varying amounts of polyphenolic and polysaccharide compounds, which varies in the different tissue types, at different times of the year, and the different phenological stages (Constable et al., 2012; Fiore et al., 2009).

The comparative studies examining different diagnostic methods also show contradictory results. Constable et al. (2012) found leaves were a more reliable virus source for both ELISA and RT-PCR testing than lignified (mature) cane material, while Ling et al. (2001) and Fiore et al. (2009) reported that mature cane was the most reliable tissue type. In this study, results from both tissue types were comparable. Differences between studies could be due to the dissimilar environmental conditions/climates, grapevine cultivar, and virus isolates present in each country (Constable et al., 2012). In addition, differences in experimental set-up including sampling strategies, RNA extraction methods, and in particular, differences in the virus detection efficiencies of the ELISA and RT-PCR assays used, could also lead to differing results (Constable et al., 2012).

Primer set(s) are an important component of a successful molecular assay, and all studies thus far have used different primer pairs, which potentially differ in GLRaV-3 strain detectability. For example, Fiore et al. (2009) used published primers
LC1/LC2, which as discussed in Section 4.4.3, are not as effective at detecting GLRaV-3 as the generic and mRT-PCR assays used in this project.

ELISA assays are dependent on the antibodies detecting epitopes on the virion coat protein (CP). A number of different monoclonal and polyclonal antibodies have been produced (Cogotzi et al., 2009; Goszczynski et al., 1995; Hu et al., 1990; Ling et al., 2000; Orecchia et al., 2008; Zee et al., 1987; Zhou et al., 2003). The different antisera may recognise different epitopes and/or recognise the epitopes on the virion surface but with different efficiencies (avidity), thus potentially leading to discrepancies between studies. This project has demonstrated that the conjugated monoclonal Bioreba antibody, MAbNY1.3, has a lower avidity to NZ-1 and NZ2 compared to other GLRaV-3 variants.

ELISA tests using the monoclonal antibody generally resulted in low reaction rates for samples with NZ-1 and/or NZ2 infections, and on several occasions this resulted in false negatives. Furthermore, 137 out of the 139 NZ-1 and/or NZ2 infected field samples from the Gimblett Gravels block (98.56%) generated high polyclonal to monoclonal reaction rate ratios, which indicates lower immunoreactivity for these samples using the Bioreba monoclonal antibody. Analogous differences in monoclonal antibody reactivity to different plant virus strains have been reported for Bean yellow mosaic virus (BYMV) (Jordan et al., 1991; Richter et al., 1995). Jordan et al. (1991) produced 30 monoclonal antibodies, raised against a mixture of 12 different potyvirus isolates, which were used to test different potyviruses and strains of BYMV. Twenty-eight out of the 30 antibodies showed varying avidity to eight BYMV strains tested and based on the antibody reactivity patterns to the 55 potyvirus isolates; at least 25 distinct epitopes, distributed in varies areas of the CP were identified.

At present, there is limited information on the identity, types, and distribution of epitopes on the GLRaV-3 CP. Based on differential monoclonal antibodies and single-chain antibody fragment reactivity to truncated maltose binding protein-GLRaV-3 fusion proteins containing varying CP lengths, a similar epitope region near the N' terminus was predicted (Orecchia et al., 2008; Zhou et al., 2003). Zhou et al. (2003) determined a region between amino acids 61 to 148 (presumed to be closer to amino acid 61) and Orecchia et al. (2008) determined a region between amino acids 59 to 78. The Bioreba, MAbNY1.3, monoclonal antibody was raised against the NY1 isolate (Hu et al., 1990) and although the two studies did not use
this particular antibody for epitope mapping, the monoclonal antibodies were raised against purified virus preparations containing a GLRaV-3 isolate from the same phylogenetic group and has 99% nucleotide identity to NY1 (Zhou et al., 2003). In addition, the primers used to obtain the recombinant coat protein to produce the single-chain antibody fragment specific to GLRaV-3 were designed against the NY1 isolate (Orecchia et al., 2008).

In this project, CP amino acid sequence comparisons between the NY1, NZ1-B, and NZ2 isolates showed that both divergent strains share five unfavourable substitutions (substitutions that involved changes to the amino acid properties) between amino acid positions 70 to 84, which are predicted to change the CP structure, refer to Chapter 3. Therefore, the reduced immunological reactivity of the MAAbNY1.3 monoclonal antibody to NZ-1 and NZ2 is most likely because of amino acid changes in the predicted epitope region and further supports the possibility of structural changes to the CP of both divergent strains. Similarly, a single amino acid determines the MCA-13 monoclonal antibody reactivity to the biological CTV strains (Pappu et al., 1993). Based on amino acid sequence analysis, the phenylalanine at position 124 is well conserved in the severe CTV strains that are MCA-13 reactive, while the MCA-13 un-reactive mild strains contain a tyrosine (Pappu et al., 1993). The importance of phenylalanine was confirmed through the construction of mutant CPs, where tyrosine was substituted for phenylalanine in severe strains and phenylalanine was substituted for tyrosine in mild strains, which resulted in the elimination and establishment of MCA-13 reactivity, respectively (Pappu et al., 1993).

4.4.7 Conclusions

The work described in this chapter has determined the CTAB RNA extraction method as the most suitable protocol for GLRaV-3 detection during this project. Although time consuming, the CTAB RNA extraction protocol was the cheapest and consistently produced high quality RNA irrespective of the type of plant material used for extraction. This chapter also described new molecular assays for the (i) **generic detection** of all known GLRaV-3 variants in New Zealand grapevines and (ii) **specific detection** of variants from group 1 to 5, and divergent strains NZ-1 and NZ2 within a single RT-PCR reaction, thus improving GLRaV-3 detection. The generic assay is useful for certification schemes as it allows for automated identification of GLRaV-3. Moreover, the field survey results from over
400 samples demonstrate the reliability and robustness of the mRT-PCR assay, providing researchers with another valuable tool that is simple and cost effective in identifying different GLRaV-3 variants in single and mixed infections, and which will assist future biological and spatial distribution studies. Comparisons between ELISA and mRT-PCR results for field samples also highlighted the reduced avidity of monoclonal antibody to divergent strains, NZ-1 and NZ2.
Chapter 5:  
Symptom development and virus distribution of  
selected GLRaV-3 variants

5.1 Introduction

Typical grapevine leafroll disease symptoms include the downward rolling of the leaf margins, premature reddening of leaves for red cultivars, the yellowing of leaves for white cultivars, reduction in growth, and the reduction in sugar levels in berries (Chapter 1). For a number of plant viruses, differences in the virus virulence, host species, rootstock/scion combination, and environmental conditions can all lead to differences in the expressed disease symptoms. For example, the GLRaV-2 PN3 variant, in single or mixed infections, expresses phenotypic symptoms, while the GLRaV-2 RG variant is consistently asymptomatic (Bertazzon et al., 2010; Rowhani et al., 2000). Based on the CP gene nucleotide sequence, the GLRaV-2 RG variant is approximately 23% divergent to the GLRaV-2 PN clade that contains the GLRaV-2 PN3 variant (Bertazzon et al., 2010).

The bulk of the GLRaV-3 studies have concentrated on understanding the genetic variation within the GLRaV-3 population, and little is known about possible biological variations, such as virus virulence, between the genetically different variants. Two studies by Habili et al. (2009) and Gouveia et al. (2012b) have indicated different GLRaV-3 variants may have differential virulence. Habili et al. (2009) reported the discovery of two biological GLRaV-3 strains in Crimson Seedless table grapes. GLRaV-3-s produced severe symptoms, associated with poor quality of grape berries, while GLRaV-3-m produced mild symptoms with larger, crisper, and heavier berries compared to virus-free vines (Habili et al., 2009). Both strains had approximately 99% CP nucleotide identity to other GLRaV-3 isolates in GenBank (Habili et al., 2009).

Gouveia et al. (2012b) compared the suppressor activity of GLRaV-3 p19.7 from phylogenetic groups 1 to 5. N. benthamiana 16C plants were infiltrated with A. tumefaciens assays carrying either 35S-GFP alone or with 35S-GFP and 35S-p19.7 from phylogenetic groups 1 to 5. Based on visual observations, northern
blots, and real-time RT-PCR analysis, a significant difference in suppressor activity was identified between variants from phylogenetic groups 3 and 4 (Gouveia et al., 2012b).

Since both the above studies were based on GLRaV-3 variants that are relatively similar to one another, it is likely that other GLRaV-3 variants that show significant genetic variation, such as NZ-1 and NZ2, will exhibit some biological differences compared to other GLRaV-3 variants. Both NZ-1 and NZ2 have considerable amino acid substitutions when compared to isolates from groups 1 to 5, which are likely to change the protein and in-turn the functionality of proteins (Chapter 3). Therefore, it is important to consider whether these genetic variations also lead to variations in biological properties such as virulence, distribution within the host, replication, and vector transmissibility, since these can influence the type of disease management programme used.

The overall objective of the work described in this chapter was to investigate the possible biological variations between three GLRaV-3 variants from group 1, NZ-1 (group 6), and NZ2. This was achieved by considering two aspects; (i) investigating differences in symptom expression/severity of the three variants in various cultivars, and (ii) investigating the distribution of GLRaV-3 within host plants. The viruses were graft inoculated to several different grapevine cultivars and monitored for visual symptoms and virus infection using the mRT-PCR assay (Chapter 4) and variant-specific real-time RT-PCR assays. Virus titre in plants was assessed by absolute or relative quantification, using variant-specific real-time RT-PCR assays in combination with either in vitro transcribed RNA standards or host-specific real-time RT-PCR assays for selected reference genes, respectively.
5.2 Materials and methods

5.2.1 Sources of virus-free and GLRaV-3 infected plant material for graft inoculations

5.2.1.1 2010 graft inoculations

The graft experiment conducted in 2010 used 194 two year old certified virus-free plants of either Sauvignon Blanc (65), Syrah (65), or Pinot noir (64) scion grafted onto 3309 (V. riparia x V. rupestris) rootstock. These bare-rooted plants were provided by the grapevine nursery Corbans Viticulture Limited.

The dormant cane material used as a source of virus inoculum was obtained from the Te Kauwhata Auckland collection. Material with five different GLRaV-3 infection combinations were used and are as follows: (i) single group 1 infection, (ii) single NZ2 infection, (iii) dual group 1 and NZ-1 infection, (iv) dual NZ-1 and NZ2 infection, and (v) a triple infection of group 1, NZ-1, and NZ2. The selection of infected cane cuttings with these infections were based on prior mRT-PCR screening results (Chapter 4 Section 4.3.6).

5.2.1.2 2011 graft inoculations

The graft experiment conducted in 2011 investigated the effect of rootstock on virus transmission. Virus-tested, dormant cane cuttings of five different virus-free rootstocks 3309 (V. riparia x V. rupestris), 101-14 (V. berlandieri x V. rupestris), Schwarzmann (V. riparia x V. rupestris), SO4 (V. berlandieri x V. riparia), and Riparia gloire (V. riparia cv. Gloire), were provided by Corbans Viticulture Limited. In addition, virus-free Pinot noir dormant cane cuttings provided by Dog Point Vineyard courtesy of Nigel Sowman, were also used as indicators.

As a source of virus inoculum dormant Cabernet sauvignon cane cuttings were obtained from the Hawke’s Bay Gimblett Gravels vineyard courtesy of Caine Thompson. Material consisted of single GLRaV-3 infections of either group 1, NZ-1, or NZ2 variants. The selection of infected cane cuttings were based on previous mRT-PCR screening results from the 2011 winter testing of dormant cane material (Chapter 4 Section 4.3.7.5).

5.2.2 Graft inoculations

The double-worked grafting method involves the plant being grafted twice, which results in a plant that consists of a rootstock, intermediate stock, and scion. The
double-worked graft set-up is generally used to overcome rootstock and scion incompatibility where an intermediate stock (interstock) is compatible to both rootstock and scion portions and is grafted in-between. In these graft inoculation experiments to investigate the virus movement up and down the plant, double-worked graft inoculations were conducted; where the GLRaV-3 infected material acted as an interstock in-between either two portions of scion (2010), two portions of rootstocks (2011), or a portion of rootstock and a portion of Pinot noir scion (2011). Growth of a bud on the terminal scion provided proof of successful graft unions with the infected scion.

5.2.2.1 Preparation of plant material

Following collection, all grafting material was kept moist and stored at 0 to 4°C until required. Just before use, material was soaked in water either overnight (as was done in 2011) or for four to five days (as was done in 2010) in order to rehydrate cuttings. The extended soaking of material in 2010 was required as the cuttings were particularly dry.

5.2.2.2 2010 graft inoculations

For the 2010 graft inoculations, two year old grapevine plants that had a pre-existing graft union between a 3309 rootstock and the scion cultivar were used. The scion on each two year old grapevine plants contained two buds. Mechanical omega cuts were conducted between these two scion buds and twice to infected cane material, generating a grafted plant consisting of the original rootstock/scion together with the new GLRaV-3 positive interstock/scion (Figure 5.1). In total 135 plants were prepared consisting of 45 plants for each of the three scion cultivars, Sauvignon blanc, Syrah, and Pinot noir, inoculated with five different GLRaV-3 infections, listed in Table 5.1.

To increase the prospect of successful graft take, similar shaped and sized diameters were matched as it increases the likelihood of cambium contact between sections. If an appropriate match could not be found (this was particularly the case with scion grafted on top of the interstock) a smaller sized cane of similar shape was selected. To protect the graft union grafting tape was applied to both unions, dipped in wax, and then in water. Grafted plants were then stood in water overnight to allow for acclimatisation and then stacked into callus boxes filled with perlite covering the new graft unions. The callus boxes were
stored in a controlled temperature room for eight weeks at 25 to 28°C and 80% relative humidity, and the boxes were watered twice a week.

Commonly used single-worked graft inoculations were also conducted for comparison with double-worked graft inoculations. The single-worked grafting method involves the plant being grafted once between a rootstock and scion (Figure 5.1). The two year old grapevine plants that were used for the double-worked graft inoculations were also used for these graft inoculations. The mechanical omega cuts were conducted between the two scion buds and once to the infected cane material generating a grafted plant consisting of the original rootstock/scion together with the new GLRaV-3 positive scion (Figure 5.1). In total 44 plants were prepared consisting of 15 Sauvignon blanc, 15 Syrah, and 14 Pinot noir plants, inoculated with five different GLRaV-3 infections (Table 5.2). The grafts were taped and plants stored in callus boxes as described above.

In addition, five healthy virus-free control plants per cultivar were used as controls. These were not graft inoculated with virus infected plant material but were otherwise treated similarly to graft inoculated plants.

Table 5.1: Number of plants generated with the corresponding graft inoculation combination for the 2010 double-worked graft inoculations.

<table>
<thead>
<tr>
<th>Graft inoculation combinationa</th>
<th>Number of plants generated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sauvignon blanc</strong></td>
<td></td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / <strong>group 1 interstock</strong> / Sauvignon blanc</td>
<td>15</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / NZ2 interstock / Sauvignon blanc</td>
<td>15</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / <strong>group 1+NZ-1 interstock</strong> / Sauvignon blanc</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / NZ-1+NZ2 interstock / Sauvignon blanc</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / <strong>group 1+NZ-1+NZ2 interstock</strong> / Sauvignon blanc</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc</td>
<td>5</td>
</tr>
<tr>
<td><strong>Syrah</strong></td>
<td></td>
</tr>
<tr>
<td>3309 / Syrah / <strong>group 1 interstock</strong> / Syrah</td>
<td>15</td>
</tr>
<tr>
<td>3309 / Syrah / NZ2 interstock / Syrah</td>
<td>15</td>
</tr>
<tr>
<td>3309 / Syrah / <strong>group 1+NZ-1 interstock</strong> / Syrah</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Syrah / NZ-1+NZ2 interstock / Syrah</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Syrah / <strong>group 1+NZ-1+NZ2 interstock</strong> / Syrah</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Syrah</td>
<td>5</td>
</tr>
<tr>
<td><strong>Pinot noir</strong></td>
<td></td>
</tr>
<tr>
<td>3309 / Pinot noir / <strong>group 1 interstock</strong> / Pinot noir</td>
<td>15</td>
</tr>
<tr>
<td>3309 / Pinot noir / NZ2 interstock / Pinot noir</td>
<td>15</td>
</tr>
<tr>
<td>3309 / Pinot noir / <strong>group 1+NZ-1 interstock</strong> / Pinot noir</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Pinot noir / NZ-1+NZ2 interstock / Pinot noir</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Pinot noir / <strong>group 1+NZ-1+NZ2 interstock</strong> / Pinot noir</td>
<td>5</td>
</tr>
<tr>
<td>3309 / Pinot noir</td>
<td>5</td>
</tr>
</tbody>
</table>

a Text in **bold** indicates the type of **Grapevine leafroll-associated virus 3** infection used as the interstock.
Figure 5.1: Diagrammatic representation of the double-worked and single-worked graft inoculations conducted in 2010. Mechanical omega cuts were performed to the Grapevine leafroll-associated virus 3 (GLRaV-3) infected material and between the two scion buds of the two year old virus-free plants (grafted onto 3309 rootstocks) to either generate a four-part grafted plant consisting of 3309/scion/GLRaV-3 positive interstock/scion separated by three graft unions (double-worked graft inoculations) or a three-part grafted plant consisting of 3309/scion/GLRaV-3 positive portion separated by two graft unions (single-worked graft inoculations). Shoots either developed above or below the GLRaV-3 infected interstock referred to as the top bud (T bud) or bottom bud (B bud), respectively.
Table 5.2: Number of plants generated with the corresponding graft inoculation combination for the 2010 single-worked graft inoculations.

<table>
<thead>
<tr>
<th>Graft inoculation combination</th>
<th>Number of plants generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sauvignon blanc</td>
<td></td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / group 1</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / NZ2</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / group 1+NZ-1</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / NZ-1+NZ2</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Sauvignon blanc / group 1+NZ-1+NZ2</td>
<td>3</td>
</tr>
<tr>
<td>Syrah</td>
<td></td>
</tr>
<tr>
<td>3309 / Syrah / group 1</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Syrah / NZ2</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Syrah / group 1+NZ-1</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Syrah / NZ-1+NZ2</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Syrah / group 1+NZ-1+NZ2</td>
<td>3</td>
</tr>
<tr>
<td>Pinot noir</td>
<td></td>
</tr>
<tr>
<td>3309 / Pinot noir / group 1</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Pinot noir / NZ2</td>
<td>2</td>
</tr>
<tr>
<td>3309 / Pinot noir / group 1+NZ-1</td>
<td>4</td>
</tr>
<tr>
<td>3309 / Pinot noir / NZ-1+NZ2</td>
<td>3</td>
</tr>
<tr>
<td>3309 / Pinot noir / group 1+NZ-1+NZ2</td>
<td>2</td>
</tr>
</tbody>
</table>

* Text in **bold** indicates the type of *Grapevine leafroll-associated virus 3* infection used for inoculations.

5.2.2.3 2011 graft inoculations

For the 2011 graft inoculations, the same graft inoculation procedure was followed as previously described in Section 5.2.2.2. However, instead of using two year old plants with a pre-existing graft union as a base, fresh cane cuttings of five different rootstocks were used. Double-worked graft inoculations were conducted with combinations of a rootstock, GLRaV-3 infected Cabernet sauvignon interstock, and either a rootstock or virus-free Pinot noir cuttings. This generated three-part grafted plants that comprised of two combinations (Figure 5.2). Due to the limited supply of virus infected scionwood, the virus-infected interstock used were approximately 2 to 4cm in length and were internodal segments without buds. The rootstock, interstock, and scion combinations that were generated are described in Table 5.3.

Negative controls consisted of five healthy virus-free control plants per rootstock. These were not graft inoculated with virus infected plant material but were otherwise treated similarly to graft inoculated plants. Also as a control, three virus-free Pinot noir cuttings were grafted onto 3309 rootstock.
Figure 5.2: Diagrammatic representation of the double-worked graft inoculations conducted in 2011. Mechanical omega cuts were performed to the Cabernet Sauvignon Grapevine leafroll-associated virus 3 (GLRaV-3) infected material, rootstock, and Pinot Noir cuttings. This generated three-part grafted plants consisting of either: (i) rootstock/GLRaV-3 infected interstock/rootstock, or (ii) rootstock/infected GLRaV-3 interstock/Pinot Noir, which were separated by two graft unions. Shoots either developed above or below the GLRaV-3 infected interstock referred to as the top bud (T bud) or bottom bud (B bud), respectively.
Table 5.3: Number of plants generated with the corresponding graft inoculation combination for the 2011 double-worked graft inoculations.

<table>
<thead>
<tr>
<th>Graft inoculation combination</th>
<th>Number of plants generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>3309 / group 1 interstock / 3309</td>
<td>5</td>
</tr>
<tr>
<td>3309 / group 1 interstock / Pinot noir</td>
<td>5</td>
</tr>
<tr>
<td>3309 / NZ-1 interstock / 3309</td>
<td>5</td>
</tr>
<tr>
<td>3309 / NZ-1 interstock / Pinot noir</td>
<td>5</td>
</tr>
<tr>
<td>3309 / NZ2 interstock / 3309</td>
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<tr>
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<tr>
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<tr>
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<td>SO4 / NZ2 interstock / SO4</td>
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<tr>
<td>SO4 / NZ2 interstock / Pinot noir</td>
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</tr>
<tr>
<td>SO4</td>
<td>5</td>
</tr>
<tr>
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</tr>
<tr>
<td>Riparia gloire / group 1 interstock / Pinot noir</td>
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</tr>
<tr>
<td>Riparia gloire / NZ-1 interstock / Riparia gloire</td>
<td>5</td>
</tr>
<tr>
<td>V Riparia gloire / NZ-1 interstock / Pinot noir</td>
<td>5</td>
</tr>
<tr>
<td>Riparia gloire / NZ2 interstock / Riparia gloire</td>
<td>5</td>
</tr>
<tr>
<td>V Riparia gloire / NZ2 interstock / Pinot noir</td>
<td>5</td>
</tr>
<tr>
<td>Riparia gloire</td>
<td>5</td>
</tr>
<tr>
<td>Schwarzmann / group 1 interstock / Schwarzmann</td>
<td>10</td>
</tr>
<tr>
<td>Schwarzmann / group 1 interstock / Pinot noir</td>
<td>10</td>
</tr>
<tr>
<td>Schwarzmann / NZ-1 interstock / Schwarzmann</td>
<td>6</td>
</tr>
<tr>
<td>Schwarzmann / NZ-1 interstock / Pinot noir</td>
<td>6</td>
</tr>
<tr>
<td>Schwarzmann / NZ2 interstock / Schwarzmann</td>
<td>8</td>
</tr>
<tr>
<td>Schwarzmann / NZ2 interstock / Pinot noir</td>
<td>7</td>
</tr>
<tr>
<td>Schwarzmann</td>
<td>5</td>
</tr>
</tbody>
</table>

* Text in **bold** indicates the type of *Grapevine leafroll-associated virus 3* infection used as the interstock.
5.2.2.4 Field planting and potting

After eight weeks in the callus boxes, grafted plants were removed and sat vertically in water for two to three days. The 2010 graft inoculated plants were planted in a field plot at Corbans Viticulture, while the 2011 graft inoculated plants were potted and grown in a glasshouse at the School of Biological Sciences, University of Auckland. To reduce the probability of secondary spread in the field and glasshouse, grafted plants with the same type of GLRaV-3 infection were grouped and planted together. In 2010, before plants were planted in the field, vines that developed long shoots (more than four buds) while in the callus boxes were pruned back to three or four buds to increase the surface area for sunlight and air circulation that encourages vine growth. In 2011, pruning of shoots before potting was unnecessary as all shoots had four buds or less. Debco Pot Power potting mix was mixed with perlite at a 1:3 ratio and graft inoculated plants were potted in 3L PB3 sized planter bags. Plants were grown in standard glasshouse conditions with temperatures between 18 to 25°C.

5.2.3 Monitoring of visual symptoms

The main visual symptoms caused by the grapevine leafroll disease are the downward rolling of the leaf margins, premature reddening of leaves in red grape varieties and a yellowing in white varieties, and stunting of growth. Symptoms on leaves in white varieties are generally harder to accurately identify than in red cultivars and rootstock cultivars generally do not exhibit visual symptoms (Krake et al., 1999; Martelli, 1993). The visual symptoms of red cultivar leaves were monitored and recorded 24 weeks after the initial graft inoculation. A qualitative scale, from 0 to 5, was used to grade the severity of the symptom development, where 0 represents a plant with no leaves with reddening or curling and 5 represents plants with leaves that have completely reddened and curled (Figure 5.3). In addition, to investigate potential stunting of plants the lengths of all newly developed shoots from all grafted plants were monitored and recorded 24 weeks after the initial graft inoculation.

To assess whether the different GLRaV-3 variant combinations lead to significant differences in symptom expression (visual leaf symptoms), the recorded data was correlated to the mRT-PCR results for GLRaV-3 infection and analysed using the non-parametric Kruskal-Wallis test and the Mann-Whitney U-test. The Kruskal-
Wallis test was used for comparisons between more than two samples, i.e. comparisons between three or more GLRaV-3 variant combinations, while the Mann-Whitney $U$-test was used for comparisons between two samples. The Kruskal-Wallis test analyses the data set as a group and therefore does not identify which pair(s) of samples are different. Although, it can be assumed that at least the two samples with the highest and lowest sum of ranks are significantly different (Fowler et al., 1998). To confirm which pair(s) of samples have significant differences, the Mann-Whitney $U$-test was conducted to sample pairs that demonstrated obvious differences based on factors such as calculated sum of ranks and means. All tests were conducted on GLRaV-3 combinations with three or more biological replicates.

![Figure 5.3: Photographs representing the qualitative scale, from 0 to 5, used to grade the severity of the leaf symptom development for Pinot noir and Syrah cultivars.](image)

To assess whether GLRaV-3 infection affected the growth of plants compared to healthy GLRaV-3 negative plants and whether the different GLRaV-3 variant combinations lead to significant differences in growth, the data was correlated with the mRT-PCR results and analysed using the non-parametric Kruskal-Wallis test and the Mann-Whitney $U$-test, as described above. The only exception was analysis of GLRaV-3 infected and healthy Sauvignon blanc plants. Since the number of infected Sauvignon blanc was greater than 20, normal distribution can be assumed and the $U$ statistic was converted into a z-score, and used to determine the p-value.
5.2.4 Testing for virus infection

Plants from the 2010 and 2011 were tested for GLRaV-3 infection using the mRT-PCR assay (Chapter 4). In addition, the 2011 plants were screened using variant-specific real-time RT-PCR assays (Section 5.2.4.3). Absolute and relative quantification was also conducted on selected 2011 grafted plants using variant-specific real-time RT-PCR assays, synthesised RNA standards and host gene-specific (reference gene specific) real-time RT-PCR assays, respectively (Section 5.2.4.4 and 5.2.4.5).

5.2.4.1 Sampling and RNA extraction

Total RNA was extracted from all leaf and cane material using the CTAB method described in Chapter 4 Section 4.2.2.3.

5.2.4.1.1 2010 graft inoculations

Molecular screening of grafted plants generated in 2010 was conducted at eight, 16, 24, and 36 weeks after the initial graft inoculation. At eight weeks, leaf material from the base of each shoot was collected. At 16 weeks two or three leaves from the base of developed shoots and at 24 weeks leaves from the base, middle, and apical positions of each developed shoot were sampled. In addition, at 36 weeks, cane phloem scrapings were taken from an area approximately 0.5cm by 2cm located 10 to 12cm below the graft union between the 3309 rootstock and scion indicator.

5.2.4.1.2 2011 graft inoculations

Molecular screening of grafted plants generated in 2011 was conducted at 16 and 24 weeks after the initial graft inoculation. At 16 weeks two or three leaves from the base of shoots were sampled. At 24 weeks leaves from the base, middle, and apical positions of each new shoot were collected.

5.2.4.2 mRT-PCR testing

The mRT-PCR screening of 2010 and 2011 plants for GLRaV-3 variants was conducted using the protocol described in Chapter 4 Section 4.2.4.2.
5.2.4.3 Real-time RT-PCR testing

At 24 weeks, the 2011 plants were also screened using variant-specific real-time RT-PCR assays. These assays were also used for the absolute (Section 5.2.4.4) and relative (Section 5.2.4.5) quantification of each GLRaV-3 variant. Depending on the GLRaV-3 variant, real-time RT-PCR assays for absolute quantification targeted a region between the 5’UTR and ORF1a, ORF1b, or ORF4 (Section 5.2.4.4.1), while additional real-time RT-PCR assays targeting ORF9 were used for relative quantification of the GLRaV-3 variants (Section 5.2.4.5.1).

5.2.4.4 Absolute quantification using real-time RT-PCR

For the 2011 graft inoculations, in addition to the mRT-PCR and real-time RT-PCR screening, at 24 weeks post-inoculation, of leaf samples from three different sites along the developed shoots, absolute quantification was conducted to investigate the titre of specific GLRaV-3 variants, and in particular the genome copy numbers, at each of the three sampling sites. This required group 1, NZ-1, and NZ2 variant-specific real-time RT-PCR assays, and a dilution series of RNA standards of known concentrations for each of the virus amplicons.

5.2.4.4.1 Primer design

GLRaV-3 has a long complex genome with 10 or 11 ORFs downstream of the ORF1a/b ‘replication gene block’ and to facilitate the expression of the downstream ORFs at the 3’ half of the genome, a nested set of 3’co-terminal sgRNAs are expressed (Chapter 1). Recently, Maree et al. (2010) and Jargulua et al. (2010) determined that GLRaV-3 sgRNA are expressed from ORF3 onwards. Thus, to enable accurate estimation of the genome copy numbers, primers were designed towards the 5’ half of the genome to exclude most non-genomic RNA. The group 1 variant-specific primer pair was designed to target a region spanning the 5’UTR and ORF1a, while the NZ-1 specific primer pair targets within ORF1b. Due to the unavailability of sequence for the NZ2 closer to the 5’terminal, this variant-specific primer set was designed in the 5’ most section of the available sequence, within ORF4.

All genomic variant-specific primer sets were designed using web-based program Primer3 (Rozen et al., 2000) based on multiple sequence alignments (targeting the different genome regions as described above), to ensure amplification of all
variants in each group and not other non-target groups, i.e. appropriate polymorphisms were present in the primer binding region of non-target groups (Table 5.4). Alignments were conducted using Geneious v5.5 (Drummond et al., 2011) and ClustalX v2.0 (Larkin et al., 2007). The primer criteria used for the design of real-time RT-PCR primers included: (i) generating an amplicon size between 70 to 250 nt, (ii) primer lengths between 19 to 27 nt, (iii) a basic melting temperature between 57 to 60°C, (iv) a G+C content of 40 to 60%, and (v) a low likelihood of forming secondary structures. The basic melting temperature was calculated using the BioMath Calculator (Promega, Madison, WI, USA) and the benchmarks used to analyse secondary structure formation for prospective primers are as described in Chapter 2 Section 2.2.3.2.1. Furthermore, high primer specificity to GLRaV-3 was confirmed using BLAST.

**Table 5.4:** Primer pairs used for real-time RT-PCR assays targeting areas of low subgenomic expression.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' to 3')</th>
<th>Positiona (nt)</th>
<th>GLRaV-3 target</th>
</tr>
</thead>
<tbody>
<tr>
<td>qNY-676F</td>
<td>TCCGTCGTCCTTCGTAACCATTATTTTCT</td>
<td>5'UTR 676-704 ORF1a 789-812</td>
<td>Group 1</td>
</tr>
<tr>
<td>qNYSA-812R</td>
<td>GTTGTACCTAACGTACTCCAAGGT</td>
<td>ORF1b 8,868-8,890 ORF1b 9,046-9,068</td>
<td>NZ1</td>
</tr>
<tr>
<td>qNZ1-8868F</td>
<td>CGCTGACTCTGCGTATTACATG</td>
<td>ORF1b 9,046-9,068</td>
<td></td>
</tr>
<tr>
<td>qNZ1-9068R</td>
<td>CGACGACTAGCCTTAGCTTTACC</td>
<td>ORF4 11,183-11,206 ORF4 11,401-11,420</td>
<td>NZ2</td>
</tr>
<tr>
<td>qNZ2-11183F</td>
<td>CAGACCGACAGCCGACGCTTTATT</td>
<td>ORF4 11,183-11,206</td>
<td></td>
</tr>
<tr>
<td>qNZ2-11420R</td>
<td>CAGCCTTCGCTCGACACAACC</td>
<td>ORF4 11,401-11,420</td>
<td></td>
</tr>
</tbody>
</table>

*a The primer nucleotide positions are based on the complete GP18 (EU259806) genome sequence.

### 5.2.4.4.2 Real-time RT-PCR protocol

The SYBR Green chemistry was used for the real-time RT-PCR assays. Due to the dsDNA-binding nature of this dye it is essential to optimise all assays to eliminate or reduce the occurrence of primer dimers and other non-specific amplification products that can lead to high background impeding virus detection and an overestimation of the target concentration (Ambion, 2001). Therefore aspects such as primer concentration, magnesium concentration, and the annealing temperature were all assessed. Primer concentrations ranged from 100 to 400 nM by a factor of 50 nM, magnesium concentrations from 3 to 5 mM by increments of 1 mM, and temperatures ranged from 60 to 65°C by increments of 1°C. Primer efficiencies were calculated using the LinRegPCR software v11.0
(Ramakers et al., 2003; Ruijter et al., 2009), and Ct and dissociation melting curves generated by Applied Biosystems 7900HT Sequence Detection Systems (SDS) software v2.3 (Applied Biosystems, Foster City, CA, USA) were used as a guide for optimisation.

The real-time RT-PCR protocol was carried out in 10µL reactions using the SuperScript® III/Platinum® SYBR® Green One-Step qPCR Kit with ROX (Invitrogen, Carlsbad, CA, USA) with 1µL of total RNA extract, 0.2µL Superscript™ III RT/Platinum® Taq Mix which includes RNaseOUT™ (Invitrogen, Carlsbad, CA, USA), and various primer and magnesium sulphate concentrations depending on the primer set used (Table 5.5). Each sample was analysed in two technical replicates and the qRT-PCR was performed using an ABI 7900HT real-time PCR thermocycler (Applied Biosystems, Foster City, CA, USA). The thermocycling conditions involved reverse transcription for 3 minutes at 55°C followed by 94°C for 2 minutes and 35 cycles of 94°C for 10 seconds, annealing and extension at 64°C for 30 seconds. The Ct and dissociation melting curves were calculated by the SDS software v2.3 (Applied Biosystems, Foster City, CA, USA). For each real-time qRT-PCR run (plate), water (no template) control, and GLRaV-3 negative controls (total RNA extracted from healthy grapevines from the same cultivar and at a similar phenological stage) were included. In addition, to remove inter-run variation, 10-fold serial-dilutions of the synthesised RNA standards (Section 5.2.4.4.4) between 10^1 to 10^8 copies of virus amplicon per µL were also run on each plate. Three technical replicates were run for all controls and RNA standards.

Table 5.5: Expected amplicon size, and final primer and magnesium sulphate concentrations used for each of the Grapevine leafroll-associated virus 3 variant-specific RT-PCR assays targeting areas of low subgenomic expression.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Amplicon size (nt)</th>
<th>Final concentration of Primer (nM)</th>
<th>MgSO₄ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qNY-676F / qNYSA-812R</td>
<td>137</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>qNZ1-8868F / qNZ1-9068R</td>
<td>201</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>qNZ2-11183F / qNZ2-11420R</td>
<td>238</td>
<td>100</td>
<td>5</td>
</tr>
</tbody>
</table>

5.2.4.4.3 Real-time RT-PCR specificity

Due to the high magnesium concentration used in real-time RT-PCR assays (at least 3mM) high primer specificity to target genes is essential. This was confirmed for all real-time RT-PCR primer sets by: (i) searching oligonucleotide sequence against the NCBI nucleotide database, (ii) testing primer sets against healthy
GLRaV-3 negative grapevine total RNA extracts, (iii) testing primer sets against total RNA extracts positive for non-target GLRaV-3 variants, and (iv) resulting PCR products amplified from total RNA extracts positive for the target GLRaV-3 variant were sequenced and compared as described in Chapter 4 Section 4.2.5.

5.2.4.4 Synthesis of the RNA standards for absolute quantification

Synthetic RNA standards were generated for the group 1, NZ-1, and NZ2 primer sets using PCR products as templates as described in Chapter 4 Section 4.2.5. The additional primer pairs with a flanking 5’ SP6 and T7 RNA polymerase binding site in forward and reverse primers, respectively, are displayed in Table 5.6. The primer pair used to generate synthetic RNA for the NZ2 variant was also used to test the sensitivity of the NZ2 variant-specific primer pair used in the mRT-PCR assay. Ten-fold serial-dilutions for all resultant synthetic RNA for each variant-specific primer set were made using healthy total RNA from 3309 rootstock leaf samples to generate a range of $10^1$ to $10^8$ copies of virus amplicon per µL and were used in each real-time qRT-PCR run to eliminate inter-run variation.

Table 5.6: Primer sets used for the generation of in vitro RNA transcripts for absolute quantification.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’n) a b</th>
<th>Position c (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NYSA-mRNA-64-F</td>
<td>ATTTAGTGACACTATAGAcTTGCTGTTCAGGGAGTTT</td>
<td>64-85</td>
</tr>
<tr>
<td>NYSA-mRNA-739-R</td>
<td>TAATACGACTCCTAGGGGACTGTTGTGGGACTGATTGC</td>
<td>719-739</td>
</tr>
<tr>
<td>NZ1-mRNA-8611F</td>
<td>ATTTAGGTGAACACTATAGAGACGTGAAGGTATTTAACCAAGC</td>
<td>8,611-8,633</td>
</tr>
<tr>
<td>NZ1-mRNA-9098R</td>
<td>TAATACGACTCCTAGGGGACAAACGGCAGGACATGGAGAC</td>
<td>9,075-9,098</td>
</tr>
<tr>
<td>NZ2-mRNA-10994F</td>
<td>ATTTAGGTGAACACTAAGAACGTGATTACGAGGAGCTGC</td>
<td>10994-11017</td>
</tr>
<tr>
<td>NZ2-mRNA-11466R</td>
<td>TAATACGACTCCTAGGGGCCGGTTGTGGGACTGACAG</td>
<td>11445-11466</td>
</tr>
</tbody>
</table>

a Sequence in bold represent the SP6 RNA polymerase binding site.

b Sequence in bold and underlined represent the T7 RNA polymerase binding site.

c Binding site positions based on the genome organisation of the GP18 isolate (EU259806) excluding NYSA-mRNA-64-F/NYSA-mRNA-739-R, which is based on the NY1 isolate (AF037268).

d Resulting in vitro RNA transcript was also used as the template for the sensitivity tests of the generic and mRT-PCR assays.

5.2.4.4.5 Analysis of results

Initial absolute quantification values generated by the SDS v2.3 software (Applied Biosystems, Foster City, CA, USA) were exported and corrected for differences in reaction efficiency, as the software assumes that all reaction efficiencies are equal. To ensure correct reaction efficiencies were used for absolute quantification, for each sample the reaction efficiencies were calculated using LinRegPCR v11.0 (Ramakers et al., 2003; Ruijter et al., 2009) and amplification curve data exported from SDS v2.3. Then to reduce potential overestimation of
within-sample variation, the calculated efficiencies were averaged for each sample and primer pair. Exported initial absolute quantification values were then corrected to account for differences in reaction efficiency, using the newly calculated amplification efficiencies for each sample reaction and serially diluted standard (Eqn. 5.1). This efficiency correction assumes that the threshold value for both the standard and samples tested are the same (Øvstebø et al., 2003).

\[ AQ_{\text{corrected}} = AQ_i \times \left( \frac{E_{\text{standard}}}{E_{\text{sample}}} \right) \]  

*Eqn. 5.1*

where, \( AQ_{\text{corrected}} \) is the corrected absolute quantification value

\( AQ_i \) is the initial absolute quantification value

\( E_{\text{standard}} \) is the efficiency of the RNA standard

\( E_{\text{sample}} \) is the efficiency of the sample

Prior to statistical analysis, the corrected absolute quantification values were normalised by \( \log_{10} \) transformation (Fowler et al., 1998), and the data from each of the three sampling sites was averaged for each sample. To determine whether there were significant differences in the genome copy number between sampling sites a one-way ANOVA test was conducted. Similar to the Kruskal-Wallis test, a significant result does not identify which pair(s) of samples is significantly different. To confirm the pair(s) of samples that have significant differences, \( t \)-tests were conducted to the different sample pairs. Prior to \( t \)-test analysis, an F-test (\( P=0.05 \)) was performed to examine whether variances were significantly different or not, and if equal variances were found a Student’s \( t \)-test was performed, while if variances were found unequal, a Welch’s \( t \)-test assuming unequal variances was performed.

To assess whether there were significant differences in the genome copy number between the GLRaV-3 group 1 and NZ-1 variants, irrespective of the sampling site, the transformed absolute quantification values of each GLRaV-3 variant across all three sampling sites were analysed as one sample using the F-test and \( t \)-test, as described above.

**5.2.4.5 Relative quantification using real-time RT-PCR**

In addition to absolute quantification of the titre of GLRaV-3 variants along the developed shoot, relative quantification was also performed. This required group 1, NZ-1, and NZ2 variant-specific and host gene-specific (reference gene-specific) real-time assays.
**5.2.4.5.1 Primer design**

In total, six GLRaV-3 variant-specific primer sets were used for relative quantification. Three of these sets target regions that are unlikely to generate high amounts of sgRNA, were also used for absolute quantification (Section 5.2.4.4). In addition, to investigate potential differences in the level of sgRNA expression, a second primer pair was designed for each GLRaV-3 variant to target ORF9 (Table 5.7). Jarugula et al. (2010) identified sgRNA(ORF9) as one of the top four highly expressed sgRNAs.

**Table 5.7:** Primer pairs used for real-time RT-PCR assays targeting areas of high subgenomic expression.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' to 3')</th>
<th>Position(^a) (nt)</th>
<th>ORF9</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>qNY-16582F</td>
<td>GCCGACTCCTCTGTTCAGCG</td>
<td>17,141-17,161</td>
<td>ORF9</td>
<td></td>
</tr>
<tr>
<td>qNY-16744R</td>
<td>CCAAATAGCTTAAGGAATCTACGC</td>
<td>17,298-17,323</td>
<td>ORF9</td>
<td>Group 1</td>
</tr>
<tr>
<td>qNZ1-17093F</td>
<td>ATGACGCTGAGACGGTTAGAGCA</td>
<td>17,093-17,116</td>
<td>ORF9</td>
<td>NZ-1</td>
</tr>
<tr>
<td>qNZ1-17203R</td>
<td>CAAAAGCGAGGGGAGGGTTCAATA</td>
<td>17,179-17,203</td>
<td>ORF9</td>
<td>NZ2</td>
</tr>
<tr>
<td>qNZ2-16957F</td>
<td>CAATGGATCGTTCTGAACACGAA</td>
<td>16,957-16,981</td>
<td>ORF9</td>
<td>NZ2</td>
</tr>
<tr>
<td>qNZ2-17114R</td>
<td>GCCTAGCTCCTTCTATCGTGAGT</td>
<td>17,091-17,114</td>
<td>ORF9</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)The primer nucleotide positions are based on the complete GP18 (EU259806) genome sequence.

To ensure that suitable reference genes are used for accurate normalisation and quantification, primers for 11 reference genes representing a range of different cellular functions were designed using *V. vinifera* sequences obtained from the NCBI database (Table 5.8). To ensure mRNA amplification, the primer sets were designed across an exon-intron-exon junction as described in Chapter 4 Section 4.2.4.1. Amplification of mRNA was confirmed by treating total RNA with DNase I (Invitrogen, Carlsbad, CA, USA) and treated RNA was used as template (Section 5.2.4.5.3).

All variant-specific and reference gene-specific primer sets (Table 5.7 and Table 5.8) were designed using web-based program Primer3 (Rozen et al., 2000) and the real-time primer criteria described in Section 5.2.4.4.1.
Table 5.8: Primer pairs used to target a selection of reference genes listing the genes targeted, NCBI Gene ID, and oligonucleotide sequence.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene Target</th>
<th>NCBI Gene ID</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vv-COX2-F</td>
<td>Cytochrome c oxidase (COX2)</td>
<td>FM179380b</td>
<td>CAATGGTATCGGACTTTATGAGTATTCCG</td>
</tr>
<tr>
<td>Vv-COX2-R</td>
<td></td>
<td></td>
<td>GACCCAATTCTGGATCATCTTCTGGA</td>
</tr>
<tr>
<td>Vv-EF2-F</td>
<td>Elongation factor 2 (EF2)</td>
<td>100261253</td>
<td>AGAGCAGTCAAGATGGTGCAAGTTTACA</td>
</tr>
<tr>
<td>Vv-EF2-R</td>
<td></td>
<td></td>
<td>GCAGCAGCCACTAAAGAATCTGTA</td>
</tr>
<tr>
<td>Vv-GAPDH-F</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>100233024</td>
<td>CTGTCTACTGATTACTGCCACCCC</td>
</tr>
<tr>
<td>Vv-GAPDH-R</td>
<td></td>
<td></td>
<td>GCCTCTCCACCTCTGAGGGTCTCCCTGGA</td>
</tr>
<tr>
<td>Vv-MDH-F</td>
<td>Malate dehydrogenase (MDH)</td>
<td>100232859</td>
<td>TCAACACTCGTTCAAGACAGGCCCCAAG</td>
</tr>
<tr>
<td>Vv-MDH-R</td>
<td></td>
<td></td>
<td>TAATCCAAAATCTGCTCCCTCCCTAGG</td>
</tr>
<tr>
<td>Vv-nad5-F</td>
<td>NADH dehydrogenase subunit 5 (NAD5)</td>
<td>FM179380b</td>
<td>GCTTCTTGGGCTTCTTGTGCTGC</td>
</tr>
<tr>
<td>Vv-nad5-R</td>
<td></td>
<td></td>
<td>TCCCTCCATCCCAAGCTAATGTGGA</td>
</tr>
<tr>
<td>Vv-PIP2-F</td>
<td>Aquaporin PIP2.2 (PIP2)</td>
<td>100233027</td>
<td>CCACGGCTATAGGTACCGAGGAGG</td>
</tr>
<tr>
<td>Vv-PIP2-R</td>
<td></td>
<td></td>
<td>TGATGTGCCCACCAAAGATGTTTGAGGCC</td>
</tr>
<tr>
<td>Vv-POLYU-F</td>
<td>Polypubiquitin-A-like (POLYU)</td>
<td>100267431</td>
<td>CATCACGCTGGAGGTTGAGAGC</td>
</tr>
<tr>
<td>Vv-POLYU-R</td>
<td></td>
<td></td>
<td>GACTCTTCTTGGATGTTTGAGGCC</td>
</tr>
<tr>
<td>Vv-PP2a-F</td>
<td>Serine/threonine protein phosphatase-like (PP2a)</td>
<td>100245244</td>
<td>CGATGCTCTTGGTACGATGTTG</td>
</tr>
<tr>
<td>Vv-PP2a-R</td>
<td></td>
<td></td>
<td>CTCCTGCTCCAAAAAAATCTCACAAGAC</td>
</tr>
<tr>
<td>Vv-SAND-F</td>
<td>SAND protein (SAND)</td>
<td>100265538</td>
<td>CGATGAGGAGATGCTCTTATTTCA</td>
</tr>
<tr>
<td>Vv-SAND-R</td>
<td></td>
<td></td>
<td>CAAATGTGCTGAGAATCTCTGCAG</td>
</tr>
<tr>
<td>Vv-SUC11-F</td>
<td>Sucrose transporter-like (SUC11)</td>
<td>100232844</td>
<td>GGGACTAGGTCAAGGGTTATCATAGG</td>
</tr>
<tr>
<td>Vv-SUC11-R</td>
<td></td>
<td></td>
<td>GCGAGTGGCCACCACCCCAAAAAGT</td>
</tr>
<tr>
<td>Vv-TATA-F</td>
<td>Transcription initiation factor subunit 7-like (TATA)</td>
<td>100258782</td>
<td>GCTGACATTGGTCAATGATGATTAGTTGT</td>
</tr>
<tr>
<td>Vv-TATA-R</td>
<td></td>
<td></td>
<td>ATCTCTTCTCAACAGGCCGCACAAAG</td>
</tr>
</tbody>
</table>

*Bracketed acronyms will be used to represent the corresponding reference genes for the remainder of the thesis.

b NCBI accession number for the complete mitochondrial genome of the Pinot noir clone ENTAV115.

5.2.4.5.2 Real-time RT-PCR protocol

The same optimisation procedure used to develop the genomic variant-specific assays was used for the sgRNA variant-specific assays and the reference gene-specific assays (Section 5.2.4.4.2). In addition, total RNA extracted from cane, leaf, and root material was also used for the development of the reference gene-specific assays, to ensure these assays could be effectively used across all types of plant tissue.

The real-time RT-PCR protocol was carried out in 10µL reactions using the SuperScript® III/Platinum® SYBR® Green One-Step qPCR Kit with ROX (Invitrogen, Carlsbad, CA, USA) as described in Section 5.2.4.4.2, with the various primer pairs and magnesium sulphate concentrations (Table 5.9). Instead of using RNA standards, virus amplification was compared to the four most stable
reference genes (Section 5.2.4.5.4). For each real-time qRT-PCR run (plate), water (no template) control, and GLRaV-3 negative control (total RNA extracted from healthy grapevines from the same cultivar and at a similar phenological stage) were included. The four reference gene assays for each sample and equivalent negative controls were also included in each run, to eliminate inter-run variation. Two technical replicates were examined for all samples and controls.

Table 5.9: Expected amplicon size, and final primer and magnesium sulphate concentrations used for each of the Grapevine leafroll-associated virus 3 (GLRaV-3) variant-specific and host-gene-specific real-time RT-PCR assays.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Amplicon size (nt)</th>
<th>Final concentration of Primer (nM)</th>
<th>MgSO₄ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLRaV-3 variant-specific primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qNY-16582-F / qNY-16744-R</td>
<td>183</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>qNZ1-17093F / qNZ1-17203R</td>
<td>111</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>qNZ2-16957F / qNZ2-17114R</td>
<td>158</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Reference gene primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vv-COX2-F / Vv-COX2-R</td>
<td>103</td>
<td>200</td>
<td>4</td>
</tr>
<tr>
<td>Vv-EF2-F / Vv-EF2-R</td>
<td>140</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>Vv-GAPDH-F / Vv-GAPDH-R</td>
<td>73</td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td>Vv-nad5-F / Vv-nad5-R</td>
<td>213</td>
<td>300</td>
<td>3</td>
</tr>
<tr>
<td>Vv-PIP2-F / Vv-PIP2-R</td>
<td>132</td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td>Vv-POLYU-F / Vv-POLYU-R</td>
<td>159</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Vv-PP2a-F / Vv-PP2a-R</td>
<td>126</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>Vv-SAND-F / Vv-SAND-R</td>
<td>130</td>
<td>200</td>
<td>3</td>
</tr>
<tr>
<td>Vv-SUC11-F / Vv-SUC11-F</td>
<td>116</td>
<td>300</td>
<td>3</td>
</tr>
<tr>
<td>Vv-TATA-F / Vv-TATA-R</td>
<td>165</td>
<td>200</td>
<td>3</td>
</tr>
</tbody>
</table>

5.2.4.5.3 Real-time RT-PCR specificity

The specificity of the GLRaV-3 variant-specific assays was tested as described in Section 5.2.4.4.3. To confirm that the reference gene-specific assays only amplified host mRNA, total RNA extracts were treated with DNase I (Invitrogen, Carlsbad, CA, USA) and treated RNA was used as template. Resultant PCR products were analysed on 2% TBE agarose gels (Chapter 2 Section 2.2.3.2.2).

5.2.4.5.4 Selection of reference genes based on expression stability

The Ct values generated by the SDS v2.3 software (Applied Biosystems, Foster City, CA, USA) were exported and analysed using three reference gene stability analysis programmes; geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004). To ensure correct reaction efficiencies were used for analysis, for each sample the reaction efficiencies were calculated using LinRegPCR v11.0 (Ramakers et al., 2003;
Ruijter et al., 2009) and amplification curve data exported from SDS v2.3. Calculated efficiencies were then averaged for each primer pair.

Prior to geNorm and NormFinder analysis, the raw Ct values were converted to relative quantities using the comparative delta-Ct method and the calculated PCR efficiencies. geNorm and NormFinder analysis was then conducted on the transformed data as per developer’s manual. In contrast, raw Ct values generated by SDS v2.3 and the calculated efficiencies for each reference gene primer pair were used as input data for the BestKeeper software, as described by Pfaffl et al. (2004).

To ensure the most stable reference genes were selected for an experimental set-up, an overall stability ranking of the candidate reference genes was generated based on comparisons between the resulting rankings from the three reference gene stability analyses. The overall top four reference genes were then used as the reference genes for relative quantification (Section 5.2.4.5.5) by geometric averaging of the four genes in the experiment (Hellemans et al., 2007; Vandesompele et al., 2002).

**5.2.4.5.5 Analysis of results**

The Ct values and amplification curve data for each reaction generated by the SDS v2.3 software (Applied Biosystems, Foster City, CA, USA) were exported. The amplification curve data was analysed using LinRegPCR v11.0 (Ramakers et al., 2003; Ruijter et al., 2009) to calculate reaction efficiencies (Section 5.2.4.4.5). Relative quantification (RQ) values for each sample were calculated using the method described by Pfaffl (2001) (Eqn 5.2) and the geometric average of the four selected reference genes was used to normalise the data, instead of using a single reference gene (Hellemans et al., 2007; Vandesompele et al., 2002).

$$RQ = \left(\frac{E_{target}^{\Delta Ct_{target}(control-sample)}}{E_{reference}^{\Delta Ct_{reference}(control-sample)}}\right)$$  

Eqn 5.2

where, RQ is the relative quantification values

- $E_{target}$ is the efficiency of the target gene transcript
- $E_{reference}$ is the efficiency of the reference gene transcript
- $\Delta Ct_{target}(control-sample)$ is the change in Ct between control and sample target gene transcript
- $\Delta Ct_{reference}(control-sample)$ is the change in Ct between control and sample reference gene transcript
To normalise the non-parametric derived data the calculated RQ values were $\log_{10}$ transformed (Fowler et al., 1998) and averaged for each of the technical replications of each sample and primer pair combination. For each GLRaV-3 variant, to assess whether there were significant differences in the RQ data when comparing more than two variables, a one-way ANOVA test was conducted. Also, to confirm the pair(s) of samples that have significant differences, Student’s or Welch’s $t$-tests were performed to the different sample pairs, as described in Section 5.2.4.4.5. To assess whether there were significant differences in the RQ data for GLRaV-3 group 1, NZ-1, and NZ2 variant genomic RNA and sgRNAs, irrespective of the sampling site, the transformed RQ values across all three sampling sites were analysed as one sample (Section 5.2.4.4.5).
5.3 Results

5.3.1 Development of GLRaV-3 variant-specific and host gene-specific real-time RT-PCR assays

The 2010 and 2011 grafted plants were all screened for GLRaV-3 using the mRT-PCR assay, described in Chapter 4. In addition, all 2011 graft inoculated plants were re-tested by real-time RT-PCR. The GLRaV-3 variant titres within 2011 grafted plants with 3309 rootstocks were also examined using real-time RT-PCR assays. The development and optimisation of the GLRaV-3 variant-specific and host gene-specific real-time RT-PCR assays are described below.

5.3.1.1 Optimisation of the GLRaV-3 variant-specific qRT-PCR assays

For all virus-specific primer sets, primer dimer formation and/or non-specific amplification was evident at primer concentrations of 200nM, 300nM, and 400nM. This was eliminated using 100nM of each variant specific forward and reverse primer. In addition, the final MgSO₄ concentrations of 3, 4, and 5mM for all virus-specific primer sets, were compared. Based on Ct values and PCR efficiencies, the best MgSO₄ concentration for all primer sets was 4mM, except for the group 1 specific primer pair targeting ORF9, and NZ2 specific primer pair targeting ORF4, which required 3mM and 5mM of MgSO₄ respectively, for optimal amplification (Table 5.5 and 5.9). All variant-specific real-time RT-PCR assays successfully detected the respective GLRaV-3 variant at all annealing/extension temperatures between 60 to 65°C (Figure 5.4 shows an example of amplification for all annealing/extension temperatures using the NZ-1 variant-specific real-time RT-PCR assay). The Ct values ranged between 15 and 30 cycles and PCR efficiencies between 1.84 and 1.97 (Table 5.10).

5.3.1.2 Specificity of virus-specific qRT-PCR assays

Each virus-specific real-time RT-PCR assay successfully amplified and detected its respective GLRaV-3 variants from all previously studied samples (Chapter 2). Non-specific amplification was not observed in healthy samples, water controls, and GLRaV-3 positive samples that were infected with other non-target GLRaV-3 variants. The specificity of each variant specific assay was further confirmed by sequencing of resulting amplicons, which resulted in 98 to 100% nucleotide identity to respective GLRaV-3 variants.
Figure 5.4: Grapevine leafroll-associated virus 3 (GLRaV-3) detection using the NZ-1 variant-specific real-time RT-PCR assay at annealing temperatures between 60°C to 65°C. For each annealing temperature, total RNA extracted from leaf material with a single infection of a NZ-1 variant (lane 1) and a NZ2 variant (lane 4), and cane material with a single infection of a NZ-1 variant (lane 2) and a group 1 variant (lane 3) were examined. Lane 5 uses total RNA extracted from healthy GLRaV-3 negative cane material, lane 6 uses water as no template controls, and lanes L are 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

5.3.1.3 Reference gene qRT-PCR assays

Ten of the 11 reference gene primer sets designed were optimised. The Vv-MDH-F/Vv-MDH-R primer set was not included as non-specific amplification of grapevine genomic DNA contaminants was observed (Section 5.3.1.5).

5.3.1.4 Optimisation of host-specific qRT-PCR assays

Reference gene qRT-PCR assays were optimised based on eliminating/reducing primer dimer formation, PCR efficiency, and effectively amplifying targets in RNA extracted from leaf, cane, and root samples. Initially, a final primer concentration of 400nM was used for both forward and reverse primers, which resulted in primer dimer formation. The primer concentration was sequentially reduced by increments of 50nM until no primer dimer formation was evident; refer to Table 5.9 for final primer concentrations.

For all primer sets, except Vv-POLYU-F/ Vv-POLYU-F and Vv-COX2-F/ Vv-COX2-R, a MgSO₄ concentration of 3mM resulted in the best PCR efficiencies and lowest Ct variation between technical sample triplicates. Vv-POLYU-F/ Vv-POLYU-R and Vv-COX2-F/ Vv-COX2-R primer pairs required 4mM (Table 5.9). Amplification was observed for all host-specific real-time RT-PCR assays at annealing/extension
temperatures between 60 to 65°C (Figure 5.5 shows an example of amplification for all annealing/extension temperatures using the NAD5 host-specific real-time RT-PCR assay) and PCR efficiencies ranged between 1.70 and 2.13 (Table 5.10).

![Image of gel electrophoresis with lanes labeled 60°C to 65°C with specific PCR results for each annealing temperature.]

**Figure 5.5:** NAD5 detection using the host-specific real-time RT-PCR assay at annealing temperatures between 60°C to 65°C. For each annealing temperature, total RNA extracted from leaf material with a single infection of a group 1 variant (lane 1), cane material with a single infection of a group 1 variant (lane 2), and root material with a dual infection of NZ-1 and NZ2 variants (lane 3) were examined. Lane 4 uses total RNA extracted from healthy GLRaV-3 negative cane material. Lane 5 uses water as no template controls, and lanes L are 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA).

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>PCR efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLRaV-3 variant-specific primers</td>
<td></td>
</tr>
<tr>
<td>qNY-676F / qNYSA-812R</td>
<td>1.87</td>
</tr>
<tr>
<td>qNZ1-8868F / qNZ1-9068R</td>
<td>1.86</td>
</tr>
<tr>
<td>qNZ2-11183F / qNZ2-11420R</td>
<td>1.90</td>
</tr>
<tr>
<td>qNY-16582F / qNY-16744R</td>
<td>1.84</td>
</tr>
<tr>
<td>qNZ1-17093F / qNZ1-17203R</td>
<td>1.97</td>
</tr>
<tr>
<td>qNZ2-16957F / qNZ2-17114R</td>
<td>1.90</td>
</tr>
<tr>
<td>Reference gene primers</td>
<td></td>
</tr>
<tr>
<td>Vv-COX2-F / Vv-COX2-R</td>
<td>1.95</td>
</tr>
<tr>
<td>Vv-EF2-F / Vv-EF2-R</td>
<td>2.03</td>
</tr>
<tr>
<td>Vv-GAPDH-F / Vv-GAPDH-R</td>
<td>1.93</td>
</tr>
<tr>
<td>Vv-nad5-F / Vv-nad5-R</td>
<td>1.96</td>
</tr>
<tr>
<td>Vv-PIP2-F / Vv-PIP2-R</td>
<td>2.13</td>
</tr>
<tr>
<td>Vv-POLYU-F / Vv-POLYU-R</td>
<td>1.97</td>
</tr>
<tr>
<td>Vv-PP2a-F / Vv-PP2a-R</td>
<td>1.75</td>
</tr>
<tr>
<td>Vv-SAND-F / Vv-SAND-R</td>
<td>1.70</td>
</tr>
<tr>
<td>Vv-SUC11-F / Vv-SUC11-R</td>
<td>2.07</td>
</tr>
<tr>
<td>Vv-TATA-F / Vv-TATA-R</td>
<td>1.82</td>
</tr>
</tbody>
</table>
5.3.1.5 Specificity of host-specific qRT-PCR assays

To confirm that the reference gene specific primer pairs only amplified plant mRNA, DNase I treated and non-treated total RNA extracts from leaf, cane, and root, were used as template and compared. For all primer pairs, except Vv-MDH-F/Vv-MDH-R, no additional amplification was observed in non-treated total RNA. For the Vv-MDH-F/Vv-MDH-R primer pair additional amplification of a ~250nt non-specific PCR product was observed in non-treated total RNA extracted from Chardonnay root material (Figure 5.6). This non-specific amplification indicates amplification of genomic plant DNA contaminants, thus the primer pair is not suitable for experiments using RNA extracted from grapevine that is not pre-treated with DNase I and was not used further in this project.

![Real-time RT-PCR products amplified from untreated total RNA extracts or total RNA extracts treated with Dnase I using four different host-specific real-time RT-PCR primer sets, (top gel) Vv-COX2-F/Vv-COX2-R and Vv-nad5-F/Vv-nad5-R, and (bottom gel) Vv-MDH-F/Vv-MDH-R and Vv-PIP2-F/Vv-PIP2-R. Lanes 1 and 4 real-time RT-PCR reactions used total RNA extracted from grapevine leaf material, lanes 2 and 5 real-time RT-PCR reactions used total RNA extracted from grapevine cane material, and lanes 3 and 6 real-time RT-PCR reactions used total RNA extracted from grapevine root material. Lane 7 is the no template control (water) and L is the 1kb Plus DNA ladder (Invitrogen, Carlsbad, CA, USA). (Bottom gel) Red dotted circle indicates the non-specific genomic DNA amplification using the Vv-MDH-F/Vv-MDH-R primer pair.](image)

5.3.1.6 Reference gene selection

Relative quantification requires standardisation against host genes (reference genes). To ensure correct quantification is achieved, the stability of the reference genes in all test samples, used for analysis, both healthy and virus-infected, is an important aspect to consider. Therefore, to ensure the most appropriate reference genes were used for the relative quantification of GLRaV-3 variants within the 2011 graft inoculated 3309 plants (Section 5.3.3.2.2), the stability of the ten
candidate reference genes (described above) were tested and compared using virally infected plant material (for each of the three GLRaV-3 variant infections, three biological replicates each) and corresponding healthy 3309 plant material (five biological replicates). In addition, three programs, geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004), were used to calculate expression stability values that were used to rank the candidate reference genes from most to least stable.

In addition, to investigate whether there is a candidate reference gene or genes that could be used for different rootstocks and/or different cultivars and type of materials, the stability of the ten candidate reference genes within healthy (five biological replicates) and virally-infected leaf material (for each of the three GLRaV-3 variant infections, three biological replicates each) from the 2011 graft inoculated plants with Schwarzmann rootstocks and Cabernet sauvignon cane material from Hawke’s Bay were also tested.

5.3.1.6.1 Stability of reference genes in 3309 leaf material

To examine whether there were differences in transcript levels between the candidate reference genes, the average Ct values for all samples were calculated and compared. The Ct values for GAPDH and NAD5 ranged between 16 and 18 cycles, for SUC11, COX2, EF2, SAND, and PIP2 ranged between 20 and 24 cycles, and for POLYU, TATA, and PP2a ranged between 27 and 29 cycles. The GAPDH gene yielded the highest transcript levels (mean Ct of 16.74 ± 0.61), while the PP2a gene yielded the lowest levels (mean Ct of 28.53 ± 0.98) (Figure 5.7a). To determine genes with the least Ct variation, the interquartile ranges were calculated. Five out of the ten reference genes had less than 1 with the GAPDH gene having the lowest, followed by genes SUC11, COX2, NAD5, and POLYU (Figure 5.7b).

For each candidate reference gene geNorm calculates an expression stability value (M value), which is the mean pairwise variation (V) between the candidate reference gene and all other genes in the analysis. A low M value indicates high expression stability, while a high M value indicates low expression stability (Vandesompele et al., 2002). To rank the candidate reference genes according to their expression stability, the program sequentially removes the least stable reference gene (gene with the highest M value) from the analysis and recalculates
the M value until the two most stable reference genes are identified (Vandesompele et al., 2002). All candidate reference genes in this current analysis of 3309 leaf material had M values less than the recommended 1.5 cut-off stability threshold (Vandesompele et al., 2002). Thus, all reference genes would be considered stable, under the current experimental conditions. The top two reference genes were COX2 and GAPDH followed by POLYU and NAD5 (Table 5.11). In contrast, the reference genes with the least stability (highest M values) were TATA and PIP2 (Table 5.11).

In addition, geNorm conducts a pairwise variation analysis ($V_{n/n+1}$) between two sequential normalisation factors (NFn and NFn+1) (Vandesompele et al., 2002), which determines the number of reference genes required for optimal normalisation, where a V value of 0.15 is the recommended cut-off threshold, i.e. a V value less than 0.15 indicates the inclusion of an additional reference gene (n+1) is not required for analysis (Vandesompele et al., 2002). Under the current experimental set-up using the top three genes, COX2, GAPDH, and POLYU, is sufficient for analysis and using a fourth gene would be optional ($V_{3/4} = 0.13$).

To determine the stability of candidate reference genes using the BestKeeper analysis, the coefficient of correlation (r) between the reference genes and BestKeeper Index (BI) is calculated (Pfaffl et al., 2004). The closer the r value is to 1, the stronger the relationship and in-turn indicates reference genes with stable expression. For each candidate reference gene, NormFinder calculates a stability value based on the combined intra- and inter-group variations in expression of the studied genes, using a model-based approach (Andersen et al., 2004). Similar to geNorm, reference genes with the lowest stability values (i.e. lowest variation) have the most stable expression (Andersen et al., 2004). BestKeeper and NormFinder also identified the COX2 and NAD5 genes within the top four most stable genes and the two least stable genes as PIP2 and TATA (Table 5.11).

Some differences were observed between the programs, for example GAPDH (one of the most stable genes identified by geNorm) is ranked third most stable gene by BestKeeper while only fifth using NormFinder. In addition, the geNorm third ranked POLYU gene ranked second by NormFinder but only seventh by BestKeeper (Table 5.11). Overall, taking into account of all the rankings from the three stability programs, the top four reference genes for infected and healthy
Figure 5.7: Plots showing the real-time RT-PCR (a) Ct values and (b) interquartile ranges for the ten candidate genes in *Grapevine leafroll-associated virus 3* (GLRaV-3) infected and healthy 3309 leaf samples. (a) Line across the box represents the median and the black cross represents the mean. The box indicates the 25% and 75% percentiles, while the whiskers represent the maximum and minimum values. (b) Interquartile ranges indicate variability of Ct values among the 25% and 75% percentiles.
### 5.3.1.6.2 Stability of reference genes in Schwarzmann leaf material

Most of the reference genes produced Ct values more than 20, `GAPDH` and `NAD5` ranged between 17 and 19 cycles, `SUC11`, `COX2`, `SAND`, and `EF2` ranged between 20 and 23 cycles, and `PIP2`, `TATA`, `POLYU`, and `PP2a` ranged between 25 and 28 cycles. The `GAPDH` gene yielded the highest transcript levels (mean Ct of 17.35 ± 0.92), while the `PP2a` gene yielded the lowest levels (mean Ct of 27.88 ± 1.32) (Figure 5.8a). High Ct variation was observed, with only two genes `GAPDH` and `SUC11`, with interquartile Ct ranges of 1 or less, while the remaining eight genes were greater than 1.5 (Figure 5.8b).

The `COX2` and `SAND` reference genes were identified as the most stable genes by all three programs (Table 5.12). `POLYU` and `NAD5` are ranked the third and fourth most stable genes respectively, by geNorm (Table 5.12). While BestKeeper ranked these genes in reverse order and NormFinder only ranked POLYU and NAD5, the fifth (stability value = 0.37) and sixth (stability value = 0.45) most stable genes (Table 5.12). For 3309 leaf material, GAPDH was the most stable gene (Section 5.3.1.6.1), but for Schwarzmann leaf material, Normfinder ranked this as the third most stable gene (stability value = 0.21), and Bestkeeper and geNorm only ranked it as the sixth and seventh stable gene, respectively.

Differences between the stability programs were observed when identifying the least stable gene. `PIP2` was identified as the least stable by geNorm and Normfinder, while Bestkeeper ranked `EF2` least stable and `PIP2` as the third least stable gene.

#### Table 5.11: The rankings of ten candidate reference genes (from most to least stably expressed gene) based on the geNorm, NormFinder, and BestKeeper analysis and the overall rankings for 3309 leaf material.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Gene</th>
<th>geNorm Average M value</th>
<th>NormFinder Stability value</th>
<th>BestKeeper Correlation coefficient (r)</th>
<th>Overall Stable Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>COX2 / GAPDH</td>
<td>0.37</td>
<td>COX2 0.42</td>
<td>NAD5 0.95</td>
<td>COX2</td>
</tr>
<tr>
<td>2</td>
<td>POLYU</td>
<td>0.46</td>
<td>NAD5 0.44</td>
<td>GAPDH 0.87</td>
<td>GAPDH</td>
</tr>
<tr>
<td>3</td>
<td>NAD5</td>
<td>0.52</td>
<td>SAND 0.48</td>
<td>EF2 0.83</td>
<td>POLYU</td>
</tr>
<tr>
<td>4</td>
<td>SUC11</td>
<td>0.59</td>
<td>GAPDH 0.50</td>
<td>SAND 0.78</td>
<td>SAND</td>
</tr>
<tr>
<td>5</td>
<td>EF2</td>
<td>0.68</td>
<td>PP2a 0.53</td>
<td>PP2a 0.78</td>
<td>PP2a</td>
</tr>
<tr>
<td>6</td>
<td>PP2a</td>
<td>0.75</td>
<td>SUC11 0.59</td>
<td>POLYU 0.76</td>
<td>EF2</td>
</tr>
<tr>
<td>7</td>
<td>SAND</td>
<td>0.84</td>
<td>EF2 0.61</td>
<td>TATA 0.58</td>
<td>SUC11</td>
</tr>
<tr>
<td>8</td>
<td>TATA</td>
<td>0.95</td>
<td>TATA 0.82</td>
<td>PIP2 0.47</td>
<td>TATA</td>
</tr>
<tr>
<td>9</td>
<td>PIP2</td>
<td>1.38</td>
<td>PIP2 1.42</td>
<td>SUC11 0.37</td>
<td>PIP2</td>
</tr>
</tbody>
</table>

* The overall top four reference genes are in bold.
stable (i.e. seventh ranked stable gene) (Table 5.12). The geNorm $V_{3/4}$ score was 0.15 and the $V_{4/5}$ was 0.12, thus this particular experimental setup would require at least four reference genes to ensure accurate analysis. Overall, based on rankings from all three programs (Table 5.12), the top four most stable genes for this analysis, in descending order, are $COX2$, $SAND$, $POLYU$, and $NAD5$.

**Figure 5.8:** Plots showing the real-time RT-PCR (a) Ct values and (b) interquartile ranges for the ten candidate genes in *Grapevine leafroll-associated virus 3* (GLRaV-3) infected and healthy Schwarzmann leaf samples. (a) Line across the box represents the median and the black cross represents the mean. The box indicates the 25% and 75% percentiles, while the whiskers represent the maximum and minimum values. (b) Interquartile ranges indicate variability of Ct values among the 25% and 75% percentiles.
Table 5.12: The rankings of ten candidate reference genes (from most to least stably expressed gene) based on the geNorm, NormFinder, and BestKeeper analysis and the overall rankings for Schwarzmann leaf material.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Gene</th>
<th>Average M value</th>
<th>Stability value</th>
<th>Gene</th>
<th>Correlation coefficient (r)</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>COX2 / SAND</td>
<td>0.34</td>
<td>0.13</td>
<td>COX2</td>
<td>0.96</td>
<td>COX2</td>
</tr>
<tr>
<td>2</td>
<td>SAND / NADH</td>
<td></td>
<td>0.95</td>
<td>SAND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>POLYU</td>
<td>0.47</td>
<td>0.21</td>
<td>GAPDH</td>
<td></td>
<td>POLYU</td>
</tr>
<tr>
<td>4</td>
<td>NADH</td>
<td>0.57</td>
<td>0.23</td>
<td>TATA</td>
<td>0.94</td>
<td>NADH</td>
</tr>
<tr>
<td>5</td>
<td>EF2</td>
<td>0.62</td>
<td>0.37</td>
<td>POLYU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>PP2a</td>
<td>0.67</td>
<td>0.45</td>
<td>NADH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>GAPDH</td>
<td>0.70</td>
<td>0.46</td>
<td>PP2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>TATA</td>
<td>0.73</td>
<td>0.48</td>
<td>SUC11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>SUC11</td>
<td>0.78</td>
<td>0.62</td>
<td>EF2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>PIP2</td>
<td>1.00</td>
<td>1.15</td>
<td>PIP2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The overall top four reference genes are in bold.

5.3.1.6.3 Stability of reference genes in Cabernet sauvignon cane material

The level of transcription for each reference gene varied, with NAD5, GAPDH, and COX2 ranging between 16 and 18 cycles, EF2, PIP2, SUC11, and SAND ranging between 20 and 22, and TATA, POLYU, and PP2a ranging between 25 and 28 cycles. The NAD5 gene yields the highest transcript levels (mean C<sub>t</sub> of 16.02 ± 0.60), while the PP2a gene yields the lowest levels (mean C<sub>t</sub> of 27.74 ± 1.33) (Figure 5.9a). Five out of the ten reference genes yielded relatively low Ct variation between samples (less than 1.1) with NAD5 having the lowest variation (interquartile range = 0.66), followed by genes SUC11, PIP2, COX2, and GAPDH (Figure 5.9b).

All candidate reference genes generated geNorm M values less than 1.2. Thus based on recommendations by Vandesompele et al. (2002), all genes would be considered stable, under the current experimental conditions. The top three reference genes with the lowest M values, in descending order of stability were GAPDH, SUC11, and SAND (Table 5.13). While the three reference genes showing the least stability (highest M values) were POLYU, PP2a, and PIP2. Therefore, under the current experimental set-up, using the most stable genes GAPDH and SUC11 would be sufficient for normalising gene expression (V<sub>2/3</sub> = 0.08).
BestKeeper proposes the same top three stable genes, but in reverse order; SAND \( (r = 0.94) \), SUC11 \( (r = 0.93) \), and GAPDH \( (r = 0.92) \) with a p-value of 0.001 (Table 5.13). In contrast, NormFinder proposed the top three reference genes as EF2 (stability value = 0.078), NAD5 (stability value = 0.11), and SAND (stability value = 0.13) (Table 5.13). GeNorm and BestKeeper only ranked EF2 and NAD5 as the fifth and fourth, and sixth and seventh most stable genes, respectively. Furthermore, NormFinder only ranked GAPDH (geNorm most stable gene) in sixth place. Reference genes POLYU and PIP2 consistently ranked with low stability, with all three programs placing both genes within the last three places (Table 5.13). Overall, the top four reference genes for this set-up would be SAND, SUC11, GAPDH, and EF2 (Table 5.13).

![Figure 5.9](image_url)

**Figure 5.9**: Plots showing the real-time RT-PCR (a) Ct values and (b) interquartile ranges for the ten candidate genes in *Grapevine leafroll-associated virus 3* (GLRaV-3) infected and healthy Cabernet sauvignon cane samples. (a) Line across the box represents the median and the black cross represents the mean. The box indicates the 25% and 75% percentiles, while the whiskers represent the maximum and minimum values. (b) Interquartile ranges indicate variability of Ct values among the 25% and 75% percentiles.
Table 5.13: The rankings of ten candidate reference genes (from most to least stably expressed gene) based on the geNorm, NormFinder, and BestKeeper analysis and the overall rankings for Cabernet sauvignon cane material.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Analysis</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Average M value</td>
<td>Gene</td>
</tr>
<tr>
<td>1</td>
<td>GAPDH / SUC11</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>NADH</td>
<td>0.11</td>
</tr>
<tr>
<td>3</td>
<td>SAND</td>
<td>0.27</td>
</tr>
<tr>
<td>4</td>
<td>COX2</td>
<td>0.31</td>
</tr>
<tr>
<td>5</td>
<td>EF2</td>
<td>0.33</td>
</tr>
<tr>
<td>6</td>
<td>TATA</td>
<td>0.43</td>
</tr>
<tr>
<td>7</td>
<td>POLYU</td>
<td>0.50</td>
</tr>
<tr>
<td>8</td>
<td>PP2a</td>
<td>0.56</td>
</tr>
<tr>
<td>9</td>
<td>PIP2</td>
<td>0.67</td>
</tr>
</tbody>
</table>

*The overall top four reference genes are in bold.*

5.3.2 Graft transmission of GLRaV-3 variants

The success of the graft inoculations in the 2010 and 2011 experiments is dependent on two processes. Firstly, the survival of graft plants during the assessment period and secondly, the formation of graft unions that allow for transmission of GLRaV-3 from the virally infected interstock to the virus-free material.

5.3.2.1 2010 graft inoculations

5.3.2.1.1 Survival of grafted plants

Of the 135 double-worked grafted plants, only 78 (57.78%) survived the entire growing season. These consisted of 28 Pinot noir (62% survival), 29 Sauvignon blanc (67% survival), and 21 Syrah (48% survival) plants. For 73 of the plants, development of shoots occurred at the bottom buds of the plants (below the interstock) while only 19 plants developed shoots at the top bud (above the interstock).

All 44 single-worked grafted plants, except one Pinot noir plant, survived the course of the season (overall survival rate of 98%). The 43 surviving plants consisted of 15 Sauvignon blanc, 15 Syrah, and 13 Pinot noir plants. Of these 39 plants developed shoots at the bottom bud while the remaining four plants only developed shoots from the virally infected interstock bud.
All 15 healthy plants for each of the cultivars, Pinot noir, Sauvignon blanc, and Syrah survived the entire growing season.

5.3.2.1.2 Virus transmission rates from the graft inoculations

Of the 78 double-worked plants that produced viable plants, 52 (66.67%) tested GLRaV-3 positive by mRT-PCR, for varying combinations of GLRaV-3 variants (Table 5.14). However, 14 of the plants did not contain the combination of GLRaV-3 variants that were expected from initial testing of the virus infected scionwood used as interstock (Table 5.15). The three cultivars were fairly evenly represented within the 52 GLRaV-3 positive plants with 15 Pinot noir, 21 Sauvignon blanc, and 16 Syrah plants (Table 5.14). Most of the 26 grafted plants that tested negative were inoculated with NZ2 (12) or a group 1 variant (9), while only 2 plants were inoculated with group 1 and NZ-1 variants, and 3 plants were inoculated with group 1, NZ-1, and NZ2 variants. All 15 healthy GLRaV-3 negative control plants tested negative by mRT-PCR.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>GLRaV-3 combination</th>
<th>Number of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinot Noir</td>
<td>Group 1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>NZ-1 + NZ2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1 + NZ2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
</tr>
<tr>
<td>Sauvignon blanc</td>
<td>Group 1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>NZ-1 + NZ2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1 + NZ2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>21</strong></td>
</tr>
<tr>
<td>Syrah</td>
<td>Group 1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>NZ-1 a</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NZ-1 + NZ2</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>16</strong></td>
</tr>
</tbody>
</table>

*a GLRaV-3 variant combinations were unexpected GLRaV-3 combinations.*
Table 5.15: Summary of the irregularities detected in double-worked graft inoculated plants.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>GLRaV-3 variant combination</th>
<th>Expected scion infection</th>
<th>Detected infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC2</td>
<td>Group 1</td>
<td></td>
<td>Group 1 + NZ-1</td>
</tr>
<tr>
<td>KC16</td>
<td>NZ2</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC21</td>
<td>NZ2</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC24</td>
<td>NZ2</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC25</td>
<td>NZ2</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC41</td>
<td>Group 1 + NZ-1</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC43</td>
<td>Group 1 + NZ-1</td>
<td>Group 1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC67</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td>Group 1 + NZ-1</td>
<td></td>
</tr>
<tr>
<td>KC98</td>
<td>NZ2</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC100</td>
<td>NZ2</td>
<td>NZ-1</td>
<td></td>
</tr>
<tr>
<td>KC108</td>
<td>Group 1</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC117</td>
<td>Group 1</td>
<td>Group 1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC122</td>
<td>NZ2</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>KC127</td>
<td>NZ2</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
</tbody>
</table>

*Differences between expected and detected infections shown in bold.

Of the 43 single-worked graft inoculated plants, 35 tested GLRaV-3 positive for various variant combinations (81%) (Table 5.16). Similar to the double-worked plants, each of the three cultivars was evenly represented within the GLRaV-3 positive plants with 11 Pinot noir, 13 Sauvignon blanc, and 11 Syrah plants. However, 12 plants did not contain the combination of GLRaV-3 variants that were expected from previous testing of the virus infected scionwood used as interstock (Table 5.17).

Table 5.16: Number of plants for each of the GLRaV-3 combinations detected in the single-worked graft plants for each of the three cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>GLRaV-3 combination</th>
<th>Number of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinot Noir</td>
<td>NZ2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>NZ-1 + NZ2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1 + NZ2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>11</strong></td>
</tr>
<tr>
<td>Sauvignon blanc</td>
<td>Group 1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NZ-1 + NZ2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1 + NZ2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>13</strong></td>
</tr>
<tr>
<td>Syrah</td>
<td>Group 1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NZ-1 a</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ2 a</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NZ-1 + NZ2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Group 1 + NZ-1 + NZ2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>11</strong></td>
</tr>
</tbody>
</table>

*a GLRaV-3 variant combinations were unexpected GLRaV-3 combinations.

The eight GLRaV-3 negative single-worked plants were inoculated with either single infections of a group 1 variant (2 plants) or a NZ2 variant (1 plant), or dual
infections of a group 1 and NZ-1 infection (1 plant) or NZ-1 and NZ2 (2 plants). In addition, two plants were inoculated with a triple infection of group 1, NZ-1, and NZ2.

**Table 5.17: Summary of the irregularities detected in single-worked graft inoculated plants.**

<table>
<thead>
<tr>
<th>Sample name</th>
<th>GLRaV-3 variant combination</th>
<th>Expected scion infection</th>
<th>Detected infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG2</td>
<td>Group 1 + NZ-1</td>
<td>Group 1</td>
<td></td>
</tr>
<tr>
<td>SG4</td>
<td>NZ2</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>SG6</td>
<td>Group 1 + NZ-1</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>SG11</td>
<td>Group 1 + NZ-1</td>
<td>Group 1</td>
<td></td>
</tr>
<tr>
<td>SG12</td>
<td>Group 1</td>
<td>Group 1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>SG13</td>
<td>NZ-1 + NZ2</td>
<td>NZ-1</td>
<td></td>
</tr>
<tr>
<td>SG15</td>
<td>Group 1</td>
<td>Group 1 + NZ-1</td>
<td></td>
</tr>
<tr>
<td>SG19</td>
<td>NZ2</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>SG24</td>
<td>Group 1</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>SG30</td>
<td>Group 1</td>
<td>NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>SG36</td>
<td>Group 1</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
<tr>
<td>SG40</td>
<td>Group 1 + NZ-1</td>
<td>Group 1 + NZ-1 + NZ2</td>
<td></td>
</tr>
</tbody>
</table>

*a Differences between expected and detected infections shown in bold.

### 5.3.2.2 2011 graft inoculations

#### 5.3.2.2.1 Survival of grafted plants

Of the 177 double-worked graft inoculated plants, 91 (51.41%) survived the entire growing season. For each of the five rootstocks, different numbers of plants survived. Regardless of the type of virally infected interstock and the cultivar used for the top portion of the graft, plants with the Schwarzmann and 3309 rootstock yielded the most plants, with 27 (57.45%) and 25 (83.33%) respectively. The remaining three rootstocks 101-14, Riparia gloire, and SO4 only yielded 15 (50%), 14 (35%), and 10 (33.33%) viable plants, respectively. Similar to the 2010 grafts, most of the shoots (85 plants) developed at the bottom buds (below the interstock) while for seven plants the buds developed above the interstock.

Only three out of the five 3309, 101-14, SO4, and Schwarzmann healthy plants, and two Riparia gloire plants survived the entire growing season.

#### 5.3.2.2.2 Virus transmission rates from the graft inoculations

Out of the 91 double-worked grafted plants that produced viable plants, 50 (54.95%) tested GLRaV-3 positive by mRT-PCR and/or real-time RT-PCR, for varying combinations of GLRaV-3 variants (Table 5.18). The five different rootstocks used as the bottom portion of grafted plants were unevenly represented (Table 5.18). Most of the plants had either a 3309 (18 plants) or Schwarzmann
(14 plants) as a base, while there were 4, 8, 6 surviving plants with 101-14, Riparia gloire, and SO4 rootstocks, respectively.

Of the 41 grafted plants that tested GLRaV-3 negative, 17 were inoculated with plant material infected with only NZ2, while 12 others were inoculated with single infections of either a group 1 or NZ-1 variant. In contrast to 2010 graft inoculated plants, there were no discrepancies between the virus variants detected in the interstocks (inoculum source) and those detected in the new shoots. All 14 healthy control plants tested negative for GLRaV-3 by mRT-PCR and real-time RT-PCR.

**Table 5.18:** Number of plants for each of the GLRaV-3 combinations detected in the double-worked graft plants for each of the five rootstocks.

<table>
<thead>
<tr>
<th>Rootstock</th>
<th>GLRaV-3 combination</th>
<th>Number of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>3309 (25 / 30)</td>
<td>Group 1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NZ-1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>18</strong></td>
</tr>
<tr>
<td>Schwarzmann (27 / 47)</td>
<td>Group 1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>NZ-1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>14</strong></td>
</tr>
<tr>
<td>101-14 (15 / 30)</td>
<td>Group 1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>NZ-1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>4</strong></td>
</tr>
<tr>
<td>Riparia gloire (14 / 40)</td>
<td>Group 1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>NZ-1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>8</strong></td>
</tr>
<tr>
<td>SO4 (10 / 30)</td>
<td>NZ-1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>NZ2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>6</strong></td>
</tr>
</tbody>
</table>

### 5.3.3 Virus distribution and replication in graft inoculated plants

Leaf material from the developing shoots of the 2010 and 2011 graft inoculated plants were sampled and tested for GLRaV-3 at different times throughout the growing season (Section 5.2.4.1.1 and 5.2.4.1.2). For both years, different test results were obtained at different testing times. Some plants that tested negative for GLRaV-3 in the first round of testing, tested positive in later tests, while in other cases the combinations of GLRaV-3 variants detected were different (e.g. some plants that initially tested positive for a single infection of NZ-1 were later tested to have a dual infection of NZ-1 and NZ2 variants). These differences could be due to uneven virus distribution and/or low virus titre (below detection limit of mRT-
PCR) at the time of the earlier testing. Therefore, to investigate the virus distribution of group 1, NZ-1, and NZ2 variants within the graft inoculated plants, leaf material from the base, middle, and apical positions along developed shoots were sampled and tested.

5.3.3.1 Virus variants detected in 2010 graft inoculated vines

Out of the 52 GLRaV-3 positive plants, 32 plants showed differences in the GLRaV-3 variant combinations at different sampling sites along the cane, with not all expected GLRaV-3 variant(s) being detected (Table 5.19). This included plants infected with only group 1 or NZ2 variants (two and four plants, respectively), dual infections (16 plants), and triple infections (ten plants) of different GLRaV-3 variants (Table 5.19). Most of the instances where GLRaV-3 variants are inconsistently detected along the cane were at the apical sampling position (38 out of 101, 38%), while there were 30 and 24 instances at the base and mid-shoot sampling positions, respectively. Testing of the phloem scrapings from the rootstock site resulted in nine instances of different GLRaV-3 variant combinations being detected.

Analysis of the particular GLRaV-3 variant involved in the inconsistent detection events, at each sampling position, showed differences in detectability between the GLRaV-3 variants (Figure 5.10). The group 1 variant had high detectability in all four sampling positions with successful detection rates of more than 90% for all sites (Figure 5.10). In addition, the NZ-1 variant had high detectability at the rootstock and mid-shoot sites with successful detection rates of more than 90%, although reductions in detectability were observed at the apical and base sampling positions with 84 and 82%, respectively (Figure 5.10). In contrast, the NZ2 variant had reduced detectability compared to the other GLRaV-3 variants. At the apical sampling position, the NZ2 detection rate was 40%, and at the basal and mid-cane positions detection rates were 62 and 66% respectively (Figure 5.10). Higher detection rates (74%) were observed at the rootstock sampling position, but this was still less than detection rates for group 1 and NZ-1.

5.3.3.2 Virus variants detected in 2011 graft inoculated vines

5.3.3.2.1 Initial mRT-PCR and real-time RT-PCR screening

Leaf material from the basal, middle, and apical positions along the developing shoots of the graft inoculated plants was tested at 24 weeks post inoculation,
using the mRT-PCR assay (Table 5.20). Of the 122 samples from plants grafted with the group 1 variant, 102 tested positive, while only 44 out of the 118 samples from NZ-1 inoculated plants tested positive for NZ-1, and none of the 80 samples from NZ2 inoculated tested positive (Table 5.20).

Because of this high number of negative results by mRT-PCR, all samples were re-tested by real-time RT-PCR. No additional group 1 variants were identified from the 122 group 1 expected samples; however additional NZ-1 and NZ2 infections were identified (Table 5.20). From the 118 samples expected to be NZ-1 positive, a further 41 NZ-1 positives were identified, and 47 out of the 80 expected NZ2 samples tested positive for NZ2.

Based on the combined mRT-PCR and real-time RT-PCR results, 34 singly infected graft inoculated plants showed non-uniform GLRaV-3 detection at all sampling positions along the developed shoot (Table 5.20). Similar to 2010, most of these inconsistent detection events occurred at the apical sampling position, as GLRaV-3 was least frequently detected from the apex of the shoot, being detected in only 46% (49 out of 107) of the apical samples. In contrast, a detection rate of approximately 75% was obtained from the mid-shoot leaves, and the highest detection rate of 93% (96 out of 103) was at the base of the shoot.

The same differential ability to detect particular GLRaV-3 variants along the shoot was observed, with high levels (>90%) of detection for all GLRaV-3 variants at the basal sampling position (Figure 5.11). Virus detectability reduced as testing continued up the shoot towards the apical position with group 1 detection rates of 83% and 76% for the mid-shoot and apical shoot positions, respectively, and 78% and 50% for the NZ-1 detection, respectively. While NZ2 had considerably lower detectability compared to the other two variants with only 59 and 27% detection at the mid- and apical shoot positions, respectively (Figure 5.11).
Figure 5.10: Plot showing the percentage of successful detections for phylogenetic group 1, NZ-1, and NZ2 Grapevine leafroll-associated virus 3 variants at 24 weeks post 2011 graft inoculations (for the base, middle, and apical sampling sites) and 36 weeks post 2010 graft inoculations (for the rootstock sampling sites) using the multiplex RT-PCR assay. The coloured bars represent the four different sampling sites as indicated in the plot.

Figure 5.11: Plot showing the percentage of successful detections for phylogenetic group 1, NZ-1, and NZ2 Grapevine leafroll-associated virus 3 variants at 24 weeks post 2011 graft inoculations using the multiplex RT-PCR and variant-specific real-time RT-PCR assays. The coloured bars represent the different sampling sites along the cane (at the base, middle and apical positions) as indicated in the plot.
### Table 5.19: Grapevine leafroll-associated virus 3 variant combinations identified for the 2010 graft inoculations by the multiplex RT-PCR assay at three sampling positions along the shoots.

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*Abbreviations used for each sampling position reads as follows: T and B represent whether the leaf samples were collected from developing shoots above (Top bud) or below (Bottom bud) the infected interstock (Figure 5.1). Numbers 1, 2, and 3 following T/B represent sampling sites along the shoot, i.e. Base, Middle, and Apical. For example B1-B represents a sample collected and tested from a one developed shoot below the interstock at the base position along the cane.  
*Coloured cells highlight the inconsistent detection of the GLRaV-3 variant(s) at a particular sampling position along the cane.*
Table 5.20: Grapevine leafroll-associated virus 3 variant combinations identified for the 2011 graft inoculations by the multiplex RT-PCR and real-time RT-PCR assays at three sampling positions along the shoots.

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Abbreviations used for each sampling site reads as follows: T and B represent whether the leaf samples were collected from developing shoots above (Top bud) or below (Bottom bud) the infected interstock (Figure 5.1). Numbers 1, 2, and 3 following T/B represents the shoot that was sampled from. B, M, and A represent the three sampling sites along the shoot, i.e. Base, Middle, and Apical. For example B1-B represents a sample collected and tested from a one developed shoot below the interstock at the base position along the cane.

Red coloured cells highlights the inconsistent detection of the GLRaV-3 variant(s) at a particular sampling position along the cane. Sites with **bold font** and blue coloured cells highlights samples that required variant-specific real-time RT-PCR re-testing to identify the GLRaV-3 combination indicated in the table.
5.3.3.2.2 Absolute and relative quantitative real-time RT-PCR

The pattern of GLRaV-3 detectability along the shoot suggests that there is a time lag in virus movement and/or replication to detectable titre as the shoot develops. Therefore, to determine whether virus titre had an effect on the detectability of GLRaV-3 variants from the 2011 graft inoculated plants, 18 grafted 3309 rootstock plants with single infections of either the group 1 (six plants), NZ-1 (six plants), or NZ2 (six plants) variants were selected for absolute and relative quantitative real-time RT-PCR.

**Absolute quantification**

Similar to the mRT-PCR testing, the NZ2 variant was not detected in any of the samples using real-time RT-PCR targeting ORF4. Based on the synthetic RNA standard, run on the same real-time RT-PCR plates, this assay could detect as little as $10^2$ copies of virus amplicon per µL. This indicates at the three sampling positions it is likely less than $10^{3.7}$ genomic copies of the NZ2 variant are present in the 100mg of plant tissue used for extractions. For the group 1 and NZ-1 variants, on average $10^{2.8}$ to $10^{5.1}$ genome copies per 100mg of plant tissue was detected for the three sampling sites (Figure 5.12). Generally, the virus titre gradually declined towards the apex of the shoot with 3 to 10-fold reductions of the group 1 variant, and 9 to 24-fold reductions of the NZ-1 variant genome copies per 100mg of plant tissue, when comparing the basal and mid-shoot, and the mid-shoot and apical sampling positions. Despite the observed reductions towards the apex, statistical analysis did not show significant differences between the sampling positions for the group 1 variant (ANOVA test, p-value ≤0.40). Differences in the NZ-1 variant titre between sampling positions were statistically significant (ANOVA test, p-value ≤0.05), specifically between the base and apical sampling positions ($t$-test, p-value ≤0.03). The overall genome copy numbers of group 1 and NZ-1 variants, across all three sites, were not significantly different ($t$-test, p-value ≤0.52).
Relative quantification

Relative real-time qRT-PCR was used for comparison purposes with absolute quantification data. To account for differences in the RNA concentrations and quality of RNA, reverse transcription efficiencies, and possible inter-run variations, the relative quantification was standardised against four stably expressed host genes (reference genes), COX2, NAD5, GAPDH, and POLYU (Section 5.3.1.6.1). In addition, to investigate the expression of one sgRNA (sgRNA(ORF9)), and in-turn virus replication between the different sampling positions and the different GLRaV-3 variants, for each GLRaV-3 variant, two different areas of the genome (5’UTR/ORF1b/ORF4 and ORF9) were targeted using two different variant-specific real-time RT-PCR assays (Section 5.2.4.5.1).

Similar to the absolute quantification analysis, virus genomic and sgRNA titres generally decreased towards the apex of the shoot (although NZ2 was not detected using ORF4 targeting primers, as mentioned above) (Figure 5.13). Significant differences in virus titre between the sampling positions on the developed shoot were only observed for NZ-1 sgRNA titres, between the basal and apical sampling sites (t-test, p-value ≤0.01).

Notably, high sgRNA expression of the NZ2 and group 1 sgRNA(ORF9) was evident at all three sampling positions (Figure 5.13) and this gave successful detection of NZ2 that was not achieved using the primers targeting ORF4.
Although, there was no significant difference in the relative NZ2 sgRNA(ORF9) expression levels across the three positions (ANOVA test, p-value ≤ 0.85), the relative group 1 variant sgRNA(ORF9) expression levels were 23-, 27- and 45-times greater than the genomic RNA levels at the base, middle, and apical sampling positions, respectively, representing significant differences between the genomic and sgRNA levels at all sites (t-test: p-value ≤ 2.29x10^{-5}, p-value ≤ 7.57x10^{-4}, p-value ≤ 3.29x10^{-5}, respectively). The NZ-1 variant generally had a higher levels of genomic RNA (approximately 4-fold) compared to the sgRNA(ORF9) (Figure 5.13), although the genomic and sgRNA relative titres at each site were not significantly different (with p-values greater than 0.17).

Finally, comparisons between the overall genomic and sgRNA levels between the different GLRaV-3 variants, irrespective of the sampling site, were performed. In contrast to the absolute quantification, the overall level of genomic NZ-1 RNA was 5-fold greater than the group 1 variant (t-test, p-value ≤ 4.83x10^{-3}). However, even though the group 1 variant had less genomic RNA than NZ-1, the group 1 variant exhibited higher sgRNA expression of sgRNA(ORF9) compared to NZ-1 and NZ2 variants, with a 30-fold difference between group 1 and NZ-1 (t-test, p-value ≤ 1.24x10^{-8}) and a 8-fold difference between group 1 and NZ2 (t-test, p-value ≤ 4.53x10^{-4}). The NZ2 variant also exhibited a higher level of sgRNA(ORF9) compared to NZ-1 (4-fold difference) (t-test, p-value ≤ 0.02).

**Figure 5.13:** Relative quantification of the group 1, NZ-1, NZ2 Grapevine leafroll-associated virus 3 (GLRaV-3) variants, 24 weeks post inoculations, using variant-specific assays targeting regions close to the 5’end of the genome (representative of genomic RNA) and within ORF9 (representative of subgenomic RNA (sgRNA) levels of sgRNA(ORF9)). Quantification was determined by real-time quantitative RT-PCR against four reference genes (COX2, GAPDH, NAD5, and POLYU). For each GLRaV-3 variant, average titre was determined for the base, middle, and apical sampling sites along developed shoots. Standard deviations are indicated by error bars.
5.3.4 Symptom development of GLRaV-3 infected plants

Generally white cultivars and rootstocks do not exhibit obvious visual symptoms, while red cultivars exhibit obvious premature reddening of the leaves, in addition to the downward rolling of the leaf margins. Therefore for this project, only the visual symptoms of red cultivar leaves and the length of the developed shoots of all plants were monitored.

5.3.4.1 2010 graft inoculations

5.3.4.1.1 Development of leaf symptoms in red cultivars infected with different GLRaV-3 variants

All GLRaV-3 infected Syrah and Pinot Noir plants, except for one Pinot Noir plant with NZ-1 and NZ2 infection, exhibited reddening and curling of the leaves of varying severity (Figure 5.14), while all virus free plants were symptomless. For statistical analysis, only GLRaV-3 variant combinations with at least three biological replicates were used. This included the following GLRaV-3 variant infections: Syrah plants infected with a single group 1 variant and dual infection of NZ-1 and NZ2, and Pinot noir plants infected with a single group 1 variant, dual infection of group 1 and NZ-1 variants, and triple infection of group1, NZ-1, and NZ2 variants. There were no statistically significant differences between the different GLRaV-3 variants for either cultivar, based on the qualitative scale used to grade the severity of the symptoms (Syrah: Mann-Whitney U-test, p-value >0.05; Pinot noir: Kruskal-Wallis test, p-value >0.05).
Figure 5.14: Photographs of a Pinot noir (a, b, c) and a Syrah (d, e, f, g,) grafted plant at eight (a, d), 16 (b, e), and 24 weeks (c, f, g) post inoculation. Both plants generate clear leafroll symptoms after 24 weeks (c, f, g) even with differing *Grapevine leafroll-associated virus 3* (GLRaV-3) infection (Pinot noir plant is infected with the GLRaV-3 group 1 variant while the Syrah plant has a dual infection of GLRaV-3 NZ-1 and NZ2 variants). Red dotted rectangle in (f) marks the area shown in (g).

### 5.3.4.1.2 Effects of GLRaV-3 infection on shoot growth

Pinot Noir GLRaV-3 infected plants showed no statistically significant difference in shoot growth compared to healthy plants (Figure 5.15) (Mann-Whitney *U*-test, p-value >0.05), while GLRaV-3 infected Syrah and Sauvignon blanc plants both had a statistically significant 1.9-fold reduction in growth compared to healthy plants (Figure 5.15) (Mann-Whitney *U*-test: p-value <0.05 and p-value =0.006, respectively). Analysis of effects of individual GLRaV-3 variants on the growth or Syrah and Sauvignon blanc plants showed no statistical support for differences in the shoot length of Syrah plants singly infected with a group 1 variant and dually...
infected with NZ-1 and NZ2 (Figure 5.16a) (Mann-Whitney U-test, p-value >0.05). In contrast, differences in the growth of differently infected Sauvignon blanc plants were observed (Figure 5.16b) (Kruskal-Wallis test, p-value <0.05), specifically Sauvignon blanc plants infected with NZ2 had a significant 2-fold reduction in shoot growth compared to plants infected with a single infection of a group 1 variant (Mann-Whitney U-test, p-value <0.05).

Figure 5.15: Plot showing the average length of the developed shoots from *Grapevine leafroll-associated virus 3* (GLRaV-3) infected (blue) and healthy control (red) plants for each of the three cultivars. Calculated averages for the Pinot noir, Sauvignon blanc, and Syrah infected plants, are based on 15, 21, and 16 biological replicates respectively. Five biological replicates were used for all healthy controls. Standard deviations are indicated by error bars.
Figure 5.16: Plots showing the average length of the developed shoots from *Grapevine leafroll-associated virus 3* (GLRaV-3) infected plants with different variant combinations (as shown in the plot) (blue) and healthy control plants (red) for the (a) Syrah and (b) Sauvignon blanc cultivars. (a) Calculated averages for the Syrah infected plants with a single group 1 or dual NZ-1 and NZ2 GLRaV-3 infection, are based on five and nine biological replicates respectively. (b) Calculated averages for the Sauvignon blanc infected plants with a single infection of Group 1 or NZ2, dual infection of NZ-1 and NZ2, and a triple infection of group 1, NZ-1, and NZ2 variants, are based on six, five, three, and four biological replicates, respectively. (a and b) Five biological replicates were used for all healthy controls. Standard deviations are indicated by error bars.

5.3.4.2 2011 graft inoculations

5.3.4.2.1 Development of leaf symptoms in red cultivars infected with different GLRaV-3 variants

Only four Pinot noir double-worked graft plants developed shoots at the top bud that were singly infected with either a group 1 (1 plant), NZ-1 (2 plants), or NZ2 (1 plant) GLRaV-3 variant. All four plants showed slight reddening and/or downward rolling of the leaf margins (Figure 5.17). No symptoms were observed in any of the healthy control plants.
5.3.4.2.2 Effects of GLRaV-3 infection on shoot growth

All GLRaV-3 positive grafted plants, on all rootstock cultivars except 3309, showed considerably reduced growth with an average shoot length ranged between 8.5cm ± 3.1cm to 20.0cm ± 8.8cm compared to the healthy rootstock controls that ranged between 33.0cm ± 1.0cm to 40.2cm ± 1.0cm (Figure 5.18) (for all comparisons: Mann-Whitney U-test, p-value <0.05). In contrast, GLRaV-3 positive plants with a 3309 base had an average shoot length of 20.3cm ± 8.8cm compared to 32.3cm ± 17.6cm for the corresponding healthy 3309 controls. Further examination of the effect of single infections of the different GLRaV-3 variants on growth, irrespective of the rootstock used, revealed no differences in growth (Kruskal-Wallis test, p-value >0.05). The effects of the individual GLRaV-3 variants on 3309 and Schwarzmann rootstock plants were also examined, as three or more biological replicates was available for both of these rootstocks. No significant differences was observed between the different variant infections in 3309 plants (Figure 5.19a) (Kruskal-Wallis test, p-value >0.05), but statistically significant differences in growth were observed for Schwarzmann rootstock plants (Kruskal-Wallis test, p-value <0.05). Schwarzmann plants infected with only NZ2 had a 2- and 1.6-fold increase in shoot length compared to plants singly infected with group 1 or NZ-1 variants, respectively (Mann-Whitney U-test: p-value <0.05 and p-value >0.05, respectively) (Figure 5.19b).
Figure 5.18: Plot showing the average length of the developed shoots from *Grapevine leafroll-associated virus 3* infected (blue) and healthy control (red) plants for each of the five rootstocks. The number of biological replicates used to calculate the average shoot length for each of the five different rootstocks varied, and are as follows: 18 for 3309, four for 101-14, eight for Riparia gloire, six for SO4, and 14 for Schwarzmann. Three biological replicates were used for all healthy controls except Riparia gloire which used only two biological replicates. Standard deviations are indicated by error bars.

(a)  
(b)  

Figure 5.19: Plots showing the average length of the developed shoots from *Grapevine leafroll-associated virus 3* infected plants with different variant combinations using (a) 3309 or (b) Schwarzmann rootstock as a base (as shown in the plot) (blue) and corresponding healthy control plants (red). (a) For each of the 3309 infected plants with a single group 1, NZ-1, or NZ2 variant, six biological replicates were used to calculate average shoot length. (b) For each of the Schwarzmann infected plants with a single group 1, NZ-1, or NZ2 variant, six, five, and three biological replicates were used to calculate average shoot length, respectively. (a and b) Average shoot length for the healthy 3309 control was calculated using three biological replicates. Standard deviations are indicated by error bars.
5.4 Discussion

The symptom expression and/or severity of symptoms of plant viruses can differ depending on the genetic variant/strain, the host species, or environmental conditions. Genetic studies of GLRaV-3, including the work from this project, have identified high genetic variability within the GLRaV-3 population with the identification of isolates that fit within at least seven different phylogenetic groups (Chapter 1, 2, and 3) (Bester et al., 2012b; Fuchs et al., 2009; Gouveia et al., 2011; Jooste et al., 2010; Sharma et al., 2011; Wang et al., 2011). However, little is currently known about the symptom severity of specific GLRaV-3 variants from these different phylogenetic groups, in particular, the highly genetically divergent isolates NZ-1 and NZ1-B (members of the newly formed phylogenetic group 6) and NZ2 (Chapter 2 and 3). Therefore, to investigate the symptom expression of GLRaV-3 variants, and potential differences in symptom severity between different GLRaV-3 variants, double-worked graft inoculations using material infected with single or mixed infections of GLRaV-3 variants from phylogenetic group 1, NZ-1, and NZ2 were conducted. To monitor the GLRaV-3 variants within the host plant, variant- and host-specific real-time RT-PCR assays were successfully developed. The effectiveness of virus detection protocols can be affected by the virus distribution and titre within the vine and subsequent testing using these assays revealed the GLRaV-3 variants were unevenly distributed in the host plant.

5.4.1 Molecular assays

5.4.1.1 Variant-specific real-time RT-PCR assays for quantification of virus

Real-time RT-PCR assays have been designed for detection of GLRaV-3 and are either based on the SYBR Green chemistry (Bell et al., 2009) or TaqMan® chemistry (Osman et al., 2007, 2008; Osman et al., 2006; Pacifico et al., 2011; Tsai et al., 2012) (Chapter 4), including two TaqMan® assays to quantify the number of GLRaV-3 genome copies (Pacifico et al., 2011; Tsai et al., 2012). To limit the amplification of sgRNAs, which can lead to an overestimation of the viral genome copies, both assays were designed to target the RdRp domain (ORF1b). Pacifico et al. (2011) designed the real-time RT-PCR primer pair and probe against sequence obtained from their study, which has high identity with the phylogenetic group 2. Tsai et al. (2012) designed two sets of primers and probes, one set was designed against an alignment of GLRaV-3 isolates including phylogenetic groups 1 and 2, while the second set was designed against isolates...
from the phylogenetic group 3, as the first primers and probe set could not detect the group 3 variant.

In this study three GLRaV-3 variants were studied and variant-specific primer pairs were specifically designed. Two primer pairs were designed for each GLRaV-3 variant to determine the RNA levels representative of genomic RNA and sgRNA. Similar to Pacifico et al. (2011) and Tsai et al. (2012), to study the virus genome titres and prevent overestimation, regions closer to the 5’end of the genome were targeted. Due to the lack of available NZ2 sequence closer to the 5’ end (sequence only begins within ORF4), the primer pair was designed to target ORF4. Though, targeting ORF4 is still likely to provide a useful representation of the NZ2 genome copy number, as according to Jarugula et al. (2010), the sg(ORF3/4) was observed to be one of the lower expressed sgRNAs, with the corresponding band barely visible in Northern blots. To evaluate possible differences in sgRNA expression between the different GLRaV-3 variants, the second variant specific primer pair was designed to target sgRNA(ORF9) as Jarugula et al. (2010) showed this was one of the top four most highly expressed sgRNAs for a group 1 variant, WA-MR.

5.4.1.2 Selection of reference genes for relative quantification

Relative quantitative real-time RT-PCR has been extensively used to investigate the changes in mRNA levels within biological samples. However, experimental errors between samples such as variability in the extraction protocol, differences in the initial RNA amount and integrity, and differences in the reverse transcription and PCR efficiencies, can affect the accuracy of this method (Andersen et al., 2004; Bustin et al., 2004; Huggett et al., 2005; Vandesompele et al., 2002). To account for these experimental errors and to enable accurate comparisons of mRNA titres across different samples to be made, normalisation of the data is essential (Andersen et al., 2004; Bustin et al., 2004; Expósito-Rodríguez et al., 2008; Huggett et al., 2005; Vandesompele et al., 2002). At present, the most common method used is normalisation to reference genes present in the same sample as the target RNA and these are subject to the same variations that may occur throughout the process (Expósito-Rodríguez et al., 2008; Huggett et al., 2005). The accuracy of the normalisation strategy is dependent on the selection of a reference gene that is expressed at relatively constant levels across all samples tested, as selection of a gene that is unevenly expressed across test
samples can lead to erroneous results (Bas et al., 2004; Dheda et al., 2005; Expósito-Rodríguez et al., 2008; Huggett et al., 2005; Tricarico et al., 2002). Therefore, the reference gene selected for normalisation must be validated for each experimental condition. In addition, the selection and validation of multiple reference genes for normalisation is recommended as it has been found to be less error-prone compared to single gene normalisation (Expósito-Rodríguez et al., 2008; Reid et al., 2006; Vandesompele et al., 2002).

Numerous computer programs have been developed to identify the best reference genes for a particular data set, based on expression stability values calculated using statistical algorithms that are then ranked from most to least stably expressed gene. In this project, the geNorm, NormFinder, and BestKeeper programs were used to select four reference genes from ten candidate genes, for the relative quantification of GLRaV-3 variants within 3309 grafted plants. Discrepancies between rankings from the three programs were observed. For instance the POLYU candidate reference gene was ranked in the top three positions by geNorm and NormFinder but only ranked as the seventh most stable gene by BestKeeper (Section 5.3.1.6.1). Ranking differences between programs have also been observed by other researchers and is most likely due to differences in the statistical algorithms used by each program (Cruz et al., 2009; Expósito-Rodríguez et al., 2008; Klie et al., 2011; Mallona et al., 2010; Reid et al., 2006).

GeNorm is based on pairwise comparisons using relative quantities, where the least stable gene is sequentially removed from the analysis until two candidate genes are selected. This approach assumes analysed genes are not co-regulated as geNorm is more likely to preferentially give a higher rank to genes with similar expression patterns (Andersen et al., 2004; Expósito-Rodríguez et al., 2008; Reid et al., 2006)). BestKeeper also uses a pairwise method though raw Ct values are used and the analysis is not reset to remove the least stable gene (Pfaffl et al., 2004). In contrast, NormFinder uses a model-based approach that accounts for different groups within a data set (for example virus infected and healthy plants), calculating and combining the inter- and intra-group variation to find the gene with the least variation (Andersen et al., 2004). Thus, to minimise potential bias introduced by the different statistical approaches, rankings from all three statistical approaches were compared to determine the most appropriate reference genes.
for relative quantification analysis of 3309 infected plants, similar to other researchers (Expósito-Rodríguez et al., 2008; Mallona et al., 2010; Reid et al., 2006). Ultimately, for this data set the $COX2$ reference gene was determined to be the most stable followed by $NAD5$, $GAPDH$, $POLYU$, $SAND$, $PP2a$, $EF2$, $SUC11$, $TATA$, and $PIP2$ in descending order.

In comparison, Reid et al. (2006) examined the expression stability of 14 reference genes in Cabernet sauvignon pericarp and the top four most stable genes in this data set were $GAPDH$, actin, $EF1-\alpha$, and $SAND$. Gutha et al. (2010) examined six different reference genes including $GAPDH$ and $SAND$, for GLRaV-3 infected and healthy Merlot leaf material, where actin and $NAD5$ were identified as the two most stable reference genes for the data set. The most stable gene from this current study for 3309 leaf material, $COX2$, was not analysed in either of these studies, whereas the third and fifth ranked reference genes, $GAPDH$ and $SAND$ respectively, were also highly ranked (first and fourth, respectively) by Reid et al. (2006). However, both of these genes were observed to have low expression stability in Merlot leaf samples infected with GLRaV-3, particularly $GAPDH$ with the lowest stability out of the six genes tested (Gutha et al., 2010). The variability between the studies emphasises the requirement for reference genes to be validated before genes are used for normalisation to ensure the best reference genes are used for a particular experimental condition (Gutierrez et al., 2008a; Gutierrez et al., 2008b; Jain et al., 2006; Radonić et al., 2004).

Therefore, it is unsurprising that differential transcription levels of the ten candidate reference genes analysed in this study was also observed when evaluating the reference genes within GLRaV-3 infected and healthy Schwarzmann leaf and Cabernet sauvignon cane material. As for all three data sets (i.e. the 3309 leaf, Schwarzmann leaf, and Cabernet sauvignon cane material data sets), no single candidate reference gene was identified as the top most stable gene. Only reference genes, $SAND$ and $GAPDH$, were ranked within the top five stably expressed genes across all data sets, although the overall rankings between the 3309 and Schwarzmann data sets were relatively similar with the identification of $COX2$ as the most stable gene and the remaining four reference genes that constitute the top five ranked genes were the same but in a different order. In contrast, the expression stability of candidate reference genes within the Cabernet sauvignon was significantly different compared to the other data sets, for instance
the POLYU reference gene was ranked within the top four positions for the 3309 and Schwarzmann data set but was only ranked ninth most stable gene (out of ten positions) for the Cabernet sauvignon data set. This greater difference in ranking is possibly due to differences in the type of tissue used (cane rather than leaf material was used) and environmental differences, as the Cabernet sauvignon material was sampled from plants grown in the field while both 3309 and Schwarzmann plants were grown in glasshouse conditions. Overall, based on the current work, if similar cultivars, plant material, and environmental conditions are used in future GLRaV-3 experiments the best reference genes for initial gene expression stability analysis are COX2, SAND, GAPDH, and NAD5.

5.4.2 Graft transmission of GLRaV-3

The 2010 and 2011 graft inoculation experiments successfully transmitted the GLRaV-3 group 1, NZ-1, and NZ2 variants, either individually or as mixtures, into Pinot noir, Sauvignon blanc, Syrah, and five different rootstocks. However, only a small proportion of the grafted plants proved to be GLRaV-3 positive. Failures to establish virus infected plants included grafted plants that did not survive the entire growing season and viable plants that tested GLRaV-3 negative. Factors that may have contributed to the low number of viable plants include the condition of the plant material used for inoculation, environmental conditions, and the grafting technique used. For the 2010 graft inoculations cane material from the Auckland collection was used as the virally-infected interstocks and material was significantly drier than what is considered ideal for grafting. In addition, some plants from this collection are known to also have additional virus infections including GLRaV-1, GLRaV-2, and GVA. It is possible these other viruses may have been present in the interstock and contributed to the low rate of successful grafts.

A low number of viable plants also resulted from the 2011 graft inoculations, even though the virally-infected interstock plant material used was of improved quality and less likely to be infected with other grapevine viruses, as cane cuttings were from the commercial Gimblett Gravels block, which is part of an on-going virus testing and control program (Chapter 4). In 2011 five different rootstocks were used. Rootstocks have varying characteristics that allow growers to select rootstocks for particular situations and environmental conditions (Shaffer et al.,
2004). It is possible the environmental conditions, such as the potting mix and the temperature of the glasshouse, may not have been conducive for some of the rootstocks. Differential survival rates for the different rootstocks were observed, with 83% of the plants using 3309 as a rootstock resulting in viable plants compared to only 35% and 33% for the Riparia gloire and S04 rootstock plants, respectively.

The technique used for grafting may also have contributed to low plant numbers. For the 2010 and 2011 graft inoculations, a double-worked grafting technique was performed in order to study virus movement up and down the plant. This double graft is likely to be more problematic than single grafts, as it requires plant material with similar size and shape to be matched twice and two graft unions need to successfully heal. Single-worked grafts were also conducted in 2010 with greater success, as only one plant died and over 80% of the plants tested positive for GLRaV-3. Another graft inoculation procedure that has been used with high success by other researchers is the chip-budding method (Cohen et al., 2004; Rosa et al., 2011). For instance, Rosa et al. (2011) graft inoculated over 720 plants, consisting of four different rootstocks, with single or mixed infections of GVA, GVB, Grapevine virus D (GVD), and GRSPaV, and only 49 plants died.

Even though phloem scrapings from a section of the source canes was pre-tested for GLRaV-3 by mRT-PCR before grafting, 26 and 41 viable plants from the 2010 and 2011 graft inoculations, respectively, tested GLRaV-3 negative (Section 5.3.2.1.2 and Section 5.3.2.2.2). Despite the positive test it is possible that the small section used as the interstock may not have carried GLRaV-3 virions due to uneven virus distribution. Alternatively, the virus may not have successfully transmitted from the interstock to the rest of the grafted plant or the infection was not detected by the diagnostic tests. The latter is possibly due to plants having a virus titre below the level required for molecular detection or uneven virus distribution, where the section tested for GLRaV-3 had no/low virus titre (discussed below, Section 5.4.4). Uneven distribution of GLRaV-3 variants was evident when different variant combinations were detected at different sampling positions along developed shoots of some grafted plants. In addition, 15 NZ2 infected 2011 grafted plants initially tested negative by mRT-PCR but tested positive in a second round of testing of the same RNA using the more sensitive
NZ2 variant-specific real-time RT-PCR assay, which is suggestive of low virus titre.

Additionally, from the 2010 graft inoculations, 14 double- and 12 single-worked grafted plants were identified as infected with a GLRaV-3 variant combinations that did not match those detected in the interstock used as inoculum (Section 5.3.2.1.2). These unexpected GLRaV-3 combinations may be the result of diagnostic testing of the initial interstock material and/or testing of resultant grafted plants being affected by the uneven distribution and titre of a specific GLRaV-3 variant(s) along a cane (discussed below, Section 5.4.4), or particular GLRaV-3 variants not moving into the virus-free material.

### 5.4.3 Phenotypic effects of GLRaV-3 variants

In this project, two characteristic symptoms of GLRaV-3 were examined, (i) the visual reddening and curling of the leaves for red cultivars, and (ii) the stunting of the plant as determined by the length of the developing shoots. Based on the typical reddening and leafroll symptoms observed in the red cultivars, there was no significant difference in the severity of symptom expression between the Syrah plants infected with a single infection of the group 1 variant and dual NZ-1 and NZ2 infected plants, or Pinot noir plants infected with group 1, group 1 and NZ-1, or the triple infection of group 1, NZ-1, and NZ2.

However, these results do not exclude the possibility that the GLRaV-3 variants may differentially affect other plant properties such as grape quality. For instance, it is known that GLRaV-3 infection can reduce anthocyanins, which are important for the retention of tannin, in Pinot noir berries compared to healthy vines (Lee et al., 2009b). Thus, it would be of interest to investigate whether GLRaV-3 variants have different effects on anthocyanin levels in grape berries.

Some GLRaV-3 variant combinations did reduce shoot growth. Shoots of GLRaV-3 infected Syrah, Sauvignon blanc, 101-14, SO4, Riparia gloire, and Schwarzmann plants had 1.6 to 4.7-fold less growth compared to corresponding healthy plants, while shoots from singly NZ2 infected Sauvignon blanc plants were approximately half the length of shoots from Sauvignon blanc plants singly infected with a group 1 isolate. Also shoots of NZ2 infected Schwarzmann plants were on average twice the length of group 1 Schwarzmann infected plants.
It is possible the difference in group 1 and NZ2 symptom expression observed in the Sauvignon blanc and Schwarzmann plants is due to different inherent host susceptibility and/or virus virulence. However, it may also be, at least in part, an environmental effect as the 2010 Sauvignon blanc grafted plants were grown in a field plot while the 2011 Schwarzmann grafted plants were grown in pots in the glasshouse. Differential symptom expression and severity have been observed in other plant viruses, such as CTV, where factors including the virulence of the CTV isolate, the host species, environmental conditions, and the rootstock/scion combination, have all been shown to effect symptom expression (Iftikhar et al., 2012; Moreno et al., 2008). For example, different CTV isolates were observed to cause different symptom severities in six pomelo cultivars, where the CTV B51 isolate caused mild stem pitting in only one of the six cultivars tested (Thong Dee cultivar), while the B31 CTV isolate caused severe stunting and stem pitting in all cultivars except the HA-Pink (Garnsey et al., 1996).

While the current work demonstrated that GLRaV-3 infection did result in stunted growth and there were differences between the effects of group 1 and NZ2 variants, further work with greater replication is required to confirm the findings as some treatments had only three biological replicates and there were large standard deviations in the shoot growth data. Also testing over more seasons would ensure growth differences were not due to seasonal fluctuations. In addition, the interstock material used for the 2010 experiment was from the Auckland collection where other grapevine viruses are known to be also present in this collection, as discussed above. These other viruses were not tested for, thus the presence of these other grapevine viruses cannot be excluded and may have influenced the 2010 graft inoculation symptom expression results.

5.4.4 Virus distribution and titre within vines and the implications for detection

False negative results by both serological and molecular diagnostic tests can result from low virus titre below the level for detection throughout the plant, absence of virus in the test sample due to uneven virus distribution, or the combination of unevenly distributed virus variants and variable virus titre within a plant. For this reason, it is important to consider the implications of virus distribution and titre to detection, as failure to detect virus when testing nursery and field plants can contribute to virus spread (Chapter 6 Section 6.3.2).
Uneven distribution of plant viruses within their host plants has been observed for a number of viruses including CTV, ASGV, PPV, GLRaV-1, GVA, GVB, and RSPaV (D'Urso et al., 2000; Jridi et al., 2006; Kominek et al., 2009; Magome et al., 1999). Previous GLRaV-3 studies, based on ELISA and real-time RT-PCR, examining virus distribution at three positions along the developing shoots/canes, observed higher virus titre (and virus detectability) at the basal position and lower virus titre at the apex (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012). Similarly in this study, for both the 2010 and 2011 GLRaV-3 grafted plants, a higher GLRaV-3 detection rate (79% and 93%, respectively) was observed at the base sampling position than the apical position (overall detection rates of 72% and 51% respectively). In addition, in 2011 using 3309 as a rootstock, both the absolute and relative virus levels of the GLRaV-3 group 1, NZ-1, and NZ2 variants decreased along the shoot from the base to the apex. However, statistical analysis only identified significant differences between the base and apical positions for the NZ-1 variant. The lack of statistical support for the observed trend is probably explained by the high variation in virus titre between biological replicates and/or the sampling of leaves nearing the end of the growing season.

In the current project, the collection of leaf samples from the three sampling positions was done 24 weeks after graft inoculation. This is equivalent to the berry ripening and harvest-ripe stages, (as described by Coombe (1995)) and corresponds to the phase in the GLRaV-3 movement where there is the least difference in virus titre between the base and apical positions, as observed by Monis et al. (1996), Teliz et al. (1987), and Tsai et al. (2012). Where the seasonal fluctuation of GLRaV-3 over a growing season was studied using ELISA (Monis et al., 1996; Teliz et al., 1987) and quantitative real-time RT-PCR (Tsai et al., 2012). At the start of the season, GLRaV-3 moved from the roots and trunks into the shoots but was initially only detected in the flower clusters and not in leaves (Teliz et al., 1987). Once inflorescences were fully developed GLRaV-3 was detected from leaves located at the basal position of the shoot first and then as the season progressed from leaves in the middle and apex of the shoot. The GLRaV-3 titre gradually increased as the season progressed with the highest virus titre found from leaves at the base of the shoot and the lowest at the tip of the shoot (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012). However, from the berry touch stage (the end of berry development where berries are still hard and green (Coombe, 1995)) until harvest, GLRaV-3 was detectable from all three sites.
including all the young apical leaves (Teliz et al., 1987; Tsai et al., 2012) and the previously significant difference in GLRaV-3 titre between the base and apex of the cane became less obvious (Monis et al., 1996).

Even though this study is only a “snapshot” of one time point in the growing season, the distribution of the three different GLRaV-3 isolates showed that in addition to sampling position, the particular GLRaV-3 variant can also affect the reliability of molecular assays, as there were significant differences in the detectability of GLRaV-3 group 1, NZ-1, and NZ2 variants. For the 2010 graft inoculations, across all sampling positions, the group 1 variant was detected from 93 to 96% of plants infected with a group 1 variant in single and mixed infections, while NZ-1 was detected from 82 to 93% of plants with NZ-1 infections. NZ2 was only detected from 74% of phloem scraping samples of NZ2 infected plants on 3309 rootstocks, and detection rates of 62, 66, and 40% for the base, mid-shoot, and apex sampling positions, respectively. A similar trend occurred in the 2011 graft inoculations, with NZ2 detected from only 59 and 27% of the NZ2 infected plants at the mid-shoot and apical sampling positions, respectively.

The initial mRT-PCR testing of the 2011 grafted plants was negative for all samples, though re-testing of the same RNA, using the variant-specific real-time RT-PCR assay targeting the ORF9, detected NZ2 in 47 samples (from 15 plants). Real-time RT-PCR also detected an additional 41 NZ-1 positives from previously mRT-PCR negative samples. This suggests that in addition to uneven virus distribution, low virus titres may also contribute to the lower detection rates for NZ2, and to a lesser extent NZ-1, compared to group 1 variants, and that the use of an alternative testing method can help improve detection.

Another example of the effects of virus titre on detection, contributing to the differential detectability of GLRaV-3 variants was when NZ2 was not detected from any RNA samples from NZ2 inoculated 3309 plants using the variant-specific real-time RT-PCR assay targeting the ORF4. This assay was able to detect up to $10^2$ copies of the virus RNA standard per µL (based on RNA standards diluted in total RNA extracted from corresponding virus-free material) and is comparable to the other group 1 and NZ-1 variant-specific real-time RT-PCR assays designed in this study, and other qRT-PCR assays for GLRaV-3 (Tsai et al., 2012) and other closteroviruses (Beuve et al., 2007; Ruiz-Ruiz et al., 2007). NZ2 was detected from the same RNA samples using the NZ2 variant-specific real-time RT-PCR
assay targeting the ORF9. This is likely due to the high expression of sgRNA, as the sgRNA(ORF9) provided a minimum 24-fold increase in template RNA compared to genomic RNA, greatly increasing the likelihood of virus detection.

Tsai et al. (2012) observed differences in the overall titre of GLRaV-3 group 1 populations compared to GLRaV-3 group 3 populations within Cabernet sauvignon plants, which they postulated was due to biological differences between the GLRaV-3 variants. Similarly, significant differences in the overall genomic and sgRNA levels between the group 1, NZ-1, and NZ2 GLRaV-3 variants, irrespective of the sampling position, were observed for graft inoculated 3309 plants. In addition, the difficulty in detecting NZ2, and to a lesser extent NZ-1, from graft inoculated plants is likely due to the combined effects of uneven virus distribution (as discussed above) and virus titre, which may indicate that these variants potentially move and replicate in the phloem tissue at a slower rate compared to the group 1 variant.

In this study, low sgRNA(ORF9) expression from NZ-1 variants compared to group 1 and NZ2 variants was observed, even though higher levels of genomic RNA were detected for NZ-1. The similarity of GLRaV-3 ORFs 8, 9, and 10 to equivalent ORFs of closteroviruses, BYV and CTV, suggested these ORFs may encode for viral silencing suppressors and systemic movement proteins (Dolja et al., 2006; Gouveia et al., 2012a). In particular, amino acid sequence analysis of ORFs 9 and 10 that generate proteins p19.6 and p19.7, showed they share a conserved amino acid motif with others from the p21-like viral suppressor of RNA silencing family (Gouveia et al., 2012a). The reduced expression of sgRNA(ORF9) may indicate potential differences in NZ-1 variant movement, replication, and/or symptom expression compared to other GLRaV-3 variants. However, more work is required to substantiate these possibilities as the suppressor activity has only been experimentally proven for p19.7 (encoded by ORF10) (Gouveia et al., 2012a; Gouveia et al., 2012b) and other additional determining factors such as protein activity and effectiveness need to be considered.

5.4.5 Conclusions

This chapter describes the symptom expression and distribution of three genetically different GLRaV-3 variants from phylogenetic group 1, NZ-1, and NZ2,
within various cultivars. Generally, successfully graft inoculated plants showed reduced shoot growth compared to healthy plants and red cultivars infected with various combinations of the three GLRaV-3 variants expressed typical premature reddening and downward rolling of the leaf margins. However, minor differences in severity of the symptom expression between the different GLRaV-3 variant combinations were observed.

To monitor and quantify GLRaV-3 variants within the host plant, mRT-PCR (Chapter 4), and six variant-specific and ten host gene-specific real-time RT-PCR assays were developed and used. These quantitative real-time RT-PCR assays are valuable tools that can be used for future diagnostic and biological studies of specific GLRaV-3 variants. It was evident that detection of particular GLRaV-3 variants was affected by the virus distribution and titre within the host plant, and that leaves sampled and tested from the basal position of shoots were more likely to detect GLRaV-3. Furthermore, the difficulty to detect NZ-1 and NZ2 variant towards the apex of the shoot, compared to the group 1 variant, suggests these genetically divergent GLRaV-3 variants may move and replicate less efficiently in the phloem tissue.
Chapter 6:

General discussion

The principal aim of this project was to investigate the genetic variability of GLRaV-3 in New Zealand to help improve the detection of GLRaV-3 by developing molecular assays. This was achieved by first screening more than 50 GLRaV-3 infected samples using SSCP and sequencing (Chapter 2), followed by more extensive characterisation of the genome (Chapter 3). Sequences from New Zealand were then compared to sequences from around the world and used to design generic and variant-specific RT-PCR and real-time RT-PCR molecular assays (Chapter 4). Molecular assays were evaluated using field and glasshouse samples (Chapter 4 and 5). With the discovery of high genetic variation, an additional project objective arose, investigating whether the divergent genetic variants also led to differences in the symptom expression and virus distribution within the plant (Chapter 5). This was achieved by graft inoculating plants with different combinations of GLRaV-3 variants, where the leaf symptoms, plant growth, and spread of the GLRaV-3 variants through the vine were monitored using the variant-specific real-time RT-PCR assays (Chapter 5). This chapter discusses the results from these studies in the context of the overall GLRaV-3 genetic variability found worldwide, the development of molecular detection methods, and the implications of these findings in an epidemiological perspective. Possible future directions from this research are also discussed.

6.1 The overall genetic diversity of GLRaV-3

An initial GLRaV-3 genetic variability study identified two phylogenetic groups (Jooste et al., 2005). Since then, more phylogenetic groups have been identified (Chapter 1). In the current study, high genetic variation was found in the New Zealand GLRaV-3 population with the discovery of GLRaV-3 variants belonging to phylogenetic groups 1, 2, 3, 5, and 6 (Chapter 2 and 3). In addition, a new variant of GLRaV-3 was identified, NZ2, which at the nucleotide level is more than 20% different from groups 1 to 6, and may represent a seventh phylogenetic group (Chapter 2 and 3). High genetic variation has also been reported for GLRaV-3 populations from a number of other countries. For instance, in South Africa GLRaV-3 isolates from groups 1 to 3 and 6 were identified (Bester et al., 2012b;
Jooste et al., 2005; Jooste et al., 2010; Maree et al., 2008), in USA isolates from groups 1 to 6 were identified (Fuchs et al., 2009; Seah et al., 2012; Sharma et al., 2011), and in Portugal group 1 to 5 isolates were identified (Gouveia et al., 2011). More recently, a genetic variability study in China identified GLRaV-3 isolates from groups 1 to 3 and 5 (Farooq et al., 2012). This worldwide distribution of GLRaV-3 from different phylogenetic groups is most likely due to the frequent movement of grapevine scion and rootstock material between countries with little or no control measures taken.

Prior to 1900, the importation of grapevine material into New Zealand was not controlled. Since then, imported scion and rootstock material was recorded and grown as a National Collection located at Te Kauwhata, though this did not include some material that was privately imported (Zuur, 1987). Material has been imported from various countries including South Africa, USA, Italy, Australia and Germany (Dick et al., 1989a). Furthermore, plants grown from imported material were known to have been virally infected and used in many New Zealand vineyards (Dick et al., 1989a; Wassilieff, 2009). For example, in the 1960s the Sauvignon blanc clone, TK00204, was used to produce New Zealand’s first Sauvignon blanc wine (Cooper, 2008). Material from a TK00204 clone was tested during this project, and contained GLRaV-3 variants from groups 1, 2, 6, and NZ2. It is unclear whether any of these GLRaV-3 variants were present in the original vines, but the resultant TK00204 plants showed severe grapevine leafroll symptoms, and had to be replaced with healthier material (Cooper, 2008).

Noticeably, most of the published GLRaV-3 sequences (>500 sequences on GenBank) fit within phylogenetic groups 1 to 5. While less than ten sequences are available for variants that differ from these five groups by more than 20%, at the nucleotide level. These include the divergent New Zealand NZ-1 isolate for which additional sequence was obtained (NZ1-B isolate) during this study (Chapter 3). NZ-1 is closely related to isolates only recently identified in South Africa and the USA, belonging to phylogenetic group 6 (Bester et al., 2012b; Seah et al., 2012). In contrast, isolates with high similarity to the NZ2 variant in other countries have yet to be reported. The limited sequence data for divergent GLRaV-3 variants, and in particular, NZ2, is possibly due to poor detection of these divergent variants with previous diagnostic primer pairs, designed against the NY1 isolate from group 1 (Chapter 4). Since isolates from groups 1 to 5 share higher sequence identity
with each other compared to the divergent isolates, previously used diagnostic primer pairs are more likely to detect and identify isolates from groups 1 to 5 (Chapter 4). Furthermore, samples are often pre-tested using ELISA to select for GLRaV-3 positive material for sequencing. Reduced detectability of NZ-1 and NZ2 variants compared to group 1 variants using DAS-ELISA and common diagnostic antibodies raised against NY1 (group 1), as observed in this project (Chapter 4), may also have placed a bias on the selection of samples used for previous molecular characterisation studies.

Recombination is one of the main contributors to virus evolution. In perennial plants with persistent viral infections, such as CTV infection in citrus (Weng et al., 2007) the possibility of recombination between virus variants is significantly enhanced. Prior to the work undertaken in this project, there was limited information about recombination events for GLRaV-3, with only one report that identified two recombinant isolates, AUSG5-2 (AJ748510) and IL1-1(AJ606355) by Turturo et al. (2005). A recent study by Farooq et al. (2012), identified recombination events within the CP, occurring between group 1-like and group 2-like variants, and between group 1-like and group 3-like variants. Similar to this study, these variants did not fit with any of the established groupings. Based on partial CP sequences in this study, recombination events were observed between group 1-like and group 3-like variants, and group 2-like and group 3-like variants (Chapter 2). In addition, within the previously known USA isolate, NY1, a complete genome analysis of GLRaV-3 isolates revealed an ancestral recombination event between a group 1-like and group 2-like variant (Chapter 3). It is likely that more evidence of recombination events will arise, as interest increases in genetic variation within the GLRaV-3 population.

With the discovery of recombinant and divergent GLRaV-3 variants (discussed above), the genetic variability within the GLRaV-3 population is highly diverse, and more complex than first thought. However, there is minimal information currently available about these variants and further genetic variability studies are required. In addition to the traditional methods of RT-PCR, cloning, and sequencing, other techniques could be used to aid these studies. In particular, NGS platforms can identify new viruses and virus strains without a need for prior sequence knowledge (Adams et al., 2012). For example, the Illumina NGS platform was used to initially identify the divergent GLRaV-3 isolates GH11 and GH30 from South Africa.
(Coetzee et al., 2010). Then, to confirm the draft genome sequence generated by the NGS analysis, traditional methods of RT-PCR, cloning, sequencing, and assembly were conducted (Bester et al., 2012b).

6.2 Molecular detection

Based on the most recent sequence data, an up-to-date diagnostic method is important to ensure tests remain accurate for certification schemes. This would reduce the probability of introducing virus-infected plants into the field, which can become a source for secondary spread. In addition, early detection of infected plants in the field using accurate and sensitive diagnostic tests, enables infected plants to be removed as early as possible before further spread occurs.

Molecular detection techniques have high reliability and sensitivity. However, it is reliant on sequence knowledge for the development of effective primer pairs and probes. Prior to this project, differential detectability of New Zealand GLRaV-3 using ELISA and RT-PCR assays was observed (D. Cohen, The New Zealand Institute for Plant and Food Research, Auckland, New Zealand pers. com.), indicating possible sequence variation within the GLRaV-3 population. This prompted the investigation of the genetic variability within the GLRaV-3 New Zealand population (Chapter 1), leading to the discovery of high sequence variability, in particular divergent variants NZ-1 and NZ2 (Section 6.1; Chapter 2 and 3). Because of the sequence variability, previous diagnostic primers were unable to detect NZ-1 and NZ2 (Chapter 4). Therefore, to improve diagnostic techniques, sequence data from this project and overseas (including the divergent NZ-1 and NZ2 variants) was used to develop a generic conventional RT-PCR and real-time RT-PCR assay (Chapter 4).

Additionally, tools that are able to differentiate between different virus variants/strains are important tools for epidemiological studies (Section 6.3). New assays were designed in this project, which use variant-specific primers and were either gel-based as an mRT-PCR assay or gel-free as real-time RT-PCR assays (Chapter 4 and 5). Based on the current work, the generic and the variant-specific assays had comparable sensitivity and accuracy at detecting GLRaV-3 variants representative of all phylogenetic groups identified thus far in New Zealand. The development of these assays was achieved by using sequence derived of three complete (groups 1, 2, and 5), and two partial genomes (NZ1-B (11,827nt) and
NZ2 (7,612nt)) (Chapter 3), in conjunction with other overseas sequences. Longer sequences representative of a number of different phylogenetic groups were used. By using these sequences, more regions of the genome could be analysed, increasing the prospects of identifying areas of high genetic variation, ideal for variant-specific primers to be designed. Conversely, if generic assays require updating because of the identification of new GLRaV-3 strains, the longer sequences increase the possibility of identifying regions of low sequence variation that are ideal for the design of new generic primer pairs.

Recently, Bester et al. (2012a) developed a real-time RT-PCR assay to detect GLRaV-3 isolates from groups 1 to 3, and 6, which in common with the assay described in this project, targets the ORF4. Analysis of the resulting melting curve was able to differentiate between variants from groups 3 and 6, but an additional primer set targeting ORF1a was required, in a subsequent real-time RT-PCR run, to differentiate between group 1 and 2 variants (Bester et al., 2012a). A two-step mRT-PCR assay was also developed to detect variants from groups 1 to 3, and 6 (Bester et al., 2012a).

The generic real-time RT-PCR assay from this project uses the SYBR green chemistry (a DNA-intercalating fluorophore), which generates a melting curve for further analysis. SYBR Green melting curve analysis has been used to differentiate between the PPV strains D and M (Varga et al., 2005). Thus, it is possible a similar approach could be performed using the generic real-time assay from this project. To ensure GLRaV-3 variants are able to be confidently differentiated, further work is required.

In addition to using variant-specific assays that only identifies the presence or absence of a virus variant, more information about the virus titres within the host plant and/or vector can be determined using real-time quantification RT-PCR assays. Six variant-specific real-time quantification RT-PCR assays were developed for this project. This was for the absolute and relative quantification of GLRaV-3 groups 1, 6 (specifically NZ-1), and NZ2 variants (Chapter 5). Absolute quantification required the synthesis of external RNA standards, while relative quantification required the design and optimisation of additional host gene (reference gene) specific real-time RT-PCR assays. To ensure accurate quantification, the reference gene assays used were carefully selected based on the expression stability of each reference gene (Chapter 5).
6.3 Epidemiology

The four main determinants that affect epidemiology of plant virus diseases are the virus, plant hosts, insect vectors, and the environmental conditions (Robert, 2001). Understanding the epidemiology of the grapevine leafroll disease caused by GLRaV-3 is highly complex, with various interrelationships between determinants that are generally multifaceted. Therefore, this project focused on investigating GLRaV-3 at the nucleotide and amino acid sequence level, in order to add to the current body of knowledge for GLRaV-3 epidemiology. Sequence data was then used to develop molecular techniques that were used to examine virus-host interrelations, in terms of symptom development and virus distribution within the plant (Chapter 5 and Section 6.3.2), and virus-vector interrelations affecting the spatial distribution of GLRaV-3 variants within a field plot (Chapter 4 and Section 6.3.3).

6.3.1 GLRaV-3 at the sequence level

Little is known about the different GLRaV-3 functions, such as replication strategies, the regulation of gene expression, and the induction of the disease. RNA viruses can contain cis-acting elements that can be involved in the aforementioned functions. Sequence conservation between significantly different virus isolates strongly indicates conservation of a feature, such as cis-acting elements important for virus functionality. Thus, nucleotide sequence from GLRaV-3 isolates representative of the various phylogenetic groups (including the New Zealand isolates NZ-PG1 (group 1), NZ-R2D4 (group 2), NZ-WCA (group 5), NZ1-B (group 6), and NZ2) were used to investigate possible cis-acting elements within the 5’UTR, 3’UTR, and each predicted sgRNA (Chapter 3).

A lack of secondary structure and overall nucleotide sequence conservation was observed in 5’UTR sequences of isolates from groups 1, 2, 3, 5, and 6. However, the first 46nt were well conserved with only four nucleotide differences, and in particular, the first 17nt were identical (Chapter 3). A recent study by Jarugula et al. (2012b), using a minireplicon cDNA GLRaV-3 clone (based on the WA-MR1 isolate, group 1), showed the first 40nt of the 5’UTR was important for virus replication. Deletion of these nucleotides completely abolished replication within N. benthamiana. Furthermore, the 5’UTRs were interchangeable, despite the differences in sequence length between groups 1 to 3. As the complete
replacement of the 5'UTR from the WA-MR1 minireplicon clone, with the complete 5'UTRs of GP18 (group 2) or PL20 (group 3) had no adverse effects to replication (Jarugula et al., 2012b). This further supports the premise that conserved cis-acting elements are needed for replication (Jarugula et al., 2012b).

The nucleotide sequence variation was unevenly distributed along the 3'UTR of isolates from groups 1, 2, 3, 5, 6, and NZ2 (Chapter 3). Low sequence conservation was observed at the 5'end, while high conservation was observed within two regions, between nucleotides 18,320 to 18,382 and 18,420 to 18,498 (Chapter 3). Within the highly conserved sequence regions, possible cis-acting elements such as three CCR initiation box motifs, and a motif for a 3'terminal pseudoknot secondary structure were identified. Additionally, the positive and negative sense 3'UTR sequences are predicted to form a well conserved SL structure, with four substructural hairpins at the 3' and 5' ends, respectively. Similar SL structures were described by Jarugula et al. (2010). Therefore, the highlighted sequence motifs and structural features (which are conserved for all GLRaV-3 isolates analysed), strongly supports the premise that there are cis-acting elements within the 3'UTR.

The expression of sgRNA of the 3'terminal genes is critical for the expression of the virus genome within a host. In total, seven sgRNAs expressing GLRaV-3 ORFs 3 to 12 have been identified (Jarugula et al., 2010; Maree et al., 2010). The regulatory sgRNA expression elements are generally sequence repeats and/or secondary structure(s) that are conserved between all sgRNAs (Gowda et al., 2001; Grdzelishvili et al., 2000; Peremyslov et al., 1998; Tatineni et al., 2009), located typically upstream of the ORF (Mandahar, 2006). Such conserved features for all sgRNAs were not found in GLRaV-3 isolates from groups 1 and 2 (Jarugula et al., 2010; Maree et al., 2010). However, for each sgRNA (for ORF6, 8, 9, and 10), comparisons of the predicted secondary structures showed similar SL structures between GLRaV-3 groups 1 and 2 isolates (Jarugula et al., 2010). This suggested that the expression of the GLRaV-3 sgRNA uses a different regulatory strategy compared to members of the genus Closterovirus, where the particular secondary structures are important for expression (Jarugula et al., 2010).
To examine whether the secondary structures between GLRaV-3 variants from recently described phylogenetic groups were also maintained, this project analysed sequences upstream of the ORFs for five different phylogenetic groups and NZ2. Sequences included NZ-PG1 (group 1), NZ-R2D4 (group 2), PL20 (group 3), NZ-WCA (group 5), GH11 (group 6), GH30 (group 6), NZ1-B (group 6), and NZ2. Nucleotide identities for the divergent GH11, GH30, NZ1-B, and NZ2 sequences ranged between 28.7% and 86.3% when compared to NY1 (group 1). Despite the high sequence variation, predicted secondary structures for four out of seven sgRNAs were conserved for all GLRaV-3 isolates analysed (Chapter 3). This further supports the premise that secondary structures may have an important role in the transcription of GLRaV-3 sgRNAs; a regulatory mechanism that is unique to GLRaV-3 (Jarugula et al., 2010).

It would be advantageous to construct a full length infectious cDNA clone of GLRaV-3, to test whether potential cis-acting elements identified from the sequence analysis were authentic. Recently, the development of a full length GLRaV-3 infectious clone has been reported (Jarugula et al., 2012a). This could be used for the generation of minireplicon cDNA GLRaV-3 clones, which are altered to contain specific mutations in identified cis-acting regions. A similar process was conducted for the analysis of the 5’UTR of GLRaV-3 (as described above) (Jarugula et al., 2012b) and for the controlling elements within the 5’UTR, 3’UTR and sgRNAs of CTV (Ayllón et al., 2004; Ayllón et al., 2005; Gowda et al., 2003a; Gowda et al., 2001; Gowda et al., 2003b; Satyanarayana et al., 2002). For example, sequence analysis of the CTV 3’UTRs revealed high conservation of ten predicted SL structures and CCR initiation boxes (Satyanarayana et al., 2002). Based on this analysis, a number of minireplicon cDNA CTV clones were constructed with different mutations. These included changes to the nucleotide sequence, which retained the SL structure and the complete removal or modification to predicted SL structures (Satyanarayana et al., 2002). To determine the effect a particular mutation had on expression, northern blots were then used to analyse the accumulation of sgRNAs (Satyanarayana et al., 2002).

The discovery of high sequence variation lead to the analysis of amino acid sequences from different genetic GLRaV-3 variants (from group 1 to 6, and NZ2). This investigated the level of difference within resulting proteins for each ORF, between the different GLRaV-3 variants (Chapter 3). High amino acid variation,
particularly substitutions that involved changes to the amino acid properties (non-conservative amino acid substitution), indicated potential differences in the predicted protein structure and/or biological functionality. For example, the *Closteroviridae* CP plays an integral role in virion assembly, replication, and movement (Dolja et al., 2006). It encapsidates approximately 95% of the viral RNA, and the remaining portion is encapsidated by a short virion tail that consists of the CPm, HSP70h, and an approximate p60 (p55 for GLRaV-3) proteins (Dolja et al., 2006). For a number of plant viruses, amino acid substitution(s) in the CP amino acid sequence have been shown to affect virus properties (Atreya et al., 1991; Atreya et al., 1995; Kaplan et al., 2007; Mochizuki et al., 2011; Shintaku et al., 1992). In this study, the predicted CP secondary structures of divergent GLRaV-3 variants from group 6 and NZ2 differed significantly, compared to GLRaV-3 variants from groups 1 to 5. This is likely due to four non-conservative amino acid substitutions, identified between amino acids 70 and 79 (Chapter 3). These divergent variants, specifically NZ-1 and NZ2, showed reduced immunological reactivity to a monoclonal antibody prepared against the NY1 isolate (group 1) (Chapter 4). This supports the premise that amino acid substitutions have altered the protein structure. Potential biological implications may include GLRaV-3 variants possessing different replication and virion movement efficiencies. Potential differences in replication and movement between GLRaV-3 variants from group 1, 6 (specifically NZ-1), and NZ2 were observed within graft inoculated plants (Section 6.3.2 and Chapter 5).

### 6.3.2 Virus-host interrelationship

GLRaV-3 causes persistent and chronic infections naturally in grapevines. As a part of the plant defence response, the virus induces changes to gene expression that controls various processes. The observed visual symptoms are a result of the molecular, cellular, and physiological changes collectively in the plant (Espinoza et al., 2007). For example, a study by Espinoza et al. (2007) examined the plant genes that are up- and down-regulated in naturally GLRaV-3 infected Cabernet sauvignon and Carménère, using DNA microarray chips to measure host gene transcription levels. A range of genes associated with different processes were affected including cell defence, metabolism, and transport. Generally, up-regulated genes were associated with the cell membrane and endomembrane systems, in particular, the genes involved in the transport of hormones, lipids, and
sugars (Espinoza et al., 2007). Alternatively, repressed genes were generally associated with the chloroplast, including genes encoding for photosynthetic proteins and chlorophyll biosynthesis enzymes (Espinoza et al., 2007).

For various plant viruses such as CTV, GLRaV-2, PPV, and *Rice dwarf virus*, the virus interactions with the host plant can induce different symptoms or differences in the severity of the expressed symptoms (such as symptomatic versus asymptomatic), depending on the virus genetic strain/variant, the host species, or the rootstock/scion combination (Bertazzon et al., 2010; Capote et al., 2010; Garnsey et al., 1996; Iftikhar et al., 2012; Moreno et al., 2008; Rowhani et al., 2000; Satoh et al., 2011). Differential symptom expression is particularly interesting, as virus strains/variants that generate mild symptoms are candidates for the mild strain cross protection control strategy. Mild strain cross protection is a technique where plants are purposefully infected with one virus strain/variant that expresses mild symptoms to protect against another strain/variant of the same virus expressing severe symptoms. This has been conducted for various plant viruses including CTV, *Papaya ringspot virus*, *Zucchini yellow mosaic virus*, and *Barley yellow dwarf virus*, with varying success (Costa et al., 1980; Lecoq et al., 1991; Wang et al., 1991; Wen et al., 1991; Yeh et al., 1988).

Thus far, there are only two reports of potential mild grapevine leafroll symptoms induced by GLRaV-3 infections (Habili et al., 2009; Rast et al., 2012). In the first instance, mild and severe GLRaV-3 strains were observed in Crimson Seedless table grapes (Habili et al., 2009). However, the mild GLRaV-3 strain was in a mixed infection with GVA and GLRaV-9, which may have influenced symptom expression. At the time of this study, both the mild and severe strains were reported to have 99% nucleotide identity to CP sequences in GenBank (Habili et al., 2009). In the second instance, another GLRaV-3 variant was observed to be symptomless in Sauvignon blanc vines (Rast et al., 2012). Although in white cultivars, leafroll symptoms are notoriously harder to identify and are less obvious compared to red cultivars. This mild GLRaV-3 variant showed high genetic variation compared to other variants, only sharing 88% amino acid identity to the CP gene of NY1 (group 1) (Rast et al., 2012).

Apart from the differential symptom development described above, little else is known about the pathogenicity of the different genetic variants, particularly the divergent GLRaV-3 variants. Therefore, in this study the pathogenicity of GLRaV-
3 variants from groups 1, 6 (specifically NZ-1), and NZ2 were investigated. This was done by graft inoculating virus-free plants with the GLRaV-3 variants in single or mixed infections, and monitoring the leaf and/or developed shoot lengths (Chapter 5). Most GLRaV-3 infected plants significantly reduced the growth of developed shoots compared to healthy controls. All GLRaV-3 infected red cultivar plants, except one Pinot noir plant, expressed obvious grapevine leafroll symptoms. However, minimal differences in the leaf symptoms and shoot lengths were observed between plants infected with different GLRaV-3 variant combinations. Sauvignon blanc and Schwarzmann GLRaV-3 infected plants with single infections of either group 1 or NZ2, showed statistically significant differences in shoot growth with each other. NZ2 infected Sauvignon blanc shoots were half the length of group 1 infected shoots, while the reverse was observed in Schwarzmann infected plants, with group 1 infected shoots half the length of NZ2 infected shoots. This discrepancy is potentially due to the differential symptom expression in different cultivars, as shown for CTV (Garnsey et al., 1996). However, as discussed in Chapter 5, further work is required to validate the results observed over a single growing season, which should consist of extending the monitoring period, increasing biological replicates, and assessing different aspects such as fruit quality.

In addition, virus-host interactions may also affect the distribution and movement of different virus strains/variants within the host plant. The sampling regime used to collect samples for diagnostic virus screens, can be affected by the uneven distribution and seasonal fluctuations of virus within a vine. The uneven distribution of different plant virus strains within a vine has been reported for CTV, ASGV, and PPV (D'Urso et al., 2000; Jridi et al., 2006; Magome et al., 1999). Previous studies have shown GLRaV-3 is also unevenly distributed, undergoing seasonal fluctuations (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012). GLRaV-3 virions move from the roots and trunks into the flower clusters, then initially to leaves at the basal position of canes (Teliz et al., 1987). As the season progresses, virus levels increase and move along the cane towards newly grown leaves. By the berry touch and harvest stages, GLRaV-3 was detected at all positions along the cane (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012). However, distribution of the different GLRaV-3 variants within the plant has not been studied, which may also affect the accuracy of diagnostic tests.
This study investigated the distribution of GLRaV-3 variants from group 1, group 6 (specifically NZ-1), and NZ2 in single or mixed infections (Chapter 5). Uneven distribution of GLRaV-3 variants was first evident when testing of graft inoculated plants infected with various GLRaV-3 variants, generated different test results at different times. For example, from the first round of testing, a sample was noted as having a single infection of NZ-1, while by the second round eight weeks later, the plant tested positive for NZ-1 and NZ2. Uneven distribution of GLRaV-3 variants was also evident when leaves from three different sampling positions along the cane (i.e. base, middle, and apical sampling positions) were collected and tested. Low detection success rates were observed at the apical sampling positions (Chapter 5). In particular, GLRaV-3 variants had differential detectability along the shoot. Twenty-four weeks after the 2010 graft inoculations, the group 1 variant was correctly identified in over 93% of infected samples at the base and mid-shoot positions, while NZ2 had significantly reduced detectability, as it was correctly identified in only 62 and 66% of infected samples, respectively (Chapter 5). Low detectability may be indicative of either an absence or low titre of virus present in a particular region of the vine, suggesting possible differences in the efficiency of the NZ2 variant to accumulate, replicate, and/or move within the phloem tissue of host plants.

Virus titres within the plant can also affect diagnostic tests. Misdiagnosed plants can be a result of low GLRaV-3 titre (Chapter 4 and 5). Thus, to examine the effects of virus-host interactions on the virus titres along the cane, absolute and relative quantification was used. This was to determine the titre of group 1, group 6 (specifically NZ-1), and NZ2 at three different sampling positions along developed shoots of 2011 graft inoculated plants that used 3309 as rootstock (Chapter 5). Generally, similar to previous studies (Monis et al., 1996; Teliz et al., 1987; Tsai et al., 2012), a higher number of group 1 and NZ-1 genome copies, were detected at the basal sampling position, compared to the other two positions. However, based on the average number of genome copies, there was a lack of statistical support for the differences between each sampling position. This is likely due to the time of sampling (as discussed in Chapter 5). In contrast, the NZ2 variant-specific real-time RT-PCR assay (targeting a region of low subgenomic expression), did not detect NZ2 from any samples. However, NZ2 was detected in some graft inoculated 3309 plants, using the NZ2 variant-specific real-time RT-PCR assay, targeting the p19.6 gene; a gene that generates a high level of
sgRNA. This further supports the premise that low levels of virus can affect the results of diagnostic testing. Though, detection can be improved by targeting regions that are likely to express high levels of RNA.

To detect NZ2 and additional NZ-1 variants from the 2011 infected plants, re-testing of RNA samples using variant-specific real-time RT-PCR assays was required (Chapter 5). The low number of NZ2 genome copies, and difficulties in detecting NZ2 (and to a lesser extent NZ-1), may be indicative of reduced replication and movement efficiencies for the divergent GLRaV-3 variants, compared to group 1. As discussed above in Section 6.3.1 and Chapter 3, the CP is involved in virus replication and movement, and the divergent GLRaV-3 variants have amino acid substitutions that are likely to change the CP structure. Furthermore, it has been suggested the virion tail of closteroviruses (that includes the HSP70h, ~60kDa (for GLRaV-3 this is a 55kDa protein, p55), and CPm proteins), is also involved in virus transportation within the host plant (Dolja et al., 2006). When the divergent NZ1-B and NZ2 variants were compared to NY1 (group 1), the amino acid identities for the three proteins (HSP70h, p55, and CPm) ranged between 72.7 and 77.1%, and 72.1 and 84.5%, respectively (Chapter 3). For the CPm and p55 proteins, 40 out of 118 and 51 out of 129 amino acid substitutions, respectively, involved a change to the amino acid property (non-conservative amino acid substitution). Thus, the high number of amino acid substitutions, observed in all four virus movement-associated proteins, are likely to influence and lead to differential GLRaV-3 variant movement and replication within the host plant.

6.3.3 Virus-vector interrelationship

Potential sources of new infection into a vineyard block includes the planting of infected material and mealybug transmission from neighbouring vines or neighbouring blocks/vineyards, or from residual roots from rogued vines (Pietersen, 2004). Spatial and temporal studies of GLRaV-3 have shown the natural spread of GLRaV-3 infection within and between different vineyards/blocks, which is commonly attributed to vector transmission by mealybugs (Cabaleiro et al., 2008; Cabaleiro et al., 2006; Charles et al., 2009; Jooste et al., 2011; Pietersen, 2004; Sharma et al., 2011). Generally, spread of GLRaV-3 within a block occurs from individual infected vines, to neighbouring vines along rows, and then to vines across rows, resulting in “GLRaV-3 clusters”
(Cabaleiro et al., 2008; Pietersen, 2004). The spread of GLRaV-3 between vineyards or vineyard blocks is generally via airborne dispersal of vectors, which is dependent of the prevailing winds and can either result in a gradient of GLRaV-3 infected vines along the block edge (Pietersen, 2004) or the random appearance of infected vines in the block (Charles et al., 2009). A number of factors can influence the spread and efficiency of GLRaV-3 spread. These include the particular virus variant infecting the plant host, the virus variant distribution within the host, the vector(s) population present in the block, vector feeding behaviour, the vector efficiency to transmit the virus, the susceptibility of the host plant to vectors, and the environmental conditions.

Studying the spatial and temporal virus distribution, in particular the distribution of the specific GLRaV-3 variants, is one approach used to understand the complex virus-vector interrelationship, and in-turn, the potential for virus spread. As discussed in Chapter 4, the spatial distribution of specific GLRaV-3 variants within a selected region of vineyard blocks in South Africa (Jooste et al., 2011) and New Zealand (this study), and across a number of different vineyards within the Napa Valley (Sharma et al., 2011) was examined using variant-specific molecular techniques. Vector transmission was evident based on the distribution patterns of specific GLRaV-3 variants.

In the Napa Valley, most of the GLRaV-3 variants were of groups 1 to 3, and unevenly distributed. Group 1 and 2 variants were mostly located in the North and Central vineyards, respectively, and the group 3 variants were generally spread throughout all vineyards (Sharma et al., 2011). Distribution patterns within the individual blocks showed evidence of short- and long-range mealybug transmission (Sharma et al., 2011). The South African study screened 14 mother blocks for different GLRaV-3 variants, and variants from group 2 were identified predominantly (Jooste et al., 2011). In a Cabernet sauvignon block, within three “GLRaV-3 clusters”, the movements of particular GLRaV-3 variants were also monitored (using SSCP analysis). Each cluster started with a single symptomatic vine at the centre, then over following years, mealybug transmission lead to the gradual development of typical grapevine leafroll symptoms to neighbouring vines (Jooste et al., 2011). In all three clusters, the GLRaV-3 variant at the centre of clusters was subsequently identified in surrounding vines (Jooste et al., 2011). In the current project, two blocks in Auckland and Hawke’s Bay were screened using
the mRT-PCR assay. At the Auckland collection, long stretches of vines infected with the same GLRaV-3 variant were observed, most likely due to mealybug transmission. As this site is a repository of known infected vines that have been retained for research purposes, disease control measures are not carried out. The Hawke’s Bay block is part of an on-going programme to control GLRaV-3 spread, which involves the annual roguing of symptomatic plants. GLRaV-3 variants from group 1, group 6 (NZ-1), and NZ2 were identified from symptomatic Cabernet sauvignon plants along and across rows, generally with the same GLRaV-3 variant infection. Similar to Jooste et al. (2011), “GLRaV-3 clusters” of NZ2, and to a lesser extent, group 1 variants were also observed.

In the study by Jooste et al. (2011), plants infected with GLRaV-3 variants from groups 1 to 3, expressed leafroll symptoms at different times. In the Hawke’s Bay Cabernet sauvignon block of this project, larger “GLRaV-3 clusters” of symptomatic NZ2 infected vines were observed, compared to more dispersed symptomatic plants infected with group 1 and 6 infected variants. Comparisons between the symptom expression of GLRaV-3 groups 1 to 3 have not been conducted. However, no differences in symptom development were observed in red cultivars, based on the graft inoculation experiments in this project. Pinot noir and/or Syrah grafted plants, singly infected with group 1, group 6 (NZ-1), and NZ2 variants, all expressed typical leaf symptoms within 24 weeks post-inoculation. However, virus variants can induce different reactions in different host cultivars, as observed in CTV (Garnsey et al., 1996). Therefore, observational differences in the Gimblett Gravels block and graft inoculation experiments may be due to the use of different cultivars.

None of the three GLRaV-3 variant distribution studies monitored the mealybug population in conjunction with the virus testing. However, results suggest possible differences in the vector transmission efficiencies, between different GLRaV-3 variants, based on the GLRaV-3 variant clustering and uneven distribution (Jooste et al., 2011; Sharma et al., 2011). The virion tail of plant viruses such as closteroviruses, has been suggested to be involved in the vector transmission in a semipersistent manner (Dolja et al., 2006; Ng et al., 2004). For instance, the CPm was proposed to be involved in the aphid transmission of CTV (Barzegar et al., 2010) and the whitefly transmission of the LIYV (Tian et al., 1999). Significant amino acid variation (between the NY1 and divergent NZ1-B and NZ2 GLRaV-3
variants) was observed for all GLRaV-3 proteins associated with the virion tail complex, particularly the CPm and p55 proteins (as discussed in Section 6.3.2). Thus, the numerous amino acid substitutions might lead to differences in vector transmissibility. Based on amino acid analysis of the CTV CPm for aphid-transmissible and non-transmissible variants, three non-conservative amino acid substitutions are proposed to have critical implications for aphid transmissibility (Barzegar et al., 2010). These substitutions are likely to either lead to changes in the protein structure, or hide important motifs needed for the interaction between the CPm and aphid stylets (Barzegar et al., 2010). In addition, amino acid substitutions may also affect the protein stability, reducing the availability of suitable CPm proteins for virion accumulation and promotion of vector transmission (Barzegar et al., 2010).

Recently, Blaisdell et al. (2012) showed GLRaV-3 variants from phylogenetic groups 1 and 6 have differential vector transmissibility. Variants from group 6 were more efficiently transmitted by P. ficus than group 1 variants. In the study, GLRaV-3 variants were transmitted from leaf cuttings of Cabernet sauvignon plants co-infected with group 1 and 6, to Pinot noir plants maintained in the glasshouse (the virus source leaf material were obtained from plants identified in the Sharma et al. (2011) distribution study). Overall, 56% of the Pinot noir plants became infected with GLRaV-3, consisting of either single infections of group 1 (2%), group 6 (29%), or co-infections of group 1 and 6 (25%). Interestingly, despite the very low transmission rate of group 1 variants, more group 1 variants were identified by Sharma et al. (2011) in the field. Blaisdell et al. (2012) suggests this difference is potentially due to the vector species and/or competition between the GLRaV-3 variants for transmission. At present, P. ficus is not found in New Zealand (the three main mealybug species are Ps. longispinus, Ps. calceolariae, and Ps. viburni), thus this study may not be directly relevant to New Zealand. However, it does support the hypothesis for differential vector transmissibility between GLRaV-3 variants that are in New Zealand, particularly with the presence of the divergent NZ-1 and NZ2 variants. This highlights the need for experiments similar to those of Blaisdell et al. (2012), to be conducted under New Zealand conditions.

The distribution of GLRaV-3 variants within the plant could also affect virus spread. For instance, variants that replicate and move quickly throughout the
plant are potentially more likely to be acquired by the mealybug, whereas slow moving or replicating variants are potentially less likely to be acquired. A positive association between virus titre and vector transmission efficiency has been shown for plant viruses such as Maize stripe virus, Tomato spotted wilt virus, and LIYV, as low virion levels lead to poor transmission efficiencies (Ammar et al., 1995; Ng et al., 2004; Rotenberg et al., 2009). A study by Tsai et al. (2011) showed the efficiency of GLRaV-3 transmission by *P. ficus* was not affected by titres between $10^3$ to $10^7$ genome copies per mg. Results from this project appear to support the study by Tsai et al. (2011). The 2011 graft inoculation experiment (using the Pinot noir and five different rootstock cultivars) indicated that the NZ2 variant replicated slowly within plants (Chapter 5, and described above in Section 6.3.2), and at the Gimblett Gravels block, large clusters of NZ2 infected plants were observed, indicating significant mealybug transmission. However, virus-host interaction can vary in different cultivars. Furthermore, *P. ficus* is not present in New Zealand, and it is possible that vectors found in New Zealand may have a lower virus titre threshold for transmission, and/or the NZ2 variant has a high transmission rate. Therefore, to examine the link between GLRaV-3 titre and vector transmission, future laboratory transmission experiments should test even lower virus titres, other mealybug species, and different GLRaV-3 variants.

Other factors that can influence the spread of GLRaV-3 include the host susceptibility to vectors, the vector feeding behaviour, the vector distribution and locations throughout the season, and the environmental conditions that can affect the plant tissue that vectors will feed on (Charles et al., 2006; Robert, 2001; Tsai et al., 2011). These aspects were outside the scope of this project. Tsai et al. (2012) reported a potential association between the seasonal fluctuations and movement of GLRaV-3 within a vine (as described in Section 6.3.2), and the seasonal abundance and distribution of some mealybug species such as *Ps. longispinus*, *Ps. viburni*, and *P. ficus* in California. This increases the likelihood of both GLRaV-3 transmission throughout the vegetative season and GLRaV-3 secondary spread (Tsai et al., 2012). Furthermore, the *P. ficus* feeding behaviour was studied in glasshouse conditions, where virus acquisition and inoculation of GLRaV-3 was not affected by the leaf tissue type or its position along the shoot (three positions tested: base, middle, apical) (Tsai et al., 2011). Work is currently in progress by other researchers, focussing on the vectors important to New Zealand, which will help understand the spread of GLRaV-3 in New Zealand.
6.3.4 Control measures

Recommended control measures for GLRaV-3 include managing vineyard hygiene practices, monitoring the vineyard for leafroll symptoms and mealybug populations, replanting using certified vines, and controlling weed growth (Chapter 1). GLRaV-3 is not known to infect plant genera other than *Vitis*, thus surrounding weeds are not potential hosts for GLRaV-3, which could act as sources of virus inoculum for secondary spread. However, weeds are still required to be managed, as they provide mealybugs and ants (which protect mealybugs against natural enemies (Charles et al., 2006)) with additional cover and protection from the environment. To reduce the chance of virus spreading, infected plants are typically removed as early as possible and replanted with certified plants. However, it is common for new infections to reappear 12 to 18 months after vine removal and replanting, as observed in this project (GLRaV-3 screening of the Gimblett Gravels block; Chapter 4) and other studies by Bell et al. (2009) and Pietersen (2004). New infections are likely due to viruliferous mealybugs moving to and/or infecting neighbouring vines before the removal of infected vines. In addition, incomplete removal of the infected plant roots may provide a source of inoculum for further spread (Bell et al., 2009; Pietersen, 2004). Twelve months after the removal of infected vines, GLRaV-3 has been found in residual roots and in *Ps. calceolariae* found on residual roots (Bell et al., 2009). In the same study, only after 12 days of planting, *Ps. calceolariae* were also found on the roots of replanted young bare-rooted vines (Bell et al., 2009). In this project, mRT-PCR testing of nine residual herbicide-treated root samples detected GLRaV-3 group 1, NZ-1, and NZ2 variants, indicating that these variants can populate and survive in roots, for further virus spread (Chapter 4). Therefore, to ensure most, if not all, potential virus inoculum is removed from the site, it is ideal to remove as much of the infected vines including the roots.

6.4 Future directions

This project has contributed additional molecular and biological information to the continuously developing body of knowledge for GLRaV-3. These findings can be used as the base for further work, to expand our understanding of GLRaV-3, particularly in the New Zealand environment.
With the identification of considerable genetic variation within the GLRaV-3 population, the question arises whether there is an even greater genetic and/or biological variation. Further screening using NGS platforms such as Illumina sequencing (which is unconstrained by the specificity of primer pairs), may be advantageous to identify more variants/strains. Additionally, in an attempt to identify potential mild strains of GLRaV-3 for mild strain cross protection, it would be particularly interesting to not only screen samples from symptomatic plants, but also from asymptomatic infected plants. Future screening will also contribute more sequence data for phylogenetic comparisons and ensure diagnostic detection methods remain up to date.

Conserved sequence and secondary structures identified in this project are potentially significant to virus functionality. Further work is required to confirm these findings, similar to studies for other plant viruses such as CTV. To test for changes in activity such as virus replication and movement, infectious virus clones can be used as the basis for site-specific mutagenesis and analysis (Ayllón et al., 2004; Ayllón et al., 2005; Gowda et al., 2003a; Gowda et al., 2001; Gowda et al., 2003b; Satyanarayana et al., 2002; Tatineni et al., 2008). In addition, it would be valuable to complete the genome sequence for the New Zealand isolates NZ1-B and NZ2. NGS platforms could be used to determine the missing sequence and confirmed by RT-PCR, cloning, and sequencing.

It would be of practical value to further test the generic molecular assays (developed in this project) against samples from different geographical regions. Particularly, from other collections worldwide that contain outlier isolates such as GLRaV-3-Tempr from Spain, as this was not available during this project. Future research may also include the design of additional mRT-PCR primer sets for the specific detection of groups 3 to 5, if this is required for a different geographical region and/or experimental protocol. Furthermore, the current multiplex assay is a gel electrophoresis-based technique, and to streamline this for larger sample sets, it could be adapted into an automated gel-free multiplex real-time RT-PCR assay, similar to assays designed for virus detection in other crops (Agindotan et al., 2007; Loconsole et al., 2010; Mortimer-Jones et al., 2009; Price et al., 2010). Alternatively, the generic real-time RT-PCR assay is a candidate for high resolution melting curve analysis, which requires further analysis to test the
viability for the generic assay to discriminate between GLRaV-3 variants (as discussed above in Section 6.2).

Biological properties of the virus are also important to consider. From the graft inoculation experiment, the genetically different variants were not observed to express differences in symptom severity. However, further biological studies should be conducted, where symptoms can be monitored over several seasons, and where berry quality can also be examined. For both red and white cultivars, features such as the bunch and berry size, Brix levels, and anthocyanin levels would be of interest to examine. In addition to monitoring the symptoms, to further understand the movement of specific GLRaV-3 variants within the plant, it would be interesting to monitor the seasonal fluctuations and distribution of each GLRaV-3 variant, alongside symptom development.

To further understand the importance of the uneven spread of GLRaV-3 variants within a block, a holistic approach to field screening over several years is needed, where the host, virus variants, and vector are all considered. Vector transmission efficiency experiments for each of the different GLRaV-3 variants and tissue types using the same mealybug species found in the block should also be conducted. Equivalent epidemiological studies have been conducted for other plant viruses such as PPV and CTV (Cambra et al., 2000; Capote et al., 2010).

6.5 Summary

In summary, this project has studied New Zealand GLRaV-3 isolates at the molecular level. It contributes to our understanding of the GLRaV-3 genetic variation present in New Zealand and worldwide. The sequence data obtained from this study has been fundamental for the (a) discovery of new GLRaV-3 variants, (b) identification of conserved regions that are of potentially important for virus processes such as replication and gene expression, and (c) development of new molecular assays that have improved the detection of GLRaV-3 in New Zealand.

Both generic and variant-specific molecular assays were developed and optimised. The generic RT-PCR and real-time RT-PCR assays are of particular importance for certification schemes, which requires reliable and sensitive diagnostic assays to ensure GLRaV-3 infected plants, are not introduced into the field. Whereas variant-specific mRT-PCR and real-time RT-PCR assays, are
important for epidemiological studies. As demonstrated in this project, the assays can be used to monitor the spread of particular GLRaV-3 variants within the field and plant host. Real-time RT-PCR assays also provide the added capability to accurately quantify a specific GLRaV-3 variant. For relative quantification, ten host gene (reference gene) specific real-time RT-PCR assays were developed and optimised, which can also be used by other researchers. However, the expression stability of the reference genes must be confirmed for the particular experimental set-up before use, as described in this project.

This project has also studied and compared three GLRaV-3 variants from group 1, group 6 (specifically NZ-1), and NZ2 in greater detail. All three variants were graft transmissible into Pinot noir, Sauvignon blanc, Syrah, and five rootstocks 3309, 101-14, Schwarzmann, SO4, and Riparia gloire. Infection lead to typical symptoms in the leaves for red cultivars, and generally reduced the growth of developed shoots of for all cultivars, compared to healthy controls. In addition, GLRaV-3 variants were found to be unevenly distributed within the graft inoculated plant. Most variants were more likely to be detected reliably from leaf material collected from the base of the cane compared to the apical position. The results also suggested that the divergent GLRaV-3 variants (NZ-1 and NZ2) move and/or replicate more slowly compared to group 1 variants in the phloem tissue. Furthermore, based on the spatial distribution observed within the Gimblett Gravels block, GLRaV-3 variants may have different vector transmission efficiencies. These variant differentiating features are important to consider and examine further, to help understand GLRaV-3 spread and the development of appropriate GLRaV-3 control regimes.
Appendix 1: Auckland collection varietal details

Table A1.1: Information about the 157 accessions of 108 red varieties and 49 white varieties in the Auckland collection including cultivar, vine position within the block, and the original source of material.

<table>
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<th>TK accession number</th>
<th>Cultivar(^b)</th>
<th>Vine position(^a)</th>
<th>Source(^b)</th>
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<td>Pinot gris</td>
<td>1-1-5</td>
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\(^a\) Vine position within the block denoted as row-bay-vine

\(^b\) As reported by Dick et al. (1989)
Appendix 2: Additional recombination information concerning New Zealand variants from minor clades a, b, and c

Minor clade a:

The break point for “clade a” variants, TK00080-A and TK00080-C, between parent isolates WG3.17-M (group 3) and CI-756 (group 1), was detected by the SiScan and 3Seq methods at nucleotides 380 and 358 respectively (SiScan p-value = 1.25x10^{-10} and 3Seq p-value = 1.82x10^{-05}). The first 380 or 358nt section of the sequence is most closely related to group 3 with 99.21% and 99.72% nucleotide identity compared to group 1 at 91.58% and 91.85% identity, respectively. However, the remaining section is more closely related to group 1 with 100% identity compared to group 3 at 88.89% and 88.64% identity respectively (Figure 2.10 and 2.11).

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Figure A2.1: Phylogenetic analysis of the recombinant Grapevine leafroll-associated virus 3 isolate, TK00080-A, based on the sequence (a) before (380nt) and (b) after (108nt) the predicted recombination break point between parent isolates CI-756 (group 1) and WG3.17-M (group 3), conducted in MEA5 (Tamura et al., 2011). (a and b) Recombinant and parent isolates are highlighted with a black triangle and circle, respectively. Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method was used to compute evolutionary distances. The percentages of bootstrap support (≥75%) from 1,000 replicates are shown at nodes. The phylogenetic groups 1 – 5 are proposed by Gouveia et al (2011). The scale represents 0.02 nucleotide substitutions per site.
Figure A2.2: Phylogenetic analysis of the recombinant *Grapevine leafroll-associated virus* 3 isolate, TK00080-C, based on the sequence (a) before (358nt) and (b) after (130nt) the predicted recombination break point between parent isolates CI-765 (group 1) and WG3.17-M (group 3), conducted in MEGAS5 (Tamura et al, 2011). (a and b) Recombinant and parent isolates are highlighted with a black triangle and circle, respectively. Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method was used to compute evolutionary distances. The percentages of bootstrap support (≥ 75%) from 1,000 replicates are shown at nodes. The phylogenetic groups 1 – 5 are proposed by Gouveia et al (2011). The scale represents 0.02 nucleotide substitutions per site.

**Minor clade b:**

For TK00080-J, WG3.17-J, and WG3.17-O sequence variants from clade b, the point of recombination was predicted at nucleotide 213 by SiScan (p-value = 2.10x10^{-10}) and 3Seq methods (p-value = 5.95x10^{-06}). The first 213 nucleotides of the “clade b” recombinants have an average nucleotide identity of 100% to CI-664 (group 1), compared to 93.90% for LN (group 3). For the remaining 275 nucleotides the relationship is reversed, with 99.39% identity to the group 3 compared to 90.30% for the group 1 variant (Figure 2.12).

Figure A2.3: Phylogenetic analysis of the recombinant *Grapevine leafroll-associated virus* 3 isolates, TK00080-J, WG3.17-J, and WG3.17-O, based on the sequence (a) before (213nt) and (b) after (275nt) the predicted recombination break point between parent isolates CI-664 (group 1) and LN (group 3), conducted in MEGAS5 (Tamura et al, 2011). (a and b) Recombinant and parent isolates are highlighted with a black triangle and circle, respectively. Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method was used to compute evolutionary distances. The percentages of bootstrap support (≥ 75%) from 1,000 replicates are shown at nodes. The phylogenetic groups 1 – 5 are proposed by Gouveia et al (2011). The scale represents 0.02 nucleotide substitutions per site.
Minor clade c:

The recombination break points for “clade c” occur earlier than for the previous two clades. The break point between parent isolates 621 (group 1) and 8-19 (group 3) for TK00080-H, WG3.17-G, and WG3.17-L variants was detected by SiScan (p-value = 2.29x10^{-11}) and 3Seq methods (p-value = 9.99x10^{-05}) at nucleotide 121. While only the SiScan method detected a break point between 621 (group 1) and PL-20 (group 3) at nucleotide 162, which results in the TK00080-T recombinant variant (p-value = 1.07x10^{-09}). Similar to “clade b”, the first section of the sequences are more closely related to group 1 variants, with an average nucleotide identity of 99.59% to the 621 isolate (group 1), compared to 93.93% to 8-19 or PL-20 (Group 3), while the remaining segment is 99.02% similar to group 3 (Figure 2.13 and 2.14).

![Figure A2.4: Phylogenetic analysis of the recombinant *Grapevine leafroll-associated virus* 3 isolates, TK00080-H, WG3.17-G, and WG3.17-L, based on the sequence (a) before (121nt) and (b) after (367nt) the predicted recombination break point between parent isolates 621 (group 1) and 8-19 (group 3), conducted in MEGA5 (Tamura et al, 2011). (a and b) Recombinant and parent isolates are highlighted with a black triangle and circle, respectively. Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method was used to compute evolutionary distances. The percentages of bootstrap support (≥75%) from 1,000 replicates are shown at nodes. The phylogenetic groups 1 – 5 are proposed by Gouveia et al (2011). The scale represents 0.02 nucleotide substitutions per site.](image-url)
Figure A2.5: Phylogenetic analysis of the recombinant Grapevine leafroll-associated virus 3 isolates, TK00080-T, based on the sequence (a) before (162nt) and (b) after (326nt) the predicted recombination break point between parent isolates 621 (group 1) and PL-20 (group 3), conducted in MEGA5 (Tamura et al, 2011). (a and b) Recombinant and parent isolates are highlighted with a black triangle and circle, respectively. Evolutionary history was inferred using the Neighbour-Joining method and the Kimura 2-parameter method was used to compute evolutionary distances. The percentages of bootstrap support (>75%) from 1,000 replicates are shown at nodes. The phylogenetic groups 1 – 5 are proposed by Gouveia et al (2011). The scale represents 0.02 nucleotide substitutions per site.
Appendix 3: Additional predicted secondary structures from the 3′UTR and selected sgRNAs

Figure A3.1: Predicted secondary structures for the 3′ untranslated region of *Grapevine leafroll-associated virus 3* isolates (a) NZ-PG1 (group 1), (b) NZ-R2D4 (group 2), (c) PL-20 (group 3), (d) NZ-WCA (group 5), (e) NZ1-B (group 6), (f) GH11 (group 6), and (g) NZ2, using the positive sense sequence. The location of the conserved “CCR initiation sites” are indicated with red dotted shapes. All secondary structures were predicted using the mfold web server (Zuker, 2003).
Figure A3.2: Predicted secondary structures for the 3' untranslated region of *Grapevine leafroll-associated virus 3* isolates (a) NZ-PG1 (group 1), (b) NZ-R2D4 (group 2), (c) PL-20 (group 3), (d) NZ-WCA (group 5), (e) NZ1-B (group 6), (f) GH11 (group 6), and (g) NZ2, using the negative sense sequence (i.e., complement sequence). All secondary structures were predicted using the mfold web server (Zuker, 2003).
Figure A3.3: Predicted secondary structures for the sequence surrounding the 5’transcription start site (5’TSS) (25nt up- and down-stream, complement sequence) of sgRNA(ORF10-12) of Grapevine leafroll-associated virus 3 isolates (a) NZ-PG1 (group 1), (b) NZ-R2D4 (group 2), (c) PL-20 (group 3), (d) NZ-WCA (group 5), (e) NZ1-B (group 6), (f) GH11 (group 6), and (g) NZ. The 5’TSS nucleotide is indicated with a red arrow. All secondary structures were predicted using the mfold web server (Zuker, 2003).

Figure A3.4: Predicted secondary structures for the sequence surrounding the 5’transcription start site (5’TSS) (25nt up- and down-stream, complement sequence) of sgRNA(ORF3-4) of Grapevine leafroll-associated virus 3 isolates (a) NZ-PG1 (group 1), (b) NZ-R2D4 (group 2), (c) PL-20 (group 3), (d) NZ-WCA (group 5), (e) GH11 (group 6), and (f) NZ1-B (Group 6). The 5’TSS nucleotide is indicated with a red arrow. All secondary structures were predicted using the mfold web server (Zuker, 2003).
Figure A3.5: Predicted secondary structures for the sequence surrounding the 5\textsuperscript{th} transcription start site (5’TSS) (25nt up- and down-stream, complement sequence) of sgRNA(ORF7) of *Grapevine leafroll-associated virus* 3 isolates (a) NZ-PG1 (group 1), (b) NZ-R2D4 (group 2), (c) PL-20 (group 3), (d) NZ-WCA (group 5), (e) NZ1-B (group 6), (f) GH11 (group 6), and (g) NZ2. The 5’TSS nucleotide is indicated with a red arrow. All secondary structures were predicted using the mfold web server (Zuker, 2003).
Appendix 4: Total RNA extraction protocols

A4.1 RNA extraction using the Sigma-Aldrich Spectrum Plant Total RNA Kit

After plant material was homogenised using the Qiagen TissueLyser II (Qiagen, GmbH, Germany), described in Chapter 2 Section 2.2.2, the remainder of the RNA extraction protocol followed the manufacturer instructions as follows. To remove residual plant debris the centrifuged samples in modified Lysis Solution buffer the lysate was transferred to a Filtration Column (blue retainer ring) (Sigma-Aldrich, St. Louis, MO, USA) placed in 2mL collection tube and centrifuged for 1 minute at 14,000xg. The resulting lysate was mixed with 500µL of Binding Solution (Sigma-Aldrich, St. Louis, MO, USA) and 700µL of the mixture was transferred into the Binding Column (red retainer ring) (Sigma-Aldrich, St. Louis, MO, USA) placed in a 2mL collection tube and centrifuged at 14,000xg for 1 minute. The flow-through was discarded. The remaining lysate-binding solution mix was added to the same column and the centrifugation and decanting steps repeated. To wash the column 500µL of the Wash Solution 1 (Sigma-Aldrich, St. Louis, MO, USA) was added to the column followed by centrifugation at 14,000xg for 1 minute and the flow-through discarded. The column was washed twice with 500µL of Wash Solution 2 (Sigma-Aldrich, St. Louis, MO, USA), centrifuged at 14,000xg for 30 seconds and flow-through discarded. To ensure removal of all residual buffers from the column, the column was further spun for 1 minute at 14,000xg. To elute RNA, each column was then placed in a new 2mL collection tube and 50µL of Elution Solution (Sigma-Aldrich, St. Louis, MO, USA) was directly added onto the centre of the column matrix and left to stand for 1 minute, followed by centrifugation at 14,000xg for 1 minute.

A4.2 RNA extraction using the Qiagen RNeasy Plant Mini Kit

After plant material was prepared and homogenised using the Qiagen TissueLyser II (Qiagen, GmbH, Germany), described in Chapter 4 Section 4.2.2.1, the remainder of the RNA extraction protocol followed the manufacturer instructions as follows. To remove excess plant debris, 750µL of the plant lysate from centrifuged samples in Mackenzie extraction buffer and 20% Sarkosyl, was transferred to a QIAshredder spin column (lilac) (Qiagen, GmbH, Germany) placed in a 2mL collection tube and centrifuged at 16,000xg for 2 minutes. The flow-through (avoiding the pellet) was transferred to a new 1.5mL microcentrifuge tube and
mixed with 250µL of 100% ethanol. This mixture was transferred to the RNeasy spin column (pink) (Qiagen, GmbH, Germany) placed in a 2 mL collection tube and centrifuged for 30 seconds at 8,000xg and the flow-through was discarded. To wash the column 700µL of RW1 buffer (Qiagen, GmbH, Germany) was added to the column and centrifuged at 8,000xg for 30 seconds and the flow-through discarded. The column was further washed with the addition of 500µL of RPE buffer (Qiagen, GmbH, Germany) and centrifuged at 8,000xg for 30 seconds with the flow-through discarded. The addition of 500µL of RPE buffer (Qiagen, GmbH, Germany) was repeated, however to remove all residual ethanol the column was centrifuged at 8,000xg for 2 minutes, flow-through discarded, and followed by an additional centrifugation at 16,000xg for 1 minute. The column was then transferred to a 1.5mL microcentrifuge tube and to elute RNA 50µL of RNase-free water was added directly to centre of the column matrix, left to stand for 1 minute, and centrifuged at 8,000xg for 1 minute.

A4.3 RNA extraction using a CTAB extraction method

After plant material was prepared and homogenised using the Qiagen TissueLyser II (Qiagen, GmbH, Germany), described in Chapter 4 Section 4.2.2.3, the remainder of the RNA extraction protocol followed the protocol described by White et al. (2008). Incubated samples in the RNA extraction buffer were centrifuged at 16,000xg for 10 minutes and 1mL of the supernatant was transferred to a new 2mL tube. To each tube, 1mL of 24:1 chloroform:isoamylalcohol (C:I) was added, vortexed, and centrifuged at 16,000xg for 15 minutes at 4°C. Being careful not to disturb the white interphase, 800µL of the top clear aqueous phase was transferred into a new 2mL tube and the C:I step was repeated with the addition of 800µL of 24:1 C:I and centrifugation at 16,000xg for 15 minutes at 4°C. 600µL of clear aqueous phase was transferred into a new 1.5mL microcentrifuge tube and 200µL of 8M lithium chloride (LiCl) was added to each sample, mixed by inversion and stored overnight at 4°C to precipitate the RNA.

Following the overnight incubation, tubes were centrifuged at 16,000xg for 1 hour at 4°C, the supernatant discarded, and the pellet washed by the addition of 800µL of 70% ethanol and mixed by inversion. Tubes were then centrifuged at 16,000xg for 5 minutes, the ethanol discarded, and to ensure all residual ethanol was removed the tubes were left to air-dry for 1 hour in an inverted position. The pellet was re-suspended with 50µL of RNAse-free water.
Appendix 5: ELISA and mRT-PCR results from the testing of the Auckland collection and winter 2011 testing of the Gimblett Grapes block

**Table A5.1:** Detection of GLRaV-3 by ELISA using monoclonal and polyclonal antibody assays and mRT-PCR in cane from the Auckland Collection.

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<sup>a</sup> Vine position within the block denoted as row-bay-vine  
<sup>b</sup> Red shading indicates ELISA negative samples. Blue shading indicates samples with high reactivity ratios (between the reaction rate of polyclonal to the reaction rate of monoclonal antibodies).
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<sup>a</sup> Vine position within the block denoted as row-bay-vine

<sup>b</sup> Red shading indicates ELISA negative samples. Blue shading indicates samples with high reactivity ratios (between the reaction rate of polyclonal to the reaction rate of monoclonal antibodies).
Table A5.2: Detection of GLRaV-3 by ELISA using monoclonal and polyclonal antibody assays and mRT-PCR in cane from the Gimlet Gravels block.

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※ Sample name denotes the vine position within the block as row-bay-vine

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