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Molecular Detection of the *Luteoviridae*

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University
by
Anastasija Chomič

Lincoln University
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Abstract of a thesis submitted in partial fulfilment of the
requirements for the Degree of Doctor of Philosophy.

Molecular detection of the *Luteoviridae*

by
Anastasija Chomič

The *Luteoviridae* is a family of single stranded positive sense RNA plant viruses which cause yield losses in many important food crops worldwide and are therefore of significant economic concern for some countries. Fast and accurate detection and identification is important for strategies designed to control the spread of *Luteoviridae* species and to reduce their economic impact.

This study offers significant advances on current molecular protocols for their detection and differentiation by being taxonomically broad-ranging, time-efficient, sensitive to asymptomatic detection and with the potential to pick up unknown sequence isolates. Current molecular detection and identification tools for the *Luteoviridae* are mainly species specific, which limits their application for fast and accurate detection and identification.

Firstly, the development of new generic primers for the family was investigated. Using the two-step reverse transcription polymerase chain reaction (RT-PCR), thirteen out of sixteen *Luteoviridae* species analysed were detected using three separate combinations of low-degeneracy generic primers, targeting the coat protein gene region. A synthetic positive control containing all primer sequence priming sites was designed as a generic tool for use with a variety of host plants and the *Luteoviridae* species. The *Luteoviridae* primers described in this study present a simple infection-detection tool which will be of benefit to biosecurity authorities in nursery-stock surveillance, disease management or outbreak prevention, and may also be useful in detection of as-yet undiscovered species within the family.

Secondly, the suitability of a two-step reverse transcription real time PCR (RT-qPCR) plus melting curve analysis (MCA) as a tool for the rapid detection and discrimination of *Luteoviridae* species was investigated. Melting temperature and shape of the melting peak were analysed for

13 *Luteoviridae* species using SYBR® GreenER™ fluorescent dye. Specific melting peaks were observed for all isolates investigated, however due to the high variability of sequences for some members of this family, different melting temperatures were also observed between different isolates of some species. Nevertheless, discrimination was achieved among 6 species. MCA, in this study, was demonstrated to be a faster and more discriminatory alternative to gel electrophoresis of end-point PCR products for the detection of *Luteoviridae* infection.

Thirdly, this study evaluated two combinations of generic *Luteoviridae* primers for the detection of an early double-infection with BYDV-PAV and CYDV-RPV in barley and oats via RT-qPCR at the early stage of virus infection (3-15 days post inoculation (dpi)). The distribution of viruses in young and old leaves for optimising plant tissue collection strategies was also considered. Quantitative data from this study indicate that in some plants the titre of both luteoviruses is comparable to the expression of the plant mitochondrial gene nad5 (used as an internal RNA control) as early as 3 dpi and that titre differs greatly between individual plants. This study also suggests that virus distribution in different parts of the plant is probably host dependent; while young barley leaves at 9 dpi had a higher titre of both BYDV-PAV and CYDV-RPV viruses than old leaves, no such trend was observed in oats over the experimental period.

Lastly, this study investigated the performance of the generic primers in several multiplex situations using both RT-PCR and RT-qPCR-MCA. Multiplex RT-PCR using seven generic primers resulted in some non-specific amplification, which although of non-viral origin, significantly impacted on the use of such an assay. Contrary to RT-PCR, multiplex RT-qPCR was shown to be a good solution for detection and discrimination of BYDV-PAV and CYDV-RPV infection in a range of samples and has the potential to be used in diagnostics.

Using this work as a model, similar assays based on more versatile generic primers could be designed for other plant virus groups or other pathogens.

Keywords: ABI PRISM®, cDNA, degenerate primers, diagnostics, *Luteovirus*, plant virus detection, *Polerovirus*, virus taxonomy.

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Abbreviations

Système International abbreviations were used for chemicals, elements and formulae. Other abbreviations used in the text are listed below.

°C	degrees Celsius
$A_{260/280}$	absorptions at 260 nm over 280 nm
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CP	coat protein
C_q	quantification cycle
cv	cultivar
DSMZ	German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
HRM	high resolution melt(ing)
ICTV	International Committee on Taxonomy of Viruses
IDC	Investigation and Diagnostic Centre
MAF	Ministry of Agriculture and Forestry
MCA	melt(ing) curve analysis
MOPS	3-(N-morpholino) propanesulfonic acid
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
ORF	open reading frame
PCR	polymerase chain reaction

qPCR	quantitative real-time PCR
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
SD	standard deviation
SPC	synthetic positive control
ss	single stranded
TAS	triple antibody sandwich
T_m	melting temperature

Clarification of ambiguous terms used

Luteoviridae, Luteovirus, Polerovirus – refers to a specific taxonomic unit, i.e. family or genus (upper case and written in *italics*).

Luteovirus/luteoviruses – used as a synonym for *Luteoviridae* and refers to family members in general (lower case and not in *italics*).

Detection - refers to a specific PCR product (of reproducible size) on a gel, or a single melting peak (of reproducible shape and melting temperature), for which the taxonomic origin is yet to be confirmed (i.e. identified).

Identification – when the taxonomic origin of a detected PCR product or melting peak is confirmed (e.g. by sequencing or a specific melting profile).

Discrimination – two or more species can be discriminated from each other, if their T_m are significantly different from each other.

Universal primers – primers which amplify/detect all members in a given taxonomic group (e.g. family or genus).

Generic primers – primers which amplify/detect many/majority of the members in a given taxonomic group.

Broad-range primers - primers which amplify/detect some (not the majority) of the members in a given taxonomic group.

Preface

This thesis has four experimental chapters. Two of these chapters are based on two published papers. Copies of the published papers are attached at the back of the thesis.

1. **A generic RT-PCR assay for the detection of *Luteoviridae***. By A. Chomič, M.N. Pearson, G.R.G. Clover, K. Farreyrol, D. Saul, J.G. Hampton and K.F. Armstrong. Published in Plant Pathology, 2010, 59, 429-442. This publication was awarded the Best Student Paper Prize 2010, by the British Plant Pathology Society.

2. **Detection and discrimination of members of the family *Luteoviridae* by real-time PCR and SYBR® GreenER™ melting curve analysis**. By A. Chomič, L. Winder, K.F. Armstrong, M.N. Pearson and J.G. Hampton. Published in Journal of Virological Methods, 2010, 171, 46-52.

These are reproduced close to their published form in Chapters 2 and 3 respectively with minor editing and a reduced Introduction section. This thesis represents my work and the contribution of any supervisors and advisers to the research and to the thesis was consistent with normal supervisory practice. External contributions to the research are acknowledged as well as specified on the pages 139-140.

Chapter 1
General Introduction

They infect most of the world's major agricultural crops and can cause devastating diseases resulting in yield losses of up to 100%. They are found almost everywhere on every continent with the exception of Antarctica. They appear to be extremely elusive and often difficult to detect due to their habit of residing deep inside the vascular tissues of plants. They are extraordinarily resistant to deep-freezing at non-natural temperature (-80°C) and can survive intact in the body fluids of aphids. They appear to be too small and too simple to cause the serious damage that they do, as they are only approximately 25 nm in diameter (Figure 1.1) and have an extremely small genome. Nevertheless, they can cause major problems for farmers and crop production by leaving the plants dwarfed and/or with no fruit. They are difficult to control and rapidly escape to other plants, their spread being greatly facilitated by aphids.

They are the *Luteoviridae*!

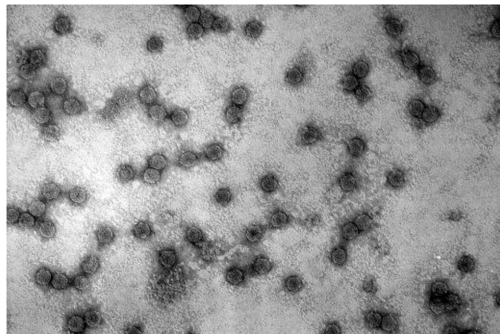


Figure 1.1 ***Soybean dwarf virus.***

Micrograph was taken and kindly provided by Dr J.W. Ashby (New Zealand Institute for Plant & Food Research Ltd, Lincoln, New Zealand).

1.1 Economic importance of *Luteoviridae*

The plant virus family *Luteoviridae* was summarised by Fauquet et al. (2005) as having 26 members (Table 1.1), but subsequently another nine new members have been discovered (Table 1.2). This number is likely to further increase in the near future because of increasing scrutiny by scientists, the demands of agricultural needs, and the availability of more sensitive detection technologies. All of the *Luteoviridae* members (luteoviruses) are of serious economic concern to global agriculture (Table 1.3). For example, *Turnip yellows virus* (TuYV) was reported to be responsible for 60-90 million pounds worth of damage for oil seed rape production in the UK (Stevens *et al.* 2008). The yield losses due to *Barley yellow dwarf* viruses (BYDVs) in Australia and New Zealand alone have an average annual value in excess of 70 million New Zealand Dollars (Johnstone 1995). Due to the spiralling increase of the world's population (1 billion in 1800 and more than 6 billion in 2011 (US Census Bureau 2011)) and the decline in growing areas suitable for staple agriculture, even a 5% crop yield loss would create a significant gap in the food supply. Luteoviruses target most of the important agricultural crops of the world: cereals (barley, oat, wheat, maize, rice, etc.), legumes, (chickpea, faba bean, groundnut, soybean, pea, etc.), potato (and sweet potato), various cucurbit crops (watermelon, melon, squash, zucchini, etc.), sugarcane and sugar beet, to name a few. These crops are the main source of proteins and carbohydrates to billions of people around the planet.

The problem of *Luteoviridae* infection became most obvious with the peak of modern agriculture, especially in the early 20th century when massive areas of land were dedicated for monoculture production. Before that, luteoviruses mostly resided in native wild plants, doing little damage to agricultural crops and the rare sporadic disease outbreaks affected relatively small areas (Oswald and Houston 1951). Crop monoculture created an excellent ecological environment for luteoviruses to inhabit, greatly facilitating their spread as neighbouring plants were much closer to each other than in the wild. Luteoviruses are also often found in mixed infections with each other and with other unrelated viruses, and these co-infections are known to cause the most devastating diseases such as groundnut rosette disease (Naidu *et al.* 1998), "virus yellows" disease of sugar beet and "yellow plaque" of cereals (Lister and Ranieri 1995).

Table 1.1 The taxonomy of the family *Luteoviridae* based on Fauquet *et al*. (2005).

Taxonomy within the family is determined by the arrangement of the genome. Unassigned species resemble *Luteoviridae* in their biological properties and coat protein gene sequences. * - species, assigned to *Luteovirus* and *Polerovirus* genera in 2009 (http://ictvonline.org/virusTaxonomy.asp?version=2009).

Nr	Species name	Abbreviation
	Genus *Luteovirus*	
1	*Barley yellow dwarf virus-MAV*	BYDV-MAV
2	*Barley yellow dwarf virus-PAV*	BYDV-PAV
3	*Barley yellow dwarf virus-PAS*	BYDV-PAS
4	*Bean leafroll virus*	BLRV
5	*Rose spring dwarf-associated virus**	RSDaV*
6	*Soybean dwarf virus*	SbDV
	Genus *Polerovirus*	
1	*Beet chlorosis virus*	BChV
2	*Beet mild yellowing virus*	BMYV
3	*Beet western yellows virus*	BWYV
4	*Carrot red leaf virus**	CtRLV*
5	*Cereal yellow dwarf virus-RPS*	CYDV-RPS
6	*Cereal yellow dwarf virus-RPV*	CYDV-RPV
7	*Chickpea chlorotic stunt virus**	CpCSV*
8	*Cucurbit aphid-borne yellow virus*	CABYV
9	*Melon aphid-borne yellows virus**	MABYV*
10	*Potato leafroll virus*	PLRV
11	*Sugarcane yellow leaf virus*	ScYLV
12	*Tobacco vein distorting virus**	TVDV*
13	*Turnip yellows virus*	TuYV
	Genus *Enamovirus*	
1	*Pea enation mosaic virus – 1*	PEMV-1
	Unassigned *Luteoviridae*	
1	*Barley yellow dwarf virus-GPV*	BYDV-GPV
2	*Barley yellow dwarf virus-RMV*	BYDV-RMV
3	*Barley yellow dwarf virus-SGV*	BYDV-SGV
4	*Chickpea stunt disease associated virus*	CpSDaV
5	*Groundnut rosette assistor virus*	GRAV
6	*Indonesian soybean dwarf virus*	ISDV
7	*Sweet potato leaf speckling virus*	SPLSV
8	*Tobacco necrotic dwarf virus*	TNDV
9	*Strawberry mild yellow edge associated virus*	SMYEaV

Table 1.2 Proposed new *Luteoviridae* members discovered and characterised since the last International Committee on Taxonomy of Viruses report in 2005.

*NCBI - National Center for Biotechnology Information

Proposed abbreviation	Proposed full name	Proposed Genera	Reference
BYDV-ORV	Barley yellow dwarf virus-ORV (oat red-leaf virus)	*Luteovirus*	(Robertson and French 2007)
CpYV	Chickpea yellows virus	*Polerovirus*	(Abraham *et al.* 2008)
CLRDV	Cotton leafroll dwarf virus	*Polerovirus*	(Corrêa *et al.* 2005)
LStV	Lentil stunt virus	*Polerovirus*	(Abraham *et al.* 2008)
PeVYV	Pepper vein yellows virus	*Polerovirus*	(Murakami *et al.* 2011)
PYLCV	Pepper yellow leaf curl virus	*Polerovirus*	(Dombrovsky *et al.* 2010)
PYV	Pepper yellows virus	*Polerovirus*	(NCBI^ Nr FN600344)
SABYV	Suakwa aphid-borne yellows virus	*Polerovirus*	(Shang *et al.* 2009)
WYDV-GPV	Wheat yellow dwarf virus-GPV	*Polerovirus*	(Zhang *et al.* 2009)

Various strategies have been implemented to control *Luteoviridae* infection (Robert and Lemaire 1999). These are mostly based either on (1) incorporating genetically modified resistance to the pathogens, which is often the most effective and cheapest method for control, for example the *Yd2* gene in barley or the *Bdv1* gene in wheat, (Burnett *et al.* 1995; Brown and Smith 2001; Makkouk and Kumari 2009) or (2) an integrated approach which includes combinations of changing sowing dates (to avoid the main migration times of aphid vectors) with the chemical or biological control of vectors (Plumb and Johnstone 1995). Where these viruses are endemic, control strategies rely on exclusion of the virus by selecting healthy plant material for propagation, using disease-resistant plants, or the use of insecticides to reduce aphid spread. Where they are absent, surveillance and quarantine activity are necessary to prevent the international movement of these viruses. Although each of these control measures can be effective in its own way, detecting the reservoir of the pathogen is the key prevention measure.

Table 1.3 Important agricultural crops infected by different *Luteoviridae* and the main regions of occurence.

* - West Asia and North Africa.

Crop	Major *Luteoviridae* affecting this crop	Countries/areas affected by this/these *Luteoviridae*	References
Canola (oil seed rape)	BWYV	Europe, WANA* countries	(Coutts *et al.* 2006; Makkouk and Kumari 2009)
Carrot	CtRLV	United Kingdom, New Zealand, USA, Belgium, Australia	(Vercruysse *et al.* 2000; Tang *et al.* 2009)
Cereals (barley, oat and wheat)	B/CYDV, WYDV-GPV	Worldwide	(Farrell and Sward 1989; Lister and Ranieri 1995; Makkouk and Kumari 2009)
Cotton	CLRDV	Brazil, several regions of Africa, Asia and Americas	(Corrêa *et al.* 2005)
Cucurbit crops (melon, watermelon, zucchini, squash, cucumber)	CABYV, MABYV, SABYV	Mediterranean basin, China, USA, Taiwan	(Xiang *et al.* 2008; Mnari-Hattab *et al.* 2009; Shang *et al.* 2009; Knierim *et al.* 2010)
Legumes (soybean, peanut, groundnut, faba bean, field pea, chickpea, lentil)	SbDV, PEMV-1, BLRV, CpCSV, CpSDaV ISDV, GRAV, CpYV, LStV.	WANA* countries, Japan, Kenya, Uganda, New Zealand, Madagascar,	(Fletcher 1993; Fortass *et al.* 1995; Wangai *et al.* 2001; Abraham *et al.* 2006; Naidu and Kimmins 2007; Kumari *et al.* 2008; Asaad *et al.* 2009; Makkouk and Kumari 2009)
Potato, sweet potato	PLRV, SPLSV	Worldwide	(Fletcher *et al.* 1996; Fuentes *et al.* 1996; Pooramini *et al.* 2010)
Sugar beet, beet, oil seed rape	BMYV, BChV, BWYV, TuYV	Europe, Australia, USA, Chile, Turkey	(Stevens *et al.* 2005; Stevens *et al.* 2008)
Sugarcane	ScYLV	North, South and Central America, Reunion, Brazil, Australia, South Africa, Mauritius, Thailand	(Schenck and Lehrer 2000; Moonan and Mirkov 2002; Lehrer *et al.* 2008)

1.2 Current status of *Luteoviridae* research

Luteoviruses are recognised as one of the most ecologically successful and economically important plant virus families (Harrison 1999). While the effects of luteovirus infection have been known for a few centuries (Harrison 1999), it was only in 1976 that these viruses became recognised as a group *per se* (Shepherd *et al.* 1975/1976). The need to classify these disease-causing agents arose at that time when their life cycle was finally understood. Since then, research on luteoviruses has constantly grown, with research papers dedicated to their detection being about 6-10% of those published on the family as a whole, especially in the last 20 years (Figure 1.2). In 1999 the book "*The Luteoviridae*" (Smith and Barker 1999) summarised recent research on this group and dedicated a whole chapter to reviewing the detection and diagnostic techniques. It was concluded that while there is a variety of such techniques available, selection of a more universal method instead of a pool of different ones would be advantageous.

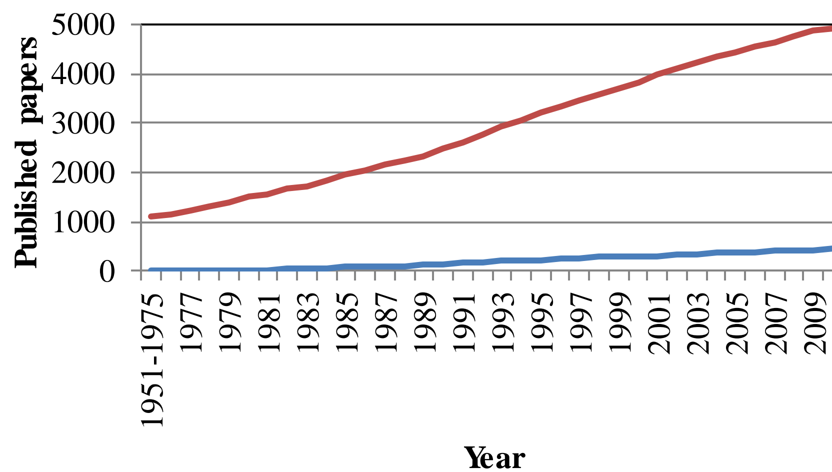


Figure 1.2 The overall number of published papers dedicated to luteovirus research in the period 1951-2010.

Red line - using the keyword "*Luteoviridae*", blue line - using the keywords "*Luteoviridae*" and "detection". Data sourced from NCBI database.

This variety of techniques available for detection and diagnosis of luteoviruses suggests the lack of an efficient approach to routine detection. As well as books and journals, conference abstracts appearing in recent years show the importance of developing more reliable detection and diagnostic methods: for example - "*Luteoviridae* viruses flying below the radar; new diagnostic protocols needed" (Loh *et al.* 2008).

1.3 Biosecurity and bioterrorism: the importance of good diagnostics

Plant biosecurity embraces measures designed to protect crops from plant pests and diseases, ensuring the safe and constant supply of food and feed which is a major indicator of a strong economy for any country. It also plays an important role in reliably ensuring the absence of pests and diseases in exported plant products. Plant biosecurity procedures are well developed in only a small number of countries (New Zealand, Australia, the United States (Magarey *et al.* 2009) and within the European Union (Gullino *et al.* 2008)). In other countries such as those within the WANA (West Asia and North Africa) region where cropping of luteovirus hosts is economically significant, they are still in their infancy (Makkouk and Kumari 2009), whereas in much of the rest of the world no biosecurity measures are implemented at all.

The plethora of new virus species discovered in recent years constantly creates a demand for more advanced detection and identification technologies. Improving diagnostics is the main focus of plant biosecurity at each point - pre-border, border and post-border (Makkouk and Kumari 2009; Rodoni 2009). The "First report" section in the "Plant Disease" journal constantly lengthens the list of countries in which *Luteoviridae* are found (Abraham *et al.* 2007; Thekkeveetil *et al.* 2007; Kundu 2008; Tornos *et al.* 2008; Tang *et al.* 2009). The "4T" rule (Gullino 2009) – Trade, Travel, Transportation and Tourism – all add to the spread of plant viruses. Re-discovery of a virus (rather than the recording of new incursions) often becomes the case as constantly improving diagnostic techniques reveal infection that has existed for years in a particular country (Zhu *et al.* 2010). In other cases, reports reflect introduction of completely new species to a region; i.e. species which had not been recorded before using the same technique. These reports represent a grave concern for a country's biosecurity, as a new pathogen could be costly to the economy of the country it has invaded (Kriticos *et al.* 2005; Murray and Brennan 2009a, 2009b; Rodoni 2009). Consequently, infection prevention measures become increasingly dependent upon reliable and rapid diagnostic protocols and/or simulation models (Davis 2008; Thackray *et al.* 2009) to be used as key strategies by biosecurity authorities to predict disease outbreak and implement appropriate control measures (Davis 2008).

In a related situation, bioterrorism (inducing outbreaks of pathogenic fungi or invasive insects) has more than 70% of Europeans considering this as a potential threat (Gullino 2009). Although plant virus "attacks" have not been recorded on a large scale, viruses transmitted by insects are convenient damaging agents, especially for developing countries which are more economically

vulnerable. Air-borne aphids, which transmit luteoviruses, are easy to disperse. Furthermore, with climate change being more apparent, aphid migration time and survival patterns might change (Stevens *et al.* 2008; Jones 2009; Thyer 2009; Trębicki *et al.* 2010). That would undoubtedly influence the dispersal and spread of luteoviruses. Reliable detection and identification tools are essential in order to control and hopefully avoid the spread of new pathogens (Jones 2009).

The importance of bioterrorism and biosecurity is highlighted by the appearance of specific journals (“Biosecurity and bioterrorism: biodefence strategy, practice, and science”, “Biosecurity” (published by the Ministry of Agriculture and Forestry (MAF) biosecurity New Zealand) and “Plant biosecurity – collaborative research initiatives” (published by CRC Plant Biosecurity Australia)) and conferences (“Bioterrorism and emerging infection conference”, 2009 in Fort Collins, USA, and “Global biosecurity conference”, 2010 in Brisbane, Australia). Indeed 35 fungal, nine bacterial and six viral species have been recognised as a potential threat to EU countries, targeting staple food (wheat and maize) as well as forest trees (Gullino 2009) that could have serious socio-economic consequences.

New Zealand, as a remote island surrounded by oceans, was for a long time fortunate to be isolated from the centres of plant-virus epidemics. The first luteovirus-aphid systems were introduced to New Zealand and Australia during colonisation by early British settlers in the late 18th and early 19th centuries (Lowe 1973). However, the situation has intensified drastically in the past few decades with increased travel of people and international exchange of plant breeding materials and tubers (seeds). For New Zealand, passive introductions have been reported with aphids arriving from Australia via jet-streams and their spread being facilitated by South-Easterly wind currents (B. Rodoni, pers. comm.). Among the more than 180 plant virus species recorded in New Zealand in 2006 (Pearson *et al.* 2006), there were eight *Luteoviridae* species (BYDV-PAV, BYDV-MAV, BWYV, CYDV-RMV, CYDV-RPV, PLRV, SbDV and TuYV). Others, such as PEMV-1 and SPLSV, are of importance to the Ministry of Agriculture and Forestry Biosecurity New Zealand (MAF New Zealand) and are included on New Zealand’s list of “Unwanted Organisms”. Unfortunately, most luteoviruses cause similar symptoms which complicate the visual differentiation of exotic species from local species that have no economic impact. To assist with management of those species already present in New Zealand and to detect and differentiate exotic species from those already present, a rapid and accurate means of identification is required.

1.4 Undiscovered plant virus species – the good and the bad

According to the International Committee on Taxonomy of Viruses, there were 2285 virus species described belonging to 87 families and 348 genera in 2009 (ICTV 2009). However, the official number of currently recognised virus species is a massive under-representation (Haenni and Mayo 2006; Roossinck 2011). This is becoming much more evident with recent studies involving next generation sequencing (NGS) which have revealed an enormous abundance of undiscovered virus species based on novel genomes. Information on the discovery of other viruses, both from the sea (Suttle 2005) and from the soil (Swanson *et al.* 2009), supports the view that host diversity parallels parasite diversity. Up to 80% of sequences of viral origin (dsRNA – double stranded ribonucleic acid) discovered by metagenomic studies in coastal waters and nucleotide sequences of sediments did not have any similarity with sequences in GenBank at all (Suttle 2005), suggesting extremely high virus genetic diversity.

Among the 2285 virus species described, plant viruses constitute just over 1000 species, belonging to 18 families and 81 genera. As technology advances, this number of plant virus species is constantly growing with new virus species discovered each year. The recent ecogenomic pyrosequencing study conducted by Roossinck et al. (2010) discovered several thousand unique plant viruses, most of which were only distantly related to known species. Among the sequences retrieved three showed a high similarity to *Luteoviridae* family nucleotide alignments and are thus likely to represent unknown luteovirus species. The most intriguing aspect of this study was that most of the plants selected for analysis were not showing any obvious symptoms and were possibly existing with their viruses in some sort of symbiotic relationships, most likely mutualistic (both organisms gain increased survivorship) (Roossinck 2008). Mutualistic viruses are found in insects (e.g. polydnaviruses of *Braconid* wasps) and are likely to exist in plants (Roossinck 2003). Other reports also support both the asymptomatic or symptomatic presence of pathogenic plant viruses in weeds and native plants (Stevens *et al.* 1994; Malmstrom and Shu 2004; Malmstrom *et al.* 2005a; Malmstrom *et al.* 2005b; Coutts *et al.* 2006; Srinivasan and Alvarez 2008; Asaad *et al.* 2009; Wylie and Jones 2010). These include BYDV, CYDV and *Cocksfoot mottle virus* (CoMV), which are the most widespread in native grasses in New Zealand (Davis and Guy 2001; Delmiglio 2008; Delmiglio *et al.* 2010). While native plants carrying the virus rarely exhibit any symptoms, they must be regarded as potential virus sources, because the efficiency of BYDV-PAV transmission from native plants via aphids to cereals has been shown to be similar to that between cereal species (Delmiglio 2008). This might be

compounded by climate change or other factors that stimulate the migration of other insect vectors from native plants to domesticated relatives grown in monoculture in near proximity. Availability of the detection and diagnosis tools for such "cryptic" plant viruses is needed to fully describe their biology and to control their spread in the future.

1.5 *Luteoviridae* detection techniques

Plants infected by luteoviruses are often stunted, their root formation, leaf initiation and elongation are inhibited, and their older leaves turn yellow or red (D'Arcy 1995). Although typical for some virus-host combinations, in nature the expression of these symptoms depends on many factors: symptoms can be very mild, or resemble those caused by other pathogens (e.g. yellows mycoplasma) or abiotic agents (such as nitrogen or phosphorus deficiency or moisture and/or temperature stress). Some host species, particularly non-cultivated hosts, may display no symptoms at all (D'Arcy 1995). Therefore, based on symptoms alone, it is often difficult to estimate whether infection has a viral origin and failure to diagnose this can lead to serious epidemiological consequences, disease outbreaks and yield losses (Shahraeen *et al.* 2003; Stevens *et al.* 2008). In order to more confidently identify infection, many different detection techniques have been employed; these include traditional biological, serological and novel molecular techniques (D'Arcy *et al.* 1999; Hull 2002).

1.5.1 Biological detection

Biological detection and diagnosis relies on knowing the aphid vector's specificity and symptomatology and was the first technique used for the luteoviruses (D'Arcy *et al.* 1999). The advantage of biological detection is its simplicity. The procedure involves three major steps (i) feeding non-viruliferous aphid species on suspected infected plants, (ii) moving aphids to a variety of healthy (indicator) plant species and (iii) observing symptoms 2-3 weeks later. Aphid vector specificity, plant species and symptoms are then used to identify the virus species/strain. However, biological techniques are no longer used for routine diagnosis, because they are time consuming, and labour- and glasshouse space–intensive (growing of plants and maintaining non-viruliferous aphid colonies in a strictly controlled environment). Another disadvantage of biological methods is that for some *Luteoviridae* species, (for example BYDV), symptom expressions often vary, depending on the plant host species, cultivar, age and physiological condition of the host, time of infection, strain and dosage of the virus as well as environmental conditions (D'Arcy 1995). Nevertheless, biological detection methods still remain a useful tool

for detecting new species or strains, which can remain undetected using other diagnostic tests developed around known viruses.

1.5.2 Serological techniques

Serological techniques provide much more specific information, are far more rapid ($\leq$ 2 days), and more sensitive than biological assays. Different ELISA versions (such as double antibody sandwich with polyclonal antibodies (DAS-ELISA with PAbs) or triple antibody sandwich with monoclonal antibodies (TAS-ELISA with MAbs) are still widely used. Other serological techniques, such as tissue-blot immunoassay (TBIA), serologically specific electron microscopy (SSEM), immunogold labelling techniques or antibodies from phage display libraries are far less popular although still used in some circumstances (D'Arcy *et al.* 1999). DAS-ELISA is useful in diagnosis of luteoviruses in both plant hosts and aphid vectors. However, PAbs often cross-react with other *Luteoviridae* species decreasing the overall accuracy of the assay. Use of monoclonal antibodies is the solution to compromised specificity, but they are not available for all luteoviruses, including many new unassigned species of this family, and are costly to produce (although development of a cheaper alternative - recombinant antibody - for PLRV detection has been recently reported (Al-Mrabeh *et al.* 2009)). Furthermore, serological detection often relies on high virus titre in symptomatic plants but the *Luteoviridae* titre in plants is usually low. Three possible reasons for this low virus titre in plants have been proposed. First is the inability of the virus to spread to plant tissues beyond the phloem (Peter *et al.* 2009). Second is the plant's defence system (based on RNA silencing) which efficiently destroys viral nucleic acids (Soosaar *et al.* 2005; Voinnet 2005; Vaucheret 2006), although several reports on both *Polerovirus* and *Luteovirus* strategies to combat plant defence RNA silencing pathways have recently been described (Soosaar *et al.* 2005; Voinnet 2005; Levy *et al.* 2008; Maclean *et al.* 2010; Waterhouse *et al.* 2010). Third is the problem associated with the difficulty of releasing the virus antigen from the juice of an infected plant (van Slogteren 1955). Nevertheless, whatever the reason, serological methods are often not sensitive enough to detect early infection in apparently asymptomatic plants. Overall, biological and serological methods are not effective for routine diagnosis of luteoviruses.

1.5.3 Standard nucleic acid techniques

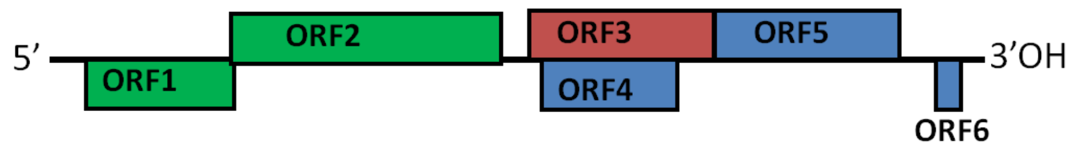
Nucleic acid techniques have revolutionised detection and diagnostic practices, not only for luteoviruses but for other plant viruses and plant pathogens as well, especially in the last decade

(Elnifro *et al.* 2000; James *et al.* 2006; Vincelli and Tisserat 2008). Being very rapid, extremely sensitive and cost-effective, nucleic acid techniques have become mainstream in most diagnostic laboratories. Reverse transcription polymerase chain reaction (RT-PCR) has become an obvious leader in detection and diagnosis of *Luteoviridae*, largely replacing the previously used serological detection.

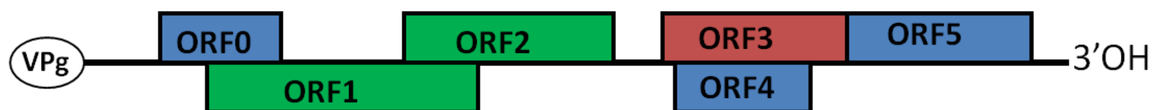
All luteoviruses have a single stranded (ss) positive sense RNA genome of about 6 kbp (Miller *et al.* 1997). For detection and diagnosis, isolation of high quality RNA is a crucial step. This is gradually becoming less problematic as more laboratories shift to robotic high-throughput nucleic acid extraction machines and commercial RNA extraction kits, although many manual RNA extraction protocols have been optimised for different species. RNA is then reverse transcribed into cDNA through the process of reverse transcription where cDNA is synthesised using random hexa-oligonucleotides or gene-specific primers. RT-PCR still requires expensive reagents and equipment, plus a contamination-free environment and skilled labour. However, the ease of final analysis and tremendous sensitivity of this assay unambiguously justify its cost in many cases.

The key to the RT-PCR process is availability of suitable primers. There are many species-specific primers available for the amplification of various luteovirus genes used in both monoplex (Chapter 2) and multiplex RT-PCR either with other luteovirus species (Hauser *et al.* 2000; Malmstrom and Shu 2004; Viganó and Stevens 2007; Deb and Anderson 2008) or other plus sense ssRNA viruses, targeting the same plant host (Peiman and Xie 2006; Viswanathan *et al.* 2009). In addition to detecting virus in plants, RT-PCR has also been used to detect viruses in individual aphid vectors (Canning *et al.* 1996; Naidu *et al.* 1998; Ortiz *et al.* 2005; He *et al.* 2006; Liu *et al.* 2006). The most targeted gene for detection and identification of luteoviruses is the coat protein gene (open reading frame 3 (ORF3)). It is about 600 nt long and is known to be the most conserved region in the *Luteoviridae* genome (probably because ORF3 also serves as a template for the overlapping movement protein gene in the different open reading frame 4), followed by the RNA-dependent RNA polymerase gene (RdRp, ORF1 + ORF2) (Figure 1.3). However, pairwise comparison of CP amino acid sequences between the three *Luteoviridae* genera showed that, whilst they are at least 60% identical among species within the *Luteovirus* and *Polerovirus* genera, they are less than 50% identical between pairs of viruses across these two genera and less than 40% between either genera and PEMV-1, the only member of the *Enamovirus* genus (Mayo and D'Arcy 1999).

Luteovirus



Polerovirus



Enamovirus

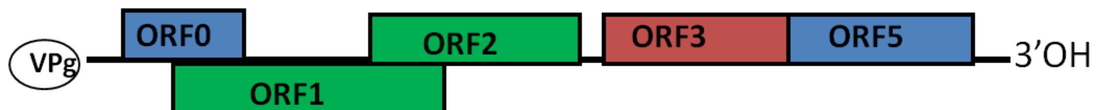


Figure 1.3 Schematic view of the genome organisation of three *Luteoviridae* genera: *Luteovirus*, *Polerovirus* and *Enamovirus*.

VPg – genome-linked protein.

However, species-specific detection is not often practical in diagnostic laboratories when multiple RT-PCR reactions are required for each plant sample to be checked for possible infection with different luteoviruses. Generic primers that enable several virus species to be amplified with the same reaction would be of great advantage; these have been developed for many other plant virus groups (reviewed in James et al. (2006)), but not for luteoviruses. The development of generic primers for *Luteoviridae* has been constrained for a very long time by the lack of appropriate gene sequence information and the huge variation of these genes (including the coat protein gene) between and within species/strains. Robertson et al. (1991) pioneered this field by designing broad-range primers based on the extremely limited sequence information that was available at the time - three nucleotide sequences of three luteovirus species.

These primers were a significant breakthrough, greatly facilitating the detection and diagnosis of up to seven species (two species in the *Luteovirus* genus (BYDV-MAV and BYDV-PAV), three

species in the *Polerovirus* genus (BWYV, CYDV-RPV and PLRV) and two unassigned species (BYDV-RMV and BYDV-SGV)), and are still widely used today. Robertson's primers are short: the forward Lu 1 primer is a 15-mer with one degenerate base position and Lu 4 is a 14-mer. They produce amplicons of similar lengths for the seven species and therefore sequence-based identification of species is required after gel electrophoresis. Two important downsides of Robertson's primers are the low annealing temperature (41°C) which sometimes results in non-specific amplification, and variable performance among different virus isolates (this study; Malmstrom and Shu (2004)).

Other potentially broad-range/generic primers were designed by Naidu *et al.* (1997), followed by Corrêa *et al.* (2005). The aim of these studies was not to create generic primers *per se*, but rather to detect the few uncharacterised *Luteoviridae* species that could not be detected using the primers designed by Robertson et al. (1991). Although these new primers were designed based on new sequence data, and were likely to be generic or broad-range, no taxonomic range studies have been undertaken with them. These primers were not considered for the current study because they were less likely to represent current nucleotide diversity of *Luteoviridae* sequences: the primers of Corrêa *et al.* (2005) were designed by using a limited number of sequences (19) and those of Naidu *et al.* (1997) were designed based on nucleotide sequences in GenBank at that time. Other advances were also made by Malmstrom and Shu (2004) and Hauser *et al.* (2000) by designing group specific primers for B/CYDV and beet/rape poleroviruses, respectively. Despite continuous research increasing our knowledge of all species, no generic *Luteoviridae* primers have been developed following these studies.

Agarose gel electrophoresis and sequencing is typically required to confirm the size of the amplification product, as an indication of the correct amplicon and virus identity. Sequencing provides most useful information (precise nucleotide sequence and size), and the high throughput capabilities of this technique makes it invaluable in ecogenomic studies (Roossinck *et al.* 2010). In addition sequencing chromatograms can be used to quantitatively distinguish between two highly similar BYDV species (PAV and PAS) in mixed infections using the PERL script on PHERD software (Hall and Little 2007). Nevertheless, even with recent advances in sequencing reagents and equipment greatly reducing the cost of the procedure, the practice of running a gel, preparing a sequencing reaction and resolving the sequencing products is still very time-consuming in a routine diagnostic situation. Furthermore, for many laboratories sequencing is

still a prohibitive option, especially in developing countries, as either specific very expensive equipment is needed on site to minimise the time for the analysis or sending samples away to sequence-service providers is too slow for routine procedures.

1.6 Alternative nucleic acid detection and identification methods

Real-time RT-PCR (RT-qPCR) is another detection method deployed for *Luteoviridae* which has become more popular in the last decade due to the more affordable equipment and reduced cost of reagents. Although this technique relies on a high quality nucleic acid template and can be affected by tissue-specific contaminants (Tichopad *et al.* 2004), it is very robust, rapid, sensitive and also provides the data in real time and therefore no gel electrophoresis is required. In qPCR, detection of the amplicon is enabled via the use of oligonucleotide probes (such as TaqMan®) or fluorescent dyes (such as SYBR® Green I), and the amount of amplicon present after each cycle can be estimated from emitted fluorescence, which is in proportion to amplicon concentration. Fractional PCR cycle, known as C_q (quantification cycle) is used for quantification, which is a cycle number at which amplified product accumulates to yield a detectable fluorescent signal. C_q value is estimated during the exponential phase of an amplification, when reagents are still abundant. Therefore the initial amount of template present in the real-time reaction can be estimated (Wilhelm and Pingoud 2003). RT-qPCR has been already been applied for detection of PLRV together with other potato viruses (Agindotan *et al.* 2007; Mortimer-Jones *et al.* 2009), BYDV-PAV and CYDV-RPV (Balaji *et al.* 2003), CYDV-RPV (Nancarrow *et al.* 2010), ScYLV (Korimbocus *et al.* 2002) in plants as well as BYDV-PAV in aphids (Fabre *et al.* 2003). RT-qPCR has been shown to be able to detect 200-400 copies of PLRV RNA in potato sap (Agindotan *et al.* 2007) and was reported as being 100 times more sensitive than conventional RT-PCR for detection of ScYLV in sugarcane leaves (Korimbocus *et al.* 2002).

A possible alternative to sequencing, which has been developed as an extension to RT-qPCR is melt(ing) curve analysis (MCA). MCA is based on the dissociation characteristics of dsDNA which in turn is dependent on the length, GC% content and complementarity of the base–base hydrogen bonds of the DNA. For MCA the amplification product is generated via RT-qPCR in the presence of a fluorescent dye. Fluorescence of the dye greatly increases when it intercalates non-specifically inside the minor groove of the dsDNA helix (Ririe *et al.* 1997). These alterations in fluorescence of free and DNA-bound fluorescence dye (e.g. SYBR Green I) are probably dependent on the structure and different interaction modes of a dye with dsDNA and ssDNA and

are explored in more details in Zipper *et al.* (2004). After 30-40 cycles of RT-qPCR a melting step is incorporated by gradually increasing the temperature from about 50°C to 95°C. This results in separation of dsDNA strands of the amplicon and release of the dye which becomes less fluorescent. These changes in fluorescence can also be viewed as a derivative curve (showing the melt peak), which summarises the "melting profile" of a particular amplicon: change in the fluorescence over the temperature range ($-\Delta F/\Delta T$) is plotted on a vertical axis and temperature on the horizontal axis. The summit of the melt peak/peaks shows the melting temperature (T_m), at which the change in fluorescence is the greatest. T_m is sequence specific and mainly depends on the length of the product and its GC% content. Sequences with a greater GC% content melt at higher temperatures. This is because more GC duplexes in a particular amplicon mean that complimentary DNA strands are held together by more hydrogen bonds and more energy is needed to disrupt them (the GC duplex has three hydrogen bonds, whereas the AT has only two). Different sequences can be identified according to their specific T_m and/or melting profile thus eliminating the need for gel electrophoresis and sequencing.

Although MCA does not provide the full information on an analysed sequence, this technique is essentially sequence-dependent and has the advantage of requiring much less time, being considerably cheaper than sequencing and can be performed on most real-time PCR equipment (Herrmann *et al.* 2006) available in many laboratories. MCA can be performed using common types of fluorescent dyes, such as SYBR Green and LCGreen and standard qPCR equipment (Herrmann *et al.* 2006). MCA is the dominant technique in medical diagnostics but is beginning to be used in other fields as well. MCA has successfully been applied to detect and differentiate among *Leishmania* (Nicolas *et al.* 2002a; Nicolas *et al.* 2002b), and *Chlamydiaceae* species (Robertson *et al.* 2009) and differentiate among animal viruses (Mouillesseaux *et al.* 2003) and single stranded RNA plant viruses such as *Plum pox virus* strains (Varga and James 2005, 2006) and *Citrus tristeza virus* isolates (Ruiz-Ruiz *et al.* 2007). RT-qPCR coupled with MCA was also used to detected asymptomatic BYDV-PAV and CYDV-RPV infection, using species-specific primers (Balaji *et al.* 2003), which demonstrates the sensitivity of this method for luteoviruses.

The sensitivity of most real time instruments makes MCA ideal for differentiation of more distantly related species or groups of species which display relatively high variability in sequence (such as RNA viruses). If minor sequence changes (such as single nucleotide polymorphism (SNP)) need to be reliably detected there are also high precision instruments available on the

market, designed specifically for the high precision melting step. This more sensitive version of MCA is called high resolution melt(ing) (HRM) and is often used for discriminating different alleles (Graham *et al.* 2005), SNP (Liew *et al.* 2004) or for mutation scanning (Zhou *et al.* 2005; Krypuy *et al.* 2007); correspondingly the equipment is much more expensive. For discrimination among more distantly related sequences, MCA sensitivity is often sufficient. Furthermore, the experimental procedure for most qPCR equipment is greatly facilitated by commercial mixtures containing all reagents at optimised concentrations, except for primers and the template. The cost per reaction using these mixtures is often comparable with conventional RT-PCR.

1.7 Improved detection and identification turns a new leaf in viral ecology

New detection and identification methods are also essential for understanding virus biology and relationships between co-infecting viruses, virus-vector and virus-host. Viruses are often viewed as harmful parasitic agents, but this view is strongly biased towards economic impacts (Wren *et al.* 2006). The view that plant viruses were entirely harmful has essentially arisen through the ecological niches of viruses being greatly expanded by human activity in the last decade. The resulting fields of monoculture crops (concentrated population of artificially bred plants with nearly identical genotypes) represent an unusual and unexplored niche for viruses and provide the opportunity "to go wild" (Jones 2009; Gibbs *et al.* 2010). In nature, plants are rarely found in monoculture (Roossinck 2003) and there is no such thing as an empty ecological niche. The species most able to adapt to any new niche as it arises will eventually occupy it, as it results in increased species variation and therefore increases the chances of survival. Plant viruses have become the big winners of this human-created lottery, along with other species which humans consider as pathogens: bacteria, phytoplasmas, fungi, nematodes and insects.

However, in recent years new evidence has emerged which emphasizes the significant role of plant viruses in native ecosystems (Roossinck 2005; Márquez *et al.* 2007; Xu *et al.* 2008). Virus ecology is a new field, which arose in response to an appreciation of viruses as "must have members" of any ecosystem, in which they usually do no significant harm, either to the host, or the vectors and may exist as beneficials. Research in virus ecology has revealed ancient evolutionary co-adaptations existing between viruses and their surroundings (Hurst and Lindquist 2000). There are multiple reports revealing plant viruses being crucial symbionts needed for surviving extreme conditions such as drought, high salinity or ice encasement (Haber 1995;

Roossinck 2005; Márquez *et al.* 2007; Xu *et al.* 2008) as well as providing an additional source of variation for plants (Melcher 2010). Moreover, according to Roossinck (2005) "symbiotic relationships are widely recognised as being responsible for major evolutionary leaps (such as acquisition of mitochondria by eukaryotic cells) and must therefore be abundant in nature".

Detection and identification are the key aspects of getting to know plant viruses, exploring their role in the ecosystem, and their evolution, biology and pathogenicity. By embracing this new information on viruses, it might be eventually possible to utilise the beneficial aspects of the host-virus relationship and grow crops in extreme environments (which are very likely to occur as a response to the changing climate) or use viruses (such as from the genus *Mycovirus*) to control fungal plant pathogens (Pearson *et al.* 2010). Maybe, one day plant viruses will be regarded as friends, helping humans to survive, rather than being treated as one of the biggest foes.

1.8 Conclusions

Positive sense ssRNA plant viruses, belonging to the *Luteoviridae* family, threaten the production of many economically important crops. To reduce the risk of introduction of exotic species in New Zealand and elsewhere, early detection and accurate diagnosis is essential. However, current detection and identification methods of the *Luteoviridae* are not optimal, mainly due to the species-specific approach of most techniques which is no longer desirable for routine sample screening. There are some new approaches that could be explored, including the development of generic nucleic acid based assays with the potential to detect unknown virus isolates. Alternative methods to sequencing, such as the use of MCA may help to streamline the diagnostic process and be more rapid. A new generic nucleic acid based assay, if developed, would significantly improve current diagnostic capabilities of biosecurity authorities as well as contribute to ecological studies and the discovery of new virus species.

1.9 Hypotheses (H) arising from the literature

<u>H1</u> Based on the successful design of generic primers for other plant virus families, and new nucleotide sequence information having become available for *Luteoviridae* in recent years, new conserved gene regions for design of generic primers can be identified.

<u>H2</u> Based on the successful application of melting curve analysis (MCA) for detection and discrimination of other pathogens, a RT-qPCR MCA using generic primers is sensitive and

accurate enough for the detection and discrimination of *Luteoviridae* species for diagnostic purposes.

H3 Given the greater sensitivity of RT-qPCR and its quantitative advantage over the RT-PCR as well as its successful application for detection of other luteoviruses, RT-qPCR is a suitable method of detecting low titres of *Luteoviridae* infection using generic primers.

1.10 Aim of this research

The aim of this study is to address the hypotheses presented in section 1.9 by exploring the development and application of generic detection and identification tools for the *Luteoviridae*.

1.11 Overview of the study

Four experimental chapters (Chapters 2, 3, 4 and 5) in this thesis describe the research conducted to achieve the above aim:

The hypothesis that new sequence information on *Luteoviridae* available since 1991 would enable identification of new and more suitable conserved regions for the design of generic primers is investigated in Chapter 2. This is addressed by analysing luteovirus nucleotide sequences available in NCBI database since 1991, designing new primers and testing various combinations for their efficacy in RT-PCR using 16 *Luteoviridae* species. Specificity of the assay is investigated by analysing two *Sobemovirus* outgroup species, which share the same hosts with luteoviruses and also have the highest CP sequence identity. A new synthetic positive control containing sequences of these new primers, which was designed to simplify the assessment of the primer performance during the RT-PCR is also described.

Application of a new technology, - MCA - as a time- and cost-efficient alternative for the detection and differentiation of the *Luteoviridae* is explored in Chapter 3. Three primer combinations are compared for their performance and suitability to detect and differentiate among 13 species. Some of the factors which might influence the performance and challenges of this assay are also considered.

In Chapter 4 the MCA method developed to address three factors that could impact on its use is applied. Namely to, (i) test the sensitivity of the assay, especially with respect to low titres of luteovirus infection, (ii) test reliability as it might be influenced by the multiplexing for

diagnosing common occurrence of multi-viral infections and (iii) determine if the age of the plant tissue sample is a major factor for the use of this method.

The multiplexing capabilities of generic luteovirus primers are then examined in Chapter 5 using RT-PCR and RT-qPCR. A simplified multiplex using two separate combinations of generic primers together with plant-specific primers is also investigated, as it could help to reduce the time and the cost, associated with additional testing of samples for success of the reverse transcription reaction.

The proposed detection and identification assays, as well as limitations of this study and future directions are further discussed and evaluated in Chapter 6.

Chapter 2

A generic RT-PCR assay for the detection of *Luteoviridae*

2.1 Introduction

A generic RT-PCR assay that would facilitate the detection and identification of the majority of *Luteoviridae* would make testing more efficient and cost effective. However, to date no such method has been published. This, as outlined in Section 1.5.3, is mainly because of the lack of sequence information of luteovirus genes/genomes and the huge variation of these genes/genomes amongst species.

Almost 600 luteovirus CP nucleotide sequences have been published as at 1st of January 2008. The aim of the research reported in this chapter is to investigate the hypothesis that this new sequence information would enable identification of new conserved regions for the design of generic primers for the members of *Luteoviridae*. The development of such primers for use in a new generic RT-PCR-based detection method is assessed here using 16 luteovirus species and two species in the genus *Sobemovirus* (a genus not assigned to any family) as an outgroup. Members of the genus *Sobemovirus* (sobemoviruses) share some hosts with luteoviruses and also have the highest CP sequence identity, compared to other unassigned genera and families, although it is still limited; for example, the CPs of PLRV and the sobemovirus *Rice yellow mottle virus* (RYMV) share 33% similarity (Terradot *et al.* 2001). Streamlining the diagnostic process with a common positive control and improving inter-laboratory comparisons would be advantageous. Therefore the design of a synthetic positive control (SPC) consisting of a plasmid containing the novel primer sequences is also described. The merits of the new assay, including the use of the SPC, are discussed.

2.2 Materials and methods

2.2.1 *Luteoviridae* and *Sobemovirus* isolates

Forty-eight viral isolates were used during this study (Table 2.1). Forty-five were isolates of 16 luteovirus species from the three genera plus unassigned viruses in the family. Three *Sobemovirus* isolates (two species: CoMV and RYMV) were used as an outgroup.

Table 2.1 List of 45 *Luteoviridae* and three *Sobemovirus* isolates used in this study.

SA – South Africa, IDC - Investigation and Diagnostic Centre. Q – RNeasy Plant Mini Kit (Qiagen), mI – modified protocol using TRIzol reagent (Invitrogen), TS – Thermo Kingfisher mL (Thermo Scientific), C - the method described by Célix *et al*. (1996), NCBI – National Center for Biotechnology Information.

	Virus	Name of isolate	Origin of isolates	Plant species	NCBI Accession Nr	Suppliers	RNA extraction method
	Genus *Luteovirus*						
1	BYDV-MAV	O1LU	NZ	*Avena sativa*	GO002360	A. Chomič	Q
2		WC5	NZ	*Triticum* sp.	GU002322	C. Delmiglio	Q
3		OA1	NZ	*Avena sativa*	GU002361	C. Delmiglio	Q
4	BYDV-PAS	DC1	NZ	*Triticum* sp.	GU002323	C. Delmiglio	Q
5		DC2	NZ	*Triticum* sp.	GU002324	C. Delmiglio	Q
6	BYDV-PAV	OA4	NZ	*Avena sativa*	GU002328	C. Delmiglio	Q
7		O2LU	NZ	*Avena sativa*	GU002326	A. Chomič	Q
8		Wh14	NZ	*Microlaena stipoides*	EF408162	C. Delmiglio	Q
9		327	NZ	*Avena sativa*	GU002325	J. Fletcher	Q
10		O3LU	NZ	*Avena sativa*	GU002327	A. Chomič.	Q
11		WC2	NZ	*Triticum* sp.	GU002330	C. Delmiglio	Q
12		PC3	NZ	*Poa cita*	GU002329	C. Delmiglio	Q
13	BLRV	DSMZ	Germany	*Pisum sativum*	GU002353	DSMZ	Q
14		AUS	Australia	*Vicia faba*	GQ906583	M. H. Loh	TS
15	SbDV	BB1	NZ	*Vicia faba*	GU002346	A. Chomič	Q
16		BB2	NZ	*Vicia faba*	GU002347	A. Chomič	Q
17		400	NZ	*Vicia faba*	GU002345	J. Fletcher	Q
	Genus *Polerovirus*						
18	ScYLV	MA3	Mauritius	*Saccharum* sp.	GU002349	S. Saumtally	Q
19		MA5	Mauritius	*Saccharum* sp.	GU002350	S. Saumtally	Q
20		SA	SA	*Saccharum officinarum*	GU002348	T. van Antwerpen	Q
21	BChV	2a	France	*Beta vulgaris*	AF167475	O. Lemaire	Q
22		MS	UK	*Beta vulgaris*	GU002352	M. Stevens	Q
23	BMYV	2ITB	France	*Beta vulgaris*	X83110	O. Lemaire	Q
24		MS	UK	*Beta vulgaris*	GU002354	M. Stevens	Q
25	BWYV	3	NZ	Not known	GU002355	IDC MAF	Q
26		4	NZ	*Pisum sativum*	GU002356	IDC MAF	Q
27		FB3	NZ	*Brassica napobrasica*	GU002357	A. Chomič	ml
28		FB4	NZ	*Brassica napobrasica*	GU002358	A. Chomič	ml
29		JF6	NZ	*Brassica napus*	GU002359	J. Fletcher	Q
30	CYDV-RPV	Kin3	NZ	*Dactylis glomerata*	EF408186	C. Delmiglio	Q
31		RL1	NZ	*Avena sativa*	GU002338	R. Lister	Q

	Virus	Name of isolate	Origin of isolates	Plant species	NCBI Accession Nr	Suppliers	RNA extraction method
32	CABYV	USA	USA	Not known	GU002335	W. Wintermantel	Q
33		ES1	Spain	*Cucumis melo*	GU002331	M. Aranda	C
34		ES2	Spain	*Cucumis melo*	GU002332	M. Aranda	C
35		ES3	Spain	*Cucumis melo*	GU002333	M. Aranda	C
36		ES4	Spain	*Cucumis melo*	GU002334	M. Aranda	C
37	PLRV	DSMZ	Germany	*Solanum tuberosum*	GU002342	DSMZ	TS
38		JF	NZ	*Solanum tuberosum*	GU002343	J. Fletcher	Q
39		5	NZ	Not known	GU002341	IDC MAF	Q
40	TuYV	FL1	France	*Lactuca sativa*	X13063	O. Lemaire	Q
41		MS	UK	*Brassica napus.*	GU002351	M. Stevens	Q
	Genus *Enamovirus*						
42	PEMV-1	DSMZ	Germany	*Pisum sativum*	GU002339	DSMZ	Q
43		USDA	USA	*Pisum sativum*	GU002340	R. Larsen	Q
	Unassigned *Luteoviridae*						
44	CtRLV	2	NZ	*Daucus carota*	GU002337	IDC MAF	Q
45	SPLSV	SF	Peru	*Ipomoea setosa*	DQ655700	S. Fuentes	Q
	Genus *Sobemovirus*						
46	CoMV	SI	NZ	*Dactylis glomerata*	EF422395	C. Delmiglio	Q
47		DSMZ	Germany	*Dactylis glomerata*	GU002336	DSMZ	Q
48	RYMV	DSMZ	Germany	*Oryza sativa*	GU002344	DSMZ	Q

They included *Luteoviridae* species not occurring in New Zealand (BChV, BLRV, BMYV, CABYV, CtRLV, PEMV-1, ScYLV and SPLSV (Pearson *et al.* 2006)), as well as TuYV, which has been recorded in New Zealand but not found among field-scored isolates during this study, plus some additional isolates which were imported or provided by IDC MAF. All overseas isolates were obtained as a positive reference material and the sourcing, as well as the initial identification of all samples was performed by other scientists (see Table 2.1) prior to shipping to New Zealand. For most species at least two isolates were tested, but only single isolates of CtRLV, RYMV and SPLSV could be obtained. New Zealand isolates were stored in a dry form over $CaCl_2$ at 4°C. All overseas isolates were stored as purified RNA in water at −80°C.

2.2.2 Generic *Luteoviridae* primer design

In order to design generic primers, a preliminary alignment of all available luteovirus nucleotide sequences on the NCBI database was carried out in 2003 by D.Saul and K.Farreyrol using Vector

NTI software (both complete genomes and CPs only). Sequences were too variable to produce a confident alignment for most, although the CP gene (ORF3) was the most easily aligned with conserved regions that looked suitable for primer design. At that time all available CP *Luteoviridae* amino acid sequences (more than 300) were then compared by phylogenetic analysis using trees constructed with PAUP (Swofford 1998); duplicate sequences were eliminated, leaving 107 distinct sequences, including 19 species: four species from the genus *Luteovirus*: (BYDV-MAV, BYDV-PAV, BLRV and SbDV), eight species from the genus *Polerovirus* (BChV, BMYV, BWYV, CYDV-RPV, CABYV, ScYLV, TuYV and PLRV), the only species from the genus *Enamovirus* (PEMV-1) and six species from the unassigned *Luteoviridae* (BYDV-GPV, BYDV-RMV, BYDV-SGV, CpSDaV, GRAV and TVDV). Two highly conserved regions were found. The first one, corresponding to 3086–3105 and 3847–3863 bp on BYDV-PAV (D01214) and PLRV (X74789), respectively (Table 2.2), was identified across 107 sequences. Another highly conserved region, corresponding to 3218–3241 and 3976–3994 bp on BYDV-PAV (D01214) and PLRV (X74789), respectively (Table 2.2), was common to *Polerovirus*, *Luteovirus* and the unassigned *Luteoviridae* species, but not *Enamovirus*.

Table 2.2 The sequences, position and the length of generic *Luteoviridae* primers used during this study.

	Name	Orientation	5' Sequence 3'	Position in genome	Length (nt)
C1 set				BYDV-PAV (D01214)	
	C1F1	Forward	GGG GTM MTC AAA TTC GGK CC	3086-3105	20
	C1F2	Forward	TCG CAA TGY CCA GCR CTT TCA G	3113-3134	22
	C1R1	Reverse	GAG TTC AAT AAA KAT WGC GCC	3218-3238	21
	C1R2	Reverse	GTC GAG TTC AAT AAA GAK WGC GCC	3218-3241	24
	C1R3	Reverse	TGG TAG GAC TTR AGT AYT C	3138-3156	19
C2 set				PLRV (X74789)	
	C2F1	Forward	TCA CKT TCG GGC CGA GT	3847-3863	17
	C2F2	Forward	TCA CKT TCG GGC CGT CT	3847-3863	17
	C2R1+2	Reverse	TCM AGY TCG TAA GCG ATK G	3976-3994	19
	C2R3	Reverse	YTC ATG GTA GGC CTT GAG	3897-3914	18

Two sets of degenerate primers, designated C1 (five primers) and C2 (four primers) (Table 2.2), were designed to accommodate the CP sequence diversity (Figure 2.1).

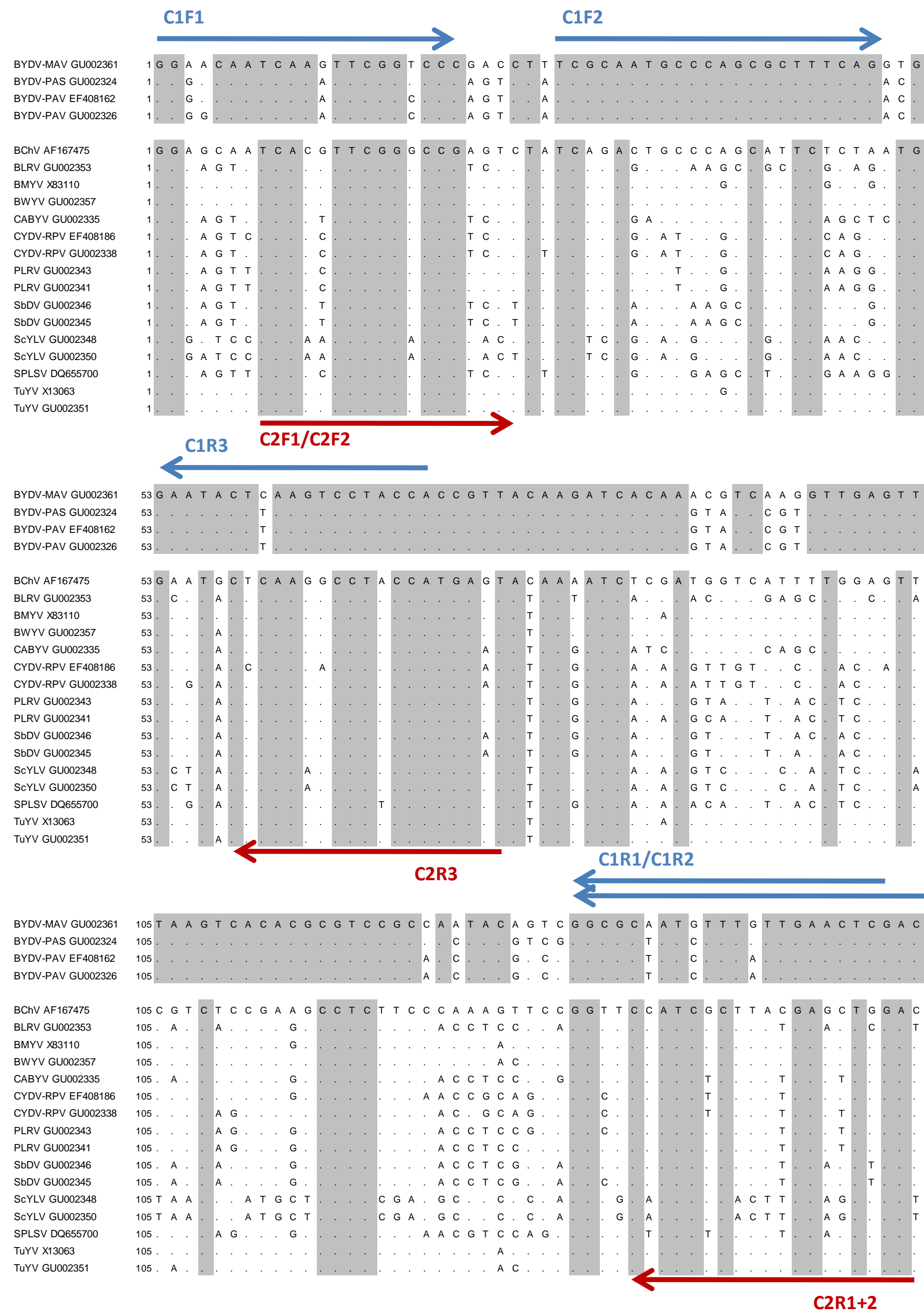


Figure 2.1 Alignment of 156 bp region of the *Luteoviridae* coat protein gene (ORF3) nucleotide sequence and location of the generic primer annealing regions.

C1 set is shown in blue, C2 set is shown in red. Conserved parts for each subalignment are shaded in grey, as performed by MEGA 4 (Tamura *et al.* 2007).

The C1F1, C1F2, C1R1 and C1R2 primers were designed using a total of 26 CP sequences of two *Luteovirus* species (BYDV-PAV (21 sequences) and BYDV-MAV (three sequences)) and one unassigned species (BYDV-SGV (two sequences)). The C2F1, C2F2 and C2R1+2 primers were designed based on 73 CP sequences for two *Luteovirus* species (SbDV (13 sequences) and BLRV (two sequences)), seven *Polerovirus* species (BChV (two sequences), BMYV (five sequences), BWYV (20 sequences), CABYV (one sequence), CYDV-RPV (two sequences), PLRV (17) and TuYV (one sequence)) and five unassigned species (BYDV-GPV (one sequence), BYDV-RMV (one sequence), CpSDaV (one sequence), GRAV (five sequences) and TVDV (two sequences)). All these 7 primers were designed and were subsequently evaluated on three luteovirus samples – BYDV-PAV, BWYV and PLRV in 2003 by D.Saul and K.Farreyrol (unpublished). In order to estimate the best primer annealing temperature, they investigated the temperature gradient of 45-55°C. The methods used and the preliminary results were not published and are available only as an internal MAFBNZ report. It was reported that successful detection and identification of all three species was achieved using these primers. Also, nad5-as/as primers were shown to work under the same PCR protocol as luteovirus generic primers. This report by D.Saul and K.Farreyrol concluded that PCR primers and the PCR protocol developed appear to be suitable for routine detection purposes and encouraged further investigation on the taxonomic range of these primers to be undertaken.

Other two primers - C1R3 and C2R3 - were designed during the current study by A.Chomič (see below). Since the 2003 study, the number of CP sequences available in the NCBI database had increased from around 300 to 597 sequences as of 1st of January 2008. Subsequent inclusion of these additional sequences in the alignment analysis indicated the presence of a further conserved region corresponding to 3138–3156 and 3897–3914 bp on BYDV-PAV (D01214) and PLRV (X74789), respectively (Table 2.2). Two additional reverse primers (C1R3 for the C1 set and C2R3 for the C2 set) were then designed.

Possible homo- and heterodimer formation was assessed for each primer by estimating thermodynamic parameters using the Vector NTI software package (Invitrogen). Subsequently, nine primers used in four combinations (Table 2.3 and Figure 2.2) were assessed for amplification of species within the *Luteoviridae*.

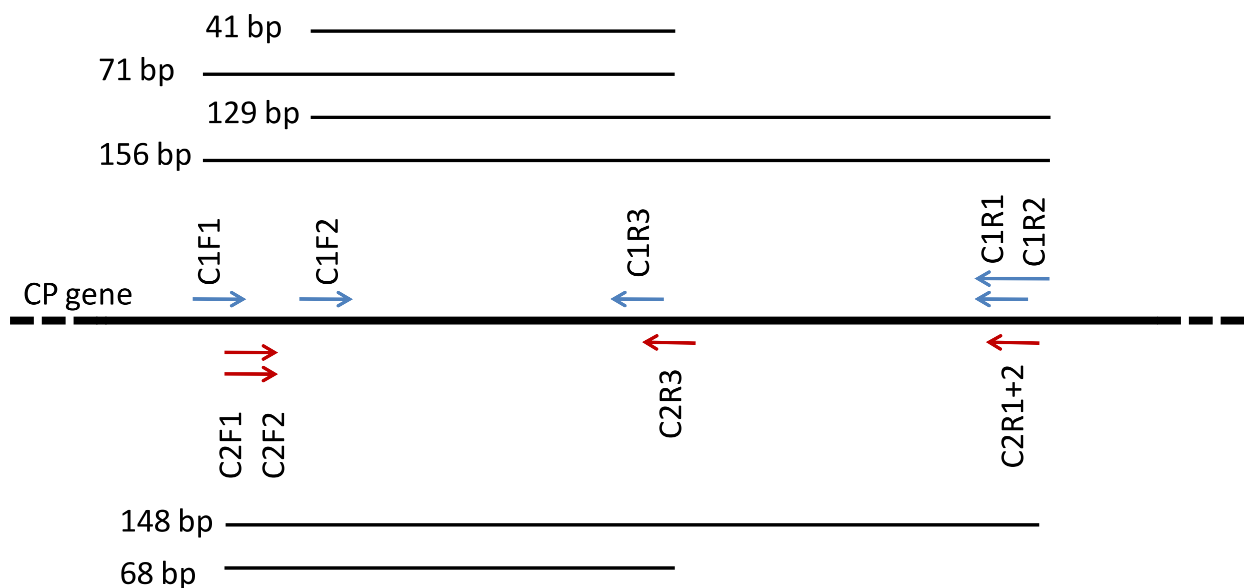


Figure 2.2 Schematic position of generic primers on the CP gene and amplicons, produced from viral template.

See Table Table 2.2 for genome position of primers

Table 2.3 The four combinations of generic primer used during this study and amplicon sizes anticipated from viral and SPC templates.

Forward primers	Reverse primer(s)	Amplicon size(s), produced from viral template (bp)	Amplicon sizes, produced from SPC template (bp)
C1F1 and C1F2	C1R1 and C1R2	129 and 156	197, 200, 217 and 220
C1F1 and C1F2	C1R3	44 and 71	237 and 257
C2F1 and C2F2	C2R1+2	148	293 and 310
C2F1 and C2F2	C2R3	68	255 and 272

2.2.3 Thermodynamic analysis of generic *Luteoviridae* primers

Because of the degenerate positions introduced into the sequences of the nine generic primers during primer design (Table 2.2), the actual number of primer sequences increased from nine to 38, with 14 sequences in the C1 set and 24 in the C2 set. Thermodynamic analysis showed that the maximum ΔG (change in Gibbs energy) of possible homo- and heterodimers formed at the 3′ end was −4.9 kcal mol^{-1}. According to Dieffenbach and Dveksler (2003) 3′-end dimers with a ΔG of −5 or higher are usually tolerated in PCR and do not interfere with the reaction. The C2R3 primer potential homodimer had a ΔG of −7.5 kcal mol^{-1}, much lower than the estimated limit of −5 kcal mol^{-1}. However, according to the computer prediction (Olygo Analyzer 3.1, Integrated

DNA Technologies (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) and Vector NTI (Invitrogen)), base pairing occurred in the central part of the primer and not at the 3′ end.

2.2.4 Total plant RNA extraction

Total RNA was extracted from 50 to 100 mg virus-infected young leaves and stems by four different methods according to the manufacturer's recommendations (see Table 2.1 for details). RNA for all New Zealand isolates except two was extracted by RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The exceptions were two BWYV isolates (FB3 and FB4) for which total RNA was extracted using a modified TRIzol Reagent (Invitrogen, Carlsbad, USA) protocol in order to explore a cheaper alternative to the RNeasy® Plant Mini Kit. Modification to the manufacturers TRIzol protocol included overnight incubation of samples at -80ºC after the homogenisation step, repeating the phase separation step twice and substituting isopropyl alcohol with 1:1 isopropyl alcohol : high salt precipitation solution (0.8M sodium citrate and 1.2 NaCl). Extraction of RNA for all imported isolates was performed by Joe Tang at Plant Health and Environment Laboratory (PHEL) at MAFBNZ using a Thermo Kingfisher mL automated nucleic acid extraction machine (Thermo Scientific, Helsinki, Finland) with InviMag Plant Kit reagents (Invitek, Berlin, Germany), except for the five CABYV isolates from Spain which were extracted by the method described by Célix *et al.* (1996) and were supplied as total RNA in 70% C_2H_5OH. RNA concentration for all samples was estimated by a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Montchanin, USA), except for the five CABYV isolates from Spain as residual C_2H_5OH might have influenced the spectrophotometer's readings. RNA was stored as a solution in water at −80°C.

2.2.5 Reverse transcription

Reverse transcription was performed using two different procedures. In the first, 5 µL random hexamer primers at 50 ng μL^{-1}, 7 µL total RNA (maximum amount of RNA added to improve chances of virus detection) and 1 µL 10 mM dNTPs (2'-deoxynucleotide 5'-triphosphates) were heated at 65°C for 5 min. The reaction was cooled on ice for 2 min and the following reagents added: 4 µL 5× First-Strand buffer, 1 µL 0.1 M DTT, 1 µL RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Carlsbad, USA) at 40 U μL^{-1} and 1 µL SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, USA) at 200 U μL^{-1}. The reaction was incubated at 25°C for 5 min, then 50°C for 60 min before inactivating the enzymes at 70°C for 15 min. In the second method, which was performed at PHEL MAFBNZ by Joe Tang for all overseas isolates, 2

μL random hexamer primers at 0.5 μg μL^{-1}, 8 μL total RNA, 8 μL 5× RT SSII buffer, 8 μL nuclease-free water, 2 μL RNasinPlus (Promega, Madison, USA) at 40 U μL^{-1} and 4 μL BSA at 10 μg μL^{-1} were heated at 70°C for 10 min followed by incubation at room temperature for 15 min. Then, 4 μL 0.1 M DTT, 2 μL 10 mM dNTPs, 1 μL SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, USA) at 200 U μL^{-1} and nuclease-free water were added to a volume of 40 μL and the reaction incubated at 45°C for 60 min.

The success of reverse transcription was checked by performing a PCR using the plant-specific nad5-s and nad5-as primers as described by Menzel et al. (2002). The nad5 primers amplify a 181 bp part of the plant gene *nad5* (NADH dehydrogenase subunit 5), which would be co-extracted with the total RNA. All 48 samples tested positive for nad5 and were used for further experiments.

2.2.6 PCR

RT-PCR reactions using generic *Luteoviridae* primers consisted of 1 mM $MgCl_2$, forward and reverse primer(s) at 300 ng each, 0.2 mM dNTPs, 1× PCR buffer, 0.525 U Expand High Fidelity (HiFi) PCR System (Roche Diagnostics GmbH, Mannheim, Germany) enzyme mixture and 0.5 μL cDNA in a total volume of 10 μL. Thermocycling conditions were 95°C for 2 min, then 40 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s, followed by 7 min at 72°C.

The presence and identity of virus sequences in the cDNA from infected plant samples were checked by performing PCR with published species- or group-specific primers (Table 2.4). All primers were synthesised by Invitrogen New Zealand Ltd, Auckland, New Zealand. For species- or group-specific PCR, 60 ng of each primer per reaction was used; other PCR reaction conditions were the same as described previously. Thermocycling conditions for species- or group-specific primers are shown in Table 2.4. The Lu4-mod primer is a modified version of the Lu4 primer (Robertson *et al.* 1991). Primers for CABYV and SbDV were designed *de novo* using sequence data in the NCBI database available in 2008. Negative controls were included in each experiment, consisting of either no template or healthy plant cDNA, and a novel synthetic positive control (SPC) as described in the following section.

All PCR products were analysed in 1–3% agarose gels, depending on the amplicon length, containing SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad, USA) in 0.5× TBE buffer. Amplicons of interest were extracted from the reaction mixture using the Wizard® SV Gel and

PCR Clean-Up System (Promega, Madison, USA) and cloned into pGEM®-T vector (Promega, Madison, USA) according to the manufacturer's recommendations. Transformation was carried out as described by Sambrook *et al*. (1989) using INVαF *Escherichia coli* competent cells (Invitrogen, Carlsbad, USA). Plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, USA). To confirm their identity inserts were sequenced at the Lincoln University Bio-Protection Research Centre, using T7 primer and ABI Big Dye (ABI, Foster City, USA) technology on ABI PRISM 3130*xl* Genetic Analyzer (ABI, Foster City, USA) according to the manufacturer's recommendations.

Table 2.4 The sequences, position, and PCR thermocycling conditions for *Luteoviridae* specific primers used during this study to check identity of isolates before using generic primers.

Primer	5' Sequence 3'	Species-specific target	Position in genome forward/reverse	PCR profile	Gene	Amplicon size (bp)	Reference
Lu1 Lu4	CCAGTGGTTRTGGTC GTCTACCTATTTGG	BWYV (X13062) TuYV (NC_003743) BYDV-PAV (D01214) BYDV-PAS (AF218798) BYDV-MAV (D01213)	81-95/601-614 3564-3578/4084-4097 2936-2950/3453-3466 2936-2950/3453-3466 2897-2911/3414-3427	95ºC - 2 min 95ºC - 30 s } x40 41ºC - 30 s } 72ºC - 30 s } 72ºC - 7 min	ORF-3	531-534	(Robertson *et al*. 1991)
LR4S LR5A	CGCGCTAACAGAGTTCAGCC GCAATGGGGGTCCAACTCAT	PLRV (X74789)	3669-368/3985-4004	94ºC - 2 min 94ºC - 30 s } x40 60ºC - 30 s } 72ºC - 30 s } 72ºC - 7 min	ORF3	336	(Singh *et al*. 1995)
CP+ CP-	ATGAATACGGTCGTGGGTAGGAG CCAGCTATCGATGAAGAACCATTG	BChV (NC_002766) BMYV (X83110)	3607-3629/4146-4169 3597-3619/4136-4159	95ºC - 5 min 95ºC - 30 s } x30 50ºC - 45 s } 72ºC - 60 s } 72ºC - 7 min	ORF-3	563	(Hauser *et al*. 2000)
BLRV-3 BLRV-5	TCCAGCAATCTTGGCATCTC GAAGATCAAGCCAGGTTCA	BLRV (AF441393)	3250-3268/3619-3638	94ºC - 5 min 94ºC - 15 s } x25 64ºC - 60 s } 72ºC - 60 s } 72ºC - 15 min	ORF-3	389	(Ortiz *et al*. 2005)
SbDV-F SbDV-R	GTTAGCAATGTCGCAATAC TCTTCACTGGTATCATGC	SbDV (L24049)	3041-3059/3523-3540	Same as for Lu1 and Lu4	ORF-3	500	*de novo* *de novo*
CABYV-F CABYV-R	CAAAGGACAATCTCACGG CTGGCACTTGATGGTGAT	CABYV (X76931)	3704-3721/4069-4086	95ºC - 2 min 95ºC - 30 s } x40 43ºC - 30 s } 72ºC - 30 s } 72ºC - 7 min	ORF-3	383	*de novo* *de novo*

Primer	5' Sequence 3'	Species-specific target	Position in genome forward/reverse	PCR profile	Gene	Amplicon size (bp)	Reference
Lu1 Lu4-mod	CCAGTGGTTRTGGTC CGTCTACCTATTTTGG	CYDV-RPV (EF521827)	3835-3849/4351-4366	Same as for Lu1 and Lu4	ORF-3	532	(Robertson *et al.* 1991) *de novo*
YLS111 YLS462	TCTCACTTTCACGGTTGACG GTCTCCATTCCCTTTGTACAGC	ScYLV (AF157029)	3827-3846/4157-4178	95ºC - 2 min 95ºC - 30 s } 50ºC - 30 s } x40 72ºC - 30 s } 72ºC - 7 min	ORF-3	352	Irey, as cited in (Abu Ahmad *et al.* 2006)
CtRLV-1 CtRLV-2	GAGGTGAGAAATCGCYTGAC MGGCGCCACARTGATAG	CtRLV (NC_006265)	2753-2772/2946-2963	95ºC - 2 min 95ºC - 30 s } 59ºC - 30 s } x40 72ºC - 30 s } 72ºC - 7 min	ORF-2	211	*(Vercruysse et al. 2000)*
PEMV-A PEMV-B	GAACCAGTGCAATTCATGGTG CCATATTGGAGGAAATGCG	PEMV-1 (L04573)	3725-3743/3849-3869	95ºC - 2 min 95ºC - 30 s } 55ºC - 30 s } x40 72ºC - 30 s } 72ºC - 7 min	ORF-2	145	(Liu *et al.* 2006)
CfCP-F1 CfCP-R2	GATGGAGCCAGTTCTTCG ATCCGTCAATCTTCAAGC	CoMV (Z48630)	3191-3208/3949-3966	Same as for PEMV-A and PEMV-B	ORF-4	776	(Delmiglio 2008)
RYMV-F RYMV-R	CAAAGATGGCCAGGAA CTCCCCCACCCATCCCGAGAATT	RYMV (L20893)	3442-3457/4428-4450	95ºC - 2 min 95ºC - 60 s } 55ºC - 60 s } x40 72ºC - 60 s } 72ºC - 7 min	ORF-4	1009	(Brugidou *et al.* 1995)

2.2.7 Synthetic Positive Control

An SPC plasmid was designed to be used with amplification of any combination of the luteovirus species and generic virus primers, plus the nad5-s-nad5-as plant primers. The SPC was made by inserting the primer sequences into the pGEM®-T vector (Figure 2.3). The position of primers in the plasmid enabled different primer pairs to produce amplicons of different lengths (Table 2.3) that could be easily distinguished by agarose gel electrophoresis. Sequences of forward and reverse primers were arranged into two fragments (F and R, respectively) (Table 2.5) and inserted in a vector as shown in Figure 2.3. Fragments F and R were 152 and 161 bp, respectively; however, synthesis of oligonucleotides greater than 100 bases was not possible. Therefore, each fragment was synthesised as two separate oligonucleotides overlapping by 20 bp: fragment F was made from F-I and F-II oligonucleotides and fragment R was made from R-I and R-II oligonucleotides (Table 2.5).

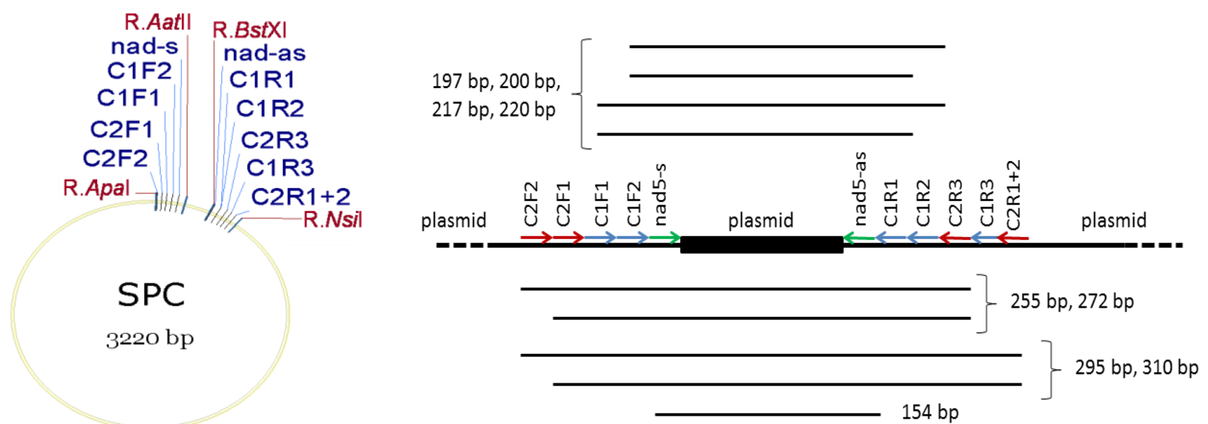


Figure 2.3 Schematic view of the SPC and position of the cloned primers.

Restriction sites shown in red *italics* indicate cloning sites.

The sequence of fragment F consisted of the SPC forward primer sequence (5′-TGTGTCTGGCTTTTGATGCT-3′), *Apa*I restriction site, C2F2, C2F1, C1F1, C1F2 and nad5-s primer sequences, *Aat*II restriction site and SPC reverse primer (5′-GAGTGCGATAGGGGCTGTT-3′) (Table 2.5). The sequence of the second fragment (R) consisted of: the SPC forward primer sequence, *Bst*XI restriction site, nad5-as, C1R2, C2R3, C1R3 and C2R1+2 primer sequences, *Nsi*I restriction site and SPC reverse primer sequence

(Table 2.5). The SPC forward and reverse primer sequences were as described by Ochoa-Corona et al. (2006) (referred to as primers for *Raspberry ringspot virus*). These primers were used only to amplify F and R fragments and were not incorporated into SPC sequence.

Table 2.5 Sequences (5' – 3') of four oligonucleotides which were synthesised for SPC construction.

The primer sequences of SPC forward and reverse primers are underlined. Restriction sites (in **bold**) are followed by primer sequences, with overlapping parts (20 nt) shown in small print.

Fragment	5' Sequence 3'
F fragment	
F-I (87nt)	TGT GTC TGG CTT TTG ATG CT**G GGC CC**G GTC ACG TTC GGG CCG TCT TCA CGT TCG GGC CGA GTG GGG Taa tca aat tcg gtc ctc gca
F-II (85nt)	GA GTG CGA TAG GGG CTG TT**G ACG TC**A ACA AGA AGC CCC AAG AAG CAT CCT GAA AGC GCT GGG CAT tgc gag gac cga att tga tt
R fragment	
R-I (91nt)	TG TGT CTG GCT TTT GAT GCT **CCA ACG CGT TGG** TTA TGC CAA TGT TGG TGA CTG GAG GGC GCT ATC TTT ATT gaa ctc gac ctc aag gcc ta
R-II (90nt)	G AGT GCG ATA GGG GCT GTT **ATG CAT** TCC AGC TCG TAA GCG ATG GTG GTA GGA CTT AAG TAT TCC TCA TGG tag gcc ttg agg tcg agt tc

The SPC was constructed via four steps:

1. Circularization of the linearised vector

The 5′ thymidines of the linearised pGEM-T plasmid were removed with Klenow enzyme (Roche Diagnostics GmbH, Mannheim, Germany). The reaction mixture consisted of 1 µL pGEM-T Easy at 50 ng μL^{-1}, 5 µL 2× Rapid Ligation Buffer (pGEM-T Easy Vector System I), 1 µL Klenow enzyme (2 U μL^{-1}), 1 µL of dATP at 10 mM and 1 µL deionised water. The mixture was incubated at 37°C for 15 min, then inactivated at 65°C for 10 min. The plasmid was circularised by adding 1 µL T4 DNA ligase (pGEM®-T Easy Vector System I) to the mixture. The reaction was stored for 24 h at 4°C. Transformation of the circularised vector and isolation of plasmids were carried out as described previously in Section 2.2.6. Sequencing (described previously) of the ligation site confirmed circularization.

2. Reconstitution of F and R fragments

Two separate oligonucleotide mixtures (10 µL) consisting of 5 µL each overlapping 50 µM oligonucleotide (F-I+F-II, or R-I+R-II) (Table 2.5) were incubated at 95°C for 10 min. The mixtures were then cooled to room temperature in a water bath to allow the annealing of 20 bp complementary 3′ ends. The 3′ ends were extended using Expand HiFi PCR System (Roche Diagnostics GmbH, Mannheim, Germany) polymerase. Two additional separate mixtures were prepared for the synthesis of F and R fragments. Mixture A (8.8 µL) contained 1× PCR buffer (Expand HiFi PCR system), 1 mM $MgCl_2$, 0.2 mM dNTP, 0.525 U Expand HiFi PCR System enzyme mixture and deionised water. Mixture B (11.2 µL) contained 1× PCR buffer and the appropriate oligonucletide mixture (10 µL). Mixture A was incubated at 94°C for 2 min to activate the Expand HiFi PCR System enzyme mixture before cooling to room temperature and adding mixture B (total volume 20 µL). Elongation was performed at 72°C for 15 min.

3. Amplification of F and R fragments for cloning

PCR was performed using the SPC primers to amplify the F and R fragments for restriction analysis. The PCR conditions were as described for the generic primers (see Section 2.2.6), except that 0.5 µL of the mixture from step 2 was used as a template and the SPC primers were used instead of the generic primers. The thermocycling conditions were: 94°C for 2 min, then 35 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 30 s, followed by 7 min at 72°C. Amplification was confirmed by agarose gel electrophoresis. Amplicons were extracted from the reaction mixture as described previously and sequencing of fragments F and R confirmed the correct annealing of the F1+F2 and R1+R2 parts.

4. Cloning of F and R fragments into the plasmid vector

The first double digestions of circularised vector and F fragment were performed separately for each template in 1× Blue Fermentas (Fermentas, Vilnius, Lithuania) buffer, using 20 U of each restriction endonucleases R.*Aat*II and R.*Apa*I (Fermentas, Vilnius, Lithuania) and 800 ng template. The reaction mixture was incubated for 2 h at 37°C following incubation for 20 min at 65°C. After restriction, digested F fragment and digested plasmid vector were extracted from the reaction mixture, ligated and transformed as described previously. The second double digestion of vector (containing F fragment) and R fragment was performed separately for each template in 1× Invitrogen buffer, using 20 U of each restriction endonucleases R.*Bst*XI (NEB, Ipswich, USA)

and R.*Nsi*I (Invitrogen, Carlsbad, USA) and 800 ng template. The reaction mixture was incubated as described previously. After restriction, digested R fragment product and digested plasmid vector (containing F fragment) were extracted from the reaction mixture, ligated and transformed as described previously. The final product was diluted 200 times in water prior to use as SPC plasmid template in further PCR reactions.

2.2.8 Phylogenetic analysis

Coat-protein gene nucleic acid sequences (156 nts) obtained from 41 *Luteoviridae* isolates (Table 2.1), together with two sequences of SPLSV (DQ655700) and BWYV-USA (AF473561) from the NCBI database, were aligned using ClustalW. An unrooted phylogenetic tree was generated by Mega 4 (Tamura *et al.* 2007) using Neighbour-Joining phylograms, based on the nucleotide Kimura–2-parameter distance matrix with complete deletion as the gap treatment. Bootstrap confidence limits were derived from 1000 replicates.

2.3 Results

2.3.1 Species- or group-specific RT-PCR

PCR products of the appropriate size were amplified using species- or group-specific primers (Table 2.4) for all 48 virus isolates used during the study. The identity of each amplicon was confirmed by sequencing and BLAST (and submitted to the NCBI database: GU002322–GU002361 and GQ906583).

2.3.2 Species detected using the generic luteovirus primers

The C2F1-C2F2-C2R3 primers amplified a 68 bp product and detected nine luteovirus species (BChV, BLRV, BMYV, BWYV, CABYV, CYDV-RPV, PLRV, SbDV and TuYV) (Figure 2.4 a). Additional bands of ~160 and ~700 bp were observed with SbDV isolates. The C2F1-C2F2-C2R1+2 primers amplified a 148 bp product from the same *Luteoviridae* species except for the CABYV isolates and CYDV-RPV isolate RL1. Both CYDV-RPV isolates (Kin3 and RL1) were detected by the C2F1-C2F2-C2R3 primers, but CYDV-RPV isolate RL1 was not detected (data not shown) when the C2R1+2 primer was used instead of the C2R3 primer. The C2F1-C2F2-C2R1+2 primers also did not amplify any of the CABYV isolates (Figure 2.4 b). The C1F1-C1F2-C1R1-C1R2 primers detected three additional species (BYDV-PAV, BYDV-PAS and BYDV-MAV) (Figure 2.4 c), producing amplicons of 129 and/or 156 bp.

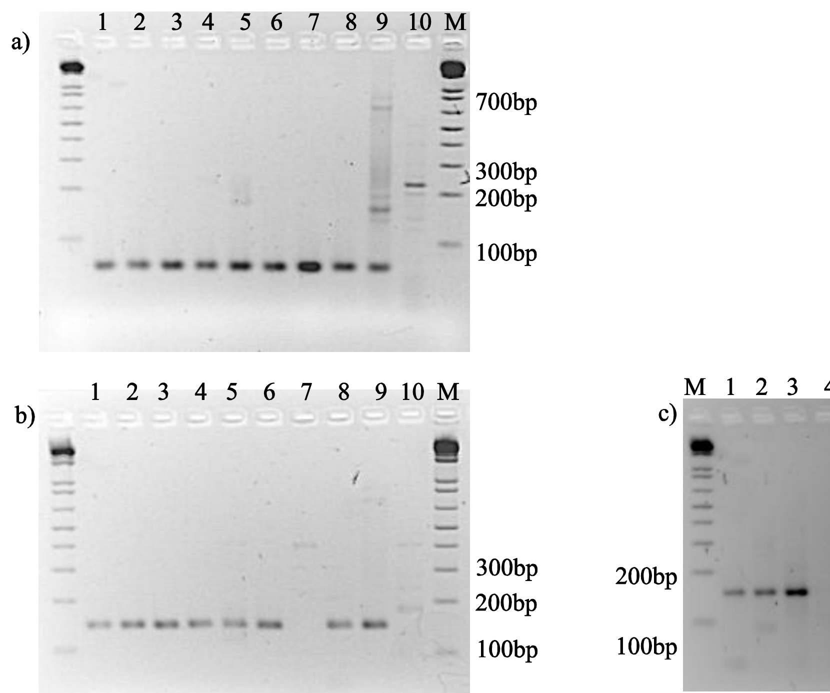


Figure 2.4 RT-PCR amplification products using generic primers.

a) C2F1-C2F2-C2R3 (68 bp amplicon) and b) C2F1-C2F2-C2R1+2 primers (148 bp amplicon); 1 – BWYV, 2 – BMYV, 3 – BChV, 4 – BLRV, 5 – PLRV, 6 – TuYV, 7 – CABYV, 8 – CYDV-RPV, 9 – SbDV, 10 – Negative (water), M – 1 kb DNA Ladder (NEB, Ipswich, USA); c) C1F1-C1F2-C1R1-C1R2 (156 bp amplicon): M – 1 kb DNA Ladder, 1 – BYDV-PAV, 2 - BYDV-MAV, 3 - BYDV-PAS, 4 – Negative (water). PCR products were analysed in 3% agarose gel.

The C1F1-C1F2-C1R3 primers also detected these species, producing amplicons of 44 and/or 71 bp. However, these amplicons appeared quite faint on agarose gels and were difficult to discriminate from primer dimers (data not shown). Therefore primer C1R3 was no longer considered for use in the generic priming system.

Multiple non-specific bands of up to 300 bp were observed in the absence of target template (negative controls) which was replaced by water or cDNA derived from healthy plants (see Figure 2.5 and Figure 2.6). However, amplicons of the size expected with viral templates were not produced.

None of the four primer combinations (Table 2.3) amplified ScYLV. The C1F1 and C2R3 primers had the highest sequence identity (85% and 92%, respectively) with ScYLV (NCBI Accession no. NC_000874). These primers belong to different generic primer sets (C1 and C2) and were initially used separately (Table 2.3). However, in combination the C1F1 and C2R3 primers amplified a 75 bp product from the CP of all three ScYLV isolates (Figure 2.6 shows

only amplification results of two ScYLV isolates). Sequencing confirmed the identity of the products.

2.3.3 Species not detected using the generic primers

None of the four primer combinations tested (Table 2.3) amplified cDNA from plant material infected with unassigned *Luteoviridae* CtRLV or the *Enamovirus* PEMV-1. The C2F1-C2F2-C2R3 primers amplified a product of *c*. 70 bp with the SPLSV template and one of *c*. 60 bp with healthy *Ipomoea setosa* (Figure 2.7), but sequencing of both amplicons was not successful. None of the four primer combinations tested (Table 2.3) amplified a specific product with the outgroups CoMV or RYMV (Figure 2.5; results for C1F1-C1F2-C1R3 primers not shown), although multiple low-molecular-weight bands were observed. The sequence similarity of the primers did not exceed 67.6% with CoMV (NCBI Accession no. NC_002618) or 69.4% with RYMV (NCBI Accession no. NC_003380).

2.3.4 Amplification of the SPC

The PCR products amplified from the SPC with the four generic primer combinations and nad5-s-nad5-as were of the expected size (Table 2.3).

2.3.5 Phylogenetic analysis

Phylogenetic analysis of a 156 bp CP gene region (obtained after sequencing of species- or group-specific amplicons, see Section 2.2.6) clearly separated the 43 *Luteoviridae* isolates into three main clusters, being C1, C2 (as indicated in Figure 2.8) with the ScYLV cluster in between. This echoed the taxonomic range of three primer combinations used (C1F1-C1F2-C1R1-C1R2, C2F1-C2F2-C2R3 and C1F1-C2R3).

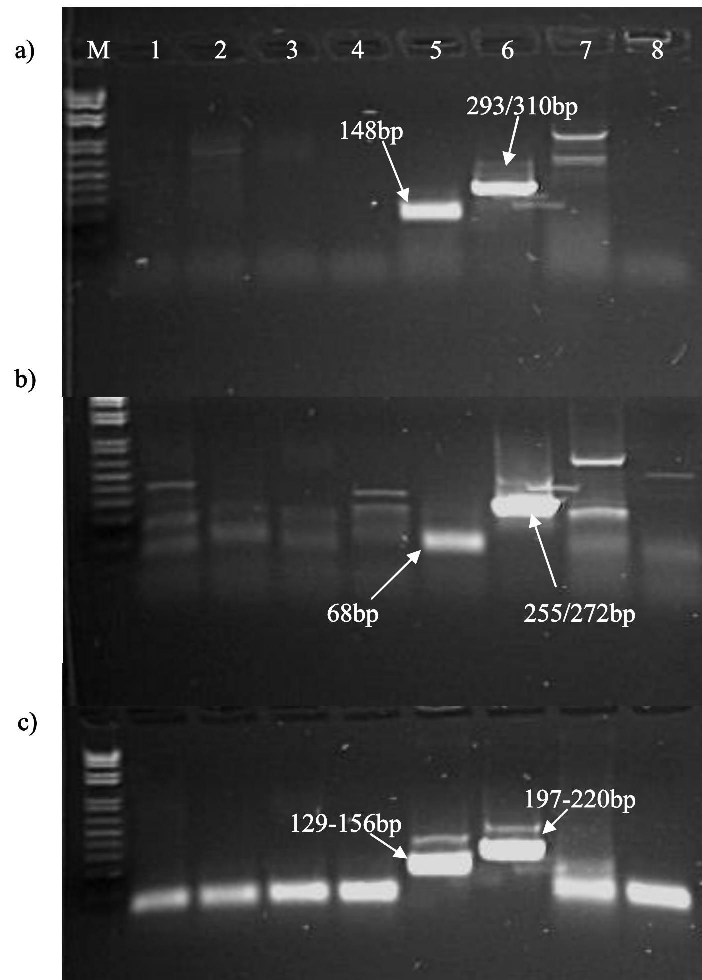


Figure 2.5 RT-PCR amplification products of out-group isolates.

a) C2F1-C2F2-C2R1+2; b) C2F1-C2F2-C2R3; c) C1F1-C1F2-C1R1-C1R2 primers. M: 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, USA); Lane 1: CoMV-DSMZ; Lane 2: CoMV-SI; Lane 3: RYMV-DSMZ; Lane 4: Negative control (water); Lane 5: Positive control (a and b –SbDV-BB2, c – BYDV-PAV-PC3); Lane 6: SPC; Lane 7: Healthy *Avena sativa*; Lane 8: Reagent control. PCR products were analysed in 3% agarose gel.

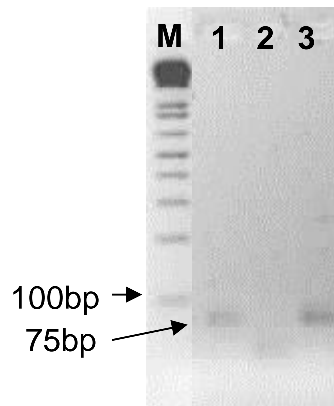


Figure 2.6 RT-PCR amplification products of two ScYLV isolates with C1F1-C2R3 primers.

M: 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, USA); Lane 1: ScYLV-MA3; Lane 2: Healthy *Saccharum* sp. Lane 3: ScYLV-MA5. PCR products were analysed in 3% agarose gel.

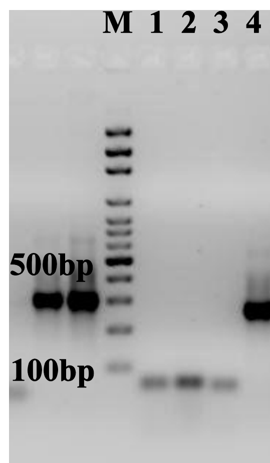


Figure 2.7 RT-PCR amplification of SPLSV with C2F1-C2F2-C2R3 primers.

M: 100 bp Ladder DNA marker (Invitrogen, Carlsbad, USA); Lane 1: SPLSV-SF; Lane 2: PLRV; Lane 3: Healthy *Ipomoea setosa*; Lane 4: SPC. PCR products were analysed in 2% agarose gel. Gel provided by S. Fuentes (see acknowledgements).

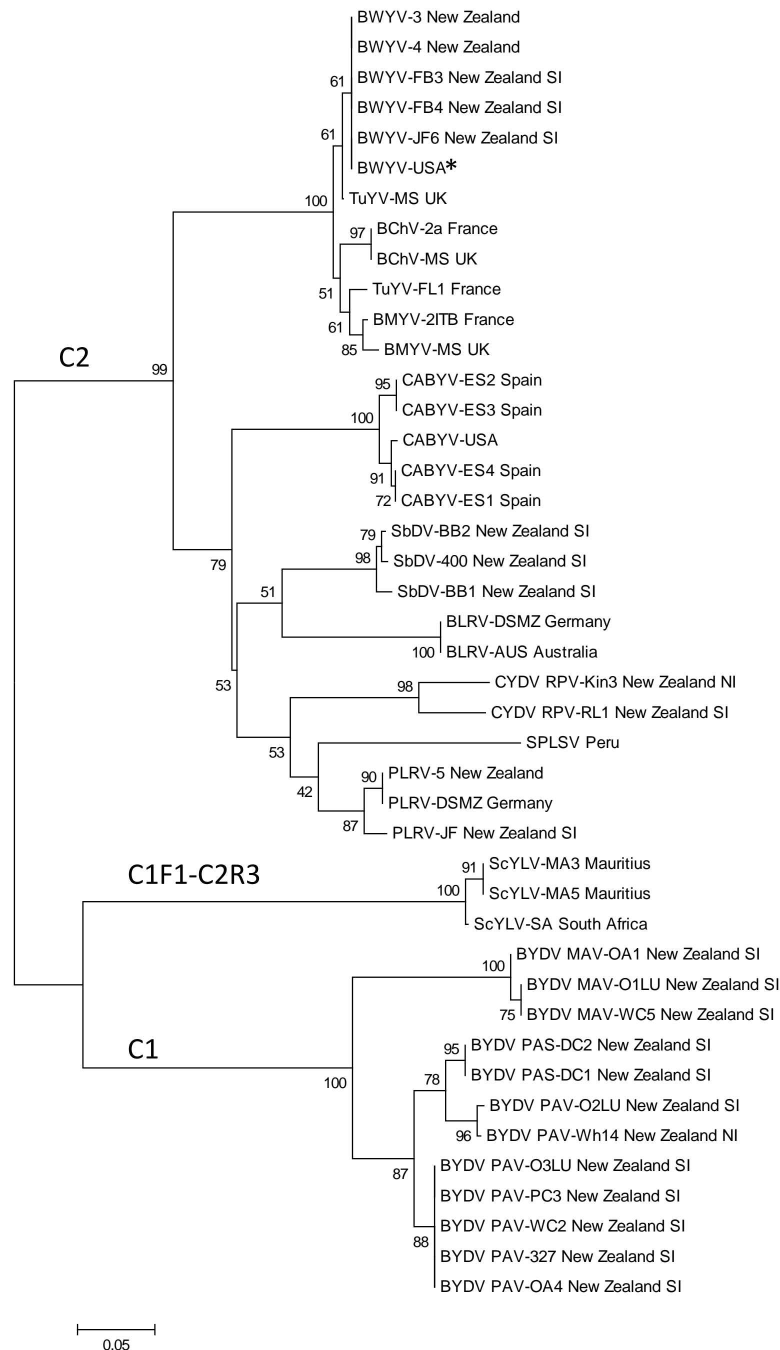


Figure 2.8 Unrooted Neighbour-Joining tree of 43 *Luteoviridae* isolates including SPLSV (DQ655700) and BWYV USA (AF473561) sequences, for the 156 bp region of the coat protein gene (annealing region of generic primers).

Bootstrap values differ from those given in Chomič *et al.* (2010) by 1-3.7%. Branches which include species also amplified by C1 and C2 primer sets, as well as with C1F1-C2R3 primers, are indicated. SI – South Island, NI – North Island (where known). * - BWYV-USA (Beuve *et al.* 2008) was used as a reference only and was not analysed during this study.

The first cluster (bootstrap support 99%) represented all nine species detected with the C2F1-C2F2-C2R3 primers together with SPLSV. The second cluster (bootstrap support 100%) contained three BYDV species which could be detected using the C1F1-C1F2-C1R1-C1R2 and C1F1-C1F2-C1R3 primers. The third cluster (bootstrap support 100%) emphasised the transient position of ScYLV, which is considered to be a recombinant virus between all three *Luteoviridae* genera (Moonan *et al.* 2000) and could be detected using the C1F1 and C2R3 primers, which belong to different sets (Table 2.2).

2.4 Discussion

No generic PCR assay for the detection of luteoviruses has been described since that of Robertson et al. (1991), with advances in this area being constrained by high sequence variability within the group and a lack of sequence data. Using luteovirus CP sequence data published since 1991, additional conserved regions were identified and used to design novel generic low-degeneracy primers. The design of multiple primer pairs proved to be the best strategy to cover the taxonomic range because of the high variability of the CP sequence, even in this relatively conserved region. Using three primer combinations (C1F1-C1F2-C1R1-C1R2, C2F1-C2F2-C2R3 and C1F1-C2R3) it was possible to detect 13 of the 16 species tested, which represented members of the two principal genera *Luteovirus* and *Polerovirus*. Specificity of the primers for luteovirus amplification was confirmed using isolates of two *Sobemovirus* species (CoMV and RYMV), which failed to amplify as anticipated from the limited sequence similarity of the generic primers with either species.

The possible interaction between the generic primers in each combination (primer dimers or primer clusters) was shown not to interfere with the successful detection of *Luteoviridae* via RT-PCR since the correct amplicon size was observed following agarose gel electrophoresis and the sequence identity of specific products was confirmed by sequencing. Amplification from templates which were free from luteovirus infection (*Saccharum* sp., *Avena sativa*, *Dactylis glomerata* and *Oryza sativa*) did not show any specific amplification products; the only bands observed were probably either primer dimers or non-specific products of different sizes to those generated from *Luteoviridae* templates (see Figure 2.5 and Figure 2.6). The formation of amplicons less than 300 bp in the water negative control reactions was probably the result of primer interactions. The generic *Luteoviridae* primers described in this study did not all have ideal thermodynamic properties (see Section 2.2.3) and were present at high concentrations (up to

300 ng each). Therefore, the formation of primer dimers might be expected. High-molecular-weight bands were also observed with SbDV (Figure 2.4a) and probably arose from non-specific priming to the host genome.

Failure of the C2F1-C2F2-C2R1+2 primers to detect CYDV-RPV-RL1 and all five CABYV isolates might be explained by lack of sequence similarity between the reverse C2R1+2 primer and viral nucleotide sequences. Sequence analysis of both CYDV-RPV isolates (RL1 and Kin3) showed that isolate RL1 had three base-pair mismatches with the C2R1+2 primer, one of which was 5 nt from the 3′ end. The C2R1+2 primer had only two mismatches with the Kin3 isolate, both located far from the 3′ end, and these did not affect cDNA amplification. Furthermore, low sequence identity between the C2R1+2 primer and CABYV (76.3%) could explain why no CABYV sequences were amplified using the C2F1-C2F2-C2R1+2 primers. The C2R3 primer had 97.2% sequence identity with CABYV and could amplify the expected 68 bp product from all five CABYV isolates when combined with the C2F1 and C2F2 forward primers. Despite not detecting the same range of species as the C2F1-C2F2-C2R3 primers, the C2F1-C2F2-C2R1+2 primers might have a supplementary value in detection of some *Luteoviridae*.

However, because of the technical difficulties (sequencing of the amplicon was not successful), it is still unclear whether the C2F1-C2F2-C2R3 primers detected SPLSV. The sequence identity between primers and SPLSV is high and an amplicon of the expected size was observed, but it was very similar to an amplicon from the healthy plant controls. The C2F1-C2F2-C2R3 primers had the highest sequence identity to SPLSV (NCBI Accession no. DQ655700), the identity of primers being: C2F1, 82.4%; C2F2, 94.1%; and C2R3, 91.7%. The phylogenetically closest species to SPLSV is PLRV, their CPs being 70% identical; it has been proposed that SPLSV and PLRV originated from an ancestral species in South America which diverged into separate species with different host ranges (Fuentes *et al.* 1996). Given the close similarity between PLRV and SPLSV, the high sequence identity of C2F2-C2R3 primers and the successful amplification of the PLRV CP sequence region, it is possible that SPLSV could also be detected.

The groups of species detected by the three primer combinations (C1F1-C1F2-C1R1-C1R2, C2F1-C2F2-C2R3 and C1F1-C2R3) were consistent with the phylogenetic clustering of the 156bp sequences of these species into three main branches (Figure 2.8). The first primer combination tested (C2F1-C2F2-C2R3) detected nine (seven *Polerovirus* and two *Luteovirus* (SbDV and BLRV)) species. Although the CP sequences of SbDV and BLRV are similar to those

of poleroviruses, they are placed in the *Luteovirus* genus, based on their genome organization. They are recombinant viruses and have a CP sequence similar to those in *Polerovirus* genus, but the polymerase gene is more *Luteovirus*-like (Domier *et al.* 2002).

The second primer combination (C1F1-C1F2-C1R1-C1R2) amplified only three BYDV species, which are phylogenetically distant from all nine species detected with the C2F1-C2F2-C2R3 primers. This phylogenetic isolation and the need for a separate primer combination to detect BYDV-MAV, BYDV-PAV and BYDV-PAS re-ignites recent debate on the taxonomic position of the BYDV species in the *Luteoviridae* family. It has been suggested that the BYDV species should be assigned to the *Tombusviridae* family, genetic analysis having revealed that BYDV species share many genetic features with this family (Miller *et al.* 2002). However their current position in the *Luteoviridae* is justified by the phylogenetic indication that BYDV movement and structural proteins were probably acquired from the genus *Polerovirus* (Miller *et al.* 2002).

The two primers in the third combination (C1F1-C2R3) targeted phylogenetically different groups of luteoviruses, but when used together they were able to detect ScYLV. This supports the recombination hypothesis of ScYLV (Smith *et al.* 2000), which is classified as a *Polerovirus* because of its genome organization. This species is most likely a recombinant species which acquired its genes from each of the three genera in the *Luteoviridae*. Despite the overall similarity of the CP sequence (ORF3) with *Luteovirus*, the N-terminal domain of the ScYLV CP (the first 40 amino acids) is most similar to the sequences of *Polerovirus* (Smith *et al.* 2000), whilst the sequence after amino acid residue 40 is similar to the *Luteovirus* BYDV-PAV. The C1F1-C2F3 primers which detected ScYLV are located after amino acid residue 40. The C1F1 primer, which detects BYDV species, anneals to the *Luteovirus*-like part of the ScYLV CP sequence with an identity of 85%, whilst the C2R3 primer annealing site has a closer affinity to a *Polerovirus*-like sequence. Phylogenetic analysis of 156 bp fragment of the CP gene region also indicated an intermediate position of ScYLV (Figure 2.8).

Although PLRV (*Polerovirus*) isolates around the world generally show low variability at the genetic level (Guyader and Ducray 2002), one New Zealand isolate (PLRV-JF) stood out as differing by four nucleotides in a 156 bp CP region from two con-specific isolates from New Zealand (PLRV-5) and Germany (PLRV-DSMZ) but was still detected by the generic primers. Further analysis and additional isolates are necessary to determine the validity of this observation.

The subclustering of isolates strongly reflected their host preferences. For example all four beet-infecting *Polerovirus* species (BWYV, BChV, BMYV and TuYV) clustered together, as did both bean-infecting *Luteovirus* species (SbDV and BLRV) (Figure 2.8). The phylogenetic position of the two European TuYV isolates (MS and FL1) was close to the BWYV isolates. This is consistent with the non-beet-infecting isolates of BWYV, found particularly in Europe, having recently been renamed TuYV (Fauquet *et al.* 2005; Beuve *et al.* 2008).

BYDV-MAV isolates were clearly more distant from BYDV-PAV and BYDV-PAS isolates than the latter two were from each other. All seven BYDV-PAV isolates originated from New Zealand and fell within two distinct clusters. This pattern was expected as it was previously shown that CP gene sequences of BYDV-PAV isolates from New Zealand fall into two subclades with low diversity within each group (Delmiglio 2008). This separation probably reflects the different geographical origin of New Zealand BYDV-PAV isolates, which cluster in two different subclades with isolates from Australia and USA.

The use of generic primers has become increasingly popular in detection of plant virus groups as more sequence data become available. However, representation of sequences in the NCBI database does not necessarily reflect the global diversity of plant viruses. The discovery and sequencing of new species is likely to reduce the apparent conservation of the primer annealing sites as identified during this study. The term "decay in conservation" of the priming sites refers to the increase in known nucleotide variation at conserved sites over a certain period of time. The primer decay issue was first investigated in the *Potyvirus* genus (Zheng *et al.* 2008). In *Luteoviridae*, the accumulation of data on nucleotide sequence variation at primer annealing sites is not only influenced by discovery of new species, as it is in *Potyvirus*, but also by the discovery of new isolates within a species (Figure 2.9). For the primers developed here, and based on the N score (showing the percentage of nucleotides that 'decay' with the accumulation of more primer-site sequence data over time), the C1F1 forward primer has had the highest rate of decay over the last nine years. This is because C1F1 not only acts as the forward primer to amplify BYDV species (together with C1R2 and C1R2 primers), but is also works in a pair with the C2R3 reverse primer to amplify ScYLV. C1F1 nucleotide sequence identity with ScYLV coat protein gene nucleotide sequences is lower (85%) than that of BYDV, and this significantly contributes to the 'decay' of C1F1 primer. If ScYLV species were excluded from the estimation of the consensus 'decay' rate, the N score of C1F1 primer would be very similar to other forward

primers analysed. Forward primers from the C2 set 'decay' much slower than the other five generic *Luteoviridae* primers. This is probably due to higher conservation in nucleotide sequences among the target *Polerovirus* species than there is among the *Luteovirus* species and ScYLV.

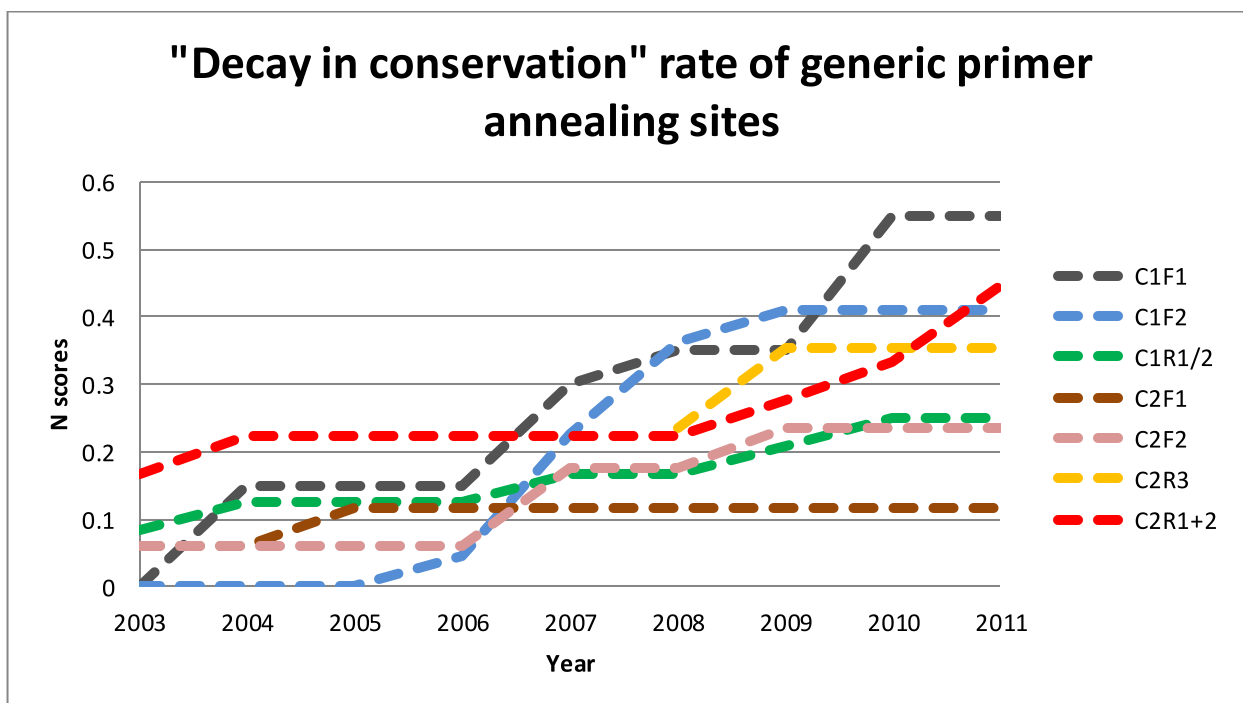


Figure 2.9 Nucleotide variant score (N score) of the seven generic *Luteoviridae* primers (decay rate) over the period 01/01/2003 – 06/04/2011.

Data sourced from NCBI database. Calculations were performed as in Zheng *et al*. (2008)

The *Luteoviridae* family is a relatively recent group, having only been described in 1976, and with twelve new species having been reported since the last ICTV report in 2005 (Table 1.2). Therefore, it is important that the sequences of the generic primers are reviewed periodically, as more CP sequences become available.

As seen from nucleotide sequence alignment on Figure 2.1, most of the primers do not have a 100% sequence identity with luteovirus isolates tested. Nevertheless, amplification of all of these isolates was successful (see Figure 2.4). As to whether primer sequences, which were designed in 2003, had to be improved (re-designed) before trying to estimate their taxonomic range in 2008, is an ambiguous issue. There will always be a tradeoff between a consensus chosen to best

represent a given region in an alignment and the number of degenerate nucleotide base positions that have to be incorporated and would not interfere with a successful performance of the primers. An ideal consensus might have included too many degenerate base positions and that would most certainly seriously impact on use of such primers (the effective concentration of each primer would decrease and the possibility of non-specific priming would increase). Therefore an "arbitrary" low-degeneracy consensus sequence, which represents only the majority, but not all of the nucleotide diversity found in an alignment, had to be chosen. This approach to generic primer design leaves out some of the luteovirus isolates, but the assay proposed during this study was shown to detect species even if nucleotide sequences of the primers do not match 100% the nucleotide sequences of the target luteovirus. There are two forward primers for each primer combination used (Table 2.3), which increases the possibility of successful annealing and extension, because one of the primers might have greater nucleotide sequence similarity to the target sequences than another, especially if it has no mismatch at its 5' end. The resulting sequences of the generic *Luteoviridae* primers used during this study, ensure a good temporary solution for amplification of the majority of the sequences used in the initial alignment (Section 2.2.2). However, the strategy used here to determine the sequences of the generic primers implies that if other consensus sequences were to be chosen (the same region, but different in sequence), they might have provided a better solution for the generic detection of luteoviruses. This certainly could be followed up in the future.

In this study the development of an SPC is also reported as a simple way to assess the performance of PCR using the generic primers. Because the SPC generates amplicons of a size different from those amplified from the luteovirus templates it can be incorporated into the same tube as the viral assay. This synthetic system is cheaper, safer, and more transferable between laboratories than the classical use of infected material, which may be difficult to obtain or pose phytosanitary risks. The main drawback of the SPC is that it assesses the performance of generic primers during the PCR only and does not provide information about the reverse transcription step. However, the success of the reverse transcription step can be checked by nad5-s/as primers as described in Section 2.2.5, as it was done in this study.

The SPC described in this study is a completely novel construct specifically designed for use with generic primers. Alternative ways of producing synthetic positive controls for the identification of endemic and exotic disease agents were described by Munro *et al.* (2005) and Smith *et al.* (2006).

Unlike the approach described in this thesis, the synthetic control developed by Smith *et al.* (2006) did not require generation of genetically modified organisms. It was based on using the pUC18 plasmid, which served as a template for incorporating a multiple cloning site in the middle of the positive control amplicon. The first round PCR used two long oligonucleotides (44 and 68 bp) containing a T7 promoter and sequences of forward or reverse virus- and pUC18-specific primers (4 in total) in a certain order. The resulting amplicon (containing the T7 promoter) was then used to synthesise RNA. Reverse transcription and second round PCR, using virus-specific primers were then performed. The resulting amplicons were then subjected to digestion by restriction enzymes (additional 2 h of analysis) to distinguish amplified synthetic control material from authentic viral amplicons, because the approach has the potential to produce false-positive amplification products. The synthetic positive control designed by Munro *et al.* (2005) was similar to the SPC described in this study, as it was based on incorporating a plasmid-derived fragment which could then be amplified using disease-specific primers that produce an amplicon different in size to the diagnostic samples. However, the large number of primers used in the assay described in this thesis (9 virus- plus 2 plant-specific) required an approach for SPC development which differed from that of Munro *et al.* (2005) and Smith et al. (2006).

Another modification relative to other studies was in the use of TRIzol protocol for total RNA extraction, including luteovirus infected samples (mostly cereals), both for RT-PCR (Deb and Anderson 2008) and RT-qPCR applications (Balaji *et al.* 2003). In this study, using the TRIzol protocol, as suggested by the manufacturers resulted in a pigmented total RNA solution with low $A_{260/280}$ (<1.5) and $A_{260/230}$ (<1) readings. Modification of the protocol thus was necessary (see Section 2.2.4) and resulted in a colourless total RNA solution and increased $A_{260/280}$ to ~2, but did not affect $A_{260/230}$. RNA preparations obtained using this modified TRIzol protocol were successfully used in RT-PCR (amplification product was seen on the gel). The modified TRIzol protocol also resulted in higher yield of total RNA (as estimated spectrophotometrically). Although delivering total RNA comparable in quality to the total RNA extracted using the RNeasy Plant Mini Kit (Qiagen) and being cheaper overall (~$5NZ vs ~16$NZ per extraction), the modified TRIzol protocol uses highly toxic material (phenol, chloroform, isopropyl alcohol) and requires overnight incubation. Therefore, although health and safety and time costs are issues, the importance of RNA purity for RT-PCR application suggests that the cheaper TRIzol method might be used, although special care should be taken regarding personal protection and

purity/quality of total RNA. Therefore, gel electrophoresis is highly advisable prior to use of TRIzol extracted total RNA in subsequent reactions, especially for RT-qPCR applications.

The generic detection method described in this thesis was able to detect a much wider range of luteoviruses than previously published for other protocols. The three novel combinations of generic primers (C1F1-C1F2-C1R1-C1R2, C2F1-C2F2-C2R3 and C1F1-C2R3) detected thirteen species, and based on sequence similarity it is likely that at least another eleven species, not tested in the current study, could also be detected. In particular, isolates of the luteoviruses BYDV-GPV, BYDV-RMV, BYDV-SGV, GRAV and TVDV could not be obtained for this study, even though their published CP gene sequences were used during the primer design. Sequence analysis also showed that some of the generic primers have at least 90% sequence identity with CYDV-RPS (genus *Polerovirus*), CpSDaV (unassigned *Luteoviridae*) and tentative species which have been reported since the last ICTV report (Table 1.2), for example: CpCSV (Abraham *et al.* 2006), CpYV and LStV (Abraham *et al.* 2008), and CLRDV (Corrêa *et al.* 2005). Apart from their use to detect and identify known species, the new primer sets described in this study are likely to be of use in detecting previously unreported luteoviruses. The generic primers may be useful not only as a detection tool, but may also allow identification to species or at least genus level when combined with sequencing. The variability of the sequences in the 148 bp, or even 68 bp amplicons, (Figure 2.2) might also be sufficient to enable differentiation of species by subsequent melting curve analysis instead of the more time consuming and costly sequencing. This approach was successfully applied for detection and differentiation of three different *Plum pox virus* strains (Varga and James 2005). This is further explored here for the luteoviruses in Chapter 3.

2.5 Conclusions

This study, using RT-PCR, is the first comprehensive assessment since 1991 of a generic detection method for the luteoviruses Thirteen *Luteoviridae* species were detected using three separate sets of low-degeneracy generic primers with RT-PCR to amplify 68-, 75- and 129⁄156-bp regions of the coat-protein gene. Species detected include all members of the genus *Luteovirus* and eight of nine species from the genus *Polerovirus*. These primers were not able to detect *Carrot red leaf virus* (recently assigned to *Polerovirus* genus, see Table 1.1), *Sweet potato leaf speckling virus* (unassigned *Luteoviridae*) and *Pea enation mosaic virus-1* (genus *Enamovirus*). This is of particular relevance to New Zealand biosecurity, because CtRLV, SPLSV and PEMV-1

were not reported in New Zealand in the last review (Pearson *et al.* 2006). Carrot, sweet potato (kumara) and lentils such as pea are all grown in New Zealand and it is important to keep these economically important crops free of exotic viruses in order to prevent yield losses. Therefore species-specific diagnostic methods have to be used for their detection as well as for other luteoviruses, which cannot be detected using the generic primers described (list of these species have yet to be fully determined). A synthetic positive control containing all primer sequence priming sites was designed to facilitate this method as a generic tool for use with a variety of host plants. The generic primers described in this study present a simple infection-detection tool of benefit to biosecurity authorities and may also be useful in detection of as-yet undiscovered species within the *Luteovirus* and *Polerovirus* genera.

Chapter 3

Detection and discrimination of members of the family *Luteoviridae* by real-time PCR and SYBR® GreenER™ melting curve analysis

3.1 Introduction

Fast and accurate detection and identification is important for helping to control the spread of the *Luteoviridae* species and reduce their economic impact (D'Arcy *et al.* 1999). The generic primers for thirteen *Luteoviridae* species developed in Chapter 2 target the coat protein (CP) gene which is the most conserved region in their genomes. Following reverse transcription PCR amplification of the CP gene using these generic primers, cDNA sequencing is able to accurately confirm virus identity. However, routine diagnosis using this method requires access to a sequencer and purchasing of expensive reagents and equipment. A potentially quicker and cheaper alternative to sequencing is to follow the real-time PCR step by melting curve analysis (MCA) (see Section 1.6).

The objectives for the research presented in this chapter were to investigate the suitability of real-time PCR plus MCA for the detection of nine *Luteoviridae* species as well as the potential of this technique to discriminate among them. No such approach for use with luteoviruses has been previously published. Only one published effort on the use of the MCA for detection and identification of plant viruses, namely *Plum pox virus* strains C, EA and W, has been reported (Varga and James 2006). In addition, because T_m values (as the key discriminating factor) have been shown to be affected by various components of the reverse transcription reaction, such as dithiothreitol (DTT) (Lekanne Deprez *et al.* 2002) or salts, as well as low template concentrations (Ririe *et al.* 1997) and template DNA type (plasmid or cDNA) (Mouillesseaux *et al.* 2003), this study included a preliminary investigation of their impact on detection of the viruses and species discrimination. A previously designed artificial SPC template, containing generic *Luteoviridae* and plant-specific primers annealing sites (see Section 2.2.7) was also evaluated as a positive control for use with MCA to monitor the success of amplification.

Various combinations of *Luteoviridae* are frequent in nature, for example mixed poleroviruses in beet or mixed luteoviruses in cereals are often found (Viganó and Stevens 2007; Deb and Anderson 2008). Therefore, a preliminary investigation on the real-time detection of mixed virus templates was undertaken in this study.

3.2 Materials and methods

3.2.1 *Luteoviridae* isolates and RNA extraction

Forty one isolates representing thirteen species (BChV, BLRV, BMYV, BWYV, BYDV-MAV, BYDV-PAV, BYDV-PAS, CABYV, CYDV-RPV, PLRV, SbDV, ScYLV and TuYV) in two genera (*Luteovirus* and *Polerovirus*) were used (Table 2.1). The range of species was based on those previously successfully amplified by PCR using generic primers (see Section 2.3.2). At least two isolates of each species were investigated. All isolates were stored as in Section 2.2.1. Total RNA extractions were performed by different methods as described in Section 2.2.4.

3.2.2 Reverse transcription

Reverse transcription reactions were performed and their success was verified by a plant-specific PCR amplification of the cDNA using nad5-s and nad5-as primers (Menzel *et al.* 2002) as described previously in Section 2.2.5.

3.2.3 Identification of *Luteoviridae* in field isolates

The species identity of isolates in the plant material provided (Table 2.1) was confirmed by sequence analysis of the PCR products as described in Section 2.2.6.

3.2.4 Real-time PCR and MCA

Two forward primers (C2F1, C2F2), that anneal to the same region but differ in their 3′ end sequence, and two reverse primers (C2R3, C2R1+2) (Figure 2.2) were used in two combinations: (a) C2F1–C2F2–C2R3 and (b) C2F1–C2F2–C2R1+2. These generated overlapping PCR products of 68 bp and 148 bp respectively. Additionally, C1F1-C1F2-C1R1-C1R2 and C1F1-C2R3 primer combinations were used for amplification of BYDV (129/156 bp amplicon(s)) and ScYLV (75 bp amplicon) isolates respectively. The real-time PCR and subsequent melting step were performed on an ABI PRISM® 7000 (ABI, Foster City, USA) using the SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen, Calrsbad, USA) with 150 or 300 nM of each forward and reverse primer, plus 1 µl of cDNA (see Section 3.2.2) template in a final reaction volume of

20 μl. As negative controls, 1 μl of healthy *Raphanus sativus* plant-derived cDNA was used as well as cDNA from two *Sobemovirus*-infected samples, CoMV-DSMZ on *Dactylis glomerata* (NCBI ID: GU002336) and RYMV-DSMZ on *Oryza sativa* (NCBI ID: GU002344). RNA extractions for these samples were performed using RNeasy Plant Mini Kit (Qiagen). Due to the limited amount of viral cDNA available from the isolates used in this study, plasmids containing the CP gene region (see Section 2.2.6) were used as additional templates at 1:200 times dilution. An initial denaturation step was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence readings were determined during the anneal/extension step at 60°C. Following real-time PCR, the amplicons were melted at 95°C for 15 s then fully annealed at 60°C for 20 s. The temperature was then increased incrementally to 85°C at the rate of 0.2°C/min (as fixed on the ABI PRISM® 7000). At least two real-time PCRs and MCAs were run for each isolate and each type of the template (cDNA and plasmid). Each isolate was tested at least in duplicate and the average T_m was calculated from the replicate MCA runs. Controls included: no template – water negative and SPC plasmid (Figure 2.3).

3.2.5 Estimation of amplification efficiency of generic primers

The estimation of amplification efficiency of generic primers was performed with a six-fold serial dilution of SPC template (Figure 2.3), which contained sequences of all generic primers. Real-time PCR was done using four combinations of generic primers (Table 2.3) as described in Section 3.2.4. Once the respective C_q were obtained at different dilutions, they were plotted against the logarithmic value of input SPC concentration. Amplification efficiency was calculated by using the formulae $E = (10^{(-1/\mathrm{slope})} - 1) \times 100$ (Pfaffl 2001).

3.2.6 Factors potentially affecting the T_m

3.2.6.1 Salt concentration and/or DTT

Two of the plasmid templates (SbDV-BB2 and BWYV-3) and three cDNA templates (CYDV-RPV-RL1, SbDV-BB2 and BWYV-JF6) were used. Plasmid templates with DTT or salts were prepared by adding DTT or 5× First Strand buffer (SuperScript™ III Reverse Transcriptase kit, Invitrogen, Carlsbad, USA) respectively to the plasmid at concentrations reflecting those used in reverse transcription reactions (5 mM DTT and 1× First Strand buffer). cDNA templates without DTT were prepared by synthesizing cDNA from total RNA as described in Section 3.2.3 but substituting DTT with water. As synthesizing cDNA without 5× First strand buffer was not possible, the effect of salts could only be investigated using the plasmid templates.

3.2.6.2 Plant host species

Two plants from each of three cereal species (*Avena byzantina* (oats, cv Coast Black), *Hordeum vulgare* (barley, cv Emir) and *Triticum aestivum* (wheat, cv Regency)) were infected with CYDV-RPV-RL1 isolate. *Rhopalosiphum padi* aphids were allowed a virus acquisition period of at least 96 h on infected CYDV-RPV-RL1 oat plants before being used to inoculate the test plants. On each plant 10 viruliferous aphids were placed in a clip-cage which was attached to the bottom part of the stem on one month-old seedlings. Aphids were left for three days to feed, then the cages were removed and aphids killed with insecticide. Leaves from clearly symptomatic plants were collected one month later for MCA analysis.

3.2.6.3 RNA extraction method

RNA was extracted from the same age oat plants, which were grown under the same conditions and infected with the same virus strain as the CYDV-RPV-RL1 virus isolate (see Section 3.2.6.2). Four different methods were used, RNeasy® (Qiagen, Hilden, Germany), TRIzol (Invitrogen, Carlsbad, USA), Thermo Kingfisher mL (Thermo Scientific, Helsinki, Finland) and the method of Célix *et al.* (1996). Three different oat plants were analysed per extraction method, and the RNA extraction and cDNA synthesis conducted as described in Sections 3.2.1 and 3.2.2.

3.2.7 Investigating the suitability of artificial SPC template as a positive control for MCA

Duplicate real-time PCR plus MCA runs were performed using 20 ng of an artificial SPC template developed previously (see Section 2.2.7 and Figure 2.3). Real-time PCR amplification was performed using each of three primer combinations: C2F1–C2F2–C2R3, C2F1–C2F2–C2R1+2 and nad5-s nad5-as. A total of six T_m readings were analysed for the amplicons generated with each primer combination.

3.2.8 Statistical analysis

Mean T_m was calculated for each isolate using data generated from the cDNA templates. A comparison of mean T_m among the different *Luteoviridae* species was performed using GenStat 11th edition and a general one way ANOVA (analysis of variance) with no blocking, together with the Fisher Unprotected least significant difference (l.s.d.) test.

3.2.9 Preparation of mixed *Polerovirus* templates and MCA

Two plasmid templates (SbDV-BB1 and BWYV-4 (Table 2.1)) at 2 ng μL^{-1} were mixed *in vitro* in four different ratios (1:1, 1:10, 1:50 and 1:100). Real-time PCR and MCA was done as described in Section 3.2.4 using C2F1-C2F2-C2R3 primers and 1 μL of template.

3.3 Results

3.3.1 Amplification efficiency of generic primers

Amplification efficiencies of three combinations of generic primers are given in Table 3.1. C1F1-C1F2-C1R1-C1R2 and C2F1-C2F2-C2R3 combination of primers had similar amplification efficiencies; in contrast the amplification efficiency of C2F1-C2F2-C2R1+2 primer combination was lower (96.1%).

Table 3.1 The slope, coefficient of determination (R^2), efficiency and C_q of generic primers estimated using SPC template.

Primer combination	Slope	R^2	Efficiency	C_q at 1:1000 SPC dilution
C1F1-C1F2-C1R1-C1R2	-3.27	0.995	102.2	18.11
C2F1-C2F2-C2R3	-3.25	0.997	103.1	18.26
C2F1-C2F2-C2R1+2	-3.42	0.995	96.1	21.53

3.3.2 Identification of *Luteoviridae* using real-time PCR with SYBR® GreenER™ and MCA

The average T_m of real-time PCR products generated from cDNA of the different species using C2F1–C2F2–C2R3 and C2F1–C2F2–C2R1+2 primers are given in Table 3.2. For most isolates a single repeatable melting peak was observed for both amplicons (Figure 3.1a, b). However, the melting peak of 68 bp amplicons from some beet-infecting virus isolates (from genus *Polerovirus*) was occasionally double-humped (Figure 3.1c, d); in those cases the T_m value of the constantly observable melting peak (always present in all independent runs) was selected for data analysis. For the 148 bp amplicons generated from cDNA templates a single melting peak was observed for 14 out of 20 isolates. The exceptions were for isolates of BMYV-2ITB, BWYV-FB3, both CYDV-RPV (Kin3 and RL1), and both TuYV isolates (FL1 and MS), where the melting curve was low and/or wide and the exact estimation of T_m was hindered. However, all of

those isolates were amplified successfully from plasmid templates, as was CYDV-RPV-RL1 which, unlike all other isolates, could not be detected using the C2F1–C2F2–C2R1+2 primers in the standard PCR as described earlier in Section 2.3.2. Considering use of different templates, the melting peak was often lower or less pronounced when cDNA was used (Figure 3.1a), compared to with melting peaks seen for corresponding plasmid templates (Figure 3.1b). The differences in T_m between different types of the templates (cDNA and plasmid) were 0.0 – 0.8°C (Table 3.2) and did not appear to be constant.

Table 3.2 GC content and mean T_m of 68 bp and 148 bp amplicons using plasmid and cDNA templates generated with C2F1-C2F2-C2R3 and C2F1-C2F2-C2R1+2 primers respectively.

Standard t-test showed no significant differences between the mean T_m from plasmid and cDNA templates for both amplicons at P=0.05.

Name of isolate	GC% of 68 bp fragment	Mean T_m of 68 bp amplicon from cDNA template	Mean T_m of 68 bp amplicon from plasmid template	GC% of 148 bp fragment	Mean T_m of 148 bp amplicon from cDNA template	Mean T_m of 148 bp amplicon from plasmid template
BLRV-DSMZ	57.4	79.8	80.3	54.1	82.0	82.5
BLRV-AUS	57.4	80.4	80.1	54.1	82.1	82.0
SbDV-BB1	45.6	75.0	74.9	45.9	78.4	78.7
SbDV-BB2	45.6	75.0	74.9	45.9	78.1	78.4
SbDV-400	45.6	75.0	74.9	46.6	78.0	78.8
BChV-2a	52.9	77.3	77.5	50.7	79.5	79.8
BChV-MS	52.9	77.3	77.5	50.7	79.5	80.1
BMYV-2ITB	57.4	79.6	79.7	51.4	N/A	80.3
BMYV-MS	57.4	79.4	79.6	52.7	80.0	80.7
BWYV-3	51.5	76.5	76.6	49.3	79.1	79.2
BWYV-4	51.5	76.5	76.6	49.3	79.1	79.2
BWYV-JF6	51.5	76.8	76.5	49.3	79.2	78.9
BWYV-FB3	51.5	76.3	76.5	49.3	N/A	78.9
BWYV-FB4	51.5	76.6	76.5	49.3	79.2	79.2
CYDV-RPV-Kin3	50.0	76.2	76.1	48.6	N/A	79.4
CYDV-RPV-RL1	51.5	77.3	77.0	48.0	N/A	79.5
CABYV-USA	51.5	77.4	77.7	50.7	-	-
CABYV-ES1	52.9	77.9	77.7	51.4	-	-
CABYV-ES2	52.9	78.1	77.9	49.3	-	-
CABYV-ES3	52.9	77.9	77.7	49.3	-	-
CABYV-ES4	52.9	77.8	77.7	51.4	-	-
PLRV-DSMZ	52.9	76.9	77.0	50.7	80.6	80.3
PLRV-JF	52.9	N/A	77.0	52.0	80.3	80.9
PLRV-5	52.9	76.8	76.7	50.7	80.1	80.0
TuYV-FL1	54.4	77.9	77.9	49.3	N/A	79.2
TuYV-MS	51.5	76.6	76.5	48.6	N/A	78.9

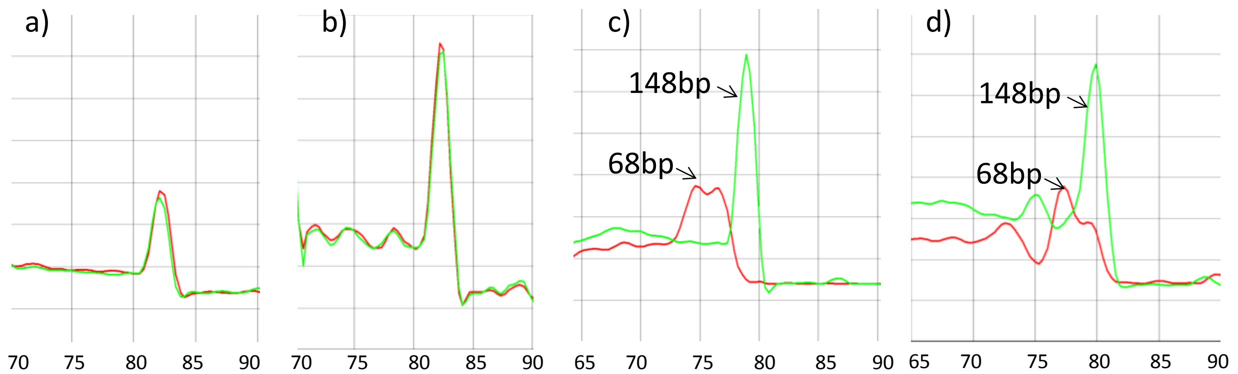


Figure 3.1 Examples of melting peaks.

Single melting peak of 148 bp amplicon of BLRV-DSMZ isolate, derived from cDNA template (a) and plasmid template (b). Melting peaks of 68 bp (double humped) and 148 bp amplicons of BWYV-4 (c) and BMYV-MS (d) isolates, derived from cDNA template.

Comparing the isolates of the same species, T_m values for the 68 bp amplicon differed for all species except BWYV and PLRV; that of the TuYV-FL1 isolate was 77.9°C ± 0.15 compared to 76.6°C ± 0.12 for TuYV-MS; two isolates of CYDV-RPV (RL1 and Kin3) had T_m of 77.3°C ± 0.05 and 76.2°C ± 0.39 respectively. Due to the lack of cDNA material the PLRV-JF isolate was not amplified with the C2F1–C2F2–C2R3 primers.

T_m values of 148 bp amplicons were higher than those of 68 bp amplicons for the corresponding templates by 0.6–3.9°C (Table 3.2).

For the BYDV species the average T_m ± SD of the additional 129/156 bp real-time PCR products generated using the alternative C1F1–C1F2–C1R1-C1R2 primer set are summarised in Table 3.3.

Amplicons of all isolates investigated produced a single repeatable melting peak from both cDNA and plasmid templates (data not shown). T_m of all three BYDV species differed by less than 0.4 ºC. The melting peak was considerably less sharp when cDNA was used, compared to with melting peaks seen for corresponding plasmid templates (same was noticed for amplicons of other species generated with C2F1-C2F2-C2R3 and C2F2-C2F2-C2R1+2).

Due to the lack of cDNA material only one ScYLV isolate (SA) was amplified with the C1F1-C2R3 primers: the 75 bp amplicon produced had the T_m of 77.23 ± 0.15. However, using plasmid templates the three ScYLV isolates (MA5, MA3 and SA) produced a uniform single melting peak of an average 77.18 ºC ± 0.16; which was higher and sharper than that of the ScYLV-SA generated from cDNA template (data not shown).

PCR amplification and MCA from healthy and the outgroup *Sobemovirus*-infected plant-derived cDNA with C2F1–C2F2–C2R3 or C2F1–C2F2–C2R1+2 primers yielded no discernible virus-associated melting peak; a single melting peak at 76.38 ± 0.32°C was observed only with host plant-specific nad5-s - nad5-as primers.

Table 3.3 Average T_m of the BYDV 129/156 bp amplicons generated with C1F1-C1F2-C1R1-C1R2 primers.

Species	Number of isolates investigated	Average T_m ± SD (cDNA templates)	Average T_m ± SD (plasmid templates)
BYDV-PAS	3	79.7 ± 0.0	79.9 ± 0.0
BYDV-MAV	2	79.5 ± 0.2	80.2 ± 0.0
BYDV-PAV	7	79.7 ± 0.1	80.1 ± 0.1

3.3.3 Discrimination of *Luteoviridae* species using MCA

The average T_m values for seven of the nine species obtained from 68 bp and 148 bp amplicons of cDNA templates are shown in Figure 3.2. T_m values for the 68 bp amplicons of all isolates of the same species were used for the statistical comparison of BChV, BLRV, BMYV, BWYV, CABYV, PLRV and SbDV. However, isolates within each of the other two species, TuYV and CYDV-RPV, had very different T_m values and therefore these species were not included in the statistical analysis. Due to the lack of data (see Section 3.3.1) only five species (BChV, BLRV, BWYV, PLRV and SbDV) were included in the comparison of the average T_m of 148 bp amplicons. Statistical analysis shows that six of the seven species presented in Figure 3.2 have significantly different T_m values and can be discriminated from each other with the 68 bp amplicons (BWYV and PLRV have overlapping T_m range and cannot be discriminated); all five species examined using the 148 bp amplicons also can be discriminated ($P < 0.05$) (Figure 3.2).

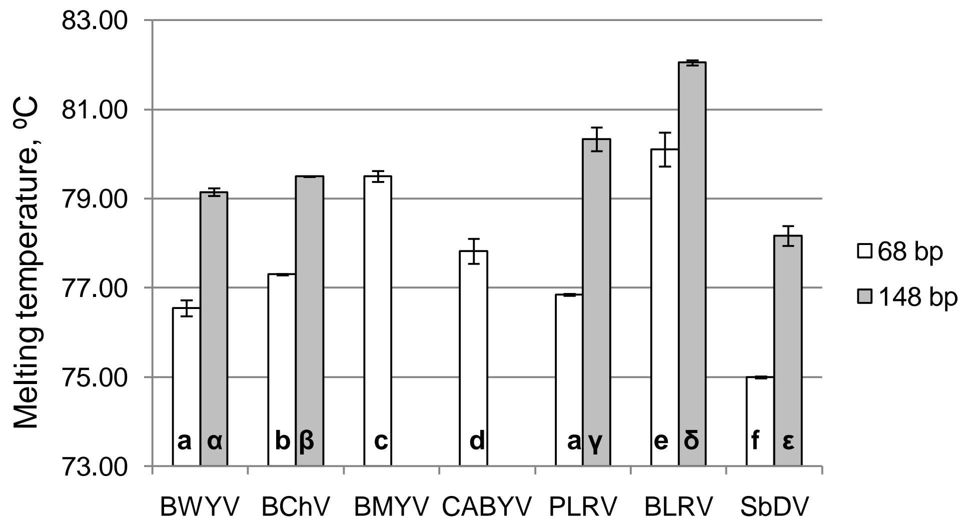


Figure 3.2 Mean T_m ±SD of 68 bp and 148 bp amplicons (n = 2-5).

Different a-f and α-ε letters/symbols show the significant differences in T_m recorded among different *Luteoviridae* species for 68 bp and 148 bp amplicons respectively. Error bars represent standard deviation.

3.3.4 Influence of salts and DTT on T_m of viral amplicons

The T_m of the 68 bp real-time PCR products derived from cDNA (BWYV-JF6, SbDV-BB2 and CYDV-RPV-RL1) or plasmid (BWYV-3 and SbDV-400) templates were unaffected by salts or DTT (see Table 3.4).

3.3.5 Influence of RNA extraction method on T_m

Isolation of total RNA from all three cereal species using the Célix RNA extraction method produced low quality RNA; this was likely due to the method being originally developed for plants from the *Cucurbitaceae* family, and is a very simple protocol with relatively few purification steps. Therefore only the other three RNA extraction methods were compared. T_m for the 68 bp product derived from cDNA of CYDV-RPV-RL1 as extracted with RNeasy® (Qiagen, Hilden, Germany), TRIzol (Invitrogen, Carlsbad, USA) or Thermo Kingfisher mL (Thermo Scientific, Helsinki, Finland) were 77.14°C ± 0.17, 77.04°C ± 0.26 and 77.02°C ± 0.24, respectively (average T_m was calculated from a total of 12 T_m readings for each RNA extraction method). Therefore, the extraction method did not significantly affect the T_m, with the l.s.d. at $P<0.05$ of 0.25 being larger than any differences between means observed.

Table 3.4 Mean T_m (°C ± S.D.) of the 68 bp fragment amplified from cDNA and plasmid templates with and without DTT or salts.

	Plasmid		
	SbDV-400	BWYV-3	
Plasmid	74.88±0.35	76.68±0.39	
Plasmid + DTT	75.05±0.35	76.90±0.35	
Plasmid + salts	74.95±0.17	76.88±0.12	
	cDNA		
	SbDV-BB2	BWYV-JF6	CYDV-RPV-RL1
cDNA - DTT	75.15±0.31	76.72±0.40	77.32±0.15
cDNA+ DTT	75.20±0.24	76.75±0.39	77.08±0.30

3.3.6 Influence of host on T_m

The T_m for the 68 bp product derived from cDNA of CYDV-RPV-RL1 virus infected barley, oat and wheat plants were 77.2°C ± 0.23, 77.0°C ± 0.13 and 76.9°C ± 0.23, respectively (average T_m was calculated from a total of eight T_m readings for each plant species). Only the difference of 0.30°C between the T_m derived from barley and wheat plants was significant (l.s.d. at $P<0.05$ = 0.26°C).

3.3.7 T_m generated from artificial SPC template

The C2F1–C2F2–C2R3 and C2F1–C2F2–C2R1+2 primers successfully generated two amplicons each for the artificial SPC template at 255 bp/272 bp and 293 bp/310 bp respectively (see Table 2.3). However, only one melting peak was observed for each of primer combination: at 85.4°C ± 0.15 and 85.12°C ± 0.13 respectively. The nad5-s - nad5-as plant reference amplicon (154 bp) generated from the SPC template, produced on average melting peak at 84.5°C ± 0.29.

3.3.8 Detection of mixed *Polerovirus* infection using MCA

Melting curve analysis of the amplification products from mixed virus plasmid templates resulted in either double-humped (Figure 3.3a) or broad peak (Figure 3.3b) for the 1:1 ratio, but that pattern was not seen with other ratios of templates: the melting peak of the less diluted template was strongly dominant, fully eliminating the melting peak of the more diluted template (Figure 3.3c).

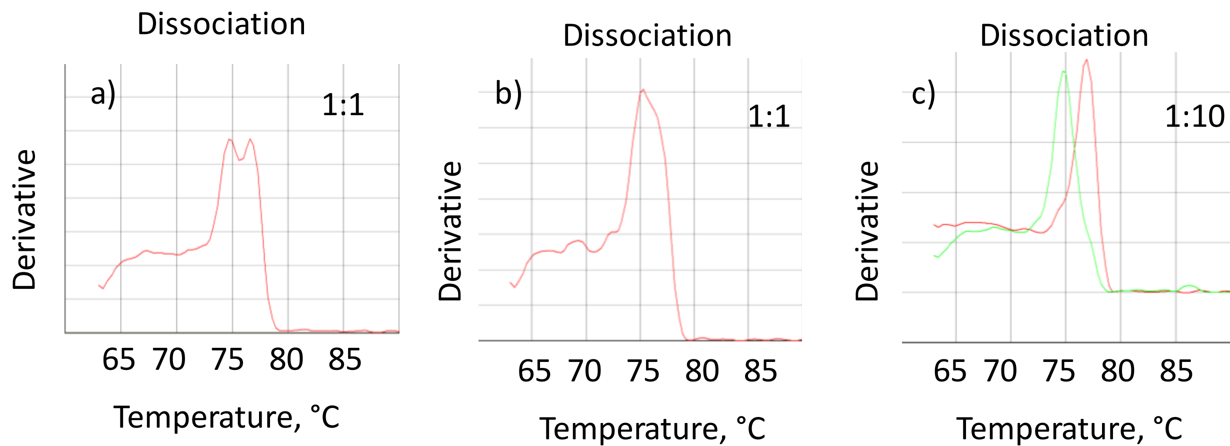


Figure 3.3 Melting profiles of SbDV (~75°C) and BWYV (~76.5°C) plasmid templates in ratios of 1:1 (a and b) and 1:10 (c).

3.4 Discussion

Melting curve analysis (Ririe *et al.* 1997; Reed *et al.* 2007) provides a much faster, more accurate and cheaper alternative to common nucleic acid detection method of viruses by gel electrophoresis (Vossen *et al.* 2009; Yvon *et al.* 2009; Winder *et al.* 2010; Winder *et al.* 2011) and in some cases to sequencing. The results presented in the current study now also support the use of MCA in detection and discrimination of species within the *Luteoviridae* family. With generic primers, MCA showed that the amplicons of each isolate for the nine species tested had a characteristic melting peak of reproducible shape and T_m. These were distinct from those of non-specific amplicons, such as primer dimers, and can be used to confirm species-specific infection.

Exceptions, however, were for the two CYDV-RPV isolates (Kin3 and RL1) had different T_m for the 68 bp amplicons because of five nucleotide differences between them. Also, for the same reason (two nucleotide differences), the melting profile of TuYV isolates (MS and FL1) also differed. Interestingly, the melting peak of TuYV-MS was strikingly similar in shape and T_m to the BWYV isolates. This is consistent with the confusion over the taxonomy of BWYV and TuYV species (see Section 2.4). Sequencing revealed significant similarity between the isolates of these two species, being 100% for the 68 bp region and 99.3% in the 148 bp region. In contrast, sequence identity between the TuYV-FL1 isolate and BWYV isolates was lower: 97.1% and 97.3% for the 68 bp and 148 bp amplicons respectively. Therefore, interpretation of MCA data has to be considered carefully in the light of established taxonomy.

However, it should be noted that the 2–5 isolates of each *Luteoviridae* species used in this study do not necessarily represent the total sequence diversity. Fast rates of mutation often occur in genomes of RNA viruses, constantly introducing new nucleotide sequence types (Holland *et al.* 1982; Castro *et al.* 2005) that are likely to influence T_m. While the results show relative intra-species consistency in T_m values, the specific values will need to be reassessed when additional sequence variants are discovered.

Of the two generic primer combinations used in this study, only that for the 68 bp fragment (C2F1–C2F2–C2R3) successfully amplified all 26 isolates (listed in Table 3.2) tested. In contrast, the C2F1–C2F2-C2R1+2 primers had lower sensitivity (C_q values at 1:1000 SPC dilution were 21.53 vs 18.26) and amplification efficiency (96.1% vs 103.1%) (Table 3.1) which probably hindered real-time PCR amplification of low copy number targets and so the 148 bp amplicon could only be generated for 15 isolates. In addition, the T_m variation for the 68 bp amplicon (for the same species) was less than that for the 148 bp amplicons and resulted in more confident discrimination between species; this unfortunately is obvious only for the T_m derived from the plasmid templates, due to insufficient data generated from cDNA templates (Table 3.2). The observation that longer amplicons tend to have more variable T_m has been made previously (Varga and James 2006) and is consistent with the increased variation of GC content that was found in 148bp amplicons. Consequently the 68 bp fragment provided a more robust method of detection and discrimination of these viruses.

Higher GC content of both the 68 bp and 148 bp amplicons correlated well with a higher T_m (Table 3.2). However, overall GC composition of a DNA fragment is not a reliable predictor of T_m (Monis *et al.* 2005). Where different sequences of the same size have the same GC content (such as 68 bp sequences of CABYV isolates from Spain and PLRV, see Table 3.2), the sequences will not necessarily melt at the same T_m and might have very different melting profiles. This is because the distribution of the GC bases in the sequence (equally distributed vs a GC clump at one end) also determines the kinetics of the amplicon melting (Ririe *et al.* 1997): sequences with uneven distribution of GC bases will have AT rich sequence regions where dsDNA melts more rapidly than at GC rich regions, whereas sequences where GC bases are distributed equally will melt in a single gradual transition.

Most isolates generated a single melting peak. However, melting peaks of 68 bp amplicons for both cDNA and plasmid templates for some beet-infecting *Polerovirus* isolates occasionally

appeared double-humped, but not for 148 bp amplicons. Double-humped melting peaks usually indicate that the sequence has two domains which melt at a different rate because of different GC% content of each region (Pangasa *et al.* 2009). Although these melting domains that cause the double-humped melting profile in the 68 bp amplicon, are also present in the 148 bp amplicon (68 bp and 148 bp amplicons overlap, because both forward primers are the same), the double-humped melting profile is not apparent when the 148 bp amplicons are analysed. Presumably the two melting domains in the 68 bp amplicon merge with the rest of the nucleotide sequence of the 148 bp region and dissolve in the sequence context (a similar observation was also made by Krypuy *et al.* (2007)). This is also supported by the POLAND DNA denaturation algorithm (Steger 1994) (often used to predict multi-peaks melting profiles) which predicts a double-humped melting profile for the 68 bp BWYV amplicon, but not for the 148 bp amplicon. Despite their less well defined shape and reproducibility, double-humped melting peaks are still clearly discernible and indicative of specific real-time PCR amplification products. Indeed, such melting profiles are often used diagnostically (Monis *et al.* 2005; Pangasa *et al.* 2009).

This study also confirmed that an artificial SPC template (Figure 2.3) can be used as a positive template to monitor the performance of two generic primer sets as well as the plant specific primers used in this study. The forward (C2F1, C2F2 and nad5-s) and the reverse (C2R3, C2R1+2 and nad5-as) primer annealing sites in the artificial SPC template are separated by an insert, therefore amplification from the artificial SPC template results in the different size amplicons than those generated from the viral templates. The T_m of amplicons generated from the artificial SPC template were higher than any of the T_m generated from the viral templates (both plasmid and cDNA), which is consistent with longer fragments tending to have a higher T_m (Mouillesseaux *et al.* 2003). Therefore, the melting peak generated from the artificial SPC template can be distinguished from amplicons of viral origins generated with the same primer combination. This is helpful if any cross-contamination occurs.

In the current study the 68 bp amplicons gave better discrimination between the *Luteoviridae* species than the 148 bp amplicons in MCA. However, the optimal amplicon size for MCA is dependent on the taxonomic group being studied. For discrimination among plant viruses such as the *Luteoviridae* species or strains of PPV, the most suitable amplicon size is ~70 bp (Mouillesseaux *et al.* 2003; Varga and James 2006); with smaller than ~70 bp amplicons the T_m can be confused with those generated from primer dimers, whilst melting of larger amplicons is

more likely to result in greater T_m variation among isolates of the same species. Elsewhere it also has been shown that 155 bp amplicons have lower resolution than 74 bp amplicons in discriminating among three strains of PPV (Varga and James 2006), and increasing the size of a desired amplicon from 50 bp to 75–100 bp was shown to facilitate discrimination by distinct T_m of the desired amplicons and primer dimers (Mouillesseaux *et al.* 2003).

Differences in the T_m values were observed during this study between different isolates of the same species using cDNA templates. This might have been influenced by plant host for which different plant derived impurities may be co-extracted and amplified during the viral cDNA preparation (Varga and James 2005). Given the sensitivity of this method, testing isolates from different hosts belonging to different plant families, such as hosts of BWYV (infecting more than 100 plant species from 21 families as cited in Coutts *et al.* (2006)), would be needed to confirm a relationship between viral host and T_m. Certainly this would be important to establish if this method were to be used diagnostically.

Small variations in T_m between replicate assays of the same sample were observed for the majority of isolates, as has been reported in other studies (Nicolas *et al.* 2002a; Nicolas *et al.* 2002b; Monis *et al.* 2005; Varga and James 2006), although the SD did not exceed 0.5°C for either the 68 bp or 148 bp amplicons. One factor generating this within-sample variation may have been the dynamic thermal uniformity of the 96-well ABI PRISM 7000 instrument. Herrmann et al. (2006) found that the T_m range for the same amplicons across the plate for a particular ABI 7000 machine was 0.66°C - that was shown for a single machine only. As the thermal uniformity of the instrument used during this study is unknown, this characteristic should be evaluated further. Another factor may have been the degeneracy of the four (C2F1, C2F2, C2R3 and C2R1+2) generic primers. Annealing of different versions of a degenerate primer (which arise because the primer is composed of a mixture of each sequence to make up the degeneracy), effectively changes the sequence of the product and can change the GC% ratio of the PCR product, both of which influence the T_m. This is especially true in the case of the C2R1+2 primer, as it has two double-degenerate base positions at the 5′ end. For the technique to be used diagnostically, the assay-specific variation needs to be measured and must be significantly smaller than the differences in T_m between species, as occurred for most of the viruses tested during this study.

Low copy number cDNA template, due to use of old cDNA stocks, was a problem during this study. This resulted in a low fluorescence signal which hindered the estimation of the T_m for some isolates, particularly when the C2R1+2 primer was used. Although long-term storage at -20 °C is accepted practice, the stocks of cDNA were gradually diminishing in quality (mainly cDNA concentration) as experiments progressed. This was probably caused by DNAses which contaminated the stock tubes as well as a consequence of frequent handling (thawing and refreezing) and could be avoided by aliquoting at the outset and using each aliquot only once. Using fresh stocks of cDNA might help to avoid the problem of low fluorescence signal. Alternatively, two strategies that have been shown to increase fluorescence based on increased success of real-time PCR amplification have been to use primers with 5′ AT-rich overhangs (Afonina *et al.* 2007) or add DMSO (dimethyl sulfoxide) to 5% (Jung *et al.* 2001) and these could be explored further. However, this approach trialled here by attaching a 5'-AT rich flap to the C2R3 primer (C2F1-C2F2-C2R3 primers) did not influence the fluorescence signal when tested with the SPC template, although it significantly reduced accumulation of the non-specific products, such as primer dimers (Figure 3.4).

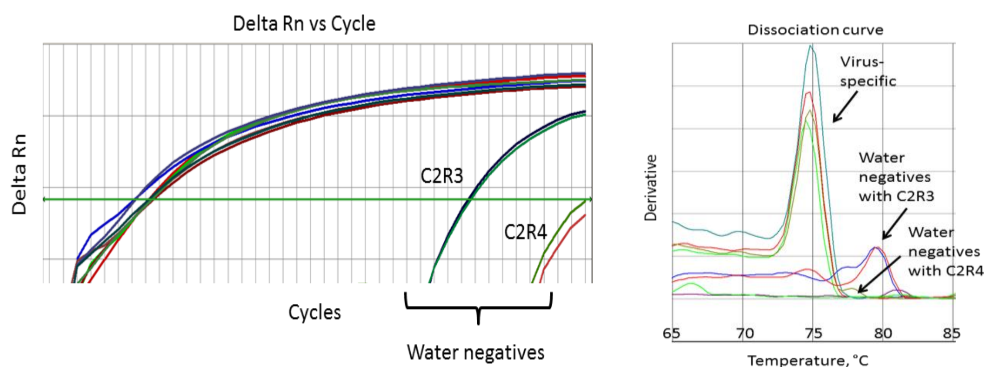


Figure 3.4 Amplification curve and melting profile with C2F1-C2F2-C2R3 and C2F1-C2F2-C2R4 primers.

Reverse primers C2R3 and C2R4 share the same sequence, but C2R4 has an additional 5'-AT-rich flap (TATAATAATATA).

Also, the addition of a 5'-flap increased the length of the primer and consequently the length of the amplicon, which theoretically, should have increased the T_m of amplicon. However, no such

effect was observed; presumably the effect of the flap was too insignificant to affect the T_m because of weak hydrogen bonds between the A and T nucleotides.

Considering the mixed template situation, the clear predominance of the melting peak of the less diluted template could be due to the melt rate used for analysis, which was 0.2°C/s (fixed on ABI PRISM). Varga and James (2006) showed that a lower melt rate (0.1°C/s) resulted in improved resolution of the melt peaks, while at a higher melt rate (0.4°C/s) only one melt peak out of two was discernible. A similar study using viral cDNA mixes would also be desirable. Nevertheless, even if differentiation of the viruses is not possible in such cases, detection of at least one virus-specific signal is possible, however, such outcome might not be acceptable for diagnostics and therefore additional tests might be required.

As this technique is relatively novel, few studies to date have applied MCA for plant virus detection and identification to demonstrate its potential and limitations. This current study therefore presents a significant contribution towards this. The virus amplicons resulted in well-pronounced virus specific peaks, clearly differentiated in temperature range from those generated by primer dimers and confirming virus infection without the need of subsequent agarose gel electrophoresis. The system was discriminatory enough to distinguish between six species using the C2F1-C2F2-C2R3 primers. Unfortunately, amplicons from the three *Luteovirus* species (BYDV-MAV, BYDV-PAV and BYDV-PAS) using primers C1F1-C1F2-C1R1-C1R2 did not show enough sequence diversity to enable their differentiation based on the T_m or their melting profiles (Table 3.3). Nevertheless, MCA is a convenient tool to detect virus infection, even though it may not always be able to differentiate closely related species. For example the third primer combination (C1F1-C2R3) used for the detection of ScYLV can also be used for MCA – a high sharp melting peak was seen for all three ScYLV virus isolates.

3.5 Conclusions

All nine *Luteoviridae* species tested were detectable using MCA, which is a faster diagnostic alternative to gel electrophoresis. It has also been shown that MCA has the potential to discriminate BChV, BMYV, CABYV, BLRV and SbDV species from each other and from either PLRV or BWYV by having discrete ranges of T_m. The use of generic primers together with MCA analysis is recommended for the evaluation of symptomatic plants for the absence or presence of the *Luteoviridae* infection. This generic priming system will also be useful in detection of

unknown isolates of *Luteoviridae* species. Further investigation on the extent of sequence variability of different isolates within a species would be useful to better define the species T_m range. Some variation in the melt profile was observed during this study, which was affected by the host plant and the genotype of the virus isolate; furthermore, some variation was seen between replicates too, therefore the method has to be thoroughly validated before it could be used as a routine diagnostic application.

Chapter 4

Evaluation of RT-qPCR using generic primers for detection of low titre of virus infection

4.1 Introduction

Plants infected with luteoviruses usually express yellowing/reddening and/or dwarfing symptoms after 2-3 weeks, although some remain symptomless much longer (D'Arcy 1995). Often, expression of symptoms positively correlates with increased luteovirus titre in a plant (Zhu *et al.* 2010). However some plants, especially grasses, manage to harbor relatively high titre of luteoviruses but remain symptomless (Fargette *et al.* 1982).

Plants harbouring low virus titre can still be considered as virus reservoirs, because, for example, it was shown that the ability of aphids (*R.padi*) to transmit CYDV-RPV is not influenced by various virus titres among source leaves (Gray *et al.* 1991). Overlooked infection of potential reservoir plants may later lead to disease outbreaks resulting in serious yield losses.

Detection of luteoviruses is well developed and is commonly performed using serological methods or species- or group-specific reverse transcription PCR (RT-PCR) (D'Arcy *et al.* 1999). However, ELISA often fails to detect low titres of luteoviruses (Balaji *et al.* 2003; Zhu *et al.* 2010) whereas RT-PCR is known to be more sensitive and to detect even lower luteovirus titre than ELISA. However, RT-PCR lacks a quantitative component, which would be valuable for more accurately assessing the temporal and spatial distribution of viruses.

RT-qPCR coupled with MCA is an example of a more sensitive technique than standard RT-PCR to detect and quantify luteoviruses in symptomless plants (see Section 1.6). Using this technique, Balaji *et al.* (2003) detected BYDV-PAV and CYDV-RPV in susceptible yet asymptomatic *Avena sativa* as early as 2 and 6 hours post inoculation, respectively, using species-specific primers. Expanding the scope of this species-specific assay by using generic primers would be very useful for routine diagnosis where several viral species could be responsible for the disease. Three combinations of generic primers have now been developed and can accurately detect and identify infection in symptomatic plants using both RT-PCR (Chapter 2) and RT-qPCR melting

curve analysis (Chapter 3). However, these assays require assessing for their sensitivity, including for the detection of low virus titre that might be present in plants.

Another important factor in early detection is to employ the most appropriate tissue sampling regime with respect to the location of infection, especially when it is not yet apparent. Although plenty of information is available on *Luteoviridae* detection (D'Arcy *et al.* 1999), most studies fail to indicate which part of the plant was used for analysis, often referring simply to "plant tissue" (e.g. Balaji *et al.* 2003). This approach is usually based only on host symptoms, sometimes leading to variation in experimental set up and poor reproducibility. For example, younger leaves often remain asymptomatic for longer (with some exceptions, such as oat plants with bright red/orange discolouration on the younger leaves, caused by novel Alaska BYDV (Robertson and French 2007), and loss of green colour is often more prominent in older leaves of infected hosts (D'Arcy 1995). The tissue sampling regime may also differ depending on the type of analysis (serological or nucleic acid based). This is because the concentration of virus particles (produced during the translation stage and detected by ELISA) and viral genomes (produced during the transcription stage and detected by RT-PCR and RT-qPCR) might significantly differ in a plant tissue sample depending on the stage of the plant virus life cycle (Mandahar 2006).

For ELISA the most symptomatic leaf of a plant, presumably infected with luteovirus, is usually selected, as the virus tends to accumulate in such leaves more than in other asymptomatic tissues (J.D. Fletcher, pers. comm.). A similar observation was made by Pereira and Lister (1989) who found that CYDV-RPV titre (as determined by ELISA) was higher in older leaves of infected *Avena sativa*. On the other hand, the majority of the plant viruses tend to reproduce more rapidly in the younger leaves and, consequently, the youngest leaves of the plant should be selected (M.N. Pearson, pers. comm.). This is consistent with the observation that the mean virus titre (as determined by ELISA) was lower in the older leaves of source plants for all three BYDV species (PAV, MAV, RPV) in a study by Gray *et al.* (1991). Hewings (1995) found that, young leaves also contained 10x more BYDV-MAV and 3x more BYDV-PAV particles than old leaves, but no differences between old and young leaves were observed for CYDV-RPV; this was explained by the fact that different BYDV/CYDVs were known to peak in the host at different times. While some indication of the distribution of luteovirus proteins/particles in host plants exist, no studies have examined the importance of host plant tissue collection strategies for nucleic acid based detection.

Few studies have investigated the distribution of luteoviruses within a plant and results are confounded by the use of different virus/host combinations and age of the plant. For example, past studies using mainly biological techniques support the view of using both old and young cereal leaves for analysis: Carrigan *et al.* (1983) reported that BYDV translocates throughout *Avena sativa* and *Triticum aestivum* plants within 24 hours, while a more recent study on the movement of ScYLV from inoculation site to other parts of the *Saccharum officinarum* shows that virus tends to accumulate in younger leaves (Lehrer *et al.* 2007). However, the Carrigan *et al.* (1983) and Lehrer *et al.* (2007) studies were done using different virus detection methods (biological techniques and immunoblot assay respectively) and therefore comparison of the results from these studies is subjective.

qPCR has been applied for luteovirus detection in both in aphids (Fabre *et al.* 2003) and plants (Korimbocus *et al.* 2002; Balaji *et al.* 2003; Agindotan *et al.* 2007; Zhu *et al.* 2010). Its phenomenal sensitivity is especially valuable and readily apparent for luteovirus detection, as concentration of luteoviruses in plants is believed to be low, in comparison with other plant viruses, presumably because they are phloem-limited and phloem only makes up a small percentage of the total leaf tissue. All the current RT-qPCR applications for luteovirus detection and quantification are based on using species-specific primers that usually restricts the use of such an approach to single virus species. Expanding the use of RT-qPCR with generic primers would significantly streamline the diagnosis, as the same protocol might be applicable for detection and quantification of many luteovirus species. Furthermore, RT-qPCR with generic primers might also be useful in providing the additional information on the sequence of an amplicon and detecting unknown virus sequence variants due to the broad-range annealing properties of primers. Consequently it was decided to investigate the hypothesis that given the greater sensitivity of the RT-qPCR and its quantitative advantage over the RT-PCR, as well as its successful application for detection of other luteoviruses (see above), RT-qPCR is a suitable method of detecting low titres of *Luteoviridae* infection using generic primers. This was assessed using double infection of BYDV-PAV and CYDV-RPV as a model in order to investigate the performance of two generic primer combinations (C1F1-C1F2-C1R1-C1R2 and C2F1-C2F2-C2R3) in detecting luteoviruses in both old and young leaves of the same plants at early stages of virus infection.

4.2 Materials and methods

4.2.1 Virus transmission

A mixed population of *Rhopalosiphum padi* and *Metopolophium dirhodum* aphids (*Hemiptera*: *Aphididae*), routinely maintained on *Hordeum vulgare* (cv Dash) plants were allowed a virus acquisition period of at least 96h on BYDV-PAV/CYDV-RPV-infected *Avena byzantina* plants before they were used to inoculate test plants. Ten random, mixed species, viruliferous aphids of different growth stages were transferred to a clip-cage. Each clip-cage was attached to the base of the stem of approximately one month old *H. vulgare* (barley, cv County – BYDV susceptible, for the October 2009 experiment; and cv Emir for the January 2011 experiment) and *A. byzantina* (oat, cv Coast Black) plants (depending on the experiment), which at this stage have 2-4 leaves (growth stage 12-14 (Tottman *et al.* 1979)). Plants were grown under natural light in insect-proofed, air-conditioned glasshouses maintained at 18–24ºC in peat sand growth mix with osmocote slow release fertilizer and watered twice daily. The selection of host plants was based on the observation that oat and barley are (in general) more severely affected by B/CYDVs than wheat (Irwin and Thresh 1990). Aphids were left for three days to feed in order to maximise the transmission of the viruses (Gray *et al.* 1991), then cages were removed and aphids killed with an insecticide; this was considered to be 'day zero' post inoculation (0 dpi).

4.2.2 Source and storage of plant material

One whole plant leaf (minimum 10 cm) was collected from 4-6 plants (biological replicates) at each of 3, 6, 9, 12 and 15 dpi. Depending on the experiment, the tissue sample collected was either the oldest (but still green) leaf (for October 2009 experiment) or both the oldest and the youngest leaves (for January 2011 experiment). Each sample was put in a separate plastic bag and stored at -80°C. At the time of tissue collection plants had 3-5 leaves (growth stage 13-15) and were noted as either asymptomatic or showing very mild virus disease symptoms (weak leaf yellowing and vein reddening in oat or weak 2-5 mm long and thin white leaf striping at the top of the leaves in barley). Plants were then left to grow for further development of the disease such that infection could be confirmed with TAS-ELISA at 19 dpi (see below).

4.2.3 Confirmation of BYDV-PAV and CYDV-RPV infection in plants with TAS-ELISA

Most of the plants were showing leaf reddening and plant dwarfing symptoms on oat or weak white leaf striping symptoms on barley at 19 dpi. Therefore, fresh and green symptomatic leaf samples (~10 cm) were collected from infected 19 dpi old greenhouse-grown plants and tested immediately by TAS-ELISA to confirm the virus species causing disease. Leaf tissue (~0.5 g) was weighed and diluted 1:10 in PBS Tween PVP (pH 7.4) extraction buffer, then crushed using Bioreba AG tissue crusher in a heavy plastic bag. The manufacturer's method (Neogen Europe Ltd – Agden Phytodiagnostics) was followed using their monoclonal antibodies for CYDV-RPV or BYDV-PAV, except, conjugate solution anti-rat IgG (Sigma–Aldrich Corp., St. Louis, USA) was used instead of that provided, because it was found to be more reliable based on previous experience (J.D. Fletcher, pers. comm.). 4-Nitro Phenyl Phosphate substrate (0.5 µg mL^{-1}) was added and absorbance at 405 nm read after 60 min using a ThermoMax microplate reader (Molecular Devices) with SOFTmaxPro software. A signal, exceeding the mean of six healthy control samples plus 3 SD, was considered to be positive.

4.2.4 Total plant RNA extraction and reverse transcription

Frozen (-80C°) leaf tissue (50-100 mg) was ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was then extracted using either RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) in April 2010 for the October 2009 experiment, or Plant Total RNA Mini kit (Viogene, Sunnyvale, USA) in February 2011 for the January 2011 experiment according to the manufacturer's directions. The different methods were used to reduce RNA extraction costs. The extracted RNA samples were stored at -80C° until further use. DNase digestion was performed only for RNA extracted using the Viogene kit, because it is not generally required with the RNeasy Kit. DNase digestion was performed using on-column DNase digestion with the RNase-Free DNase set (Qiagen, Hilden, Germany). Additional changes to the DNase digestion protocol included (i) substituting 350 µL of buffer RW1 (Qiagen) with 250 µL of buffer WF (Viogene) and (ii) centrifuging for 1 min at 14000 g instead of 15 sec at > 8000 g. The yield and purity of RNA samples were checked with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Montchanin, USA).

The integrity of all RNA samples was checked by loading 10 µL of total RNA (prepared as in Sambrook *et al.* (1989) onto 1.2% agarose MOPS (3-(N-morpholino) propanesulfonic acid) /formaldehyde gels. Gel electrophoresis was run in MOPS buffer for 2-3h at 40 V and 500 mA.

Reverse transcription was done using the SuperScript™ III Reverse Transcriptase, as recommended by the manufacturer, with 250 ng of random hexamer primers and 500 ng of total RNA in 20 µL reaction volume. To compare the virus titre in young and old leaves (January 2011 experiment), the reverse transcriptase was removed, as it has been shown that its presence in the subsequent RT-qPCR reaction may contribute to the overestimation of the amplification efficiency (Suslov and Steindler 2005). Reverse transcriptase was removed by treating each sample with 30 µg of Proteinase K (Sigma–Aldrich Corp., St. Louis, USA) and adding 1.5 µL of 60 mM EDTA (ethylene diamine tetra-acetic acid) to chelate extra magnesium ions present in the 5x First Strand Buffer (15 mM $MgCl_2$) that might otherwise influence downstream RT-qPCR. The mixture was incubated at 56°C for 15 min and Proteinase K was deactivated at 95°C for 1 min. The success of the reverse transcription was checked by performing a RT-PCR with 250 nM plant-specific nad5-s and nad5-as primers using GoTaq® Green Master Mix (Promega, Madison, USA), 2 µL of cDNA template in 20 µL volume. The PCR profile was as in Menzel *et al.* (2002). Additionally, the presence of genomic DNA in cDNA preparations was checked by preparing 'no-RT controls' for each sample. Reverse transcription for these was done the same way as described above, substituting only RNaseOUT™ Recombinant Ribonuclease Inhibitor and SuperScript™ III Reverse Transcriptase with 50% glycerol. 'No-RT controls' were checked for plant-specific amplification with nad5-s - nad5-as primers as described above. Aliquots of cDNA were stored at -20C°.

4.2.5 Optimization of primer concentration for RT-qPCR

The performance of three separate primer combinations (C1F1-C1F2-C1R1-C1R2, C2F1-C2F2-C2R3 and nad5-s-nad5-as) was optimised using three different primer concentrations (50, 150 and 300 ng) of each forward and reverse primer. This was done in order to determine the primer concentration for a real-time reaction which results in the maximum fluorescence intensity and the absence of primer dimmers. From the pool of isolates that were used for this study (see Section 4.2.2), the cDNA of two barley and two oat samples with a high titre of BYDV-PAV and CYDV-RPV were selected for analysis. The minimum forward and reverse primer concentration that yielded the maximum fluorescent intensity (ΔR_n) was 150 ng per primer. A single high

melting peak, with no additional peaks indicative of primer dimer, was observed on the melting profile for all reactions. Reactions were also run on 4% agarose gel to confirm the absence of primer dimers.

4.2.6 RT-qPCR assay

For RT-qPCR the following primers were used: C2F1-C2F2-C2R3 (for the detection of a CYDV-RPV 68 bp amplicon), C1F1-C1F2-C1R1-C1R2 (for the detection of a BYDV-PAV 129-156 bp amplicon(s)) (Table 2.3) and mRNA-specific nad5-s - nad5-as primers (plant mitochondrial *nad5* 181 bp amplicon). The RT-qPCR and subsequent melting step was performed as in Section 3.2.4 with 150 nM of each primer (determined by RT-qPCR optimization as described above). The temperature profiles of RT-qPCR and the melting step were as in Section 3.2.4. Reaction mixtures were pipetted manually using barrier tips (RNase/DNase & Pyrogen safe from Axygen). To minimise any errors due to pipetting, duplicates of each sample were performed on each plate. Reactions were carried out in a 96 well, clear microplate (Axygen, Union City, USA, Cat. Nr PCR-96-AB-C) using adhesive UltraClear Film for plate sealing (Axygen, Union City, USA, Cat. Nr UC-500). The RT-qPCR and MCA runs were repeated twice. Controls included: healthy plant cDNA, NTC (no template control - water negative) and SPC plasmid (contains C1, C2 and nad5 priming sites (Figure 2.3)).

Three selection criteria were adopted for determining that a sample under investigation was virus-positive: (i) exponential shape of the amplification curve, (ii) C_q value (quantification cycle (formerly C_t – threshold cycle)) compared to the healthy control (see below) and (iii) virus-specific melting peak (~80°C for BYDV-PAV and ~76°C for CYDV-RPV). Samples that did not meet all three criteria were considered to be inconclusive and were removed from quantification analysis.

4.2.7 Normalization of RT-qPCR and relative quantification

In this study the *nad5* (NADH dehydrogenase subunit 5) gene was selected for normalization of the RT-qPCR assay. The *nad5* mitochondrial DNA gene contains two exons separated by an 848 bp intron and was first characterised in apple by Kato *et al.* (1995). Menzel *et al.* (2002) developed primers for that gene in apple and prune hosts, with the forward primer annealing exactly to the two-exon splicing site making these primers mRNA specific. This enables the

specific amplification of RNA, even in the presence of genomic DNA, and decreases the cost of the assay because of the absence of additional treatment of samples with DNase.

Relative quantification of BYDV-PAV and CYDV-RPV in young and old leaves was done according to the modified amplification plot method described by Peirson *et al.* (2003). The theoretical fluorescence value (R_0) and the amplification efficiency of each virus-positive sample were calculated by setting the threshold line automatically, to generate C_q values by the Applied Biosystems Sequence Detection Software Version 1.2.3. (SDS v1.2), and omitting the water negative and healthy samples from the analysis. The C_q value for healthy samples was determined by setting the threshold line manually to be the same as the automatically generated (see above) fluorescence level determined for virus-positive samples. Samples where the C_q was higher than 'the mean C_q of healthy samples minus 2 SD from the mean' were not included in the subsequent analysis and considered as inconclusive results; higher C_q corresponds to lower virus titre, (i.e. lower template concentration) in a sample and *vice versa*. Replicates where the C_q differed by more than 1.5 cycles (i.e. template concentration between replicates differed more than twofold) were considered to be outliers and were also removed from the data analysis. Normalised R_0 values from two separate runs were averaged for data analysis.

4.2.8 Virus population studies

The investigation into the genotypes of BYDV-PAV and CYDV-RPV present in individual plants after inoculation with viruses was done by comparison of their nucleotide sequences of 475 bp and 301 bp coat protein gene regions respectively. The BYDV-PAV and CYDV-RPV-specific products were amplified with Lu1-Lu4 and CYDV-1 – CYDV-2 primers (Table 2.4) respectively using GoTaq® Green Master Mix (Promega, Madison, USA) and 2 µL of cDNA template, PCR profiles were as in Table 2.4. BYDV-PAV–specific amplicons were amplified from the two oat (9-1Y and 3-4Y) and two barley (9-2Y and 3-1Y) samples, and the CYDV-RPV-specific amplicons were amplified from the two oat (9-1Y and 3-1Y) and four barley (9-3Y, 9-4O, 9-1O and 9-1Y) samples. Sequencing was performed as described in Section 2.2.6 using 0.5 µL of the PCR mixture. CP gene sequences of the 475 bp (corresponding to 27-502 bp of BYDV-PAV-327 (GU002325)) and the 301 bp (corresponding to 1-301 bp of CYDV-RPV-RL1 (GU002338)) were obtained for BYDV-PAV and CYDV-RPV respectively. These sequences were edited manually and aligned with Mega 4 (Tamura *et al.* 2007) using ClustalW algorithm.

4.3 Results

4.3.1 Integrity of the RNA extraction and success of the reverse transcription step

Four ribosomal RNA bands and the absence of a smear indicated purification of intact RNA; in addition to the plant cytosolic 25S (svedberg) and 18S ribosomal RNA (rRNA) bands, the plastid rRNA bands common to plant samples were also present (most likely being 16S and smaller, according to their size) (Figure 4.1 and Figure 4.2). Estimation of the RNA integrity is important in order to ensure availability of the intact RNA templates for the subsequent reverse transcription step so that complete, full-length cDNA could be obtained at the end of the reaction.

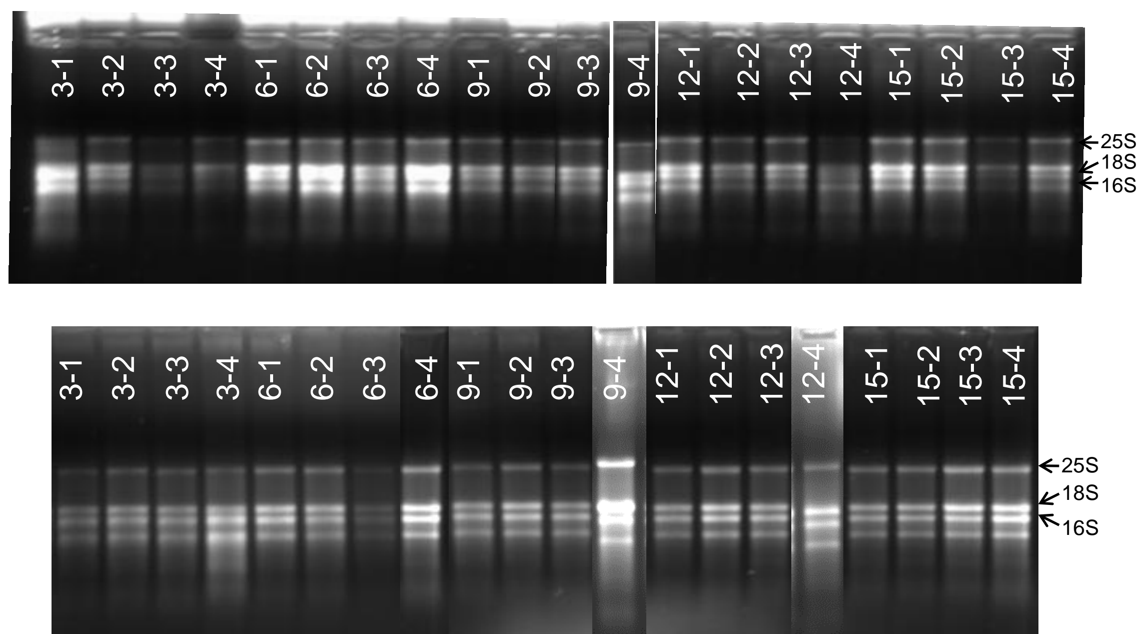


Figure 4.1 MOPS/formadehyde gel of oat (above) and barley (below) old leaf total RNA samples collected in October 2009.

First digit (3, 6, 9, 12 and 15) indicates the day post inoculation (dpi) at which sample was collected, second digit (1, 2, 3, 4) – number of the biological replicate.

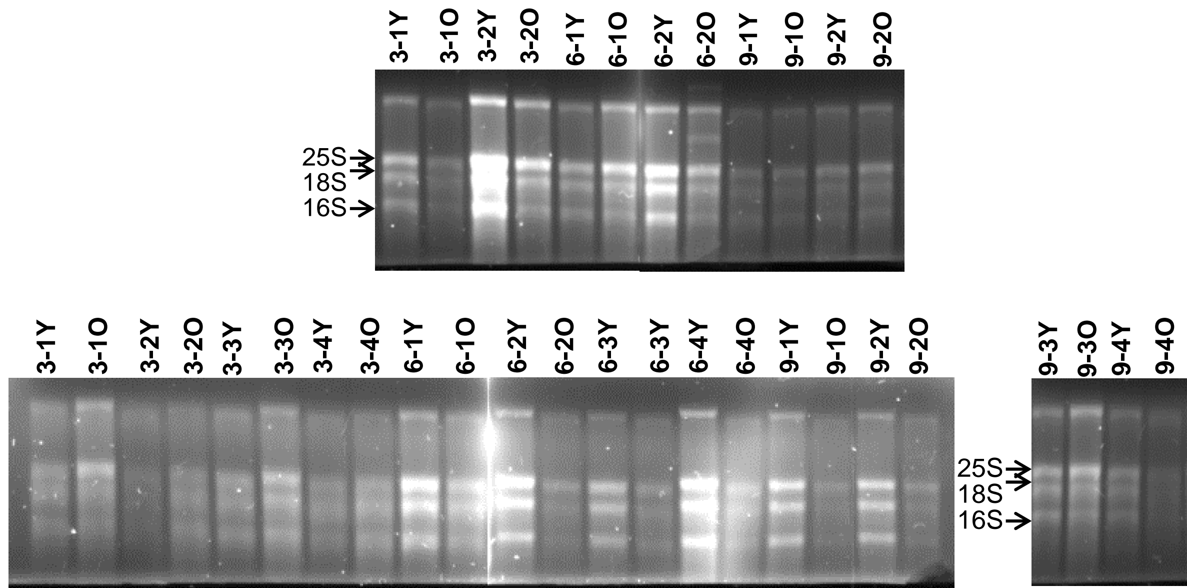


Figure 4.2 MOPS/formadehyde gel of oat (above) and barley (below) old and young leaf total RNA samples collected in January 2011.

First digit (3, 6, 9) indicates the day post inoculation (dpi) at which sample was collected, second digit (1, 2, 3, 4) – number of the biological replicate, letter (O or Y) indicates the old and the young leaf respectively.

Amplification with plant-specific primers (nad5-s – nad5-as) produced the uniform sharp specific 181 bp bands for all samples (see Figure 4.3 and Figure 4.4) confirming isolation of intact RNA and successful reverse transcription steps.

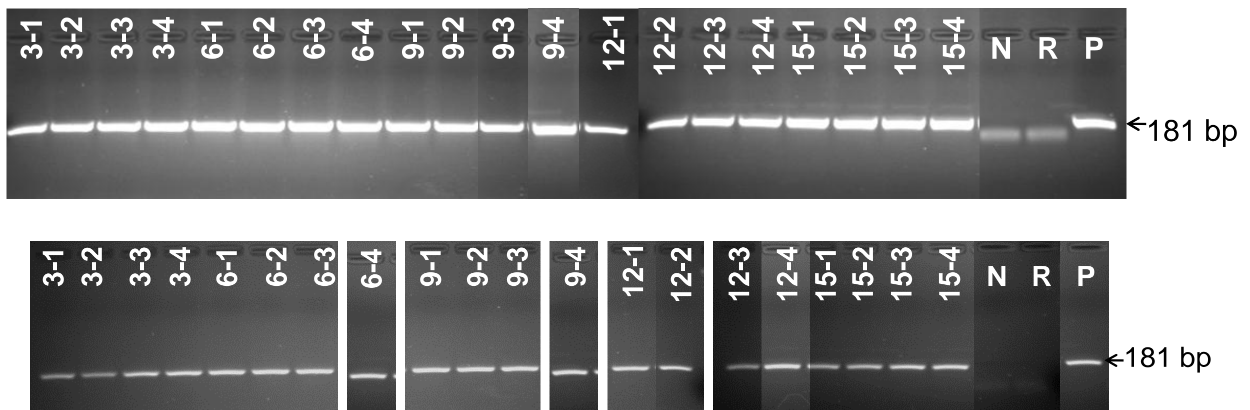


Figure 4.3 Amplification of oat (above) and barley (below) cDNA with plant-specific nad5s-as primers (October 2009 experiment).

First digit (3, 6, 9, 12 and 15) indicates day post inoculation (dpi) at which sample was collected, second digit (1, 2, 3, 4) – number of biological replicate, N – water negative, R – reagent, P – positive control.

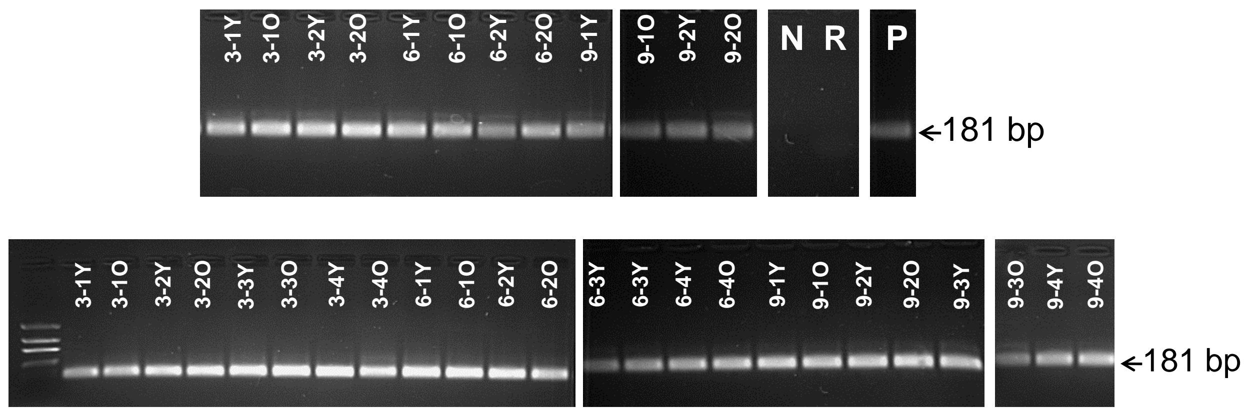


Figure 4.4 Amplification of oat (above) and barley (below) cDNA with plant-specific nad5s-as primers (January 2011 experiment).

First digit (3, 6, 9) indicates day post inoculation (dpi) at which sample was collected, second digit (1, 2, 3, 4) – number of biological replicate, letter (O or Y) indicates old and young leaf respectively. N – water negative, R – reagent, P – positive control.

4.3.2 Detection of BYDV-PAV and CYDV-RPV infection in old leaf (October 2009 experiment).

Twenty separate samples (Figure 4.5) of oat and barley (four biological replicates per sample collection day), which showed a positive TAS-ELISA signal for both BYDV-PAV and CYDV-RPV at 19 dpi, were selected for analysis of their tissue (old leaf) collected at 3, 6, 9, 12 and 15 dpi, and which had been frozen at -80°C.

The RT-qPCR amplification of BYDV-PAV in oat and barley confirmed the virus infection in all but one oat sample at 6 dpi. CYDV-RPV was detected in 2-3 out of the four biological replicates over the 3-15 dpi period in oat; whereas in barley CYDV-RPV was detected in all biological replicates at 3 and 15 dpi but with an apparent dip in virus detection at 6-9 dpi (Figure 4.5).

The amplification plot obtained for negative control (healthy) plants as well as water negatives, was different to that from infected material; the amplification curve was non-exponential and had a very late C_q (Figure 4.6). Also, although a melting peak indicating a virus specific amplification was discernible, it was less pronounced than that observed in the virus infected samples. Some water negatives produced a broad primer dimer peak at about 72-74°C. All SPC positives produced melting peaks as described in Section 3.3.7.

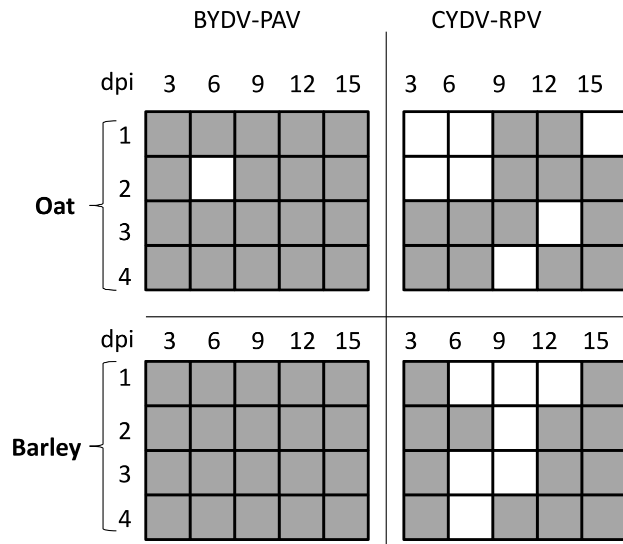


Figure 4.5 RT-qPCR detection of BYDV-PAV and CYDV-RPV in the oldest leaf (October 2009 experiment).

Asymptomatic oat and barley plants (20 of each) at 3, 6, 9, 12 and 15 dpi were analysed. Four biological replicates for each data-collection day are shown as 1, 2, 3, 4. White squares show no virus detection, grey – positive detection of virus.

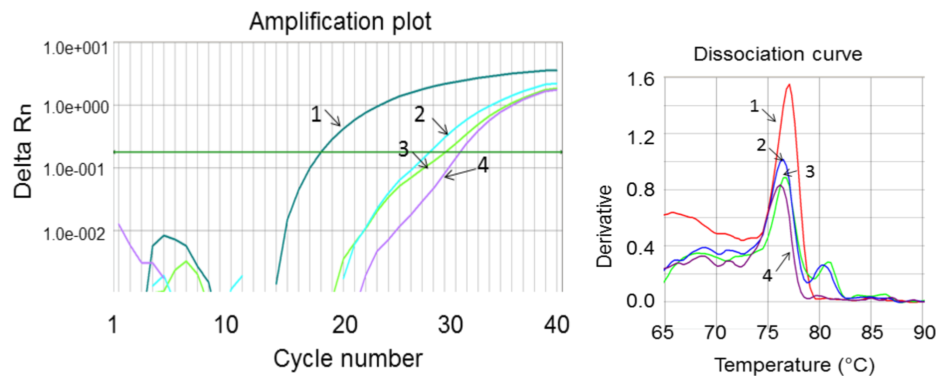


Figure 4.6 Examples of amplification plot and dissociation curves obtained during the RT-qPCR with C2F1-C2F2-C2R3 primers (CYDV-RPV detection).

1 – CYDV-RPV positive sample, 2 –CYDV-RPV negative sample , 3 – healthy barley, 4 – water negative.

Virus titre within each dpi varied greatly between individual oat and barley isolates (Figure 4.7) and there was no clear distribution pattern for either virus over time across dpi. While C_q of viral amplicons varied greatly among the samples within a RT-qPCR run, all C_q values generated with the nad5-s – nad5-as primers were in a stable range of 0.3-0.9 SD.

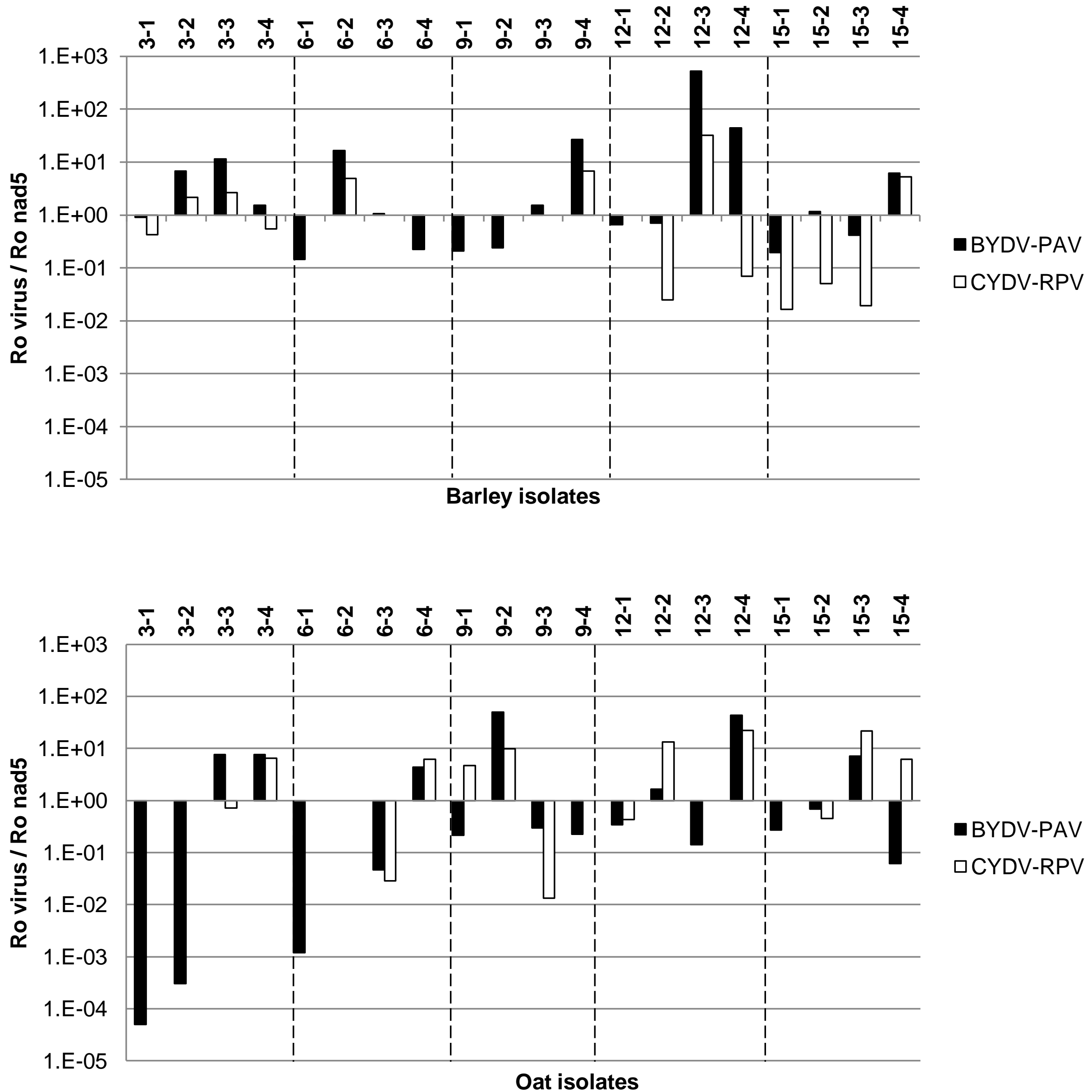


Figure 4.7 Relative quantification of BYDV-PAV and CYDV-RPV titre relative to nad5 plant gene in barley (above) and oat (below) (October 2009 experiment).

Y axis shows a logarithmic expression of R_o virus / R_o nad5. First digit (3, 6, 9, 12, 15) indicates dpi, at which sample was collected, second digit after the dash - 1, 2, 3, 4 – number of biological replicate.

Both viruses showed transcription levels both greater and less than the *nad5* in 13 oat and 13 barley plants. In oat no clear prevalence of one virus titre over another was found, whereas in barley BYDV-PAV was clearly more prevalent than CYDV-RPV in all 13 double-infected plants.

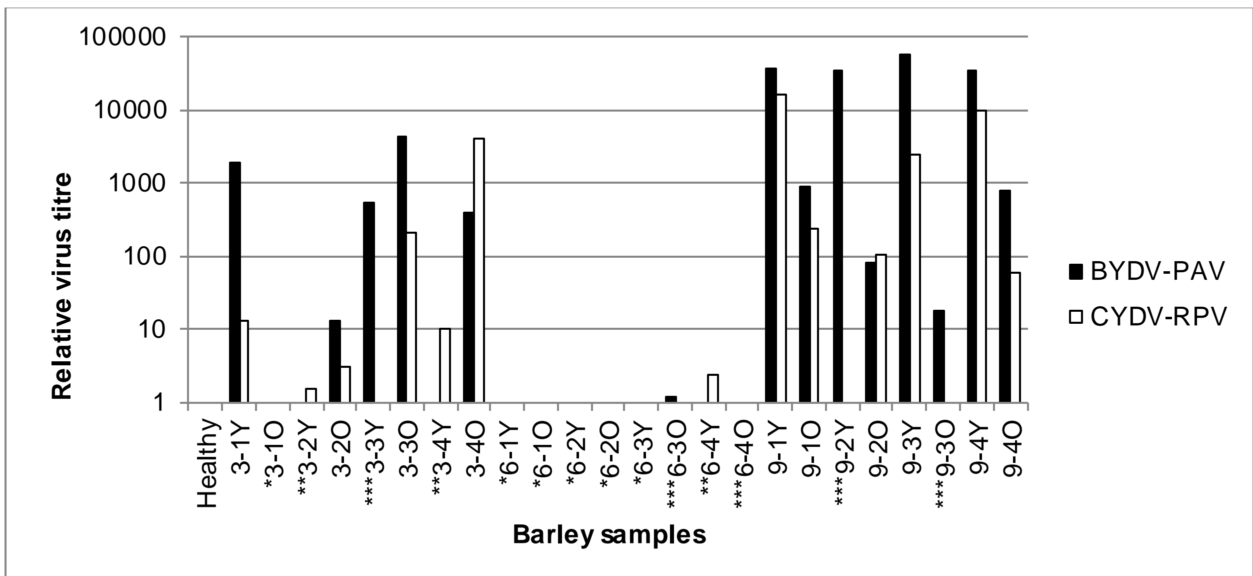
4.3.3 Comparison of the virus titre in the young and old cereal leaves (January 2011 experiment).

Twelve separate samples of oat and barley (four biological replicates per sample collection day), which showed a positive TAS-ELISA signal for both BYDV-PAV and CYDV-RPV at 19 dpi, were selected for analysis of their tissue collected at 3, 6, 9 dpi which had been frozen at -80°C.

Virus transmission in oats was less efficient than in barley, resulting in fewer infected plants (2 instead of 4 biological replicate samples per collection day) for RT-qPCR analysis. However, at the time of sample collection (starting at 3 dpi), barley plants were showing symptoms of both net blotch and powdery mildew fungal diseases (J.D. Fletcher, pers. comm.). The plants were therefore sprayed at 4 dpi with Amistar fungicide, although at a 50% lower dosage than that recommended by the manufacturer in order not to damage soft leaf growth of these glasshouse grown plants. Consequently, infection with net blotch and powdery mildew may have influenced the rate of virus infection in barley (Figure 4.8).

RT-qPCR detected BYDV-PAV in 10 (out of 12) oat and 15 (out of 24) barley samples, whereas CYDV was detected in 11 and 13 oat and barley samples respectively. Of these, double infection was detected in 10 (out of 12) oat and 10 (out of 24) barley samples; among these, the BYDV-PAV titre was higher than that of CYDV-RPV in eight barley samples, while the opposite was observed in nine oat samples (Figure 4.8).

In barley, no trend amongst young and old leaves was noticed for 3 dpi infected plants and almost no infection was detected at 6 dpi for either virus. At 9 dpi, however, a clear trend appeared with young leaves having a higher mean titre of BYDV-PAV than that in old leaves in all four biological replicates. A higher titre of CYDV-RPV was also observed in the young leaves in three out of four biological replicates respectively (no CYDV-RPV was detected in the 9-2Y barley sample meaning that the old leaf had higher CYDV-RPV titre). In oat, no consistency between virus titre in young and old leaves was apparent (Figure 4.8).



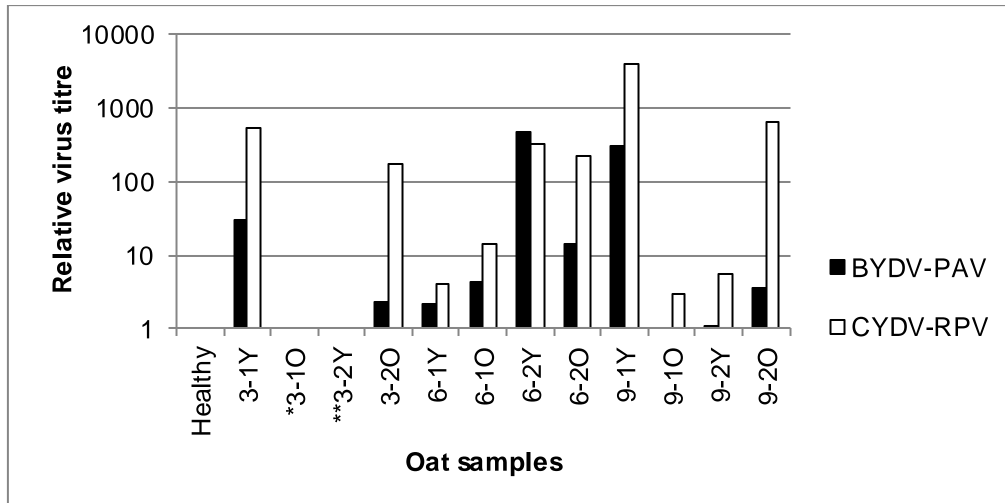


Figure 4.8 BYDV-PAV and CYDV-RPV titre in young and old leaves of asymptomatic barley (above) and oats (below) (January 2011 experiment).

First digit (3/6/9) indicates dpi, at which sample was collected, second digit – (1/2/3/4) – number of biological replicate, last letter (Y/O) indicates either young or old leaf. * - BYDV-PAV and CYDV-RPV negative sample, ** - BYDV-PAV negative sample, *** - CYDV-RPV negative sample. ("Negative" meaning no virus present at the time of measurement by RT-qPCR despite the positive ELISA signal at 19 dpi).

The fact that virus titre for some samples exceeded the *nad5* gene expression levels even at 3 dpi (Figure 4.7) suggests that both BYDV-PAV and CYDV-RPV accumulates to relatively high concentration in plants. *Nad5* gene is easily detected by standard PCR in many plant hosts (Chapter 2) because of multiple copies of this mitochondrial gene present in a single plant cell. In

fact, virus gene expression levels similar to that of *nad5* can be easily detected by the standard RT-PCR at 3 dpi as is shown for four oat samples in Figure 4.9.

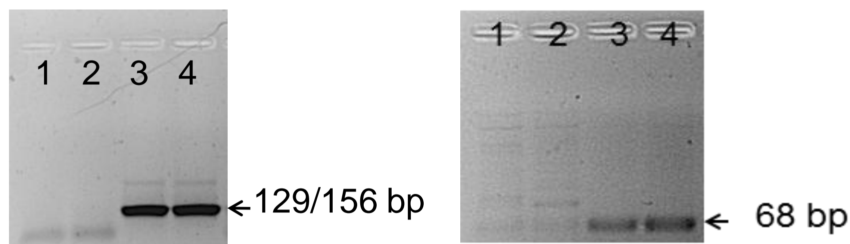


Figure 4.9 Detection of BYDV-PAV (left) and CYDV-PAV (right) at 3 dpi in oats.

The digits 1, 2, 3, 4 indicate the number of the biological replicate and correspond to the numbers 3-1, 3-2, 3-3 and 3-4 in Figure 4.7. RT-PCR was performed using GoTaq® Green Master Mix (Promega) (primer concentration and amplification conditions as described in Section 2.2.6).

4.3.4 Virus population studies

100% sequence similarity was observed among all four 475 bp BYDV-PAV coat protein gene sequences, as well as among all six 301 bp CYDV-RPV coat protein gene sequences (data not shown). This showed that oat and barley samples, representing plants from the same population, were infected with identical BYDV-PAV or CYDV-RPV genotypes (at least in the genome region sequenced).

4.4 Discussion

Nucleic-acid based detection methods are recognised as being very powerful, rapid, specific and highly sensitive techniques for plant virus detection (James *et al.* 2006; Boonham *et al.* 2008; Olmos *et al.* 2008; Vincelli and Tisserat 2008). Use of RT-qPCR in plant virus detection is well established, although studies on detection of *Luteoviridae* are rare (Korimbocus *et al.* 2002; Agindotan *et al.* 2007). Even fewer studies have investigated the detection of *Luteoviridae* in plants with low luteovirus titre and have relied on the use of species-specific-primers (Balaji *et al.* 2003). With the aim of producing a more convenient and robust method for routine diagnostics, this study investigated the use of recently described generic primers (Table 2.3) for detection of low titres of luteovirus infection using RT-qPCR. Infections with two cereal viruses (BYDV-PAV and CYDV-RPV) in oat and barley were used as model systems, with the assumption that because of the broad-specificity of these primers, the same protocol could be subsequently

applied to detect at least the other 11 *Luteoviridae* species (Section 2.3.2), although other luteovirus/host species combinations may require optimisation.

RT-qPCR detection of BYDV-PAV was possible as early as 3 dpi in all but one plant sample. Detection of CYDV-RPV on the other hand was not so successful, and may be consistent with the often lower titre of this virus in comparison with BYDV-PAV when in mixed infections with CYDV-RPV (Balaji *et al.* 2003). Nevertheless RT-qPCR is, in general, much more precise and discriminatory than conventional RT-PCR because detection is determined by strict drop-off criteria; therefore the decision as to whether a sample is infected or not is not as subjective as when assessing the intensity of the PCR product on an agarose gel. However, the results are complex, indicating that the virus titre differs between individual plants. Different titre dynamics may be explained by (i) different location of feeding aphids on a source plant and different availability of biologically active virus particles in different parts of that plant (Gray *et al.* 1991) and (ii) the fact that the ability to transmit virus differs between individual aphids within a given population due to genetic variation, developmental stage and morphs (Power and Gray 1995; Gray 1999).

With the model systems used here, the results indicate that both BYDV-PAV and CYDV-RPV tend to accumulate to a higher titre in young barley leaves (Figure 4.8). However, the same or a similar pattern was not seen in oats. Sequencing of randomly chosen oat and barley isolates revealed 100% nucleotide sequence identity among 475 bp and 301 bp coat protein gene sequences of BYDV-PAV and CYDV-RPV respectively, meaning that the observed difference between barley and oat is probably not due to the potential differences in susceptibility to different virus strains, although only a small part of the genome was sequenced and other regions not investigated might have revealed the differences in nucleotide sequence that could be responsible for differences in susceptibility.

Whilst the reason for this difference is unclear, it is consistent with the observation of Carrigan *et al.* (1983) that BYDV translocates throughout the oat plants within the first day; consequently infection appears almost equally present in all leaves of the oat plant. Indeed, very little is known about luteovirus movement in infected plants. Viral proteins involved in luteovirus movement have been shown to interact with plant cell structures (plasmodesmata or various organelles (Schmitz *et al.* 1997) and proteins (protein kinases (Sokolova *et al.* 1997)). Therefore luteovirus movement in the plant might also be plant species-dependent, influenced by factors such as (i)

proteins comprising plasmodesmata of phloem cells, (ii) sap-derived polypeptides, (iii) plant defence system or (iv) resistance-factors (Taliansky and Barker 1999).

The apparently spurious result in barley in the January 2011 experiment, showing a dip in BYDV-PAV titre at 6 dpi (Figure 4.8), coincided with the plants being sprayed with fungicide at 4 dpi. However, as the quality and yield of the total RNA of 6 dpi barley plants was high, and the C_q values for amplification with plant specific primers did not differ from the subsequent samples, there is no evidence that the fungicide directly inhibited the RT-qPCR *per se*. A possible explanation is that the plant's defence response was induced by the fungicide. Amistar's active ingredient – azoxystrobin - belongs to a class of fungicides named strobilurins, which have been shown to enhance the resistance of tobacco against *Tobacco mosaic virus*, acting either via a salicylic acid signaling mechanism or separately from it (Herms *et al.* 2002). A similar response induced in barley could therefore have resulted in a greater plant defence against the virus, seen here as temporarilly low titre at 6 dpi. The effect of strobilurins on virus accumulation in plants could be further explored as a potential means of chemical control of virus infection.

In contrast, in a different experiment (October 2009), the apparent dip in CYDV-RPV detection in barley at 6 and 9 dpi (Figure 4.5) is not related to fungicide treatment, as these plants were free from fungal infection over the period of that experiment. Furthermore, the dip was seen in CYDV-RPV detection only, and did not affect BYDV-PAV (which was prevalent in the same plant samples) detection as it did in the other experiment, where plants were treated with fungicide (January 2011, Figure 4.8). This dip in detection was temporary, although the CYDV-RPV virus titre in most of the barley samples never reached the same concentration at 12 and 15 dpi as before the dip at 3 dpi. Low virus titre (undetectable by nucleic acid techniques) may indicate that replication of CYDV-RPV was exclusively suppressed at 6 and 9 dpi. As this effect was found in barley only, it might be linked to plant defence response (such as post-transcriptional gene silencing) which is pathogen specific and is known to vary between different plant species (Soosaar *et al.* 2005).

For the experiments described in this Chapter, natural wild type field-obtained BYDV-PAV and CYDV-RPV isolates were used. The selection of these particular viral species was influenced by three factors. Firstly, the aphid colony and source plants were already established and being maintained to support these two viral species. Secondly, choosing two viruses amplified by two different primer sets enabled a comparison of the primers for detection of early virus infection to

be made at the same time. Thirdly, investigation of naturally occurring mixed luteovirus infection provides the opportunity to compare virus titres in the same plants. Both viruses were maintained in typical Canterbury-grown oat and barley cultivars that usually show mild symptoms. The viruses had been maintained by mixed aphid populations for many years under glasshouse conditions and using such a system proved to be robust for low-scale trials. Essentially, these experiments were echoing the field situation using naturally occurring and typical aphids and viruses with commercially grown cereal cultivars. This is different from some of the previous RT-qPCR studies on plant viruses, investigating either highly resistant or susceptible plants (Balaji *et al.* 2003) or virus strains with different pathogenicity characteristics (Ruiz-Ruiz *et al.* 2007). Therefore symptoms expressed by the plants used for this study, even a few months after inoculation with viruses, were obvious, but still relatively mild, intuitively suggesting low virus titre in these plants. The study examined the RT-qPCR detection of luteoviruses only from 3 dpi onwards, as it was based on the assumption that virus concentration in plants during the first few days are low. However, considering the results of this study, further investigation of virus titres in plants at 0-3 dpi would be advantageous.

Duplication of natural field conditions in the glasshouse was found to be hard to maintain as many biological aspects were difficult to control. Therefore the experimental set up used here took several attempts to establish. The first problem was maintenance of the aphid colonies on non-infected fresh and young barley plants – the aphids often multiplied poorly and did not reach sufficient numbers required for the experiments. Secondly, although the literature suggests that virus-infected plants are more palatable to aphids for feeding (Jensen and D'Arcy 1995), the opposite effect was found here when aphids were placed to feed on virus infected oat/barley source plants. Different plants of different growth stages (1-2 months old) were therefore tried as the virus-source. However, despite several attempts to establish an aphid colony on virus-infected plants, many individuals apparently starved to death although normally (and usually during the warmer summer months) feeding on the same virus-infected hosts is successful. Thirdly, over several attempts, virus transmission was not efficient and in some cases up to 100% of the plants infested with aphids previously maintained on virus-infected plants, were found to be virus-negative at 19 dpi as determined with ELISA (as well as being symptomless at this stage). As aphids were held under controlled indoor environment conditions under an artificial day/night regime, the colder months of the year (New Zealand winter and spring) cannot fully explain the reason behind such problems and may be attributed to the aphid-specific factors. Because of the

nature of large glasshouse trials, many aphids are used at once, leaving only a fraction of the population to reproduce further. Therefore, the genetic bottleneck of this aphid population could be the reason behind the poor aphid fitness and transmission as well as the unusual repellence of their natural host. Poor aphid survival is most likely not related to virus infection, as there is no direct evidence that virus presence in the aphid body is harmful to aphids (Gildow 1999).

A key aspect to undertaking a comparative, quantitative analysis of viral infection is to be able to account for non-related variation in RNA concentration by normalizing the data across samples (Bustin 2002; Bustin *et al.* 2009). This is achieved by making reference co-extraction of non-target plant genes. There are many different plant reference genes used for plant virus detection studies, such as the phenyl alanine lyase gene for normalization in winter barley (Mumford *et al.* 2004) and the 3-o-methyltransferase gene in sugarcane (Korimbocus *et al.* 2002). However, selection of the reference gene for this study was complicated by the absence of sequence information for these genes in the experimental plant host, oat (*Avena* sp). In this study, the quantification of viral nucleic acid was relative to amplification of *nad5* as a plant reference gene. The design of these nad-5s – nad5-as primers was originally based on the sequences of apple (*Malus domestica*) (Menzel *et al.* 2002), but other studies have also shown their utility for internal control amplification of mRNA in many additional plant hosts, both monocotyledons and dicotyledons (Varga and James 2005; Chomič *et al.* 2010). Furthermore, no-RT controls for each sample were tested with *nad5* primers to ensure the absence of specific RT-PCR amplification in the barley and oat plant model systems used. Although no validation of the *nad5* gene was done to ensure its expression stability within the experimental situation used, low variation in C_q within the run (0.3-0.9SD) for amplicons generated with nad5-s – nad5-as primers (see Section 4.3.2), as well as visually uniform amplicon quantity from samples collected at different dpi (Figure 4.3 and Figure 4.4), suggest that expression of *nad5* is not greatly influenced by virus infection. While the expression of the *nad5* gene was found to be more variable than that of 18S and actin genes, (the other most commonly used reference genes for RT-qPCR normalization (Jarošová and Kundu 2010)), nucleotide sequence data for these genes was also not available for barley and oat and the universality of the *nad5* gene primers provided a good option for this study.

There were a few other concerns regarding qPCR. Although the amplification with C1F1-C1F2-C1R2-C1R2 primers resulted in typical exponential amplification, the same was not observed

with the C2F1-C2F2-C2R3 primers. The latter produced lower fluorescence signals and were flatter in their "S" shape than that with C1F1-C1F2-C1R2-C1R2 primers. There are possibly two explanations for this. Firstly, the different length amplicons (68bp and 129/156 bp derived with C2F1-C2F2-C2R3 and C1F1-C1F2-C1R2-C1R2 primers respectively) bind different quantities of fluorescent dye, therefore emitting different amounts of fluorescence. Secondly, different priming efficiencies between the two combinations may be involved (Chapter 3). The amplification efficiency (i.e. slope of calibration curves) of both primer combinations was found to be similar to each other when investigated on a universal SPC template. However, the nature of that synthetic template might have had an impact on the amplification efficiency, being a clean DNA template with 100% sequence similarity to the generic primers. In comparison, the viral cDNA might not have had the same level of nucleotide sequence similarity to the primers, effectively lowering the amplification efficiency. In turn a less similar template means that fewer primer molecules bind, fewer amplicons are produced, and a lower fluorescence signal results.

While all plants with infection were confirmed by TAS-ELISA after 19 dpi, some tested negative by RT-qPCR up to 15 dpi (white squares in Figure 4.5). However, the results from these two techniques are not directly comparable because ELISA detects coat proteins and RT-qPCR detects nucleic acids (protein translation step usually occurs after the nucleic acid translation step (Mandahar 2006) therefore these two processes are likely to be isolated in time). Furthermore, different amounts of plant tissue were used for each technique: ~0.1 g of plant material for 1 TAS-ELISA reaction vs ~0.001g material for 1 µL of total RNA. Confirmation of virus infection at 19 dpi (see Section 4.2.3) using RT-qPCR (and not TAS-ELISA) would undoubtedly produce more comparable results, but would significantly increase the analysis cost (~NZD20 per 1 RT-qPCR reaction vs ~NZD1 per 1 ELISA reaction).

RT-qPCR-MCA is extremely sensitive and therefore, the manual set up of experiments, even with the greatest precautions and use of sterile plasticware, meant that carry-over contamination from other plate wells or plasticware was still likely and detectable. Virus-specific melting peaks were occasionally seen in healthy control samples after MCA and were more pronounced after repeated use of the sample. In contrast, water samples for the negative controls that were changed prior to every experiment revealed no virus-specific melting peaks on their melt profiles; given sufficient availability of cDNA, single-use aliquots would also be a recommended strategy for test samples.

Use of such a sensitive method as RT-qPCR plus MCA for detection of low titre of *Luteoviridae* infection was first demonstrated by Balaji *et al*. (2003) who detected cereal BYDV-PAV and CYDV-RPV as early as 2h post inoculation. However this was using species specific primers which showed a clear advantage of RT-qPCR over ELISA. A similar approach was undertaken in this study, using RT-qPCR with generic primers. Using RT-qPCR, amplification can also be monitored in real time. This enables assessment of data quantitatively as well as the identification of specific amplicons and spurious results by melting curve and $T_{m.}$ It was also less time consuming than standard RT-PCR combined with amplicon identification by gel electrophoresis and sequencing. RT-qPCR was also shown to be sensitive enough to account for differences in virus titre in different parts of the plant and therefore can be used for tracking BYDV-PAV or CYDV-RPV virus infections through plants. This might be even of greater interest with tracking of other luteoviruses in dicotyledonous plants and grasses that have tillers with multiple growing points.

Lastly, the fact that the cost of RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) is NZD16 per extraction was the main reason why an alternative and cheaper (NZD5 per extraction) RNA extraction protocol was explored, as has been explored elsewhere for ScYLV (Korimbocus *et al.* 2002). The Plant Total RNA Mini extraction delivered total RNA comparable in quality, but not quantity, to that extracted using the RNeasy Plant Mini Kit (Qiagen); this might be of great concern for studies which rely on the maximum total RNA yield (such as for virus diagnostics).

4.5 Conclusions

This study investigated the detection of double BYDV-PAV and CYDV-RPV infection in young oat and barley plants 3-15 days after inoculation using RT-qPCR and two combinations of generic primers (described in Chapter 2). The advantage of RT-qPCR in providing real-time data enabled relative quantification analysis that was applied for estimation of virus titre in different parts of the plant. Prior to the study it was assumed that absence of visual disease symptoms implied low titres of luteoviruses in plants. However quantitative data from this study indicate that in some plants the titre of both luteoviruses as early as 3 dpi, is comparable to the expression of the plant mitochondrial gene *nad5*, suggesting relatively high concentration of virus long before symptom expression. Results also indicate that the titre of both luteoviruses investigated differs greatly between individual plants, suggesting involvement of individual virus transmission efficiency of aphids or plant immunity. Furthermore, virus distribution in a plant is probably host

dependent; while young barley leaves at 9 dpi had higher titre of both BYDV-PAV and CYDV-RPV viruses than old leaves, no such trend was observed in oats over the experimental period. Therefore efficacy of diagnosis using generic primers probably needs to be considered for each luteovirus-host combination, however this is not necessarily feasible in a quarantine situation or wild host study. Therefore any negative results obtained in other uncharacterised host system must be interpreted with care and/or re-tested using other parts of the plant over the few weeks period. This study also suggests that even mild strobilurin-based fungicide treatment can have an immediate, but reversible effect on the accumulation of virus in both oat and barley, suggesting that plant defence response may play a role in regulating titre of CYDV-RPV in barley.

Chapter 5

Investigation into multiplexing capacity of generic primers

5.1 Introduction

In comparison with bacterial or fungal plant pathogens, which have the advantage of highly conserved ribosomal gene regions, there are no universally conserved priming sites among viruses. In fact, universal priming sites within virus families are also extremely rare, and most of them are likely to be temporary as more species become described and reveal new sequences. Certainly, the increasing use of sequencing technologies (Roossinck *et al.* 2010) might expand our knowledge of virus sequences and exponentially influence the “decay in conservation” (Section 2.4) of such universal priming sites. This is why current broad range diagnostic assays for many positive sense RNA plant viruses are mainly based on use of species-specific primers in multiplexed RT-PCR or (on even rarer occasions) on either broad-range primers, or generic primers (James *et al.* 2006).

As outlined in Chapter 1, there are many multiplexed RT-PCR protocols developed for luteovirus detection. Many of them are based on use of species- or group-specific primers and target B/CYDVs or beet poleroviruses. Recent work by Deb and Anderson (2008) describes using five specific primer pairs to detect five B/CYDV species; similar work by Malmstrom and Shu (2004) describes detection of six B/CYDV species in two multiplexes with seven primers of different specificity. Multiplexed RT-PCR for beet poleroviruses was described by Hauser *et al.* (2000) and Viganó and Stevens (2007) detecting two and three *Polerovirus* species respectively.

This study, using seven primers grouped into three separate combinations and detecting 13 species, provides to date the most extensive method currently available for the *Luteoviridae*; no other such generic assay of this taxonomic range has been available before. Three sets of primers were shown to perform well individually in both RT-PCR (Chapter 2) and RT-qPCR-MCA (Chapters 3) and therefore multiplexing these primers is appealing due to the opportunity to detect any of the 13 species as well as mixed infections in one reaction. However, due to their degeneracy, multiplexing is challenging as it increases the chances of even more pronounced non-specific amplification than was noticed in Chapter 2. The number of primers used in such a multiplex would be 26 (non-degenerate), which makes for a complex priming environment.

To avoid being absolutely lost in a seemingly endless list of recommended guidelines for multiplex PCR design (Elnifro *et al.* 2000; Dieffenbach and Dveksler 2003; Wei *et al.* 2008), key strategies, such as matching the annealing temperature of primers or their GC% content are usually tried first. The outcome is then evaluated critically and predictions for future improvement are made. Such empirical and trial-and-error testing is the most practical approach for assessing the efficacy of mixed primers, as there are no proven methods to predict their performance even if the general parameters of primer design are satisfied.

Recently, a theoretical prediction of the ideal universal assay for 79 virus families, including *Luteoviridae*, was made by Gardner *et al.* (2009) who determined family-specific primers for viruses using a novel Multiplex Primer Prediction algorithm. This programme predicts that for successful amplification of all *Luteoviridae* species, at least 28 non-degenerate primers (17-21-mers) were required. This number of primers is equivalent to 14 separate species-specific primer pairs currently available for luteoviruses (some examples are given in Table 2.4). Interestingly, this part of the conserved coat protein at the C2F1/C2F2 primer sites (majority of the C2F1 and C2F2 primers sequences overlap), was selected by Gardner et al. (2009), for one of their 28 predicted primers.

The Gardner *et al*. (2009) primers were designed to amplify several regions across the genome and were not restricted to the coat protein gene. Furthermore, their analysis was based only on full nucleotide genome sequences that were available before 25th of April 2007 and included *Luteoviridae* species that were not used for primer design in this study. The number of primers predicted for luteoviruses by Gardner *et al*. (2009) is in fact less than they predicted for the majority of other plant virus families with positive stranded ssRNA genomes (e.g. *Comoviridae* – 90 primers, *Potyviridae* – 196 primers, *Tombusviridae* – 70 primers), although the *Sequiviridae* required only 10 primers (non-degenerate) which could therefore be amplified in a feasible multiplex. Predicted 16-plex for *Poxviridae* was used to prove the concept by successfully detecting a 617-bp vaccinia virus specific amplicon (confirmed by sequencing).

The predictions by Gardner *et al* (2009) for *Luteoviridae* have not been laboratory tested and the majority of their primers were also not investigated for their specificity against virus populations and species. Indeed, assessment of the nucleotide sequence similarity of the Gardner *et al*. (2009) primers, performed during this study using BLAST revealed high sequence similarity (up to 100%) to some nucleotide sequences of plants as well as to members of other Kingdoms of life,

suggesting that further optimisation might be required to avoid non-specific amplification. Conversely, the specificity of the seven generic primers used in three combinations (C1F1-C1F2-C1R1-C1R2, C2F1-C2F2-C2R3 and C1F1-C2R3) developed during this study was illustrated by their inability to amplify nucleotide sequences of a related plant virus genus, sobemoviruses (currently unassigned to a family) and presumably the host plants (Figure 2.5). Therefore, multiplexed PCR with seven generic primers specific to the *Luteoviridae* appears to be more likely to amplify any of 13 species than those proposed by Gardner *et al* (2009). Such a multiplex would be especially valuable for detection of naturally occurring mixed luteovirus infections in cereals (e.g. one of the most common virus combinations BYDV-PAV and CYDV-RPV) which could be distinguished either by agarose gel electrophoresis based on the different sizes of amplicons or via MCA. Using qPCR-MCA other mixed infections (e.g. mixed infection of beet poleroviruses) could also be detected and discriminated using such a multiplex, similarly to the singleplex described in Section 3.2.9. This Chapter will investigate whether multiplexing all seven generic primers is possible. Such a multiplex will be investigated using both RT-PCR and RT-qPCR. A simplified multiplex using two separate combinations of generic primers C1F1-C1F2-C1R1-C1R2 (detecting BYDV-PAS, BYDV-PAV and BYDV-MAV) or C2F1-C2F2-C2R3 (detecting BChV, BLRV, BMYV, BWYV, CABYV, CYDV-RPV, PLRV, SbDV, TuYV) together with plant-specific nad5-s/as primers will also be investigated as it could help to reduce the time cost associated with additional testing of samples for success of the reverse transcription reaction. Virus- and plant-specific amplification products (129-156 bp or 68 bp and 181 bp, respectively) are of different sizes and therefore are anticipated to be distinguished by agarose gel electrophoresis.

5.2 Materials and Methods

5.2.1 Multiplex RT-PCR with seven generic primers

Two combinations (C1F1-C1F2-C1R1-C1R2 and C2F1-C2F2-C2R3) of generic primers (total of 7 individual primers) were used for the multiplex RT-PCR. Primer concentration was optimised first, by using 100 nM or 250 nM of C1F1-C1F2-C1R1-C1R2 primers with 250 nM or 500 nM of C2F1-C2F2-C2R3 primers per reaction in GoTaq® Green Master Mix (Promega, Madison, USA) using 1 µL of template and PCR profile as in Section 2.2.6. Subsequently, annealing temperatures of 52°C, 54°C and 56°C were compared using 0.5 $\mu g\ \mu L^{-1}$ BSA and two extension temperatures: 65°C and 68°C. Optimization strategies were selected based on previous research by Wei *et al.*

(2008), who suggested that (i) addition of BSA at 0.5 μg μL^{-1} concentration was determined as optimal for eliminating inhibitors and enhancing efficiency of PCR and (ii) lower extension temperatures than those suggested by the manufacturer, deliver better amplification efficiency using GoTaq® Green Master Mix. Controls included: water negative control, healthy plant sample, and SPC (Figure 2.3) in order to monitor performance of primers. In order to check the origin of any non-specific bands, MCA analysis of some RT-PCR products was carried out: 10 μL of RT-PCR product was cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and eluted in 15 μL instead of the recommended 50 μL in order to concentrate the RT-PCR product. Eluted RT-PCR product (15 μL) was then mixed with 15 μL of SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen, Calrsbad, USA). The mixture was subjected to MCA using the equipment and melting profile as described in Section 3.2.4.

5.2.2 Multiplex RT-PCR using two separate generic primer combinations with nad5-s/as primers

This multiplex RT-PCR was conducted to assess the ability of two primer combinations (C1F1-C1F2-C1R1-C1R2 and C2F1-C2F2-C2R3) to work together with plant-specific primers. All primers were used at a 250 nM concentration using GoTaq® Green Master Mix (Promega, Madison, USA), 2 μL of cDNA template in 20 μL volume and the PCR profile as in Section 2.2.6. Controls were as in Section 5.2.1.

5.2.3 Multiplex RT-qPCR with seven generic primers

The qPCR-MCA was preformed as described in Section 4.2.6 using seven generic primers at optimal concentration (Section 4.2.5) and 1 μL of template. Two types of templates were used: plasmid and cDNA. Two plasmids – BYDV-PAV-O3LU and BWYV-4 (Table 2.1) were used at 20 ng μL^{-1} concentration in the following ratios: 1:1, 10:1 and 1:10. Three cDNA samples harbouring different concentrations of BYDV-PAV and CYDV-RPV as determined by relative virus titre (Figure 4.7) were selected, being oat samples 6-2Y (similar concentration of both viruses) and 3-2O (CYDV-RPV predominant) and barley sample 9-3Y (BYDV-PAV predominant). Controls were as in Section 5.2.1.

5.3 Results

5.3.1 Evaluation of multiplex RT-PCR with seven generic primers

For multiplex RT-PCR, primer concentrations of 100 nM for C1F1-C1F2-C1R1-C1R2 and 500 nM for C2F1-C2F2-C2R3 primers were found to be optimal, generating two clearly visible amplification products for double infected samples (see Figure 5.1). However, bands of a similar size to the virus-derived products were produced by the water negative and healthy plant samples. These were confirmed by MCA to be different to the target amplicons probably being large primer dimers or primer clusters as no virus-specific peaks were derived from these samples (Figure 5.2), but this introduces potential ambiguity with use of this approach.

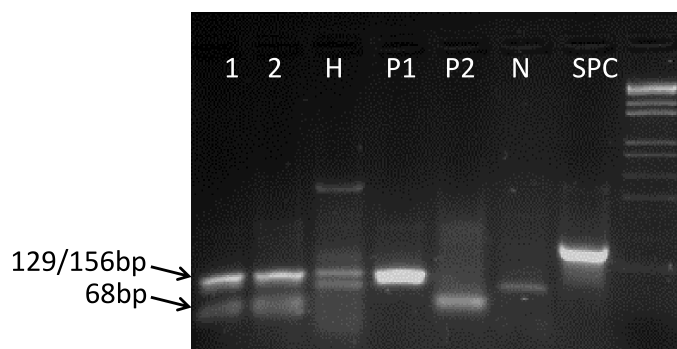


Figure 5.1 Agarose gel, showing results of multiplex PCR optimisation outcome using C1F1-C1F2-C1R1-C1R2 (100nM of each primer per reaction) and C2F1-C2F2-C2R3 primers (500nM of each primer per reaction).

1 and 2 – double infected (BYDV-PAV and CYDV-RPV) cDNA samples, H – healthy oat cDNA, P1 – BYDV-PAV positive (plasmid), P2 – CYDV-RPV positive (plasmid), N – water negative control, SPC – Synthetic Positive Control (Figure 2.3).

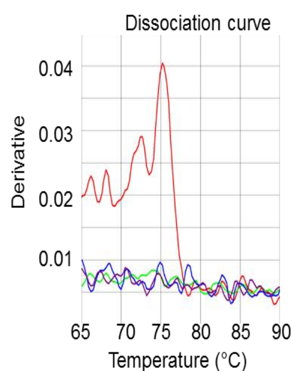


Figure 5.2 MCA analysis of multiplex RT-PCR produts.

BYDV-PAV and CYDV-RPV infected sample (shown in red) with clear melting peak of ~75.5ºC; healthy *H.vulgare*, and two water samples are shown in green, purple and blue respectively. Melting runs were re-run 12 times, but appearance of the melting peaks did not change, indicating that there was no dye translocation occurring (Varga and James 2006).

Attempted optimization of the thermal cycling conditions and relative primer concentrations did not help to eliminate this non-specific priming and only served to reduce the appearance of the C2F1-C2F2-C2R3 amplification product (Figure 5.3).

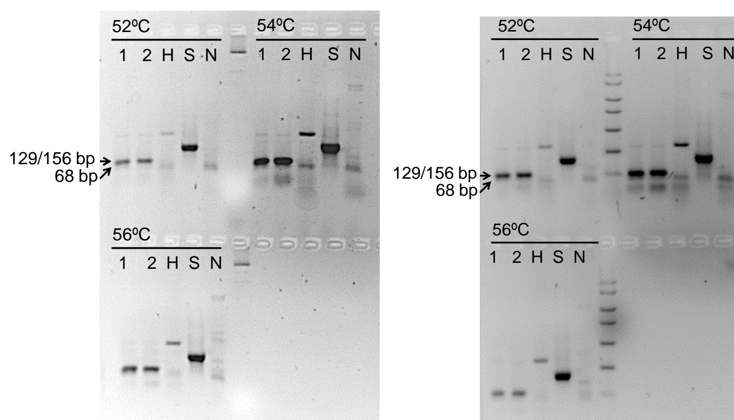


Figure 5.3 Optimization of multiplex RT-PCR with C1F1-C1F2-C1R1-C1R2 and C2F1-C2F2-C2R3 primers.

Three annealing temperatures (52ºC, 54ºC, 56ºC) and two extension temperatures - 65ºC (left picture) and 68ºC (right picture) were investigated; 1 and 2 – BYDV-PAV and CYDV-RPV-infected barley cDNA samples (3-4 and 6-2, Figure 4.5), H – healthy barley cDNA, S – SPC – Synthetic Positive Control (Figure 2.3), N – water negative control.

Sequences of both BYDV-PAV and CYDV-RPV could be amplified from the two barley samples used for multiplex optimizations (3-4 and 6-2) when C1F1-C1F2-C1R1-C1R2 and C2F1-C2F2-

C2R3 primers were used separately (Figure 5.4) thus confirming that the less intense band of 68 bp product in Figure 5.3 (at three annealing and two extension temperature combinations) in comparison to that seen on Figure 5.1 was not due to absence of CYDV-RPV infection.

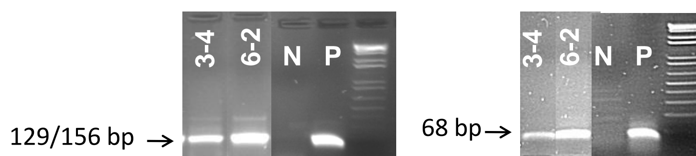


Figure 5.4 Amplification of 3-4 and 6-2 barley samples with C1F1-C1F2-C1R1-C1R2 (left) and C2F1-C2F2-C2R3 primers (right).

N – water negative control, P – positive control. RT-PCR performed as described in Section 2.2.6.

5.3.2 Evaluation of multiplex RT-PCR of two sets of generic primers with nad5-s/as primers

Co-amplification of nad5-s/as with the C1F1-C1F2-C1R1-C1R2 primers resulted in multiple amplification products of similar sizes for double-infected BYDV-PAV plus CYDV-RPV (shown as 1 in Figure 5.5a) as well as for single-infected BYDV-PAS cDNA sample (shown as 2 in Figure 5.5a). Therefore virus specific amplicon(s) (129-156 bp) could not easily be discriminated from the plant specific amplicon (181 bp). Amplification of SbDV and healthy plant cDNA samples resulted in a single 181 bp plant specific amplification product and extensive primer dimers. Primer dimers were also present in the water negative control (Figure 5.5a).

Co-amplification of the nad5-s/as and C2F1-C2F2-C2R3 primer set resulted in two clear amplification products of 181 and 68 bp for double-infected BYDV-PAV plus CYDV-RPV (shown as 1 in Figure 5.5b) as well as for the single-infected SbDV cDNA sample (shown as 3 in Figure 5.5b); BYDV-PAS (shown as 2 in Figure 5.5b) infected cDNA sample produced only 181 bp amplicon as well as all three non-infected cDNA samples. No amplification products were observed in water negative samples (Figure 5.5b).

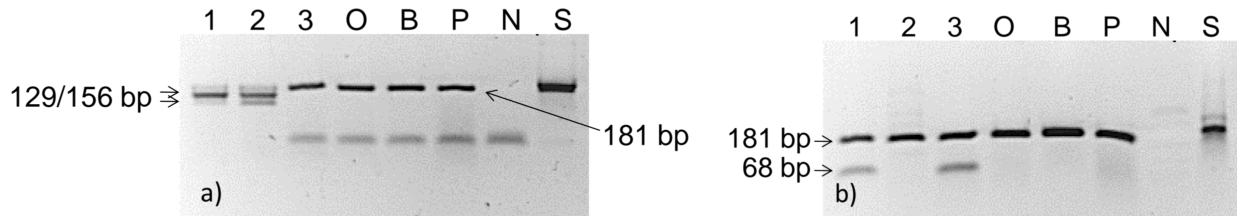


Figure 5.5 Multiplex RT-PCR with nad5-s/as and C1F1-C1F2-C1R1-C1R2 primers (a) and with nad5-s/as and C2F1-C2F2-C2R3 primers (b).

1 – BYDV-PAV and CYDV-RPV infected oat 15-4 cDNA sample (Figure 4.5), 2 – BYDV-PAS - DC1 (Table 2.1) cDNA, 3 – SbDV-BB2 (Table 2.1) cDNA, O – healthy oat cDNA, B – healthy barley cDNA, P – healthy potato cDNA, N – water negative control, S – SPC (Figure 2.3).

5.3.3 Evaluation of multiplex RT-qPCR with seven generic primers

For both DNA templates (plasmid (Figure 5.6) and cDNA (Figure 5.7)) amplification of similar concentration of BYDV-PAV and CYDV-RPV viruses resulted in two clear melting peaks of similar height and sharpness that were consistent with the appropriate virus. However for two other virus ratios (10:1 and 1:10 (Figure 5.6) and predominance (Figure 5.7)) the melting peak of the predominant virus was higher and sharper than that of the less predominant virus).

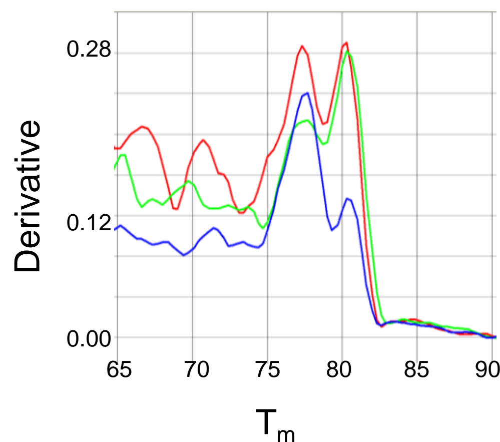


Figure 5.6 Multiplex qPCR-MCA using plasmid templates

1:1 BYDV-PAV : CYDV-RPV ratio is shown in red, 10:1 – in blue and 1:10 in green.

For cDNA templates at 1:10 and 10:1 ratios this lower melting peak spread over a greater temperature range, hindering precise estimation of T_m for that peak (Figure 5.7). Also, the T_m of virus-specific melting peaks for all three ratios did not match exactly.

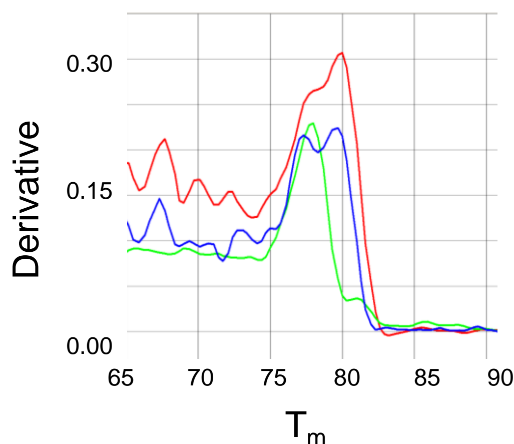


Figure 5.7 Multiplex qPCR-MCA using cDNA templates

Sample with similar concentration of PAV and CYDV-RPV is shown in blue, green shows sample where BYDV-PAV is predominant, red – where CYDV-RPV is predominant.

Amplification of a water negative control resulted in a very early C_q (Figure 5.8 left), but MCA did not show well pronounced specific melting peaks, except for a small peak at ~85ºC (Figure 5.8 right).

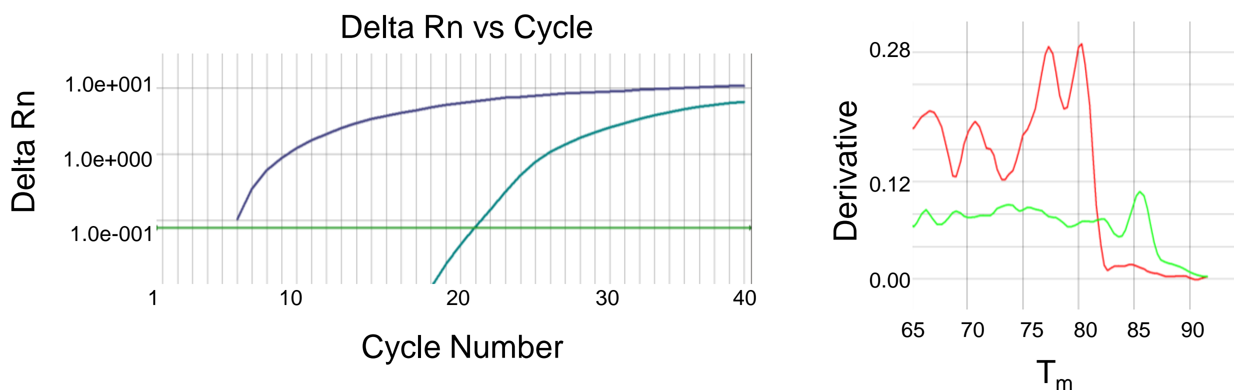


Figure 5.8 Amplification plot (left) and melting profile (right) of water negative control in comparison to 1:1 plasmid mixture in multiplex RT-qPCR using seven generic primers

Water negative control and plasmids (Section 5.2.3) are shown in the amplification plot in blue and purple, respectively, and in the melting profiles - in green and red, respectively.

5.4 Discussion

Multiplex PCR has been shown to be a valuable tool in diagnostic virology (James *et al.* 2006; Olmos *et al.* 2008). Once developed, it is very informative and produces a considerable savings in time and effort. Although it is a tempting tool to utilise, development is often not an easy process and unfortunately, in many cases, not possible. Although there are many guidelines provided for successful multiplex PCR design, often these guidelines cannot be met due to the nature of the primers or immense amount of time and effort required to improve on an existing technique. Using degenerate primers within a multiplex PCR, as was attempted in this study, is often even more ambitious due to the increased number of primer sequences in the reaction introduced by every degenerate position. Due to the variability of the luteovirus nucleotide sequences, many crucial guidelines, such as avoiding degeneracy and large G/C repeats, had to be broken when primers were designed. Although being the optimal size (17-24 nt) to ensure specific amplification, the generic primers also contain long G/C segments that increase the chances of self-annealing and of nonspecific amplification.

Development of the multiplex RT-PCR with generic *Luteoviridae* primers was a challenging and complex task. Despite being robust when used in separate combinations, seven degenerate primers did not produce satisfactory results when used together in multiplex RT-PCR. Although the amplicons for the two virus species were distinguishable on an agarose gel, non-specific amplification in the water negative or healthy control plants was impossible to eliminate. Furthermore, some of the non-specific bands obtained from the healthy control plants were almost indistinguishable in size from the virus-specific amplification products. The risk of calling false positives is therefore increased, and compromises the anticipated value of a multiplex assay.

Failure to optimise this multiplex RT-PCR assay and avoid non-specific amplification was most likely influenced by the large number of individual primers used, since the total level of degeneracy was equivalent to 26 primers. Given such a large number of primers in one reaction in the absence of a preferential template to bind, generation of primer dimers or primer clusters is very likely. Therefore, although multiplexing primers is often seen to be a more efficient method than singleplex RT-PCR for diagnosis, in this case, using two sub-sets of primers separately, which reduced the level of degeneracy and effective number of primers from 26 to 20 for C1F1-C1F2-C1R1-C1R2 and 6 for C2F1-C2F2-C2R3, was the most feasible solution. Although the effective number of primers in each primer combination was reduced, some non-specific

amplification still occurred (Figure 2.4). Excessive primer dimer formation was also seen when multiplexing C1F1-C1F2-C1R1-C1R2 (primer combination with the highest level of degeneracy) with plant-specific primers (Figure 5.5). Consequently, using separate primer combinations (with or without plant-specific primers) rather than a complex multiplex of seven generic primers in RT-PCR, might be the only strategy for the *Luteoviridae*.

Contrary to RT-PCR, non-specific amplification can be resolved with MCA when using such multiplex in RT-qPCR as it was shown in Section 5.3.3. Preliminary investigation of the suitability of multiplex RT-qPCR to detect double BYDV-PAV and CYDV-RPV infection resulted in two virus-specific melting peaks. However, the melting peak of the less predominant virus was less pronounced when cDNA templates were used, hindering precise estimation of T_m. This was not the case for plasmid templates, suggesting that purity of the template might be the issue (a similar observation was also made in Section 3.3.2).

Variation in T_m between replicates was shown to be as high as 0.5°C for cDNA template (Section 3.4) and therefore is most likely to cause some variation in T_m of virus-specific amplicons between three different isolates (Figure 5.7). Several randomly selected virus genotypes examined in Section 4.3.4 had 100% sequence similarity and therefore virus specific amplicons for the three samples from the same population investigated for the multiplex RT-qPCR in Section 5.3.3 are expected to have matching T_m. However, since the virus-specific amplicons from the three double-infected cDNA samples used for multiplex RT-qPCR (Section 4.2.8) were not sequenced, the assumption that all oat and barley samples in the January 2011 experiment (Section 4.3.3) had a single dominant (among quasispecies) virus genotype might not be correct and could be the reason behind the T_m variation among these three samples. A more diverse range of samples than was possible during this study, with different ratios of virus titres, should be investigated for complete evaluation of such multiplex RT-qPCR, both for single- and mixed infections. Furthermore, as RT-qPCR is a very sensitive technique, detection of carry-over contaminants (e.g. SPC – specific melting peak at ~85ºC in water negative control in Figure 5.8) even with the most stringent precautions is possible, due to the manual experimental set up (a similar problem was described in Section 4.4) and should be accounted for.

What remains, is to examine the performance of each of the virus specific primer combinations with plant-specific primers in an RT-qPCR-MCA situation, in order to investigate if virus-specific amplicons could be distinguished from plant-derived amplicons via MCA. As was shown

in Chapter 4, plant-specific amplicons produced by nad5-s – nad5-as primers had a T_m of ~ 76.4°C, which implies that it might not be distinguishable from the CYDV-RPV-specific amplicon; the two CYDV-RPV isolates investigated for their T_m in this study were CYDV-RPV-RL1 and CYDV-Kin3, and had T_m of ~77.3°C and ~76.2°C respectively (Chapter 3). It might be possible, however, to distinguish BYDV-specific amplicons from amplicons generated by nad5-s – nad5-as primers, because of the greater differences between their T_m (~80°C vs ~76.4°C).

The computer software, predicting multiplex primers such as one described by Gardner *et al.* (2009), is a powerful and useful tool for elucidating the potential primer sites which might be missed by manual processing of nucleotide sequence data and arbitrary selection of genome regions for primer design. However, as this research has shown, these arbitrary chosen primer sequences, although not detecting all 26 species, provided a good solution for *Luteoviridae* detection not only in single, but also in mixed infection situation. Manual testing of primers remains a crucial step and the knowledge obtained during this study might give important hints towards improvement of multiplex PCR predictive methods, e.g. development of additional algorithms which would test sequence identity of the primer to non-viral nucleotide sequences (to avoid non-specific priming) or prediction of the minimum numbers of primers required to detect a particular set of viruses (e.g. the most epidemiologically important ones or of a particular importance to the biosecurity laboratories).

5.5 Conclusions

This study investigated the performance of generic primers in several multiplex situations using both RT-PCR and RT-qPCR-MCA. Multiplex RT-PCR using seven generic primers resulted in some non-specific amplification of similar size to that of the target, which although of non-viral origin, significantly impacts on the use of such an assay. Contrary to RT-PCR, RT-qPCR has resolved the occurrence of non-specific amplification and detected both viruses producing two melting peaks, although the one derived from the amplicon of the less predominant virus in a given sample was less pronounced and less sharp than that of the more prevalent virus. Nevertheless, multiplex RT-qPCR was shown to be a good solution for detection and discrimination of BYDV-PAV and CYDV-RPV infection in a range of samples and has the potential to be used in diagnostics, provided additional validation tests are performed. Similarly, the simplified multiplex RT-PCR using C2F1-C2F2-C2R3 primer combinations with plant-specific primers also provided good discrimination of the both amplicons via gel electrophoresis

and might be recommended for routine diagnosis. Although analogous multiplexing of plant-specific primers with C1F1-C1F2-C1R1-C1R2 failed to deliver optimal discrimination of the amplicons via gel electrophoresis, discrimination of amplicons might be possible via MCA due to a difference of ~3.5ºC between their T_m. However this remains to be examined.

Chapter 6
General discussion

6.1 The value of generic primers for detection of luteovirus species

As outlined in Chapter 1, current detection and identification methods for the *Luteoviridae* are not optimal. In the current study generic (and potentially universal) molecular detection and identification tools for luteoviruses were developed and their performance and sensitivity assessed, as described in chapters 2-5, testing the three hypotheses listed in Section 1.9. The molecular assays described in this study offer significant advances on current protocols for *Luteoviridae* detection and differentiation by being taxonomically broad-ranging, time-efficient, sufficiently sensitive to detect low titres of viruses and with the potential to detect isolates with previously unknown sequences. Each of these aspects builds on a wealth of information from a range of other taxa improving the ability to study and diagnose a broad-range of luteoviruses.

New sequence information, available since the last documented attempt to design generic luteovirus primers (Robertson 1991), enabled identification of four novel conserved regions (Figure 2.1). These were located on the most conserved part of the *Luteoviridae* genome – the coat protein gene. However, to accommodate the nucleotide sequence diversity among the different species and isolates, even at this most conserved part of the genome, design of 1-3 primers for each conserved region was necessary. That resulted in the development of nine primers of low degeneracy. These were tested in five combinations (the four shown in Table 2.3, plus C1F1-C2R3) on 16 out of the 26 currently described *Luteoviridae* species, as well as on two species in the related *Sobemovirus* genus, to assess specificity of the assay (Chapter 2). Three out of the five primer combinations tested enabled the detection of 13 species (Figure 2.4), representing the two principal genera: *Luteovirus* (five species) and *Polerovirus* (eight species), confidence in their specificity was enhanced by their failure to amplify *Sobemovirus*-infected or healthy plants tested (Figure 2.5). Amplification of two out of the three remaining species tested (CtRLV and PEMV-1) was presumably not successful due to the relatively low nucleotide sequence similarity between the primers and their coat protein gene sequences (78.9% and 69.4%, respectively). However, non-amplification of the last species - (SPLSV), for which the coat protein sequence exhibited high sequence similarity with primers - requires a more thorough examination than was possible during the study (Figure 2.7). The H1 hypothesis - "**based on the**

successful design of generic primers for other plant virus families, and new nucleotide sequence information having become available for *Luteoviridae* in recent years, new conserved gene regions for design of generic primers can be identified" - was therefore confirmed and the results emphasised the importance of sufficient nucleotide sequence information for the development of a generic assays for plant virus families. This study provides the best detection method currently available for the *Luteoviridae*, no other such generic assay of this taxonomic range having been available previously.

Using standard PCR with low-degeneracy primers (Table 2.2) to detect a broad-range of species effectively replaces the suite of species-specific primers currently available for the majority of known species (Table 2.4). Low degeneracy (1-3 double degenerate nucleotide positions per primer) configured in three different combinations proved to be a good strategy and resulted in the successful amplification of a central 68-156 bp region of the coat protein in the majority of *Luteoviridae* species tested (Figure 2.4). What remains, however, is to (i) test the primers with the other 13 known (definite) *Luteoviridae* species (Table 1.1) as well as recently discovered ones (Table 1.2) that were not available during the study, plus possibly a wider range of virus families that also occur on the same host(s) and (ii) further investigate the amplification of non-target sequences in virus-free hosts as some non-specific amplification was noticed in an SbDV-infected sample (Figure 2.4).

Designing degenerate primers has been a common choice for the amplification of other plant virus families, as well as viroids (James *et al.* 2006). With a few exceptions, these degenerate primers target the coat protein gene or the RdRp gene, which are known to be the least variable regions in plant virus genomes (Hull 2002). However, as RNA plant virus genomes are generally highly variable due to their error-prone mechanism of replication (Holland *et al.* 1982; Pita *et al.* 2007), the majority of primers designed have degenerate positions. Some also contain either inosine (when sequence variation is too high) or oligo (dT)s (for plant viruses which have a poly-A tail at their 3'-end) (James *et al.* 2006). There are many primer selection and analysis software programmes available (for example Geneious, DNAMAN, IDT Olygo Analyzer), but different programmes use a range of different algorithms and therefore often generate different primer predictions (Chavali *et al.* 2005). Manual selection of primer sequences from a sequence alignment (as was done in this study), although more laborious, is often performed as an alternative to give an instant understanding of the nucleotide sequence diversity in a particular

genome region. The sequences of the generic primers were hard to design and, it was not always possible to comply with all of the recommended guidelines (Dieffenbach and Dveksler 2003); for example most of the primers (except C1R3) had three or more C/G nucleotides in a row. All primers were used at high concentration (150-300 ng/primer) to ensure greater amplification and improve visibility of low molecular weight PCR products on an agarose gel. These factors may have influenced non-specific amplification observed during this study.

For the preliminary work where the taxonomic range of the primers was not known, the use of random hexamers (rather than species-specific or generic primers) for the reverse transcription step (Section 2.2.5) proved a useful approach and enabled the same cDNA synthesis conditions to be used for all samples. In addition, it was not biased by the individual sensitivities of species-specific primers. Different species-specific primers (i) work under different reaction conditions and (ii) may not be equally identical to all sequence variants (i.e. isolates) of the species to which they were designed. Random hexamer primers (N_6) are shorter than conventional primers and anneal to RNA across its entire length, rather than to the few limited regions. This potentially enables recovery of the full length cDNA, rather than a particular region that generic primers anneal to (for example the coat protein gene) and makes the cDNA step universal for future applications. However, using random hexamers, rather than species-specific primers for the reverse transcription reaction, generally results in a lower yield of target cDNA. Nevertheless, there is always the possibility to change/update the reverse transcription protocols using generic luteovirus primers.

Following the amplification of the viral amplicons with generic primers there are several different ways of sequence identification. Sequencing (with or without cloning of the amplification product) accurately confirms virus identity and is ideal for a low-throughput situation. However, it is not optimal for routine diagnosis due to high operational costs and the often limited equipment availability. Melting curve analysis (MCA) was investigated during this study (Chapter 3) as a potentially quicker and cheaper alternative to sequencing (Section 1.6). Amplification using generic primers is conducted using fluorescent dye on qPCR equipment, and the melting profile of the amplicon is then determined by MCA (Section 3.2.4). Because MCA is sequence-dependent, different amplicons might be distinguished via their different melting profiles (shape of the melting curve and T_m). This was therefore the first ever attempt to apply MCA for detection and discrimination of *Luteoviridae* family members. During this study, qPCR-

MCA was assessed for its suitability for detection and identification of 13 *Luteoviridae* species, using generic primers (C2F1-C2F2-C2R3, C2F1-C2F2-C2R1+2, C1F1-C1F2-C1R1-C1R2 and C1F1-C2R3) (Chapter 3). Detection of all the *Luteoviridae* species tested was possible, but differentiation of species was complicated by high intra-species nucleotide sequence diversity among different isolates of some species (high intra-species nucleotide sequence diversity among different isolates of the same species was also noticed in other plant viruses, e.g. *Apple stem pitting virus* (*Betaflexiviridae*) (Komorowska *et al.* 2011) or *Grapevine fanleaf virus* (*Comoviridae*) (Bashir *et al.* 2007)). Consequently, of the 13 species tested, confident differentiation was possible for only six of them (Figure 3.2). Therefore the results partially confirmed the H2 hypothesis - "**based on the successful application of melting curve analysis (MCA) for detection and discrimination of other pathogens, a RT-qPCR-MCA using generic primers is sensitive and accurate enough for the detection and discrimination of *Luteoviridae* species for diagnostic purposes**" and showed the potential of MCA for detection and discrimination of luteoviruses as well as emphasising the need for further investigations of additional virus isolates for better understanding on how nucleotide sequence diversity impacts on the use of MCA.

However, there were a number of issues that hindered differentiation of some species. As was shown in Chapter 3, some poleroviruses (TuYV-MS and BWYV isolates) had very high nucleotide sequence similarity for both the 68 bp and 148 bp regions. This was shown to hinder the discrimination of these two species via MCA because of their identical melting profiles. This highlights the importance of the region chosen for amplification as it implies a potential challenge of finding conserved regions for priming, which would at the same time span regions variable enough to allow identification.

Different luteoviruses might not have the same epidemiological impact and therefore it is important to differentiate between them. Among seven species presented in Figure 3.2 five (BChV, BMYV, CABYV, BLRV and SbDV) could be differentiated from each other and from BWYV or PLRV using MCA. This is epidemiologically significant, because, BWYV, SbDV and PLRV are present in New Zealand, and BChV, BMYV, CABYV and BLRV – are not (Pearson *et al.* 2006). Therefore it is of benefit to biosecurity in New Zealand to identify these exotic species with confidence as they can infect the same commercial plant hosts as other *Luteoviridae* already

present in the country (Table 1.3). Therefore the generic primed MCA assay offers a very useful means for the detection of the unwanted species.

Although BWYV and PLRV could not be differentiated from the T_m of their 68 bp amplicon, they could be differentiated from the T_m of their 148 bp amplicon (Figure 3.2); there might be greater T_m variation due to the bigger size of the amplicon. However, having this option to differentiate these species is useful as they present a potentially different epidemiological problem. These viruses share the same aphid vector and both infect the broad-leafed weed *Solanum nigrum* (Robert 1999; Coutts *et al.* 2006). However, PLRV is a specialist virus infecting about 20 plant species, largely from the family *Solanaceae* (Taliansky *et al.* 2003), whereas BWYV has a much broader host range including many arable weeds and volunteer crop plants (Stevens *et al.* 1994; Coutts *et al.* 2006) which could serve as a reservoir for the latter. Some of these plant species may serve as a common host for these two *Polerovirus* species. Similarly to BWYV and PLRV, none of the three BYDV species tested (PAV, PAS, MAV) could be differentiated via their melting profiles using their 129/156 bp fragments Table 3.3.

The virus detection methods described in this study are based on PCR, which requires isolation of high purity RNA uncontaminated by plant-derived compounds. The RNA isolation step for the *Luteoviridae* is especially difficult and time consuming. They are positive sense ssRNA viruses, residing deep inside the vascular tissues of the plant, and extraction therefore requires thorough grinding of the plant material. Automated nucleic acid extraction machines (such as Thermo Kingfisher mL (Thermo Scientific, Helsinki, Finland)) are available and, while they are high cost, they usually skip the tissue-grinding step which is the most laborious and also contamination prone. RNA purity was shown here to be critical to MCA, because host-derived compounds might influence the T_m of the virus-specific amplicon (Section 3.3.6). To eliminate plant compounds that inhibit RT-PCR/RT-qPCR, alternative RNA extraction kits to that used here, which deliver purer RNA by eliminating enzyme inhibitors (for example Spectrum^{TM} Plant Total RNA Kit (Sigma–Aldrich Corp., St. Louis, USA), should be tested. The alternative would be to target isolation of dsRNA, which is more stable than ssRNA and the extraction procedures are much simpler (although might be quite lengthy and/or require large quantities of sample). Roossinck *et al* (2010) has successfully demonstrated the suitability of dsRNA for plant virus discovery confirming the availability of sufficient amount of dsRNA enough to serve as a diagnostic target. The majority of known plant viruses have ssRNA genomes and generate

dsRNA at some point in their life cycle. dsRNA is the form of nucleic acid that is generally unique to viruses and therefore cannot be confused with non-viral nucleic acid. Furthermore, extraction of dsRNA has the potential to provide more comprehensive information on the collection of viruses present in a plant, especially from asymptomatic hosts as indicated in a recent study which detected dsRNA of viral origin in most of the asymptomatic wild plants tested (Roossinck *et al.* 2010). This provides the opportunity to examine a number of issues of ecological and biological interest and at present is being investigated using next generation sequencing facilities (Roossinck 2008; Roossinck *et al.* 2010), although this involves additional bioinformatic and resourcing costs.

The MCA assay developed in this study could be modified by using different reaction chemistries, fluorescent dyes and real-time equipment. However care must be taken when interpreting results as modifications could result in a different T_m or a melting curve for the same target sequence. Firstly, different fluorescent dyes interact differently with dsDNA and ssDNA and therefore saturation and dissociation kinetics of different fluorescent dyes differ (Monis *et al.* 2005). Secondly, different real-time equipment performs at different resolutions, melting rates, and data interpretation/averaging software (Herrmann *et al.* 2006). Thirdly, different commercial kits may have dissimilar components that increase shelf life or enhance qPCR, which potentially may result in altered binding characteristic of a dye (Giglio *et al.* 2003).

The recent adaptation of qPCR–MCA (or its even more sensitive version - HRM) – is now extremely popular in medical research (Krypuy *et al.* 2007; Pangasa *et al.* 2009; Robertson *et al.* 2009) and is gradually gaining recognition in the plant virus field (Section 1.6). qPCR is being recognised as the most effective generic technology platform for detection of many plant pathogens due to its phenomenal sensitivity (reviewed in Boonham *et al.* 2008, Olmos *et al.* 2008, Vincelli and Tisserat 2008). Besides the potential to identify organisms to the species level, MCA, being sequence dependent, might also provide additional information not apparent with species-specific PCR, such as non-specific amplification or amplification of new sequence variants. The real-time PCR process, which precedes MCA, is also useful in providing information on amplification efficiency of the samples.

One of the biggest advantages of the qPCR is its ability to detect minute quantities of the target DNA which otherwise would not be sufficient to see on an agarose gel following standard PCR or to produce a positive signal with ELISA. Characteristically, the *Luteoviridae* are restricted to

the phloem and usually do not accumulate to a high titre in their host plants. Therefore detection with RT-qPCR becomes an attractive option, especially if analysis at the early stages of infection needs to be performed. RT-qPCR has already been applied for luteovirus detection and quantification (Section 1.6) using species specific primers and is known to be much more sensitive than RT-PCR or ELISA. Expanding its use with generic primers would streamline the diagnosis of luteoviruses as well as provide additional information on the sequence of an amplicon and may assist with discovery of unknown sequence variants.

Using double BYDV-PAV and CYDV-RPV infection and appropriate generic primers as a model system, RT-qPCR was assessed for detection of these two viruses in plants, supposedly present at low titres, during the 3-15 days post inoculation (dpi) period (Chapter 4). Results demonstrated that virus titre in some individual plants was higher than the expression of the plant's mitochondrial *nad5* gene (Figure 4.7) as early as 3 dpi. This indicated that titre of both viruses is not as low as it was assumed, even though plants were asymptomatic or showing only mild disease symptoms at the time of tissue collection. The results confirmed the H3 hypothesis - "**given the greater sensitivity of RT-qPCR and its quantitative advantage over the RT-PCR, as well as its successful application for detection of other luteoviruses** (see Section 1.6)**, RT-qPCR is a suitable method of detecting low titres of *Luteoviridae* infection using generic primers**" and demonstrated that RT-qPCR with generic primers is a suitable method of detecting *Luteoviridae* infection in double-infected plants at 3-15 dpi (in contrast to ELISA, which was not always reliable, unless performed using symptomatic tissue at 19 dpi). Furthermore, because of its quantitative aspect RT-qPCR can be used for tracking virus infection in different parts of the plants – this was shown by demonstrating that virus titre in young barley leaves is higher than in old leaves at 9 dpi (Figure 4.8) - and therefore could have epidemiological implications (such as it would be useful to know which parts of the asymptomatic plants are best to sample in quarantine in order to increase the possibility of virus detection). This RT-qPCR assay, being generic, might be applicable for detection of other luteoviruses as well. Testing of other virus combinations beyond that of the two virus species used during this study would be necessary to better gauge how broadly useful this approach is using generic primers (given that they fall within the taxonomic range of the primers). RT-qPCR works over a wider range of template concentrations than RT-PCR and therefore is more likely to detect other luteoviruses that might be present at a broad-range of titres in host plants. This RT-qPCR assay with generic primers may even be applicable for detection of luteoviruses in wild/non-cultivated hosts which often remain

asymptomatic through their whole life cycle and/or harbour very low titres of viruses but act as a reservoir of infection. Additional investigation into (i) the virus titre at different time points after infection in plants, including leaves of different ages and (ii) the comparison of the virus concentration between different hosts would shed more light on luteovirus titre dynamics in different hosts, epidemiology, and their general biology.

Detection of luteoviruses with either RT-PCR or RT-qPCR during this study (Chapters 2, 3 and 4) was mostly performed using separate combinations of generic primers (Table 2.3). As the primers were shown to work under the same PCR condition (Section 2.2.6), using them together in one multiplex PCR (or qPCR) is an appealing option to have for detection of any of 13 luteovirus species (Section 2.3.2). Furthermore, such a multiplex would be a significant step forward, given that all current multiplex PCR protocols developed and used are often restricted to a limited number of viruses residing in a single host (Section 5.1). However, developing a multiplex PCR using species-specific luteovirus primers is often hindered by the large number of different primers that need to be used together and the different thermodynamic parameters or secondary structures they might form. Nevertheless, theoretical analysis of thermodynamic parameters of the generic luteovirus primers here showed high, but still acceptable ΔG values of possible homo- and heterodimers for all nine generic primers (Section 2.2.3). This suggested that these primers might be possible to multiplex.

In order to explore if such streamlining of diagnostic process is feasible, several multiplexing options were investigated during this study (Chapter 5). This was assessed using seven generic primers (which work in three different combinations: C1F1-C1F2-C1R1-C1R2, C2F1-C2F2-C2R3 and C1F1-C2R3) using RT-PCR and RT-qPCR for the detection of mixed BYDV-PAV and CYDV-RPV infection. Multiplex RT-PCR resulted in some non-specific amplification from healthy samples and water negative controls (Figure 5.1 and Figure 5.3), which was confirmed to be of non-viral origin (Figure 5.2). This suggests intensive primer interactions and was also supported by RT-qPCR which showed early C_q value of water negative controls (Figure 5.8). Despite this, both viruses can be detected by MCA (Figure 5.6 and Figure 5.7) although the results indicate some possible challenges for detection of the less prevalent virus in double-infection situation (melting peak of the less prevalent virus is less pronounced). Multiplex RT-qPCR combined with MCA has the potential to be used in diagnostics following appropriate validation tests, as it delivered a good solution for detection and discrimination of mixed BYDV-

PAV and CYDV-RPV infection in a range of samples. Results also emphasized the advantage of MCA in resolving the origin of non-specific amplification (primer dimers, amplification from host genome) or potential cross-contamination, as well as the need to investigate additional naturally occurring plant samples beyond these available to this study, which harbour other mixed infections of luteoviruses.

6.2 Implication for the detection of mixed infection of luteoviruses

Mixed infections of luteoviruses are frequent in nature (Table 1.3) and are often much more economically important than single infections. Mixed infections offer several ecological advantages over a single infection. For example, genomes of the co-infecting viruses can be packaged in the same capsid and thus have a possibility to exchange some parts of the genome (thus increasing the biodiversity of viruses or providing functional benefits regarding pathogenicity/access to a plant by replacing the missing parts of the virus genome or parts, containing harmful mutations) or be transmitted to a new host, as well as supplement each other's missing functions such as providing products to circumvent the plant defence response or alternative form of the movement proteins (Barker 1987). Some luteoviruses that exist in mixed infections may also share the same aphid vector, although often the transmission efficiencies differ within an aphid species. For example beet co-infecting BChV and BMYV are both transmitted most efficiently by *Myzus persicae* (Stevens *et al.* 2004), whereas legume co-infecting BLRV and SbDV species may share the same vector (*Acyrthosiphon pisum*) (Makkouk and Kumari 2009). However, some strains of SbDV have other aphid vectors which deliver better transmission efficiency (Terauchi *et al.* 2001). These specific luteovirus-aphid species combinations have been established during co-evolution of the luteovirus, aphid, and host plant in order to ensure the most efficient transmission of a virus as well as the benefits or absence of impact on the fitness of its vector and plant.

Some of these co-infecting luteoviruses might be of very different epidemiological significance. For example SbDV and BLRV are found co-infecting some cool season food legumes in the Middle East (Abraham *et al.* 2006) and share common vectors *Acyrthosiphon pisum* and *Aulacothum solani* (Makkouk and Kumari 2009). However, SbDV was recorded for the first time in New Zealand in 1979, while BLRV is still absent from the country (Pearson *et al.* 2006). This is epidemiologically significant, because BLRV has already been recorded in Australia (Schwinghamer *et al.* 1999) and both vectors - *A.pisum* and *A.solani* - have been recorded in New

Zealand (Teulon and Stufkens 2002). Plant hosts of BLRV include beans, faba beans and pea (Ashby 1984) the latter of which is a major export crop. Current practise for discrimination between virus species with low epidemiological risk from those of biosecurity concern involves species- or group-specific detection. Although well established, these methods are not optimal due to the double effort required for the diagnosis of each disease causing agent and may not detect all population variants. The molecular detection and identification assays presented in this study offer a solution to these problems. The potential of these assays for detecting multiple luteovirus infection using generic primers and RT-qPCR and MCA has been demonstrated here, with mixed luteovirus infection detected using primers either in multiplex PCR (Chapter 5) or in separate combinations (Chapter 3 and 4). Using the primers in multiplex, CYDV-RPV and BYDV-PAV mixed infection could be detected and discriminated (Figure 5.7). Also, discrimination between BWYV and SbDV was demonstrated when their cloned CP products were present at similar ratios (Figure 3.3), although an analogous study using double-infected cDNA samples (for example in the common hosts *Erodium cicutarium* or *E. moschatum* (Pearson *et al.* 2006)) would be beneficial to investigate detection and discrimination of these viruses in natural situations.

6.3 Future trends

Alternatives to standard PCR amplification techniques are gradually becoming popular detection tools in applied plant pathology (Vincelli and Tisserat 2008). Some of these more future-oriented technologies include different isothermal amplification methods (e.g. nucleic acid sequence-based amplification (NASBA) (Olmos *et al.* 2008), and loop-mediated isothermal amplification (LAMP) (Tomlinson and Boonham 2008)), surface plasmon resonance for detection and quantification of *Tobacco mosaic virus* (Boltovets *et al.* 2004) and quartz crystal microbalance immunosensors for detection of orchid viruses (Eun *et al.* 2002). A few studies using LAMP (Zhao *et al.* 2010) and NASBA have been described for detection and identification of *Luteoviridae* in plants. NASBA was first applied in 1997 for detection of PLRV (Leone *et al.* 1997) and later in 2002, combined with molecular beacons for sensitive detection of ScYLV (Gonçalves *et al.* 2002). However, based on the most popular research carried out in the last decade, future trends will probably focus on (i) improvement of the existing technologies that increase simplicity, such as the use of dsDNA binding dyes in qPCR or chip technologies (such as in Sip *et al.*(2010)), or (ii) techniques that combine different methods such as PCR, array

technologies and immunoassays (James *et al.* 2006). In the immediate future increased uptake of DNA sequencing (such as demonstrated in Pallett *et al.* (2010), using the small interfering RNA approach to detect CYDV, and Kreuze *et al.*(2009)) is likely to have the biggest impact in the plant health arena as assays become more affordable, more rapid and easier to analyse bioinformatically.

6.4 Practical aspects and applications

Although results presented in this study indicate that the assays described are specific, sensitive, rapid and cost-effective, a more thorough examination of these assays is needed before recommending them for routine diagnostics as a reliable test. Several attributes of a good diagnostic technique need to be considered: accuracy (expressed through sensitivity and specificity), repeatability, reproducibility, rapidity and low cost. Estimation of rapidity and overall cost of the assay is straightforward, but other attributes of the diagnostic test have to be estimated during the much more laborious process of validation. That includes the processing of a much greater range of virus-infected isolates than it was possible to obtain during this study, plus confirmation of their infection and estimation of exact numbers of false and true negatives and positives detected by these assays. When such a validation procedure is accomplished – the real value and potential applications of these assays in routine diagnostics could be outlined more precisely.

The assays described here were evaluated in plant hosts only, but this could also be extended for detection of the *Luteoviridae* in their vectors - aphids. Together with the SPC to monitor the performance of the primers, the assays described here present a convenient and much improved tool for diagnosticians and researchers to adopt. During this study SPC was proven to work well as a positive control. Amplification using this plasmid template always resulted in high amplification signal, both via PCR and qPCR, suggesting that is should be used highly diluted, especially if used in one tube with viral template in order not to overpower significantly weaker amplification from the latter. Special care should also be taken to prevent carry-over contamination, such as was shown in Figure 5.8. Serious contamination issues with SPC were not encountered during this study, probably due to the separate storage and careful handling of tubes containing the plasmid which is highly advised as a mean of precaution for further studies.

By using the assays described in this study, a number of areas of applied research might be accomplished more cost- and time-efficiently. For example in biosecurity routine virus screening

surveys, assessment of the material in post-entry-quarantine or nursery stocks, detection of an incursion and its control, investigation of incursion pathways, assessing virus resistance traits in plant breeding procedures, developing virus reference collection and validation of molecular-based diagnostic tests. Also, to assess their role as reservoirs of virus inoculums by exploring the spatial and temporal aspects of populations of viruses in wild and asymptomatic plants and assessment of their role as reservoirs of virus inoculum. These generic detection methods are especially useful in small diagnostic laboratories as they are applicable to high-throughput situations and dramatically reduce the numbers of tests that need to be performed. This also implies that less staff needs to be involved, which makes the service more cost-effective. Some of these testing methods might be more suitable for some applications than others (e.g. testing small numbers of samples in a quarantine situation versus epidemiological studies).

Another application is amplification of ancient RNA, such as of old herbarium samples to study their evolution. Successful amplification of BYDVs from herbarium samples of invasive and native grasses dating from as far back as 1917 has been described, using multiplex PCR with species- and group-specific primers (Malmstrom *et al.* 2007). Given the short sizes of amplicons (68 bp - 156 bp), degraded RNA has a high chance of recovery when using the generic primers described in this study, as long as appropriate RNA extraction protocols are employed to include the RNAs < 200 nt which are typically excluded by the standard protocols that use RNA enrichment technology. Short amplicon size also enables the application of a rapid PCR cycle, which involves shortening the template elongation and melting steps and reduces the overall time of a RT-qPCR-MCA run to as little as 15 minutes (Reed *et al.* 2007).

The economic impact of the luteovirus diseases likely to become of greater consequence with an increasing world population (Bloom 2011), a declining land area suitable for agriculture and monoculture susceptibility all putting pressure on sustainable food production. Efficient and early detection of infection is therefore critical to minimise crop damage. Improved technologies continue to allow the discovery of many new plant viruses, including within the *Luteoviridae* (Roossinck *et al.* 2010) and so a prescribed species-specific approach to detection and identification, which would in all likelihood fail to detect these new sequences and isolates, has distinct limitations. The potential of the generic assays, as developed here, to detect previously unknown virus isolates is also becoming of greater use in ecological studies. Viruses, as an integral part of any ecosystem, is a relatively new concept (Hurst and Lindquist 2000; Astier *et*

al. 2007). Discovery of symbiotic and non-pathogenic viruses using improved versions of conventional techniques (as in this study) would be of great advantage for small-scale ecological studies. Furthermore, due to the unusual genome structure of *Luteoviridae*, and their expression and complex relationship with the host, this family of viruses is increasingly gaining interest as a model for solving some of the fundamental questions in biology, such as genome organization and functioning both in plants and vectors, and plant-pathogen interactions. The convenient detection tools described here could greatly facilitate this type of research.

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Conferences and workshops attended

1. 8th Australasian Plant Virology Workshop, 19-22 November 2008, Rotorua, New Zealand. Presentation "Molecular Detection of the *Luteoviridae*".
2. New Zealand Plant Protection Society Conference, 11-13 August 2009, Dunedin, New Zealand. Poster "Towards universal detection of *Luteoviridae*".
3. Lincoln University Postgraduate Conference, 31 August – 1 September, 2009, Lincoln, New Zealand. Presentation "Towards universal detection of *Luteoviridae*".
4. Australasian Plant Pathology Society Conference, 29 September – 1 October 2009, Newcastle, Australia. Presentation "Towards universal detection of *Luteoviridae*".
5. COMBIO, 6 – 10 December 2009, Christchurch, New Zealand. Presentation "Degenerate primers and melt curve analysis as tools for biosecurity".
6. 9th Australasian Plant Virology Workshop, 16 – 19 November 2010, Melbourne, Australia. Presentation "New diagnostic tools for the *Luteoviridae*".

Copies of publications

1) Chomič A, Pearson MN, Clover GRG, Farreyrol K, Saul D, Hampton JG, Armstrong K.F. (2010). A generic RT-PCR assay for the detection of *Luteoviridae*. *Plant Pathology,* **59**, 429–442.

Contributions of authors:

Chomič A – design and performance of the experiments, acquisition of data, analysis and interpretation of data, manuscript writing, preparation of figures and tables, submission of the manuscript, addressing revisions.

Pearson MN – providing access to previous research, editorial help*.

Clover GRG – ensuring successful importation of overseas isolates and their processing (RNA extraction and reverse transcription).

Farreyrol K and Saul D – design of primers (C1F1, C1F2, C1R1, C1R2, C2F1, C2F2, C2R1+2) and initial evaluation of these on three luteovirus species (see Section 2.2.2).

Hampton J – editorial help, assistance with manuscript preparation.

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2) Chomič A, Winder L, Armstrong KF, Pearson MN, Hampton JG. (2011). Detection and discrimination of members of the family *Luteoviridae* by real-time PCR and SYBR® GreenER™ melting curve analysis. *Journal of Virological Methods,* **171**(1), 46-52.

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Chomič A – design and performance of the experiments, acquisition of data, analysis and interpretation of data, manuscript writing, preparation of figures and tables, submission of the manuscript, addressing revisions.

Winder L – discussion over experimental design, editorial help.

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