Using new tools to detect and characterise plant viruses

by

Mr Hao Luo

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School of Biological Sciences and Biotechnology

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DECLARATION

The work described in this thesis was undertaken while I was an enrolled student for the degree of Doctor of Philosophy at Murdoch University, Perth, Western Australia. I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution. To the best of my knowledge, it contains no material or work performed by others, published or unpublished without due reference being made within the text.

SIGNED____________________    DATE____________________
ABSTRACT

Executive summary:

The overall aim of this study was to develop new methods to detect and characterise plant viruses. Generic methods for detection of virus proteins and nucleic acids were developed to detect two plant viruses, *Pelargonium zonate spot virus* (PZSV) and *Cycas necrotic stunt virus* (CNSV), neither of which were previously detected in Australia. Two new approaches, peptide mass fingerprinting (PMF) and next-generation nucleotide sequencing (NGS) were developed to detect novel or unexpected viruses without the need for previous knowledge of virus sequence or study. In this work, PZSV was found for the first time in Australia and also in a new host *Cakile maritima* using one dimensional electrophoresis and PMF. The second new virus in Australia, CNSV, was first described in Japan and then in New Zealand. In this work it was detected and characterised as a new strain in Australia using NGS analysis and was found in a lily plant (*Lilium longiflorum*) with symptoms. Patterns of infection of a native virus *Hardenbergia mosaic virus* (HarMV) and the introduced virus *Cucumber mosaic virus* (CMV) were studied in natural and recent host plants using real-time reverse transcription polymerase chain reaction. For different virus isolates and symptoms, the virus concentration in plants varied and interaction between two co-infected viruses (such as HarMV and CMV) altered the accumulations of both viruses. Field studies were done to measure the potential impact of natural infection by HarMV on an economically-important legume crop plant, *Lupinus*
angustifolius (narrow-leafed lupin). In field studies, HarMV was spread by naturally occurring aphids, with up to 31% of the lupin plants infected. Grain yield of affected lupin plants was substantially reduced, but seed-borne infection of HarMV was not detected.

Summary

Peptide mass fingerprint (PMF) analysis of plant viruses

A generic assay to detect and partially characterise viruses from plants was developed. Proteins extracted from virus-infected and uninfected plants were separated by one dimensional SDS polyacrylamide gel electrophoresis. Expressed coat protein bands not presented in uninfected plants were eluted after trypsin digestion and resulting peptide fragments separated according to their masses by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Resulting PMFs were compared with those present or predicted in protein databases. This assay strategy was used to identify four known viruses: the potyviruses Zucchini yellow mosaic virus (ZYMV) and Turnip mosaic virus (TuMV), an alfamovirus Alfalfa mosaic virus (AMV), and a cucumovirus (CMV). It was also used to identify a virus that manifested symptoms in wild C. maritima plants, tentatively identified as PZSV (genus Anulavirus) by its PMF, which was subsequently confirmed by Reverse transcription polymerase chain reaction (RT-PCR) and nucleotide sequencing. The detection of PZSV constitutes a first record of this virus in Australia and in this host. It is proposed that this simplified assay is a useful approach for analysis of plant samples known to harbour viruses, particularly for viruses which cannot be identified readily using antisera or nucleic acid-based assays. Although five viruses from different families and genera were identified successfully by this method, it was not a high-throughput and low cost
technique for sample screening, since protein extraction procedures were time-consuming, and protein identification based on PMF requires access to a high quality MALDI-TOF mass spectrometer. Nevertheless, this assay is a valuable complementary detection technique to support the identification of unexpected viruses to the species level, with further characterisation by other methods.

Detection and characterisation of CNSV by NGS

A lily plant (*L. longiflorum*), growing locally in Perth, Western Australia, with symptoms of chlorosis and streaking of leaves, was collected. Total RNA was extracted and sequenced using Illumina GA IIx technology. After assembly of reads, contigs representing the partial genome of CNSV RNA 1 and RNA2 were identified using Blastn and Blastx software. This virus was also found in bulb tissues of infected lily plants by RT-PCR and nucleotide sequencing (primers designed from the NGS sequences). This indicates that the virus can be transmitted through vegetatively propagated plant materials. Phylogenetic analysis suggested that the virus was a new strain of CNSV (family *Secoviridae*, genus *Nepovirus*). Comparison of the sequences of this virus to the CNSV (cycas strain) showed homology with identity of 88% and 96% at the nucleotide and amino acid levels, respectively, and to CNSV (gladiolus strain) of 88% and 94%, respectively; also provided in this work is a new strain of CNSV (lily strain). Another ten lily plants and nineteen cycas plants were tested for the virus by RT-PCR but none appeared to be infected. This virus has been recorded to be transmitted by seeds, nematodes and vegetative propagation, and has a very wide host range in ornamental and crop plants. This result is the
first record of this virus in Australia, which indicates that screening for CNSV and other nepoviruses in imported ornamental plants is essential to protect the Australian horticulture industry from incursion of new non-indigenous viruses.

**Natural spread of HarMV to narrow-leafed lupin**

Two field experiments were undertaken to study the potential threat of the Australian indigenous potyvirus, HarMV, to spread from its natural host, *Hardenbergia comptoniana* into narrow-leafed lupin (*L. angustifolius*) crops. Plants were grown in field conditions in two different years. Field plots of narrow-leafed lupin were established and interplanted with *H. comptoniana* plants infected with HarMV. Wild aphids were allowed to colonise the plots and spread the virus. Plants were monitored for aphids and symptoms of virus infection. Infection was confirmed by enzyme-linked immunosorbent assay (ELISA), RT-PCR and nucleotide sequencing. The first year established a pilot study, and this showed that HarMV spread naturally to 4.7% of *L. angustifolius* plants in the field. All infected lupin plants died within 20 days after virus symptoms became visible. In the second year a full field experiment was undertaken, and 30.7% of lupins became infected with HarMV. The majority of infected plants remained alive during this growing season, but showed symptoms of stunting, necrotic stem streaking and tip wilting. Three species of aphids were identified on plants during the experiment, including *Myzus persicae* (green peach aphid), *Acyrthosiphon kondoi* (bluegreen aphid) and *Rhopalosiphon padi* (oat aphid). A total of 761 seeds collected from infected plants were sown, and seedlings were tested for virus infection by ELISA. However, none of them were
found to be infected with HarMV, indicating that the virus was possibly not seed-borne in L. *angustifolius*. This aspect should be verified further by testing more seeds for HarMV from infected plants. The conclusion is that HarMV, a virus confined largely to a single native wild host, is capable of naturally extending its host range to an introduced grain legume under field conditions. Since adaption to an alternative host, as the case of HarMV invading lupin crops, is likely to be a driver of virus evolution, this pathosystem represents an ideal opportunity to study evolution of this virus in real time as it encounters new hosts, at the interface between an ancient ecosystem and a recent agroecosystem.

**Virus quantification using real-time quantitative PCR**

In this study, virus quantification by real-time quantitative PCR was used to titrate HarMV (isolate WHP-1, WHP-2 and MU-4) and CMV (Sn strain, subgroup II) expression in host L. *angustifolius*, *H. comptoniana* and *Nicotiana benthamiana*. A glasshouse experiment was done that showed HarMV isolate WHP-1 induced a non-necrotic (NN) response, while WHP-2 induced systemic necrosis (N) on lupin plants. Lupin cv Belara infected with WHP-2 isolate harboured a virus at a concentration of $1.34 \times 10^8$ copies/µl, approximately 18% higher than WHP-1 ($1.13 \times 10^8$ copies /µl). Although typical symptoms caused by HarMV on wild *H. comptoniana* include: chlorosis, leaf mosaic, leaf distortion, yellow spots and blotches, the symptom severity is variable on different plants, and even different branches of the same plant show inconsistent symptoms. Their within-plant titres varied, and the highest concentration ($1.75 \times 10^8$ copies /µl) was more than 19-fold that of the lowest ($9.16 \times 10^6$ copies /µl). The interaction between two co-infecting viruses (HarMV and CMV) infecting *N. benthamiana*
plants was studied. In doubly-infected *N. benthamiana* plants there was a strong synergistic increase in symptoms, with severe yellowing, stunting and chlorosis, and a higher overall virus titre (2.24×10⁸ copies/µl) than for single virus infected plants (at 6.89×10⁷ copies/µl for HarMV single infection and 4.05×10⁷ copies/µl for CMV single infection). Compared to the single infection, CMV accumulation was enhanced (at 2.18×10⁸ copies/µl in doubly-infected plants and 4.05×10⁷ copies/µl in single-infected plants four weeks after inoculation), while HarMV was suppressed (at 5.66×10⁶ copies/µl in doubly-infected plants and 6.89×10⁷ copies/µl in single-infected plants four weeks after inoculation) in mixed infection. This study used real-time quantitative PCR to investigate the virus within-plant titre, and showed that the virus concentrations were variable in hosts depending on the virus isolate, symptoms and interaction with co-infected viruses.

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**Viruses:**

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<td>Alfalfa mosaic virus</td>
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<td>WMV</td>
<td>Watermelon mosaic virus</td>
</tr>
<tr>
<td>WSMV</td>
<td>Wheat streak mosaic virus</td>
</tr>
<tr>
<td>WVMV</td>
<td>Wisteria vein mosaic virus</td>
</tr>
<tr>
<td>ZYMV</td>
<td>Zucchini yellow mosaic virus</td>
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</tbody>
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**Other abbreviations used in the text:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ammonium bicarbonate</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Long Form</td>
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<tr>
<td>mRNAs</td>
<td>messenger RNAs</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<tr>
<td>N</td>
<td>necrotic</td>
</tr>
<tr>
<td>NGS</td>
<td>next generation sequencing</td>
</tr>
<tr>
<td>NN</td>
<td>non-necrotic</td>
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<tr>
<td>NTB</td>
<td>nucleotide-binding protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PMF</td>
<td>peptide mass fingerprinting</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2</td>
<td>protein phosphatase 2</td>
</tr>
<tr>
<td>PVPP</td>
<td>polyvinylpolypyrrolidone</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid-amplification of cDNA ends</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RT</td>
<td>reverse-transcribing</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RuBisCO</td>
<td>ribulose-1,5-bisphosphate carboxylase oxygenase</td>
</tr>
<tr>
<td>SA</td>
<td>sinapinic acid</td>
</tr>
<tr>
<td>SBS</td>
<td>sequencing by synthesis</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
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ssRNA-  negative sense single-stranded RNA
ssRNA+  positive sense single-stranded RNA
TAE      Tris-acetate acid-EDTA
TBIA     Tissue blot immunoassay
TCA      trichloroacetic acid
TEM      Transmission electron microscopy
TFA      trifluoroacetic acid
UTRs     untranslated regions
VPg      viral protein genome-linked
WCM      wheat curl mite
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CHAPTER 1 LITERATURE REVIEW
1.1 Overview of plant viruses

This study aims to develop new tools, peptide mass fingerprinting (PMF) and next-generation nucleotide sequencing (NGS), to detect novel or unexpected viruses without prior knowledge of them. Two field experiments were undertaken to study the potential threat of the Australian indigenous potyvirus, *Hardenbergia mosaic virus* (HarMV), to spread from its natural host, *Hardenbergia comptoniana* into narrow-leafed lupin (*Lupinus angustifolius*) crops. Real-time quantitative PCR was used to titrate the necrotic (N) and non-necrotic (NN) isolates of HarMV, and study the interaction between two co-infecting viruses, HarMV and *Cucumber mosaic virus* (CMV) in lupin plants.

Plant viruses are nucleoproteins which can only multiply in living plant cells or their vectors. Their particle sizes range from 20 - 950 nm. A complete virion consists of nucleic acid surrounded by a protecting capsid (coat) protein (CP) which is encoded by the genome of the virus. Viruses are traditionally considered to be parasites, which employ host cell machinery to replicate their own genomes. Plant viruses in the genus *Tospovirus* (family *Bunyaviridae*) and family *Rhabdoviridae* also have an outer bilayer envelope containing lipid, resembling envelopes of animal viruses (Whitfield *et al.*, 2005).
Viruses are classified into six groups based on the nature of their genome: double-stranded DNA (dsDNA) viruses, single-stranded DNA (ssDNA) viruses, reverse-transcribing viruses (RT), double-stranded RNA (dsRNA) viruses, negative sense single-stranded RNA (ssRNA-) viruses and positive sense single-stranded RNA (ssRNA+) viruses. The only plant viruses in the group of dsDNA viruses are the Caulimoviridae family, classified in reverse-transcribing viruses. Two plant virus families, the Geminiviridae and Nanoviridae constitute the group of ssDNA viruses. Their genomes often consist of two or more circular components (Chasan, 1995; Vetten, 2008).

Two plant virus genera in virus family Partitiviridae, Alphacryptovirus and Betacryptovirus, have dsRNA genomes. The majority of plant viruses are included in the ssRNA+ group with fewer in the ssRNA- group (such as viruses in the families Bunyaviridae and Rhabdoviridae). dsRNA and ssRNA viruses all use a template-specific RNA-dependent RNA polymerase (RdRp), also known as RNA replicase, for genome replication. The difference between ssRNA+ and ssRNA- viruses is that ssRNA- viruses translate genes into proteins from an RNA strand complementary to that of the genome while ssRNA+ viruses translate directly from RNA sequences of the genome.

Reverse-transcribing virus replication includes the synthesis of DNA from RNA by the enzyme reverse transcriptase (via an RNA intermediate), such as viruses in family Caulimoviridae, with genomes arranged with a single molecule or two segments of open circular dsDNA (Hull, 1999).

The International Committee on Taxonomy of Viruses (ICTV) is the committee which authorises and organises the taxonomic classification of viruses. The objective of the ICTV is to communicate taxonomic decisions to the international community of virologists and develop an
internationally agreed taxonomy for viruses, which classify viruses on levels of order, family, subfamily, genus and species. According to the most recent report of the ICTV (8th) published in 2005 (Fauquet et al., 2005), plant viruses are divided into 16 families, with 61 genera in these families, and 17 unassigned genera, giving a total of 797 recognized species. There are two families of the circular ssRNA viroids, with 7 genera and 28 species, and 4 satellite virus species (Rybicki, 2005). New taxonomic proposals are always kept open, discussed and accepted by ICTV, especially for creating novel virus species, genera and families (Carstens, 2009).

1.2 Plant viruses in Australia

Since prehistoric times wild plants have been used for food, fibre, ornaments and medicine by mankind. As wild plants were domesticated ten to fifteen thousand years ago, human activities impacted both on plant evolution by plant selection, and on the plant viruses that infect them. Unlike the situation that existed in most other continents, agriculture was not developed by the original human inhabitants of Australia. Cultivated plants were introduced into Australia only about 200 years ago when Europeans and others colonised the continent. Plant viruses of the native flora, therefore, have not had a long duration to evolve with introduced crops, and many of them have not yet been shown to invade cultivated plants and remain restricted to wild plants (Gibbs & Guy, 1979; Cooper & Jones, 2006). Therefore Australia provides a unique model for research on virus evolution and the effects of human intervention, providing an ideal
environment to study encounters between endemic viruses and introduced crops, and vice-versa (Webster et al., 2007).

The greatest genetic diversity of an organism is normally present at the location where the organism first evolved (Stenger et al., 2002; Webster et al., 2007; Gibbs et al., 2008) (Fig. 1.1). Potyviruses native to Australia are a case in point. The genetic diversity of Australian endemic potyviruses is greater within Australia (Gibbs et al., 2008). In 2007, Webster et al. characterised a previously undescribed potyvirus from a native legume *H. comptoniana* and named it HarMV. The nucleotide sequences of CP genes of 28 HarMV isolates showed up to 21% diversity and clustered into eight distinct clades (Fig. 1.2). In contrast, most potyviruses isolated from crops in Australia originated from other regions of the world, and this is confirmed by phylogenetic analysis, which shows that for such introduced viruses, their genetic diversity is much lower in Australia. This tendency can be explained by the fact that plant viruses that affect domesticated plants in other parts of the world have evolved for up to ten thousand years, in the centres of origin of cultivated crop species in other continents (Cooper & Jones, 2006), while both the crops and crop viruses were introduced into Australia only within the last 200 years, and grown on a large scale only since the 1920s. Gibbs *et al.* (2008) suggested that the potyviruses in Australia were divided into two groups: one group was introduced to Australia only two centuries ago by European migrants, and the constituent viruses are genetically closely related to, but less variable than potyviruses present in other regions of the world. For the other group, all members belong to the *Bean common mosaic virus* (BCMV) lineage, mostly isolated from
native plants, and these are more variable than those of the crop potyviruses, since they are indigenous and only found in Australia (Fig. 1.1).

**Figure 1.1** Neighbour-joining tree calculated from the aligned ‘coherently evolving capsid’ (cCP) sequences from various isolates of *Bean yellow mosaic virus* (BYMV), *Rye grass mosaic virus* (RGMV) and *Clover yellow vein virus* (CYVV) together with those of 45 other potyviruses found outside Australia. Arrows indicate the positions of cCPs from Australian isolates. Numbers of cCPs are given when there is more than one. The other 12 viruses found in Australia are *Apium virus Y* (ApVY), *Bean common mosaic virus* (BCMV), *Celery mosaic virus* (CeMV), *Ornithogalum mosaic virus* (OrMV), *Papaya ringspot virus* (PRSV), *Pea seed-borne mosaic virus* (PSbMV), *Potato virus Y* (PVY), *Sweet potato feathery mottle viruses* (SPFMV), *Sweet potato virus Y* (SPVY), *Turnip mosaic virus* (TuMV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV). ud/s is uncorrected pairwise sequence difference per site.
Figure 1.2 Neighbour-joining tree calculated from the aligned ‘coherently evolving capsid’ (cCP) sequences from (a) viruses of Australia Bean common mosaic virus (BCMV) lineage, I to VIII are eight clades of Hardenbergia mosaic virus (HarMV) (b) the rest of the BCMV lineages (c) non-BCMV group members of the genus Potyvirus known only from Australia. HarMV was identified as the first endemic plant virus in Western Australia because of its great diversity in the Western Australian native flora. 28 isolates of HarMV were determined and divided into eight clades in the neighbour-joining tree with divergences of 5.4–21.1% in nucleotide identity (Webster et al., 2007). PWV, Passionfruit woodiness virus. S1VY, Siratro 1 virus Y. HiVY, Hibbertia virus Y. S2VY, Siratro 2 virus Y. CliVY, Clitoria virus Y. CerMV, Ceratobium mosaic virus. BCMNV, Bean common mosaic necrotic virus. PCIV, Passiflora chlorosis virus. EAPV, East Asian passiflora virus. CABMV, Cowpea aphid-borne mosaic virus. PSTV, Potato spindle tuber virus. DiVY, Diurus virus Y. WMV, Wisteria vein mosaic virus. SMV, Soybean mosaic virus. WMV, Watermelon mosaic virus. PFVY, Passiflora foetida virus Y. ZYMV, Zucchini yellow mosaic virus. SarVY, Sarcococlis virus Y. EVY, Eustrephus virus Y. DsMV, Dasheen mosaic virus. RhoVY, Rhopalanthe virus Y. ApVY, Apium virus Y. CarVY, Carrot virus Y. PleVY, Pleione virus Y. PtVY, Pterostylis virus Y. BYMV, Bean yellow mosaic virus. NSW, New South Wales. Qld, Queensland. WA, Western Australia.
1.3 Detection, identification and characterisation of plant viruses

Although some beneficial viruses have been discovered in plant hosts (Roossinck, 2011), most plant viruses cause a range of diseases, which are detrimental both to ecosystems and to agricultural production. Identification of virus pathogens is often a first step in control. Two or more techniques are often required to identify viruses, especially for unexpected or novel viruses. The main methods available to detect and identify plant viruses currently include symptom diagnosis and host range studies of experimental host plants, transmission electron microscopy (TEM), antisera-based immunoassay (e.g. enzyme-linked immunosorbent assay - ELISA and tissue blot immunoassay - TBIA), nucleic acid hybridization (e.g. microarray), reverse transcription polymerase chain reaction (RT-PCR) or polymerase chain reaction (PCR) for DNA viruses, cloning and nucleic acid sequencing, mass spectrometry (MS) and NGS.

1.3.1 Experimental host plants

Experimental host plants are often chosen because they are susceptible to a range of viruses, or they permit high titre infections or are species which are readily infected and maintained (propagation hosts), or they show a strong physical response to infection, such as local lesions (local lesion hosts). Many commonly-used experimental host plants are found in the genera Nicotiana, Solanum, Chenopodium, Cucumis, Phaseolus, Vicia and Brassica (Hull, 2002).
The selection of an indicator plant for a virus is based mainly on the ease of infection after inoculation or after exposure to the vectors, the time taken to show symptoms and the consistency of visual symptoms on the leaves. For instance, Allen & Matteoni (1991) tested eight species for use as indicator plants for the thrips transmitted *Tomato spotted wilt virus* (TSWV), genus *Tospovirus*, family *Bunyaviridae*. They found petunia and gloxinia developed the most viral lesions in the shortest time, i.e. 2-3 days after exposure to thrips. Petunia consistently showed the highest percentage of infection. Symptoms of plants infected by different viruses in the field are usually inadequate to distinguish one virus responsible for the disease from another, although some virus infections have characteristic symptoms (e.g. sugar beet infected with *Beet necrotic yellow vein virus* (BNYVV)). Viruses often induce very similar symptoms, such as the yellowing symptoms on beet (*Beta vulgaris*) caused by *Beet yellows virus* (BYV), *Beet mild yellowing virus* (BMYV), and *Beet western yellows virus* (BWYV) (Mouhanna et al., 2002). *Bean yellow mosaic virus* (BYMV) and HarMV both induce necrotic stem streaking and leaf downcurling in infected narrow-leafed lupin (*L. angustifolius*). In other cases different virus isolates in a species could result in completely different symptoms and susceptibility, such as CMV isolate LY2 does not systemically infect two commonly-used propagation hosts for CMV, tomato and cucumber plants (Francki & Hatta, 1980; Lee et al., 2007).

1.3.2 Transmission electron microscopy (TEM)
Since the size, shape, pattern of symmetry and other surface features are basic criteria that distinguish one virus particle from another, it is valid first to attempt to identify viruses by particle morphology. TEM is a standard approach to identify some viruses based on their morphological characteristics. For examination of virus particles in crude extracts or purified preparations, a negative-staining procedure is usually used. The most commonly used electron dense negative stains are sodium phosphotungstate, ammonium molybdate or uranyl acetate, depending on the stability of the virus to these stains.

One of the keys to identifying viruses according to morphological characteristics is to determine particle dimensions, especially for rod-shaped viruses. However, this can be influenced by preparation and stain. Thus when researchers define the size of a specific kind of virus the method of preparing and staining samples must be stated as well. The structure of the virus CP is another indicator used to identify or characterise virus particles. According to the appearance and organisation of the CP there are four virus morphologies: helical, icosahedral, enveloped and complex. The CPs of helical particles consist of identical subunits which lie outside the central coiled RNA forming a helix structure. The virions of this kind of virus are rod like or filamentous (e.g. Potexvirus and Potyvirus, respectively). There are two kinds of rod-shaped viruses: those with rigid rod viruses, such as Tobacco mosaic virus (TMV), and flexible rod viruses, such as Potato virus Y (PVY). Flexible rod virus particles are often longer but narrower than rigid rod viruses. The subunits of viruses with icosahedral symmetry are constructed in an optimum way to form a closed sphere (e.g. Cucumovirus). Some virus species envelope
themselves in membrane of host-origin, such as the outer cell membrane or nuclear membrane. This type of virus is called an enveloped virus (e.g. Tospovirus).

Apart from observation of particle characteristics, the recording of ultrastructural alterations in host plant cells induced by virus infection is another way to use TEM to diagnose diseases. For example, characteristic pinwheel inclusion bodies are present in cells infected by potyviruses (Roberts et al., 1998). However, preparation of samples for ultrastructural studies using TEM takes several days. A microwave irradiation method was developed to reduce the preparation time to shorten this whole procedure to less than half day (Zechmann & Zellnig, 2009). They investigated two cases: Nicotiana tabacum infected with TMV, and Cucurbita pepo infected with Zucchini yellow mosaic virus (ZYMV). Large regions of virions aligned parallel in the cytosol of TMV-infected leaf cells, and cylindrical inclusions induced by ZYMV in the cytosol of infected leaf cells were clearly observed. Virions were also detected by negative stain. This research contributed to a rapid virus diagnostic method using TEM.

1.3.3 ELISA and TBIA

Before the ELISA method had been developed, immunoassays were done mainly using radioactively labeled antigens and antibodies (Yalow & Berson, 1960). The principle of ELISA is to use the specific binding of antibodies to antigens, with an enzyme linked to the antibodies. From a subsequent enzyme-substrate reaction, colour or fluorescence is generated, which can
be interpreted both qualitatively and quantitatively. ELISA has been used widely as a diagnostic tool in medicine and plant pathology because it is versatile, relatively sensitive, specific and easy to automate.

Since ELISA was first described as a detection tool for plant viruses it has become one of the most widely used and reliable techniques in plant virus research (Clark & Adams, 1977). In this approach, a labeled secondary antibody is used to bind the specific one, which avoids the expensive process of linking every specific probe selected to detect different antigens with enzyme, but instead enables the use of the same secondary antibody, in a process called ‘sandwich ELISA’. Since then, researchers have developed many plant virus antibodies specific to various levels: isolates, species, strains, sub-groups and genera (e.g. potyvirus) (Jordan & Hammond, 1991).

TBIA shares the principle of ELISA, in which one of the reactants (usually the antigen) is bound to a membrane, such as nitrocellulose, and then detected by a labeled antibody probe. TBIA requires simple equipments and is sensitive (especially with application of a secondary antibody) and fast. However, it cannot provide quantitative information on the test samples, and background colour may lead to confusion (Garnsey et al., 1993).
1.3.4 Reverse transcription PCR and Real time PCR

PCR is the most widely used method to amplify particular DNA for subsequent analysis (e.g. sequencing). Most plant virus genomes are RNA based so that viral RNA is first reverse transcribed into complementary DNA (cDNA) by reverse transcription. Primers used in PCR are designed from known sequences of the target viral genome, most of which are highly species-specific. A few group-specific primers are available, such as potyvirus degenerate primers (Langeveld et al., 1991; Webster et al., 2007). (RT) PCR is in general a much more sensitive diagnostic method than ELISA, although care must be taken to prevent contamination. New PCR primers are also much easier to design and synthesize than novel antibodies.

RT-PCR is used to detect and quantify a targeted DNA or RNA molecule. Both non-specific fluorescent dyes (e.g. SYBR Green) that bind with any double-stranded DNA and sequence-specific DNA probes are used to detect the amplification. Compared with conventional PCR it allows the detection of PCR amplification during the early phases of the reaction. It is much more sensitive and the amplified DNA is detected as the reaction progresses in real time. Multiplex RT-PCR was developed to detect different RNA targets simultaneously in a single reaction (Osiowy, 1998), which makes this approach a useful detection tool because plants are often infected with more than one virus. A multiplex RT-PCR assay was developed to detect six RNA viruses in olive trees (Bertolini et al., 2001). Real-time RT-PCR can be used to detect a small amount of viral nucleic acid in various biological backgrounds, such as in irrigation waters.
(Boben et al., 2007), vectors (Boonham et al., 2002) or seeds (Zhang et al., 2010), because of its high sensitivity. Mortimer-Jones et al. (2009) detected and quantified four potato viruses from tuber tissue extracts in a multiplex quantitative real-time RT-PCR assay. Apart from virus detection, real-time PCR has also been applied to investigate the influence of virus infection on host gene expression by quantifying messenger RNAs (mRNAs) and microRNAs (miRNAs) in host plants (Feng et al., 2009). Immunology is sometimes used in combination with RT-PCR to enhance its sensitivity, such as in immunocapture RT-PCR used in plant virus detection (Žižytė et al., 2006).

### 1.3.5 Detection and characterisation of plant viruses by MS

MS has become more widely used in biological research primarily because of the development of matrix-assisted laser desorption-ionization (MALDI) and electrospray ionization (ESI) MS, which enable accurate analysis of large biomolecules, such as proteins, peptides, nucleic acids and carbohydrates. MS is widely used in research on protein-protein interactions, protein-DNA interactions (Kriwacki & Siuzdak, 2000), post-transcriptional modification of biomolecules (Taniguchi & Hayashi, 1998), protein mapping (Manabe et al., 2007) and protein identification (Webster & Oxley, 2005).

Most current virus detection tools require some previous knowledge of the virus, and this is a drawback for identifying unknown or unexpected viruses. For antisera-based techniques such
as ELISA and TBIA a wide range of species-specific antibodies are commercially available, as well as some antisera that are group-specific (Kiratiya-Angul & Gibbs, 1992). However, such antibodies are not available for all known viruses, and certainly not for unknown viruses. The most widely used nucleic acid-based assay for virus identification is enzymatic amplification of fragments of the virus genome by PCR (preceded by RT in the case of RNA viruses), often followed by nucleotide sequencing. Microarrays are less commonly used because of inherent sensitivity and reliability issues (Boonham et al., 2007). In both cases primer/probes are designed from known sequences of the target genome to be either highly species/strain-specific or group-specific, depending on the whether identification of one specific virus or a group of related viruses is desired (Gibbs & Mackenzie, 1997; Webster et al., 2007; Wylie et al., 2008). Where primer sets amplify a range of viruses, restriction fragment length polymorphisms within PCR amplicons can be used to identify specific genotypes by electrophoresis or MS (Michael et al., 2009). Traditional diagnostic methods of symptom evaluation, vector specificity, host range studies, and electron microscopy to visualise particle and inclusion size and shape tend to be more generic than molecular methods. Often, two or more assays are combined as part of the diagnostic process (Cooper et al., 2003). In most plant virology research MS provides complementary information to plant virus identification. For instance, Seifers et al. (2005) found a novel virus in sorghum that weakly reacted with *Johnsongrass mosaic virus* (JGMV) antiserum. Tandem mass spectrometry (MS/MS) analysis of the CP sequence suggested that it was a new strain of JGMV, and explained the weak binding to antiserum: the CP sequence of the new virus strain was rather different from other strains at the position of the antigen-antibody interaction.
MS has been used to study a wide range of biological processes of viruses, including virus-antibody binding (Siuzdak, 1998), virus-host interactions (Di Carli et al., 2010), protein–protein interactions (Brizard et al., 2006) and protein dynamics (Lan et al., 2010). Application of MS to characterise CPs of plant viruses is based on its wide mass measurement range (She et al., 2001; Padliya & Cooper, 2006), and on PMF (Lewis et al., 1998) and protein sequencing (She et al., 2001). MS has been used accurately and rapidly to identify viruses based on the molecular weight of their CPs, especially for prevalent viruses of specific hosts (Thomas et al., 1998; Tan et al., 2000; Padliya & Cooper, 2006). However, not all CP masses are well characterised for plant viruses, and the actual protein mass can differ from that predicted from its genomic sequence because of post-translational modifications. To achieve better sensitivity and specificity, PMF was developed as a reliable technique to identify different proteins (Pappin et al., 1993). A protein is digested by a site-specific proteolytic enzyme, such as trypsin or chymotrypsin, to generate a profile of peptides each with unique masses. The mass data obtained by MS is subsequently compared with theoretical mass data of proteolytic fragments from a protein database, which then gives the protein identity (Fig. 1.3). This technique can be used to identify CPs of plant viruses and therefore resolves different species or mutants of the virus (Lewis et al., 1998). We have demonstrated its application for detection of unknown viruses in a host using one-dimensional electrophoresis to separate virus proteins followed by PMF analysis (Luo et al., 2010) or peptides sequencing (Blouin et al., 2010). Search engines and databases are available, with which to search for homologous proteins: SEQUEST (MacCoss et al., 2002), MOWSE
(Savitski et al., 2005), Mascot (Hirosawa et al., 1993) and Profound (Zhang & Chait, 2000).

Unknown viruses whose sequence is not in a protein database will still remain unidentified, and such viruses require further analysis by peptide sequencing. MS/MS peptide sequencing can be used to detect and characterise strains, isolates and minor mutants of virus CPs. Compared to PMF analysis, sequencing of proteins or peptides provides more accurate information on a specific virus. For example, She et al. (2001) reported deviations of several Brome mosaic virus (BMV) isolates in CP amino acid sequences, when these isolates multiplied in different host.
Figure 1.3 The principle of identifying plant virus proteins using peptide mass fingerprinting (PMF). Comparison between infected and uninfected plant protein composition indicates suspected coat protein band, which is cut from the polyacrylamide gel and digested by trypsin or other proteinase. Mass spectrometry (MS) is used to accurately measure the peptides’ masses. The masses matching those in protein databases are analysed by an algorithm.
1.3.6 Other techniques for plant virus detection

1.3.6.1 NGS: 454 sequencing/illumina sequencing

Recently, competing new ‘massively parallel’ DNA sequencing platforms have been developed. They are based on attaching hundreds of thousands of relatively short DNA sequences to beads or slides, and then measuring addition of sequential bases to DNA strands from primers complementary to target sequences. DNA sequencing technology developed by 454 Life Sciences (now Roche) called 454 sequencing (Fig. 1.4) has been used in research on human genomes, metagenomics, genomic structure, ecology, evolution and paleobiology. There have been routine improvements in library preparation, template preparation and sequencing, and this technology enables massive parallel sequencing reactions to be done at relatively low cost (Rothberg & Leamon, 2008). This high throughput pyrosequencing system has been used to sequence approximately 400-600 megabases of DNA per run, with the latest upgrade providing reads that average 800 megabases. The second more widely used NGS platform is the Illumina/Solexa Genome Analyzer by ‘sequencing by synthesis (SBS) technology’. This technology is based on adding specific nucleotides complementary to target DNA sequences. As each base is added to each DNA fragment, a highly sensitive camera is used to take the images of the flash of the fluorescence as the nucleotide is added to record the nucleic sequences. The Illumina sequencer is able to generate 20 – 25 Gb of high quality data per run and 2.5 Gb per day. Recent improvements, such as in base-calling approaches (the process of bases being
recorded from light signals), makes this technology more accurate, high throughput and cost-effective (Ledergerber & Dessimoz, 2011).

Several projects have demonstrated the utility of the NGS in detecting and classifying unexpected pathogens, such as viruses, from a complex biological background. For instance, a new arenavirus transmitted through solid-organ transplantation was identified using NGS from three patients who received visceral-organ transplants from a single donor and died 4 to 6 weeks after transplantation (Palacios et al., 2008). The metagenomic analysis of healthy and infected hives using NGS indicated that Israeli acute paralysis virus presented in unhealthy honeybees with colony collapse disorder (Cox-Foster et al., 2007). However, Israeli acute paralysis virus was found not to be the causative agent of colony collapse disorder. It is essential to have fully biological understanding of the studied disease when using metagenomics.

Unknown viruses are readily sequenced and detected in complex backgrounds using 454 or Illumina sequencing without cloning and pre-amplification. Continuous improvements make these techniques easier to use and less costly (Rothberg & Leamon, 2008). NGS was usually used with metagenomic analysis to produce a large amount of viral cDNA sequences in complex background. A previously uncharacterised cucumovirus Gayfeather mild mottle virus was detected in plant Liatris spicata and its full genome was obtained, developing a routine assay
for new viral pathogens (Adams et al., 2009). Roossinck et al. (2010) analysed thousands of plant samples from northeastern Oklahoma and northwestern Costa Rica for viruses in metagenomic studies. They discovered a few thousand new plant viruses and linked all of them to their hosts by using molecular tags at the end of each virus cDNA. By searching using BlastN and BlastX they concluded that 70% of the samples had putative virus sequences in 11 families. Most virus sequences were classified to the family level, but it was hard to specify them all to a known strain or species. Wylie et al. (2010) applied Illumina NGS to analyse infected plants, both domesticated and wild, and characterised several novel full virus genomes from them by subsequent bioinformatics analysis. These included Passionfruit woodiness virus (PWV) (full genome 9,858 nucleotides) (Wylie & Jones, 2011b), Hardenbergia virus A (full genome 6,936 nucleotides) (Wylie & Jones, 2011c) and Shallot latent virus (SLV) (full genome 8,371 nucleotides) (Wylie et al., 2011), and the genomes of another 19 viruses were partially characterised.
Figure 1.4 Overview of 454 sequencing. (a) Purified and fragmented single strand DNA is ligated to adapters. (b) Each fragment is bound to one single bead in a droplet of a PCR-reaction-mixture-in-oil emulsion where PCR amplification occurs. (c) Enriched single-stranded DNA templates are deposited into wells of a fiber-optic slide. (d) Beads with immobilized enzymes for sequencing reactions are deposited into wells. (e) A view of the surface of a fiber-optic slide by electron microscope (f) Diagram of the major components of a 454 sequencing instrument: (i) a fluidic assembly; (ii) fiber-optic slide in a flow cell; (iii) a highly sensitive camera used to record sequencing results as flashes of light and a computer for instrument control and data analysis (Rothberg & Leamon, 2008).
1.3.6.2 Microarrays

Microarrays provide parallel, specific and high-throughput tests for various viruses at different levels, strains, species, genera or families depending on the specificity of the immobile probes. Each probe can be bound to the solid support at an individual small spot on the chip, allowing different DNA fragments to be investigated simultaneously in a highly parallel fashion. In practice this is useful for screening material for the presence of viruses, for example, from a quarantine list, or against a list of viruses that should be absent from the material (Boonham et al., 2007).

One improvement in microarray technology was that synthetic oligonucleotides could be used to substitute for PCR products as probes, to enhance the sensitivity and specificity for plant virus identification. For instance, Deyong et al. (2005) successfully distinguished sub-groups of CMV, with only 8% nucleotide sequence difference between them. Microarrays have been used to rapidly screen common viruses infecting important crops (Boonham et al., 2003; Call et al., 2003; Vora et al., 2004; Call, 2005). Microarrays have also been used to provide information on interactions between host plants and their pathogens, including plant defense responses, by measuring gene expression via cDNA, i.e. by expression analysis or expression profiling (Wan et al., 2002). In addition, with improved automation, sensitivity and high-throughput of microarrays, they are a powerful tool for virus detection (Bystricka et al., 2005; Boonham et al., 2007). However, NGS is likely to supercede microarray technology as the costs are reducing.
Some NGS systems provide both in depth sequence data and quantification of viruses present in a sample. In addition, the complexities of microarray production and control of hybridisation conditions make microarrays technically more difficult to use for virus diagnostics.

1.4 Transmission of plant viruses and their vectors

Viruses are described as ‘molecular parasites’ in cells, using bio-molecules and energy of the hosts for replication, and whose spread and transmission from one host to another is necessary for survival. However, the presence of cell walls and the immobility of plants are the two main barriers to plant virus transmission. Some viruses are transmitted in seeds or via pollen and spread by wind. However, most plant viruses are spread by vectors that can introduce viruses across cell walls and also aid dispersal of viruses. These include fungi, nematodes, arthropods and arachnids (Hull, 2002). Mechanical inoculation and grafting are the most convenient and commonly used methods for virus transmission in laboratory or glasshouse studies (Dijkstra & de Jager, 1998).

1.4.1 Insects

Insects are the most common vectors of plant viruses. Seven orders of the Class Insecta transmit plant viruses, most in Thysanoptera and Hemiptera. They have piercing and sucking
mouthparts. Other vectors are in Orthoptera, Dermaptera, Coleoptera, Lepidoptera and Diptera, which have chewing mouthparts (Raccah & Fereres, 2009).

Depending on the way they are transmitted, plant viruses are divided into three groups: nonpersistent, semipersistent and persistent. Nonpersistent viruses have a short retention time (usually a few minutes to hours) in the vector, in which virus particles are attached to the stylet of the insect and are transmitted to the next plant it probes or feeds on. Persistent viruses remain viable in the insect salivary gland to either replicate (propagative) or not (circulative). Semipersistent viruses have an intermediate retention time in their vector.

The CP is usually important for virus transmission by insects. Mutations in the CP gene can often change the transmissibility of the virus (Perry et al., 1998). Many viruses encode helper components (HC) to facilitate the interaction between virions and their vectors (Kassanis & Govier, 1971). Pure virions of potyviruses and caulimoviruses cannot be transmitted by their vectors unless they are present in the mixture of nonstructural virus-encoded protein (HC) (Raccah & Fereres, 2009).

Piercing and sucking mouthparts, their polyphagous nature and global distribution, make aphids vectors of many plant viruses. Fifty percent of insect-vectored plant viruses were reported to be transmitted by aphids (Nault, 1997; NG & Perry, 2004), such as the majority of
potyviruses. Thrips are considered to be pests not only because they feed on many commercial crops but also because they transmit some damaging viruses, such as TSWV, one of the most damaging viruses of horticultural crops (Martinez, 2005). Leafhoppers feed on a wide range of plants and are vectors of plant pathogens, including viruses (Todd et al., 2010), phytoplasmas (Weintraub & Orenstein, 2004) and bacteria (Almeida et al., 2005). Some mastreviruses and curtoviruses (family Geminiviridae) are spread by different leafhopper species, and affect several important crops, such as maize, chickpea and cotton (Redinbaugh et al., 2002; Akhtar et al., 2011).

1.4.2 Nematodes

Although four orders of nematodes feed on plants, only a few species in the migratory ectoparasitic families Longidoridae and Trichodoridae are vectors of plant viruses. Since 1958, several nepoviruses of the family Comoviridae were reported to be transmitted by Longidorid nematodes in America and Europe, including Arabis mosaic virus (ArMV), Tomato black ring virus (TBRV) and Grapevine fanleaf virus (GFLV) (Brown et al., 1995). So far, of the 38 known nepoviruses, 13 have been shown to be naturally transmitted by Longidoridae, seven by Longidorus species, one by Paralongidorus and nine by Xiphinema species, while all tobraviruses are transmitted by Trichodoridae vector species (Decraemer & Robbins, 2007).
With the availability of molecular techniques, research on nematodes that transmit viruses has focused on mechanisms of plant-nematode-virus interactions. The CPs of nematode transmitted viruses play an important role in nematode-virus interactions, and there is a high degree of specificity between vector species and their associated viruses, but CPs are not the sole determinant for transmission. All the nematode vectored nepoviruses and tobraviruses have bipartite genomes consisting of two single-stranded RNAs. CP and other proteins encoded by the RNA-2 are involved in the transmission process (Brown et al., 1995).

Nematodes are widely distributed worldwide, including in Australia. Some are capable of transmitting harmful viruses to a wide range of fruit and vegetable crops. Since some nematode species may have long life-cycles or quiescent stages (2 to 5 years) (Lamberti et al., 1975) they might act as another reservoir of crop viruses apart from wild plants present in fields or their vicinity.

1.4.3 Fungi

Fungal species of three genera are virus vectors: genus *Olpidium* in family *Olpidiaceae*, genus *Polymyxa* and genus *Spongospora* in family *Plasmodiophoraceae*. These fungi survive as obligate parasites in the roots of plants, and produce and release zoospores to infect other healthy plants. The zoospores present at healthy roots withdraw their flagellae and start to generate cysts whose infection tube can penetrate root cell walls to introduce both fungal
material and infective viruses (Kakani et al., 2003). Sometimes zoospores form thick walls and act as resting spores, remaining in root debris for a substantial time. They can germinate subsequently and release new zoospores, depending on environmental conditions. Some viruses enter the resting spores when they form and remain infective. Viruses can then be transmitted to host plants via zoospores produced by the reactivated virus-containing resting spores (Dijkstra & de Jager, 1998), making them a long-term repository of crop viruses.

The fungus species Polymyxa graminis, genus Polymyxa, family Plasmodiophoraceae, transmits cereal viruses causing crop production losses worldwide, including viruses that infect rice, barley, oats and wheat. For example, Barley yellow mosaic virus (BaYMV) and Barley mild mosaic virus (BaMMV) cause barley disease that is widespread in Europe and East Asia, which is of great concern to the agricultural industry (Kanyuka et al., 2003). In one study, the yield of barley crops infected with BaYMV was reduced by 15% for cv. Maris Otter and 50% for cv. Igri (Plumb et al., 1986).

1.4.4 Seed- and pollen- borne viruses

In the order of 20% of plant viruses can be transmitted from generation to generation in seeds either via infected gametes or invasion by the embryo via the ovule. Virus testing is destructive to seeds and only samples can be tested, giving an infection incidence. It is more difficult to detect viruses in contaminated seeds, but seed infection is significant because viruses can
rapidly extended their geographic distribution through international trade of virus infected seeds.

It is usually the case that seed-borne viruses are not lethal to the hosts which produce the seeds in which they are transmitted, and this facilitates virus spread through the crop by providing an ‘internal infection source’, such as when CMV infects narrow-leaf lupin (L. angustifolius) (Jones, 2005). Lupin seeds infected with CMV were tested initially using ELISA for large-scale samples and this was superceded by using RT-PCR for more sensitive detection (to 0.1% infection rate) (Wylie et al., 1993). Testing farmers’ samples and removing this source of infection from the field is an effective way to reduce crop losses. However, some seed-borne viruses do not necessarily cause obvious symptoms, such as Pea seed-borne mosaic virus (PSbMV), which does not produce characteristic symptoms on peas, but nevertheless causes production loss (Khetarpal & Maury, 1987).

Virus invasion of the embryo via the ovule is related to the host maternal genotype (Wang & Maule, 1992). Virus invasion of embryo cells occurs via the transient suspensor cells, whose function is nutritional support of the growing embryo, and which become a route for virus invasion. Multiple host genes are involved in resistance to seed transmission of PSbMV, such as maternal genes which prevent the virus from reaching the suspensor before its programmed degeneration (Wang & Maule, 1994). In addition, pollen transmitted viruses sometimes cause
fertilisation failure. For instance, *Tobacco ringspot virus* (TRSV)-infected soybean produces less pollen than virus-free plants and germ tubes do not grow normally (Yang & Hamilton, 1974).

### 1.4.5 Transmission through soil

Plant viruses can be transmitted through soil both in a biotic or an abiotic manner. Biotic transmission is mainly by nematodes and fungi living in the soil (1.4.2 and 1.4.3). Although it is not a major pathway for virus spread, some plant viruses are physically held in the soil or in soil water and can infect healthy roots through wounds (Cadman, 1963; Dijkstra & de Jager, 1998). Only a few viruses have been found to be transmitted in this way (Harrison, 1960). For example, *Rice yellow mottle virus* (RYMV) was detected from guttation fluid collected from infected rice plants and irrigation water collected at the base of infected plants. RYMV containing soil remained infective two weeks after soil contamination (Traoré *et al.*, 2008).

### 1.4.6 Mechanical inoculation and grafting

Spread of plant viruses by direct contact between infected and healthy plants rarely takes place in nature, and is mainly found in roots. TMV can infect healthy tomato seedlings from infected tomato debris through contact to the roots (Gergerich, 2004). However, in glasshouse studies, mechanical inoculation is the method used more often for plant virus diagnosis, host range tests, assessment of infectivity, characterising symptoms and so on. The infected plant tissues
are ground in phosphate buffer (Chapter 2). The sap is mixed with diatomaceous earth (celite) and rubbed manually against healthy plants to cause wounds on leaves, which provide a route for virus invasion. Effective and quick manipulation during inoculation is important since viruses do not remain infective for a long period after isolation.

Grafting is another method that is often used for virus inoculation, especially for viruses that are unstable in sap or for which no biological vector is available, such as for phloem-limited viruses (e.g. *Citrus tristeza virus* (CTV) (Rocha-Peña *et al.*, 1993)). Methods include bud grafting, wedge grafting and tuber grafting. Grafting can be used as a complementary inoculation method to mechanical inoculation. It also provides a greater inoculation ‘pressure’, in which a continuous supply of virus inoculum is delivered from the graft. Grafting with an infective scion is therefore the most comprehensive method to test whether the stock is susceptible or resistant to infection by a particular virus.
1.5 Impact of plant viruses on agricultural production and natural ecosystems

1.5.1 Losses caused by plant viruses

With the increasing globalisation of trade, particularly over the last 20 to 30 years, plant products, including edible produce, propagules, ornamental plants and other plant materials have become widely traded internationally. Inevitably, plant pathogens infecting them are also spread to distant regions and continents. Plant viruses and phytoplasmas were identified as the cause of 51% of the emerging infectious diseases of plants worldwide during the period of 1996–2002 (Rodoni, 2009).

The main motivation for the study of plant viruses is the impact of diseases they cause to crops and native plants. It is difficult to obtain reliable data on global economic losses from plant virus diseases because much of the data is from small scale experiments rather than comprehensive global surveys (Hull, 2002). Furthermore, visual symptoms of virus infection do not necessarily correlate with yield losses or product quality, because effects can be caused by indirect and more subtle means, which are often not considered. For example, some pollen-borne viruses can prevent normal germination and growth of the germ tube, which then results in failure to fertilise the egg, for example in soybean, which results in a reduction of soybean yield without obvious external symptoms (Yang & Hamilton, 1974).
TSWV is the most well studied virus in the genus *Tospovirus*, family *Bunyaviridae*, and is transmitted by the western flower thrips (*Frankliniella occidentalis*), tobacco thrips (*F. fusca*) and some other thrip species. It can infect a range of crops, including tomato, peanut, tobacco and pepper. As the most damaging disease of peanut crops in America, TSWV caused a 7.5% loss of peanut production in 2005 in Georgia, valued at $ 31.7 million (Martinez, 2005), and annual losses for all crops caused by TSWV infection are estimated at $ 100 million in Georgia (Jain *et al.*, 1998).

Western Australia produces about 80% of the world production of lupin seed, which amounted to 920,000 t produced on 650,000 ha area in 2005 (Palta *et al.*, 2008), although production has dropped considerably since then. Of viruses infecting the lupin crop in Australia, BYMV is one of the most significant (Jones & McLean, 1989). Narrow-leafed lupins show two distinct responses to BYMV infection, either systemic necrosis (N) or non-necrotic reactions (NN). Infection by the common N strain of BYMV kills young lupin plants quickly, while infection of older plants decreases their yields by 55-80% (Jones *et al.*, 2003), depending on the stage of maturity at infection. Total crop yield losses resulting from the NN strain of BYMV were higher (27-98%) because affected plants remained infective for the growing season resulting in a polycyclic spread pattern (McKirdy & Jones, 1995; Cheng & Jones, 2000; Jones, 2005). CMV, transmitted through lupin seeds, is another economically important virus of lupins. Seedlings infected from seed planted amongst healthy plants act as the major source of CMV infection in lupin crops,
and act as foci for spread of this virus via aphid vectors to the current season’s previously healthy plants.

Although most viruses are regarded to be detrimental to their hosts, viruses can also be mutualists and beneficial to their hosts (Roossinck, 2011). In some cases, virus infection improves plant tolerance to drought and freezing. For example, CMV improved drought tolerance of various host plants and enhanced freezing tolerance of beet plants (Xu et al., 2008). A persistent plant virus, *White clover cryptic virus* (WCCV), encodes a gene that can help nodulation of its legume plant host (Nakatsukasa-Akune et al., 2005).

### 1.5.2 Climate change and plant virology

Climate change, a feature of the earth’s periodic cycles of warming and cooling that is accelerated by human activities (Cox et al., 2000), such as increasing greenhouse gas emission since the mid-twentieth century, will have a substantial impact on plant-virus interactions. Climate change results in altered and more extreme climates including changes in temperature (both warmer and cooler), rainfall (both drier and wetter), and wind patterns.

Temperature and rainfall patterns both affect virus multiplication. Some viruses, their vectors, and hosts grow in tropical zones, whereas others grow in temperate or cooler zones. With
altered temperatures, viruses adapted to warmer climates will establish in regions that are now cooler, and this will generate encounters between new hosts and viruses. Some resistance genes are only effective in temperate zones, but are ineffective with warmer temperatures, which may reduce the effectiveness of resistance genes in warmer climates (Jones, 2009). For example, the N gene is a single dominant gene conferring a hypersensitive response (HR) to TMV infection. It develops necrotic local lesions that prevent a systemic necrotic response when the temperature is below 28 °C, but fails in higher temperatures (Kang et al., 2005). Both plant density and the duration of leaf water films, with the latter affected by rainfall patterns, influence host infection, mainly by influencing the behavior of virus vectors (Garrett et al., 2006). Other changes to climate, such as more frequent drought and higher wind speeds, will also have an impact on virus epidemics (Jones, 2009). For example, drought reduces soil moisture, which in turn decreases the activity of zoospores of fungal vectors. Wheat curl mite (WCM; *Aceria tosichella*) is the vector of *Wheat streak mosaic virus* (WSMV), and the wind speed affects its movement in crops. The spread of plant viruses is not only affected directly by climate change, but also in indirect ways, such as by changes of virus vector distribution. Aphid vectors are predicted to increase in numbers, expand their geographical distributions and advance the timing of spring migrations as a result of climate change (Harrington *et al.*, 1995; Norse & Gommes, 2003), thus facilitating aphid-borne virus spread.

Early on in the domestication of crops, such crops stayed at their original locations together with their wild relatives, most within the eight major crop domestication centers (Jones *et al.*, 2005).
Over the years, world trade in plant products has expanded the original geographical range of crops and their pathogens. New encounters of plants and viruses present a significant threat for both agricultural production and ecological diversity. An aggressive virus that a crop has not previously encountered before could result in a new damaging disease. In addition, native flora could be destroyed by new introduction of a virus to a new area, systematically threatening local biodiversity, especially for those places with there is a delicate ecosystem which has been geographically isolated from external pathogens for long time (Anderson et al., 2004). For example, whitefly-borne geminiviruses had been found mainly in East and West Africa infecting Cassava (*Manihot esculenta*) in the 1970s (Fondong et al., 2000). There have now been serious geminivirus diseases in the Western Hemisphere since the 1990s, and these viruses now widely infect tomato, cotton, cassava, cucurbits, and beans. This is attributed to worldwide expansion of its vector, and has caused substantial economic losses (Polston & Anderson, 1997). Geminiviruses destroyed 95% of the tomato harvest in the Dominican Republic, and caused $140 million loss for tomato production in 1992 in Florida (Moffat, 1999). Cotton leaf curl disease (CLCuD), caused by a geminivirus *Cotton leaf curl virus* (CLCuV), became the major limitation to cotton production in Pakistan in the early 1990s, and it has now spread into India (Briddon & Markham, 2000).

### 1.6 Aims and Objectives of this research

#### 1.6.1 Optimising Mass Spectroscopy-based methods to detect viruses
Many methods are available that can be used to detect and identify plant viruses in plant tissues, including symptom diagnosis, host range studies, electron microscopy, serology hybridization, PCR and DNA sequencing techniques. However, there is no single method that can be used to identify novel viruses reliably. For example, PCR-based methods, conventional virus genome sequencing, and ELISA require some prior knowledge of the virus to be detected. Virologists often have to apply many procedures in order to identify an unknown virus. It is valuable, therefore, to develop generic approaches to characterise both known and unknown viruses.

MS provides a new approach to virus detection, identification and characterisation. Detection of viral proteins, their identification by peptides mass fingerprinting (PMF) and characterisation with MS/MS protein sequencing are the three most common strategies to detect plant virus using MS. However, most plant virus research is focused on further characterising known viruses rather than the detection of new viruses (Tan et al., 2000; She et al., 2001; Padliya & Cooper, 2006).

**Aim 1:** A primary aim of this project was to optimise plant virus detection using MS. The strategy used was one-dimensional gel electrophoresis to separate proteins extracted from infected plant tissues, to isolate one or more extra protein bands, and to identify novel viruses in virus infected plants using PMF and MS/MS protein sequencing.
1.6.2 Investigating natural spread of an indigenous virus to an introduced crop plant in the field

HarMV was recently identified and characterised in Western Australia (Webster et al., 2007; Wylie & Jones, 2011a). Its principle wild host is believed to be *H. comptoniana*. In glasshouse experiments HarMV infects *L. angustifolius* (narrow-leafed lupin), *L. cosentinii* (sandplain lupin) and *L. luteus* (yellow lupin). No research has been done to investigate natural spread of HarMV to lupins in the field by vectors.

**Aim 2:** The aim of this experiment was to investigate whether wild aphid vectors can transmit HarMV to lupins under field conditions, and to find out whether this virus is a potential threat to the lupin crop.

1.6.3 Symptom responses of lupin genotypes to infection by a range of isolates of HarMV

HarMV infection of narrow-leafed lupin (*L. angustifolius*) was thought to induce a NN response similar to that of the NN strain of BYMV (Webster *et al.*, 2007). Isolate MU-4 of HarMV was not one of the isolates used in the previous glasshouse study by Webster *et al.* (2007). Isolate MU-4 induced necrotic response in the 2009 pilot field experiment and it belongs to phylogenetic
group V of HarMV, none of whose members caused a necrotic reaction in narrow-leafed lupin in the glasshouse study.

**Aim 3:** The aim of this experiment was to assess responses of four different genotypes of narrow-leafed lupin (cvs Mandelup, Belara, Tanjil and Tallerack) infected with three HarMV isolates (WHP-1, WHP-2 and MU 4) to characterise the responses on the host plants.

### 1.6.4 Detecting viruses using next generation sequencing

NGS is a new approach to nucleic acid sequencing (Margulies et al., 2005). Because of its power, NGS has rapidly become a major new technology for genomics research in biology, with the potential to detect plant pathogens in complex backgrounds. This technique has been used recently to detect novel viruses, and single or multiple infection of plants (Adams et al., 2009; Roossinck et al., 2010). Lily plants growing locally were observed to have virus-like symptoms, including mosaic and chlorotic streaking on the leaves, and they were chosen as a source for application of NGS.
**Aim 4:** The aim of this experiment was to identify the virus in a symptomatic lily plant using NGS and bioinformatic analysis, and to characterise the detected virus further by determining its host range and species distribution in the Perth region.

**1.6.5 Quantify virus in single or doubly-infected hosts using real-time PCR**

Narrow-leafed lupin (*L. angustifolius*) cv Belara infected with HarMV isolate WHP-1 showed NN symptom, and when infected with WHP-2 showed systemic necrosis 22 days after inoculation. The major host of HarMV is *H. comptoniana* and infected plants show variable symptoms with differing severity. *N. benthamiana* plants with mixed infections of CMV and HarMV exhibit a strongly synergistic increase in severity of viral symptoms.

**Aim 5:** The aim of this experiment was to investigate whether HarMV is present in different titres in plants with differing symptoms, and to determine how plants react to double infection with HarMV and CMV by quantification of virus presence using real-time PCR.
CHAPTER 2 GENERAL MATERIALS AND METHODS
2.1 Introduction

To obtain virus-infected plants and retain the virus in indicator plants in the glasshouse, mechanical inoculation and graft inoculation was used. DNA sequencing analysis of viruses was done by extracting total RNA from plant tissues, RT to obtain cDNA, PCR to amplify target regions and nucleic acid sequencing. For PCR amplicons with more than one component or where low sequencing quality was obtained using direct sequencing reactions, DNA cloning or extraction from agarose gels was done. Protein components from infected or uninfected plant tissues were separated using one-dimensional polyacrylamide gel electrophoresis and stained by Coomassie blue.

2.2 Inoculations

2.2.1 Mechanical inoculation

Virus indicator hosts and cultivated plants were grown in insect-proof and temperature-controlled glasshouses (23 °C). When the experimental hosts were in two-leaf stage, infected leaf tissue was ground in cold (4 °C) 0.05 M potassium phosphate buffer (pH 7.2) with a mortar and pestle, which was mixed with the diatomaceous earth ‘Celite’ (10 g per 200 ml buffer) before being rubbed onto young leaves of indicator plants. Inoculated plants were covered by plastic bags for one or two days to maintain humidity for the plants to recover.
2.2.2 Graft inoculation

Wedge-grafting of *H. comptoniana* was used as a more potent method of inoculation for plants that were not infected by mechanical inoculation. Stems were cut and a 1 cm vertical incision was made in the center of the stem. Fresh scions were collected from young infected plants, primary leaves were cut off to reduce evaporation. The lower end of the scion was cut into a wedge shape with a length of approximately 1 cm. The scion was inserted into the rootstock incision and an elastic tape was wound around the graft site tightly so that the combined layer of scion and rootstock were in close contact. Grafted plants were enclosed with plastic bags to keep them humid for at least one week.

2.3 RNA extraction

Plant and virus total RNA in infected plants was extracted using a RNeasy Plant Mini™ extraction kit (Qiagen, # (catalog number) 74904) according to the manufacturer’s instructions as described below:

- Weigh the amount of plant material (usually less than 100 mg for leaves) and put it in a 2 ml tube.
- Place the tube in liquid nitrogen and grind the tissue thoroughly into a fine powder.
- Before tissue thaws add 450 µl buffer RLT (supplied by the kit) and vortex vigorously.
- Transfer the entire lysate to a QIAshredder spin column and centrifuge for 2 min at 20,000 × g.
- Transfer the supernatant of the flow-through to a new RNase-free tube.
- Add 220 µl 100% ethanol to the lysate and pipette to mix.
- Transfer the sample to an RNeasy spin column with collection tube. Close the lid gently and centrifuge for 15 s at 20,000 × g.
- Discard the flow-through. Add 700 µl buffer RW1 (supplied by the kit) to the RNeasy spin column, close the lid gently and centrifuge for 15 s at 20,000 × g.
- Discard the flow-through. Add 500 µl buffer RPE (supplied by the kit) to the RNeasy spin column, close the lid gently and centrifuge for 15 s at 20,000 × g.
- Discard the flow-through. Add 500 µl buffer RPE to the RNeasy spin column, close the lid gently and centrifuge for 15 s at 20,000 × g.
- Discard the flow-through together with the collection tube. Place the RNeasy spin column in a new 2 ml collection tube, close the lid gently and centrifuge for 1 min at 20,000 × g.
- Discard the collection tube. Place the RNeasy spin column in a new 2 ml collection tube. Add 40 µl RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at 20,000 × g to elute the RNA.
- RNA is stored at -80 °C.
2.4 Reverse transcription

Reverse transcription of total RNA was done using a High-Capacity cDNA Transcription Kit (Applied Biosystem, #4368814):

- Prepare the 2× RT reaction mix (per 20 µl reaction) on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× RT buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>25× dNTP mix (100mM)</td>
<td>0.8</td>
</tr>
<tr>
<td>10× random primers(^a)</td>
<td>2.0</td>
</tr>
<tr>
<td>MultiScribe(^{TM}) reverse transcriptase</td>
<td>1.0</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>1.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10.0</strong></td>
</tr>
</tbody>
</table>

\(^a\) Random primer was used in general plant virus diagnosis. Reverse primers in quantitative PCR are specified and listed where they were used.

- Place the 2× RT mix on ice and mix gently.
- Pipette 10 µl 2× RT mix into each reaction tube and then pipette 10 µl RNA sample to the tube, pipetting to mix.
- Thermal cycling conditions as follows:
<table>
<thead>
<tr>
<th></th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25 °C</td>
<td>37 °C</td>
<td>85 °C</td>
<td>12 °C</td>
</tr>
<tr>
<td>Time</td>
<td>10 min</td>
<td>90 min</td>
<td>5 min</td>
<td>∞</td>
</tr>
</tbody>
</table>

- cDNA was stored at -20 °C.

### 2.5 PCR reaction

#### 2.5.1 Promega GoTaq® Flexi DNA Polymerase

PCR was carried out using Veriti® Thermal Cycler PCR ABI (Applied Biosystems), GoTaq® Flexi DNA Polymerase (Promega, #M8291), 5× Colourless GoTaq® Flexi Buffer (Promega, #M890A), Nucleotide Mix (dNTPs) (10 mM each dNTP) (Promega, #C1141), Nuclease-Free Water (Promega, # P1193) and Magnesium Chloride Solution 25 mM (Promega, #A351B), following recommended conditions (for each 25 µl reaction):

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× Colourless GoTaq® Flexi Buffer</td>
<td>5</td>
<td>1×</td>
</tr>
<tr>
<td>Magnesium Chloride Solution 25mM</td>
<td>1</td>
<td>1.0 mM</td>
</tr>
</tbody>
</table>
dNTPs, 10 mM each
Forward primer, 10µM
Reverse primer, 10µM
GoTaq® DNA Polymerase (5u/µL)
Template DNA
Nuclease-Free Water to

0.5 0.2 mM each dNTP
1 0.4 µM
1 0.4 µM
0.125 0.625 u/25 µl
1.0-3.0 < 0.25 µg/25 µl
25

- Thermal cycling conditions as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>10 s</td>
<td></td>
</tr>
<tr>
<td>Annealing(^a)</td>
<td>50-55 °C</td>
<td>30 s</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>30 s/kb</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td>hold</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Annealing temperature was determined by melting temperature of primers, specified below.

2.5.2 KAPA HiFi\textsuperscript{TM} HotStart DNA Polymerase

KAPA HiFi\textsuperscript{TM} HotStart Kit (KAPA, #KK2501) was used to amplify DNA product as well, following the manufacturer’s instructions (for each 25 µl reaction):
<table>
<thead>
<tr>
<th>Component</th>
<th>Vol (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water to</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>5× KAPA HiFi GC buffer</td>
<td>5.00</td>
<td>1x</td>
</tr>
<tr>
<td>dNTP mix, 10 mM each</td>
<td>0.75</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>Forward primer, 10 µM</td>
<td>0.75</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Reverse primer, 10 µM</td>
<td>0.75</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.00-3.00</td>
<td>&lt;50 ng/25 µl</td>
</tr>
<tr>
<td>KAPA HiFi HotStart DNA Polymerase (1 u/µl)</td>
<td>0.5</td>
<td>0.5 u/25 µl</td>
</tr>
<tr>
<td>Final vol</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

The thermocycling conditions for KAPA HiFi\textsuperscript{TM} HotStart PCR was as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98 °C</td>
<td>20 s</td>
<td></td>
</tr>
<tr>
<td>Annealing\textsuperscript{a}</td>
<td>50-55 °C</td>
<td>15 s</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>30 s/kb</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td>hold</td>
<td>1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Annealing temperature was determined by melting temperature of primers, specified where they were used.
2.6 Agarose gel electrophoresis

Tris- acetic acid-EDTA (TAE) electrophoresis buffer (50× TAE: 2 M Tris-Acetate; 0.05 M EDTA; pH 8.0 (Sambrook et al., 1989)) was used to make 1% or 2% agarose gels and used as running buffer. SYBR Safe DNA Gel Stain (Invitrogen, #S33102) was added to agarose gel solution 1 µl/100 ml to visualize DNA amplicons. Each gel well was loaded with 5 µl PCR products with 1 µl glycerol loading buffer (30% glycerol; 0.25% bromophenol blue). 5 µl 100bp DNA molecular ladder (Promega, #G2101) was loaded to estimate the size of DNA product. The gel was run on Bio-Rad Mini-Sub Cells™ or Bio-Rad Wide Mini-Sub Cells™ at 80 V for 20 min or until products were separated. DNA components were then visualized on a UV transilluminator.

2.7 Purification of PCR products

2.7.1 Ethanol precipitation

PCR products were purified and concentrated using ethanol precipitation:

- Add 2.5 vol of ice cold ethanol and 0.1 vol of 3 M Sodium acetate (pH 5.2) to PCR product. Vortex to mix.
- Place the PCR product on ice for 20 min to precipitate DNA.
- Centrifuge for 10 min at max speed at 4 °C.
- Pipette out the supernatant without touching the bottom of tube.
• Add 100 µl 70% ethanol and remove it without touching the bottom of tube.

• Dry pellet at 37 °C for 10 min and resuspend in Milli-Q water (purified by Milli-Q Advantage A10 Ultrapure Water Purification System).

### 2.7.2 Cleanup using MinElute PCR Purification Kit

PCR product was cleaned up for subsequent analysis, using MinElute PCR Purification Kit (QIAGEN, #28004) as follow:

• Add 5 vol of PB buffer (supplied by the kit) to the PCR reaction and mix.

• Check that the colour of the mixture is yellow. If not, add 10 µl of 5 M sodium acetate (PH 5.2) and mix.

• Apply the sample to MinElute column and centrifuge for 1 min at 13,000 ×g to bind DNA. Discard the flow-through.

• Add 750 µl PE buffer (supplied by the kit) to the MinElute column and centrifuge for 1 min at 13,000 ×g to wash. Discard the flow-through.

• Centrifuge for additional 1 min at 13,000 ×g and discard the flow-through with collection tube.

• Place the MinElute column in a clean tube. Add 10 µl water to the center of the membrane.

• Let the column stand for 1 min and centrifuge for 1 min at 13,000 ×g to elute DNA.
2.8 DNA quantification

DNA products were quantified using a Nanodrop UV-Vis spectrophotometer (Nanodrop Technologies) following the manufacturer’s instructions.

2.9 DNA extraction from agarose gel

PCR products with more than one component were separated by agarose gel electrophoresis. The target band was excised and the DNA was extracted and purified by MinElute PCR Purification Kit (QIAGEN, #28004) as described in 2.7.2.

2.10 Cloning of PCR products

For sequencing PCR amplicons containing two or several components with similar molecular size, they were ligated into a plasmid and transformed into E. coli using pGEM-T easy cloning kit (Promega, #A1380):

- Mix 5 µl 2× rapid ligation buffer, 1 µl T4 DNA ligase, 1 µl pGEM-T easy™ vector and 3 µl purified PCR amplicon. Incubate for 1-2 h at room temperature or overnight at 14 °C water bath.
- Add 50 µl competent E. coli cells suspension to 2 µl ligated plasmid. Pipette to mix and incubate on ice for 15 min.
• Transfer to water at exactly 42 °C for exactly 45 s.

• Transfer to ice bath for 1-2 min.

• Add 500 µl Luria Broth (LB) media (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0) and incubate with shaking at 37 °C for 1 h.

• Plate 200 µl transformed E. coli cells on LB agar plates containing 100 mg/ml ampicillin.

• Incubate at 37 °C overnight.

2.11 Screening for recombinant plasmids

The colonies on the agar plate were screened for recombinant plasmids using PCR:

• Cells were marked and picked up from individual colonies and introduced to 20 µl clean water.

• PCR reactions were carried out using GoTaq® Flexi DNA Polymerase (see 2.5.1).

• PCR amplicons with expected sizes were visualized and selected using agarose gel electrophoresis (see 2.6).

2.12 Plasmid extraction

5 ml LB media with 100 mg/ml ampicillin was used to culture the cells from a selected colony at 37 °C overnight. The plasmids with target fragments were purified using QIAprep Spin Miniprep Kit (Qiagen, #27104):
• Transfer 1.5 ml culture to centrifuge tube and pellet the cells at 15,000 xg for 2 min.

• Pipette out the supernatant and resuspend the cells in 250 µl P1 buffer (supplied by the kit).

• Add 250 µl P2 buffer (supplied by the kit) and mix by inverting 6 times.

• Add 350 µl N3 buffer (supplied by the kit) and mix by inverting 6 times.

• Centrifuge at 15,000 xg for 10 min.

• Apply supernatant to QIAprep spin column and centrifuge for 1 min at 15,000 xg. Discard the flow-through.

• Add 500 µl PB buffer (supplied by the kit) to wash. Centrifuge at 15,000 xg for 1 min and discard the flow-through.

• Add 750 µl PE buffer (supplied by the kit) and centrifuge at 15,000 xg for 1 min. Discard the flow-through.

• Centrifuge at 15,000 xg additional 1 min to remove residual wash buffer. Discard the flow-through with collection tube. Place the column in a new clean centrifuge tube.

• Add 50 µl water directly to the column membrane and let it stand for 1 min. Centrifuge 1 min at 15,000 xg to elute the DNA.

---

### 2.13 Clone storage

_E. coli_ cells transformed with plasmid containing a target fragment were stored as glycerol stocks:

- Add equal vol of 30% glycerol to 0.5 ml fresh liquid culture.
• Incubate at room temperature for 30 min to allow glycerol to be taken up by cells.
• Liquid nitrogen was used to freeze the mixture rapidly.
• Cells were stored at -80 °C.

2.14 DNA sequencing reaction

DNA products purified from PCR reaction, agarose gel or transformed cloning cells were used to carry out subsequent sequencing reaction. Reaction mix was prepared as follows using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied biosystems, # 4337455) (for each 10 μl reaction):

<table>
<thead>
<tr>
<th>Components</th>
<th>Vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× sequencing reaction buffer</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Primer(^a), 3.2 pmoles/μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>BigDye® version v3.1 terminator mix</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>DNA product</td>
<td>100 ng</td>
</tr>
<tr>
<td>Water to</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

\(^a\) For sequencing directly from PCR amplicons primers used in PCR reaction were used as a dilute. Sequencing was done from both directions (forward and reverse) or sequencing only from one side was used for diagnostic purpose. Primers M13R and M13F (Promega) were used in plasmid sequencing.

The thermocycling set for sequencing reaction was follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
</table>

74
Annealing temperature was determined by melting temperature of primers, specified where they were used.

Sequencing reactions were cleaned up using ethanol precipitation as follows:

- Add 25 µl ethanol, 1 µl 3 M sodium acetate (pH 5.2) and 1 µl 125 mM EDTA to each 10 µl sequencing reaction.
- Leave at room temperature for 20 min to precipitate DNA.
- Centrifuge at 13,000 ×g for 30 min at 4 °C.
- Pipette out the supernatant without disturbing the pellet at the bottom.
- Rinse pellet with 125 µl 70% ethanol.
- Centrifuge at 13,000 ×g for 5 min at 4 °C.
- Remove the supernatant and incubate at 37 °C to evaporate ethanol.
- Stored in -20 °C before capillary electrophoresis.

### 2.15 Protein polyacrylamide gel electrophoresis
Polyacrylamide gel electrophoresis was used to separate protein components extracted from plant tissues and visualize differential expressed proteins between infected and uninfected plants. Gel solution preparation and electrophoresis procedure was as follows:

- Prepare separating gel solution as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide/Bis Solution 37.5:1 (Bio-Rad, #161-0148)</td>
<td>15.0</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>12.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.5</td>
</tr>
<tr>
<td>Water</td>
<td>21.75</td>
</tr>
</tbody>
</table>

- Mix the gel solution. To remove bubbles degas the solution for 60 min.
- Set up the vertical gel slab apparatus (Bio-Rad PROTEAN™ II, # 351084)
- Before casting separating gel add 250 µl 10% ammonium persulfate and 25 µl TEMED (MP Biomedicals, # 04805615). Mix gently and pour the gel solution to the apparatus.
- Prepare the stacking gel solution as below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide/Bis Solution 37.5:1 (Bio-Rad, #161-0148)</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl (pH 6.8)</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>240 µl</td>
</tr>
</tbody>
</table>
• Mix the gel solution. To remove bubbles degas the solution for 60 min.

• Before casting the stacking gel add 120 µl 10% ammonium persulfate and 24 µl TEMED (MP Biomedicals, # 04805615). Mix gently and pour the gel solution to the apparatus. Allow it to stand and solidify for at least 2 h for solidification.

• Mix 10-20 µl protein solution with 2× loading buffer and load to the gel. Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363) was used to estimate protein molecular weight.

• The electrophoresis was done at 75 V for 18 h using 1× TGS running buffer (10× TGS: 3% Tris, 14.4% glycine, 1% SDS).

• Gels were stained with 0.2% Coomassie® Brilliant Blue R (Sigma, #27816) in solution of methanol/water/acetic acid: 45/45/10 for 1 h and destained with solution of methanol/water/acetic acid: 25/65/10 for about 1 h. Remove the destain solution and add fresh solution to destain the gel for 1 h. Repeat 3 times.

• Store gels in water at 4 °C.
CHAPTER 3 DEVELOPING UNBIASED VIRUS DETECTION ASSAYS
3.1 introduction

3.1.1 Mass Spectrometry in detection of plant viruses

Plant viruses are widespread in plants grown as agricultural crops and in native ecosystems, and some cause damaging diseases. Since the disease management strategy used depends on the virus species responsible, it is crucial to accurately identify the virus(es) causing the disease and then to implement appropriate management strategies.

The assays used most widely to detect plant viruses are based on antiserum affinity and nucleic acid hybridization, amplification and sequencing. For both approaches, some previous knowledge of the virus is required and this has been a serious drawback to identifying unknown or unexpected viruses. MS-based protein profiling has been applied to diagnose various plant pathogens and pests including aphids (Perera et al., 2005a), nematodes (Perera et al., 2005b), and viruses (Blouin et al., 2010). A possible output of MS analysis of virus proteins includes the total mass and resolution of amino acid sequences using MS/MS (Padliya & Cooper, 2006). She et al. (2001) determined the complete amino acid sequence of the CP of BMV using MS/MS and distinguished four isolates of the virus. TMV and Potato virus X (PVX) were identified using two-dimensional SDS polyacrylamide gel electrophoresis (PAGE) to compare proteomes of infected and uninfected plant tissues. Differential spots were excised, digested by trypsin, sequenced by High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) (Cooper et al., 2003). Blouin et al. (2009) partially purified plant virus particles using ultra-centrifugation,
visualized viral protein bands on one-dimensional electrophoresis, digested the viral proteins with trypsin and applied HPLC-liquid chromatography electrospray tandem MS system to determine peptide sequences. Using this approach, they identified four common and two novel viruses.

The major limitation of this approach is the amount of purified target peptide needed, which may require chromatographic purification. PMF is a more rapid MS-based analytical technique for protein identification (Henzel et al., 1993; James et al., 1993; Mann et al., 1993; Pappin et al., 1993; Yates et al., 1993). Trypsin cleaves proteins at specific sites (the carboxyl side of the amino acids lysine or arginine), thereby generating a unique series of peptides whose masses can be determined by MS analysis. The mass values are then compared to entries in a protein sequence database with calculated mass values obtained by applying cleavage rules. By using an appropriate scoring algorithm, the closest match or matches are identified.

In this chapter, identification of known and unknown plant viruses was done by one-dimensional electrophoresis coupled with PMF. ZYMV, *Turnip mosaic virus* (TuMV), PWV, *Alfalfa mosaic virus* (AMV), TSWV, CMV which are regarded to exist in abundance in their hosts, were used to optimise this identification assay. Four protein extraction methods were used to optimise the identification of virus protein bands on polyacrylamide gel. Two protein in-gel digestion and peptides recovery protocols were tried to maximise the performance of PMF.
identification. Bovine serum albumin (BSA) (Sigma, #A7906) was digested in solution and identified by PMF using a prOTOF 2000 MALDI O-TOF mass spectrometer (PerkinElmer) in order to optimise protein identification by PMF. An unknown virus originated from *Cakile maritima* was detected by this assay.

### 3.1.2 Second generation sequencing used in plant virus identification

In 2005 publication of the sequencing-by-synthesis technology developed by 454 Life Sciences (Margulies *et al.*, 2005) presented a revolutionary change to sequencing compared with the chain-termination method (Sanger *et al.*, 1977). This second - generation sequencing has been used in several areas of biological research, including novel virus detection, and is becoming a more economical sequencing technology since the development of short read method by Solexa, and later aquired by Illumina. NGS has already shown enormous potential for detecting unexpected and novel viruses in complex backgrounds. Adams *et al.* (2009) isolated a new cucumovirus in *L.spicata* (Gayfeather) named ‘*Gayfeather mild mottle virus*’ using NGS (GS-FLX Genome Sequencer) coupled with metagenomic analysis. Roossinck *et al.* (2010) analysed individual plant sample for virus in metagenomic studies by NGS (454 GS-FLX) data and concluded that ~60% of samples from a Costa Rican rainforest community had sequences with identity to viruses of fungi. Recently, small interfering RNA (siRNA) derived from viruses was characterised by NGS to identify plant viruses in a complex background (Kreuze *et al.*, 2009; Yan *et al.*, 2010). NGS was used as a high-throughput and low-cost method for viromics study.
(Harris et al., 2008). Wylie and Jones (2011a, b, c) applied Illumina NGS to both domesticated and wild plants, characterising several novel viral genome sequences from them by subsequent bioinformatics analysis. They characterised the full genomes of PWV (full genome 9,858 nucleotides) (Wylie & Jones, 2011b), *Hardenbergia virus A* (full genome 6,936 nucleotides) (Wylie & Jones, 2011c) and SLV (full genome 8,371 nucleotides) (Wylie et al., 2011), and another 19 viruses were characterised for part of their genomes. These examples show that NGS is now one of the cutting edges of plant virus diagnostics. Aim 4 of this project was to use NGS and bioinformatics to characterise a virus previously unidentified in Australia. Raw data of NGS consists of millions of short reads (each one 25 to 300 bp or more depending on the platform used). Bioinformatic analysis of NGS enables assembling and identifying the origins of sequences, and obtaining the full genomes of the viruses. NGS can also be used to quantify virus levels in hosts (Wylie & Jones, 2011a).

In international plant trade quarantine is an important issue, and application of quarantine principles is required in Australia to protect farming systems and native biodiversity from introduction of exotic viruses. The nepoviruses, a group of viruses in the family *Secoviridae*, include 46 species, are nematode transmitted polyhedral viruses that infect a wide range of plant families, and may be viewed as the most serious virus diseases of horticultural crops by quarantine authorities worldwide. Nine exotic nepoviruses have been listed as strict quarantine pathogens and strategies to control nepovirus-infected plant materials entering or spreading within Australia are in place (http://ictvdb.bio-mirror.cn/Aussi/ausiname.htm (2002)).
Easter lily (*Lilium longiflorum*) originated from Japan and Taiwan and is a widely traded as ornamental plant. An easter lily plant growing in a garden in Perth, Western Australia showed mosaic symptoms on leaves, and was chosen as a source to identify infecting viruses by NGS approaches in this study.

3.2 Materials and methods

3.2.1 Detection of plant viruses using PMF

3.2.1.1 Plant and virus samples

Samples from young leaves with symptoms were collected from wild plants or test plants in the glasshouse (Table 3.1). Fresh plant material was used to extract protein or RNA within one day of sampling.

**Table 3.1** Plants and viruses used for protein extraction

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Wild host</th>
<th>Experimental host</th>
<th>Virus (if known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Cucurbita pepo</em></td>
<td></td>
<td>Zucchini yellow mosaic virus (ZYMV)</td>
</tr>
<tr>
<td>2</td>
<td><em>Brassica juncea</em></td>
<td></td>
<td>Turnip mosaic virus (TuMV)</td>
</tr>
<tr>
<td>3</td>
<td><em>Nicotiana benthamiana</em></td>
<td></td>
<td>Passionfruit woodiness virus (PWV)</td>
</tr>
<tr>
<td>4</td>
<td><em>Medicago truncatula</em></td>
<td></td>
<td>Alfalfa mosaic virus (AMV)</td>
</tr>
</tbody>
</table>
3.2.1.2 Extraction of plant and virus proteins

Four extraction protocols were tested to determine the best method for extracting proteins from uninfected and infected plants.

**Method 1: Methanol/ chloroform precipitation of proteins**

Method modified from Tan *et al.* (2000)

- Weigh 200 mg fresh leaves, freeze in liquid nitrogen and grind thoroughly in a mortar and pestle.
- Add 1.5 ml SDS-Tris buffer (125 mM Tris, 7% SDS, pH 7.0, with 0.5% polyvinylpolypyrrolidone (PVPP) (Sigma, #P6755) added immediately before extraction and 1 mM dithiothreitol (DTT) (Sigma, #D9779)) and vortex vigorously.

- Incubate at room temperature for 30 min and centrifuge at 10,000 × g for 5 min.

- Transfer the supernatant (usually 1 ml) to a new centrifuge tube.

- Add 4 vol methanol and vortex. Add 1 vol chloroform and vortex. Add 2.5 vol water and vortex.

- Centrifuge at 10,000 × g for 5 min and discard the upper phase.

- Add 2.5 vol methanol and vortex to break up protein pellet. Centrifuge at 10,000 × g for 10 min.

- Discard the supernatant and let the pellet dry at room temperature. Resuspend the protein pellet using 250-500 µl MSS solution (5 M urea, 2 M thiourea, 2% SDS, 2% Triton X-100 (Sigma, #X100), immediately before use add 2 mg/ml DTT (Sigma, #D9779)).

**Method 2: Acetone/β-mercaptoethanol precipitation**

Method modified from Cooper et al. (2003)

- Weigh 100 mg fresh leaves, freeze in liquid nitrogen and grind thoroughly to a fine powder by mortar and pestle.

- Add 10 vol of ice-cold acetone containing 10% wt/vol trichloroacetic acid (TCA) (Sigma, #T8657) and 0.07% vol/vol β-mercaptoethanol (BioRad, #161-0710).

- Incubate at -20 °C for 1 h.
• Centrifuge at 14,000 × g for 30 min and discard the supernatant.

• Wash pellet three times with 10 vol of ice-cold acetone containing 10% wt/vol TCA, 0.07% vol/vol β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride (PMSF) (Sigma, #P7626), and 2 mM EDTA, and dried at room temperature.

• Resuspend the protein pellet using 150-300 µl MSS solution, immediately before use add 2 mg/ml DTT (Sigma, #D9779)).

Method 3: Acetone/DTT precipitation
Method modified from Jacobs et al. (2001)

• Prepare 20% TCA (Sigma, #T8657) in acetone, stored at -20 °C.

• Weigh 3 g fresh leaves, freeze in liquid nitrogen and grind to a fine powder by mortar and pestle. Transfer the powder into a centrifuge tube.

• Add 0.2% DTT (Sigma, #D9779) to acetone containing 20% TCA before use (precipitation solution).

• Add 10 ml of precipitation solution to the centrifuge tube and keep at -20 °C overnight to allow complete protein precipitation.

• Centrifuge at 30,000 × g for 20 min at 4 °C.

• Carefully remove the supernatant and wash the pellet in cold acetone (-20 °C) containing 0.2% DTT.

• Centrifuge at 30,000 × g for 20 min at 4 °C. Repeat to wash 2 more times.
Dry pellet at room temperature for 3 h to overnight.

Resuspend pellet in 2 ml rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (Sigma, #C9426), immediately before use add 1% DTT). The sample was vortexed and maintained on an orbital shaker for overnight.

Centrifuge at 30,000 × g for 60 min at 20 °C.

Transfer supernatant to a clean tube, stored at -20 °C.

Method 4: Methanol/acetone precipitation
Method based on plant total protein extraction kit (Sigma, #PE0230) protocol, with modifications

Grind 200-300 mg fresh leaves in liquid nitrogen to a fine powder in centrifuge tube.

Add 1.5 ml freshly prepared methanol solution (1% protease inhibitor cocktail in methanol).

Briefly vortex and incubate at -20 °C for 10 min with vortexing every 1 min.

Centrifuge at 20,000 × g for 5 min at 4 °C.

Remove the supernatant without disturbing the pellet.

Repeat washing with methanol for 2 more times.

Dry pellet at room temperature for 1 h. Any visible methanol on the pellet should be avoided.

Add 1.5 ml ice-cold acetone and vortex.
• Incubate 10 min at -20 °C and centrifuge at 20,000 × g for 10 min at 4 °C.
• Remove the supernatant without disturbing the pellet.
• Dry pellet at room temperature for 1 h.
• Add 300-500 µl reagent type 2 working solution in the kit to resolve protein. The pellet should be completely broken up by vortexing. Incubate at room temperature for 2 h.
• Centrifuge at 20,000 × g for 30 min and remove the supernatant to a clean tube.

3.2.1.3 One-dimensional polyacrylamide gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis was done using the voltage described in Chapter 2 and 10 to 20 µl protein extract was mixed with an equal volume of 2× loading buffer (0.5 M Tris-HCl pH 8.8, 10% SDS 20% glycerol 1% bromophenol blue, 0.2% DTT) and loaded into each well. Gel photos were taken by ProXPRESS™ 2D Proteomic Imaging System (PerkinElmer).

3.2.1.4 Protein in-solution digestion and PMF analysis

BSA (Sigma, #A7906) was used as a test protein for digestion in solution and identification by PMF using a prOTOF 2000 MALDI O-TOF mass spectrometer (PerkinElmer) in order to optimise PMF identification parameters using this mass spectrometer.
250 µl 0.01% trifluoroacetic acid (TFA) (MP Biomedicals, #152166) was added to a Roche trypsin vial (Roche, #11418475001) to a final concentration of 0.1 µg/µl. 10 mg BSA was dissolved in 10 ml 100 mM Tris-HCl pH 8.5 to make the BSA solution. 50 µl of BSA solution was mixed with 10 µl trypsin solution (pH 8.2) and incubated at 37 °C for 4 h.

A solution of α-Cyano-4-hydroxycinnamic acid (CHCA) (Sigma, #C2020) was used as the matrix to assist in molecular desorption and ionization of peptides. 10 mg CHCA was added in 500 µl 0.1% TFA and 500 µl acetonitrile (ACN) to make the matrix solution. 1 µl peptide solution was mixed with 9 µl matrix solution and 1 µl of the mixture was loaded for each spot on the plate. The samples were allowed to dry and crystallise for 10 min at room temperature.

When using the prOTOF™ 2000 MALDI O TOF Mass Spectrometer (PerkinElmer) to generate PMF profiles, calibration was done following instructions of ProteoMass™ Peptide MALDI-MS Calibration Kit (Sigma, # MSCAL2). The parameter sets for acquiring data were as follows: 100 laser shots, laser energy 75%, laser rate 100.0 Hz, declustering 30.0 V, and cooling Nitrogen gas flow 190.0 ml/m. Binning size was set to 4 for peak analysis. The resulting PMFs generated were used to search NCBI nr and Swiss-Prot databases for proteins sharing identity using ProFound (Zhang & Chait, 2000). The search parameters were as follows: taxonomic category, all taxa; protein mass, 0.0–3,000.0 kDa; protein pl, 0.0–14.0; missed cleavages, 3; enzyme, trypsin; tolerance average, 30 ppm, mono, 30 ppm; charge state, MH+. 
3.2.1.5 Protein in-gel digestion and peptide recovery

Two protocols were used for in-gel digestion and peptide recovery. One of the most abundant enzymes in plants - ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) - was used to optimise in-gel digestion and peptide recovery protocols compatible with the prOTOF™ 2000 MALDI O-TOF mass spectrometer before analysing bands of interest.

Protocol 1 Sonication/Iyophilisation

- The band of interest was excised using a clean razor blade and cut into 1mm² fragments. Fragments were placed into a clean tube.
- Add 100 µl destain solution (25 mM ammonium bicarbonate (ABC) in 50% ACN) to gel pieces. Briefly vortex and incubate at 37 ºC for 20 min.
- Remove the destain solution and add another 100 µl of destain solution. Repeat this procedure until the gel is colourless.
- Dry the gel pieces using lyophilisation for 30 min.
- 100 µl 0.01% TFA (MP Biomedicals, #152166) was added to Roche trypsin vial (Roche, #11418475001) to final concentration of 0.25 µg/µl.
- 20 µl trypsin solution was added to 40 µl ACN, 50 µl 200 mM ABC (pH 8.5) and 290 µl water to make the trypsin working solution.
• Add 50-100 µl trypsin working solution to the dried gel pieces according to the size of the band.
• Incubate the digestion solution at 37 °C for 16 h.
• Pipette the digestion solution to tube A.
• Crush the gel pieces using a pestle and add 100 µL 5% TFA/50% ACN.
• Incubate in sonication bath (frequency 20 kHz) for 15 min.
• Pipette the solution to tube A.
• Add 100 µL 1% TFA in ACN to the gel and incubate for 20 min and transfer the solution to tube A.
• Add 100 µL 0.1% TFA to the gel and incubate at -20 °C for 20 min until frozen
• Incubate at 37 °C for 10 min to thaw. Repeat freeze-thaw cycle 3 times.
• Transfer the solution to tube A.
• Use lyophilisation to completely dry tube A.
• Add 10 µL 0.1% TFA to the tube to resuspend the peptides.
• The peptides can be stored at -20 °C for 2-3 days.

Protocol 2 Ziptip

This protocol is based on the Trypsin Profile IGD Kit (Sigma, #PP0100) protocol, with modifications.
• Use clean razor blade to excise the gel band of interest and cut into 1 mm$^2$ fragments. All fragments were placed into a clean tube.

• Add 100 µL destain solution (25 mM ABC in 50% ACN) to gel pieces. Briefly vortex and incubate at 37 °C for 20 min.

• Remove the destain solution and add another 100 µL destain solution. Repeat this procedure until the gel was colourless.

• Remove the destain solution, add 100 µL ACN to shrink the gel piece for 20 min and remove it.

• Add 100 µL Trypsin Solubilization Reagent (1 mM HCl, provided by the kit) to one vial of trypsin (20 µg trypsin for each vial, provided by the kit). Add 1 part of acidic trypsin solution to 9 parts of Trypsin Reaction Buffer (40 mM ABC, 9% ACN, pH 8.2, provided with the kit) to make the trypsin working solution (trypsin final concentration 20 µg/mL).

• Add 20-30 µL trypsin working solution to the gel according to its size. Incubate on ice 20 min to let the enzyme reach the protein in gel.

• Add 50-70 µL Trypsin Reaction Buffer to gel. Make sure the gel was covered by the buffer.

• Incubate at 37 °C for 4-16 h.

• After digestion collect the liquid to a new clean tube. Add 0.5 µL TFA and vortex. Make sure sample solution pH<4.

• Use ZipTip (Millipore, #ZTC18P096) to concentrate and desalt the peptide solution using supplied protocol.
• The peptides can be stored at -20 °C for 2-3 days.

### 3.2.1.6 In-gel digested PMF analysis using prOTOF 2000 MALDI O-TOF mass spectrometer

The matrix CHCA (Sigma, #C2020) was used to help molecular desorption and ionization. 10 mg CHCA was added in 500 µL 0.1% TFA and 500 µL ACN to make matrix solution. 1 µL peptide solution was mixed with 9 µL matrix solution and 1 µL of the mixture was added to each spot on the plate. Allow to crystallise for 10 min at room temperature.

Use the prOTOF™ 2000 MALDI O TOF MS to generate PMF profiles. Calibration was done following the instructions of the ProteoMass™ Peptide MALDI-MS Calibration Kit (Sigma, #MSCAL2) or MALDI Calibration Kit (PerkinElmer, #6771000). The general parameters set for acquiring data were as follows: 100 laser shots, laser energy 75%, laser rate 100.0 Hz, declustering 30.0 V, and cooling Nitrogen gas flow 190.0 ml/m (minor adjustment for some samples were specified in results). Binning size was set to 4 for peaks analysis.

The resulting PMFs generated were used to search NCBI nr and Swiss-Prot databases for proteins sharing identity using ProFound (Zhang & Chait, 2000) general search parameters: taxonomic category, all taxa; protein mass, 20.0–50.0 kDa; protein pl, 0.0–14.0; missed
cleavages, 1; enzyme, trypsin; tolerance average, 30 ppm, mono, 30 ppm; charge state, MH+
(minor adjustment for some samples were specified in result).

3.2.1.7 Detection and characterisation of viral CP by Voyager mass spectrometry

Intact viral CP was characterised on a mass spectrometer: Voyager-DE™ PRO Workstation
(Applied Biosystems). 1 µL protein extract using method 3 (see 3.2.1.2) was diluted with 100 µL
50% ACN/0.1% TFA to make it compatible with the matrix and voyager. Sinapinic acid (SA)
(Sigma, #S8313) was used as matrix to assist in molecular desorption and ionization. 40 mg SA
was added to 360 µL methanol, 80 µL water and 600 µL ACN. 1 µL diluted protein solution was
mixed with 9 µL SA matrix solution.

Add 1 µL of the mixture to each spot on the plate. Allow crystallization for 5 min at room
temperature. Parameters were as follows: accelerating, 25000 v; grid, 92%; guide wire, 0.2%;
delay time, 700 nsec; matrix, sinapinic acid; shots, 100; mass range set was according to the
target protein.
3.2.1.8 Virus protein sequencing

Virus CP PMF analysis was based on generating unique peptide mass spectra that matched sequences in the database. The analysis is accurate as long as accurate masses can be determined for each tryptic peptide fragment that ionizes. MS/MS peptide sequencing was done to verify that the peptide sequences predicted by PMF were accurate.

Differentially expressed protein bands (Fig. 3.10) were excised and sent to Proteomics International Pty Ltd in Western Australia to be sequenced. Protein samples were trypsin digested and peptides extracted according to standard techniques (Bringans et al., 2008). Peptides were analysed by MALDI TOF/TOF mass spectrometer using a 4800 Proteomics Analyzer (Applied Biosystems). Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with the Ludwig NR Database and Taxonomy set to ‘all’. Search parameters were set as follows: variable modifications: oxidation (M); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 0.6 Da; fragment mass tolerance: ± 0.6 Da; max missed cleavages: 1; instrument type: default; number of queries: 60.
3.2.2 Second generation sequencing

3.2.2.1 Virus detection using NGS

A *L. longiflorum* plant with symptoms of mottle on the leaves was collected and used to extract total RNA using a RNeasy Plant Mini extraction kit (Qiagen, #74904). The RNA, stored in ethanol, was sent to Macrogen Inc (Seoul, S. Korea) to synthesise cDNA, construct libraries, and sequence 78 nt reads using an Illumina GAIIx platform. Geneious v5.3.4 (Drummond *et al.*, 2010) was used to assemble contigs *de novo* using the ‘Assembly’ program (sensitivity was medium sensitivity/ fast). The preliminary contig sequences were compared with those in GenBank using BlastN (National Centre for Biotechnology Information, Bethesda, MD) (Altschul *et al.*, 1997). Where virus related sequences were found, the sequence of that virus was used as scaffold against which to assemble all sequence reads with medium sensitivity. The resulting consensus sequence was used as scaffold to do a second assembly with all reads using the ‘Assembly’ program (sensitivity was medium sensitivity/ fast; the consensus sequence was set as reference sequence).

3.2.2.2 Detection of virus using PCR

Presence of a virus detected using NGS was confirmed by RT-PCR of RNA from the host plant using specific primers. Total RNA was extracted from bulb material of the infected lily plant using the NucleoSpin RNA Plant Kit (Macherey-Nagel, #740949).

- Grind 80 mg of bulb tissue in clean tube using pestle.
• Add 350 µl RA1 and 3.5 µl β-mercaptoethanol to the ground tissue, and vortex vigorously.

• Place NucleoSpin Filter units in collection tubes, apply the mixture, and centrifuge for 2 min at 11,000 × g. Transfer the filtrate to a new tube without disturbing the pellet at the bottom.

• Add 350 µl 70% ethanol and mix by pipetting up and down.

• Apply the mixture to NucleoSpin RNA Plant column and centrifuge for 1 min at 11,000 × g. Discard the flow-through.

• Add 350 µl MDB and centrifuge at 11,000 × g for 2 min to dry the membrane. Discard the flow-through.

• Add 200 µl RA2 to the NucleoSpin RNA Plant column and centrifuge for 1 min at 11,000 × g. Discard the flow-through.

• Add 600 µl RA3 to the NucleoSpin RNA Plant column and centrifuge for 1 min at 11,000 × g. Discard the flow-through.

• Add 250 µl RA3 to the NucleoSpin RNA Plant column and centrifuge for 4 min at 11,000 × g. Discard the flow-through.

• Place the column into a nuclease-free tube. Elute the RNA in 50 µl RNase water and centrifuge at 11,000 × g for 2 min. RNA was stored under -80 °C.

Reverse transcription of total RNA was done using High-Capacity cDNA Transcription Kit (Applied Biosystem, #4368814) by random primers. Primers for PCR reactions were designed
according to the sequence obtained from NGS. The annealing temperature used was 58 °C and others PCR sets were described in chapter 2. Sanger sequencing of the amplicon was done using both primers.

Phylogenic relationships were determined as follows: the most conserved region of nepoviruses RdRps (Han et al., 2002) was used to undertake phylogenetic analysis of Cycas necrotic stunt virus (CNSV) (cycas strain/lily strain), seven nepoviruses reported in Australia and other five nepoviruses, by Geneious v5.3.4 using program Tree Builder. The parameters of Tree Builder were set as follows, genetic distance model: jukes-cantor; tree build method: neighbour-joining; outgroup: Satsuma dwarf virus (SDV) was set as outgroup.

3.3 Results

3.3.1 Detection of plant viruses using PMF

3.3.1.1 In-solution digestion and optimising PMF using MS

BSA was digested by trypsin in solution and resulting peptides were analysed using the prOTOF™ 2000 MALDI O TOF MS to optimise the trypsin digestion conditions, matrix used and MS parameter settings for PMF analysis (Fig. 3.1). The peaks that matched those in the protein database are listed in Table 3.2.
Figure 3.1 Peptides mass fingerprinting spectrum of trypsin in-solution digested Bovine Serum Albumin (BSA).

Table 3.2 The masses of Bovine Serum Albumin (BSA) trypsin digested peptides that matched the predicted masses in the protein database

<table>
<thead>
<tr>
<th>Measured peptide masses (Da)(^a)</th>
<th>Predicted masses (Da)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>507.250</td>
<td>507.244</td>
</tr>
<tr>
<td>648.344</td>
<td>648.326</td>
</tr>
<tr>
<td>648.344</td>
<td>648.326(^c)</td>
</tr>
<tr>
<td>664.376</td>
<td>664.369</td>
</tr>
<tr>
<td>688.372</td>
<td>688.365</td>
</tr>
<tr>
<td>711.373</td>
<td>711.366</td>
</tr>
<tr>
<td>846.503</td>
<td>846.496</td>
</tr>
<tr>
<td>926.492</td>
<td>926.486</td>
</tr>
<tr>
<td>973.459</td>
<td>973.450</td>
</tr>
</tbody>
</table>
PMF analysis of the peaks identified the profiles as belonging to BSA. The GenBank accession code that most closely matched the PMF was P02769 (Serum Albumin) with an expectation value of 3.50710e-9, coverage 41%.

3.3.1.2 Identification of RuBisCO from polyacrylamide gel

Plant protein extracts were separated using one-dimensional gel electrophoresis. A bright band containing abundant protein at 50 kDa, presumed to be the large subunit of RuBisCO, was
digested by trypsin in gel and identified by PMF. Protocol 1 (see 3.2.1.5) was used to do in-gel digestion and recover the peptides. In searching data parameters the protein mass was set to the range 0.0-30,000.0 Da and the missed cleavage parameter set to 3. The remaining parameters used were the general parameters described in 3.2.1.6. The protein band analysed is indicated in Fig. 3.4 (band 1), and the resulting spectrum obtained is shown in Fig. 3.2. The peaks that matched those in the protein database are listed in Table 3.3.

**Figure 3.2** Peptide mass fingerprint spectrum of trypsin in-gel digested band 1 from Fig. 3.4 (host *Cucurbita pepo*).

**Table 3.3** The masses found from trypsin digested band 1 (Fig. 3.4) of peptides matching predicted masses in the protein database for RuBisCO large subunit

<table>
<thead>
<tr>
<th>Measured peptide masses (Da)</th>
<th>Predicted masses (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>984.582</td>
<td>984.571</td>
</tr>
<tr>
<td>1020.533</td>
<td>1020.524</td>
</tr>
<tr>
<td>1186.661</td>
<td>1186.657</td>
</tr>
<tr>
<td>1381.729</td>
<td>1381.712</td>
</tr>
<tr>
<td>1406.677</td>
<td>1406.660</td>
</tr>
</tbody>
</table>
The GenBank accession code that most closely matched peptide mass fingerprints of the proteins tested was AAK71006 (RuBisCO large subunit) with expectation $1.707943 \times 10^{-2}$, coverage 36%. This result indicated the molecular mass of the most closely matched protein to be 51 kDa, which was approximately the same mass that was estimated by protein mass standards on the protein gel. The other nine matches were RuBisCO large subunits from a range of plant species.

In conclusion, the in-gel digestion and PMF analysis assay based on prOTOF™ 2000 MALDI O TOF Mass Spectrometer successfully identified both the BSA and RuBisCO test samples.
3.3.1.3 Identification of viruses

Five known viruses infecting their hosts were used to test and optimise this assay strategy. Three of them were successfully identified. An unknown virus was then identified using PMF and confirmed by RT-PCR and MS/MS protein sequencing. Double infection of CMV and HarMV was also used to test the assay.

**Zucchini yellow mosaic virus**
Zucchini (*C. pepo*) leaves infected with ZYMV showed symptoms of severe stunting, yellowing and distortion (Fig. 3.3). Infected and uninfected leaves were used to extract protein by methanol/ chloroform precipitation of protein (method 1 in 3.2.1.2), acetone/β-mercaptoethanol precipitation (method 2 in 3.2.1.2), acetone/DTT precipitation (method 3 in 3.2.1.2). The gel image of proteins extracted from the zucchini leaves are shown in Fig. 3.4. Comparison of the protein bands on the gel suggested that these three methods extracted different protein components, for example an additional band is seen only in lane 1 (arrow 3) and another one is present only in lane 5 (arrow 2). Extraction method 3 acetone/DTT precipitation generated the most protein bands, especially in the size range between 25-50 kb. Putative viral proteins were visualised by comparison of gels loaded with protein extracts from uninfected and infected plants.
Two putative virus coat protein bands (arrow 2 and arrow 3, Fig. 3.4) were removed. Both bands were analysed by PMF using protocol 1 sonication/lyophilisation (3.2.1.5). The parameters used were the general parameters described in 3.2.1.6 (spectra in Fig. 3.5). Four peptides digested from band 2 matched known accessions and are listed in Table 3.4 (ZYMV). The closest match was the ZYMV (Singapore strain) CP from GenBank. The matching protein mass was 31 kDa, which agreed with the mass estimated from the protein mass standard on the gel (Fig. 3.4). Band 3 gave no reliable identity, and so could have originated either from the plant itself or from an unknown virus. The peaks in the spectrum are listed in Appendix 1.

Proteins from infected and uninfected plants extracted using method 3 were analysed using the Voyager-DE™ PRO Workstation to obtain a mass value for the intact CP of ZYMV. For data
The mass range was set to 30,000 Da to 40,000 Da. The other parameters used were as described in 3.2.1.7. The spectra are shown in Fig. 3.6. By comparing the spectra of infected and uninfected protein extracts, the additional peak at 3,4864.60 Da was identified as the CP of ZYMV.

Protein extraction method 3 (acetone/DTT precipitation) was the only method of protein extraction tested by which viral CPs were identified by PMF. It was therefore used as the main protein extraction method for subsequent analysis.
Figure 3.4 One-dimensional polyacrylamide gel electrophoresis image of infected and uninfected zucchini (Cucurbita pepo). Lane 1 and 2: Infected/uninfected zucchini protein extracted using method 1 methanol/ chloroform precipitation; Lane 3 and 4: Infected/uninfected zucchini protein extracted using method 2 acetone/β- mercaptoethanol precipitation; Lane 5 and 6: Infected/uninfected zucchini protein extracted using method 3 acetone/DTT precipitation; Lane 7: Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363). Arrow 1 indicates putative RuBisCO band, arrows 2 and 3 indicate differential bands.
**Table 3.4** Peptide mass data for three known viruses (ZYMV, TuMV and AMV) and an unknown virus

<table>
<thead>
<tr>
<th>Virus tested (host plant)</th>
<th>Measured peptide masses$^a$ (Da)</th>
<th>Predicted peptide masses (Da)</th>
<th>Predicted identity$^b$</th>
<th>CP mass (kDa)$^c$</th>
<th>Coverage (%)</th>
<th>Expectation valued</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZYMV (Cucurbita pepo)</td>
<td>1381.615</td>
<td>1381.619</td>
<td>ZYMV</td>
<td>31</td>
<td>12</td>
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<tr>
<td></td>
<td>1823.856</td>
<td>1823.853</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1850.846</td>
<td>1850.847</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>2289.098</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TuMV (Brassica juncea)</td>
<td>1175.555</td>
<td>1175.550</td>
<td>TuMV</td>
<td>33</td>
<td>36</td>
<td>2.83567e−007</td>
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<tr>
<td></td>
<td>1428.610</td>
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<td>2773.373</td>
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<td>AMV (Medicago truncatula)</td>
<td>921.468</td>
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<td>AMV</td>
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<td>2383.156</td>
<td>2383.141</td>
<td></td>
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<tr>
<td>Unknown (Nicotiana glutinosa)</td>
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<td>2007.113</td>
<td>2007.110</td>
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<td></td>
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</tr>
</tbody>
</table>

$^a$ Measured peptide masses after calibration.

$^b$ GenBank accession codes of viruses that most closely matched peptide mass fingerprints of the proteins tested: *Alfalfa mosaic virus* (AMV) P03591, *Turnip mosaic virus* (TuMV) CAA70331, *Zucchini yellow mosaic virus* (ZYMV) CAA44529, and *Pelargonium zonate spot virus* (PZSV) NP 619773.

$^c$ CP masses predicted from amino acid sequences.

$^d$ According to default settings in ProFound, expectation value <1e-003 is a high confidence identification while an expectation value <1e-001 is a marginal match. An expectation value >1e-001 is considered to be a failed search.
Figure 3.5 Spectrum of trypsin digested protein band 2 as shown in Fig. 3.4 (putative viral protein in *Cucurbita pepo* infected with *Zucchini yellow mosaic virus* (ZYMV)).

Figure 3.6 Spectra of infected and uninfected plant protein extracts using the Voyager Mass Spectrometer (30-40 kDa range). a: infected *Cucurbita pepo* with *Zucchini yellow mosaic virus* (ZYMV); b: uninfected *C. pepo*. 
**Turnip mosaic virus, Passionfruit woodiness virus, Tomato spotted wilt virus and Alfalfa mosaic virus**

Infected and uninfected host plants were grown separately in an insect-proof glasshouse. These were: *Brassica juncea* plants infected with TuMV and uninfected, *N. benthamiana* infected with PWV and uninfected, *Medicago truncatula* infected with AMV and uninfected, *Solanum lycopersicum* plants infected with TSWV and uninfected. Proteins were extracted from these plants using method 3.

The infected *B. juncea* protein extract showed a highly-expressed additional protein band below 37 kDa (band 4 in Fig. 3.7). This band was excised and analysed by PMF using protocol 2 ziptip, a modified in-gel digestion and peptides recovery protocol (see 3.2.1.5). The spectrum is shown in Fig. 3.8a and has 8 peaks matching the NCBI database, the expectation and the accession number of the closest match to TuMV is listed in Table 3.4. The PMF results indicated that the closest match of this protein was the CP of TuMV (isolate GK1). A differentially expressed band (lane 8) was present between 50 kDa and 75 kDa, but was not analysed because most coat proteins of plant viruses are less than 50 kDa (Blouin et al., 2010). This may be the CI protein that accumulates to high levels in potyvirus infections, resulting in pinwheel inclusions.

In infected *M. truncatula* plants, in the extracted proteins separated by gel electrophoresis there is a clearly differentially expressed protein band (band 5 arrow). In-gel digestion using
protocol 2 and the standard parameters were used for PMF analysis of band 5 (Fig. 3.7 lane 4). In the spectrum (Fig. 3.8 b) there were 6 peaks matching the database, and the expectation and the accession number of the closest match are listed in Table 3.4. The PMF indicated that the closest match was the CP of AMV (isolate Leiden).

Figure 3.7 One-dimensional polyacrylamide gel electrophoresis image of infected and uninfected plant protein extracts. Lane 1 and lane 10: Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363); Lanes 2 and 3: infected/uninfected *Solanum lycopersicon* with *Tomato spotted wilt virus* (TSWV); Lanes 4
and 5: infected/uninfected *Medicago truncatula* with *Alfalfa mosaic virus* (AMV); Lanes 6 and 7: infected/uninfected *Nicotiana benthamiana* with *Passionfruit woodiness virus* (PWV); Lanes 8 and 9: infected/uninfected *Brassica juncea* with *Turnip mosaic virus* (TuMV). Band 4 and 5 were analysed by peptide mass fingerprinting (PMF).

When the infected and uninfected *S. lycopersicon* and infected and uninfected *N. benthamiana* protein extracts were compared, most plant proteins in infected plant leaves accumulated at slightly lower levels than in uninfected plants (e.g. RuBisCO), but there was no obvious virus CP band. The reason for this could be that the virions were not present at a high enough concentration in the plant tissues to be visualised on the gel, or that the extraction method was suitable to extract CPs from some viruses but not for others, or the virus CP band could have been masked by a plant protein. In addition, there were some other differences in bands between lane 6 and 7 in Fig. 3.7 at about 75 kDa. These differences may reflect other virus proteins, or plant responses to virus infection.

The Voyager-DE™ PRO Workstation was also used to characterise the intact CP of TuMV and AMV. Comparison of spectra (Fig. 3.9) of infected and uninfected plant extracts indicated that the additional peak at 33295.98 Da was from the CP of TuMV (Fig. 3.9 1a) and that at 24329.51 Da was from the CP of AMV (Fig. 3.9 2a). Peaks common to infected and uninfected extracts were probably plant proteins, such as the peaks at 26437.23 Da and 26472.18 Da (Fig. 3.9 2a and 2b respectively). The Voyager MS accurately determined the CP mass, which was close to that estimated from comparison with the protein standard on the gel.
Figure 3.8 Spectra of trypsin in-gel digested bands (a) Profile of trypsin digested protein band 4 from Fig. 3.7 (putative viral protein in infected Brassica juncea); (b) Profile of trypsin digested protein band 5 from Fig. 3.7 (putative viral protein in infected Medicago truncatula).
Figure 3.9 Spectra from infected and uninfected plant protein extracts (Voyager DE RoMs). 1a and b: infected/uninfected *Brassica juncea* with *Turnip mosaic virus* (TuMV); 2a and 2b: infected/uninfected *Medicago truncatula* with *Alfalfa mosaic virus* (AMV). Additional peaks in infected extracts in 1a and 2a were from virus CP.

**Unknown virus**

A *C. maritima* plant (Family *Brassicaceae*, coastal rocket) showing faint yellow mottle on the leaves was collected at the high tide mark at the beach at Woodman Point Recreation Reserve, Cockburn Sound, Western Australia. *Nicotiana glutinosa* was mechanically inoculated with *C. maritima* sap and after 20 days the plant showed symptoms of stunting and the leaves had a chlorotic mosaic, indicating the presence of a virus(es). Total proteins from inoculated and uninoculated *N. glutinosa* proteins were extracted using method 3 and separated on a gel (Fig. 3.10).
**Figure 3.10** One-dimensional polyacrylamide gel electrophoresis image of infected and uninfected plant protein extracts. Lane 1 and 4: uninfected *Nicotiana glutinosa*; Lane 2 and 3: infected *N. glutinosa*; Lane 5: Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363). Differentially expressed band 6 is shown by arrow.

Infected and uninfected protein extracts shared most bands and infected plant extracts appeared to contain less protein, exemplified by a lower intensity of staining of the 50 kDa RuBisCO. A differentially expressed protein (band 6) of approximately 24 kDa is clearly visible in
lanes 2 and 3 in the infected plant extracts. This band (band 6) was removed and analysed by PMF. The general parameters were used for data acquisition. For the spectrum (Fig. 3.11 and the 4 peaks matching the database), the Expectation Value (i.e. the likelihood of predicted mass being correct) and the accession number of the closest match are listed in Table 3.4. The closest match for these peptide fragment sizes is the CP of *Pelargonium zonate spot virus* (PZSV) (tomato strain).

![Figure 3.11](image)

**Figure 3.11** Spectrum of trypsin in-gel digested band 6 from Fig. 3.10 (putative viral protein in infected *Nicotiana glutinosa*).
Figure 3.12 Spectra of infected and uninfected plant protein extracts as detected by the Voyager MS. a and b: infected/uninfected *Nicotiana glutinosa* with *Pelargonium zonate spot virus*.

The Voyager-DE™ PRO Workstation was used to characterise the intact CP of PZSV (Fig. 3.12). The additional peak in infected extracts at 22997.77 Da was characteristic of the CP mass of PZSV, whose calculated CP mass is 23084.58 Da. Two peaks present in both infected and uninfected extracts, approximately 24938.83 Da and 26887.88 Da, were interpreted as plant proteins common to both extracts.

To further confirm the identity of the virus present in the *C. maritima* plant, RNA was extracted and RT-PCR was done as described in Chapter 2. PZSV-specific oligonucleotide primers that annealed within the putative movement protein (MP) of the genome (Liu & Sears, 2007) (R3-F 5′ CTCACCAACTGAATGCTCTGGAC 3′ and R3-R 5′ TGGATGCGTCTTTCCGAACC 3′) were used to amplify part of RNA3 using an annealing temperature of 54 °C (Fig. 3.13). The resulting amplicon was purified (MinElute PCR kit, Qiagen, #28004) and sequenced directly with the primers used in their amplification. The virus sequence was assigned an accession number by GenBank (GU046705). This sequence was analysed using NCBI BlastN and the sequence was confirmed to be part of the PZSV genome, with 93–95% identity to other published PZSV RNA3 sequences (Finetti-Sialer & Gallitelli, 2003). Fig. 3.14 showed the phylogenetic relationship of PZSV (Woodman point isolate and Tomato strain) with other viruses in the family *Bromoviridae*. The CP nucleotide sequence of PZSV (Woodman point isolate) is 49%-52% identical to other
viruses in the family, and 95% identical to PZSV (Tomato strain). In 2005, PZSV was classified as the only member of a new genus in the family: *Anulavirus* (Gallitelli *et al*., 2005).

![Figure 3.13](image)

**Figure 3.13** Lane 1: Amplified part of RNA3 sequence of *Pelargonium zonate spot virus* genome. Lane M: BenchTop 1kb DNA Ladder (promega, #G7541).
Figure 3.14 Phylogenetic tree constructed with partial CP nucleotide sequences of members of the family Bromoviridae. AMV: Alfalfa mosaic virus; EmoV: Elm mottle virus; OLV-2: Olive latent virus 2; PZSV-T: Pelargonium zonate spot virus (Tomato strain); PZSV-W: Pelargonium zonate spot virus (Woodman point isolate); PSV: Peanut stunt virus; CMV-Mi: Cucumber mosaic virus (Mi isolate); CMV-Z: Cucumber mosaic virus (Z strain); BMV: Brome mosaic virus; MYFV: Melandrium yellow fleck virus; SBLV: Spring beauty latent virus; BBMV: Broad bean mottle virus; CCMV: Cowpea chlorotic mottle virus; CYBV: Cassia yellow blotch virus; TMV (Tobacco mosaic virus) was used as outgroup.
Detection of double infection

Plants are often infected with more than one virus. Further optimisation of the system was done to detect double infections of individual plants. *N. benthamiana* is a host of both CMV and HarMV so this host was inoculated with both of these viruses. Controls were plants inoculated with either CMV or HarMV, and uninoculated *N. benthamiana*.

Two weeks after inoculation, symptoms on *N. benthamiana* inoculated with CMV and HarMV included mosaic, leaf distortion and stunting. However, *N. benthamiana* inoculated either with CMV or HarMV alone showed only mild mosaic (Fig. 3.15). Fresh leaves of the plants inoculated with either CMV, HarMV or with both viruses were used to extract protein using method 3. The protein extracts were then separated by one dimensional electrophoresis (Fig. 3.16).
Figure 3.15 Symptoms of Cucumber mosaic virus (CMV) and Hardenbergia mosaic virus (HarMV) infected Nicotiana benthamiana (arrows). (1) Four pots from left to right were N. benthamiana infected with CMV and HarMV, N. benthamiana infected with CMV, N. benthamiana infected with HarMV and N. benthamiana uninfected. Plants with mixed infection showed stunting compared with single infected plants and the uninfected control. (2) Mix infection plant leaves showed severe mosaic and leaf distortion. (3) CMV infected plant leaves did not show obvious symptoms. (4) HarMV infected plant leaves showed mild mosaic.
When the intensity of protein bands from uninoculated plants are compared with those from plants inoculated with CMV (Fig. 3.16 lane 2) or with both CMV and HarMV (Fig. 3.16 lane 3), the lanes that have protein from a CMV infected plants have protein bands at just above 25 kDa (band 7 and 8) with much higher levels of expression.

**Figure 3.16** One-dimensional polyacrylamide gel electrophoresis image of inoculated and uninoculated *Nicotiana benthamiana* protein extracts (using extraction method 3 with the same weight of starting
materials and loading the same volume of protein extracts for each lane). Lane 1: *N. benthamiana* inoculated with *Hardenbergia mosaic virus* (HarMV); Lane 2: *N. benthamiana* inoculated with *Cucumber mosaic virus* (CMV); Lane 3: *N. benthamiana* inoculated with CMV and HarMV; Lane 4: uninoculated *N. benthamiana*. Lane 5: Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363). Arrow indicated bands were highly expressed in inoculated plant extracts and were applied to peptide mass fingerprinting (PMF).

PMF analysis of these two bands was done using in-gel digestion protocol 2 (Table 3.5). The protein mass range was set from 10 to 37kDa when searching within the database. Other parameters used were as described previously.

**Table 3.5 Peptide mass fingerprinting (PMF) analysis of CMV CP**

<table>
<thead>
<tr>
<th>Band number</th>
<th>Measured peptide masses&lt;sup&gt;a&lt;/sup&gt; (Da)</th>
<th>Predicted masses (Da)</th>
<th>Predicted identity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CP mass (kDa)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Coverage (%)</th>
<th>Expectation value&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1138.572, 1208.622, 1479.746, 1587.807, 2316.177</td>
<td>1138.569, 1208.619, 1479.745, 1587.800, 2316.170</td>
<td>CMV 23.5</td>
<td>25</td>
<td>6.51819e-003</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured peptide masses after calibration.

<sup>b</sup> GenBank accession codes of viruses that most closely matched peptide mass fingerprints of the proteins tested: *Cucumber mosaic virus* (CMV) AAF28361.1 (No. 8), CMV CAC81741.1 (No.7).

<sup>c</sup> CP masses predicted from amino acid sequences.
Band 7 and 8 were identified to be from the CP of CMV as expected. The intensity of the bands on Fig. 3.16 indicates that the concentration of CMV was higher in the single infected plant than in the doubly infected plant. The reason for this difference is discussed in Chapter 5. However, in both plants inoculated with HarMV (Fig. 3.16 lane 1 HarMV alone, and lane 3 HarMV and CMV) no additional band corresponding to HarMV CP was visible. Degenerate primers LegpotyF (5’-GCWKCHATGATYGARGCHTGGG-3’) (Webster et al., 2007) and LegpotyR (5’-AYYTGYTYMTCHCCATCCATC-3’) were used to amplify the 3’ terminal end of the NIb gene and the 5’ region of the CP gene of HarMV to confirm infection. Bands with approximately 600 bp indicated that the plants were infected with HarMV (Fig. 3.17).

Figure 3.17 DNA amplicons of *Hardenbergia mosaic virus* (HarMV) inoculated to *Nicotiana benthamiana* plants. Lane M: 100 bp DNA Ladder (Axygen, #M-DNA-100bp); Lane 1: *N. benthamiana* inoculated with HarMV and *Cucumber mosaic virus* (CMV); Lane 2: *N. benthamiana* inoculated with HarMV.

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\(^d\) According to default setting in ProFound, expectation value < 1e-003 is a highly confident identification while 1e-003<expectation value <1e-001 is as marginal match. Expectation value >1e-001 is considered to be failed search.
3.3.1.4 Non-viral protein Peptide Mass Fingerprinting analysis

Several other plants (*H. comptoniana, N. glutinosa, Kennedia coccinea, Anigozanthos manglesii* and *Phaseolus vulgaris*) with symptoms of unknown virus infection were used to search for identity of an infectious virus using the MS assay strategy described above (Table 3.1 6, 8, 9, 10 and 11). Differentially expressed proteins were found in most of the infected plant tissue extracts. However, these proteins were either identified to be plant proteins or remain to be identified. The gel images (Fig. 3.18 to Fig. 3.20) show differentially expressed bands and these were analysed by PMF. Proteins identified are listed in Appendix 1. Unidentified proteins are listed in Appendix 2. No viruses were identified in these experiments.
Figure 3.18 One-dimensional polyacrylamide gel electrophoresis images of protein extracts from infected and uninfected plants. Lane 1 and 2: symptomatic/symptom-free Kennedia coccinea; Lane 3 and 4: symptomatic/symptom-free Nicotiana glutinosa; Lane 6 and 7: symptomatic/symptom-free K. coccinea; Lane 5 and 8: Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363). Additional bands 9, 10, 11, 12 (arrows) were isolated to do in-gel digestion and peptide mass fingerprinting (PMF) analysis. Band 13 was much more highly expressed in symptom-free plant extracts using both extraction methods and was analysed by PMF. Band 10: unknown; band 9: 1,3,-beta-D-glucanase; band 11: 1,3,-beta-D-glucanase; band 12: oxygen-evolving enhancer protein 1; band 13: ribulose 1,5-bisphosphate carboxylase (RuBisCO) small subunit.
Figure 3.19 One-dimensional polyacrylamide gel electrophoresis images of infected and uninfected plant protein extracts. Lane 1 and 8: Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363); Lane 2 and 3: symptom-free/symptomatic <i>Anigozanthos manglesii</i>; Lane 4 and 5: symptom-free/symptomatic <i>A. manglesii</i>; Lane 6 and 7: infected/uninfected <i>Hardenbergia comptoniana</i> with <i>Hardenbergia mosaic virus</i> (HarMV). Arrows indicate bands from symptomatic <i>A. manglesii</i> extracts that were analysed by peptide mass fingerprinting (PMF). No visible differentially-expressed band was seen in ‘infected’ and uninfected <i>H. comptoniana</i>. Band 14: unknown; band 15: unknown.
Figure 3.20 One-dimensional polyacrylamide gel electrophoresis image of infected and uninfected plant protein extracts. Lane 1 to 6: symptomatic *Phaseolus vulgaris* named BB2, SB1, 4B2, 4B1, J52, J51; Lane 7: uninfected *P. vulgaris*; Lane 8: Precision Plus Protein Unstained Standards (Bio-Rad, #161-0363). Differentially expressed protein bands 16 and 17 were analysed by peptide mass fingerprinting (PMF). Band 16: beta-1,3-endoglucanase; band 17: cytosolic ascorbate peroxidase.
3.3.1.5 MS/MS sequencing of the coat protein of *Pelargonium zonate spot virus*

Band 6 (Fig. 3.10) from a plant infected by an unknown virus later determined to be PSZV was excised and analysed by MS/MS sequencing by Proteomics International Pty Ltd in Western Australia. Fig. 3.21 shows the matching probability distribution based Mowse Score (Savitski *et al.*, 2005) in the report. According to the MS/MS report protein hits scored above 54 were confident identities. This figure shows that only one peptide had a match with a significant score at 233 (>54), which was to the CP of PZSV (GenBank accession codes: Q9DUT0). Both PMF and MS/MS successfully identified band 6 (Fig. 3.10) as the CP of PZSV. The sequenced peptides by MS/MS are listed in Table 3.6. Fig. 3.22 shows the full sequence of PZSV CP from NCBI database, peptides detected by PMF and sequenced peptides by MS/MS analysis.

The amino acid sequences of four peptides of PZSV CP were sequenced by MS/MS, three of which were detected by PMF, thus validating the accuracy of PMF identification based on the molecular mass. The peptide SRQQALAK sequenced by MS/MS was not detected by PMF and peptide EFSGLSMSVER detected by PMF was not identified by peptide sequencing, which probably derived from different resulting peptides under different conditions of trypsin digestion, such as pH, temperature, digestion duration.
Table 3.6 MS/MS amino acid sequencing results of *Pelargonium zonate spot virus* CP

<table>
<thead>
<tr>
<th>Observed mass (Da)</th>
<th>Calculated mass (Da)</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>901.4973</td>
<td>900.5141</td>
<td>R.SRQQALALK.</td>
</tr>
<tr>
<td>1170.5408</td>
<td>1169.5982</td>
<td>K.GHVIVHYPGKT.</td>
</tr>
<tr>
<td>1885.8575</td>
<td>1884.9985</td>
<td>K.IFVEHEKIGQAEGYIPL.-</td>
</tr>
</tbody>
</table>

\(^a\) Calculated mass based on the peptide sequence.

Figure 3.21 Score = -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 54 indicate identity or extensive homology (p<0.05).
3.3.2 Detection of plant viruses using Next Generation Sequencing

Illumina sequencing of the cDNA prepared from total RNA extracted from leaf tissue of a lily plant with viral symptoms generated a total of 36 million individual sequences with a read length of 78 nt, giving a total of about 2.8 billion (2.8 Gb) nt of DNA sequence. Contig assembly using Velvet short read assembler (Zerbino & Birney, 2008) gave 171, 920 contigs. BlastN (Altschul et al., 1997) analysis showed that one of them had 88% identity with protein phosphatase 1 (PP1) gene for polyprotein 1 of CNSV cycas strain (host *Cycas revoluta*) (accession number AB073147.1), and the other one was 88% similar to protein phosphatase 2
(PP2) gene for polyprotein 2 of CNSV cycas strain (host *C. revoluta*) (accession number AB073148.2). The complete genome sequences of CNSV cycas strain (host *C. revoluta*) RNA 1 (accession number NC_003791.1) and RNA 2 (accession number NC_003792.2) were used as scaffold sequences with which to align the entire data set against (Fig. 3.23). In total, 1402 reads were assembled to RNA 1 and 1242 to RNA 2. The coverage (the number of times the sequence of nucleic acid was sequenced) for RNA 1 was minimum 0, maximum 142 and mean 15 (pairwise identity 98.5%) and for RNA 2 it was minimum 0, maximum 141 and mean 21 (pairwise identity 98.1%). The consensus sequence of the new strain of CNSV was then used as a scaffold sequence with which to align the entire data set against (Fig. 3.24). In total, 1406 reads were assembled to RNA 1 and 1241 to RNA 2. The coverage for RNA 1 was minimum 0, maximum 141 and mean 17 (pairwise identity 99.0%) and for RNA 2 it was minimum 1, maximum 141 and mean 23 (pairwise identity 98.7%). For RNA 1 6259 nt (accession number JN127336) of full sequence (7471 nt) (84% of the full sequence) and for RNA 2 4206 nt (accession number JN127337) of full sequence (4667 nt) (90% of the full sequence) were obtained. Comparison of CNSV Lily strain in Western Australia with CNSV Cycas strain in Japan, sequences of RNA 1 were 89.5% identical and sequences of RNA 2 were 88.6% identical. This is the first report of CNSV in lily plants and in Australia. Part of the genome organisation of CNSV (lily strain) is shown in Fig. 3.25, and this is similar to that of other nepoviruses (Ritzenhalter *et al.*, 1991; Karetnikov & Lehto, 2007).
Figure 3.23 *Cycas necrotic stunt virus* (CNSV) RNA 1 (accession number NC_003791.1) (A) and RNA 2 (accession number NC_003792.2) (B) were used as reference sequences to assemble the entire illumina data in Geneious v5.3.4. Bars below reference sequences were next generation sequencing (NGS) data reads (78 nt in length). The confidences were higher where the nucleic acids were sequenced more frequently. Arrows indicated positions with low confidence.
Figure 3.24 *Cycas necrotic stunt virus* (CNSV) (Lily strain) RNA 1 consensus sequence (Fig. 3.23) (A) and RNA 2 consensus sequence (Fig. 3.23) (B) were used as reference sequences to assemble the entire data in Geneious v5.3.4. Bars below reference sequences were next generation sequencing (NGS) data reads (78 nt in length). The confidences were higher where the nucleic acids were sequenced more frequently.

![RNA 1 and RNA 2 sequences](image)

**Figure 3.25** Part of the genome organisation of *Cycas necrotic stunt virus* (CNSV) (Lily strain) based on alignment with CNSV (Cycas strain). Genes and domains are indicated as boxes; lines indicate untranslated regions (UTRs); (A), and horizontal dashed lines represent poly(A) tail; nucleotide positions of the borders of the genes or domains are shown above. CNSV polyprotein 1 is predicted to be cleaved into: nucleotide-binding protein (NTB); viral protein genome-linked (VPg); cysteine protease (CysPro); RNA-dependent RNA polymerase (RdRp). CNSV polyprotein 2 is predicted to be cleaved into: movement protein (MP) and coat protein (CP).

Presence of the virus in the bulb tissue of infected lily plants was confirmed by RT-PCR. Primers for PCR reactions were designed according to the sequence obtained from the NGS assembly (RNA 1): CNSVF 5’- TGCCATGGCTGGTTTTGGAG-3’; CNSVR 5’- GCAGCATGGCAATCCATAGG-3’ with expected amplicon size 915 bp. A DNA band of approximately 1000bp (expected size 915 bp) is present on the gel (Fig. 3.26). Sanger sequencing of this amplicon (915 bp) confirmed that it was part of the CNSV RNA 1 sequence, which was the same as sequence obtained from NGS.
Figure 3.26 Lane M: BenchTop 1kb DNA Ladder (Promega, #G7541); Lane 1: amplicon of part of *Cycas necrotic stunt virus* (CNSV) RNA 1 sequence (915 bp); Lane 2: water control.

The phylogenetic analysis of 14 nepoviruses indicated that the newly detected virus had 96% amino acid identity to CNSV cycas strain in the conserved region of RdRp, and 32% to 49% amino acid identity to other nepoviruses listed, confirming that the virus characterised in this study belonged to the species of CNSV (Fig. 3.27, 3.28).
Figure 3.27 Multiple alignment of the conserved region of the RNA dependent RNA polymerase (RdRp) of *Cycas necrotic stunt virus* (CNSV) (lily strain and cycas strain) and other 12 nepoviruses (amino acid sequences). Virus species names and accession numbers used are listed below: SDV (*Satsuma dwarf virus*, AB009958), RpRSV (*Raspberry ringspot virus*, NC_005266.1), PRMV (*Peach rosette mosaic virus*, AF016626), ArMV (*Arabis mosaic virus*, AY303786.1), GFLV (*Grapevine fanleaf virus*, D00915), CLRV (*Cherry leaf roll virus*, GU167974.1), ToRSV (*Tomato ringspot virus*, L19655), TRSV (*Tobacco ringspot virus*, U50869), GCMV(*Grapevine chrome mosaic virus*, X15346), TBRV (*Tomato black ring virus*, AY157993.1), CNSV (*Cycas necrotic stunt virus*, NC_003791.1 (cycas strain)/ next generation sequencing (NGS) data (lily strain)), CRLV (*Cherry rasp leaf virus*, AJ621357.1), SLRSV (*Strawberry latent ringspot virus*, NC_006964.1). Shaded regions represent residues with high identity.
Figure 3.28 Evolutionary relationships of amino acid sequences of RNA dependent RNA polymerase (RdRp) conserved region of 11 nepoviruses. Evolutionary history was inferred using the neighbour-joining method. The virus species in bold indicated nepoviruses reported in Australia and others were included as outgroups. The scale bar shows the number of substitutions per aa. The numbers at the nodes showed the confidence of the estimated branch (%). Virus species names and accession numbers used are listed below: SDV (Satsuma dwarf virus, AB009958), RpRSV (Raspberry ringspot virus, NC_005266.1), PRMV (Peach rosette mosaic virus, AF016626), ArMV (Arabis mosaic virus, AY303786.1), GFLV (Grapevine fanleaf virus, D00915), CLRV (Cherry leaf roll virus, GU167974.1), ToRSV (Tomato ringspot virus, L19655), TRSV (Tobacco ringspot virus, U50869), GCMV (Grapevine chrome mosaic virus, X15346), TBRV (Tomato black ring virus, AY157993.1), CNSV (Cycas necrotic stunt virus, NC_003791.1 (cycas strain)/next generation sequencing (NGS) data (lily strain)).
3.4 Discussion

3.4.1 Using Peptide Mass Fingerprinting to identify plant viruses

A simplified assay for identifying viruses in infected plant material using one-dimensional SDS PAGE to separate total proteins, followed by trypsin digestion of differential bands and PMF identification of viral proteins, was developed. Four known viruses, ZYMV, TuMV, AMV and CMV were identified correctly, confirming application of the assay. An unknown virus infecting an invasive coastal dune weed *C. maritima* (Rodman, 1986) was identified as PZSV, the first report of its presence in Australia and in this host species. When plants doubly infected with two known viruses were tested, only one virus was identified.

3.4.1.1 Selection of propagation hosts for virus and protein extraction protocols

Although the virus assay showed that in some cases both known and novel viruses could be identified by PMF, some others could not be identified. One of the main limitations to identification is that virus concentration appeared to vary considerably. For example, in *B. juncea* infected with TuMV it showed a high load of TuMV CP on the gel, whereas in *N. benthamiana* infected with PWV it showed no visible viral protein band (Fig. 3.7). *N. benthamiana* was a good propagation host for CMV (Fig. 3.16). The reason why HarMV CP not being visualised in infected *N. benthamiana* protein extracts (Fig. 3.16) was either that the extraction method was not optimal for HarMV or that the infected *N. benthamiana* plants did
not accumulate enough virions that could be extracted and seen on an one-dimensional
polyacrylamide gel. In support of this explanation, further research (Chapter 5) showed that
two weeks after inoculation HarMV had a much lower accumulation in *N. benthamiana* than CMV.

It is important to select proper propagation hosts for viruses to be studied to obtain high virus
titres in host plant tissues. Plants containing high concentrations of tannins, gums, or phenolic
compounds, which interfere with virus purification, are also problematic. Plant species in the
following genera have been found to be suitable propagation hosts for purification of a large
number of viruses: *Chenopodium, Cucumis, Nicotiana, Petunia* and *Vigna* (Dijkstra & de Jager,
1998). For most viruses their titre decreases in plants after reaching a peak. Some plant viruses
multiply slowly over relatively long period of time, while others reach a peak titre in a short
period (Kokkinos & Clark, 2006; Malapi-Nelson *et al*., 2009). For example, *Barley yellow dwarf
virus* (BYDV) PAV isolate can multiply to the peak level in oat plant roots in 7-8 days after
inoculation (Irwin & Thresh, 1992). In doubly infected (CMV and HarMV) *N. benthamiana* plant
CMV accumulation reaches a peak level one month after inoculation (Chapter 5 Fig. 5.12). The
best sampling time is when the viruses multiply to maximum level (Cafrune *et al*., 2006). Virus
titres in different tissues of plants vary considerably as well. Young and vigorously growing
leaves usually contain virus at a higher titre (Chapter 5).
It is also evident from this research that one single protein extraction method cannot be applied to all plant species or viruses. Successful extraction depends on the plant species, the maturity of tissues selected and the properties of the target protein (e.g. pl, stability etc). Although many plant sample preparation protocols for proteomic analysis have been established (Hurkman & Tanaka, 1986; Meyer et al., 1988; Wang et al., 2004; Wang et al., 2006), no common or simple protocols can be applied for all plant species. For extraction of plant viruses, most protocols are specific to a virus or a group of viruses (Gallitelli, 1982; Palukaitis et al., 1992), partly because different virus particles remain stable at different pH and ion conditions. A ‘minipurification’ has been developed and shown to be useful to extract proteins of several plant viruses (Blouin et al., 2010), including CMV, TSWV, *Tobacco streak virus* (TSV), AMV, *Citrus leaf blotch virus* (CLBV) and a novel potexvirus. However, the extraction conditions, such as the concentration and pH of Na citrate buffer, depend on the stability of the virions of the species involved, and the technique of Blouin et al. (2010) needs an ultracentrifugation step.

In this study, four protocols were used to extract both plant and virus proteins from plant tissues. The extraction method that could extract most virus proteins despite their different stability and properties is desired for the assay. Different protocols in fact extracted different protein components and this was reflected in the different protein bands in the SDS PAGE gels (Fig. 3.4). Band 3 was subsequently identified to be CP of ZYMV, however only extraction method 3 (acetone/DTT precipitation) showed a virus band at about 33 kDa. The acetone/DTT precipitation method was then applied for subsequent research and successfully used to extract proteins of four known viruses and one novel virus, from 5 different hosts. However, this method was not suitable to extract virus protein from several other plants with presumed
virus symptoms (*H. comptoniana, N. glutinosa, Kennedia coccinea, Anigozanthos manglesii* and *Phaseolus vulgaris*). The reason could be that this extraction method was not optimised to extract virus proteins from these hosts, or that the infected plants did not contain a high enough virus titre that its proteins could be extracted and seen on a gel. Another possibility is that some of the apparently symptomatic plants were not in fact infected with viruses. But this can not be tested by ELISA/PCR because no prior knowledge was available for these plants with symptoms.

### 3.4.1.2 Visualisation of viral proteins using one-dimensional PAGE

Using this assay strategy, it was possible to identify some of the viruses tested, but further optimisation is needed in cases where mixed infection occurs with viruses that have closely similar CP masses, or where CP masses are similar to those of abundant plant proteins. The large subunit of RuBisCO, especially, is present in leaves in large amounts, and viral proteins of approximately 50 kDa would be masked by its presence. According to predicted CP masses of plant viruses (Blouin *et al.*, 2010), most virus CPs have masses lower than that of RuBisCO, although some fall within its mass range. Another consideration is that viral CPs can be resistant to trypsin digestion because of post-translational modification (Shukla *et al.*, 1988), which may explain why coverage of tryptic fragments of viral CPs was relatively low (11–36%). It is worth noting that viral proteins other than CPs were not observed on the gels or detected by PMF, probably because they were in quantities below the resolution of the Coomassie stain used. A
protein band below 75 kDa in Fig. 3.7 lane 8 may be a viral CI protein. The work also showed that plant proteins such as RuBisCO were present in lower amounts in infected plants compared to uninfected control plants (Fig. 3.4, 3.7 and 3.10). There are two possible explanations for this: (i) virus infection lowers accumulation of plant proteins, including RuBisCO, (ii) in infected plants, virus proteins account for a portion of the total extracted protein mass, therefore a proportionally lower amount of plant protein is loaded on the gel. It is probable that both explanations are correct to a greater or lesser extent (Luo et al., 2010).

In addition to virus CPs, other differentially expressed proteins were found in some infected and uninfected plant tissue extracts (3.3.4). However, these proteins were either identified to be plant proteins or remained unidentified. The plant proteins were possibly expressed in response to virus invasion, while the unidentified proteins could be plant proteins or other pathogen proteins (including viral protein) that are not present in the protein database. Since all samples were suspected to be infected with virus based on infection-like symptoms (except *H. comptoniana* known to be infected with HarMV) the reason could be either that they were not infected with virus and the symptoms were due to other environmental or biotic factors, such as nutrient deficiency, or that the virus did not multiply to a concentration high enough for detection by one-dimensional PAGE. Differentially expressed plant protein components also gave clues to investigate the plant defense responses to pathogen invasion, such as a peroxidase reported to be related to cell wall enhancement for developing a stronger physical barrier preventing further spread of the pathogen from the site of infection, and 1,3-\(\beta\)-D-
glucanase has been reported as part of the defence mechanism to fungal infection in previous studies (Bol et al., 1990).

In Fig. 3.7, band 5 was not an extra band but a much more highly expressed protein, indicating that this band probably included more than one component. Two-dimensional gel electrophoresis could also be used to separate such proteins in extracts, although two-dimensional gel electrophoresis is technically demanding and would require larger protein extracts for separation. However, in this work it was demonstrated that in a multiple-component protein band, the more highly expressed protein can be identified by PMF analysis. One of the main advantages that this assay has over those published previously is rapidity, since it requires less than two full days to complete. In this approach viruses were identified from an extract of total plant proteins, without the need for lengthy or complicated methods of purification of virus particles from host proteins, such as gel filtration chromatography (GFC) (Wang et al., 2007) or ultracentrifugation (Blouin et al., 2010). It does not require two-dimensional separation by SDS PAGE (Cooper et al., 2003). However, in the double infection (CMV and HarMV) experiment, only one virus, CMV, reached a high accumulation in host plant *N. benthamiana* that can be extracted and visualised on one-dimensional gel. The protein extract from *N. benthamiana* plant only infected with HarMV did not reveal the virus protein either, indicating HarMV existed in this host in low accumulation. Application of two-dimensional electrophoresis or change of host plant may help detect double infection using this assay.
3.4.1.3 Peptide Mass Fingerprinting and tandem MS/MS sequencing

PMF predicts peptide identity by the accurate measurement of peptide masses and a statistical procedure. Both PMF and MS/MS sequencing were used to identify the protein in band 6 (PZSV CP) in Fig. 3.10. They were both used successfully to identify the protein and correctly indicated the species of the virus. The correspondence of peptide sequences predicted by PMF and those sequenced further justifies the use of PMF to identify virus protein from in-gel digestion, although one of the peptides detected by PMF was not sequenced, probably because of the different digestion conditions used to do PMF (3.1.2.5 protocol 1) and sequencing (done by Proteomics International Pty Ltd). The assay optimised in this study utilises a rapid in-gel trypsin digestion procedure. Further, single MS analysis rather than tandem MS (Cooper et al., 2003) was sufficient for identifying the viruses tested.

Despite being relatively simple and rapid, the assay does not currently lend itself to high throughput analysis of samples, and availability of an appropriate MALDI-TOF mass spectrometer may be limiting in many laboratories. An appropriate protein extraction procedure has to be optimised and several suspected bands on the gel may have to be analysed. Its primary use is in identifying viruses whose presence is certain but whose identities have proven difficult to determine using more rapid methods. Once possible PMF matches are established, other techniques such as those based on nucleic acid analysis or serology can be
used to confirm identity. Where viruses are detected that do not share identity with known viruses, a peptide sequencing approach using MS/MS can be used to gain enough understanding of the genome to facilitate design of degenerate primers. RT-PCR and sequencing provide more specific and confident information of virus identities.

Using this approach, four plant viruses were identified successfully to the species level from two families and three genera with distinct CP sizes and particle morphology (filamentous and bacilliform). This improved and simplified assay may facilitate wider use of PMF for plant virus identification, and possibly for other plant pathogens.

3.4.1.4 Pelargonium zonate spot virus

PZSV is the only species in the genus Anulavirus, family Bromoviridae. It was originally isolated from Pelargonium zonale and first reported in Italy in 1982 (Gallitelli, 1982) and subsequently from tomato crops in other parts of Europe (Hanssen et al., 2010). The economic importance of this virus is a result of the tomato disease it induces, with concentric chlorotic/necrotic rings symptoms on leaves and fruits. It was recently reported in the east coast of North America (Liu & Sears, 2007) infecting tomato plants. It was listed as a potential threat to the Australian horticultural industry before its report in this study. This is the first report of the presence of this virus in Australia and in the host C. maritima. Other hosts of PZSV include tomato (S. lycopersicon) (Gallitelli, 1982), Pelargonium (Pelargonium zonale), pepper (Capsicum annuum),
Rubia tinctorum (Escriu et al., 2009). Since the virus was collected from the coastal dune weed C. maritima growing along the coast of Perth, and this virus has been shown to be seed-borne in tomato (Lapidot et al., 2010), it is suggested that PZSV may be transmitted to Western Australia by ocean current with floating viruliferous seeds. An antibody to PZSV CP is available commercially, making monitoring of this virus possible using ELISA as well as PCR, should this virus become a problem in Australia.

3.4.2 Using Next Generation Sequencing to identify plant viruses

NGS and conventional Sanger sequencing were used to obtain the partial genome sequence of a new strain of CNSV isolated from L. longiflorum. This host is a new host of this virus and this is the first report of CNSV in Australia. CNSV was first isolated and characterised in Japan in 1986 from sago cycad (C. revoluta) with symptoms of dwarfing and twisting of young leaves and chlorotic or necrotic spots on mature leaves (Kusunoki et al., 1986), and in 2006 from gladiolus (Gladiolus spp.) (Hanada et al., 2006). In a glasshouse based inoculation test, the host range of sago cycad strain is limited whereas the gladiolus strain is wider, mainly infecting ornamental plants, such as aucuba (Aucuba japonica), daphne (Daphne odora), allium (Allium fistulosum), globe amaranth (Gomphrena globosa) and zinnia (Zinnia elegans), but also some economically important crops, including soybean (Glycine max), peanut (Arachis hypogaea) and spinach (Spinacia oleracea) (Hanada et al., 2006). Chenopodium amaranticolour and C. quinoa were used as indicator hosts of this virus in which infected leaves showed chlorotic mottle or
chlorotic spots. In addition to nematode transmission, many nepoviruses can be transmitted through seeds, pollen or vegetatively propagated plant materials (Brown et al., 1995). CNSV has been reported to be both nematode-transmitted and seed-borne (Han et al., 2002). In 2003 CNSV was recorded in New Zealand in sago cycad (C. revoluta) and peonies (Paeonia lactiflora) (Ochoa-Corona et al., 2003), indicating that this virus is probably widespread internationally.

Unlike other diagnostic assays, such as ELISA, PCR or hybridization methods, NGS does not require any virus-specific reagent, therefore it can be used as a universal identification technique. Full genome sequences can be obtained readily through NGS and subsequent bioinformatic analysis (Adams et al., 2009; Wylie & Jones, 2011b; Wylie & Jones, 2011c). Although the majority of the genome sequence (86%) was obtained by NGS, the sequence of the 5' prime end of CNSV lily strain RNA 1 was not obtained (Fig. 3.23, arrows indicated). This might result from a biased library preparation. Future work should include sequencing the remainder of the genome by conventional sequencing or rapid-amplification of cDNA ends (RACE).

As for the genome organisation, CNSV has common features of viruses in the Comoviridae: a bipartite genome; RNA 1 encodes a helicase, 3-C like proteinase and RdRp to form a conserved replication block; RNA 2 polyprotein includes a MP immediately followed by the CP. All nepoviruses have two domains upstream of the helicase in RNA 1, which is different from the
other two genera in the same family: *Comovirus* and *Fabavirus*. CNSV shares this feature in nepoviruses. Furthermore, CNSV RNA 1 and RNA 2 have identical 5’ untranslated (UTR) and 3’ UTR regions, which is typical of nepoviruses (Sanfacon *et al.*, 2009). By analysing the RdRp conserved regions of the lily virus and 13 other nepoviruses, the lily virus had 89% and 96% identity to CNSV (Cycas strain) at the nucleotide sequence and amino acid sequence levels, respectively. However, this virus showed limited homology to other nepoviruses with identity of 53%–65% and 29%–64% at the nucleotide and amino acid levels, respectively. According to the criteria defining species within *Comoviridae* (two species have less than 75% nucleic acid sequence identity and less than 80% amino acid sequence identity) (Sanfacon *et al.*, 2009) the virus from the lily plant belongs to CNSV at the species level. Therefore, we propose that the detected virus is a new strain of CNSV, named lily strain (RNA 1, accession number JN127336; RNA 2, accession number JN127337).

Detection of the virus in the lily bulb tissues suggests that CNSV is not only transmitted by seed and nematodes, but also through vegetatively propagated plant materials, which significantly increases the chance of its spread within Australia, because many ornamental plants, including lily, are vegetatively propagated in nurseries and planted in home gardens. Furthermore, the same viral symptoms reported here have already been observed for more than 10 years in the lily plant tested in this study, so it is probable that CNSV is already established in Australia. It has been reported that different CNSV strains have a much wider host range than generally recognized (Hanada *et al.*, 2006). The economic importance of CNSV, distribution and potential
to infect native flora still needs to be assessed, to determine its quarantine status. CNSV appears to be a broad host range virus because the three natural hosts reported (cycas, lily and peony) are distantly related. It is therefore a possible threat to other commercial species and indigenous plants. CNSV isolates in Japan have been shown to be highly variable and have a rather wide host range, including ornamental and crop plants, including soybean (*Glycine max*), peanut (*Arachis hypogaea*) and spinach (*Spinacia oleracea*) (Hanada *et al.*, 2006). Future work should focus on the incidence of CNSV infection in naturalised and domesticated lily plants, other ornamental plants, such as cycas and native plants. RT-PCR/sequencing appears to be the best way to detect CNSV, since serological properties of different CNSV isolates can be variable (Ochoa-Corona *et al.*, 2003). The results reported here will assist in detecting other strains of the virus (Wylie *et al.*, 2011).
CHAPTER 4 A NEW ENCOUNTER BETWEEN A VIRUS AND A GRAIN LEGUME AT THE INTERFACE OF AN ANCIENT ‘BIODIVERSITY HOTSPOT’ AND A RECENT AGROECOSYSTEM
4.1 Introduction

Plant viruses co-evolved with wild plants long before crop domestication (Lovisolo et al., 2003). When man first domesticated plants about 10,000 years ago, some plant viruses were favoured when host plant species were selected for domestication traits and cultivated over large areas (Vaughan et al., 2007). Humans inadvertently extended the geographical and host ranges of viruses infecting their crops when they migrated to new lands carrying infected plants with them. In new locations viruses encountered new hosts, new vectors, and other viruses (Thresh, 1980). Perhaps more commonly, domesticated species encountered new viruses from the local flora when they were established in new regions. Passage within an alternative host was likely to be major driver of evolutionary change (Kearney et al., 1999; Lovisolo et al., 2003), which, in some cases, led to host specialisation through mutation and recombination (Wylie et al., 2008; Wylie & Jones, 2009). Reproductive success of a virus upon encountering a new host depended on the type of resistance encountered and the number of resistance genes present (Harrison, 2002). In modern times, reports of new encounters between viruses and plants have increased with the rapid trade of plant products around the world, and with better detection assays (Gibbs & Mackenzie, 1997; Luo et al., 2010). Further, it is anticipated that climate change will increase the incidence of new encounter scenarios between plants, viruses, and their vectors. Encounters of native viruses and introduced crops were reported in Africa. For example, Cassava brown streak disease was first reported in Tanzania in 1936 (Storey, 1936) and it was recently reported to be caused by an African native whitefly-borne virus Cassava brown streak virus (CBSV) (Monger et al., 2001; Maruthi et al., 2005) which induced necrotic roots and stems.
on cassava originally from Brazil. Replacement of vulnerable cultivars with relatively tolerant ones has reduced US$ 15–20 million loss caused by viruses in production of cassava in Africa (Thresh, 2006). Studying viruses in wild plant communities and at the interface between wild and cultivated vegetation is a neglected area that has the potential to provide critical information on threats to biodiversity and cultivated species, and on virus evolution (Lovisolo et al., 2003; Jeger et al., 2006).

Narrow-leafed lupin (L. angustifolius) is a grain legume originating from the Mediterranean basin where it has been utilised as a food source for millennia. Today, cultivars are grown in several regions of the world (Europe, the Americas, southern Africa, southern Australia). In the ‘wheatbelt’ area within the Southwest Australian Floristic Region of Western Australia, low alkaloid lupin cultivars were first grown commercially in the early 1970s (Gladstones, 1972). They now constitute a critical component of the cropping system where they are sown in rotation with wheat. The region grows around 80% of world lupin grain production, amounting to 920,000 t produced on 650,000 ha in 2005 (Palta et al., 2008). The bulk of the grain is processed into stock feed.

Several viruses are reported to infect lupin crops in Western Australia (Jones & McLean, 1989), but the most economically significant are CMV (family Bromoviridae, genus Cucumovirus) and BYMV (family Potyviridae, genus Potyvirus), neither of which originated in Australia. BYMV also
infects at least four wild legumes indigenous to the Southwest Australian Floristic Region (McKirdy et al., 1994). Narrow-leafed lupin cultivars respond to BYMV infection either by systemic necrotic or non-necrotic reactions. Infection by the necrosis-inducing strain of BYMV kills young lupin plants quickly (Jones et al., 2003) and spreads through the crop in a monocyclic pattern because there is only a brief window of time for virus acquisition and transmission to other plants by aphid vectors before the plant dies. Symptoms of infection by the non-necrosis-inducing strain include tip necrosis, stunting, and flower abortion, but not plant death. Affected plants remain infective for the duration of the growing season resulting in a polycyclic spread pattern (Cheng & Jones, 2000). Early infection of necrotic strain of BYMV killed lupins therefore there was no seed production while the late infection decreased the number of seeds by 55% to 80%. The yield losses resulting from the non-necrotic strain of BYMV depended on the infection stage. The number of seeds decreased by 27% to 98% as infection duration increased (Jones et al., 2003).

The Southwest Australian Floristic Region is one of 25 global ‘biodiversity hotspots’, where 7380 species of indigenous vascular plants have been described, half of them endemic (Myers et al., 2000; Brooks et al., 2002; Hopper & Gioia, 2004). Webster et al. (2007) described 29 isolates of a novel plant virus from the region, HarMV (genus Potyvirus), that symptomatically and asymptptomatically infected plants of H. comptoniana (Australian native wisteria), an endemic wild species whose provenance is a narrow coastal strip about 800 km in length within the region. H. comptoniana has an attractive climbing growth habit and panicles of purple flowers...
which makes it a valuable amenity plant that is traded around the world, and the subject of selective breeding. Although the authors identified other natural hosts of HarMV, the level of infection amongst them appeared to be very low (unpublished data). On the other hand, the level of HarMV infection of wild *H. comptoniana* plants was high. At seven collection sites within its natural range, 13-72% (mean 45%) of *H. comptoniana* individuals were infected with HarMV. Nucleotide sequences from a collection of isolates from throughout its natural range showed CP genes were genetically diverse (over 21% nucleotide diversity), placing isolates in eight distinct phylogenetic groups that corresponded mainly to geographic location (Webster *et al.*, 2007). Because genomic divergence is roughly proportional to the evolutionary distance from a common ancestor (Van Regenmortel, 2000), a high degree of sequence diversity over a small geographic range, as was the case with HarMV, is typical of a virus that co-evolved with indigenous wild plants over a long period of time (Spetz *et al.*, 2003). Webster *et al.* (2007) described a glasshouse-based experiment where HarMV isolates representing six of the eight described phylogenetic groups were inoculated manually or by aphids to four species of lupins: *L. cosentinii* (sandplain lupin), *L. luteus* (yellow lupin), *Lupinus mutabilis* (pearl lupin) and *L. angustifolius* (narrow-leafed lupin). All lupin species became infected and symptoms observed ranged from symptomless local infection in *L. mutabilis* to plant death in *L. cosentinii*. Reactions in infected narrow-leafed lupin plants resembled those of the non-necrotic strain of BYMV: leaf mosaic, leaf distortion, leaf down-curling, stunting, and bunching of young leaves, but not plant death.
Despite the clearance of more than 75% of the native vegetation of the ‘wheatbelt’ region of the Southwest Australian Floristic Region since agriculture first began there 180 years ago (Coates & Dixon, 2007), *H. comptoniana* remains a common component of the indigenous flora that survives on road verges and perimeters of fields in part of the area where lupins are cultivated, notably the coastal strip from Perth to about 200 km north of Perth. The region provides an interface between the remnants of an ancient ecosystem and a recent agroecosystem that is ideal for studying interactions between native and introduced plant viruses and their native and introduced hosts. Although the study by Webster et al. (2007) indicated there was potential for HarMV to spread naturally via vectors to lupin crops, this was not tested in the field and using *H. comptoniana* as a source plant. In this Chapter a two-years field study is described in which *H. comptoniana* plants infected with HarMV were grown within a field plot of narrow-leafed lupin plants and monitored for natural spread of HarMV via wild aphids.
4.2 Methods and Materials

4.2.1 Virus resource, inoculation and grafting

*H. comptoniana* seedlings were germinated in seedling trays in an insect-proofed glasshouse. When seedlings were eight weeks old leaf samples from each plant were tested for potyviruses by indirect ELISA (Torrance *et al.*, 1986) using a generic potyvirus monoclonal antibody (Agdia Inc). Actively growing shoots were taken from a naturally infected (isolate MU-4), wild *H. comptoniana* plant growing on campus at Murdoch University (Fig. 4.1 A), Perth, inoculated and grafted on virus-tested *H. comptoniana* seedlings (Fig. 4.1) (Fig. 4.2) in the glasshouse (described in Chapter 2).

Four weeks after grafting, young *H. comptoniana* leaves were tested by ELISA. Where a potyvirus was detected by ELISA it was further characterised using RT-PCR and nucleotide sequencing. Total RNA was extracted using an RNeasy Plant Miniprep kit (Qiagen, #74904). Degenerate primers used were LegpotyF (5’- GCWKCHATGATYGARGCHTGGG-3’) (Webster *et al.*, 2007) and LegpotyR (5’- AYYTGYTYMTCHCCATCCATC-3’) (Wylie *et al.*, 2010) (annealing temperature 50 °C). These primers generate an amplicon of approximately 630 bp from most potyviruses, which corresponded to the 3’ terminal end of the NIb gene and the 5’ region of the CP gene. For amplification and sequencing of the whole HarMV CP gene, primer sequences used were HarMVC PF (5’- CYCCTTACATTGCTGAATCAGC-3’) and HarMVC PR (5’- GACTACGAGCCAATAACTGTG-3’) (annealing temperature 50°C). RT-PCR conditions and direct
nucleotide sequencing of the resulting amplicons were as described in Chapter 2. Sequences were edited within Geneious Pro v4.8.3 (Drummond et al., 2010), then compared with those in GenBank (National Centre for Biotechnology Information, Bethesda, MD) using BlastN (Altschul et al., 1997). The CP sequence of HarMV isolate MU-4 was assigned an accession code by GenBank.

Figure 4.1 A: Hardenbergia comptoniana plant naturally infected with Hardenbergia mosaic virus (HarMV) on campus at Murdoch University, Perth. B: Symptoms on naturally infected (MU-4) wild H. comptoniana leaves include mosaic and leaf distortion. C: Two month old healthy H. comptoniana seedlings. D: Uninfected H. comptoniana leaves in the glasshouse.
Figure 4.2 A, B, C: Grafted shoots infected with *Hardenbergia mosaic virus* (HarMV) on stock plants. D: Symptoms of infection of *Hardenbergia comptoniana* leaves after grafting in the glasshouse.

4.2.2 Field plot layout, field data collection, seed transmission

Pilot study 2009

*L. angustifolius* cv Mandelup seed was obtained from the Department of Agriculture and Food, Western Australia. The land used for this experiment was located on the campus of Murdoch University, Perth, Western Australia. A plot 9 x 5 metres in area and consisting of a sandy loam was prepared by rotary hoeing. Nitrophoska blue fertiliser (12-12-17-2+TE) was broadcast at the rate 2 kg/100 m². Six rows of lupin cv Mandelup were sown by hand at a density of 7/m² in July 2009. Ten *H. comptoniana* plants infected with HarMV were planted amongst the lupins throughout the plot (Fig. 4.3 and 4.4).
Figure 4.3 Experimental plot in 2009 15 weeks after planting showing the position of virus source plants (*Hardenbergia comptoniana*) (▲), uninfected (●) and infected lupin plants (■). Numbers represent the distance in metres of the infected plant to the north end of the row. Numbers in parentheses represent the order in which plants developed symptoms of virus infection.
Figure 4.4 Picture of layout of field trial in 2009. A: Lupins started to germinate one week after planting. *Hardenbergia comptoniana* plants infected with HarMV were planted at random intervals in the plot. B: Lupins grew to two leaf stage at week two. C: Field trial layout eight weeks after planting.

Plants were watered regularly. No pesticides were applied during the trial so that wild aphids could freely colonise plants. Lupin seedlings were examined twice weekly for symptoms of virus infection and for aphids. Every 10-12 days, leaves of plants with symptoms resembling virus infection were collected and tested by ELISA for potyvirus and RT-PCR/sequencing using
degenerate primers LegpotyF and LegpotyR as described before. Aphids were collected from *H. comptoniana* and lupin plants and stored in a solution of 70% ethanol for subsequent identification. On week 15 leaves were sampled from each asymptomatic plant, watering was discontinued and the plants allowed to dry. Leaves were tested in batches of ten by ELISA with the general potyvirus antibody. Seed was harvested from the dried plants at week 16 and 1,000 seeds were planted in trays in an insect-proofed glasshouse. Seedlings were observed for symptoms of virus infection over six weeks.

**2010 trial**

The same field was used for a full field trial in July 2010. The experiment was redesigned and control plots were introduced into the field (Fig. 4.5 and 4.6). Twelve square plots, each 1x1 metre, were arranged in four rows 1 m apart. Fifty plants per plot (5 rows × 10 plants/row) of lupin cv Mandelup were sown at the end of July. Insect-proof netting was used to cover six of the plots to prevent aphids colonising them. Eight HarMV-infected *H. comptoniana* plants were planted evenly throughout the plots. Plants were watered daily and Nitrophoska blue fertiliser (12-12-17-2+TE) was broadcast at the rate 2 kg/100 m² once a week. No pesticides were applied during the trial. Symptoms of infection and aphids were recorded three times a week. Lupins showing symptoms of virus infection were tagged and tested by ELISA, RT-PCR and direct sequencing as described above at week 5 and 10. 12 weeks after planting leaves collected from control plants were tested in batches of ten by ELISA with the general potyvirus antibody. In week 14 all experimental plot lupin plants were tested for presence of CMV and BYMV by ELISA.
Seeds, harvested from infected lupins plants in week 16, were planted in trays in an insect-proofed glasshouse. Seedlings were observed for symptoms of virus infection and tested by ELISA for potyvirus two weeks after germination.

Figure 4.5 Field experiment layout in 2010. Symbol ▲ showed position of virus source plants (*Hardenbergia comptoniana*) with the number under them indicating the distances they were away from the south end of the field. Each square represents a plot planted with 5 rows of lupin plants, each row 10 individuals. T1 to T6 were experimental plots while C1 to C6 were control plots covered with insect-proof netting. ● indicates uninfected and ○ infected lupin plants. The total infection rate was 39.1% at the margin and 24.4% at the centre of all the plots.
Figure 4.6 Field experiment in 2010. A: Layout of the field trial. Control plots were covered by insect-proof netting. Infected *Hardenbergia comptoniana* plants were planted between plots. Lupins with suspected symptoms were marked by blue paper tags and tested. B: Lupins growing under the aphid proof covers as controls, where no potyvirus infection was found.

4.2.3 Interactions of three HarMV isolates against four lupin cultivars

Two isolates of HarMV (WHP-1, WHP-2) collected from wild *H. comptoniana* plants growing at Wireless Hill Park, Perth, were sequenced using primer HarMVCpf and HarMVCpr as described
above. Sequences were compared with those in GenBank using BlastN to sort out the virus isolates. *L. angustifolius* cvs Mandelup, Belara, Tanjil and Tallerack seeds were obtained from the Department of Agriculture and Food, Western Australia and planted in insect-proofed glasshouse. Fresh leaves of *H. comptoniana* infected with three HarMV isolates (WHP-1, WHP-2 and MU-4) were collected and inoculated to lupin plants in the glasshouse when the seedlings were at the two-leaf stage (each inoculation with a repeat pot and a control pot). Infected leaf tissue was ground in cold 0.05M potassium phosphate buffer (pH 7.2) with a mortar and pestle, which was mixed with the diatomaceous earth ‘celite’ before being rubbed onto young leaves. Viral symptoms were recorded daily to week 6.

### 4.3 Results

The ELISA test of 20 eight-week-old seedlings of *H. comptoniana* showed that none of them were infected by potyviruses. Four weeks after shoots derived from a wild *H. comptoniana* plant infected with HarMV isolate MU-4 were grafted to each seedling, some plants showed virus like symptoms of systemic infection on young leaves in ungrafted shoots (Fig. 4.2). Grafted and ungrafted shoots of all plants were tested for potyviruses by ELISA. Ten grafted plants were systemically infected with a potyvirus, confirmed to be HarMV MU-4 by nucleotide sequencing of PCR amplicons (accession number GU812281). A phylogenetic analysis of the CP sequence of the isolate showed it to be a member of HarMV group V (Webster et al., 2007). Symptoms were typical of naturally infected *H. comptoniana* plants: chlorosis, mosaic patterns on the leaves and leaf distortion (Fig. 4.2). Aphids were first observed on lupin seedlings in the field plot (both
two weeks after planting and on new growth on *H. comptoniana* plants six weeks after planting. Both winged and wingless *Myzus persicae* (green peach aphid), *Acyrthosiphon kondoi* (bluegreen aphid) and *Rhopalosiphon padi* (oat aphid) were collected from both species (Fig. 4.7).

In the 2009 pilot study leaves of lupins with suspected symptoms of virus infection, including leaf down curling, stunting, necrotic stem streaking, tip wilting, leaf abscission, and necrosis (Fig. 4.8) were collected on weeks 8, 10, 12, 13 after planting. In total, 15 plants were infected by potyviruses over the course of the experiment, five at week 8, four each at weeks 10 and 12, and an additional two at week 13. Nucleotide sequences showed that 14 of these plants (4.67% of the total lupin plants) were infected with HarMV MU-4 (Fig. 4.3), the same isolate present in the *H. comptoniana* plants. The remaining lupin plant was infected with the necrotic strain of BYMV, presumably transferred from a wild plant growing nearby. All infected plants died within 20 days of symptoms becoming visible and none produced viable seed. About 4-5 infected plants were identified during each of the first three samplings. Only two infected plants were identified on the final sampling day in October 2009, probably because the exceptionally hot and dry weather during this time inhibited aphid activity. By then, *H. comptoniana* plants had stopped active growth and aphids could not be found on them. In general, lupins that became infected occurred close to *H. comptoniana* plants, both along rows and across rows (Fig. 4.3 and 4.9). Nine of the 14 infected lupins were located within 1 m of a *H. comptoniana* plant. No potyviruses infected the asymptomatic lupins that were all tested before the plants dried off.
Figure 4.7 Wingless green peach aphids feeding on a young shoot of a *Hardenbergia comptoniana* plant (A) and lupin plant (D); Winged oat aphids feeding on young leaves of a *H. comptoniana* plant (B) and lupin plant (C).

Seed was collected from all surviving plants, mixed and a sample of 1000, representing about 25% of the number harvested, was planted. By six weeks after planting, none of the 724 seedlings that germinated showed virus-like symptoms.
Figure 4.8 Typical viral symptoms of lupin plants induced by *Hardenbergia mosaic virus* (HarMV) infection. (A) The plant showed typical necrotic stem streaking, stunting and seed production failure by early infection; (B) Late infection induced no stunting and seed production failure but common necrotic stem streaking; (C) Typical tip necrosis occurred on a lupin plant; (D) Healthy lupin plants growing vigorously under the cover net.
Figure 4.9 Number of lupin plants infected with *Hardenbergia mosaic virus* (HarMV) MU-4 decreases with distance (metres) to the nearest virus source plant (*Hardenbergia comptoniana*). The trend line fits $y = 8.3255e^{-0.921x}$.

In the experiment in 2010, the first lupin plant showing the same suspected symptoms described above (Fig. 4.8) were sampled and tested to be infected with HarMV (isolate MU-4) by nucleotide sequencing 5 weeks after planting. The leaves of symptomatic lupin plants were all collected and tested by ELISA for potyvirus 10 weeks after planting, of which 92 were positive (infection rate 30.67%). 25 of them were randomly selected and further characterised by nucleotide sequencing, confirmed to be infected with HarMV (isolate MU-4). Although they initially developed symptoms resembling those present in 2009, infected plants usually did not die before uninfected plants (3 infected plants died). Spread was also much greater in 2010, probably because infected lupin plants acted as foci of infection to other lupins. Lupin plants on the margins of the plots initially became infected before those in the middle of the plots (Fig. 4.5), suggesting that lupin to lupin spread may have been occurring. In week 12 no lupin plants
in the control plots were infected with potyvirus. None of the lupin plants in the experimental plots was confirmed to be CMV or BYMV infected in week 14. If non-necrotic HarMV infection became widespread in lupin crops it could impact on yields. Seed from all 92 HarMV infected *L. angustifolius* plants and another 92 uninfected plants were collected and dried in paper bags at room temperature for 10 days before analysis. HarMV infection reduced individual seed weight by 32% (Fig. 4.10A), seed number by 79% (for 92 plants) (Fig. 4.10B), and reduced total seed yield of 92 plants by 86% (Fig. 4.10C). The seed was often discoloured (Fig. 4.10D). None of the 761 seedlings grown from seed collected from infected parent plants were positively tested to be HarMV infected by ELISA. Like BYMV, the virus does not appear to be seed transmitted in the same manner that CMV is. The question remains why the symptom phenotypes were different in 2009 and 2010.

Four lupin cultivars showed different levels of resistance to three HarMV isolates (isolate MU-4, WHP-1 and WHP-2) infection (Table 4.1 and Fig. 4.11). Necrotic stem streaking and tip leaf necrosis were common symptoms of lupin plants with HarMV infection. Slight necrotic stem streaking was visible close to the tip of the plants about two weeks after inoculation and this then spread to the bottom along stem. Although most tip necrosis was not lethal to lupin plants, it did cause plant stunting and leaf downcurling. Lupin cvs Mandelup and Belara infected with HarMV isolate WHP-2 showed systemic necrosis and died 22 and 32 days after inoculation respectively. Although no lupin cultivar was found to induce a systemic hypersensitive response to all three HarMV isolates, lupin cvs Mandelup and Belara showed necrotic responses to isolate WHP 2.
Figure 4.10 Data from 2010 field plot: comparison of average grain weight of single seed (A), seed number produced per 92 *Lupinus angustifolius* plants (B), seed weight produced per 92 *L. angustifolius* plants (C) and appearance (D) between *Hardenbergia mosaic virus* (HarMV)–infected (dark grey) and uninfected (light grey) *L. angustifolius* plants.
Figure 4.11 Symptom variation among 4 genotypes of *Lupinus angustifolius* infected with 3 isolates of *Hardenbergia mosaic virus* (HarMV) (WHP-1, WHP-2 and MU-4). Photos were taken 25 days after inoculation. Belara infected with WHP-2 isolate exhibited systemic necrosis 22 days after inoculation and Mandelup infected with WHP-2 isolate died 32 days after inoculation. Symptoms are described in Table 4.1.
**Table 4.1** The responses of four lupin cultivars to different isolates of *Hardenbergia mosaic virus* (HarMV)

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Isolates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WHP-1</th>
<th>WHP-2</th>
<th>MU4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belara</td>
<td>LC, TN, NSS</td>
<td>LC, S&lt;sup&gt;b&lt;/sup&gt;, D&lt;sup&gt;c&lt;/sup&gt;, NSS</td>
<td>S, LC, TN, LD, NSS</td>
<td></td>
</tr>
<tr>
<td>Tallerack</td>
<td>LC, TN, LD, S, NSS</td>
<td>LC, TN, LD, S, NSS</td>
<td>LC, TN, S, NSS</td>
<td></td>
</tr>
<tr>
<td>Tanjil</td>
<td>LC, TN, NSS</td>
<td>TN, LC, NSS</td>
<td>S, TN, LD, NSS</td>
<td></td>
</tr>
<tr>
<td>Mandelup</td>
<td>LC, TN, LD, NSS</td>
<td>LC, D&lt;sup&gt;d&lt;/sup&gt;, NSS</td>
<td>LC, TN, LD, NSS</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Leaves were inoculated with 3 HarMV isolates infected lupins.

<sup>b</sup> Coded symptom descriptions: S stunting; LC leaf downcurling; D plant death; LD leaf distortion; NSS necrotic stem streaking; TN tip leaves necrosis.

<sup>c</sup> Plant was dead 22 days after inoculation.

<sup>d</sup> Plant was dead 32 days after inoculation.
4.4 Discussion

Field trials done in 2009 and 2010 are described which showed that natural infection can occur between HarMV, a virus of indigenous plants in south-western Australia, and narrow-leafed lupin, an important component of the cropping system of the region. Because symptoms of HarMV infection of lupins usually resemble those of non-necrotic BYMV (Cheng & Jones, 2000) and may have been mistaken for it, it is possible that HarMV infection has occurred naturally since lupin production began in the region in the early 1970s. HarMV was only characterised in 2007 (Webster et al., 2007) and there is no specific antiserum for it, making high-throughput testing problematic.

In 2009 the lupin response to HarMV infection resembled that of a necrotic strain of BYMV, and the reasons for this are unclear. It may have been because there was less rain in 2009 than 2010 so the plants were under more physiological stress. Three of 92 infected lupin plants in 2010 died probably because of infection with other pathogens or pests. Isolate MU-4 was not one of those used in the previous glasshouse study by Webster et al. (2007). The nucleotide sequence of isolate MU-4 placed it in phylogenetic group V, none of whose members caused a necrotic reaction in narrow-leafed lupin in the glasshouse study (Webster et al., 2007). In the glasshouse based experiment Mandelup infected with HarMV MU-4 also developed non-necrotic symptoms, suggesting that the response of lupins to infection in 2009 was atypical. The stage of infection of the plants may have been a factor. In lupins, infection by BYMV at the seedling stage induces more stunting and loss of seed production failure (Cheng & Jones, 2000).
These authors tested differential responses of two lupin genotypes to BYMV infection and distinguished two subgroups within the necrotic strain, one of which induced systemic necrosis in both lupin genotypes and the other that killed one but induced a non-necrotic response in the other. Here a range of lupin genotypes was challenged against a panel of HarMV isolates and a similar interaction occurred between HarMV and narrow-leafed lupin. Isolate WHP-2 was shown to cause a necrotic reaction in cvs Mandelup and Belara but a non-necrotic response on cvs Tanjil and Tallerack. Isolate WHP-1 and MU-4 induced non-necrotic symptoms to all four lupin genotypes. A study by Wylie et al. (2008) showed that comparisons of CP nucleotide sequences of BYMV did not distinguish between necrotic and non-necrotic strains, indicating the genetic determinant of those phenotypes did not reside in the CP. It appears that this also applies to HarMV.

The experiments here showed that the HarMV isolate which induced necrotic or non-necrotic symptoms on lupin plants had a strong impact on the virus spread pattern and infection rate. In 2009 the infection rate of HarMV was low (4.67%), as expected with a virus that induces rapid death of its host. The infection rate in 2010 was much higher (30.67%). In 2010, some plants infected early remained infective in the field for more than eight weeks, possibly acting as viral reservoirs for further spread. In these experiments, the rate of spread would also have been influenced by several factors including the density of lupin plants, the density of infected H. comptoniana plants, and the number, species, and activity of aphids. In the 2009 trial, plant density of seven plants/m² was lower than the usual agricultural rates in the region of 25-45 plants/m² (O'Connell et al., 2003). Higher incidences of virus infection occur at lower lupin plant
densities, attributed partly to incoming viruliferous vector aphids being more attracted to plants with bare earth around them than to a plant canopy (Jones, 1993). In 2010 the plant density in the trial was closer to that in agricultural fields (50 plants/m\(^2\)). In agricultural fields, *H. comptoniana* plants occur around the margin of the crop, not within it as they did in the 2009 trial, so infection of lupins is expected to occur mainly near the margins of fields. This prediction was studied in 2010 by planting *H. comptoniana* plants around the plots instead of among the plants. It was observed that symptomatic plants first appeared in the margin of each plot and more plants showed common necrotic stem streaking inside the plot later. This was the case with the necrotic strain of BYMV, which is spread mainly from infected clovers in surrounding fields (Cheng & Jones, 2000).

Potyviruses are spread by aphid vectors. The three species of aphids recorded colonising both *H. comptoniana* and lupin plants (*M. persicae, A. kondoi, R. padi*) are all naturalised species originally from the northern hemisphere. This is not surprising because of the around 150 species of Aphididae estimated to be present in Australia, only 13 indigenous species are known (Valenzuela *et al*., 2007). All three species associated with both plant species are known vectors of other potyviruses (Bradley & Ganong, 1955; Castle *et al*., 1992; Keldish *et al*., 1998) and are therefore potentially to be responsible for spreading HarMV. Most infected lupins occurred close to *H. comptoniana* plants so viruliferous aphids may have walked between them. However, winged aphids were present and would be capable of spreading viruses further, as evidenced by the occurrence of BYMV in one plant in 2009 that must have been brought in by a winged adult. It had been recorded that aphid activity had an impact on crop yield loss from
virus infection in the region. In Western Australia rainfall during late summer and early autumn (February-April) is a determinant for aphid epidemics that year by influencing soil moisture and the availability of green plants on which aphids build up before moving into crops (from website http://www.agric.wa.gov.au/PC_90884.html?s=1001). The overall rainfall from February to April in 2009 was 23.2 mm while in 2010 it was substantially higher at 79.4 mm, which resulted in more aphid activity in 2010, and this could be another factor contributing to the higher infection rate in 2010.

As it stands, HarMV does not appear to pose a significant threat to the Western Australian lupin industry because the natural range of *H. comptoniana* covers only a small part of the lupin growing area of the ‘wheatbelt’, that constitutes a narrow band along the coast from Perth to 200 kms north of it, an area less than 10% of the lupin production area. In an agricultural context, infection of lupins by the necrotic strain of HarMV is likely to be restricted to the margins of fields where *H. comptoniana* grows and cause minimal production losses. However, the non-necrotic strain could spread rapidly in lupin fields within the vulnerable region of the ‘wheatbelt’ with much higher infection rates.

It is concluded that HarMV is capable of naturally extending its host range under field conditions to a recently introduced grain legume with the assistance of introduced aphid species as vectors, but it is probable that this does not pose a significant threat to the lupin industry at present. *H. comptoniana*, the major host of HarMV, occurs naturally in only about 10% of the lupin growing area. The virus is potentially not seed-borne so it must be spread from
wild plants each year, and it is unlikely be spread to new parts of the wheatbelt because of
restriction to its wild host’s range. However, HarMV is spread by introduced aphids that may
probe a range of native and introduced plant species, thereby offering the virus opportunities
to extend its host range. Further studies should test other native and introduced legumes for
the virus. A high throughput antiserum-based test would simplify screening for HarMV. *H.
comptoniana*, which is now widely traded around the world as an ornamental plant, and this
too provides opportunities for HarMV to extend its geographical range where it will encounter
a range of potential new hosts, although no studies have been made to determine the extent of
infection of plants internationally. The differential responses to infection of narrow-leafed lupin
by HarMV indicates that this pathosystem represents an ideal opportunity to study evolution
and epidemiology of a virus in real time as it encounters new vectors and hosts at the interface
of an ancient natural ecosystem and a recent agroecosystem.
CHAPTER 5 DETECTION AND QUANTIFICATION OF VIRUSES IN SINGLE OR MIXED INFECTED PLANTS USING REAL-TIME PCR
5.1 Introduction

Real-time PCR is a technique used to detect and quantify template DNA by amplifying PCR products in the presence of a fluorescent reporter molecule (e.g. SYBR Green or a dye-labeled sequence specific probe) in which fluorescence increases as the PCR reaction proceeds. This enables the progress of the sequence-specific reaction to be quantified in real time. Conventional RT-PCR is able to detect specific viruses only, while real-time RT-PCR is used to detect and quantify specific virus nucleic acid sequences, such as in the detection of Tomato mosaic virus (ToMV) in irrigation waters (Boben et al., 2007), TSWV in individual thrips (Boonham et al., 2002) and Maize chlorotic mottle virus (MCMV) in maize seeds (Zhang et al., 2010). Compared with conventional RT-PCR, real-time RT-PCR is timesaving, allowing the detection of viruses during the RT-PCR reaction. Real-time RT-PCR is also highly automated and sensitive that it does not need to do the electrophoresis at the end of reactions. Real-time multiplexed assays have been developed to detect simultaneously two or more viruses in plant tissues (Eun et al., 2000; Mortimer-Jones et al., 2009) using virus sequence-specific probes labeled with fluorescence probe that transmits at different wavelengths. An advantage of this method over conventional diagnostic tests is that it is much more sensitive than ELISA and does not require electrophoretic separation of products as is the case for standard PCR test. This makes real-time PCR suitable for a high-throughput virus diagnostics. By using highly specific fluorescence probes, real-time PCR can be used to distinguish different strains or isolates of a virus and even to detect point mutations (Jacquot et al., 2005; Balme-Sinibaldi et al., 2006; Kogovsek et al., 2008).
Because of its high sensitivity, specificity and ability to detect target nucleic acids in complex backgrounds, real-time PCR has been developed to quantify amounts of plant viruses (Seal & Coates, 1998) rather than, for example, using an immunoassay. When a virus infects its host systemically the virus titre can impact on epidemiology by affecting symptom severity (van den Bosch et al., 2007), transmission rate (Jimenez-Martinez & Bosque-Perez, 2004), the rapidity of onset of death (Magbanua et al., 2000) and yield loss. Virus titre varies according to temperature (Jensen et al., 1985), virus strain (Varma & Malathi, 2003; Jimenez-Martinez & Bosque-Perez, 2004; Seal et al., 2006), presence of inherent resistance genes, and any applied control strategy (e.g. propagation in vitro/vivo, breeding for resistance/tolerance) (van den Bosch et al., 2007).

Narrow-leafed lupin (L. angustifolius) has been cropped in Western Australia since the early 1970s and became an important component of the cropping system mainly for stock feed. Its growth has also had a significant positive impact on yields of grain crops. For example, lupin is planted with wheat in rotation to increase wheat yield by contributing to soil nitrogen. The most economically important virus diseases of lupin plants in Western Australia are caused by BYMV (family Potyviridae, genus Potyvirus) and CMV (family Bromoviridae, genus Cucumovirus). Work in Chapter 4 showed that HarMV is naturally transmitted to lupin plants from H. comptoniana by wild aphids. In a glasshouse, three HarMV isolates were inoculated to four lupin cultivars and both necrotic and non-necrotic responses were recorded. Lupin cv Belara
plants infected with isolate WHP-1 showed symptoms of leaf downcurling, tip leaf necrosis and necrotic stem streaking, while plants of the same cultivar infected with isolate WHP-2 showed stunting, leaf downcurling, necrotic stem streaking and death about 22 days after inoculation (Fig. 4.11 and Table 4.1). A possible reason for the symptom discrepancy was that there were different titres of the two virus isolates within the plants infected. It is generally recognised that higher virus titre often generates more severe symptoms (van den Bosch et al., 2007). In this study, real-time PCR was used to quantify two HarMV isolates (WHP-1 and WHP-2) titres in lupin crop (cv Belara). This study aims to use real-time quantitative PCR to elucidate whether the virus titres contribute to the symptom discrepancy of lupin plants infected with two different HarMV isolates.

Mixed infections of plants with more than one virus strain or virus species are common in both cultivated and wild plants (Hammond et al., 1999; Wylie et al., 2011; Wylie & Jones, 2011c). For example, wild populations of *Nicotiana glauca* were reported to be infected with seven viruses (Dodds, 1993). Vegetatively propagated crops are considered to have a higher risk of infection with multiple viruses (Hammond et al., 1999; Wylie et al., 2011). Mixed infections sometimes do not exhibit obvious changes in symptoms (Martin & Elena, 2009), but in most cases symptoms are either enhanced or moderated in some way (Hammond et al., 1999). Other epidemiological traits affected by the mixed infections include the efficiency of virus transmission (Wintermantel et al., 2008), host resistance (García-Cano et al., 2006) and the titre of individual infecting viruses (Malapi-Nelson et al., 2009; Martin & Elena, 2009). It is therefore
usually not possible to predict the effects of interactions between two co-infecting viruses on symptom severity and virus titre, as these will depend on the different virus species and host. The same co-infection of viruses in different hosts has been shown to induce different symptom modification and viral titres (Kamei et al., 1969; Martin & Elena, 2009). Most species in the family Potyviridae, one of the largest families of viruses, are in the genus Potyvirus. They infect a wide range of plants and cause significant losses in agricultural production. CMV, a virus with a worldwide distribution, has the widest host range of any known plant virus, and infects more than 1,000 plant species in 100 families (experimental and natural host range) (Hobbs et al., 2000). Interactions between potyviruses and CMV in co-infected plants (e.g. ZYMV, Watermelon mosaic virus (WMV) co-infected with CMV in Cucurbit) (Wang et al., 2002) commonly induced synergistic symptoms, and in plant CMV titre increased while the potyvirus titre decreased or remained the same compared with singly infected hosts (Taiwo et al., 2007; Mascia et al., 2010). The CMV 2b gene and potyvirus HC-Pro gene that mediate RNA-silencing suppression are both involved in this interaction (Martin & Elena, 2009; Mascia et al., 2010). The expression of the CMV 2b gene inhibits the production of small interfering RNAs (siRNAs) directed against virus sequences, generated by Dicer-like enzymes, whose expression acts to suppress the host antiviral silencing (Diaz-Pendon et al., 2007). HC-Pro, decreases siRNA stability by methylation (Fukuzawa et al., 2010; Mascia et al., 2010), and is also a suppressor of virus RNA silencing that promotes potyvirus accumulation. In this study, real-time quantitative PCR was used to determine both CMV and HarMV titres in doubly or singly infected N. benthamiana plants. This study is to investigate the interaction between two co-infection virus, HarMV and CMV, in host plant N. benthamiana.
In the study described in this Chapter, HarMV isolates WHP-1 and WHP-2 were quantified in lupin cv Berala leaf tissues to investigate the relationship between symptom severity and virus titre. In its natural host, the symptom severity of HarMV different isolates infection varies for different plants, and even different branches of the same plant infected with the same HarMV isolate show inconsistent symptoms (Fig. 5.1). The leaves of a wild *H. comptoniana* plant infected with HarMV isolate WHP-2 showed different symptoms and was collected. The virus titres in these leaves were quantified using real-time quantitative PCR to determine whether different virus titres contributed to differences in this symptom development.
Figure 5.1 Symptom variation in wild *Hardenbergia comptoniana* plants infected with *Hardenbergia mosaic virus* (HarMV) isolate WHP-2 located on the Murdoch campus (A). The leaves shown in (B) to (F) in this figure were from this single plant; (B) Leaves showed bright yellow spots and blotches, distortion and mosaic at the front compared with asymptomatic leaves behind; (C) Leaves on the right showed severe chlorosis and distortion compared with asymptomatic leaves on the left; (D, E) Leaves showed mosaic and are distorted; (F) Leaves showed yellow spots and mild distortion.
5.2 Methods and Materials

5.2.1 Quantifying necrotic and non-necrotic HarMV isolates in lupin plants

Wild *H. comptoniana* leaves (10-20) infected with HarMV isolates WHP-1 and WHP-2 were collected and inoculated to lupin plants in an insect-proofed glasshouse (temperature 23 °C) as described in Chapter 4 (4.2.3). Lupin cv Belara infected with isolates WHP-1 and WHP-2 were collected from young leaves (one leaf for each) two weeks post inoculation. Total RNA was extracted from the collected leaves individually using an RNeasy Plant Miniprep kit (Qiagen, #74904) and quantified using a Nanodrop UV-Vis spectrophotometer (Nanodrop Technologies). Reverse transcription of total RNA was carried out using a High-Capacity cDNA Transcription Kit (Applied Biosystems, #4368814) (using reverse primer). Two sets of primers for RT-PCR were designed to amplify part of the CP gene (HarMVQ3F 5’ ATGGTGTGGTGCATTGA 3’ and HarMVQ3R 5’ TCAATGTACGCTTCAGCTGC 3’) (annealing temperature 52 °C)/ (HarMVQ4F 5’ AATGCAAAGACAGCAGTGCG 3’ and HarMVQ4R 5’ TCCAAGCCAAACAATCTTAGT 3’) (annealing temperature 55 °C). PCR was done using GoTaq® Flexi DNA Polymerase (Promega, #M8291) to confirm infection and evaluate primer specificity and viability. All other conditions were as described in Chapter 2.

Products of PCR were concentrated using ethanol precipitation and quantified using the Nanodrop UV-Vis spectrophotometer. Amplicons were diluted $1 \times 10^{-3}$, $1 \times 10^{-4}$, $1 \times 10^{-5}$, $1 \times 10^{-6}$,
$1 \times 10^{-7}$ times to make a standard curve. The following formula was used to calculate cDNA molecule number (Fronhoffs et al., 2002):

$$N \text{ (molecules per } \mu l) = \frac{C \text{ (cDNA } \mu g/\mu l)}{K \text{ (fragment size } \text{bp})} \times 182.5 \times 10^{13}$$

Young leaves 16 days post inoculation were collected from lupin cv Belara showing necrotic symptoms with WHP-2 infection and non-necrotic symptoms with WHP-1 infection (Fig. 4.11). Three leaf samples were collected for each isolate from different infected lupin plants and treated independently. Total RNA was extracted and quantified as described above. 5 µg total RNA was used for reverse transcription using primer HarMVQ4R. The concentration of primers was decreased from 200 nM to 50 nM to minimise primer-dimer formation. All the reverse transcription reactions were done using the same thermal cycler in one batch.

Real-time PCR reaction mix (20 µl for each reaction) was prepared using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems, #KK4601):

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>20 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water</td>
<td>8.8 µl</td>
<td></td>
</tr>
<tr>
<td>KAPA SYBR® FAST qPCR Master Mix (2×) Universal</td>
<td>1× 10</td>
<td>10 µl</td>
</tr>
<tr>
<td>Forward primer (10 µM) (HarMVQ4F)</td>
<td>50 nM</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Reverse primer (10 µM) (HarMVQ4R)</td>
<td>50 nM</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>&lt;20 ng/20 µl</td>
<td>1 µl</td>
</tr>
</tbody>
</table>
The thermal cycler used for real-time PCR reaction was Rotor-Gene Q (QIAGEN, # 9001580) with the thermocycling set as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activation</td>
<td>95 °C</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>3 s</td>
<td>40</td>
</tr>
<tr>
<td>Annealing /Extension</td>
<td>55 °C</td>
<td>30 s</td>
<td></td>
</tr>
</tbody>
</table>

Standard dilutions $1 \times 10^{-3}$, $1 \times 10^{-4}$, $1 \times 10^{-5}$, $1 \times 10^{-6}$ and $1 \times 10^{-7}$ of the RT-PCR amplicon (primers used were HarMVQ4F and HarMVQ4R) were run with cDNA samples in the Rotor-Gene Q real time thermal cycler simultaneously, all of which were done in triplicate. Healthy lupin leaves were used as control. The threshold for quantification analysis was set to ‘Auto-Find’.

5.2.2 Quantification of HarMV from wild *H. comptoniana* leaves showing symptom variation

Isolate WHP-2 was collected from a wild *H. comptoniana* plant located on Wireless Hill, Booragoon, Perth City (Fig. 5.1). Four groups (A, B, C, D) of leaf samples (three leaves with similar symptoms and leaf age for each group) with different symptoms from this plant were collected (Fig. 5.2) and extracted for total RNA. Three leaves in each group were extracted for RNA individually and then quantified using a Nanodrop UV-Vis spectrophotometer. RT-PCR was
carried out as described to evaluate the viability of the primers in *H. comptoniana* (using HarMVQ4R for reverse transcription and HarMVQ4F/ HarMVQ4R for PCR).

For real-time RT-PCR, 2 µg total RNA was reverse transcribed using primer HarMVQ4R. All reverse transcription reactions were done using the same thermal cycler in one batch. cDNA standards, reaction mix preparation and cycler setting used were as described before (5.2.1).

![Image of leaves showing symptoms](image)

**Figure 5.2** Symptoms of leaves of *Hardenbergia mosaic virus* (HarMV) isolate WHP-2 infected wild *Hardenbergia comptoniana*. Leaves showed (A) distortion and severe mosaic; (B) severe chlorosis; (C) severe leaf curling and vein clearance; (D) mild mosaic. All the leaves were collected from one infected plant. A, B, C are younger leaves while D are mature leaves (three leaves for each group).

### 5.2.3 Quantification of CMV/HarMV in leaf tissues of single/double infected *N. benthamiana* plant

The leaves (10-20) of *M. truncatula* infected with CMV (Q strain) (subgroup II) obtained from the Department of Agriculture and Food Western Australia were collected and inoculated to
three *N. benthamiana* seedlings. The leaves (10-20) from a wild *H. comptoniana* plant naturally infected with HarMV isolate MU-4 (Chapter 4) on the Murdoch campus were collected and inoculated to three *N. benthamiana* seedlings. Both viruses were also inoculated to three *N. benthamiana* seedlings simultaneously. All the inoculations were done in an insect-proofed glasshouse (temperature 20 °C). Two pots of *N. benthamiana* seedlings were not inoculated for control. The symptoms were recorded 4 weeks post inoculation.

One leaf was collected from each plant and total RNA was extracted. Primers to amplify part of the CMV subgroup II CP gene were designed by Integrated DNA Technologies online software (CMVQF 5’ TTCGCGTCTTAGTGTGCCTATGGA 3’ and CMVQR 5’ ATCTGCTGAGTCAAAGCACGCAAC 3’) (annealing temperature 55 °C). RT-PCRs were done to confirm infection and evaluate primer specificity and viability using primer pairs CMVQF/ CMVQR and HarMVQ4F/ HarMVQ4R. RT-PCR product was concentrated using ethanol precipitation and quantified using a Nanodrop UV-Vis spectrophotometer. The concentrated product was diluted 1×10⁻³, 1×10⁻⁴, 1×10⁻⁵, 1×10⁻⁶, 1×10⁻⁷ times to make the standard stock for CMV real-time PCR assay.

Young and symptomatic leaf samples (one leaf from each plant) from all infected plants were collected 1, 2, 3, 4 weeks post inoculation, and total RNA extracted within 2 hours of harvest. All RNA samples were stored at -80 °C. At week 5, RNA samples were quantified using a Nanodrop UV-Vis spectrophotometer and for each sample 1 µg total RNA was used to do
reverse transcription, using CMVQR or HarMVQ4R. All the reactions were done using the same thermal cycler in one batch. In real-time PCR primer pairs HarMVQ4F/ HarMVQ4R were used to amplify HarMV sequence and CMVQF/ CMVQR were used to amplify CMV sequence. HarMV real-time PCR standards made in 5.2.1 were used as standards. Reaction mix and cycler setting were as described before (5.2.1).
5.3 Results

5.3.1 Quantitation of necrotic and non-necrotic isolates of HarMV in lupin

Necrotic (N) and non-necrotic (NN) symptoms were induced by HarMV isolates WHP-1 and WHP-2 (Fig. 4.11 and Table 4.1). An RT-PCR assay indicated that primers HarMVQ4F/HarMVQ4R amplified the target sequence (92 bp) of both WHP-1 and WHP-2 isolates much more efficiently than primers HarMVQ3F/HarMVQ3R (target sequence 170 bp) (Fig. 5.3). Therefore, primers HarMVQ4F/HarMVQ4R were selected for use in subsequent real-time PCR assays. The amplicons were sequenced to confirm that they originated from HarMV.

Figure 5.3 2% agarose gel electrophoresis of RT-PCR products. Lane M: 100 bp DNA Ladder (Axygen, #M-DNA-100bp); Lane 1: WHP-1 using primer HarMVQ3F/HarMVQ3R; Lane 2: WHP-2 using primer HarMVQ3F/HarMVQ3R; Lane 3: water control using primer HarMVQ3F/HarMVQ3R; Lane 4: WHP-1 using primer HarMVQ4F/HarMVQ4R; Lane 5: WHP-2 using primer HarMVQ4F/HarMVQ4R; Lane 6: water control using primer HarMVQ4F/HarMVQ4R.
PCR products amplified by primers HarMVQ4F/ HarMVQ4R were concentrated and quantified at 248.8ng/µl. The copies of molecules/µl at dilutions $1\times10^{-3}$, $1\times10^{-4}$, $1\times10^{-5}$, $1\times10^{-6}$, $1\times10^{-7}$ were calculated according to the formula in 5.2.1 to be $4.93\times10^9$, $4.93\times10^8$, $4.93\times10^7$, $4.93\times10^6$, $4.93\times10^5$. The standard curve (Fig. 5.4) had a correlation coefficient $R^2 > 0.99$, making it valid for quantification analysis. The healthy control had Ct values $>30$, indicating that samples with Ct values above 30 could be considered negative. The sample concentrations were calculated automatically by Rotor-Gene 6 software (QIAGEN) (Table 5.1 and Fig. 5.5).

**Figure 5.4** (A) Fluorescence data of *Hardenbergia mosaic virus* (HarMV) standards. Healthy control had average Ct value 30.8. (B) Standard curves for 10-fold serial dilution of stock solutions using $4.93\times10^5$ to $4.93\times10^5$ copies of cDNA.
**Table 5.1** Relative virus titres of necrotic and non-necrotic isolates of *Hardenbergia mosaic virus* in lupin plants (cv. Belara)

<table>
<thead>
<tr>
<th>Lupin sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(copies/µl)a</td>
<td>(copies/µl)</td>
<td>(copies/µl)</td>
<td>(copies/µl)</td>
<td>(copies/µl)</td>
</tr>
<tr>
<td>Isolate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHP-1 (non-necrotic)</td>
<td>1.1127×10⁸</td>
<td>1.1611×10⁸</td>
<td>1.1214×10⁸</td>
<td>1.1317(±0.0258)×10⁸</td>
</tr>
<tr>
<td>WHP-2 (necrotic)</td>
<td>1.3856×10⁸</td>
<td>1.3090×10⁸</td>
<td>1.3264×10⁸</td>
<td>1.3403(±0.0402)×10⁸</td>
</tr>
</tbody>
</table>

a The titres listed were average values of three reactions in the real-time PCR assay.

**Figure 5.5** Virus titre of *Hardenbergia mosaic virus* (HarMV) necrotic (WHP-2) and non-necrotic (WHP-1) isolates in lupin plants. Error bars indicate the standard deviation.
The relative within-plant virus titre measurements showed that at 16 days post inoculation the necrotic isolate WHP-2 was present at a titre 18.4% higher than the non-necrotic isolate (WHP-1) (Table 5.1 and Fig. 5.5). This corresponded with the symptom severity in that the non-necrotic strain induced leaf downcurling, tip leaf necrosis and necrotic stem streaking but not death, while the necrotic strain induced leaf downcurling, stunting, necrotic stem streaking and plant death 6 days after sample collection.

### 5.3.2 HarMV WHP-2 quantification in wild *H. comptoniana*

Symptom variation of a wild *H. comptoniana* infected with HarMV WHP-2 isolate is shown in Fig. 5.1 and 5.2. RT-PCR indicated primers HarMVQ4F/ HarMVQ4R amplified the target sequence (92 bp) consistently with 12 samples (Fig. 5.6), demonstrating that they are appropriate to use to measure HarMV infection of *H. comptoniana* using a real-time PCR assay.

The standard real-time PCR assay curves (Fig. 5.7) had a correlation coefficient $R^2 > 0.99$, demonstrating the test is valid for quantification analysis. The samples with Ct values above 28 were considered negative since healthy control showed Ct values >28. Four groups (A, B, C, D) of leaf samples (three leaves with similar symptoms and leaf age for each group) were collected (group A symptoms: distortion and severe mosaic; group B symptoms: severe chlorosis; group C symptoms: severe leaf curling and vein clearance; group D symptoms: mild mosaic). The sample
concentrations were calculated automatically by Rotor-Gene 6 software (QIAGEN) (Table 5.2 and Fig. 5.8).

**Figure 5.6** 1.5% agarose gel electrophoresis of RT-PCR products of *Hardenbergia mosaica virus* (HarMV) infected *Hardenbergia comptoniana*. Lane M: 100 bp DNA Ladder (Axygen, #M-DNA-100 bp); Lane 1 to 12: amplicons of part of HarMV CP gene (length 92 bp); Lane 13: water control using primers HarMVQ4F/ HarMVQ4R.
Figure 5.7 Fluorescence data of *Hardenbergia mosaic virus* (HarMV) standards. Healthy control had average Ct values 28.8. (B) Standard curves for 10-fold serial dilution of stock solutions using \(4.93 \times 10^9\) to \(4.93 \times 10^5\) copies of cDNA.
Table 5.2 Relative virus titre of *Hardenbergia mosaic virus* (HarMV) isolate WHP-2 in a *Hardenbergia comptoniana* plant

<table>
<thead>
<tr>
<th>Sample</th>
<th>1 (copies/µl)(^a)</th>
<th>2 (copies/µl)</th>
<th>3 (copies/µl)</th>
<th>Mean (±SD) (copies/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: distortion and severe mosaic (young leaves)</td>
<td>1.6688×10(^7)</td>
<td>1.8135×10(^7)</td>
<td>1.6898×10(^7)</td>
<td>1.7240(±0.0782)×10(^7)</td>
</tr>
<tr>
<td>B: severe chlorosis (young leaves)</td>
<td>8.8827×10(^6)</td>
<td>9.3083×10(^6)</td>
<td>9.2788×10(^6)</td>
<td>9.1566(±0.2377)×10(^6)</td>
</tr>
<tr>
<td>C: severe leaf curling and vein clearance (young leaves)</td>
<td>1.7130×10(^8)</td>
<td>1.7684×10(^8)</td>
<td>1.7751×10(^8)</td>
<td>1.7522(±0.0341)×10(^8)</td>
</tr>
<tr>
<td>D: mild mosaic (mature leaves)</td>
<td>7.0254×10(^7)</td>
<td>6.5862×10(^7)</td>
<td>6.6088×10(^7)</td>
<td>6.7401(±0.2473)×10(^7)</td>
</tr>
</tbody>
</table>

\(^a\) The titres listed were average values of three reactions in a real-time PCR assay.

\(^b\) Symptoms are shown in Fig. 5.2.
Figure 5.8 Virus titre of *Hardenbergia mosaic virus* (HarMV) isolate WHP-2 in wild *Hardenbergia comptoniana* plant. Symptoms of A leaves: distortion and severe mosaic (young leaves); Symptoms of B leaves: severe chlorosis (young leaves); Symptoms of C leaves: severe leaf curling and vein clearance (young leaves); Symptoms of D leaves: mild mosaic (mature leaves) (Fig. 5.2). All four groups of leaves were collected from different branches of one *Hardenbergia comptoniana* plant. Three biological replicates were used by testing three different leaves for each leaf group. The concentration above the bars showed the mean value of three biological replicates of each leaf group. The variations between three biological replicates were shown in Table 5.2.

The quantification analysis indicated that the wild *H. comptoniana* infected with HarMV WHP-2 not only showed variable symptoms but also harboured different virus titres in different leaves of the plant. Leaves of sample group D (three leaves tested) showed much less severe distortion and mosaic than did leaf group A and B (three leaves tested for each), but contained a 3.9-fold virus greater accumulation of virus than did leaves A, and a 7.4-fold greater virus accumulation compared to leaves B. Leaves C had different symptoms of severe leaf curling and vein clearing, not comparable to mosaic symptoms in terms of severity (Fig. 5.2). They contained the highest
virus titre of the four leaf groups, about 10.2-fold more than in leaves A, 19.1-fold more than in leaves B, and 2.6-fold more than in leaves D. The conclusion is that this wild *H. comptoniana* infected with HarMV WHP-2 showed diverse symptoms and within-plant titres in the leaves, and that virus titre and leaf symptoms were not directly correlated, with the highest titre being more than 19 times that of the lowest.

### 5.3.3 CMV/ HarMV quantification in single/ double infected *N. benthamiana*

CMV/ HarMV mixed infection of *N. benthamiana* plants strongly enhanced some viral symptoms, such as chlorosis, and induced new symptoms that differed from single infection, such as severe yellowing and stunting (Fig. 5.9).

The results of RT-PCR using primers CMVQF/ CMVQR and HarMVQ4F/ HarMVQ4R indicated that all of the nine *N. benthamiana* plants tested were infected with both viruses and these two primer sets were able to amplify efficiently both CMV and HarMV target viral sequences in *N. benthamiana* plants (Fig. 5.10). Concentrated CMV PCR product was quantified using the Nanodrop UV-Vis spectrophotometer to be 83.5 ng/μl. The numbers of molecules in dilutions $1 \times 10^{-3}$, $1 \times 10^{-4}$, $1 \times 10^{-5}$, $1 \times 10^{-6}$, $1 \times 10^{-7}$ were calculated according to the formula in 5.2.1 to be $1.13 \times 10^9$, $1.13 \times 10^8$, $1.13 \times 10^7$, $1.13 \times 10^6$, $1.13 \times 10^5$ (copies/μl).
Figure 5.9 Virus symptoms of *Cucumber mosaic virus* (CMV), *Hardenbergia mosaic virus* (HarMV) and CMV/ HarMV infected *Nicotiana benthamiana* plants compared with a healthy plant. CMV/ HarMV mixed infection showed severe stunting, yellowing, leaf distortion and chlorosis; CMV single infections showed mild mosaic on leaves; some HarMV infection showed mild mosaic, others mild chlorotic spots on leaves.
Figure 5.10 2% agarose gel electrophoresis of RT-PCR products of Cucumber mosaic virus (CMV) and Hardenbergia mosaic virus (HarMV) infected Nicotiana benthamiana plant. Lane M: 100 bp DNA Ladder (Axygen, #M-DNA-100bp); Lane 1 to 3: CMV infected N. benthamiana using primers CMVQF/CMVQR (length 135 bp); Lane 4 to 6: CMV/ HarMV infected N. benthamiana using primers CMVQF/CMVQR (length 135 bp); Lane 7: healthy N. benthamiana using primers CMVQF/CMVQR as negative control; Lane 8 to 10: HarMV infected N. benthamiana using primers HarMVQ4F/HarMVQ4R (length 92 bp); Lane 11 to 13 CMV/ HarMV infected N. benthamiana using primers HarMVQ4F/HarMVQ4R (length 92 bp); Lane 14: healthy N. benthamiana using primers HarMVQ4F/HarMVQ4R as negative control.

The standard curves for both CMV and HarMV real-time assays had a correlation coefficient $R^2 > 0.99$ (Fig. 5.11), making them valid for quantification analysis. For the CMV real-time assay the samples with Ct values above 28 were considered negative since healthy controls showed Ct values $>28$. For the HarMV real-time assay the samples with Ct values above 36 were considered negative since healthy control showed Ct values $>36$. The sample concentrations were calculated automatically by Rotor-Gene 6 software (QIAGEN) (Table 5.3 and Fig. 5.12).

Three biological replicates were applied by testing three N. benthamiana plants infected with CMV, three N. benthamiana plants infected with HarMV and three N. benthamiana plants
infected with both CMV and HarMV (one leaf was tested for each plant). For single infections with CMV, the virus titre reached a peak value of $1.6 \times 10^8$ copies/µl in plants about 2 weeks post inoculation, while the values stayed at $4 \text{ to } 6 \times 10^7$ copies/µl for 1, 3 and 4 weeks post inoculation. When the hosts were co-infected with CMV and HarMV, CMV accumulation increased slightly more in the first two weeks compared with single infection. Two weeks later CMV accumulation increased slightly in the double infection (week 3 to $1.9 \times 10^8$ copies/µl, week 4 to $2.2 \times 10^8$ copies/µl) whereas it decreased in the single infection, suggesting that co-infection with HarMV helped increase and maintain high CMV accumulation levels in *N. benthamiana*. On the other hand, HarMV titre in single infected plants increased consistently each week over all 4 weeks after inoculation from a low level ($4.6 \times 10^5$ copies/µl) in week 1 to about 150-fold higher level ($7.0 \times 10^7$ copies/µl) in week 4. In the plants with mixed infection, HarMV titre was more than 10 times higher than that in single infection in week 1. However, unlike the surge in the single infected host, the HarMV titre showed a rather stable level at $5.48 \times 10^6$ copies/µl to $6.00 \times 10^6$ copies/µl for all 4 weeks, indicating that HarMV accumulation was suppressed in the presence of CMV in the same host. In terms of total viral titres, mixed infection was at $2.2 \times 10^8$ copies/µl made up of HarMV at $5.7 \times 10^6$ copies/µl and CMV at $2.2 \times 10^8$ copies/µl, while for HarMV single infection the virus titre was at $6.9 \times 10^7$ copies/µl and for CMV single infection it was at $4.1 \times 10^7$ copies/µl (week 4) (Table 5.3 and Fig. 5.12).
Figure 5.11 Fluorescence spectra and standard curves of *Cucumber mosaic virus* (CMV) (A) and *Hardenbergia mosaic virus* (HarMV) (B) real-time assays. For CMV assay healthy control had an average Ct value of 36.5 and for HarMV the value was 28.5.
Table 5.3 *Cucumber mosaic virus* (CMV) and *Hardenbergia mosaic virus* (HarMV) with-in plant titres in single and double infected *Nicotiana benthamiana*

<table>
<thead>
<tr>
<th>Infection</th>
<th>Plant</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(copies/µl)³</td>
<td>(copies/µl)³</td>
<td>(copies/µl)³</td>
<td>(copies/µl)³</td>
</tr>
<tr>
<td><strong>CMV</strong> (single infection)</td>
<td>Week 1</td>
<td>5.7669×10⁷</td>
<td>5.8898×10⁷</td>
<td>5.6088×10⁷</td>
<td>5.7552(±0.1407)×10⁷</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>1.6063×10⁸</td>
<td>1.7237×10⁸</td>
<td>1.5725×10⁸</td>
<td>1.6342(±0.0794)×10⁸</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>6.1825×10⁷</td>
<td>5.8378×10⁷</td>
<td>6.0964×10⁷</td>
<td>6.0389(±0.1794)×10⁷</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>4.1361×10⁷</td>
<td>4.1841×10⁷</td>
<td>3.8351×10⁷</td>
<td>4.0518(±0.1892)×10⁷</td>
</tr>
<tr>
<td><strong>HarMV</strong> (single infection)</td>
<td>Week 1</td>
<td>4.8772×10⁵</td>
<td>4.9467×10⁵</td>
<td>4.0070×10⁵</td>
<td>4.6103(±0.5236)×10⁵</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>1.5505×10⁷</td>
<td>1.6914×10⁷</td>
<td>1.4192×10⁷</td>
<td>1.5537(±0.1361)×10⁷</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>3.6369×10⁷</td>
<td>3.9621×10⁷</td>
<td>3.9307×10⁷</td>
<td>3.8432(±0.1794)×10⁷</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>7.4763×10⁷</td>
<td>7.0011×10⁷</td>
<td>6.1984×10⁷</td>
<td>6.8919(±0.6459)×10⁷</td>
</tr>
<tr>
<td><strong>CMV</strong> (double infection)</td>
<td>Week 1</td>
<td>5.9868×10⁷</td>
<td>6.6256×10⁷</td>
<td>6.0445×10⁷</td>
<td>6.2190(±0.3533)×10⁷</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>1.8173×10⁸</td>
<td>1.8656×10⁸</td>
<td>1.9070×10⁸</td>
<td>1.8633(±0.0449)×10⁸</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>1.9867×10⁸</td>
<td>1.9619×10⁸</td>
<td>1.8602×10⁸</td>
<td>1.9363(±0.0670)×10⁸</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>2.2088×10⁸</td>
<td>2.2132×10⁸</td>
<td>2.1152×10⁸</td>
<td>2.1791(±0.0554)×10⁸</td>
</tr>
<tr>
<td><strong>HarMV</strong> (double infection)</td>
<td>Week 1</td>
<td>5.6958×10⁶</td>
<td>6.2203×10⁶</td>
<td>6.0747×10⁶</td>
<td>5.9970(±0.2708)×10⁶</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>5.0700×10⁶</td>
<td>5.5652×10⁶</td>
<td>6.4123×10⁶</td>
<td>5.6825(±0.6788)×10⁶</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>5.6750×10⁶</td>
<td>5.1919×10⁶</td>
<td>5.5658×10⁶</td>
<td>5.4776(±0.2533)×10⁶</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>5.7015×10⁶</td>
<td>5.7756×10⁶</td>
<td>5.5090×10⁶</td>
<td>5.6620(±0.1376)×10⁶</td>
</tr>
</tbody>
</table>

³ The titres listed are average values of three reactions in real-time PCR assay.
Three biological replicates were applied by testing three *N. benthamiana* plants infected with CMV, three *N. benthamiana* plants infected with HarMV and three *N. benthamiana* plants infected with both CMV and HarMV (one leaf was tested for each plant).
The relative virus titres for *Cucumber mosaic virus* (CMV), *Hardenbergia mosaic virus* (HarMV) and CMV/HarMV infected *Nicotiana benthamiana* first 4 weeks after inoculation are shown in Figure 5.12. A: CMV titres in CMV and CMV/HarMV infected *N. benthamiana* plant; B: HarMV titres in HarMV and CMV/HarMV infected *N. benthamiana* plant; C: total virus titres in CMV, HarMV and CMV/HarMV infected *N. benthamiana* plant. Total virus titres in double infection were CMV titre+HarMV titre and total virus titres in single infection were calculated from CMV and HarMV infections respectively. Three biological replicates were applied by testing three *N. benthamiana* plants infected with CMV, three *N. benthamiana* plants infected with HarMV and three *N. benthamiana* plants infected with both CMV and HarMV (one leaf was tested for each plant).
5.4 Discussion

In this study real-time RT-PCR was applied to titrate HarMV (isolates WHP-1, WHP-2 and MU-4) and CMV (subgroup II) in the host *L. angustifolius, H. comptoniana* and *N. benthamiana*. In the quantification of necrotic and non-necrotic HarMV isolates in lupin plants, three leaf samples (one leaf for each) were collected for each isolate from different infected lupin plants and tested independently (three biological replicates). In the quantification of HarMV from wild *H. comptoniana* leaves showing symptom variation, four groups (A, B, C, D) of leaf samples (three leaves with similar symptoms and leaf age for each group) with different symptoms from one plant were tested (three biological replicates). In the quantification of CMV/HarMV in leaf tissues of single/double infected *N. benthamiana* plant, three biological replicates were applied by testing three *N. benthamiana* plants infected with CMV, three *N. benthamiana* plants infected with HarMV and three *N. benthamiana* plants infected with both CMV and HarMV (one leaf was tested for each plant). HarMV necrotic isolate WHP-2 was at a titre 18.4% higher than the non-necrotic isolate (WHP-1). A *H. comptoniana* plant infected with HarMV isolate WHP-2 which exhibited a range of symptoms in different leaves of the same plant was found to harbour inconsistent levels of virus titres in leaves with different symptoms, with the highest concentration more than 19 times that of the lowest. Visible symptoms did not correlate well with virus titres. A comparison of single infection of *N. benthamiana* with CMV and HarMV, and CMV/HarMV double infection not only showed strong synergistic enhancement of symptoms, but also had an impact on the accumulation of both viruses over time. In combination, CMV accumulation was enhanced whilst HarMV was suppressed. The total virus levels of CMV plus
HarMV in double infected *N. benthamiana* plants was 3 to 5.5 times higher that for single infected plants.

Real-time RT-PCR has been used to measure and compare the viral load of RNA viruses and real-time PCR has been used for DNA viruses (Martin & Elena, 2009), based on the copy number of target virus sequence being related to the number of viral genomes in total nucleic acid pool. For the quantification of RNA viruses most research has been done using a one-step RT-quantitative PCR (RT-qPCR) for absolute quantification (Mortimer-Jones et al., 2009). However, since the RT reaction efficiency is highly reproducible when applying the same primer to the same target RNA (Stahlberg et al., 2004). This study used real-time qPCR to quantify the viral cDNA by doing RT using a conventional thermal cycler rather than real-time PCR thermal cycler. T7 RNA polymerase was not required to synthesise RNA standard stocks, the latter were established by serial dilutions of PCR products. However, a drawback of this approach is that it is a relative quantification method, and direct comparison of the the amounts of two viruses must be made with caution because the RT efficiency may vary from 70%-90% with different primers (Stahlberg et al., 2004). With this in mind RT reactions for each experiment were done using the same thermal cycler in one batch.

The differences in virus titre in single and doubly infected *N. benthamiana* plants with HarMV and CMV (Fig. 5.12) were not only influenced by the other virus infection, but also by the stage
of infection. For example, at week 1 and week 2 CMV accumulations in the single infection and double infection were almost at the same level (week 1 single infection at $5.76 \times 10^7$ and double infection at $6.22 \times 10^7$; week 2 single infection at $1.63 \times 10^8$ and double infection at $1.86 \times 10^8$). However, at week 3 CMV accumulation in single infection decreased more than 50% to $6.04 \times 10^7$ while in double infection the CMV accumulation slightly increased to $1.94 \times 10^8$, which was more than three times of the CMV accumulation in single infection. Thus the measurement of virus titre in plant hosts over a period of time provided more information on virus-host and virus-virus interactions than quantifying virus at a single infection time point.

The lupin cv Belara infected with HarMV isolate WHP-2 (necrotic isolate) was shown to harbour slightly higher mean virus titres than when infected with WHP-1 (non-necrotic isolate) 16 days post inoculation, but this result does not strongly support the hypothesis that this virus induced more severe symptoms because of the higher virus accumulation. Instead it is likely that a gene-for-gene systemic hypersensitive reaction occurred. Such cases have been reported for lupin plants, such as the single dominant hypersensitivity gene Nbm-1 that controls the systemic necrotic response for *L. angustifolius* to infection with BYMV-N (Jones & Smith, 2005). A presumption was that the systemic necrosis-induced virus concentration would increase to a peak in the host and start to decrease after a certain stage. This phenomenon was observed in many other viruses and their hosts (Mas & Pallas, 1996; Kathiria *et al.*, 2010). Therefore, experimental bias was introduced when the virus was titrated only to day 16. Lupin plants infected with the necrotic isolate died at day 22, before which the virus titre could have been
much higher than for non-necrotic isolate. In future studies, it is recommended that the virus titre be measured at 5, 10, 15, 20 days post-inoculation.

Virus titre has been shown to influence transmission rates by vectors. Studies suggest a positive correlation between the virus titer in the source leaf and the rate of transmission (Froissart et al., 2010). For example, the transmission efficiency of BYDV in wheat by *R. padi* was greater when the virus was present at a higher titre (Jimenez-Martinez & Bosque-Perez, 2004). Another study showed that the comparison between *Maize stripe virus* (MStV) US isolate and the CR isolate suggested that CR isolate of MStV multiply faster and reach higher levels in, and are transmitted more efficiently by *Peregrinus maidis* (Ammar et al., 1995). Study of virus accumulation of HarMV in *H. comptoniana* not only helped understand the interaction between the virus and its wild host but also provided a perspective of assessing risk of the virus transmitted to new introduced crops.

A wild *H. comptoniana* plant infected with HarMV WHP-2 showed variable symptoms and harboured different virus titres in different parts of the plant. Leaf age could influence different titres. Leaves A and B were old while leaves C were new growing young leaves and leaves D were typical mature leaves (Fig. 5.2). The trend found here was that leaves C (severe leaf curling and vein clearance) had the highest virus titre while leaves A (distortion and severe mosaic), B (severe chlorosis) and D (mild mosaic) were lower. Severe mosaic was usually
observed on aged leaves but these harboured lower levels of virus. On the other hand, young leaves mostly exhibited mild symptoms, but the virus was present at a much higher titre. Others have found similar trends in which young leaves were more susceptible to infection and also contained more virus particles (Fargette et al., 1987). Since young shoots were more attractive to aphids and had a higher virus accumulation level, actively growing H. comptoniana plants probably act as the main reservoir or source of spread of HarMV. The high titre of virus particles in the young shoots could be a strategy for virus to spread to other plants. In addition, actively dividing cells in young leaves tend to contain more virus particles because viruses rely on cell multiplication machinery to reproduce. In the glasshouse experiment, the HarMV titre was high (on average 1.2×10^8 copies/µl) in lupin plants compared with H. comptoniana (on average 6.7×10^7 copies/µl), which suggested that this virus, present in new host, may replicate to a higher level.

The interactions between CMV and potyviruses, such as enhanced virus titre and synergism in mixed infections, have been mainly attributed to silencing suppressor activities (HC-Pro for potyviruses and 2b for CMV) (Wege & Siegmund, 2007; Wang et al., 2009; Fukuzawa et al., 2010). Both HC-Pro and 2b have been found to bind dsRNA to inhibit DCL (Dicer-like) processing. Methylation of short interfering RNAs (siRNAs) by HC-Pro helps decrease their stability (Fukuzawa et al., 2010), whereas the product of CMV gene 2b can inhibit the activity of the Argonaute 1 protein complex to inhibit cleavage activity (Fukuzawa et al., 2010). In N. benthamiana infected with CMV compared with doubly infected with CMV and HarMV, CMV
titres were higher from week 1 to week 2; at week 1 the double infection titre was 7.8% higher and by week 2 it was 14.7% higher. However, CMV single infection titre was reduced in weeks 3 and 4 possibly because of host silencing while in the double infection CMV titre slightly increased until it was 3.2-fold higher than in single infected plants in week 3, and 5.4-fold more by week 4. A possibility was that CMV gene 2b worked as a suppressor of gene silencing and so helped increase virus accumulation in the early stage of infection (week 1 and 2), while HC-Pro was more active at later stages (weeks 3 and 4). In HarMV single and doubly infected plants, CMV presence enhanced HarMV accumulation at the early stage (week 1) (12.9-fold higher in double infection). HaMV titre strongly increased in weeks 2, 3 and 4, possibly because HC-Pro helped suppress host gene silencing. However, in the HarMV double infection the titres were very stable and did not show an expected increase, which could involve virus-virus competition in the mixed infection (Martin & Elena, 2009). Synergistic enhancement of CMV/ HarMV double infection in the *N. benthamiana* plant was shown in this study. Further experiments might focus on investigating CMV/ HarMV double infection in lupin plants. This would be important because CMV may be considered as the most important virus disease for the lupin industry in Western Australia, and HarMV is a native virus in the region found only recently to be transmitted to lupins. The concern here is that CMV could multiply to a much higher titre in co-infected crops, thereby increasing the vector transmission rate and yield loss in the region (Yates *et al.*, 1993). In addition, CMV is a seed-borne virus. If the coinfection of CMV and HarMV can increase CMV titre level in lupins it may enhance seed transmission of CMV. Further research should be done to find out whether increasing CMV virus titre could induce a higher rate of seed transmission in lupins.
CHAPTER 6 GENERAL DISCUSSION
6.1 Overview of major outcomes

In this study, peptide mass fingerprinting (PMF), MS/MS protein sequencing, RT-PCR/nucleotide sequencing, ELISA, real-time qPCR, and bioinformatics of next generation sequencing data were used to detect, identify and characterise viruses infecting native and introduced plants in Western Australia. PMF coupled with one dimensional electrophoresis was used to detect unexpected plant viruses by their CPs and NGS was powerful in novel viruses discovery and characterisation by full genome sequencing. Two exotic viruses, *Pelargonium zonate spot virus* (PZSV) and *Cycas necrotic stunt virus* (CNSV), were found for the first time in Australia and both were infecting new host plant species. Transmission of plant viruses to new hosts is likely result in host specialisation (mutation and recombination), regarded to be main power of virus evolution. International trade of crops and other plant products makes the quarantine of exotic plant viruses a biosecurity concern. Surveillance of virus transmission from their native hosts to crops, especially in some areas where agriculture was introduced only recently, was important to protection both on crops production and native ecology. The presumed indigenous potyvirus *Hardenbergia mosaic virus* (HarMV) was transmitted from its natural host *H. comptoniana* to an introduced legume, *L. angustifolius*, under field conditions via introduced aphid species. Interactions between viruses and plants were studied and characterised in single- or doubly- infected plants.

In detail, the major tasks undertaken and results gained were:
Four protocols were used to extract proteins from infected and uninfected plant leaves, one of which was particularly useful for distinguishing viral coat proteins (CP) from plant proteins. This method, originally developed for extracting proteins from cotyledons, was modified to extract proteins from mature leaves.

Viral coat proteins were visualised and isolated after one dimensional gel electrophoresis.

Test protein (BSA) was used in experiments to optimize the method by which protein was digested in solution by trypsin and the peptide masses determined by MS in a prOTOF 2000 MALDI O-TOF mass spectrometer (PerkinElmer). The matrix and all the parameters were optimised for PMF analysis. Calibration was essential before acquiring data.

Two in-gel digestion protocols, one modified from an in-gel digestion kit and the other modified from published protocols, were used to identify proteins of interest. Whilst the protocol based on a commercial kit was more rapid and generated less “noise” in spectra, the cost of the other protocol was much less.

Four known viruses were used to test the MS based diagnostic procedure: Zucchini yellow mosaic virus (ZYMV), Turnip mosaic virus (TuMV), Alfalfa mosaic virus (AMV) and Cucumber mosaic virus (CMV). All four viruses were identified correctly, and this result confirms application of the assay. An unexpected virus found in an invasive coastal dune weed Cakile maritima was identified as PZSV, and this is the first report of its presence in Australia and in this host species.

MS/MS protein sequencing of PZSV CP peptides confirmed its identification.
• The intact CPs of four viruses were characterised using the Voyager-DE™ PRO Workstation to accurately measure their total masses. This approach was undertaken to explore the possibility of identifying these viruses by detecting their intact CPs.

• A pilot field study in 2009 showed that HarMV was naturally transmitted from its native host *H. comptoniana* to *L. angustifolius* by naturally occurring aphids. A larger trial with control plots in 2010 gave a higher infection rate and possibly a polycyclic spread pattern. Control plants growing within insect-proof cages did not become infected with the virus. Seeds from infected plants were free of HarMV, indicating that the virus may not be seed-borne in *L. angustifolius*.

• In a glasshouse-based study using manual inoculation, both necrotic and non-necrotic symptoms were recorded when three HarMV isolates were inoculated to four lupin cvs. WHP-2 induced systemic necrosis on lupin cvs Belara and Mandelup and non-necrotic response on cvs Tallerack and Tanjil, resembling the interaction between BYMV isolates to lupin cvs.

• Three species of aphids: *M. persicae* (green peach aphid), *A. kondoi* (bluegreen aphid) and *R. padi* (oat aphid) were collected in the field trial and were identified as possible vectors of HarMV transmission.

• Real-time qPCR was used to measure HarMV isolates WHP-1, WHP-2 and MU-4, and CMV (Q strain) (subgroup II) in *L. angustifolius, H. comptoniana* and *N. benthamiana* plants. Isolate WHP-2 occured at 18% higher concentration than WHP-1 in lupin cv Belara. Virus titres differed in different *H. comptoniana* leaves showing a range of symptoms and the highest was more than 19-fold greater than the lowest. In doubly
infected *N. benthamiana* plants the overall virus titre increased to a higher level than in singly infected plants. Four weeks after inoculation with CMV/ HarMV, CMV accumulation was enhanced while HarMV accumulation was suppressed.

- A new strain of CNSV was identified after analysis of NGS data. The partial genome of RNA1 and RNA2 was obtained. Virus was found in vegetatively propagated materials, the bulb tissue of infected lily plants (*L. longiflorum*). This is the first report of this virus in Australia and in the host *L. longiflorum*.

The overall aim of detecting and characterising plant viruses present in Western Australian native flora and introduced plants, using a range of techniques, was achieved. The conclusion that identification and characterisation of a virus, especially an unknown virus, usually requires use of several methods, was supported.

### 6.2 Techniques to identify and characterise plant viruses

In plant virology, it is generally necessary to characterise new viruses using several different techniques. ELISA is one of the most common virus identification tools, and it is favoured where high-throughput assays of well characterised viruses are required. Although ELISA is about 1000 times less sensitive than PCR (Boben *et al.*, 2007), it is a rapid, economical and reliable method to identify wide range of viruses. In the lupin field trial, leaves from control plots were collected in batches of ten to test for the presence of potyviruses by ELISA using potyvirus antibody. In
several batches, leaves were collected from one positive plant and nine negative plants. It was found that ELISA could be used to detect batches containing one positive sample in a total of ten combined samples. The availability of a potyvirus group-specific antibody facilitated identifying HarMV. In the past, potyvirus-positive ELISA tests of lupins were considered to be solely the result of infection by BYMV, one of the most important virus diseases of the lupin crop. However, the field trial showed convincingly that another potyvirus, HarMV, can also infect lupin plants, and the generic potyvirus antibody cannot be used to distinguish between them. Symptoms of HarMV infection of lupin usually resemble those of non-necrotic BYMV (Cheng & Jones, 2000) and in the past could well have been mistaken for it. In such a case, antibodies specific for both species of virus are desirable for virus diagnosis in lupin crops in Western Australia. The development of specific antiserum for HarMV would be very useful for future surveillance of HarMV spread in native flora and crops in Western Australia. Another solution would be screen potyviruses using the universal antibody and also run a BYMV ELISA alongside; any potyvirus positives that are BYMV negatives could be tested by PCR for HarMV.

PCR or RT-PCR is used to detect viruses in most laboratories worldwide because of its sensitivity, specificity and rapidity. In addition, sequencing of the PCR amplicon provides the basis for many phylogenetic studies on the relatedness and origins of plant viruses, including those on HarMV (e.g. Webster et al., 2007; Wylie & Jones, 2011). In this study, RT-PCR/sequencing was used to confirm the identity of PZSV and also to identify HarMV isolates infecting L. angustifolius and H. comptoniana. For both the ELISA and RT-PCR/sequencing approaches previous knowledge of
virus sequence or access to virus particles is required to design specific primers or to raise specific antisera, respectively. This is clearly a major limitation when novel or unexpected viruses are encountered.

One-dimensional electrophoresis coupled with trypsin digestion and MS analysis to identify proteins was developed to address the need for a strategy to assay and detect plant viruses in an unbiased and generic manner. This strategy was developed successfully to identify PZSV, a virus which was not expected to be found in Western Australia or in the host species in which it was found. Blouin et al. (2010) have compiled a useful overview of plant virus CP sizes for 19 families and the genera in them (Fig. 6.1). All the viruses identified in this study had coat protein sizes within the expected mass ranges listed in this Table (ZYMV about 33 kDa/ AMV about 23 kDa/ TuMV about 31 kDa/ CMV 25 kDa/ PZSV 25 kDa). This overview is a useful guide for the expected sizes of virus CP which can then be confirmed by subsequent MS analysis. However, the major limitation of the approach found in this study was that not all the hosts tested contained a suitably high viral titre to be visualised on a protein gel under the protein extraction conditions used. Furthermore, for confirmation of PMF of novel viruses protein sequencing may well be required. As a result, the general approach is not high-throughput or simple to do, but it is a significant complementary method that can be used to identify viruses when other routine techniques have failed.
Moving from protein-based to nucleic acid-based detection, NGS is even more generic in nature and does not require any information on any virus that is present in plant tissues. Therefore NGS can be used as a universal technique for identification and characterisation of new viruses, at least for RNA viruses. The full genome of a virus can often be generated using NGS, and depending on the platform used, may also provide information on virus accumulation. The sequence information obtained can then be used to design primers for subsequent routine amplification-based assays.
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**6.3 Infection rate and spread pattern of HarMV in field trials**

In the initial field test in 2009 and the full experiment in 2010, HarMV was transmitted from its native host under field conditions to *L. angustifolius*, probably by the introduced aphid species found on the plants. However, the crop infection rate (4.7% in 2009 and 30.7% in 2010) and virus spread pattern (monocyclic in 2009 and probably polycyclic in 2010) were different in the two years.
One reason for different HarMV incidences was that the symptoms of lupin plants induced by HarMV differed in these two trials. In the pilot experiment in 2009 lupin plants were killed about three weeks after the necrotic stem streaking symptom became visible. In the following year infected lupin plants showed tip leaf necrosis, stunting and necrotic stem streaking but usually did not die, allowing infected lupin plants to act as foci of infection to other lupins. In the initial study in 2009, L. angustifolius plants infected with HarMV died after expressing symptoms of systemic necrosis of the N strain of BYMV. In the 2010 field experiment and the glasshouse experiment, L. angustifolius (cv. Mandelup) infected with HarMV (isolate MU-4) were seldom killed. The reason for these different symptoms is not obvious. One possibility is that there were quantitative or qualitative differences which could have influenced the level of inoculum pressure and in this way modified the symptoms. In P. vulgaris, low temperatures (25 °C) protected plants against mechanical inoculation of potyviruses but at higher temperatures (above 33 °C) necrotic lesions and veinal necrosis developed on inoculated leaves, followed by systemic veinal necrosis and plant death (Fisher & Kyle, 1994). In contrast, high ambient temperatures at the field site do not appear to be a causative factor for the observed differences in symptoms in L. angustifolius, although individual days with high temperatures could have been one factor. In 2009, when all infected plants died, the climatic conditions for the July- November period were cooler (mean 20.7 °C) than they were in 2010 (mean 22.4 °C) (Bureau of Meteorology, Australian Government). It is possible that there is a genetic basis for the observed differences in symptoms in 2009 and 2010, although the experiment was designed to minimise this potential. Viruses exist in genetically heterogeneous populations within a host and the dominance of particular genotypes may fluctuate over time (Elena et al.,
A powerful driver of plant virus evolution is infection of a new host (Wylie et al., 2008; Wylie & Jones, 2011a). It is possible, therefore, that during the process of movement from *H. comptoniana* plants to *L. angustifolius* plants over two growing seasons, subtly distinct virus genotypes became dominant in the new lupin hosts and this could underlie the differential host response. The samples may be the first of an establishment of a tissue set that determines genetic changes of HarMV in lupin plants over time. Indeed, the observation that a few lupin plants died after infection in the 2010 season supports the hypothesis that different viral genotypes were being transferred between species. Although amplicons representing viral CPs were sequenced in both years and were identical, other viral genes that have a more direct influence on host response were not sequenced. It is also possible that an undetected virus(es) was present and caused symptoms. For instance, a novel flexivirus, *Hardenbergia virus A*, has since been described co-infecting *H. comptoniana* plants from the region (Wylie & Jones, 2011c), although its infectivity in lupins has not been tested. Furthermore, in the glasshouse real-time quantitative PCR showed that HarMV titre was at a high level (>1×10^8 copies/µl) in lupin plants compared with *H. comptoniana*, which could increase transmission rate as well. Therefore, removing plants with viral symptoms represents one of the best disease management strategies by reducing the chance of secondary virus spread.

Another possible reason for variable incidence of this virus disease was the activity of aphids in the two years. Although in both trials all three aphid species were observed, in 2010 the aphids, especially green peach aphids, were in greater numbers. In areas with a Mediterranean climate,
like Western Australia, survival of aphid populations over the hot, dry season (November to February) is a critical factor that influences aphid outbreaks and virus epidemics in subsequent crops that year. In Western Australia, rainfall during late summer and early autumn (February-April) is a determinant for high aphid numbers that year by increasing soil moisture and the availability of green plants on which aphids build up before moving into crops (from website http://www.agric.wa.gov.au/PC_90884.html?s=1001). The overall rainfall from February to April in 2009 was 23.2 mm while in 2010 it was substantially greater at 79.4 mm, corresponding to the observation that there were more aphids present in 2010, which possibly contributed to the higher incidence of virus disease. Some aphids migrate to Western Australia on the wind from other places in early autumn (Thackray et al., 2004). This indicates that monitoring climate data and aphid activity can further assist in decisions on aphid control strategies for prevention and management of aphid vectored virus diseases in crops both before and during the growing season.

6.4 Future research

The following points address possible future research following on from this study:

- The one-dimensional electrophoresis/MS strategy to identify plant viruses can be optimised further to detect double or multiple infections using protocols optimised to extract a wide range of viruses that exist in plants at low titre.
• The use of protein separation by one-dimensional electrophoresis coupled with PMF can be tested further to investigate plant responses to virus invasion by identifying differentially expressed plant proteins, or detect other virus protein (e.g. CI protein of potyviruses).

• Develop a high throughput assay for HarMV infection to facilitate surveillance of virus incidence - one approach would be to produce HarMV specific antiserum for ELISA testing, and another one would be real-time RT-PCR. ELISA or RT-PCR based methods should be applied to survey and quantify the potential incidence of HarMV as a pathogen in lupin crops.

• There should be further testing for the presence of HarMV in other introduced economically important legumes, such as in chickpea, faba bean, peanut and pasture legumes (clovers, medics and vetches, etc).

• There is a similar need to characterise and survey for the presence of PZSV in the Perth area, and to assess the risk of this virus infecting crops in other regions. This might also invoke using commercially available antibody/ ELISA methods.

• There is a need to study CNSV epidemiology in Australia, including host range, distribution and economic importance.

• NGS has evolved as a very powerful new method of obtaining full genomic sequence data in a cost effective manner, and this will undoubtedly reveal the presence of many new virus species, both in native and crop plants.
Domesticated crops were cultivated at their origins with their wild relatives during the early time of agriculture. In modern times, world trade in plant products has expanded the original geographical range of crops and their pathogens. New encounters of plants and viruses present a significant threat for both agricultural production and ecological diversity. An aggressive virus that a crop has not previously encountered before could result in a new damaging disease. In addition, native flora could be destroyed by new introduction of a virus to a new area, systematically threatening local biodiversity. Discovery of novel and unexpected viruses in the native flora and introduced crops all over the world and further characterise host-virus new encounters (such as symptoms and production lost caused by new encounters, or virus seed-borne rate in new hosts).
Appendix 1 Peptide mass fingerprinting (PMF) analysis of differential expression plant protein

<table>
<thead>
<tr>
<th>Band number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Identity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Measured peptide masses (Da)</th>
<th>Predicted masses (Da)</th>
<th>Coverage (%)</th>
<th>Expectation value&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>endo-1,3-beta-glucosidase</td>
<td>990.547, 1202.648, 1471.781, 2638.364, 2831.318</td>
<td>990.549, 1202.644, 1471.778, 2638.355, 2831.303</td>
<td>24%</td>
<td>1.07741e-002</td>
</tr>
<tr>
<td>11</td>
<td>endo-1,3-beta-glucosidase</td>
<td>990.555, 1202.651, 1471.787, 2638.371, 2831.325</td>
<td>990.549, 1202.644, 1471.778, 2638.355, 2831.303</td>
<td>24%</td>
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<tr>
<td>12</td>
<td>oxygen-evolving enhancer protein 1</td>
<td>963.588, 1235.660, 1292.646, 1759.890, 2269.098, 2293.143</td>
<td>963.579, 1235.650, 1292.640, 1759.873, 2269.099, 2293.122</td>
<td>21%</td>
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<tr>
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<td>ribulose 1,5-bisphosphate carboxylase (small subunit)</td>
<td>774.439, 1020.523, 1464.745, 2395.159</td>
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<td>990.549, 1202.644, 1471.778, 1890.024, 2301.109, 2340.141, 2638.355, 2813.270, 2831.303, 2984.458, 3583.809</td>
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<td>17</td>
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<td>1518.728, 2030.887, 2248.096, 2305.061</td>
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<td>30%</td>
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<sup>a</sup> Bands numbered in 3.3.4

<sup>b</sup> GenBank accession codes of plant protein that most closely matched peptide mass fingerprints of the proteins tested: P23535.1 (No.9), P23535.1 (No.11), P14226.1 (No.12), CAC04296.1 (No.13), P23535.1 (No.16), AAB03844.1 (No.17).
c Measured peptide masses after calibration.

d All the searching parameters used were general parameters. According to default setting in ProFound, expectation value < 1e-003 is highly confident identification while 1e-003 < expectation value < 1e-001 is marginal match. Expectation value > 1e-001 is considered to be failed search.

**Appendix 2 Peptide mass fingerprinting (PMF) peaks of unidentified protein**

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<th>Peaks&lt;sup&gt;b&lt;/sup&gt; (Da)</th>
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<td>691.9754 2266.1655 1261.7016 693.9766 1278.6428 1845.9903 2850.4500</td>
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<td>3542.7611 3125.5067 3109.5165 3441.6819 3526.7441</td>
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<td>524.1444 1929.0676 3472.5719 3832.9770 506.1342 1895.9617 550.1236</td>
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<td>650.0434 1809.9198 1830.8343 1872.9925 522.1288 2159.0985 2584.3142</td>
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<td>2780.4295 3528.7657 3514.7258 3091.4713 2788.9495 3807.8459</td>
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<sup>a</sup> Bands numbered in 3.3.3 and 3.3.4.

<sup>b</sup> Measured peptide masses after calibration.
REFERENCES


Bradley, R. H. E., & Ganong, R. Y. (1955). Evidence that potato virus Y is carried near the tip of the stylets of the aphid vector *Myzus persicae* (s.l.). *Canadian Journal of Microbiology, 1*, 775-782.


