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Authors: H. Luo, S.J. Wylie, M.G.K. Jones

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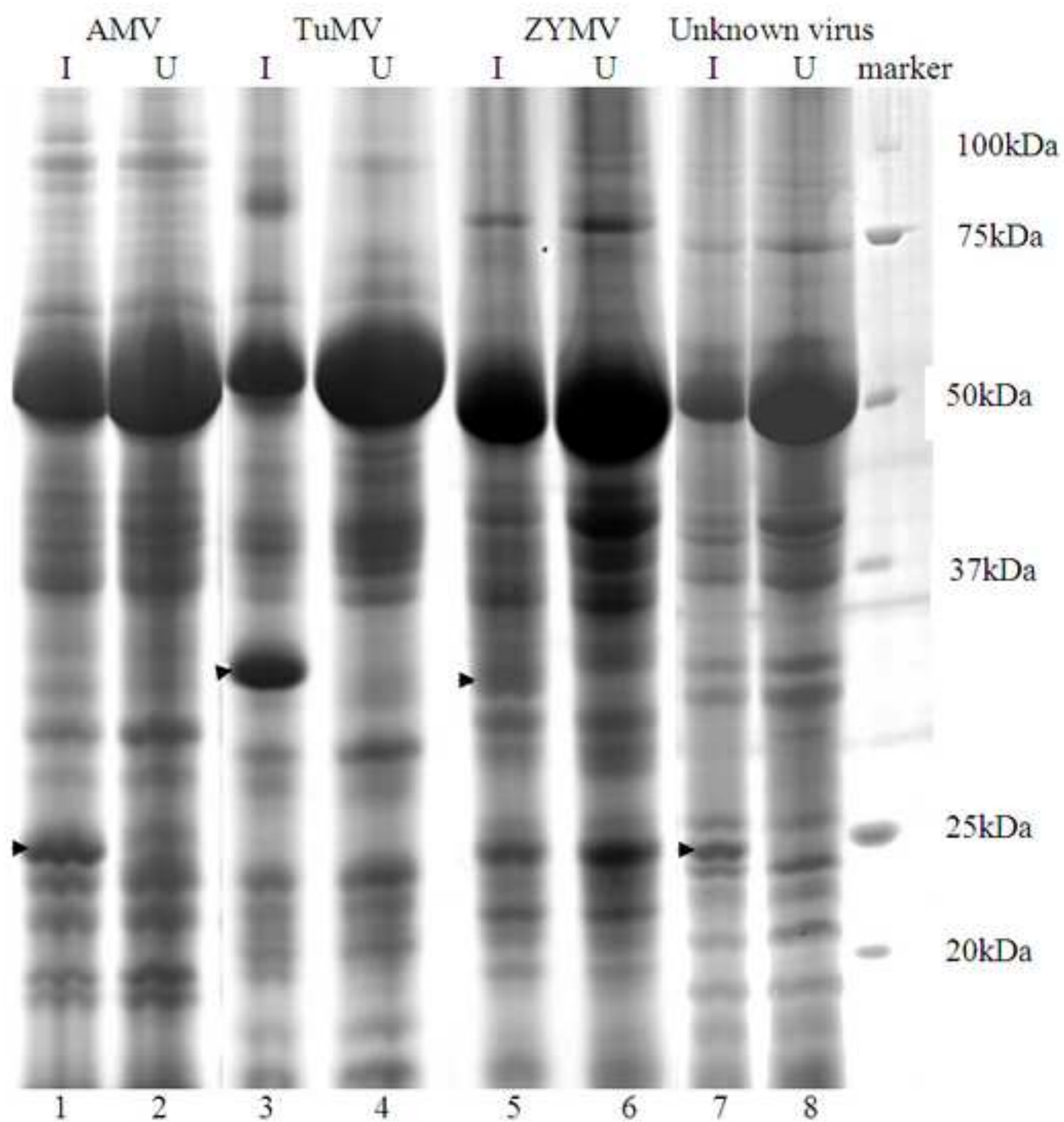

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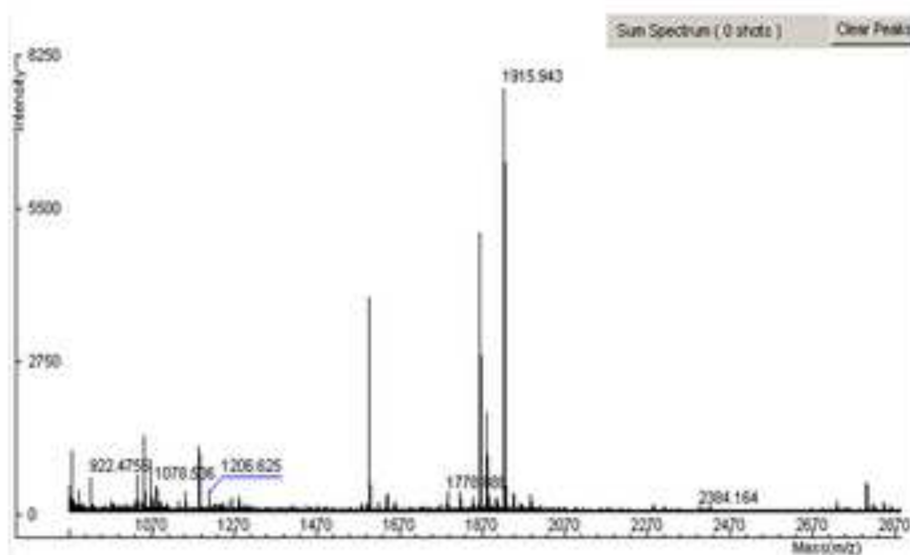

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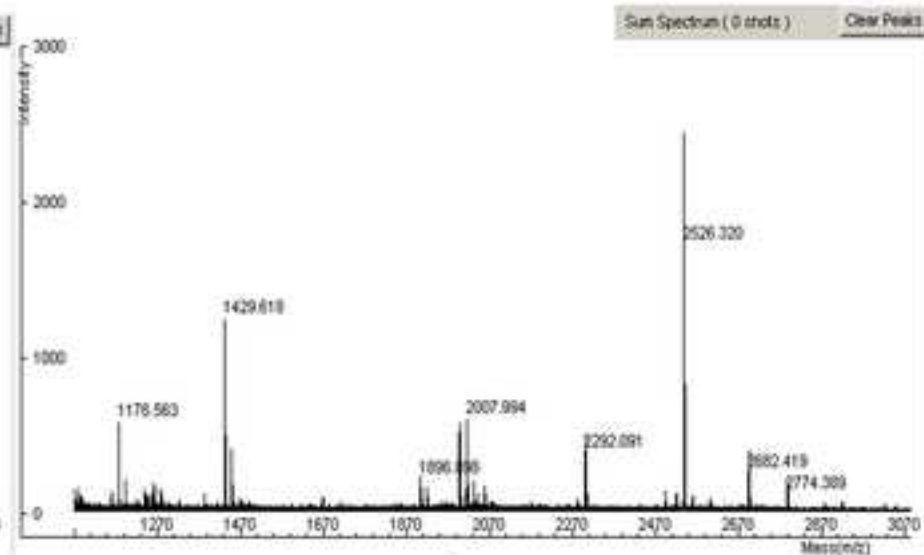

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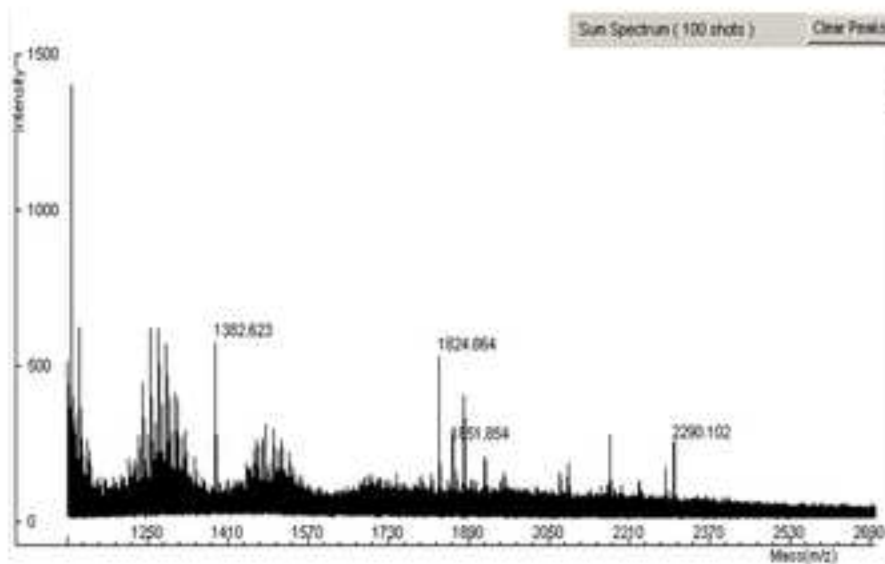

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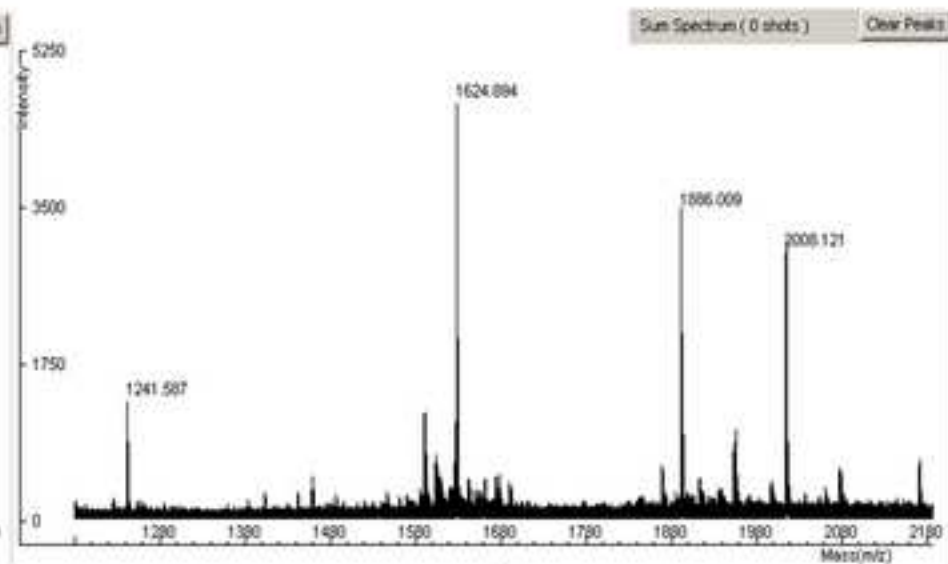

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Identification of plant viruses using one-dimensional gel electrophoresis and peptide mass fingerprints

H. Luo, S. J. Wylie, M.G.K. Jones

Plant Biotechnology Research Group, Western Australian State Agricultural Biotechnology Centre, School of Biological Sciences and Biotechnology, Murdoch University, Perth, WA 6150, Australia

Abstract A generic assay for detection and partial characterization of unknown viruses from plants was developed. Proteins extracted from virus-infected and uninfected plants were separated in one dimension by SDS polyacrylamide gel electrophoresis. Differentially-expressed protein bands were eluted after trypsin digestion and resulting peptide fragments separated according to their mass by matrix-assisted laser-desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry. Resulting peptide mass fingerprints (PMF) were compared with those in protein databases. The assay was used to identify three known viruses: the potyviruses *Zucchini yellow mosaic virus* and *Turnip mosaic virus*, and an alfamovirus *Alfalfa mosaic virus*. It was also used to identify a virus that manifested symptoms in wild *Cakile maritima* plants, tentatively identified as *Pelargonium zonate spot virus* (PZSV) (genus *Anulavirus*) by its PMF, and then confirmed by nucleotide sequencing. The detection of PZSV constitutes a first record of this virus in Australia and in this host. It is proposed that this rapid and simple assay is a useful approach for analysis of plant samples known to harbor viruses that could not be identified using antisera or nucleic acid-based assays.

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Keywords: virus detection, diagnosis, MALDI-TOF, *Pelargonium zonate spot virus*, coat protein

Corresponding author at: WA State Agricultural Biotechnology Centre, Murdoch University, Perth, Western Australia 6150, Australia

Tel: +61893606600; Fax: +61893606303

E-mail address: s.wylie@murdoch.edu.au (Steve Wylie)

Plant viruses are widespread in plants grown as agricultural crops and in native ecosystems, and many cause damaging diseases. Correct identification of viruses when they are recorded is often needed for their control, because identification often helps with predicting the likely mode of spread. The assays used most widely to detect plant viruses are based on antiserum affinity and nucleic acid hybridization, amplification and sequencing. For both, some previous knowledge of the virus is required and this can be a drawback to identifying unknown or unexpected viruses. For antisera-based techniques such as enzyme-linked immunosorbent assay (ELISA) and tissue blot immunoassay (TBIA) a wide range of species-specific antibodies are commercially available, as well as some antisera that are group-specific (Kiratiya-Angul and Gibbs, 1992; Thiea et al., 2002). The most widely used nucleic acid-based assay for virus identification is enzymatic amplification of fragments of the virus genome through the polymerase chain reaction (PCR) (preceded by reverse transcription in the case of RNA viruses), often followed by nucleotide sequencing. Microarrays are used less commonly because of inherent sensitivity and reliability issues (eg 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 (eg Gibbs and 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 mass spectrometry (MS) (Michael et al., 2009). Traditional diagnostic methods of symptom evaluation, vector specificity, host range studies, and electron microscopy to visualize 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 diagnosis process (Cooper et al., 2003).

MS-based protein profiling has been applied to diagnosing various plant pathogens including nematodes (Perera et al., 2005), aphids (Perera et al., 2005), and viruses (eg Blouin et al., 2009). A possible output of MS analysis of virus proteins includes the total mass and resolution of amino acid sequences using tandem mass spectrometry (MS/MS) (Padliya and Cooper, 2006). She et al. (2001) determined the complete amino acid sequence of the coat protein (CP) of *Brome mosaic virus* (BMV) using MS/MS and distinguished four isolates of the virus. The major limitation of this approach is the amount of purified target peptide needed, which may require chromatographic purification. Peptide mass fingerprint (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, 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. Cooper et al. (2003) identified *Tobacco mosaic virus* and *Potato virus X* using two-dimensional SDS PAGE to compare proteomes of infected and

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uninfected plant tissues. Differential spots were excised, digested by trypsin, analyzed by MALDI-TOF MS and PMFs generated. 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 high-resolution electrospray MS/MS to determine PMFs. Using this approach, they identified four common and two novel viruses.

In this study, we simplify MS-based virus diagnosis assays described previously by eliminating complicated and time-consuming centrifugation steps to purify virus particles, by using one-dimensional SDS polyacrylamide gel electrophoresis (PAGE) to detect viral proteins, and by using a single MS step to generate PMFs. These modifications reduced significantly the time and cost of virus diagnosis. This assay was tested on three known viruses from two families and used to identify a fourth, unknown, virus. The assay accurately identified each virus to the species level. The PMF assay described here provides a generic approach for detection of plant viruses.

Virus indicator hosts and cultivated plants were grown in insect-proof glasshouses. For sap inoculations, infected leaf tissue was ground in 0.05M potassium phosphate buffer (pH 7.2) with a mortar and pestle and the sap mixed with the diatomaceous earth ‘Celite’ before being rubbed onto leaves. A culture of *Zucchini yellow mosaic virus* (ZYMV) was maintained in zucchini (*Cucurbito pepo*), *Turnip mosaic virus* (TuMV) was maintained in mustard (*Brassica juncea*), and *Alfalfa mosaic virus* (AMV) was maintained in alfalfa (*Medicago sativa*). A *Cakile maritima* (coastal rocket) plant showing faint yellow mottles on the leaves was collected at the high tide mark at the beach at Woodman Point Recreation Reserve, Cockburn Sound, Western Australia.

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Nicotiana glutinosa was mechanically inoculated with ground leaves and after twenty days the plant showed symptoms of stunting and the leaves had a chlorotic mosaic, indicating the presence of a virus(es).

The method used for total protein extraction was modified from that described by Jacobs et al. (2001). Two to three grams of leaves were ground in liquid nitrogen to a fine powder and transferred to a 50 ml polyallomer centrifuge tube (#363420, BeckmanCoulter). To the ground leaf material was added 20% (w/v) Trichloroacetic acid (TCA) (#91228, Sigma) and 0.2% Dithiothreitol (DTT) (#D9779, Sigma) dissolved in 10 ml cold acetone. This mixture was vortexed and incubated overnight at -20 °C, then centrifuged for 25 000 xg at 4 °C, 20 min. The pellet was washed three times in 10 ml cold acetone containing 0.2% DTT, dried at 4 °C, and the pellet resuspended in fresh rehydration buffer (Urea, 4.2 g; Thiourea 1.52 g; CHAPS, 0.4 g; Tris, 0.049 g; DTT, 100 mg; H_2O to 10 ml). The sample was vortexed and maintained on an orbital shaker overnight at room temperature then centrifuged 25 000 xg at 20 °C, 60 min. Supernatant was collected and transferred to a 1.5 ml centrifuge tube. One-dimensional SDS PAGE was done in a vertical gel apparatus (Bio-Rad Protean™ II) using a 5% acrylamide stacking gel above a 12% acrylamide separating gel at 75 V, 18 h. Protein bands were visualized after staining with Coomassie brilliant blue R-250 (#27816, Sigma) and destained in 200 ml 25% methanol/65% water/10% acetic acid 1 h and repeated twice more with fresh staining solution. In-gel digestion was done for 5 h using a Trypsin Profile IGD Kit (#PP0100, Sigma) according to the manufacturer's instructions.

PMFs were generated on a prOTOF™ 2000 MALDI orthogonal time of flight Mass Spectrometer (PerkinElmer). Data was acquired with 100 laser shots, laser energy 75%, laser rate 100.0 Hz, declustering 30.0 V, and cooling N_2 gas flow 190.0 ml/m. Calibration of the

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output was done with a MALDI Calibration Kit (#6771000, PerkinElmer).

α-Cyano-4-hydroxycinnamic acid (CHCA) (#C2020, Sigma) was applied as matrix at a ratio of 9:1 CHCA/peptides. Analyses of peptide spectra were with ProFound (Zhang and Chait, 2000). Total RNA extraction from infected *N. glutinosa* leaves was done using an RNeasy Plant Mini Kit (#74904, Qiagen) as described in the manufacturer's protocol. cDNA synthesis was done using a High-Capacity cDNA Reverse Transcription Kit (#4374966, Applied Biosystems) with random hexamer primers, and PCR was with GoTaq® Flexi DNA Polymerase (#M8295, Promega) using PZSV-specific primers R3-F 5' CTCACCAACTGAATGCTCTGGAC 3' and R3-R 5' TGGATGCGTCTTTCCGAACC 3' (Liu and Sears, 2007) that anneal within the putative movement protein of the genome. Cycle conditions were as follows: one step of denaturation at 94 ^{o}C for 2 min, followed by 30 cycles of 94 ^{o}C 10 s, 54 ^{o}C 30 s, and 72 ^{o}C for 30 s. Both strands of the resulting amplicon were purified using a MinElute PCR kit (#28004, Qiagen) and sequenced directly with the primers used in their amplification. Automated sequencing was done using an Applied Biosystems Industries/Hitachi 3730 DNA Analyzer using BigDye terminator V3.1 chemistry (Applied Biosystems Industries). The virus sequence was assigned an accession number by GenBank (**GU046705**). The nucleotide sequence was compared with those in GenBank using BlastN (National Centre for Biotechnology Information, Bethesda, MD).

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Protein components were separated from infected and uninfected host plants and compared by one-dimensional SDS PAGE electrophoresis within the same gel. Protein bands expressed differentially in infected plants were identified. For example, putative viral CPs of approximately 26 kDa (AMV), 33 kDa (TuMV), and 31 kDa (ZYMV) were visualized (Fig. 1,

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lanes 1, 3, 5, respectively), excised, and digested with trypsin.

The resulting PMFs generated (Fig. 2 a, b, c) were used to search NCBInr and Swiss-Prot databases for proteins sharing identity using ProFound search parameters: taxonomic category, all taxa; protein mass, 20.0-50.0 kDa; protein pI, 0.0-14.0; missed cleavages, 1; enzyme, trypsin; tolerance average, 30 ppm, mono, 30 ppm; charge state, MH^{+}. Matching peptide masses were recorded and the identities of the three control viruses were confirmed with high confidence and coverage values (Table 1), thereby confirming the validity of the testing procedure. The procedure was then used to analyse *N. glutinosa* plants infected with an unknown virus originally isolated from a *C. maritima* plant. A protein band of approximately 23 kDa from an infected plant (Fig. 1, lane 7) was not present in an uninfected plant, and a PMF was obtained from it (Fig 2 d). To analyse the PMF of the unknown virus, search parameters used in ProFound were as above with the exceptions of: protein mass, 20.0-30.0 kDa; missed cleavages, 3. The closest match to known viruses was *Pelargonium zonate spot virus* (PZSV), but the predicted identity had a low expectation value and coverage (11%) making it a marginal match. To confirm the identity, oligonucleotide primers designed for PZSV (Liu and Sears, 2007) was used to amplify and sequence part of RNA3 of the genome. The nucleotide sequence was analyzed using BlastN and the sequence was confirmed as part of PZSV with 93-95% identity to other published PZSV RNA3 sequences (Finetti-Sialer and Gallitelli, 2003). This virus had not been recorded previously in Australia or infecting *C. maritima*.

A simplified assay of identifying viruses infecting 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. Three known viruses, ZYMV, TuMV and AMV

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were identified correctly, confirming application of the system. An unknown virus infecting an invasive coastal dune weed *C. maritima* (Rodman, 1986) was identified as PZSV, a first report of its presence in Australia and in this host species.

This assay successfully identified the viruses tested but it may need further optimization 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, so that viral proteins of approximately 50 kDa would be masked by it. According to predicted CP masses of plant viruses (Blouin et al., 2009), most virus CPs have masses lower than 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. Figure 1 shows that plant proteins such as Rubisco were present in lower amounts in infected plants compared to uninfected control plants. There are two possible explanations for this: i. Virus infection lowers expression 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 possible that both explanations are correct to a greater or lesser extent.

The main advantage this assay has over those published previously is rapidity, requiring less than two full days to complete. In this assay the virus was identified from an extract of total plant proteins, and it does not require lengthy or complicated purification of virus particles from host proteins (eg Wang et al., 2007; Blouin et al., 2009). It does not require two-dimensional

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separation by SDS PAGE (eg Cooper et al., 2003) and it utilises a rapid in-gel trypsin digestion procedure. Further, single MS analysis rather than tandem MS (eg Blouin et al., 2009) 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. 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. Using this approach, we identified successfully four plant viruses to the species level from two families and three genera with distinct CP sizes and particle morphology (filamentous and bacilliform). We believe that this improved and simplified assay will facilitate wider use of PMF for plant virus identification, and possibly for other plant pathogens.

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Fig. 1. SDS-PAGE of protein extracts of host plants infected with a virus isolate and uninfected host plants. Lane 1, *Medicago sativa* infected with *Alfalfa mosaic virus* (AMV), Lane 2, uninfected *M. sativa*; Lane 3, uninfected *Brassica juncea*; Lane 4, *B. juncea* infected with *Turnip mosaic virus* (TuMV); Lane 5, uninfected *Cucurbito pepo*; Lane 6, *C. pepo* infected with *Zucchini yellow mosaic virus* (ZYMV)*;* Lane 7, uninfected *Nicotiana glutinosa*; Lane 8, *N. glutinosa* infected with an unknown virus. CP bands are indicated by arrows.

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Fig. 2. Spectra of viral coat protein peptides. a: *Alfalfa mosaic virus* coat protein; six peptides corresponding to the masses of AMV coat protein fragments. b: *Turnip mosaic virus* coat protein; eight peptides corresponding to the masses of TuMV coat protein fragments. c: *Zucchini yellow mosaic virus* coat protein; four peptides corresponding to the masses of ZYMV coat protein fragments. d: unknown virus coat protein; four peptides corresponding to the masses of *Pelargonium zonate spot virus* (PZSV) coat protein fragments. Masses shown are pre-calibration.

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Table 1 Peptide mass data of three known viruses (ZYMV, TuMV and AMV) and an unknown virus.

Virus Tested	Measured Peptide Masses (Da)[a]	Predicted Masses (Da)[b]	Predicted Identity[c]	Coat protein mass (kDa)[d]	Coverage (%)	Expectation value
AMV	921.468	921.466	AMV	26	24	7.09133e-005
	1077.528	1077.524				
	1205.617	1205.619				
	1777.880	1777.873				
	1914.936	1914.936				
	2383.156	2383.141				
TuMV	1175.555	1175.550	TuMV	33	36	2.83567e-007
	1428.610	1428.601				
	1895.890	1895.887				
	2006.986	2006.988				
	2291.083	2291.077				
	2525.313	2525.311				
	2681.411	2681.412				
	2773.381	2773.373				
ZYMV	1381.615	1381.619	ZYMV	31	12	1.70971e-007
	1823.856	1823.853				
	1850.846	1850.847				
	2289.094	2289.098				
Unknown	1240.576	1240.575	PZSV	23	11	1.78929e-004
	1623.886	1623.882				
	1885.004	1884.998				
	2007.113	2007.110				

[a] Measured mass after calibration.
[b] Predicted mass calculated from amino acid sequences.
[c] 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**.
[d] Coat protein masses predicted from amino acid sequences.

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