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Identification of two plant viruses using partial purification and mass spectrometry

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Abstract Serological and PCR techniques failed to detect Tomato spotted wilt virus (TSWV) infecting stunted tomato plants exhibiting leaf curling, purpling and crumpling. In contrast, Mass Spectrometry rapidly identified peptides generated from a ~28kD protein cut from PAGE gels as TSWV N-protein within 5 days. The results indicate that the incidence of TSWV may be underestimated in the field. *Cocksfoot mild mosaic virus* (CMMV) was identified infecting *Bromus diandrus* using serological techniques. Peptides from a ~25kD protein failed to identify with any protein on GenBank until a CMMV sequence was published. The use of Mass Spectrometry as an adjunct for virus detection and identification is briefly discussed.

Keywords Virus identification · Mass spectrometry · Cocksfoot mild mosaic virus · Tomato spotted · Wilt virus

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Virus detection and identification have evolved steadily over the last 100 years. Early techniques, including mechanical and insect transmission tests, do not rely on prior knowledge of the virus concerned (Kado and Agrawal 1972; Wilson 2014). Electron microscopy does not rely on prior knowledge of the virus and generally provides information on the size, shape and some surface features of virus particles (Hull 2002; Wilson 2014). In contrast serology and PCR based techniques rely on prior knowledge of which viruses are likely to be present in a sample and may require many rounds of diagnosis before a successful identification (Cooper et al. 2003; Zheng et al. 2011). More recently Next Generation Sequencing has revolutionised the identification of viruses and other organisms (Wilson 2014). Another technique which does not rely on prior knowledge is Mass Spectrometry (MS). Increasing numbers of viruses have been identified and interactions with host proteomes have been characterised (Zheng et al. 2011). MS has been used to study a few plant viruses (Cooper et al. 2003; Seifers et al. 2005; Luo et al. 2010) but this technique is under-utilised (Blouin et al. 2010). In this paper we examine the utility of using MS to identify two plant viruses.

Tomato plants (cv Sub Arctic Plenty) showing leaf curling, crumpling, purpling and stunting (but not yellowing) and a wild grass (*Bromus diandrus*) showing mosaic symptoms were collected from a farm at Waitati, East Otago NZ. The lack of virus particles in leaf dip preparations suggested that a low titre virus was present in the tomato samples. The presence of isometric particles without distinct subunit structure suggested the presence of a bromo-, sobemo- or tombus-virus in the grass samples.

Tomato samples were tested for 15 viruses (Table 1) by ELISA, using kits (Agdia, Adgen, Loewe) in accordance with

Table 1 Tomato sample ELISA

Antisera	Source (Catalog no.)	Viruses detected according to the manufacturer	Results: all negative for the tomato sample
Alfalfa mosaic virus	Agdia (SRA 87601)	AMV	
Arabis mosaic virus	Agdia (SRA 23201)	ArMV	
Begomoviruses	Adgen (1073)	Tomato and others	
Cucumber mosaic virus	Agdia (SRA 44501)	CMV	
Potyviruses	Agdia (SRA 27200)	many	
Strawberry latent ringspot virus	Agdia (SRA 14000)	SLRV	
Tobacco ringspot virus	Agdia (SRA 64000)	TRSV	
Tomato bushy stunt virus	Agdia (SRA 454000)	TBSV	
Tomato ringspot virus	Agdia (SRA 22000)	ToRSV	
Tospoviruses			
Tomato spotted wilt &Impatiens necrotic spot viruses	Agdia (SRA 30400)	TSWV & INSV	
Tospo Broad Range	Loewe (07507)	TSWV, TCSV, GRSV, INSV	
Iris yellow spot virus	Loewe (07508)	IYSV	

the manufacturers' instructions. The tomato samples tested negative for all these viruses. Two isolates of *Tomato spotted wilt virus* (TSWV) from dahlia were used as positive controls. The Alfonso (Dahlia cultivar) isolate caused chlorotic ringspot and oakleaf patterns in infected leaves. The Pam Howden (Dahlia cultivar) isolate caused a diffuse chlorotic mottle. Both dahlia isolates tested positive in TSWV ELISA tests.

The brome grass samples were tested for 8 viruses by ELISA and 20 viruses using Ouchterlony double diffusion tests done in sodium acetate (50 mM sodium acetate, 0.15 M sodium chloride, 0.2 % sodium azide, 0.75 % agarose; pH6) gels using antisera diluted 1/10 to viruses known to infect the Poaceae and to a selection of sobemoviruses (Table 2). The grass samples tested positive for *Cocksfoot mild mosaic virus* (CMMV), CMMV-Phleum mottle and *Panicum mosaic virus* (PMV).

RNA was purified from tomato and dahlia leaf tissue. Samples (ca. 3–20 mg) of leaves were homogenized with 200 μl STE buffer (0.1 M NaCl, 10 mM Tris, pH 8.0, 1 mM ETDA, pH 8.0) with 500 μl of Trizol (Sigma) in a Beadbeater™. After 5 min incubation at room temperature, 100 μL of chloroform was added to the homogenates and, after vigorous shaking, the homogenates were centrifuged at 14,000 g for 10 min. A half volume (ca. 250 μL) of isopropanol was added to the aqueous phase to precipitate RNA from the supernatant. Samples were incubated at room temperature for 10 min. After centrifuging at 14,000 g for 8 min the supernatants were discarded and the pellets were washed twice in 75 % ethanol. Air dried pellets were resuspended in DEPC-treated water. Each sample was processed separately; sterilized barrier pipette tips, freshly autoclaved solutions and DEPC treated water (negative controls) were used for each procedure. Total RNA (1-5uL) was used to produce cDNA and amplified via PCR using the Superscript III One-Step RT-PCR System (Invitrogen) (50 °C for 30 min, 94 °C for 2 min then 35 cycles: 94 °C 30 s, 54 °C 35 s, 72 °C 1 min; 72 °C 2 min). A two-step RT-PCR was also used. Reverse transcription was done using AMV and RNAsin (Promega) and reverse primer (Table 3) for 55 min in a thermal cycler at 42 °C. Following the RT reaction PCR was done using Platinum Taq (94 °C for 1 min then 30 cycles: 94 °C 1 min, 55 °C 1 min, 72 °C 1 min; 72 °C 10 min). The PCR was then repeated using the same cDNA and primers (Table 3) at a lower annealing temperature (94 °C for 5 min then 35 cycles: 94 °C 30 s, 48 °C 40 s, 72 °C 40 s; 72 °C 5 min). Freeze-dried tomato leaf material was also sent to Agdia Inc USA for RT-PCR analyses.

None of the tomato samples tested positive for any of the viruses in house or at the Agdia testing service. The dahlia isolates were used as positive controls in house and an unspecified isolate was used as a positive control at the Agdia testing service.

Leaf material ground in liquid nitrogen was combined with 0.1 M potassium phosphate containing 1 % 2-mercaptoethanol and 0.75 % sodium sulfite (*w*/*v*) (tomato) or 0.5 M acetate buffer (Brome grass) and emulsified with 1/10 volume of chloroform. The aqueous phase was made to 0.2 M sodium chloride and polyethylene glycol 6000 was added (6 %*w*/*v*). The precipitate was collected by low speed

Table 2 Brome grass serology

Antisera	Source (Catalog no.)	Viruses detected[a]	Result[b]
Agropyron mosaic virus	R. Plumb, UK	AgMV	
Brome mosaic virus	Agdia (SRA 29300)	BMV	
BMV-Julius	R. Sward, Australia	BMV	
BMV-Rhdolf	R. Sward, Australia	BMV	
Brome streak mosaic virus	Loewe ((07123)	BStMV	
Cocksfoot streak virus	L Torrance, Scotland	CSV	
Cocksfoot mild mosaic virus	W. Huth, Germany	CMMV	++
CMMV-Phleum mottle	W. Huth, Germany	CMMV	++
Cocksfoot mottle virus	W. Huth, Germany	CoMV	
Foxtail mosaic virus	B. Falk, USA	FMV	
Ginger chlorotic fleck virus	J. Thomas, Australia	GCFV	
Lucerne transient streak virus	R. Forster, NZ	LTSV, CoMV	
Maize chlorotic mottle virus	B. Falk, USA	MCMV	
Maize stripe virus	Agdia (SRA 17300)	MSpV	
Maize white line virus	B. Falk, USA	MWLV	
Panicum mosaic virus	B. Falk, USA	PMV, CMMV	+
Potyviruses	Agdia (SRA 27200)	many	
Rice yellow mottle virus	American Type Culture Collection (ATTC 516)	RYMV	
Rice necrosis mosaic virus	R. Plumb, UK	RNMV	
Ryegrass mosaic virus	D. Webster, NZ	RGMV	
Solanum nodiflorum mottle virus	R. Greber, Australia	SNMV	
Soilborne wheat mosaic virus	Agdia (SRA 42001)	SBWMV	
Southern bean mosaic virus	American Type Culture Collection (ATTC 298, 300)	SBMV	
Sugarcane bacciliform virus	Agdia (SRA 72200)	SCBV	
Subclover mottle virus	R. Jones, Australia	SCMV	
Velvet tobacco mottle virus	J. Randles, Australia	VTMoV	
Wheat dwarf virus	Loewe (07082)	WDV	
Wheat streak mosaic virus	Agdia (SRA 47001)	WSMV	

[a] according to the source, [b] blank cells represent no reaction to brome grass extracts: ++ distinct precipitin line in double diffusion tests, + less distinct line in double diffusion tests

centrifugation (5,000 g) after stirring for 3 h at 4 °C. Pellets were resuspended in buffer and after low speed centrifugation (5,000 g) were re-pelleted in a Sorvall Ultracentrifuge (100, 000 g) for 2 h. The final high speed pellets (and other fractions) were resuspended in distilled water and Laemli loading buffer and analysed using BioRad minigel polyacrylamide gel

Table 3 Primers used for tomato sample RT-PCR

Target virus	Primers (Reverse primer)	Expected size	Reference
Tomato spotted wilt virus	TCTGGTAGCATTCAACTTCAA (GTTTCACTGTAATGTTCCATAG)	628 bp	Roberts et al. 2000
	TGTAITGITCCATIGCA (AGAGCAATIGTGTCA)	871 bp	Mumford et al. 1996
	GTGTCATACTTCTTTGGGTC (GGGAGAGCAATYGWGKYR)	709 bp	Kuwabara et al. 2010

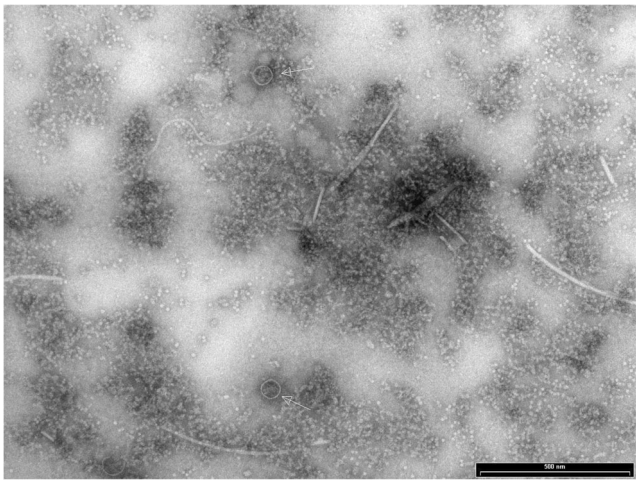

Fig. 1 TEM image of purified tomato extract: arrows indicate *Tomato spotted wilt virus* particles

electrophoresis (PAGE) on 10–20 % polyacrylamide gradient gels. Bands were cut from Coomassie R250 stained/destained gels for analysis by mass spectrometry (MS) at the University of Otago, Centre for Protein Research.

A prominent ~28kD band (tomato) and a ~25kD band (brome grass: only band on gel) were cut from gels for analysis by mass spectrometry (MS). Samples of high speed pellets resuspended in distilled water were mixed with phosphotungstate and examined in a Philips transmission electron microscope. The tomato samples contained damaged membrane bound particles ~75 nm in diameter associated with nucleoprotein resembling tospovirus particle preparations (Fig. 1) and the grass samples contained numerous ~30 nm particles (Fig. 2) mentioned above.

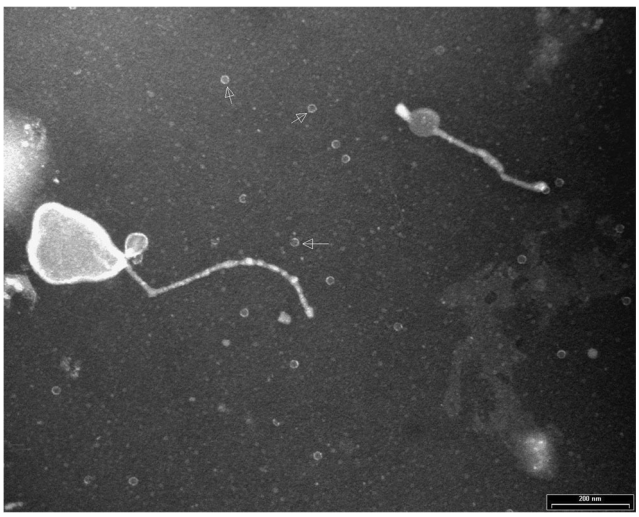

Fig. 2 TEM image of purified brome grass extract: arrows indicate *Cocksfoot mild mosaic virus* particles

The proteins of interest were digested in-gel using protease (Table 4) and the resulting peptides were subjected to MALDI tandem Time-of-Flight (TOF/TOF) MS. Fragment ion spectra were searched against the NCBI nr database using the Mascot search engine (www.matrixscience.com). For the grass sample, which was not identified by Mascot, a de novo sequencing mass spectrometry approach (Chen et al. 2004) was applied. Following tandem mass spectrometry peptide ions were fragmented sequentially using laser desorption and each individual amino acid was identified by its mass to charge ratio. These data were interpreted manually and assembled into peptide sequences (Table 4). The assembled peptides were searched against the NCBI database.

Partial purification followed by PAGE and MS rapidly identified a ~28kD band as the Mr=28.8kD N-protein of TSWV. The nine identified peptides (Table 1) covered 48 % of the N-protein target sequence. Many contaminating peptides from photosynthetic proteins, mostly Rubisco, were also identified (data not shown). The successful detection and identification of TSWV and other tospoviruses is often time consuming and involves multiple techniques (Mumford et al. 1996; Dietzgen et al. 2005). This current study shows that MS is a useful tool for detecting and identifying TSWV.

Initial MS data on a ~25kD band from the grass sample PAGE produced no matches with the NCBI nr databases and sequence tags derived from de novo sequencing. Following the publication of the CMMV sequence (Ziegler et al. 2009) the ~25kD band was identified as the 26.5kD coat protein of CMMV. The five identified peptides (Table 1) covered 20 % of the target sequence. Seven of the 56 amino acids in the 5 peptides were mismatched with the target sequence indicating variation between the Scottish and NZ isolates of CMMV.

Considerable time and effort is required to identify plant viruses using serological and PCR techniques (Wilson 2014). Each candidate virus has to be tested individually by buying in antibodies and primers and ordering more antibodies and primers when other possible diagnoses become apparent. Extensive testing of the tomato samples failed to detect TSWV. It is uncertain which of the TSWV structural proteins react with the antibodies in the Agdia and Loewe kits. The RT-PCRs were designed to amplify segments on the N-protein gene but the in-house assays and the Agdia testing service failed to detect the

Table 4 Summary of peptide sequences detected by mass spectrometry

Virus targeted	Digest	Peptides identified	Published seq. length	Coverage
TSWV gi 535099 (and 17 other TSWV N-protein sequences)	Trypsin	ESIVALLTQGK DLEFEEDQNLVAFNFK TFCLENLDQIKK MSVISCLTFLK IGATDMTFR LVEETGNSENLNTIK IASHPLIQAY GLPLDDAK AFEMNEDQVKK GSIAMEHYSETLNKFYEMFGVK	258	48 %
CMMV NC 011108	Papain+Trypsin Trypsin	RPLAYgQaVGP TQPEIILR LHSQHeWR VVLKFFPNYR LEDLMDtSsLTfSPYEkHT	245	20 % (lower case letter codes were mismatches with the published amino acid sequence)

tomato isolate of TSWV but succeeded in detecting two Dahlia isolates in-house and an unknown isolate at Agdia Inc. PCR assays vary in their robustness (Wilson 2014) and unexpected sequence variation is a major limitation for PCR based techniques (Zheng et al. 2011). For example, Osman et al. (2007) found using multiple isolates of six Grapevine leafroll associated viruses that individual primer pairs detected 54–89 % of their collected isolates. Osman et al. (2007) used nine pairs of primers in their RT-PCR study. The relatively simple partial purification, PAGE electrophoresis followed by MS identified TSWV within 5 days. A similar effort was mounted to test the grass samples serologically. CMMV was more readily purified than TSWV however, initially, the identified peptides did not match any sequence on GenBank. After the publication of a CMMV sequence, the peptides were identified as part of the CMMV coat protein. The speed and utility of using MS to identify plant viruses is limited by the number of virus species' sequences lodged with databases. As modern shotgun sequencing techniques become more accessable and cheaper these databases become more comprehensive and MS will become an increasingly useful adjunct in virus identification. Mass Spectrometry analysis has the advantage that it analyses proteins, rather than nucleic acids, and therefore does not rely on the analysis of both DNA and RNA to be complete.

The tomato plants did not display the bronzing pattern on leaves typically associated with TSWV infection. Chamberlain (1954) noted that occasionally TSWV infected tomatoes show the symptoms outlined for the Waitati samples. The fact that the tomato isolate remained undetected by ELISA and RT-PCR indicates that the incidence of this virus may be under estimated in the field.

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