Red clover vein mosaic virus—A Novel Virus to New Zealand that is Widespread in Legumes

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Abstract

Fletcher, J., Tang, J., Blouin, A., Ward, L., MacDiarmid, R., and Ziebell, H. 2016. *Red clover vein mosaic virus*—A novel virus to New Zealand that is widespread in legumes. *Plant Dis.* 100:890-895.

Red clover vein mosaic virus (RCVMV) is an important virus of leguminous crops that can cause devastating losses. During a routine survey of legumes conducted on the South Island of New Zealand, RCVMV was found in mixed infections in clover plants with *Alfalfa mosaic virus* and *White clover mosaic virus*. The full-length sequence of the New Zealand isolate RCVMV-NZ

from clover shared 96% nucleotide sequence identity with a chickpea isolate previously described from Washington (United States). Targeted surveys of pea, faba bean, and pasture crops showed that RCVMV-NZ is widespread on the South Island in New Zealand. This isolate is causing mild if any symptoms on experimental hosts and naturally infected plants.

Legumes and pasture plants are important agricultural crops for New Zealand, providing food for human consumption and fodder for cattle and sheep. Recent surveys have shown the widespread occurrence of different plant viruses affecting these crops (Guy 2014; Guy et al. 2013). More than 23 virus species have been found to have infected pasture grasses and legumes such as white, red, subterranean, and Caucasian clover and lucernes as well as other legumes (e.g., pea, *Phaseolus* bean, lentil, and broad bean) in New Zealand on both the South and North Islands (Denny and Guy 2009; Fletcher 1993; Guy 2014; Pearson et al. 2006). In the Canterbury region (South Island, the primary target of this report) pea, faba bean, and dwarf bean crops cover approximately 7,300 ha. Clover seed production covers 5,240 ha whereas lentil production is insignificant and chickpea is not grown. A further 1,136,000 ha are covered by mixed grass and legume pastures.

Red clover vein mosaic virus (RCVMV) was first reported from red clover in the United States by Osborn (1937) but has since been found in other legumes in many countries (Bos et al. 1972; Edwardson and Christie 1986, 1991; Hagedorn et al. 1959; Varma et al. 1970; Wetter et al. 1959). RCVMV belongs to the genus *Carlavirus* in the family *Betaflexiviridae* (Adams et al. 2004, 2012). Its flexuous particles contain a single-stranded (+)-sense RNA molecule with six open reading frames (Adams et al. 2004; Larsen et al. 2009). Thus far, only one complete genome sequence of an RCVMV chickpea isolate from Washington State, U.S.A., has been published (Larsen et al. 2009). RCVMV can cause vein mosaic or vein chlorosis as well as plant stunting depending on host species and virus isolate, although latent infections have also been reported (Bos et al. 1972; Graves and Hagedorn 1956; Hagedorn et al. 1959; Wetter et al. 1959). Economic losses caused by RCVMV infection have been up to 88% of the grain weight of moderately diseased pea (Khan and Singh 1997a) but yield losses in chickpea have been up to 100% depending on time of infection (Larsen and Miklas 2001).

Several aphid species can transmit RCVMV in a nonpersistent manner; mechanical transmission and seed transmission have also been reported (Freeman 2007; Kraft et al. 1998; Osborn 1937; Sander 1959; Weber and Hampton 1980). Resistance to RCVMV in red clover has been reported (Hanson and Hagedorn 1961; Khan et al. 1978; Stuteville and Hansen 1964; Stuteville and Hanson 1964) but little information is available on resistant pea cultivars (Hagedorn 1968; Kraft et al. 1998).

Having accidentally discovered RCVMV in a white clover (*Trifolium repens* L.) sample during a routine survey of legumes for viruses, we aimed to characterize the biological and molecular properties of this virus. Because this was also the first report of RCVMV for New Zealand, we conducted surveys to investigate the incidence and distribution of RCVMV in the vicinity of the original finding (Dromore, Mid-Canterbury in the South Island). The surveys included a selection of locally grown annual legume crops (pea, broad bean, and dwarf bean) as well as mixed legume and grass pastures and clover weeds. The small area of lentil crops grown in Canterbury was not included in these surveys.

Materials and Methods

Annual crop surveys. With the cooperation of growers, seed merchants, and processors of broad bean (*Vicia faba* L.) and pea (*Pisum sativum* L.), 16 faba bean seed crops, 21 process and seed pea crops, as well as 12 process dwarf bean crops (*Phaseolus vulgaris* L.) were surveyed in Canterbury between 2011 and 2014. Based on the method used in past legume crop surveys (Fletcher 1993), 100 leaf samples were randomly collected in a “W” pattern for each crop. Leaves were stored at 4°C and serologically assayed for RCVMV and other legume viruses in groups of 10 leaves. Each bulked sample was tested by enzyme-linked immunosorbent assay (ELISA) for the following viruses: *Alfalfa mosaic virus* (AMV), *Bean common mosaic virus*, *Bean yellow mosaic virus* (BYMV), *Broad bean stain virus*, *Broad bean true mosaic virus*, *Broad bean wilt virus 2*, *Broad bean mottle virus*, *Cucumber mosaic virus* (CMV), *Faba bean necrotic yellows virus*, *Pea seed-borne mosaic virus* (PSBMV), *Soybean dwarf virus* (SbDV), *Turnip yellows virus* (TuYV; formerly known as *Beet western yellows virus*), and RCVMV. Double-antibody sandwich (DAS) and triple-antibody sandwich ELISA were completed using kits supplied by Leibniz-Institute-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH following the manufacturer’s instructions for all legume viruses, apart from the DAS-ELISA detection of SbDV, which used antibodies produced at Plant & Food Research (PFR), Lincoln, New Zealand.

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure is published online.

Accepted for publication 24 November 2015.

<http://dx.doi.org/10.1094/PDIS-04-15-0465-RE>
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Perennial pasture and clover weed surveys. Two mixed pastures and two white clover pastures were sampled as described above and tested for RCMV. A further 19 samples of weed clover from roadsides, farmland, and reserves around Canterbury were also collected and tested for RCMV.

Indirect plate-trapped antigen (PTA)-ELISA. Indirect ELISA was completed at PFR, as described before (Fletcher 1993). For the detection of RCMV, a polyclonal antiserum prepared at the Julius Kühn Institute (JKI), Braunschweig, Germany was used at a dilution of 1:1,000 [vol/vol]. A positive reaction was recorded when the mean optical density (OD) was greater than the mean of six negative controls plus three times the standard deviation. Virus incidence in surveyed crops was estimated using the method described by Fletcher (1993) and a table derived using the formula of Gibbs and Gower (1960). Survey results of other virus species detected are not reported here.

Direct DAS-ELISA. At JKI and the Plant Health and Environment Laboratory (PHEL), Auckland, New Zealand, direct DAS-ELISA was used according to Clark and Adams (1977). At JKI, the production of coating antibodies and conjugate was initially carried out with RCMV-specific immunoglobulin G (IgG) derived from a German isolate of RCMV; later, IgG derived from RCMV-NZ was used (at a dilution of 1:1,000 [vol/vol]).

At PHEL, DAS-ELISA was performed using an AC Diagnostics kit (Fayetteville, AR) following the manufacturer's instructions. Several RCMV isolates from different host species and countries were used as positive controls. These included, in total, three isolates from *T. repens* and *V. faba* from New Zealand, one isolate from *T. pratense* L. from the American Type Culture Collection (ATCC, Manassas, VA), and one isolate from an unknown host species from AC Diagnostics. Healthy plants of *T. repens* and *V. faba* were also included as negative controls. A positive reaction was recorded when the mean OD was greater than three times the mean of the negative controls.

Electron microscopy. Transmission electron microscopy (TEM) was performed using a crude sap preparation, as described by Tang et al. (2013). Grids were examined using a JEOL JEM-1230 transmission electron microscope (JEOL Ltd., Tokyo).

Virus propagation and purification. The original white clover sample infected with RCMV-NZ, AMV, and *White clover mosaic virus* (WCIMV) was ground in buffer (0.05 M phosphate, 20 mM sodium diethyldithiocarbamate (DIECA), 5 mM sodium thioglycollate, 1 mM sodium EDTA [pH 7.0], and 0.75% [wt/vol] activated charcoal) and mechanically inoculated onto virus-free *Nicotiana benthamiana* Domin., *Chenopodium quinoa* Willd., *P. sativum*, and *V. faba* 'Scirocco' plants in duplicate in order to find a suitable propagation host. The plants were kept under pest-free greenhouse conditions (20°C with additional light during the winter months for 16 h). Subsequently, *C. quinoa* plants were chosen for propagation of RCMV because this plant is a nonhost plant for WCIMV (Brunt et al. 1996) but showed chlorotic lesions upon inoculation with RCMV or AMV. Despite using excised local lesions for repeated inoculations of *C. quinoa*, separation of RCMV and AMV was not successful using this assay. Therefore, it was decided to separate the two viruses by differential centrifugation during virus purification. Infected leaf material was frozen at -20°C and stored until use. The material was mixed with three volumes of homogenate buffer (0.01 M sodium phosphate [pH 8.0], 0.01 M DIECA, and 0.5% [vol/vol] β -mercaptoethanol). A half-volume of chloroform was added and the solution was stirred for 5 min. The solution was centrifuged for 10 min at 5,858 $\times g$ (Sorvall GSA rotor). The upper supernatant was filtrated and the filtrate subject to centrifugation at approximately 115,000 $\times g$ for 90 min (Beckmann 46Ti rotor). The pellets were resuspended in 100 ml of borate buffer (0.01 M boric acid phosphate [pH 8.3] and 0.2 M urea) and stored at 4°C for 4 h. The solution was centrifuged for 10 min at 5,200 $\times g$ (Sorvall SA 600 rotor). The supernatant was mixed with 0.5% Triton X-100 and stirred at 4°C overnight. The solution was carefully placed over sucrose cushions (25% sucrose in borate buffer) and centrifuged at 76,000 $\times g$ for 2 h. The pellets were resuspended in 30 ml of borate buffer and centrifuged for 10 min at approximately 5,200 $\times g$ (Sorvall SA 600 rotor). The supernatant was subject

to cesium chloride gradient centrifugation (400 mg/ml) for 18 h at 120,000 $\times g$ and 10°C. The desired band containing virus particles was extracted and resuspended in borate buffer. The solution was centrifuged for 4 h and 25 min at 100,000 $\times g$ (Beckmann 70Ti rotor). The viral pellet was resuspended in 500 μ l of borate buffer. Purity of the virus preparation was assessed by TEM.

Production of polyclonal antiserum. For the production of polyclonal antiserum, purified virus solution (virus at 400 μ g/ml in 0.9% [wt/vol] sodium chloride) was mixed 1:1 with Freund's complete adjuvant and injected intramuscularly into one cross-bred rabbit. This procedure was repeated twice after 3 weeks, each time using Freund's incomplete adjuvant. Bleeding started 12 days after the last injection and was repeated every week for 2 months. IgG and conjugate production was carried out according to Clark and Adams (1977).

Reverse-transcription polymerase chain reaction for sequencing. Viral RNA was extracted from purified virions using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified viral RNA or purified virions themselves were used as templates for a random reverse-transcription polymerase chain reaction (RT-PCR) approach, as described by Froussard (1992). First-strand cDNA synthesis was carried out using SuperScript III reverse transcription (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. Klenow polymerase (Life Technologies) was used for second-strand synthesis. The double-stranded cDNA was purified using the Zymo DNA clean kit (Zymo Research, Freiburg, Germany), according to the manufacturer's instructions. For PCR, 5.0 μ l of 10 $\times$ buffer, 5.0 μ l of magnesium chloride (25 mM), 1.5 μ l of UN primer (10 mM), 5 U of Axon Taq (Axon Labortechnik, Kaiserslautern, Germany), 8.0 μ l of purified double-stranded cDNA, and water to 50 μ l were added. PCR conditions were 1 min at 94°C; 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 90 s; followed by a final extension step at 72°C for 10 min. PCR products were subjected to gel electrophoresis in 1.5% [wt/vol] agarose gels. PCR products visualized on these gels were cut and purified using the Zymoclean Gel Recovery kit (Zymo Research), following the manufacturer's instructions. Purified PCR products were cloned using the Qiagen PCR Cloning Kit (Qiagen), following the manufacturer's instructions. Electrocompetent *Escherichia coli* DH5 α cells were transformed with the pDRIVE plasmids and selected on Luria-Bertani media plates containing ampicillin at 50 μ g/ml. Colonies were screened by blue-white selection and checked for appropriate inserts by colony PCR using the GoTaq Green master mix (Promega, Mannheim, Germany) and T7/SP6 primers. Colonies with desired inserts were propagated and plasmids purified using the Zymo Plasmid Prep Kit (Zymo Research). Sequencing was carried out by Eurofins Genomics (Ebersberg, Germany). Based on initial sequence information, specific RT-PCR primers were developed and used in standard PCR to amplify missing regions of the RCMV genome. PCR products were sent directly to Eurofins Genomics for sequencing.

To obtain the 3'-end sequence, purified virus RNA was used for A-tailing with the A-Plus Poly (A) Polymerase Tailing Kit (Cellscript, Madison, WI), according to the manufacturer's instructions. The product was phenol/chloroform extracted and precipitated using one volume of ammonium acetate solution (5 M) and resuspended in 20 μ l of sterile water. Reverse transcription of the tailed RNA was carried out with an oligo(d)T-primer Mix (Pappu et al. 1993) using SuperScript III reverse transcriptase. PCR was carried out using RCMV-specific primer pairs or an oligo(d)T primer (cctcgaggcagctccttttttttttttt) in combination with an RCMV-specific primer. The PCR products were sequenced directly.

To obtain the 5'-end sequence, cDNA using an RCMV-specific primer located at positions 353 to 372 was produced and purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany). cDNA (5 μ l) was incubated with 4 μ l of TdT-buffer (5 $\times$), 1 μ l of terminal transferase (Promega, Mannheim, Germany), and 0.5 μ l of dATP (100 μ M) or dGTP (100 μ M) at 37°C for 60 min. The reaction was stopped at 70°C for 10 min. Standard PCR assays with tailed cDNAs were carried out using a second RCMV-specific primer located at positions 297 to 316 and an oligo(d)T and oligo(d)C primer. Sequence assembly and subsequent analysis

was carried out with the CLC Workbench (package 7, CLC bio; www.clcbio.com). The sequence was deposited in GenBank as accession number KR108251.

Development of standard RT-PCR protocol. RCVMV-specific primers RCVMV-5217F (5'-TGCACTGAATCGGATTATGA-3') and RCVMV-5728R (5'-GCATAAGACACTTCAATAGCAT-3') were designed based on the RNA-dependent RNA polymerase coding region of the only available RCVMV sequence in GenBank (accession number FJ685618) (Larsen et al. 2009), with a predicted amplicon of 512 bp. Total RNA was extracted using the InviMag Plant DNA mini Kit/KFml (Stratag Molecular GmbH, Berlin) and a Kingfisher milliliter semiautomated nucleic acid extraction instrument (Thermo Scientific, Waltham, MA), as per the manufacturers' instructions. One-step RT-PCR was carried out with the SuperScript III one-step RT-PCR system with Platinum Taq polymerase (Invitrogen, Life Technologies, Carlsbad, CA) using 2 µl of RNA template, 1 µl each of 5 µM forward and reverse primers, 1 µl of a 10 mg/ml bovine serum albumen solution, 0.5 µl of SSIII RT/Platinum Taq mix, and 0.2 µl of RNasin Plus (Promega Corp., Madison, WI), and made up to a final volume of 20 µl with nuclease-free water. RT-PCR was performed on a GeneAmp PCR system 9700 (Applied Biosystems, Waltham, MA) with the following cycling conditions: 94°C for 5 min; then, 40 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s; followed by a final extension at 72°C for 5 min. The PCR products were sequenced in both directions on an ABI 3130xl genetic analyzer (Applied Biosystems, Ecogene, Auckland, New Zealand). Sequences were edited and analyzed using Geneious 7.1.5 software (Biomatters Ltd., Auckland, New Zealand; http://www.geneious.com).

Results

Detection and purification of RCVMV, experimental host range, and TEM. During the annual legume survey of 2011, a symptomatic clover sample was detected in the Rakaia region of New Zealand's South Island (Fig. 1A). Analysis of the symptomatic white clover sample by ELISA and immunosorbent electron microscopy using antibodies from the JKI stock collection confirmed that the plant was infected with AMV and WCIMV (data not shown). The sample also contained RCVMV, which had not been found in New Zealand until then. In order to separate RCVMV from the other viruses, mechanical transmission to virus-free differential hosts such as *C. quinoa* was used. After several rounds of mechanical transmission, including the use of single lesions from *C. quinoa*, host plants still were infected by both AMV and RCVMV,

as confirmed by ELISA (data not shown). Therefore, purification including cesium chloride density centrifugation was carried out to separate particles of AMV and RCVMV. RCVMV particles were successfully separated from AMV and used for further virus propagation and host range determination.

Using mechanical inoculations of diluted virus preparations (at approximately 100 µl/ml), a range of virus-free experimental host plants was successfully inoculated in duplicate with RCVMV (Fig. 1B

Table 1. Red clover vein mosaic virus host and non-host plants as determined by mechanical inoculation using purified virions and detection by enzyme-linked immunosorbent assay (ELISA)^a

Plant species	Symptoms	ELISA reaction ^b
<i>Chenopodium amaranticolor</i>	Chlorotic lesions	+++
<i>C. foliosum</i>	No data	+
<i>C. murale</i>	Chlorotic lesions	+
<i>C. quinoa</i>	Chlorotic lesions	+++
<i>Cicer arietinum</i>	None	0
<i>Datura metel</i>	None	0
<i>D. stramonium</i>	None	0
<i>Gomphrena globosa</i>	None	0
<i>Lens culinaris</i>	None	0
<i>Medicago sativa</i>	None	0
<i>M. trunculata</i>	None	0
<i>Nicotiana benthamiana</i>	None	0
<i>N. clelandii</i>	None	0
<i>N. debneyi</i>	None	0
<i>N. glutinosa</i>	None	0
<i>N. occidentalis 37b</i>	Mosaic	++
<i>N. occidentalis P1</i>	Mosaic	+++
<i>N. tabacum samsun-NN</i>	None	0
<i>N. tabacum xanthii</i>	None	0
<i>Physalis floridana</i>	None	0
<i>Pisum sativum</i>	None	+++
<i>Trifolium incarnatum</i>	Mosaic	+++
<i>T. pratense</i>	None	0
<i>T. resuspinatum</i>	None	+
<i>Vicia faba</i>	None	0

^a Symptoms on *Pisum sativum* and *Trifolium resuspinatum* were very mild and indistinguishable from noninfected plants.

^b Scale: 0 = no reaction, + = mild reaction, ++ = intermediate reaction, and +++ = strong reaction.

Fig. 1. Symptoms of Red clover vein mosaic virus (RCVMV, NZ isolate) on different host plants. **A**, *Trifolium repens* specimen in which RCVMV was detected for the first time in New Zealand in 2011. The plant displayed prominent symptoms of interveinal chlorosis and was subsequently found to be infected with RCVMV, Alfalfa mosaic virus (AMV), and White clover mosaic virus (WCIMV). The obvious symptoms induced by AMV and WCIMV infection might have masked the very mild RCVMV-specific symptoms. **B**, RCVMV symptoms on *Trifolium incarnatum* displaying mild mottling. **C**, RCVMV symptoms on *Nicotiana occidentalis* 37b showing chlorotic ring-spots.

and C; Table 1). Susceptible host species included *N. occidentalis* Wheeler PI and 37b, *C. quinoa*, and *C. murale* L., as well as *Trifolium* spp. and garden pea (Table 1). Overall, symptoms were very mild (Fig. 1B and C; Table 1) and virus titers differed on different hosts (Table 1). Other leguminous experimental hosts such as faba bean or *Medicago sativa* L. and *M. truncatula* Gärtn. could not be mechanically infected under greenhouse conditions.

Slightly flexuous, filamentous particles approximately 650 by 12 nm in size (Supplementary Fig. S1) were observed from the original *T. repens* and inoculated *C. quinoa* plants. The particles were similar in morphology and dimensions to carlaviruses.

Antiserum production. Purified virions were used for the production of polyclonal antisera as outlined in the Materials and Methods section. Extraction of IgG and the production of conjugate were successfully used for routine detection of RCVMV in direct DAS-ELISA formats (JKI, experimental host range) and indirect DAS-ELISA formats (PFR, crop surveys in New Zealand).

Crop survey in New Zealand. After the first find of RCVMV on clover plants in New Zealand, virus surveys of legume and pasture crops included tests for RCVMV. This virus was found in pea, faba bean, green bean, *T. pratense*, *T. repens*, and mixed grass and clover pasture crops (Tables 2 to 4). Highest incidences in annual legume crops surveyed were in *V. faba* with up to 66% of process crops (0 to 20% range), *P. sativum* up to 36% of process crops (0 to 3.5% range), and *P. vulgaris* 41% of process crops (0 to 9% range). In Lincoln and mid-Canterbury, only clover pastures older than 3 to 5 years appeared infected with RCVMV (2 to 20%), and 47% of the 32 individual clover weed plants surveyed were found infected with RCVMV.

Full sequence of RCVMV. Several attempts to obtain sequence information from the RCVMV-NZ isolate failed. This included the extraction of double-stranded RNA from infected plant material and subsequent random PCR (Froussard 1992) as well as immunocapture PCR using polyclonal trapping antibodies and a range of carlavirus-specific primers (Badge et al. 1996; Gaspar et al. 2008). Even the production of cDNA and subsequent PCR using oligo(d)T primers (Gaspar et al. 2008; Pappu et al. 1993) repeatedly failed to produce a PCR product. Random PCR (Froussard 1992) was only successful when viral RNA was purified directly from extracted virions. The amplicon fragments were cloned and sequenced. The sequences were used to design specific primers to amplify the sequence gaps using viral RNA in RT-PCR. The determination of

the 3'-end sequence with oligo(d)T primer (Gaspar et al. 2008; Pappu et al. 1993) failed repeatedly (data not shown). The remaining sequence gaps at the 3'-end finally was determined by A-tailing using the A-Plus Poly (A) Polymerase Tailing Kit. However, with this method, it is not possible to distinguish whether the poly (A)-tail sequences are of viral origin or were added during the procedure. The sequence was deposited at GenBank (accession number KR108251). BLAST analysis showed 96% identity on the nucleotide level to the Washington state isolate, the only published full-length sequence of RCVMV (Larsen et al. 2009).

Phylogenetic analyses. Due to the limited availability of RCVMV sequences in public databases, further analyses were conducted using PCR-amplified fragments of 512 bp in length that were consistently amplified from RNA extracts of different RCVMV-infected host species (including *T. pratense*, *T. repens*, *P. sativum*, and *V. faba*) from the North Island and South Island, New Zealand, and the United States. Phylogenetic analysis based on the obtained 512-bp nucleotide sequences (neighbor-joining algorithm using Kimura 80 distance measure and 1,000 bootstrap replicates) revealed that all New Zealand RCVMV isolates, regardless of the host species from which the isolate was derived, appeared to be monophyletic (94% bootstrap support; Fig. 2). The closest relative is the American chickpea isolate (Fig. 2) (Larsen et al. 2009). However, one isolate obtained from ATCC (PV-110) was quite distinct, sharing only about 75% nucleotide identity and 91% amino acid identity to the other isolates.

Discussion

This is the first report of RCVMV in New Zealand. The virus was purified for antiserum production and its genome fully sequenced (GenBank accession number KR108251). Although the occurrence of RCVMV in New Zealand had not yet been documented, subsequent surveys of legume and pasture crops showed that RCVMV is widespread in surveyed crop legumes. *V. faba* showed the highest infestation of around 60% of crops infected and up to 20% incidence in certain locations; in *P. vulgaris*, 40% crops were infected, with up to 9% incidence; and with *P. sativum*, around 30% crops were infected, with up to 3.5% incidence within a crop. This is surprising because attempts to mechanically infect *V. faba* or *T. pratense* under greenhouse conditions failed (Table 1). One can speculate that, under field conditions, effective transmission occurred through aphid vectors.

Table 2. Survey results on incidence of *Red clover vein mosaic virus* (RCVMV) in three annual legume vegetable crops from Canterbury, New Zealand, 2011 to 2014

Plant species	Crop type	Number of crops	Total RCVMV incidence (%)	Range of incidence (%)
<i>Vicia faba</i>	Seed	10	60	0–20
	Process	6	66	0–20
<i>Pisum sativum</i>	Seed	7	28	0–3.5
	Process	14	36	0–3.5
<i>Phaseolus vulgaris</i>	Process	12	41	0–9

Table 3. Survey results on incidence of *Red clover vein mosaic virus* (RCVMV) in pasture crops from Lincoln and mid-Canterbury, 2014

Type of crop	Location	Number of crops	Age of crop (in years)	RCVMV incidence (%)
White clover seed crop	Boundary Rd Lincoln	1	1	0
Mixed clover grass pasture	Lincoln University Dairy	1	20–30	2
White clover seed crop	Tancred Rd	1	3–5	0
Mixed clover grass pasture	Coe's Ford Reserve	1	50	20

Table 4. Survey results on incidence of *Red clover vein mosaic virus* (RCVMV) in clover weed species from Mid-Canterbury (MC), Kaikoura (KA), South Canterbury (SC), and North Otago (NO), 2014

Plant species	Location	Region	RCVMV positive
<i>Trifolium repens</i>	Dromore (original isolation site of RCVMV)	MC	8/8
<i>T. repens</i>	Foley Rd, Arowhenua	SC	0/1
<i>T. pratense</i>	Foley Rd, Arowhenua	SC	0/1
<i>T. repens</i>	Pleasant Valley Rd, St Andrews	SC	1/1
<i>T. pratense</i>	Pleasant Valley Rd, St Andrews	SC	1/1
<i>T. repens</i>	Springfield Rd, Totara	NO	1/1
<i>T. pratense</i>	Gerald St Lincoln	MC	0/1
<i>T. repens</i>	Gerald St, Lincoln	MC	0/1
<i>T. pratense</i>	Springs Rd, Lincoln	MC	1/2
<i>T. repens</i>	Springs Rd, Lincoln	MC	2/2
<i>T. repens</i>	Birches Rd/Tancred Rd	MC	0/1
<i>T. repens</i>	Goulds Rd, Irwell	MC	0/1
<i>T. repens</i>	Rakaia	MC	1/2
<i>T. pratense</i>	Lake Pearson	MC	0/1
<i>T. pratense</i>	Lake Sarah	MC	0/1
<i>T. pratense</i>	Lake Hawdon	MC	0/1
<i>T. repens</i>	Lake Hawdon	MC	0/1
<i>T. pratense</i>	Quailburn	SC	0/2
<i>T. pratense</i>	Oaro	KA	0/3

In the field, no visible symptoms were observed in any crop and the impact on yield was unclear but probably negligible, unless present with other viruses. This is in contrast to reports from the United States and India where RCMV can cause severe crop losses on pea, chickpea, and lentils (Hagedorn and Walker 1949, 1954; Khan and Singh 1997a,b; Larsen and Miklas 2001; Larsen et al. 2009). ELISA analysis of host plants revealed the presence of numerous viruses, including AMV, CMV, BYMV, SbDV, TuYV, and PSbMV. Because RCMV-induced symptoms are very mild (such as very mild systemic mottle on *T. incarnatum* L. or mild systemic chlorotic spots on *N. occidentalis* 37b; Fig. 1B and C), these symptoms might have been masked by mixed infection with one or more of the other viruses that induce more severe symptoms.

In the four clover crops surveyed around Lincoln, RCMV was only detected in crops over 20 years old and, even then, never exceeding 20% incidence level. In the sampled clover weeds, apart from at the Dromore site where RCMV was first observed, only 33% of plants were found to be infected. In the broad bean crop adjacent to the Dromore clover sample, 20% incidence of RCMV was recorded.

From the survey of four clover crops, it seems likely that the spread of RCMV may be quite slow in clover given its apparent absence in newly planted crops and low incidence in clover weeds from other regions. On the other hand, seasonal infection in annual legumes can reach up to 60% of crops, with up to 20% incidence, which implies high levels of infection from the legume aphids moving from clover weeds and pasture to these crops. Graves and Hagedorn (1956) and Freeman (2007) recorded *Acyrtosiphon pisum* (pea aphid), *Aphis fabae*, *Cavariella aegopodii*, *C. theobaldi*, *Myzocallis onomidis*, *Myzus persicae*, and *Therioaphis maculata* as vectors of RCMV. *Acyrtosiphon pisum*, *T. trifolii* subsp. *maculata*, and *M. persicae* are common aphid species found in New Zealand clover pastures. Other potential virus vectors such as *A. kondoi*, *Aphis craccivora*, *Aulacorthum solani*, and *M. ornatus* are also found on clover and other legume crops in New Zealand. *C. aegopodii* is present in New Zealand but is not reported on clover or legumes (Ayong and Gordon 2010). Aphid transmission of RCMV may be particularly important in broad bean because the crop in Canterbury is exposed to

both late autumn and spring aphid flights. Pea and dwarf bean crops are only exposed to spring aphid flights. Seed transmission of RCMV has been reported in red clover, broad bean, and field pea (Freeman 2007), which may also contribute to the spread of RCMV in New Zealand.

Although RCMV has been reported to cause leaf chlorosis and yield loss in susceptible cultivars of red clover (Khan et al. 1978), the effect of RCMV on clover is not very clear. That is because other viruses (e.g., AMV, BYMV, *Clover yellow vein virus*, or WCIMV, all present in New Zealand) also naturally infect clover and are often found in mixed infections (Alconero et al. 1986; Sherwood 1997). For example, Gibbs et al. (1966) found that white clover plants were symptomless when infected with RCMV alone unless also infected with *Clover yellow vein virus*. RCMV has also been reported to cause pea stunt diseases with severe reduction of growth and yield in some pea cultivars in India (Khan and Singh 1997a,b). However, our investigations show that RCMV-NZ causes only very mild symptoms in pea and other host plants (Fig. 1; Table 1). RCMV is likely to be established and widespread in New Zealand. This conclusion is based on its seed transmissibility, and the fact that most of its reported aphid vectors are present in New Zealand and common on reported RCMV host plants, especially the pea aphid. Furthermore, the perennial cultivated or wild legume species can play a role as a whole-year reservoir for RCMV to infect other crops in new seasons.

Because New Zealand runs one of the strictest plant health and quarantine regimes in the world, one can only speculate about the introduction of RCMV to New Zealand. The nucleotide sequence of RCMV-NZ differs by only 4% from the isolate from Washington State; therefore, one could assume the same origin of the two isolates. Because natural transmission of the virus by insect vectors over the distance from Washington State to New Zealand is unlikely, it is possible that RCMV was introduced unknowingly by humans, most likely by imported seed or plant material. As has been shown, RCMV causes only very mild symptoms on certain hosts (pea and clover; Fig. 1B and C); therefore, it is reasonable to expect that infected plant material might have accidentally been imported but not detected.

Fig. 2. Phylogenetic relationship of *Red clover vein mosaic virus* (RCMV) isolates based on approximately 520-bp nucleotide sequences generated by primers RCMV-5217F/5728R and from GenBank (the reference sequence). The tree was created using neighbor-joining algorithm with Kimura 80 distance measure and 1,000 bootstrap replicates. The tree was rooted with *Narcissus symptomless virus* (NSV)-AM182596 and *Trifolium-American Type Culture Collection* (ATCC)-PV110. Only bootstrap values above 70% are indicated. Interestingly, the isolate obtained from ATCC is distinct from others; that is, it shares only approximately 75% nucleotide (91% on the amino acid sequence) identity to other isolates, while the latter have >98% nucleotide identity to each other.

Attempts to amplify RNA extracted from purified RCVMV virions using oligo(d)T primers for cDNA production and generic carlavirus primer for PCR repeatedly failed in both the New Zealand and German laboratories. Sequencing of the 3' end was finally achieved by A-tailing of the 3' end and RT-PCR using oligo(d)T primers; however, this did not allow us to distinguish whether the A-tail was of viral origin or was artificially added. It might be possible that RCVMV-NZ does not have a poly-A tail as described for other carlaviruses or that the tail is truncated in such a way that cDNA production using oligo(d)T primers fails. The biological relevance of an RCVMV isolate without a poly-A tail remains to be elucidated.

Acknowledgments

Parts of this work were funded by a bilateral cooperation grant by the Federal Office of Agriculture and Food (Germany) and the Royal Society of New Zealand. We thank A. Sieg-Müller and P. Lüddecke for excellent technical support; and W. Maier, K. Richert-Pöggeler, and W. Menzel for discussion.

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