Molecular characterisation of viruses from Kiwifruit

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Abstract

In 2003 Apple stem grooving virus was discovered in Actinidia accessions from China, being held in quarantine in Auckland. Subsequent examination of kiwifruit germplasm from the same source has detected several additional viruses, including a ~300 nm rigid rod related to Ribgrass mosaic virus (Tobamovirus), a 700-750 nm flexuous virus related to Citrus leaf blotch virus (Flexiviridae) and a novel vitivirus. Currently these viruses have not been reported from commercial kiwifruit crops in New Zealand or elsewhere. The biological properties of the viruses from kiwifruit and their phylogenetic relationships with similar viruses from other plants will be described, and the possible implications for the international movement of Actinidia germplasm are discussed.

Introduction

Although kiwifruit has been grown commercially since the 1930's (Ferguson and Bollard, 1990) until recently very little has been published on viral diseases. In 1983 in New Zealand, local lesions, of presumed viral origin, were observed on C*henopodium quinoa* mechanically inoculated with leaf extracts from imported *Actinidia* germplasm (Gary Wood, DSIR, pers. comm.). Other reports include a sap transmissible (presumed) virus disease of *Actinidia* in China (Lin and Gao, 1995) and a report of viral-like symptoms in in *A. deliciosa* grafted to *A. polygama* rootstocks in Japan (Nitta and Ogasawara, 1997). The first definitive identification of a virus from *Actinidia* was *Apple stem grooving virus* (ASGV) in germplasm of Chinese origin being held in quarantine in New Zealand (Clover et al., 2003). Following the discovery of ASGV we examined the quarantine material for further viruses.

Given the lack of information on viruses of Actinidia and the absence of any closely related crops, initial screening of Actinidia germplasm used non-specific virus detection methods such as symptoms in Actinidia itself, mechanical transmission to herbaceous indicators and electron microscopy (EM). Where particle morphology could be determined by EM specific PCR tests for likely families or genera were performed. Where no virus particles were seen and/or subsequent test did not provide an identification, we adopted an approach involving mass spectrometry of potential viral proteins from the presumed virus infected plants.

Materials and methods

Source plants: Woody cuttings of Actinidia accessions from Shaanxi province, China, were grafted onto healthy rootstock of A. chinensis cv. Hort 16A in post-entry quarantine in New Zealand. In addition, samples of Actinidia glaucophylla, A guilinensis and A fortunatii were obtained from Plant and Food Research germplasm collections.

Sap transmission to herbaceous indicators: Leaf tissue (1–2 g) from both young and mature leaves of Actinidia was homogenized using a pestle and mortar in 4 ml 0.1 M phosphate buffer, pH 7.5 (Sweet 1975) containing 5 % polyvinylpyrrolidone and 0.12 % sodium sulphite. The homogenate was mechanically inoculated to 3-4 leaf seedlings of *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. occidentalis* 37B, and *Phaseolus vulgaris* cv. 'The Prince'. The inoculated plants were grown in a greenhouse at 20–22 °C for up to 6 weeks and observed for viral symptoms.

Transmission electron microscopy (TEM): Leaf extracts in 0.01 M Sørensen's phosphate buffer, pH 7 were negatively stained with 2 % (w/v) aqueous potassium phosphotungstate, pH 7 and observed using a Phillips Tecnai 12 electron microscope (FEI, Eindhoven, Netherlands). Particle lengths were determined using the microscope's internal calibration and by comparison to Tobacco mosaic virus.

Enzyme-linked immunosorbent assay (ELISA): Leaf samples from Actinidia and herbaceous indicators with were tested for Alfalfa mosaic virus (AMV) and Cucumber mosaic virus using antisera from Bioreba (CMV) and Prime Diagnostics (CMV), according to the manufacturer's instructions.

RT-PCR and sequencing: RNA was extracted from 100 mg leaf samples of Actinidia and indicators using an RNeasy® Plant mini kit (Qiagen) with the lysis buffer modification of MacKenzie et al. (1997) or a SpectrumTM (Sigma-Aldrich) kit, according to the manufacturer's protocol. RT was performed using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's protocol using anchored oligo(d)T primers or virus

specific reverse primers. PCR amplification was performed with either specific or generic primers, Platinum® Taq polymerase (Invitrogen) and 2.5 mM dNTPs (Invitrogen) according to the manufacturer's instructions. PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide. DNA was purified using 'Perfectprep Gel Cleanup' purification kit (Eppendorf,

Hamburg, Germany), and sequences cloned in E. coli using pGEMT easy vector (Promega, Madison, WI, USA). Plasmids were purified using a FastPlasmid®Mini kit (Eppendorf, Hamburg,Germany) and the inserts sequenced with an ABI PRISM automated DNA sequencer (University of Auckland, New Zealand).

Sequence analysis: Consensus nucleotide sequences were compiled using Sequencher 4.5 (Gene Codes Corporation, Michigan 48108, USA) and translated into amino acid sequence using BioEdit (Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008). Sequence comparison and phylogenetic analyses were conducted using BLAST (Altschul et al. 1997; Schäffer et al. 2001) and MEGA version 3.1 (Kumar et al. 2004). Neighbour joining trees were constructed with Poisson corrected amino acid distances and pairwise gap deletion options and 50,000 bootstrap replicates. Consensus sequences were analysed for recombination events using RDP3 Beta 24-Recombination (Martin et al. 2005).

Virus partial purification and protein separation: Virus isolates were maintained in Nicotiana occidentalis and virions were partially purified from approximately 1 g of plant tissue using a protocol based on that of Lane (http://lclane.net/minipur.html). Denatured total soluble protein (15 µg) from the partially purified extracts was separated on a 12 % polyacrylamide gel (NuPAGE® Novex Bis-Tris Gels, Invitrogen) and molecular weights determined against Benchmark (Invitrogen) protein standards. Gels were stained with Coomassie blue G250 (SimplyBlue™ SafeStain, Invitrogen) following the manufacturers' instructions.

Tryptic digestion of proteins and mass spectrometry: Protein bands were excised from stained gels and digested with trypsin as described by Blouin et al. (2009). The resulting peptides (10 µl sample) were subjected to liquid chromatography electrospray tandem MS using a Finnigan™ LTQ-FT™ mass spectrometer coupled to a Surveyor HPLC system (ThermoFisher Scientific. Resulting peptide tandem (MS/MS) data were analyzed using TurboSEQUEST from the BioWorks v3.3 Suite (Thermo Fisher), against a virus database extracted from the National Centre for Biotechnology Information (NCBI) GenPep database (March 2008) containing non-human virus protein sequences (107,951 sequence entries). All resulting matched peptides were then confirmed by visual examination of the individual spectra.

Results

Infections in herbaceous hosts and electron microscopy: Mechanical inoculations to herbaceous indicators from both symptomatic and asymptomatic Actindia resulted in a range of virus-like symptoms, including mottles, mosaics, ring spots, vein clearing, and leaf distortion. EM examination of negatively stained leaf extracts from the herbaceous hosts revealed the presence of both ~300nm rigid rods and ~700-750 nm flexuous rods in several samples. However, virus particles were not observed in all symptomatic samples, suggesting the likely presence of other, less easily detectable particle types.

PCR and sequencing using generic and virus specific primers: Based on the particle morphology and size we tested for tobamoviruses (~300 nm rigid rod) using the Tob Uni-1 and Tob Uni-2 primers of Letschert et al. (2002) and carlaviruses (610-700nm flexuous rods) using Agdia carlavirus group PCR mix (Agdia Inc, Elkhart, USA). The Tob Uni-1 and Tob Uni-2 primers yielded a ~650bp product and the sequence confirmed this was a tobamovirus. Further PCR and sequencing, using primers of Zhu et al (2001) plus primers designed using alignments of 10 closely related tobamoviruses confirmed the virus as belonging to sub-group 3 of the genus Tobamovirus (Figure 1), most closely related to the R14 strain of Ribgrass mosaic virus (Heinze et al., 2006; Chavan et al., 2009).

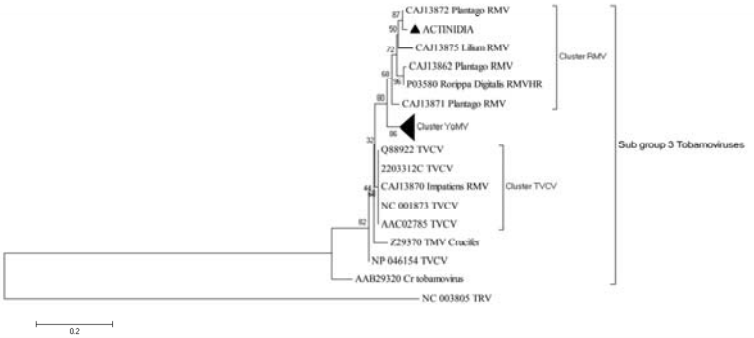

Fig. 1 Eighbour joining tree showing phylogenetic relationship of of putative coat protein gene amino acid sequence of *Actinidia* tobamovirus with CP aa sequences of sub group 3 tobamoviruses.

A ~300bp product was generated from the presumed carlavirus but the sequence was found to be most closely related to Citrus leaf blotch virus (CLBV) (Flexiviridae) rather than to carlaviruses. Subsequent sequencing using primers based on the published coat protein gene sequences of CLBV plus conserved regions from trichovirus, carlavirus, potexvirus, and vitivirus, confirmed this relationship (Table 1).

Tab. 1 Nucleotide identity of the partial replicase, complete movement and coat proteins and partial 3' UTR of a flexivirus from *Actinidia chinensis* with representative members of the *Flexiviridae*.

Virus	GenBank Accession No	% nucleotide identity
Citrus leaf blotch virus [Citrivirus]	NC_003877.1	80%
Citrus leaf blotch virus NZ_G18 [Citrivirus]	EU857539.1	80%
Citrus leaf blotch virus_G78 [Citrivirus]	EU857540.1	79%
Dweet mottle virus_932 [Citrivirus]	FJ009367.1	80%
Apple stem grooving virus [Capillovirus]	D14995.2	15%
Apple chlorotic leaf spot virus [Trichovirus]	M58152.1	16%
Apple stem pitting virus [Foveavirus]	D21829.1	14%
Grapevine virus A [Vitivirus]	AF007415.2	15%
Indian citrus ringspot virus [Mandarivirus]	AF406744.1	12%
Hop mosaic virus [Carlavirus]	NC_010538.1	15%
Shallot virus X [Allexivirus]	M97264.1	14%

Protein sequences from mass spectrometry: Where no particles were seen by EM, and consequently there was no indication of which viruses to test for, protein profiles from infected indicators were examined for proteins not present in healthy plants. Several of these were sequenced by mass spectrometry together with a sample known to contain the CLBV-like virus. In addition to the expected CLBV proteins indicative of the presence of Alfalfa mosaic virus (AMV) and Cucumber mosaic virus (CMV) and an apparently novel vitivirus were identified. The identity of AMV was confirmed from three Actinidia species by ELISA and by PCR using primers designed to amplify the CP and MP (Table 2). The Actinidia AMV coat protein and movement protein sequences showed >96 % nucleotide identity with each other and < 98% nucleotide identity with other AMV isolates (Table 2). Peptides from the vitivirus showed closest identity to Grapevine virus B (GVB) and subsequent sequencing showed 66 % and 69 % to the RdRp and 60 % and 69 % to the CP of GVA and GVB, respectively (Table 3). The CMV isolates were confirmed by ELISA but are yet to be sequenced.

Tab. 2 Nucleotide identity (%) of movement protein and coat protein sequences of *Actinidia* isolates of *Alfalfa mosaic virus* (AMV) with other isolates of AMV. Coat protein identity above the diagonal (italicized), movement protein below the diagonal.

	A. glaucophylla	***A. guilinensis***	***A. fortunatii***	**X00819**	**AF015717**	**M59241**	**AB126031**
A. glaucophylla		96.0	98.4	93.5	95.4	94.2	93.7
A. guilinensis	96.0		96.5	94.2	98.4	95.4	94.5
A. fortunatii	98.6	96.2		94.0	95.8	94.7	94.2
X00819	95.1	94.7	95.1		93.9	97.7	97.2
AF015717	96.2	98.0	96.4	94.0		94.4	93.5
M59241	95.0	94.4	94.7	97.2	94.9		97.7
AB126031	94.2	93.0	93.9	96.2	93.5	95.4	

Tab. 3 Amino acid identity (%) of *Actinidia* vitivirus with other vitiviruses.

Virus	ORF 1			ORF 2	ORF 3	ORF 4	ORF 5
	MeTr 336aa	Hel no data	RdRp 310aa	? 219aa	MP 297aa	CP 198aa	NB 105aa
Grapevine virus A	63	-	66	22	42	60	45
Grapevine virus B	63	-	69	17	38	69	11
Grapevine virus E	43	-	60	19	34	42	17
Mint virus 2	-	-	72	19	36	61	35

Discussion

Since 2003 six different viruses have been identified in kiwifruit germplasm that originated in China. The first was clearly an isolate of ASGV (Clover et al., 2003). The nucleotide sequence (~2900nt of 3' end) of the Actinidia isolate differs by ~15 % from both citrus and apple strains, which differ from each other by approximately the same amount. It is not known whether the Actinidia isolate of ASGV infects citrus and apple and vice versa.

The second virus to be described was a tobamovirus (Pearson et al, 2007) which clusters with cruciferous infecting isolates in sub-group three of the tobamoviruses (Chavan et al., 2009). Based on the coat protein and movement protein genes it is most similar to Ribgrass mosaic virus (RMV), a widely distributed virus. The host range of the Actinidia isolate has not been determined but RMV isolates have been found in a wide range of species, including common vegetables.

The Actinidia flexivirus sequences showed greatest identity with CLBV, the single member of the (proposed) genus Citrivirus (Mayo & Haenni, 2006). However, the Actinidia isolates may be sufficiently different to represent a distinct strain or species, but there are currently no guidelines for species differentiation within the genus Citrivirus (Fauquet et al., 2005). The host range of the Acitinidia isolate has not been determined but citrus isolates of CLBV are only known to infect citrus, are not mechanically transmitted in the field and have no known insect vectors. The vitivirus is most closely related to GVA and GVB but based on ICTV criteria (Fauquet et al., 2005) is sufficiently different to be considered a novel species. As with the other viruses isolated from Actinidia little is known about the host range of this virus but vitiviruses such as GVA and GVB typically have restricted host ranges. The other two viruses identified from Actinidia, AMV and CMV, both have very wide host ranges and are readily transmitted by a range of polyphagous aphid species. Since the sequences of the Acitinidia isolates show high similarity to isolates from other hosts it is quite likely that these viruses have moved into Actinidia from other hosts.

All six of the viruses were detected during routine screening of germplasm lines and to date no viral diseases have been reported from commercial crops in New Zealand. There have been reports of viral-like diseases in Actinidia from China and Japan but no specific virus identifications have been published and the impact of these is unknown. Although the Actinidia viruses detected so far appear to belong to previously known virus genera and species, some may be sufficiently different to be considered as distinct species or strains. Since the viruses were detected in individual germplasm accessions and have not been assessed in replicated infection trials very little is known about cultivar susceptibility and the effects, if any, on plant productivity. With traditional, long established crops the detection of viruses has mostly been driven by the presence of distinct disease symptoms in the crop and characterisation has advanced along with development of new technologies. The situation for kiwi fruit is quite different since we have detected several viruses in imported germplasm but so far have not observed any viral disease problems in commercial crops. Thus, modern molecular techniques have allowed us to detect and identify viruses but tell us little about pathogenicity and potential host range. This poses problems when comparing the virus status of kiwifruit in different countries, since in the absence of obvious disease problems in commercial crops researchers and government agencies are unlikely to actively look for viruses.

Since we have not observed any viral disease problems in commercial kiwifruit orchards in New Zealand the current focus is to provide high health material for breeding programmes. Since we have found viruses in material originating from China it is likely that these or other viruses are also present in germplasm collections in other countries. Consequently we would encourage growers and researchers in China and other kiwifruit growing countries to investigate the presence of viruses in order to: (a) understand the full range of kiwi fruit viruses, (b) determine what risk these may pose to the industry, and (c) take necessary action to mitigate any potential virus problems.

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