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Molecular confirmation of New Zealand garlic yellow streak virus as *Leek yellow stripe virus*

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Abstract. Garlic yellow streak virus (GYSV) was first reported in New Zealand by Mohamed and Young in 1981 as a new potyvirus distinct from *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV), based on host range and serological differences. Recent examination of garlic showing the typical yellow streak symptoms, using serological and molecular methods, clearly identifies the associated virus as a strain of LYSV.

Additional keyword: Allium sativum.

Introduction

Mohamed and Young (1981) reported that many *Allium sativum* (garlic) plants in New Zealand exhibited yellow streak symptoms and identified the cause as a *Potyvirus* which they named garlic yellow streak virus (GYSV). They concluded that GYSV was a distinct virus from two other *Allium* potyviruses, *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV), based on their different host range and serological reactions. More recently, Fletcher and Davison (J. D. Fletcher and J. E. B. Davison, unpublished data) surveyed 16 garlic crops in New Zealand and concluded that GYSV was serologically related to OYDV. In contrast, Van Dijk (1993), who used serology to test and characterise potyviruses from 5700 *Allium* plants, representing 22 species from the Netherlands and other parts of the world, concluded that garlic yellow streak disease was probably caused by a mixture of OYDV and LYSV.

The viruses infecting garlic have been widely studied over the past three decades (Walkey and Antill 1989; Helguera 1997; Lot 1998; Conci *et al*. 2002, 2003). However, identification based on traditional methods, such as serology, host range and symptoms has sometimes resulted in controversial or incorrect conclusions for particular isolates (Mohamed and Young 1981; Atreya 1992; Conci *et al*. 1992; Riechmann *et al*. 1992; Tsuneyoshi *et al*. 1998; Chen *et al*. 2001; J. D. Fletcher and J. E. B. Davison, unpublished data). In contrast, sequence comparison provides a more definitive tool for pathogen classification and identification (Atreya 1992; Riechmann *et al*. 1992; Tsuneyoshi *et al*. 1998; Chen *et al*. 2001), especially for potyviruses where serological cross-reactions are common. For RT–PCR of uncharacterised potyviruses there are a number of universal primers, including PV2I/T7 and PV1/SP6 (Mackenzie *et al*. 1998) which amplify a 1.7–2.1 kb fragment across the NIb gene (RNA-dependent RNA polymerase), coat protein gene and 3′ UTR regions, plus U335 and D335 (Langeveld *et al*. 1991) that amplify a fragment of ~335 bp from the central part of the coat protein gene.

We analysed field-grown garlic showing typical GYSV symptoms from Pukekohe (New Zealand) by ELISA, RT–PCR and sequencing to compare GYSV with LYSV and OYDV.

Methods

Source of plant material

Garlic leaves with typical yellow streak symptoms were collected from the field at Pukekohe, Auckland, New Zealand in November 2003, freeze-dried, and stored at 4°C until used. Dried leaf tissue of *Allium porrum* (leek) infected with *Leek yellow stripe virus* and *A. cepa* (onion) infected with *Onion yellow dwarf virus* were provided by Dr John D. Fletcher, Crop & Food Research, New Zealand. Leaves of *Colocasia esculenta* (taro) infected with *Dasheen mosaic virus* (DsMV), healthy taro, *Cucurbita pepo* subsp. *pepo* (zucchini) and *Nicotiana benthamiana*, used as positive and negative controls, were obtained from plants maintained in the greenhouse.

Virus purification

Viruses were partially purified from garlic leaves with obvious yellow streak symptoms, using the method of Pearson *et al*. (1994) with minor modifications. The leaves were ground to a fine powder in liquid nitrogen using a pestle and mortar and then homogenised in 200 mM sodium borate buffer (pH 8.0) containing 0.3% mercaptoethanol. After

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centrifugation at 2000 *g* for 10 min, the supernatant was mixed with an equal volume of chloroform, and Triton X-100 was added to a final concentration of 5% (v/v). The mixture was stirred at 4°C for 30 min, centrifuged at 10 000 *g* for 20 min and the supernatant further centrifuged at 250 000 *g* for 2 h. The resultant pellet was re-suspended in 50 mM sodium borate buffer (pH 8.0) for 2 h at 4°C, and then centrifuged at 10 000 *g* for 10 min. The supernatant was centrifuged at 250 000 *g* for 2 h through a 200 g/L sucrose cushion. The resultant pellet, containing extracted virus, was re-suspended in sterile distilled water and stored at −80°C.

Enzyme-linked immunosorbent assay

The following antisera were used: *Potyvirus* universal monoclonal antibody (Agdia Inc., Elkhart, Indiana, USA); OYDV polyclonal antiserum (DSMZ, Braunschweig, Germany); GYSV and LYSV polyclonal antisera produced by Mohamed and Young (1981) (provided by Dr Francisco Ochoa-Corona, Ministry of Agriculture and Forestry of New Zealand).

Partially purified virus and sap extracts from virus-infected garlic leaves were tested for potyviruses by antigen-coated plate–enzyme-linked immunosorbent assay (ACP–ELISA) using the *Potyvirus* universal monoclonal antibody (McAb) according to the manufacturer's protocol. For GYSV, LYSV and OYDV, the same protocol was used with virus specific polyclonal antibodies. Healthy leaves of taro, zucchini, and *N. benthamiana* were used as negative controls. DsMV-infected taro, LYSV-infected leek and OYDV-infected onion were used as positive controls. All samples were tested in duplicate and optical density (OD) values were read at 405 nm. Readings with an A_{405} value more than three times the mean of the negative controls were considered positive.

RNA extraction and cDNA synthesis

Total RNA was extracted from a single freeze-dried infected garlic leaf using an RNeasy Plant Mini Kit (QIAGEN Sciences, Maryland, USA), following the manufacturer's instructions. Virus cDNA was synthesised from 1–3 µg of total RNA and 5 µL of purified GYSV using M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, Canada) and an oligo $d(T)_{17}$ primer PV1/SP6 (Mackenzie *et al*. 1998).

PCR, cloning and sequencing

PCR products were generated using potyvirus universal primers PV2I/T7 and PV1/SP6 (Mackenzie *et al*. 1998) which amplifies a fragment of ∼1.7–2.1 kb, and primers U335 and D335 (Langeveld *et al*. 1991) which amplify a fragment of ∼335 bp from the central conserved region of coat protein gene. In addition, the primer combinations PV2IT7/D335 and U335/PV1SP6 were used to amplify two fragments, of G13 (∼1.3 kb) and G10 (∼1.0 kb) with an overlap of ∼335 bp.

The 25-µL PCR reaction mixture contained reaction buffer, 2.5 mM of $MgCl_2$, 0.2 mM dNTP, 1 unit of Taq polymerase (Applied Biosystems, Foster City, USA), and 5 pmol of each primer. The following cycling parameters were used: 94°C for 3 min, 30 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 90 s, followed by a 6 min extension at 72°C. PCR products were separated by electrophoresis on 1% agarose–TBE gels and detected by ethidium bromide staining.

PCR products were purified from the agarose gel using a PerfectPrep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) and cloned into the vector pGEM–T Easy (Promega Corporation, Madison, WI, USA), then transformed to Max Efficiency DH5α chemically competent cells (Invitrogen, Carlsbad, Canada), according to the manufacturer's instructions. Ten white colonies were selected from each transformation and were screened for an insert by PCR using T7 and SP6 primers. Plasmids with insert were extracted using a FastPlasmid Mini Kit (Eppendorf, Hamburg, Germany) and the insert sequenced using M13F and M13R primers.

Phylogenetic analysis

Nucleotide sequences of the NZ garlic virus isolates were compared with selected LYSV and OYDV sequences from GenBank. Phylogenetic analyses were conducted using BioEdit version 7.0.4 (Hall 1999), ClustalX version 1.83 (Thompson *et al*. 1997) and PAUP version 4.0 Beta 10 (Swofford 2000). Neighbour-joining trees were constructed using *Oat mosaic virus* as the outgroup sequence. Putative coat protein amino acid sequences of garlic isolates were compared with GenBank LYSV coat protein sequences using BioEdit.

Results

ELISA

The ELISA results are summarised in Table 1. As anticipated, all of the virus-infected samples reacted positively with the *Potyvirus* McAb. Both GYSV-infected garlic sap and purified GYSV reacted strongly with the antisera to GYSV and LYSV, but not with antiserum to OYDV (purified GYSV gave a marginal positive). LYSV-infected leek sap reacted strongly with LYSV and GYSV antiserum but only weakly with the OYDV antiserum. The OYDV-infected onion sap reacted strongly with the OYDV antiserum, weakly with the LYSV antiserum (marginal positive) but not with the GYSV antiserum.

PCR, cloning and sequence analysis

Using primer sets PV2IT7/D335 and U335/PV1SP6, two overlapping fragments of ∼1.3 kb (G13) and ∼1.0 kb (G10) were amplified, from both total RNA extracted from four individual plants and purified GYSV from bulked material from more than ten plants (Fig. 1). A minimum of three clones were sequenced for each fragment from each plant, in total 26 G10 and 32 G13 clones were sequenced.

Alignment of the G13 and G10 sequences revealed two distinct nucleotide sequences of 2085 bp and 2099 bp, designated as nzLYSVg1 (GenBank accession: AY842134)

Table 1. Reaction of GYSV, LYSV, and OYDV to various antisera

Antigens	Mean ELISA A_{405} after two hours			
	Potyvirus McAb	GYSV antiserum	LYSV antiserum	OYDV antiserum
GYSV-infected garlic sap	0.204	0.396	0.327	0.012
Purified GYSV	0.948	1.614	1.085	0.117
LYSV-infected leek sap	2.554	0.779	1.071	0.227
OYSV-infected onion sap	0.881	0.026	0.116	0.572
DsMV-infected taro sap	0.997	0.065	0.078	0.090
Healthy taro sap	0.032	0.022	0.073	0.077
Healthy zucchini sap	0.056	0.022	0.030	0.029
Healthy *N. benthamiana* sap	0.032	0.067	0.052	0.026

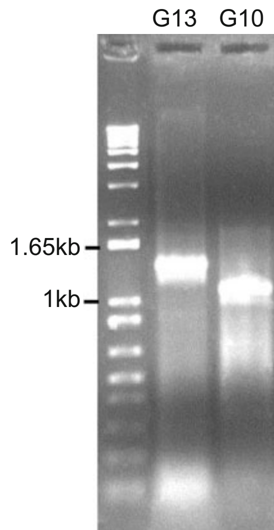

Fig. 1. Potyvirus PCR products G13 and G10 amplified from garlic using primer sets PV2IT7/D335 and U335/PV1SP6, respectively.

and nzLYSVg2 (GenBank accession: AY842136), respectively. Both sequence variants were found in all four of the individual plants and the purified virus from the bulked sample. The similarity of the nucleotide sequences of nzLYSVg1 and nzLYSVg2 is 83.4%, whereas the similarity of their putative amino acid sequences is 85%. The phylogenetic comparison showed that both sequences have a high level of similarity (77.4–96.8%) to isolates of LYSV from GenBank and a low similarity (34.8–48.8%) to OYDV isolates. A neighbour-joining tree (1000 bootstrap replicates) showed that both of the garlic isolates were clustered closely with isolates of LYSV (Fig. 2).

The putative amino acid sequences for the NIb and coat protein genes of nzLYSVg1 and nzLYSVg2 were compared with that of other LYSV from GenBank. The N-terminal amino acid sequences of the coat protein were very variable (Fig. 3), with nzLYSVg2 possessing three amino acids (positions 27–29 of the N-terminal region) not

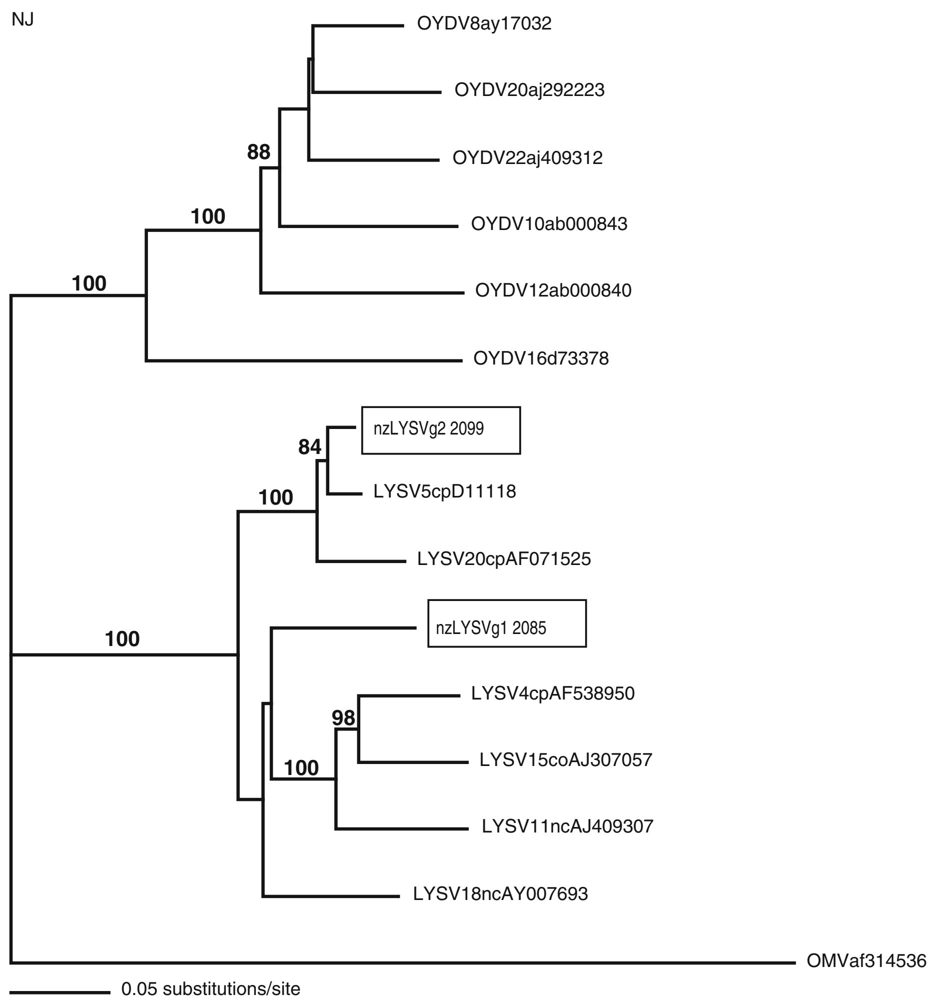

Fig. 2. Neighbour-joining tree for nzLYSVg1, nzLYSVg2 sequences from NZ garlic and LYSV, OYSV sequences from GenBank. *Oat mosaic virus* (OMV, GenBank accession # AF314536) was used as the outgroup (numbers on branches are bootstrap values, 1000 replicates).

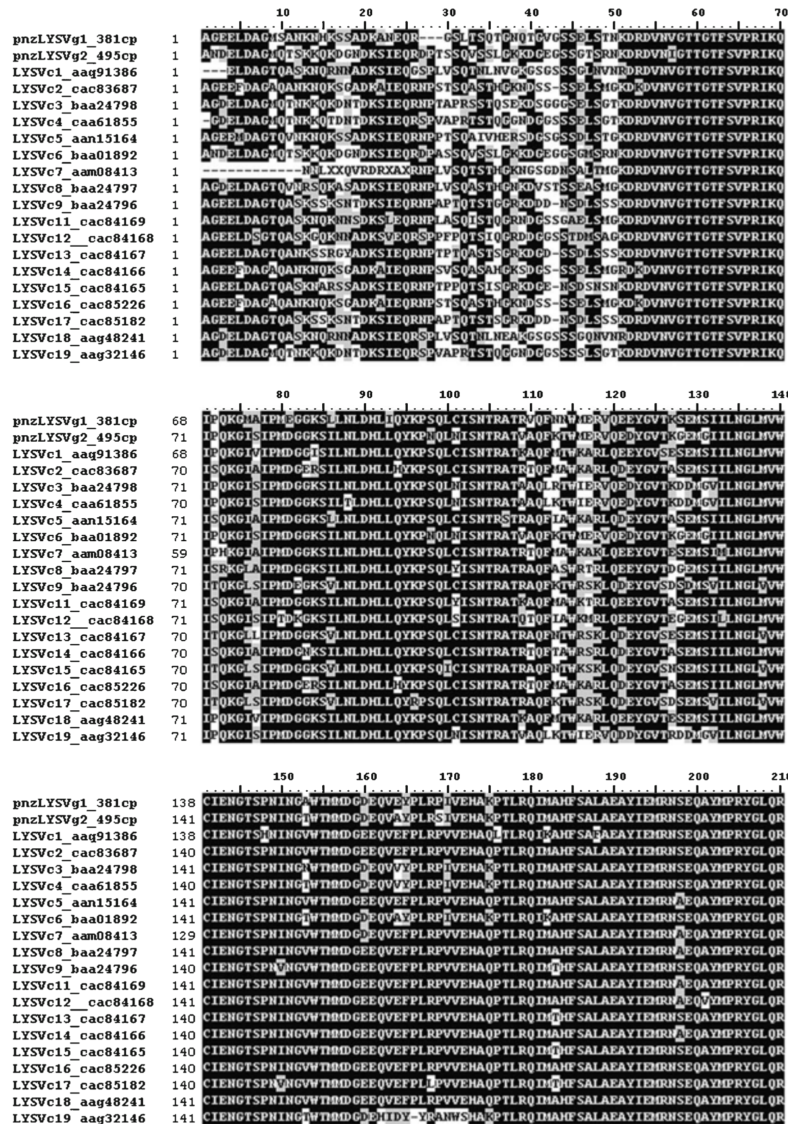

Fig. 3. Alignment of coat protein amino acid sequences of nzLYSVg1, nzLYSVg2 with selected LYSV isolates from GenBank.

present at the nzLYSVg1 sequence. In contrast the NIb gene sequences were highly conserved, as were the 3′ UTR sequences (data not shown).

Discussion

Mohamed and Young (1981) found that in microprecipitin tests, purified GYSV reacted only weakly with OYDV and LYSV antisera, and LYSV from leek did not react with antisera to either OYDV or GYSV. On the basis of this serological evidence and host range data, they concluded that GYSV was distinct from OYDV and LYSV. However, GYSV was not subsequently accepted by the International Committee on Taxonomy of Viruses (ICTV) as a new species (Van Regenmortel *et al*. 2000) and its precise identity remained unclear.

In our current investigation using ELISA, which is more sensitive than microprecipitin tests, both partially purified GYSV and leaf extracts from GYSV-infected garlic plants reacted strongly with the same GYSV and LYSV antisera used by Mohamed and Young (1981) but not with OYDV antiserum, indicating that GYSV is closely related to LYSV. The strong reaction between current samples of GYSV and the Mohamed and Young antiserum (produced in 1979) suggests that the virus has not changed substantially during the last 25 years.

Two distinct strains were found in all four of the individual plants and the purified virus sample showing that these two strains commonly co-exist in the field.

One of the properties of GYSV that led Mohamed and Young (1981) to conclude it was distinct from LYSV was

their inability to infect leeks with GYSV. However, restricted host ranges are a common feature of potyviruses. Van Dijk (1993), in a review of *Allium* potyviruses, comments that poor infectivity on most other plant species is a common characteristic of garlic viruses, and Lot (1998) found that even different garlic cultivars demonstrated different susceptibility to LYSV.

Amino acid sequence of the coat protein, especially the N-terminal region, has proved useful in *Potyvirus* taxonomy (Shukla *et al*. 1988; Jordan 1989; Usugi *et al*. 1989; Riechmann *et al*. 1992) and on this basis our sequence data clearly identify both of the garlic yellow streak associated viruses as strains of LYSV (nzLYSVg1 and nzLYSVg2). Although LYSV has been recorded in garlic in the UK (Walkey *et al*. 1987) and in other countries (Van Dijk 1993; Barg *et al*. 1994; Yamashita *et al*. 1995; Conci 1997; Conci *et al*. 2002), the New Zealand garlic isolates are quite distinct from the other garlic LYSV sequences in GenBank. These differences may well be reflected in their biological properties, as various studies have shown that even point mutations in the potyviral genome can affect properties such as host range and symptom induction (Riechmann *et al*. 1992) as well as aphid transmissibility (Atreya *et al*. 1990, 1991).

In summary we conclude that the virus named garlic yellow streak virus by Mohamed and Young (1981), and to which they produced their antiserum, is in fact a strain of *Leek yellow streak virus*. We did not detect OYDV in any of our samples, but our results do not preclude the possibility that some garlic plants in New Zealand may also be infected by this virus.

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