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ORIGINAL ARTICLE

Turnip yellows virus variants differ in host range, transmissibility, and virulence

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Abstract

Turnip yellows virus (TuYV; family *Solemoviridae*, genus *Polerovirus*, species *Turnip yellows virus*) is a genetically diverse virus that infects a broad range of plant species across the world. Due to its global economic significance, most attention has been given to the impact of TuYV on canola (syn. oilseed rape; *Brassica napus*). In Australia, a major canola-exporting country, TuYV isolates are highly diverse, with the most variation concentrated in open reading frame 5 (ORF 5), which encodes the readthrough domain (P5) component of the readthrough protein (P3P5), which plays an important role in host adaptation and aphid transmission. When analysing ORF 5, Australian TuYV isolates form three phylogenetic groups with just 45 to 49% amino acid sequence identity: variants P5-I, P5-II, and P5-III. Despite the possible implications for TuYV epidemiology and management, research examining phenotypic differences between TuYV variants is scarce. This study was designed to test the hypothesis that three TuYV isolates, representing each of the Australian P5 variants, differ phenotypically. In particular, the host range, vector species, transmissibility, and virulence of isolates 5414 (P5-I_{5414}), 5509 (P5-II_{5509}), and 5594 (P5-III_{5594}) were examined in a series of glasshouse experiments. Only P5-I_{5414} readily infected faba bean (*Vicia faba*), only P5-II_{5509} infected chickpea (*Cicer arietinum*), and only P5-I_{5414} and P5-III_{5594} infected lettuce (*Lactuca sativa*). *Myzus persicae* transmitted each isolate, but *Brevicoryne brassicae* and *Lipaphis pseudobrassicae* did not. When using individual *M. persicae* to inoculate canola seedlings, P5-I_{5414} had significantly higher transmission rates (82%) than P5-II_{5509} (62%) and P5-III_{5594} (59%). As indicated by enzyme-linked immunosorbent assay absorbance values, P5-I_{5414} reached higher virus titers in canola than P5-II_{5509}, which, in turn, reached higher titers than P5-III_{5594}. P5-I_{5414} was also more virulent in canola than P5-II_{5509} and P5-III_{5594}, inducing more severe foliar symptoms, stunting, and, in one of two experiments, seed yield loss. Results from this study compared to those of previous studies suggest that analysis of ORF 5 alone is insufficient to assign isolates to coherent strain categories, and further sequencing and phenotyping of field isolates is required.

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Introduction

Turnip yellows virus (TuYV; family *Solemoviridae*, genus *Polerovirus*, species *Turnip yellows virus*) was first described in Belgium in 1950 [59] and was initially considered to be a strain of beet western yellows virus (BWYV) due to their close serological relationship and biological similarities [18, 19]. TuYV and BWYV were initially distinguished from one another by their host range; in particular, that TuYV did not infect *Beta* species [28]. However, it was not until 2002 that TuYV was officially classified as a member of a separate species [42], a decision subsequently

supported by molecular analysis [54]. Prior to this, many studies used the term 'BWYV' to refer to TuYV and relied solely on serological assays that are unable to distinguish between the two viruses, posing a challenge to interpreting older literature. In Australia, it was not until 'BWYV' isolate WA-1 was sequenced in 2006, and further isolates were sequenced and confirmed to be TuYV that the name TuYV came into common usage [37]. The availability of high-throughput sequencing uncovered further complexity when isolates thought to be TuYV infecting leguminous crops were found to be distinct members of the genus *Polerovirus*, such as phasey bean mild yellows virus (PBMYV) and faba bean polerovirus 1 (FBPV-1) [21, 52].

Poleroviruses have a monopartite single-stranded linear RNA genome ~ 5.6 to 6.2 kb in length containing seven overlapping open reading frames (ORFs). Variability in regions influencing host adaptation and vector transmission, such as ORFs 0 and 5, likely mediates their broad host and vector ranges [40]. Sequencing and phylogenetic analysis of 35 Australian TuYV isolates revealed substantial intraspecific genetic diversity, concentrated most in ORFs 0, 3a, and 5 [20]. In ORF 0, which encodes the silencing suppression protein P0, isolates formed one monophyletic group with 77 to 100% amino acid (aa) sequence identity. In ORF 3a, which encodes protein P3a, which is involved in systemic virus movement, isolates formed two monophyletic groups sharing 69 to 73% aa sequence identity [16, 20]. Most notably, phylogenetic analysis of ORF 5, which encodes the readthrough domain component (P5) of the readthrough protein (P3P5), revealed three variants – P5-I and P5-III – sharing just 45–49% aa sequence identity, and P5-II, which is a recombinant of the other two (Fig. 1A) [20]. TuYV variant P5-III isolates formed a monophyletic group with isolates of brassica yellows virus (BrYV), which has been proposed to represent a separate species and is reported to occur throughout Asia and Australia [20, 58]. Protein P3P5 is involved in vector specificity and transmission, long-distance trafficking, phloem location, and accumulation of the virus in the host plant and subsequent symptom development [16]. Therefore, it is likely that phenotypic differences

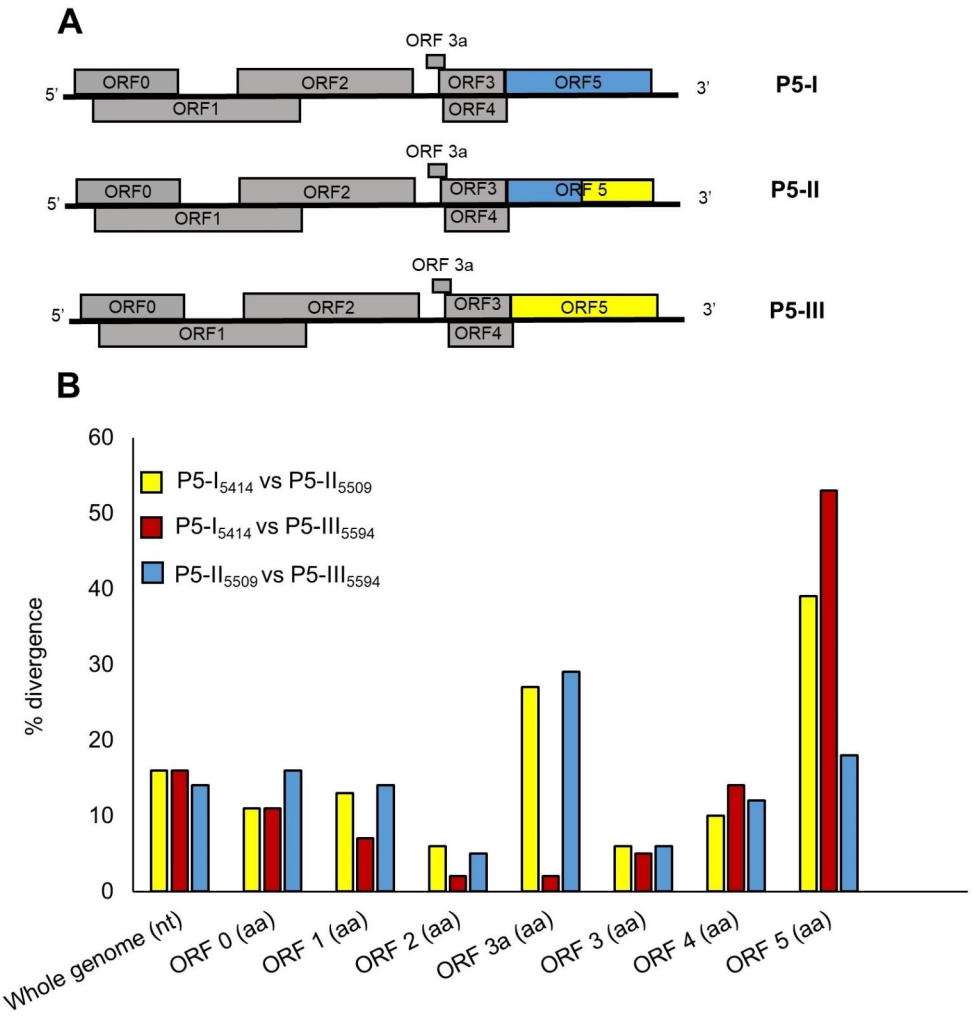

Fig. 1 Schematic representation of the seven open reading frames (ORFs) of turnip yellows virus (TuYV) highlighting the three Australian P5 variants identified by Filardo et. al (2021) (**A**) and percentage divergence across the nt sequence of the whole genome and aa sequence of each ORF between the three TuYV isolates used in this study. P5-I, '5414' (MT586591); P5-II, '5509' (MT586587); P5-III, '5594' (OQ377541) (**B**)

exist between genetic variants of TuYV, and these differences are linked to the function of the proteins in which they vary.

TuYV has a broad host range representing at least 15 plant families [35]. The most researched TuYV host pathosystem is canola (syn. oilseed rape; *Brassica napus*), reflecting the importance of this crop to the global economy and the frequent seed yield and quality damage that TuYV causes [5, 11, 34]. In Australia, TuYV infection also reaches high infection incidences and can have a significant impact on grain legume species (*Fabaceae*) including chickpea (*Cicer arietinum*), pea (*Pisum sativum*), lentil (*Lens culinaris*), and faba bean (*Vicia faba*) [8, 20, 39, 44, 51]. Lupins, especially narrow-leafed lupin (*Lupinus angustifolius*), are also economically important but have not yet been reported to be TuYV hosts. Few studies have compared the host range of different TuYV variants. In a PhD thesis undertaken in the United Kingdom, Newbert [45] identified potential host range differences when challenging four plants from each of five crop species with a representative isolate from each of three TuYV P0 groups identified in an earlier PhD thesis [4]. One of three isolates infected sugar beet (*B. vulgaris* subsp. *vulgaris*), and two of three infected faba bean. Kehoe and Coutts [37] found some minor variation when challenging five to ten plants of 15 *Brassica* cultivars against two Australian isolates representing variants P5-I and P5-II. Therefore, if the epidemiology of TuYV is to be better understood and management strategies to be more effective, a deeper understanding of host specificity of different TuYV variants is important.

TuYV is limited to the phloem of its host, in which it is acquired and transmitted solely by aphids (Hemiptera: Aphididae) in a circulative, nonpropagative manner [46]. The common polyphagous species *Myzus persicae* (green peach aphid) is considered the primary TuYV vector due to its broad overlapping host range, high transmission efficiency, and capacity to rapidly colonise vast areas of crop [11, 48]. Transmission efficiency is defined as the probability of virus transmission by a single aphid, measured by using single-aphid inoculations or multiple vector-transfer tests [27]. However, the TuYV transmission efficiency of 96% commonly cited for *M. persicae* is based on a transmission study done using five aphids per plant for inoculation, which resulted in infection rates that were too high to validly estimate transmission efficiency [26, 48]. In a study testing the reaction of different canola cultivars to inoculation with an Australian TuYV isolate, *M. persicae* transmitted TuYV to up to 20 plants of a susceptible canola cultivar with 12% efficiency in one experiment and 58% in another [13]. Testing *M. persicae* transmission efficiency using single-aphid inoculations and a larger number of plants is required for a more thorough estimation. Australian canola crops are colonized by two other aphid species: *Brevicoryne brassicae* (cabbage aphid) and *Lipaphis pseudobrassicae* (turnip aphid). No studies have specifically tested whether these species transmit TuYV in Australia despite scientific articles and local management guides from Australia referring to *B. brassicae* as a vector, based on studies from abroad. In studies conducted in Eurasia, *B. brassicae* transmitted TuYV to canola at transmission efficiencies of 2 to 3% [2, 3, 48]. However, in another European study, over 270 individuals of *B. brassicae* failed to transmit TuYV, and the virus was no longer detectable in the aphid six days after acquisition access [31]. To the best of our knowledge, no published studies have tested *L. pseudobrassicae* as a TuYV vector, although it has been shown to transmit BWYV [38]. If *B. brassicae* and *L. pseudobrassicae* were able to transmit TuYV, they could be epidemiologically important by providing primary infection foci for secondary spread by *M. persicae* but would likely be insignificant contributors to secondary spread due to their propensity for dense vertical colonization concentrated on crop edges [50].

Variants belonging to the same virus species commonly differ in transmissibility and virulence (degree of damage caused to a host) [15]. The trade-off hypothesis assumes that higher transmissibility and virulence correlate with a higher virus titer and predicts the existence of an optimal virus titer, because an over-virulent infection causes damage that harms the ability of the virus to spread under field conditions, i.e., plant death and a shorter infection duration [23]. Surprisingly, few studies have examined the aspects of this hypothesis. In one such study, some isolates of the polerovirus potato leafroll virus were found to be more transmissible and virulent, and this correlated with an increase in virus titer as measured by enzyme-linked immunosorbent assay (ELISA) [55, 56]. As the polerovirus P3P5 protein is involved in aphid transmission, virus titer, and symptom development [7, 43], the variation in this protein between TuYV variants could translate into differences in virus accumulation, transmissibility, and virulence.

This study was designed to test the hypothesis that isolates representing the three TuYV P5 variants present in Australia have significant phenotypic differences, in particular (i) host range among cultivated species, (ii) transmission by *M. persicae*, *B. brassicae*, and *L. pseudobrassicae*, and (iii) virus titer, transmissibility, and virulence in canola.

Materials and methods

Virus cultures, aphid colonies, and inoculation

Plants of canola cvs. Bonito and 970CL, used for maintenance of aphid colonies and virus cultures, were grown

in potting mix and maintained at 16 to 25°C (daily min. to max.) in a naturally lit insect-proof air-conditioned glasshouse. The *M. persicae* clone was originally collected from a field site in Horsham, Victoria (GPS 36°43'14.8"S 142°09'55.1"E), the *B. brassicae* clone A from Nunile, Western Australia (GPS 31°27'15"S 116°31'27"E) and clone B from Kendenup, Western Australia (GPS 34°26'20.9"S, 117°28'34.2"E), and the *L. pseudobrassicae* clone from Bentley, Western Australia (GPS 32°00'20"S 115°55'49"E). All colonies were maintained inside aphid-rearing cages (BugDorm, Australia) located in an air-conditioned controlled environment room held at 20°C with a 16-h photoperiod. TuYV isolate '5414' (P5-I_{5414}, accession no. MT586591) was collected in 2016 from canola at Deniliquin, New South Wales (35°52'82"S, 144°9566"E). TuYV isolate '5509' (P5-II_{5509}, accession no. MT586587) was collected in 2017 from canola at Jerramungup, Western Australia (GPS 33°57'09"S, 118°53'47"E). TuYV isolate '5594' (P5-III_{5594}, accession no. OQ377541) was collected in 2019 from Chinese cabbage (*Brassica rapa*) at Lowood, Queensland (GPS 27°27'24"S, 152°34'33.7"E). The differences between these three isolates at the whole-genome level (nt sequence) and across each ORF (aa sequence) are represented in Fig. 1B. For all inoculations, aphid apterae were transferred to TuYV-infected plants for a 72-h acquisition access period (AAP) before third- to fourth-instar nymphs were transferred individually to test plants using a fine-tipped paintbrush and given a 24- to 72-h inoculation access period (IAP), depending on the experiment. Pots were then drenched with imidacloprid (0.125 g/liter) to terminate the IAP.

Tissue blot immunoassay (TBIA)

Sap from each plant stem was blotted onto a nitrocellulose membrane (Amersham™ Proton™ 0.45 µm NC, Merck, USA) and tested for TuYV by TBIA using polyclonal antiserum (DSMZ, Germany, cat. no. TuYV-AS-0049) as described by Freeman et al. [22].

ELISA

Two leaf discs (approximately 40 mg of leaf material) were taken from the midrib of the newest fully formed leaf of each plant. Samples were extracted in 1 mL phosphate-buffered saline (PBS), pH 7.4 (10 mM potassium phosphate, 150 mM sodium chloride, Tween 20 at 5 mL/liter, and polyvinyl pyrrolidone at 20 g/liter) using a mixer mill (Retsch, Germany). Of each sample extract, 200 µL was tested for TuYV by double-antibody sandwich ELISA [10] using BWYV polyclonal antiserum (Sediag, France, cat. no. BWY-SRA 5000). All samples were tested in duplicate wells in microtiter plates. The substrate was *p*-nitrophenyl phosphate at 1.0 mg/mL in diethanolamine (pH 9.8) at 100 mL/liter. ELISA absorbance values at 405 nm (E405) were measured in a microplate reader (Bio-Rad Laboratories, USA) at 4 h after addition of substrate.

Total RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted using a QIAGEN RNeasy Plant Mini Kit according to manufacturer instructions (QIAGEN, Australia). To obtain cDNA, reverse transcription was performed using an ImProm-II™ Reverse Transcription System with random primers (Promega, Australia). The cDNA was used to perform PCR amplification using goTaq® DNA polymerase (Promega, Australia) to differentiate between the three TuYV P5 variant isolates. The primer pairs used were TuYV-4841F/TuYV-5328R for TuYV P5-I, TuYV-4841F/BrYV-5476R for P5-II, and BrYV-4680F/BrYV 5476R for P5-III [20]. The three primer sets were used together in a multiplex RT-PCR format, with a 0.2 µM final concentration of each primer under the following cycling conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 50 s, followed by a final extension step at 72°C for 10 min. The PCR bands for TuYV variants were distinguished by size on a 1% agarose gel: P5-I was 487 bp, P5-II was 637 bp, and P5-III was 772 bp.

Host range of TuYV P5 variants

Experiments 1a, 1b, and 1c were done to test the host range of the three TuYV P5 variant isolates by challenging several cultivated plant species with isolates P5-I_{5414}, P5-II_{5509}, and P5-III_{5594}. Plants were grown in potting mix in small pots (100 mm high by 70 mm wide), and seedlings were grown in an air-conditioned glasshouse kept at 16 to 24°C.

In experiment 1a, canola cv. Bonito, field pea cv. Kaspa, lentil cv. Hurricane, faba bean cv. Fiord, chickpea cv. Hattrick, and narrow-leafed lupin cvs. Mandelup and Jurien were inoculated using 10 *M. persicae* apterae per plant and a 72-h IAP. The two narrow-leafed lupin cultivars were chosen based on preliminary evidence suggesting differences in susceptibility to TuYV infection. Inoculations with P5-II_{5509} and P5-III_{5594} were done at the Department of Primary Industries and Regional Development in Western Australia (DPIRD WA), and the plants were tested by ELISA and PCR. Ten plants per species were inoculated, and the experiment was replicated three times. Inoculations with P5-I_{5414} were done at Agriculture Victoria, Horsham, the plants were tested by TBIA, and the experiment was replicated twice. In replicate one, seven to 12 plants per species were inoculated,

and in replicate two, 32 to 40 plants per species were inoculated. Plants were tested 28 and 42 days after inoculation (DAI). This was the only experiment in which an isolate was tested at a different location from DPIRD WA.

Experiment 1b, with all three isolates tested at DPIRD WA, was done to confirm host range differences identified in experiment 1a. Five plants each of faba bean and chickpea were inoculated using 10 *M. persicae* apterae per plant and a 72-h IAP. Plants were tested for the presence of TuYV infection by ELISA and PCR 21 and 35 DAI. This experiment was replicated twice; however, in the second replicate experiment, 15 aphids per plant were used for inoculation.

In experiment 1c, 10 plants each of lettuce cv. Great Lakes, carrot (*Daucus carota*) cv. All Year Round, and chard (*B. vulgaris* subsp. *vulgaris*) cv. Fordhook Giant were inoculated using 10 *M. persicae* apterae per plant and a 72-h IAP. Plants were tested for the presence of TuYV infection by ELISA and PCR 21 and 35 DAI. This experiment was replicated twice; however, in the second replicate experiment, five plants per species were inoculated using 15 aphids per plant.

Ability of three aphid species to transmit TuYV P5 variants

Experiments 2a and 2b were done to assess the capacity of *M. persicae*, *B. brassicae*, and *L. pseudobrassicae* to transmit the three TuYV P5 variant isolates. Canola cv. Bonito plants were grown in potting mix in small pots (100 mm high by 70 mm wide), and seedlings were grown in an air-conditioned glasshouse kept at 16 to 24°C. In experiment 2a, five *M. persicae* apterae per plant and 15 *L. pseudobrassicae* and *B. brassicae*-clone A apterae per plant were used to inoculate plants. In experiment 2b, five *M. persicae* and 20 *B. brassicae*-clone B apterae per plant were used to inoculate plants. A 72-h IAP was used for both experiments. Ten plants were inoculated in each experiment, and both experiments were replicated twice. Plants were tested for TuYV infection by ELISA at 21 and 35 DAI, and by PCR at 35 DAI.

Transmissibility of TuYV P5 variants to canola

Experiment 3 was done to assess the transmissibility of TuYV isolates P5-I_{5414}, P5-II_{5509}, and P5-III_{5594} using single-aphid inoculations of canola seedlings. Three seedling trays, each with 100 cells, were sown with canola cv. Bonito seed to grow up to 100 seedlings for inoculation with each isolate. At the cotyledon stage, each plant was inoculated using a single *M. persicae* aptera per plant and a 24-h IAP. At the end of the IAP, aphids remaining on each seedling were counted to assess movement between seedlings in each tray, but no significant difference in movement was observed between trays in any experiment. At 28 DAI, each plant was tested by ELISA for TuYV infection. This experiment was replicated four times. Z-tests were used to analyse differences between transmission rates for each replicate experiment. Analysis of variance (ANOVA) and Fisher's least significant difference were used to analyse differences in E405 between infected plants for each replicate experiment.

Virulence of TuYV P5 variants on canola

Experiment 4 was done to assess differences in virulence between TuYV isolates P5-I_{5414}, P5-II_{5509}, and P5-III_{5594} by inoculating canola plants and growing them to maturity. Each isolate was inoculated onto 10 plants of canola cv. Bonito at GS12 using 10 *M. persicae* apterae per plant and a 72-h IAP. A further 10 plants were mock inoculated with 10 non-viruliferous *M. persicae* apterae per plant as controls. The experiments were conducted in a naturally lit air-conditioned glasshouse kept between 16 and 26°C. Plants were grown in large pots (230 mm high by 270 mm diameter) in premium potting mix (Baileys, Australia) organised into a factorial randomized block design. Plants were tested for TuYV by ELISA and PCR at the beginning of stem elongation (GS30, leaf samples) and examined for symptoms at GS65. Symptoms were rated on a scale of 0 (no stunting or foliar symptoms) to 5 (stunted plant with severe yellowing and purpling of majority of foliage). Once plants had senesced, the branches of each plant were counted. Then, the pods were removed and counted, and the length of 10 pods per plant was measured. The remaining above-ground biomass (AGB) was put in a drying oven for at least 48 h and then weighed. Pods were then threshed to extract the seed, and the weight of 50 seeds and total seed yield were measured. This experiment was replicated twice.

Results

Host range of TuYV P5 variants

TuYV was detected in almost all canola, field pea, and lentil plants inoculated with each of the three isolates (experiment 1a; Table 1). TuYV was only detected in faba bean plants inoculated with P5-I_{5414} by TBIA and ELISA (19/58 plants across experiments 1a and 1b) and not with P5-II_{5509} and P5-III_{5594}. The occasional faint band was obtained when testing faba bean plants inoculated with P5-II_{5509} by PCR. TuYV was only detected in chickpea plants inoculated with P5-II_{5509} (22/40 plants in experiments 1a and 1b) by both ELISA and PCR, and not with P5-I_{5414} or P5-III_{5594}. TuYV

Table 1 Infection rates of three turnip yellows virus (TuYV) P5 variant isolates inoculated onto plants of nine cultivated plant species using *Myzus persicae*

					TuYV variant[1]		
Species		Cultivar	Expt.	No. of aphids used[2]	P5-I_{5414}	P5-II_{5509}	P5-III_{5594}
Brassica napus	Canola	Bonito	1a	10	39/39[3]	30/30	30/30
Pisum sativum	Field pea	Kaspa	1a	10	28/39	26/30	30/30
Lens culinaris	Lentil	Hurricane	1a	10	48/52	30/30	30/30
Vicia faba	Faba bean	Fiord	1a	10	15/48	0/30	0/30
			1b	10	2/5	0/5	0/5
			1b	15	2/5	0/5	0/5
Cicer arietinum	Chickpea (desi type)	Hattrick	1a	10	0/49	19/30	0/40
			1b	10	0/5	1/5	0/5
			1b	15	0/5	2/5	0/5
Lupinus angustifolius	Narrow-leafed lupin	Mandelup	1a	10	0/36	0/30	0/30
		Jurien	1a	10	29/30	29/30	30/30
Lactuca sativa	Lettuce	Great Lakes	1c	10	6/10	0/10	4/10
			1c	20	5/5	0/5	2/5
Daucus carota subsp. *sativus*	Carrot	All Year Round	1c	10	0/10	0/10	0/10
			1c	20	0/5	0/5	0/5
Beta vulgaris subsp. *vulgaris*	Chard	Fordhook Giant	1c	10	0/10	0/10	0/10
			1c	20	0/5	0/5	0/5

[1]Isolates used in this study and their GenBank accession numbers: '5414', MT586591; '5509', MT586587; '5594', OQ377541

[2]*M. persicae* were given a 72-h acquisition access period on a TuYV-infected canola cv. Bonito plant before being transferred to test plants for a 72-h inoculation access period

[3]Number of plants infected/number of plants inoculated. Plants inoculated with isolate 5414 in experiment 1a were tested by tissue blot immunosorbent assay. All other plants in all other experiments were tested by enzyme-linked immunosorbent assay

Table 2 Infection rates of three turnip yellows virus (TuYV) P5 variant isolates in canola (*Brassica napus*) plants following inoculation with three aphid species

				TuYV variant[1]		
Aphid species	Experiment	Clone	No. of aphids used[2]	P5-I_{5414}	P5-II_{5509}	P5-III_{5594}
Myzus persicae	2a	A	5	20/20[3]	20/20	20/20
	2b	A	5	20/20	20/20	20/20
Brevicoryne brassicae	2a	A	15	0/20	0/20	0/20
	2b	B	20	0/20	0/20	0/20
Lipaphis pseudobrassicae	2a	A	15	0/20	0/20	0/20

[1]Isolates used in this study and their GenBank accession numbers: '5414', MT586591; '5509', MT586587; '5594', OQ377541

[2]Aphids were given a 72-h acquisition access period on a TuYV-infected canola cv. Bonito plant before being transferred to test plants for a 72-h inoculation access period

[3]Number of plants infected/number of plants inoculated. Pooled results of two replicates of 10 plants each

was not detected in any inoculated narrow-leafed lupin cv. Mandelup plants (experiment 1a), although occasional faint bands were obtained when these plants were tested by PCR. In contrast, TuYV was detected in almost all narrow-leafed lupin cv. Jurien plants inoculated with each of the three isolates. TuYV was only detected in lettuce plants inoculated with P5-I_{5414} (11/15 plants in experiment 1c) and P5-III_{5594} (6/15 plants) by ELISA and PCR, and not with P5-II_{5509}. TuYV was not detected by serology or PCR in any carrot or chard plants inoculated with TuYV.

Ability of three aphid species to transmit TuYV P5 variants

All canola plants inoculated with each of the three P5 variant isolates using five *M. persicae* apterae became infected (Table 2). In contrast, no canola plants (180 plants total) became infected with any of the three P5 variants when inoculated using either 15 apterae per plant of *B. brassicae*-clone A, 20 per plant of *B. brassicae*-clone B (total of 2100 aphids of this species), or 15 per plant of *L. pseudobrassicae* (total of 900 aphids).

Transmissibility of TuYV P5 variants to canola

In each of the four experimental replicates, the transmission rate of P5-I_{5414} was significantly higher compared to P5-II_{5509} and P5-III_{5594}, which were not significantly different from each other (Table 3, Supplementary Fig. S1). In each of the four experimental replicates, the E405 was significantly higher in plants infected with P5-I_{5414} than with P5-II_{5509} and P5-III_{5594}, and significantly higher in plants infected with P5-II_{5509} than with P5-III_{5594} (P5-I_{5414} > P5-II_{5509} > P5-III_{5594}).

Virulence of TuYV P5 variants on canola

The E405 was significantly higher in P5-I_{5414}-infected plants than in P5-II_{5509}- and P5-III_{5594}-infected plants in both experiment replicates, and significantly higher in P5-II_{5509}-infected plants than P5-III_{5594}-infected plants in the first replicate, but not in the second (Table 4, Supplementary Fig. S2A). Plants infected with P5-I_{5414} consistently produced symptoms that were significantly more severe than those in plants infected with P5-II_{5509} and P5-III_{5594}, which were often symptomless (Fig. 2B, Supplementary Fig. S2B). Symptom severity positively correlated with E405 in both replicates ($R^2 = 0.72$ and 0.43, respectively). Plants infected with P5-I_{5414} were significantly shorter than plants infected with P5-II_{5509} and P5-III_{5594} and uninfected control plants in both replicates (Fig. 2A, Supplementary Fig. S2C). P5-I_{5414}-infected plants had significantly fewer branches, fewer pods, and shorter pods than P5-III_{5594}-infected plants and uninfected control plants in replicate 1, but not in replicate 2. P5-I_{5414}-infected plants had lower seed weight than P5-II_{5509}-infected plants and uninfected control plants, and P5-III_{5594}-infected plants had significantly lower seed weight than uninfected control plants in the first replicate, but not the second. In the first experimental replicate, the seed yield of P5-I_{5414}-infected plants was 38% less than that of uninfected control plants and 24% less than that of P5-II_{5509}-infected plants but was not significantly different from the seed yield of P5-III_{5594}-infected plants (Supplementary Fig. S2D). Seed yields of P5-II_{5509}- and P5-III_{5594}-infected plants were 24% and 19% less, respectively, than those of uninfected control plants but were not significantly different from each other. In the second replicate, seed yields in plants infected with P5-I_{5414}, P5-II_{5509}, and P5-III_{5594} were 33%, 32%, and 31% lower, respectively, than those of uninfected control plants, but there was no significant difference between each variant. The remaining dry AGB did not differ significantly between infected and uninfected plants in either replicate.

Table 3 Transmission rate and mean enzyme-linked immunosorbent assay absorbance readings (E405) of three turnip yellows virus (TuYV) P5 variant isolates when inoculated onto canola (*Brassica napus*) seedlings using a single *Myzus persicae* per plant

	Rep 1			Rep 2			Rep 3			Rep 4		
TuYV variant[1]	Plants infected[2]	%[3]	E405[4]	Plants infected	%	E405	Plants infected	%	E405	Plants infected	%	E405
P5-I_{5414}	65/70	93 α	1.10 α	81/100	81 α	1.39 α	78/96	81 α	0.94 α	60/82	73 α	0.50 α
P5-II_{5509}	64/82	78 β	0.80 β	55/100	55 β	0.92 β	58/96	60 β	0.62 β	47/84	56 β	0.22 β
P5-III_{5594}	56/78	72 β	0.54 γ	44/100	44 β	0.69 γ	65/96	68 β	0.44 γ	40/73	55 β	0.11 γ

[1]Isolates used in this study and their GenBank accession numbers: '5414', MT586591; '5509', MT586587; '5594', OQ377541

[2]Aphids were given a 72-h acquisition access period on a TuYV-infected canola cv. Bonito plant before being transferred to test plants for a 24-h inoculation access period

[3]Greek letters indicate significance at $P < 0.05$ based on Z-tests

[4]Mean enzyme-linked immunosorbent assay absorbance readings at 405 nm of infected plants only. Greek letters indicate significance at $\alpha = 0.05$ based on ANOVA and Fisher's least significant difference

Table 4 Virus titer as indicated by E405 and infection symptoms on canola (*Brassica napus*) cv. Bonito plants inoculated with three turnip yellows virus (TuYV) P5 variants. Mean values are from 12 plants per treatment per experiment replicate

	Replicate 1				Replicate 2			
Variable	Uninfected	P5-I_{5414}[1]	P5-II_{5509}	P5-III_{5594}	Uninfected	P5-I_{5414}	P5-II_{5509}	P5-III_{5594}
E405[2]	0.11d	1.18a	0.6b	0.28c	0.16c	1.42a	0.58b	0.55b
Symptom severity[3]	0c	3.8a	1b	0.5bc	0c	2.9a	1.0b	0.4bc
Height (cm)	129a	118.5b	126.3a	128a	132.4a	115.1b	126.9a	126.8a
No. of branches	26a	21b	24ab	25a	56a	55a	56a	52a
No. of pods	379a	319b	339ab	367a	603a	519a	513a	501a
Pod length (cm)	6.54a	5.99b	6.49ab	6.52a	6.1a	5.8a	6.0a	6.1a
50 seed weight (mg)	219a	191c	211ab	198bc	257a	242a	252a	255a
Seed yield (g)	13.04a	8.03c	10.51b	9.93bc	20.4a	13.7b	13.9b	14.0b
Dry AGB[4] (g)	16.09a	15.53a	14.85a	16.48a	32.4a	33.1a	28.9a	31.5a

[1]Isolates used: '5414', P5 variant 1 (MT586591); '5509', P5 variant 2 (MT586587) ; '5594', P5 variant 3 (OQ377541)

[2]Enzyme-linked immunosorbent assay absorbance readings at 405 nm at the beginning of stem elongation

[3]At full flowering, symptom severity for each plant was rated on a scale of 0 (no stunting or foliar symptoms) to 5 (stunted plant with severe yellowing and purpling of majority of foliage)

[4]Above-ground biomass (AGB) of all remaining stem material after pods were removed

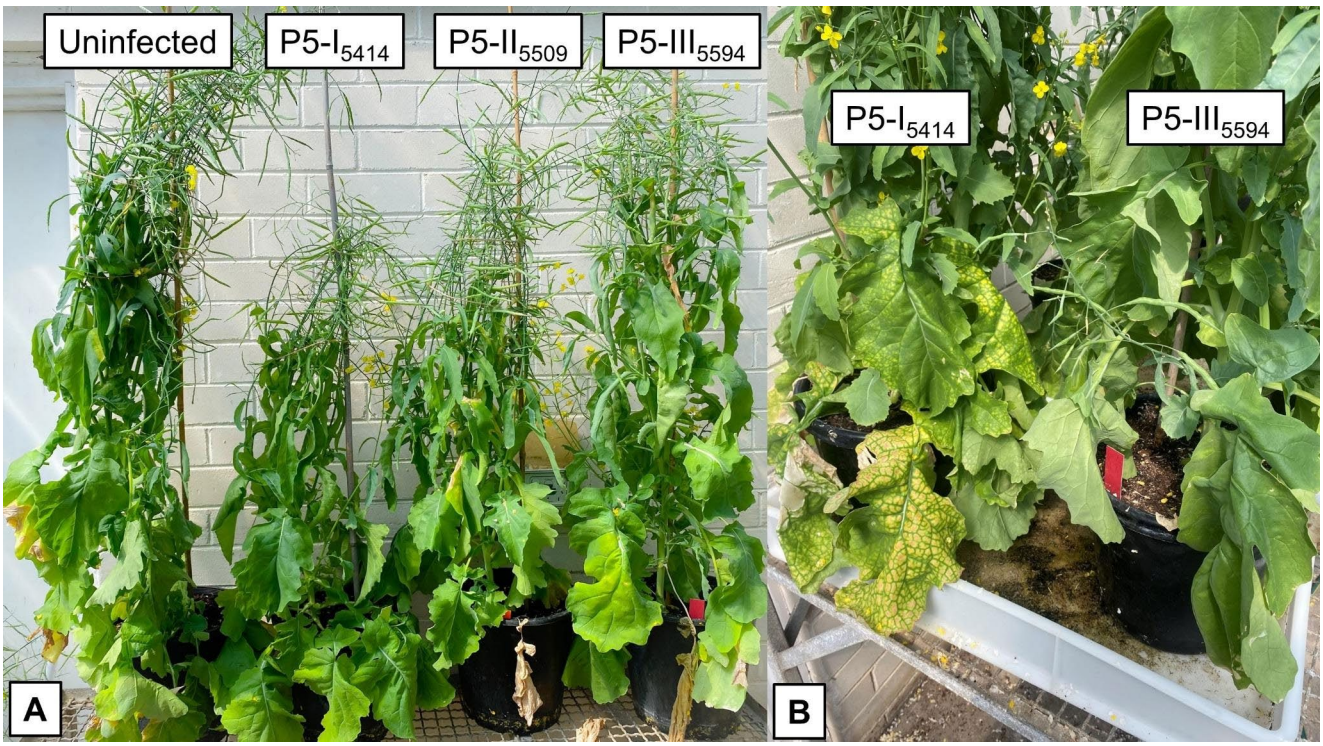

Fig. 2 TuYV infection symptoms were always more severe in canola (*Brassica napus*) plants infected with TuYV P5 variant I isolate '5414' (MT586591) than in plants infected with P5 variant II isolate '5509' (MT586587) and P5 variant III isolate '5594' (OQ377541) or uninfected control plants. Predominant symptoms were plant stunting (**A**) and yellowing and purpling symptoms beginning in the lower leaves (**B**)

Discussion

This study uncovered phenotypic differences between representative isolates of the three Australian P5 variants. The isolates differed in their ability to infect some important cultivated plant species; only P5-I_{5414} readily infected faba bean, only P5-II_{5509} infected chickpea, and only P5-I_{5414} and P5-III_{5594} infected lettuce. Furthermore, all isolates infected narrow-leafed lupin cv. Jurien but not cv. Mandelup, providing the first report of this species as an experimental host and evidence of strong resistance in its gene pool. Secondly, when using single-aphid inoculations to assess the transmissibility of each isolate, P5-I_{5414} had a higher mean transmission rate (82%) across four replicate experiments than

P5-II_{5509} (62%) and P5-III_{5594} (59%). This also provides a more accurate measure of *M. persicae* transmission efficiency than previous studies. Finally, when canola cv. Bonito plants were inoculated and allowed to grow to maturity, P5-I_{5414} induced more-severe foliar symptoms and stunting than P5-II_{5509} or P5-III_{5594}. In one of two replicate experiments, this difference also translated to a greater reduction in seed yield. Consistent across the study, P5-I_{5414} reached a higher E405 than P5-II_{5509}, which, in turn, reached a higher E405 than P5-III_{5594}. Although the differences in E405 most likely represent differences in *in planta* virus titers between the three isolates, differing antibody affinities for each isolate cannot be ruled out as another source of variation. Further questions have been raised by the results of this study that warrant future research and are discussed below.

One objective of this study was to investigate whether the P5 variant grouping correlated with phenotypic differences and thus whether it could be a coherent method for strain classification. As just one isolate from each P5 variant group was used, comparisons with previous studies are required to provide an initial assessment. As mentioned above, a key phenotypic distinction between the isolates tested in this study was host specificity; only TuYV isolate P5-II_{5509} infected chickpea, and only P5-I_{5414} infected faba bean. However, Filardo et al. [20] detected many P5-I and P5-III isolates infecting chickpea and one P5-III isolate infecting faba bean in field crops. One of the variant P5-I isolates infecting chickpea, '5511' (accession no. MT586596), was fully sequenced and shares only 88 to 90% whole-genome nt sequence identity with the other eight P5-I isolates sequenced, including 79 to 80% aa identity in P0, 83 to 87% in P1, and 89 to 91% in P5. (Interestingly, '5511' has 99% aa sequence identity in P5 to FBPV-1 isolate '5253' [NC_055495].) The ability of '5511' to infect chickpea, unlike P5-1_{5414}, may be associated with '5511' having more aa sequence similarity in P0 and P1 to the chickpea-infecting isolate P5-II_{5509}. Moreover, at the whole-genome sequence level, '5511' is most closely related to P5-II isolates. Therefore, it is likely that variation in regions other than P5 determines the infectivity of TuYV in chickpea. In this study, none of the three TuYV isolates infected chard or carrot. However, all three European TuYV isolates described in Newbert's 2016 PhD thesis infected carrot, and one isolate infected sugar beet [45]. TuYV was also detected coinfecting sugar beet with other poleroviruses in Sweden [47]. Unfortunately, whole-genome sequences of these isolates are not available online for comparative analysis. Yoshida and Tamada [60] showed that 'BrYV' isolate CC1 could infect one sea beet (*B. vulgaris* subsp. *maritima*) accession, but not three other sea beet accessions, four sugar beet cultivars, or a chard cultivar. Furthermore, Filardo et al. [20] detected and sequenced a TuYV P5-I isolate that was able to infect table beet, but this isolate varied genetically from other Australian P5-I isolates and had a high degree of nt sequence similarity to some European isolates. The 'BrYV-like' TuYV isolate P5-III_{5594} infected lettuce in this study, but in a study in Japan, the investigators failed to infect lettuce with either of two different 'BrYV' isolates, one of which (CC1) shares 98% whole-genome sequence nt identity with P5-III_{5594} [60]. The inability of 'BrYV' to infect lettuce has been proposed as feature for distinguishing species [62]. The evidence presented above suggests that a combination of both host and viral genotype may explain inconsistencies in TuYV infectivity among the studies discussed. Given the likely importance of other proteins for determining host adaptation [40], it would be unsurprising if host specificity were being driven by genetic variation not represented by P5 grouping. Moreover, the host genotype/cultivar is highly likely to influence TuYV infectivity (i.e., host resistance), especially in genetically diverse species such as *B. vulgaris* [24]. The influence of the host cultivar was exemplified in this study by the contrasting infectability of narrow-leafed lupin cvs. Jurien *versus* Mandelup, which have significantly different lineages [14]. Sequencing and phenotyping of isolates found infecting chickpea, faba bean, lettuce, and *B. vulgaris* will help elucidate the relationship between TuYV genetic variation and host specificity. Challenging more cultivars of the diverse range of grain and pasture legume species grown in Australia with multiple TuYV variants may reveal further complexities in host specificity as well as host resistance useful for breeding programs. Coinfection with multiple TuYV variants seems to be common in canola crops, especially in the south-east of Australia [20], and coinfection with viruses of multiple species is a regular occurrence in Australian grain legume crops [39]. Transmission of one virus or virus variant can be facilitated by the presence of another and thus allow an otherwise unsuitable host or vector to be utilized, and this promotes development of new variants *via* recombination [1, 25]. Therefore, the role of mixed infections in TuYV phenotypic expression needs to be seriously considered.

Of the three aphid species that colonise canola, just *M. persicae* was able to transmit the three TuYV variant isolates, and *B. brassicae* and *L. pseudobrassicae* were not. As mentioned in the Introduction, two to three in 100 *B. brassicae* could transmit TuYV in studies conducted in Europe and Iran [2, 48]. However, in experiments 2a and 2b in our study, no transmission occurred despite inoculating plants with a total of 2100 *B. brassicae* and 900 *L. pseudobrassicae* apterae. The inconsistencies between various studies examining the capability of *B. brassicae* to transmit TuYV could be due to the use of (i) *B. brassicae* clones unable to transmit TuYV, as observed with the 'red race' of *Acyrthosiphon pisum*, which was unable to transmit TuYV whereas

the 'green race' could in a study described by Schliephake et al. [48], and (ii) TuYV variants that are not transmissible specifically by *B. brassicae*, i.e., intraspecies vector specificity, observed in related viruses and driven by genetic differences in P5 [e.g., 6, 57]. This is the first study to test *B. brassicae* and *L. pseudobrassicae* as TuYV vectors in Australia and highlights the risks of making assumptions about virus biology in geographically distinct growing regions like Australia based on studies from abroad. TuYV reaches high infection incidences in grain legume crops, which are colonised by several other aphid species for which little is known about their ability to transmit TuYV. *Aphis craccivora* caught near a faba bean field and immediately transferred to faba bean cv. Fiord seedlings transmitted a virus that reacted to BWYV antisera [49], but the virus could have been PBMYV [52]. Other legume-colonising species, such as *A. pisum* and *Aulacorthum solani*, have been shown to transmit TuYV under experimental conditions [32, 48]. Therefore, the capacity of aphid species other than *M. persicae* to transmit different TuYV variants to leguminous hosts should be investigated, especially given the broad diversity in P5 and its role in aphid transmission.

Inoculating canola cv. Bonito plants (as an indicator host) with more TuYV isolates representing each of the P5 variants and measuring E405, transmissibility, and virulence would increase confidence in the relationships observed in this study and test the hypothesis that P5 variation is playing a key role in the variation. Furthermore, to determine if differences between variants are consistent across host genotypes or whether there is host-specific adaptation, similar experiments should be conducted with other important hosts, such as field pea and lentil. The observed correlation between higher E405 and higher transmissibility and virulence of P-I_{5414} supports the assumptions of the trade-off hypothesis described in the Introduction. However, isolate P5-II_{5509} had similar transmissibility and virulence to P5-III_{5594}, despite the former consistently reaching a higher E405, suggesting a non-linear relationship between virus titer, transmissibility, and virulence under the experimental conditions used. To better understand the relationship between virus titer, acquisition, and transmission, future studies should test a range of AAPs and measure the quantity of virus in the source leaves and that subsequently acquired by the aphid [23]. Identifying virus titer thresholds at which both acquisition/transmission and virulence is reduced will help to define levels of TuYV resistance that can guide resistance screening and breeding efforts. Furthermore, other factors that govern titer, transmissibility, and virulence of TuYV, such as host tolerance, time of infection, presence of other stressors, and climatic factors, should also be considered [12, 53, 61]. As no plants died from infection in this study, no conclusion can be made regarding the fitness cost (reduced success in the field) that might be incurred by isolate P-I_{5414} associated with its higher virulence. Fitness costs may be more obscure and situation-dependent, e.g., severely stunted canola plants may be shaded out by healthier plants in thick canopy crops and be less available as a source [33]. In addition, a higher virus titer and more-intense foliar symptoms may influence the attractiveness or palatability of infected plants for aphids, thus impacting their rate of spread in a crop [9, 41].

The genetic and phenotypic variation among members of the species *Turnip yellows virus* is still poorly understood, and different conceptual frameworks should be considered when designing future experiments in this area of research, i.e., the quasispecies concept [17]. Nevertheless, a better understanding of this variation is essential to develop management strategies that are effective long term. For example, developing broad-spectrum TuYV resistance must include challenging lines with genetically diverse isolates. Studies that have identified, characterised, and/or created prebreeding tools for sources of TuYV resistance thus far have used just a single isolate, which risks development of less-durable strain-specific resistance [12, 29, 30, 36].

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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