Biological and Molecular Properties of a *Turnip mosaic virus* (TuMV) Strain that Breaks TuMV Resistances in *Brassica napus*

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Abstract

A new resistance-breaking strain of *Turnip mosaic virus* (TuMV) overcomes TuMV resistance genes that currently suppress spread of this virus in *Brassica napus* crops in the Liverpool Plains region of eastern Australia. Isolates 12.1 and 12.5 of this strain and three other isolates in TuMV pathotypes 1 (NSW-2), 7 (NSW-1), and 8 (WA-Ap1) were inoculated to plants of 19 *B. napus* cultivars and one breeding line. All plants of these cultivars and the breeding line proved susceptible to 12.1 and 12.5 but developed only resistance phenotypes with WA-Ap1 or mostly resistance phenotypes with NSW-1 and NSW-2. Five different TuMV resistance phenotypes occurred either alone or segregating in different combinations. When these five isolates were inoculated to plants of nine other crop or wild Brassicaceae spp. and four indicator hosts in other families, 12.1 and 12.5 resembled the other three in inducing TuMV resistance

phenotypes in some Brassicaceae spp. but not others, and by inducing extreme resistance phenotypes in all inoculated plants of *B. oleracea* var. *botrytis* and *Raphanus sativus*. Therefore, the overall resistance-breaking properties of 12.1 and 12.5 were restricted to *B. napus*. When isolates 12.1, 12.5, and WA-Ap1 and additional Australian isolate WA-EP1 were sequenced and complete genomes of each compared, 12.1 and 12.5 grouped separately from the other 2 and from all 23 Australian isolates with complete genomes sequenced previously. In addition, there was evidence for at least six separate TuMV introductions to Australia. Spread of this *B. napus* resistance-breaking strain poses a significant threat to the *B. napus* oilseed industry. Breeding *B. napus* cultivars with resistance to this strain constitutes a critical priority for *B. napus* breeding programs in Australia and elsewhere.

Turnip mosaic virus (TuMV; family *Potyviridae*; genus *Potyvirus*) causes damaging disease in many kinds of *Brassica* and other crops worldwide, including economically important vegetable, oilseed, biofuel, forage, and ornamental crops (Edwardson and Christie 1991; Hardwick et al. 1994; Tomlinson 1987). It also commonly infects a wide range of weed species. It is transmitted nonpersistently by more than 40 different aphid species and has a wide natural host range. The disease symptoms it causes depend upon TuMV strain, host plant, and environmental conditions. They are diverse and often include vein clearing, mosaic, necrosis, plant stunting, and plant death (Tomlinson 1987; Edwardson and Christie 1991). Considerable TuMV-induced losses in yield and quality of produce are recorded in diverse crop species (Hardwick et al. 1994; Hunter et al. 2002; Jiang et al. 2010; Shattuck and Stobbs 1987; Spence et al. 2007; Walsh and Tomlinson 1985). Examples of *Brassica* spp. that TuMV infects include *Brassica napus* var. *napus* (canola or rapeseed), *B. napus* var. *napobrassica* (swede), *B. carinata* (Ethiopian mustard), *B. juncea* (Indian mustard), *B. nigra* (black mustard), *B. oleracea* var. *botrytis* (cauliflower), *B. oleracea* var. *capitata* (cabbage), *B. oleracea* var. *italica* (broccoli), *B. oleracea* var. *gemmifera* (Brussels sprout), *B. oleracea* var. *acephala* (kale), *B. rapa* var. *oleifera* (oilseed rape), *B. rapa* var. *rapa* (turnip), and *B. rapa* var. *pekinensis* (Chinese cabbage).

Brassica spp. have one or two of three types of genomes (A, B, and C) and these occur in different combinations: AA (*B. rapa*), BB (*B. nigra*), CC (*B. oleracea*), AABB (*B. juncea*), AACC (*B. napus*), and BBCC (*B. carinata*) (U 1935). Single dominant resistance genes giving pathotype-specific TuMV resistance have been identified on the A genome of *B. napus* and *B. rapa* and include *TuRB01*, *TuRB03*, *TuRB04*, and *TuRB05* in *B. napus* and *TuRB01b* in *B. rapa*. A recessive

TuMV resistance gene (*retr01*) and a dominant TuMV resistance gene (*conTR01*), which provide broad-spectrum resistance to most TuMV pathotypes in *B. rapa*, are also found on the A genome (Rusholme et al. 2007; Walsh and Jenner 2002, 2006; Walsh et al. 2002). Quantitative trait locus *TuRB02* has been mapped to the C genome of *B. napus* (Walsh and Jenner 2002, 2006). A dominant TuMV resistance gene (*TuBJU01*) was identified in *B. juncea* (Nyalugwe et al. 2015a, 2016c) but whether it is on its A or B genome is unknown. A TuMV strain pathotyping system based on symptom reactions in plants of *B. napus* differentials carrying TuMV pathotype-specific resistance (Jenner and Walsh 1996; Walsh 1989) has been widely used. Another TuMV strain pathotyping system based on symptom differences in *B. rapa* var. *pekinensis* differentials is also available (Providenti 1980; Yoon et al. 1993).

TuMV occurs in all Australian states, where it infects plants of oilseed, forage, and vegetable *Brassica* spp.; other species of the family Brassicaceae; and various weeds, grain legumes, and ornamentals (Buchen-Osmond et al. 1988; Coutts and Jones 2000; Gibbs et al. 2008; Latham et al. 2003; Schwinghamer et al. 2014). The phylogeny of Australian TuMV isolates has been studied extensively using complete genomes which all fitted into the currently recognized World-B or Basal-B within-species phylogroups (Nyalugwe et al. 2015b; Yasaka et al. 2015). In the southwest of Western Australia (WA), surveys of *B. napus* and vegetable *Brassica* crops and the weed *Raphanus raphanistrum* (wild radish) found widespread infection with TuMV in *B. napus* and *R. raphanistrum*. However, incidences in *B. napus* were generally low (Coutts and Jones 2000; Latham et al. 2003). These low incidences were attributed to widespread occurrence of strain-specific TuMV resistances in Australian *B. napus* cultivars (Coutts et al. 2007), which reflects the situation in *B. napus* cultivars and germplasm on other continents (Hughes et al. 2002; Nyalugwe et al. 2014). In northern New South Wales (NSW), crop surveys undertaken between 1999 and 2007 in the Liverpool Plains region found frequent infection in *B. juncea* and the virus was also detected in *B. rapa* subsp. *rapa* but never in *B. napus*. TuMV infection was frequent in the Brassicaceae family weeds *Rapistrum rugosum* (turnip weed) and *Hirschfeldia incanum* (Buchan weed) but infrequent in *Raphanus raphanistrum* plants (Schwinghamer et al. 2014). However, in surveys undertaken in 2012 and 2013, van Leur et al. (2014) reported high incidences of TuMV infection associated with obvious leaf mosaic and leaf deformation symptoms in *B. napus* crops in

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the Liverpool Plains region but little or no TuMV infection of *B. napus* crops elsewhere in NSW. Preliminary inoculations with isolates belonging to this strain onto diverse *B. napus* germplasm reproduced these symptoms but found no virus resistance, suggesting the presence of a new virulent, resistance-breaking TuMV strain (Guerret et al. 2016). When one isolate of this strain (12.5) was inoculated to plants of *B. carinata* with a known localized resistance phenotype, this resistance was overcome (Nyalugwe et al. 2016b). By contrast, when this and another isolate of the same strain (12.1) were inoculated to *B. juncea* plants carrying resistance gene *TuBJU01*, they did not overcome it (Nyalugwe et al. 2016a,c).

This article explores the effectiveness of the new resistance-breaking strain from the Liverpool Plains against TuMV resistances present in plants of (i) 20 *B. napus* cultivars or breeding lines produced by different plant breeding programs and (ii) nine other Brassicaceae spp. For this, the phenotypic responses of plants infected with unpathotyped isolates 12.1 and 12.5 were compared with those obtained with isolates belonging to pathotypes 1 (NSW-2), 7 (NSW-1), and 8 (WA Ap-1). In addition, the complete genomes of isolates 12.1, 12.5, and 2 other previously unsequenced Australian TuMV isolates (WA-Ap1 and WA-EP1) were obtained and their molecular characteristics compared with those of 23 Australian TuMV isolates sequenced previously.

Materials and Methods

Glasshouse-grown plants, virus isolates, and inoculations. Seed of the culture host *Sinapis alba* (white mustard), and experimental plants consisting of 19 cultivars and one breeding line (06-63777) of *B. napus* and 12 lines of nine other Brassicaceae spp. were obtained from a Brassicaceae seed collection maintained at the University of Western Australia. Seed were sown and plants grown in square pots (8.8 by 8.8 by 17.2 cm) filled with pasteurized potting mix composed of crushed pine bark/peat/sand (2.5:1.0:1.5). Plants were grown in insect-proofed, air-conditioned glasshouses maintained at 22 to 23°C. The TuMV isolates used were WA-Ap1 from pathotype 8 (Coutts and Jones 2000), NSW-1 from pathotype 7, NSW-2 from pathotype 1 (Kehoe et al. 2010), unpathotyped isolates 12.1 and 12.5 (Nyalugwe et al. 2016c), and unpathotyped isolate WA-EP1 obtained in 2013 from an infected plant of *B. oleracea* var. *acephala* plant growing in East Perth, Western Australia. All isolates were maintained by repeated manual inoculation onto *S. alba* plants; isolate WA-EP1 was only used in molecular studies. To inoculate plants, leaves from infected culture plants were ground in phosphate extraction buffer containing monosodium phosphate (NaH_2PO_4) at 3 g liter⁻¹ and disodium hydrogen phosphate (Na_2HPO_4) at 11.5 g liter⁻¹, and the infective sap was mixed with celite (diatomaceous earth) before being rubbed onto leaves of healthy plants.

Host responses to inoculation. For the *B. napus* study, infective leaf sap containing TuMV isolates NSW-1, NSW-2, WA-Ap1, 12.1, and 12.5 was inoculated at the two- to three-true-leaf stage to plants of each of 19 cultivars and breeding line 06-63777 (four to seven replicate plants per line). Two of these cultivars ('Ag Muster' and 'AV Opal') and 06-63777 had also been used in a previous study of host responses of diverse cultivars to inoculation with isolate WA-Ap1 (Nyalugwe et al. 2014). Two control plants per cultivar or breeding line were mock inoculated with healthy leaf sap and their appearance compared with that of symptomatic plants. All plants were observed for virus symptoms at weekly intervals for a 6-week period after inoculation. Samples from inoculated leaves were tested for TuMV by enzyme-linked immunosorbent assay (ELISA) 2 weeks after inoculation and from tip leaves tested similarly 3 to 4 weeks after inoculation. Uninoculated leaves recorded as uninfected were retested after 6 weeks. For the study with nine other species of Brassicaceae and four indicator host species, infective leaf sap containing each of isolates WA-Ap1, 12.1, and 12.5 was inoculated to each of them (four to seven replicate plants per line); host response data for the same species following inoculation with isolates NSW-1 and NSW-2 was obtained from Nyalugwe et al. (2015b) and used for comparison. When each inoculation was done, a single control plant of each of the other Brassicaceae spp. and the four indicator host species was left uninoculated as a healthy control. The growth stage of plants inoculated and symptom observations were as described above. Samples

from inoculated and uninoculated leaves were tested for TuMV by ELISA 3 to 4 weeks after inoculation. No virus was ever detected in leaf samples from mock-inoculated or uninoculated control plants.

Resistance phenotype classification. For each of the host response investigations, after symptoms had been recorded, the reactions of each species to TuMV infection were classified according to susceptibility and resistance phenotypic codes described previously for *Brassica* spp. by Jenner and Walsh (1996), and added to subsequently by Coutts et al. (2007) and Kehoe et al. (2010). The coded phenotypic responses that developed in this study were + = systemic infection without necrosis, O = extreme resistance with no detectable infection, R = infection of inoculated leaves without necrosis but no detectable systemic infection, R_N = local necrotic spot lesions in inoculated leaves but no detectable systemic infection, R_{N/+} = local necrotic spot lesions in inoculated leaves and systemic infection without necrosis, +_N = systemic infection with necrosis, and +_{ND} (systemic hypersensitivity and plant death).

ELISA. Leaf tip samples from inoculated or uninoculated top leaves were tested for the presence of TuMV antigen by double-antibody sandwich ELISA (Clark and Adams 1977). For this, they were extracted (1 g per 20 ml) using a mixer mill (Retsch) in phosphate-buffered saline (10 mM potassium phosphate and 150 mM sodium chloride, pH 7.4) and Tween 20 with polyvinylpyrrolidone (Retsch) at 5 ml liter⁻¹ and 20 g liter⁻¹. All samples were tested in duplicate wells in microtiter plates, and sap from TuMV-infected and healthy *S. alba* leaf samples was included in paired wells to provide positive and negative controls, respectively. The substrate used was *p*-nitrophenyl phosphate at 0.6 mg ml⁻¹ in diethanolamine, pH 9.8, at 100 ml⁻¹. Absorbance values at 405 nm were measured in a Bio-Rad microplate reader (model 680; Bio-Sys). Absorbance values of positive samples were always more than three times those of the healthy sap control. Polyclonal antiserum specific to TuMV obtained from DSMZ was always used.

RNA extraction. Total RNA was extracted from frozen-dried specimen samples of the four Australian TuMV isolates lacking complete genomes: WA-Ap1, 12.1, 12.5, and WA-EP1. These extractions were done using a ZR Plant RNA MiniPrep™ kit (Zymo Research) according to the manufacturer's instructions. Crude RNA was treated with RNase-free DNase (Invitrogen) and further cleaned using a ZR column. The concentration and purity of extracted RNA were measured with a spectrophotometer (ND-1000, NanoDrop Technologies) and Qubit (Invitrogen). RNA integrity was confirmed before library preparation using RNA screen Tape (TapeStation 2200; Agilent Technologies).

High-throughput RNA sequencing. The libraries were prepared from 1 µg of total RNA using a TruSeqstranded Total RNA Sample Preparation kit with RiboZero Plant (Illumina). This involved subjecting the RNA to depletion of ribosomal RNA using rRNA Removal Mix-Plant. RNA was then fragmented and primed with random hexamers. First-strand cDNA synthesis was done using a mixture of Superscript II and Actinomycin D to allow RNA-dependent synthesis but inhibit DNA-dependent synthesis, respectively. The second-strand cDNA synthesis was done using dUTP (instead of dTTP) to create stranded cDNA. Libraries were then adenylated at the 3' end, and set A and B adaptors containing the identifier sequences and flow cell binding sequences were ligated to both ends. The cDNA fragment was enriched by 15 cycles, as described by Maina et al. (2016). The final size and concentration of each library was verified using Qubit and D1000 ScreenTape (TapeStation 2200; Agilent). The prepared libraries were sent to Macrogen Inc., where they were subjected into HiSeqEquation 2500 using a TruSeq SBS KIT V4 (Illumina) with 151 cycles to generate paired-end reads in a multiplex of 24 samples in one lane.

Viral sequence and genome analysis. After sequencing, RNA-Seq reads were first subjected to quality control using CLC Genomics Workbench 6.5 (CLCGW) (CLC Bioinformatics; Qiagen), with the quality scores limit set to 0.01, maximum number of ambiguities to two, and removing any reads shorter than 60 nucleotides (nt). Contigs were assembled using the *Denovo* assembly method of CLCGW, with settings of automatic word size, automatic bubble size, minimum contig length 1,500, mismatch cost two, insertion cost three, deletion cost three, length fraction 0.5, and similarity fraction 0.9. Contigs were sorted by length and the longest subjected to a BLAST search and a pairwise sequence comparison (Altschul et al. 1990;

Bao et al. 2014). In addition, trimmed reads from CLCGW were imported into Geneious 8.1.7 (Biomatters Ltd.) (Kearse et al. 2012) and provided with a reference sequence obtained from GenBank. Mapping was performed with minimum overlap 10%, minimum overlap identity 80%, allow gaps 10%, and fine tuning set to iterate up to 10 times (Kehoe et al. 2014). The consensus sequence integrity was observed and its full length used with the contig of interest from CLCGW in Geneious by alignment with MAFFT (Katoh et al. 2002), to create a final sequence. The TuMV coding region was improved by aligning nucleotide sequences to the aligned deduced amino acid sequences using MUSCLE available in Geneious. Open reading frames were predicted and annotations made using Geneious. Finalized sequences were designated as complete genome coding sequences based on comparison with the reference sequences used in the mapping process. Only one TuMV genome was obtained from each sample sequenced. However, a second virus, “*Brassica campestris chrysovirus 1*” (Zhang et al. 2015), was found occurring as a minor component of a mixture within the isolate 12.1 sample sequenced. Because chrysovirus infect fungi, it may have come from a fungus associated with the 12.1-infected *B. campestris* plant sampled (S. Maina and R. A. C. Jones, unpublished). Final TuMV sequences were deposited in GenBank under accession numbers KX610929 (12.1), KX610930 (12.5), KX61931 (WA-Ap1), and KX610932 (WA-EP1).

Phylogenetic analysis. The 27 Australian sequences retrieved from GenBank included 5 identical sequences resulting from the same isolates having been sequenced twice by two different research groups (Supplementary Table S1). In our analysis, we included sequences NSW-1 to NSW-4 and NSW-6 from Nyalugwe et al. (2015b) but excluded the five duplicated isolate sequences AUST1 to AUST4 and AUST6 from Yasaka et al. (2015). The remaining 23 Australian isolates included 21 recombinants and 2 nonrecombinants (NSW-5 and AUST21) (Nyalugwe et al. 2015b; Yasaka et al. 2015). Before phylogenetic analysis, the coding regions of the 4 new TuMV genome sequences

were aligned with those of the remaining 23 Australian sequences and 37 nonrecombinant sequences from other countries using Clustal W in MEGA version 7.0.14 (Kumar et al. 2016). These 37 nonrecombinant sequences from GenBank were selected because of their previous use to represent all of the currently recognized TuMV phylogenetic groups (Nguyen et al. 2013; Nyalugwe et al. 2015b). The new sequences were aligned using Clustal W in MEGA 7 (Kumar et al. 2016) and, prior to phylogenetic analysis, further alignments were also compared using MAFFT (Katoh et al. 2002). Phylogenetic analysis compared the coding regions of all 64 TuMV genome sequences using the neighbor-joining method implemented in MEGA with 1,000 bootstrap replicates. The tree was rooted with an isolate of *Japanese yam mosaic virus* (AB016500). Two within-virus species phylogroup classification systems were used. The first was based on the region of the world where each isolate originated and its ability to infect *R. sativus* and *Brassica* spp. (Nguyen et al. 2013; Yasaka et al. 2015). The second removes misleading names and ambiguities based on geography or biology (e.g., host range and pathogenicity) that are inherent in such classification systems and replaces them with a neutral nomenclature system involving use of Latinized numerals (Jones and Kehoe 2016; Nyalugwe et al. 2015b).

Results

Symptoms and phenotypes in *B. napus*. Isolates 12.1 and 12.5 induced obvious mosaic and leaf deformation in all plants of the 19 cultivars and one breeding line (06-63777) of *B. napus* inoculated (Table 1; Fig. 1A to C). Thus, they always induced systemic susceptibility phenotype + (Table 2). With the other isolates, when the same isolate was inoculated to plants of the same cultivar or breeding line, in many instances there was segregation for two phenotypes within the plants inoculated. In one instance with WA-Ap1 (in ‘Clancy’) and two with NSW-1 (in ‘Nuseed GT 50’ and ‘Tarcoola’), there was segregation for three resistance phenotypes. Such segregation is a common phenomenon for heterozygous seed lots of individual lines in outcrossing species of Brassicaceae (Cowling 2007).

Table 1. Symptoms induced by five isolates of Turnip mosaic virus (TuMV) in plants of 19 cultivars and one breeding line (06-63777) of *Brassica napus*^a

<i>B. napus</i>	Isolate ^b				
	NSW-1	NSW-2	WA-Ap1	12.1	12.5
Canola					
Ag Muster	4/5, SL/M; 1/5, –/–	3/5, SL/M; 2/5, SL/M, SN	5/5, O	5/5, SL/M, LD	5/5, SL/M, LD
Ag Outback	4/5, SL/M; 1/5, LNS/SN, NST	4/4, LNS/–; 1/4, SL/M, SN, NST	4/5, –/–; 1/5, LNS/–	5/5, SL/M, LD	5/5, SL/M, LD
Bonanza TT	5/5, –/–	5/5, –/–	5/5, –/–	5/5, SL/M, LD	5/5, SL/M, LD
Bravo TT	5/5, –/–	4/5, SL/M; 1/5, LNS/M	4/5, LCS/–; 1/5, LNS/–	5/5, SL/M, LD	5/5, SL/M, LD
CB Argyll	5/5, –/–	5/5, LNS/–	1/5, –/–; 4/5, LNS/–	5/5, SL/M, LD	5/5, SL/M, LD
CB Nitro HT	5/5, LNS/–	2/5, –/–; 3/5, LNS/–	2/5, –/–; 3/5, LNS/–	5/5, SL/M, LD	5/5, SL/M, LD
CB Sturt TT	5/5, –/–	2/5, SL/M; 3/5, –/–	2/5, –/–; 3/5, LCS/–	5/5, SL/M, LD	5/5, SL/M, LD
CB Tango	3/5, –/–; 2/5, LNS/–	3/5, –/–; 2/5, LNS/–	5/5, –/–	5/5, SL/M, LD	5/5, SL/M, LD
Clancy	2/4, LNS/–; 2/4, –/M, SN	4/4, LNS/–	1/4, –/–; 2/4, LCS/–; 1/4, SL/M, SN, NST	5/5, SL/M, LD	5/5, SL/M, LD
Cobbler	4/5, –/–; 1/5, LNS/–	3/5, –/–; 2/5, LNS/–	4/5, –/–; 1/5, LNS/–	5/5, SL/M, LD	5/5, SL/M, LD
Crusher TT	5/5, –/–	5/5, –/–	5/5, –/–	5/5, SL/M, LD	5/5, SL/M, LD
Fighter TT	3/4, SL/M; 1/4, –/–	4/4, SL/M	5/5, –/–	5/5, SL/M, LD	5/5, SL/M, LD
Flinders	4/7, LNS/–; 3/7, LNS SN ^c	7/7, LNS/– ^c	7/7, LNS/–	7/7, SL/M, LD	7/7, SL/M, LD
Jackpot TT	3/5, SL/M; 2/5, –/–	3/4, SL/M; 1/4, –/–	3/5, –/–; 2/5, LCS/–	5/5, SL/M, LD	5/5, SL/M, LD
AV Opal	5/5, –/–	5/5, –/–	5/5, –/–	5/5, SL/M, LD	5/5, SL/M, LD
Nuseed GT 50	1/5, –/–; 3/5, LNS/–; 1/5, LNS/SN, NST	5/5, LNS/–	2/5, –/–; 3/5, LNS/–	5/5, SL/M, LD	5/5, SL/M, LD
Tarcoola	3/5, –/–; 1/5, LNS/–; 1/5, LNS/SN	4/5 SL/M; 1/5, LCS/–	4/5, –/–; 1/5, LNS/–	5/5, SL/M, LD	5/5, SL/M, LD
06-63777	5/5, –/–	5/5, –/–	5/5, –/–	5/5, SL/M	5/5, SL/M
44C79	5/5, –/–	3/5, –/–, 2/5, LNS/–	5/5, –/–	5/5, SL/M, LD	5/5, SL/M, LD
Swede					
Invitation	7/7, SL/SL ^c	6/6, SL/SL ^c	7/7, LCS/–	7/7, SL/M, LD	7/7, SL/M, LD

^a With each cultivar or breeding line, plants were inoculated with infective sap and two control plants were mock inoculated with sap from healthy plants. Symptoms were recorded weekly for 6 weeks after inoculation, and inoculated and leaf-tip samples were tested for TuMV by enzyme-linked immunosorbent assay 2 and 3 to 4 weeks after inoculation, respectively.

^b Numbers indicate number of plants with each type of response/numbers of plants inoculated and letters indicate inoculated leaf symptoms/uninoculated leaf symptoms. Coded responses: – = no TuMV infection, LCS = local chlorotic spot lesions, LNS = local necrotic spot lesions, M = mosaic, LD = leaf deformation; SN = systemic necrosis, SL = symptomless infection, and NST = necrotic stem streaking.

^c Data for isolates NSW-1 and NSW-2 in Flinders taken from a previous study (Nyalugwe et al. 2015b).

WA-Ap1 differed from 12.1 and 12.5 in always inducing one or more of four different resistance phenotypes when inoculated to plants of the same 19 cultivars and single breeding line (Tables 1 and 2). With WA-Ap1, the most common phenotype consisted of no detectable infection in inoculated or uninoculated leaves (i.e., extreme resistance phenotype O). This developed either alone or segregating with one to three other phenotypes, except in 'Bravo TT' and 'Flinders' and swede 'Invitation'. The second most common plant response to inoculation with WA-Ap1 was necrotic local lesions in inoculated leaves without systemic invasion (i.e., resistance phenotype R_N), which developed in all plants of Flinders and some plants of eight other cultivars or breeding lines. Chlorotic spot local lesions in inoculated leaves without systemic invasion (i.e., resistance phenotype R) developed only in all plants of Invitation and some plants of Bravo TT, 'CB Sturt TT', Clancy, and 'Jackpot TT'. Also, one plant of Clancy developed a combination of systemic mosaic, necrotic stem streaking, and necrosis (i.e., resistance phenotype $+_N$).

With NSW-1 and NSW-2, phenotype + developed in all plants of Invitation and, with NSW-2, in all plants of 'Fighter' (Tables 1 and 2). Some inoculated plants of Ag Muster and Jackpot TT developed phenotype + with both isolates, while some of Fighter and 'Ag Outback' did so with NSW-1, and Bravo TT, CB Sturt TT, and Tarcoola did so with NSW-2. As with WA-Ap1, phenotype O developed most often with NSW-1 and NSW-2. With all isolates, it was the only phenotype induced in inoculated plants of 'Bonanza TT', 'Crusher TT', AV Opal, and breeding line 06-63777. It occurred alone with NSW-1 in Bravo TT, 'CB Argyll', 'CB Nitro HT', and '44C79'. It segregated with other phenotypes in plants of seven (NSW-1) or six (NSW-2) cultivars. With isolate NSW-2, R_N developed alone in CB Argyll, Clancy, Flinders, and Nuseed GT 50 and together with other phenotypes in five other cultivars. With NSW-1, it developed alone only in CB Nitro HT but segregated with other phenotypes in six other cultivars. Phenotype $+_N$ occurred more commonly with these two isolates than with WA-Ap1, segregating with other phenotypes in seven (NSW-1) (Fig. 1D and E) or two (NSW-2) cultivars but never occurring alone. When plants with $+_N$ grew older, they often died prematurely. In contrast to its more frequent occurrence with WA-Ap1, R was absent with NSW-1 and present in only one inoculated plant of Tarcoola with NSW-2. The phenotype involving necrotic spots in inoculated leaves followed by systemic invasion (phenotype $R_{N/+}$) was found only in one Bravo TT plant inoculated with NSW-2.

No TuMV was ever detected in leaf samples from mock-inoculated control plants, which always remained asymptomatic.

Symptoms in other host species. Among the additional Brassicaceae spp. tested with the five TuMV isolates, the phenotypic responses in plants of *B. oleracea* var. *italica* 'Romanesco' caused by *B. napus* resistance-breaking isolates 12.1 and 12.5 differed from those of all three other isolates, because they were the only ones to cause systemic susceptibility phenotype + in this cultivar. No infection was ever detected in inoculated or upper leaves of plants of *B. oleracea* var. *botrytis* or *R. sativus*; therefore, all developed extreme resistance phenotype O (Tables 3 and 4). Also, all five isolates caused systemic susceptible phenotype + in plants of *B. carinata* PI 390133, *B. rapa* 'Evigwe 2013', and *S. alba*. In *B. juncea* JM 06006, apart from one of seven plants inoculated with NSW-1 which developed local necrotic spot lesions and systemic invasion without necrosis ($R_{N/+}$), all plants inoculated developed a susceptible phenotype. Similarly, except for isolate WA-Ap1, which caused a localized resistance phenotype without necrosis or systemic invasion (phenotype R) in 4 of 15 plants of *B. rapa* var. *chinensis*, all inoculated plants of this species developed a systemic susceptible phenotype. Isolate WA-A-1 also behaved differently in plants of *B. oleracea* var. *capitata*. It caused extreme resistance phenotype O in all inoculated plants. However, plants inoculated with the other four isolates developed this same phenotype O in some plants and either a localized resistance phenotype without any necrosis or systemic invasion (NSW-1 and NSW-2; phenotype R), or systemic susceptible phenotype + (12.1 and 12.5) in the remainder of the plants. Three isolates (WA-Ap1, 12.1, and 12.5) induced a systemic hypersensitive response (phenotype $+_{ND}$) in plants of *Camelina sativa* 'China' but systemic susceptible phenotype + developed with the two others (NSW-1 and NSW-2). *Eruca sativa* plants

responded differently to each of the five isolates: three of the above phenotypes were found (+, O, and R_N) along with systemic infection with necrosis (phenotype $+_N$); one to three phenotypes developing per isolate. In *S. arvensis*, the three isolates tested (WA-Ap1, 12.1, and 12.5) only caused a systemic susceptible phenotype.

All five TuMV isolates caused necrotic spot local lesions in inoculated leaves without systemic invasion in inoculated plants of the indicator hosts *Chenopodium amaranticolor*, *C. quinoa*, and *Gomphrena globosa* (Table 3). Four isolates also infected *Nicotiana glutinosa* but only in its inoculated leaves, causing chlorotic spot local lesions. No infection was detected in this species with isolate NSW-1. In contrast, all five isolates caused systemic invasion in *N. benthamiana*, with NSW-1 causing mild mosaic but the other four causing systemic necrosis and plant death.

No TuMV was ever detected in leaf samples from uninoculated control plants, which always remained without symptoms.

HTS. Numbers of reads obtained from HTS were 14,946,756 to 17,237,762 but they reduced to 14,785,015 to 17,056,849 after quality control (removal of low-quality and adaptor-contaminated reads; Supplementary Table S2). Following *Denovo* assembly of each individual sample using CLCGW, 160 to 1,591 contigs were produced. The lengths of the contigs of interest were 8,120 to 9,500 nt, with average coverage 1,018 to 48,523 times, and 74,065 to 2,606,971 reads mapped to each contig. After mapping to a reference genome in Geneious, the lengths of the consensus sequences were 9,902 to 9,996, with average coverage of 17,130 to 47,999, and the numbers of reads mapped to the reference sequence were 1,258,983 to 3,275,078. Final sequence lengths consisted of the consensus of the contig from CLCGW and the consensus from Geneious, and were 9,802 to 9,949 nt. All the sequenced samples had a Q30 (=Illumina sequencing quality score, 1 in 1,000 base call accuracy) of more than 95%.

Sequence identities. Of the four new sequences, 12.1 and 12.5 were sister isolates, with 99.9% nucleotide identities, and were closest to isolate Czech from the Czech Republic (97.7% nucleotide identity). WA-Ap1 was closest to its sister isolate WA-Ap, obtained from a different plant of the same host species growing nearby in the same location (99.6% nucleotide identity). WA-EP1 was closest to isolate Cal-1 from Italy (94.5% nucleotide identity). These percent identities were determined using final genomic sequences. Further analysis was undertaken by comparing the sequences of each isolate after trimming off all the UTR, leaving only their polypeptides, which resulted in sequences of 9,495 nt. When these trimmed sequences were compared, their nucleotide identities still remained as shown above, except with the WA-Ap and WA-Ap1 comparison, which gave 99.5 instead of 99.6% identity.

Phylogenetic analysis. When the coding regions of the four new complete TuMV genomes were compared with those of the 23 Australian and 37 others from GenBank, they fitted within three major phylogenetic groupings (Fig. 2). The sequences obtained from isolates 12.1 and 12.5 were closest to each other in phylogroup II within the overall World-B grouping. There were 19 other Australian isolate sequences within World-B. Two of these (AUST19 and AUST23) were also within phylogroup II but some distance away from isolates 12.1 and 12.5. The 17 other Australian sequences in World-B grouped together within phylogroup I. They included isolates NSW-1 to NSW-6, which were mostly from the same location in New South Wales. This large Australian isolate grouping also contained two other isolates, KWB778J from Japan and PV134 from California. The sequence obtained from new isolate WA-EP1 fell within the Basal-BR grouping within phylogroup III. It was closest to isolate Cal-1 from Italy. No other Australian isolates were present in this phylogroup. The sequence obtained from new isolate WA-Ap1 was in phylogroup IV within the overall Basal-B grouping. It was closest to its sister Australian isolate WA-Ap. Three other Australian isolates (AUST10, AUST13, and BRSA1) grouped within Basal-B phylogroup IV but some distance away. The remaining three TuMV groupings—Asian-BR (within phylogroup III), Basal-B (phylogroup V component), and Orchis (phylogroup VI)—lacked any Australian isolates.

Discussion

This research describes the biological and molecular properties of unpathotyped sister TuMV isolates 12.1 and 12.5, which belong to a

B. napus resistance-breaking strain currently causing concern to the Australian oilseed industry. All plants of 19 *B. napus* cultivars and one breeding line inoculated with these isolates developed a susceptible phenotype involving obvious foliage symptoms, whereas isolates belonging to pathotypes 1 (NSW-2), 7 (NSW-1), and 8 (WA Ap-1) developed resistance phenotypes in all (pathotype 8) or most (pathotypes 1 and 7) of the same cultivars and breeding line. Thus, all strain-specific TuMV resistances revealed by inoculating pathotypes 1, 7, and 8 to *B. napus* cultivars from different plant breeding programs were overcome. This not only confirms that 12.1 and 12.5 represent a resistance-breaking strain but also explains why high *B. napus* crop incidences of TuMV are being found in the Liverpool Plains region of New South Wales. When the same five isolates were inoculated to plants of nine other Brassicaceae spp., the resistance-breaking strain resembled the other three pathotypes in inducing both resistance and susceptibility phenotypes. Occurrence of these phenotypes depended on the combination of virus isolate and host species or subspecies. Also, only with *B. oleracea* var. *italica* did the two resistance-breaking isolates induce systemic susceptibility phenotype + when the other three isolates all induced resistance phenotypes. This did not occur in plants of *B. oleracea* var. *botrytis* and *capitata* or the other eight additional Brassicaceae spp. Thus, their unique resistance-breaking properties only applied to *B. napus*. When the complete genome sequences of isolates 12.1 and 12.5 were compared with those of 25 other Australian TuMV isolates, they grouped together but separately from all the other Australian isolates.

The complete genomes of isolates 12.1 and 12.5, 2 other new Australian genome sequences, and the genomes of 23 other previously sequenced Australian TuMV isolates grouped within phylogroups I and II of World-B, Basal-BR of phylogroup III, or phylogroup IV of Basal-B. They were located in four distinct positions within World-B and two within Basal-B, suggesting the likelihood of at least six TuMV introductions to the Australian continent after agriculture commenced following European colonization in 1788. The new resistance-breaking strain may be the most recent introduction because it was only detected in 2012 and, thus far, has only been found in one region. In contrast, the presence of the other TuMV pathotypes identified thus far in Australia was demonstrated earlier.

Because it is most closely related to an isolate from the Czech Republic (97.7% nucleotide identity), it may be of European origin. How it was first introduced is unknown. Although TuMV is not known to be seedborne in *Brassica* crop species or its other host species, seed transmission might still occur occasionally at very low levels in some crop species and weeds, as with several other potyviruses. This would provide a possible entry route into Australia. Other possible entry routes include importation of vegetable or other crop produce inadvertently carrying infection, and unknowingly infected planting material of host crops imported illegally. Interestingly, when the complete genome sequences of five Australian TuMV isolates sequenced previously using different approaches by different research groups (Nyalugwe et al. 2015b; Yasaka et al. 2015) were compared between each of the duplicated pairs, HTS with *Denovo* sequence assembly gave exactly the same result as the traditional sequencing approach using stepwise reverse-transcription polymerase chain reaction. This demonstrates its accuracy and reliability when obtaining virus genomes for use in phylogenetic and evolutionary studies.

In addition to TuMV susceptibility phenotype +, five previously recognized phenotypic resistance responses (O, R_N, R, R_{N/+}, and +_N) developed in *B. napus* and in one or more of five of nine other Brassicaceae spp. Within the same isolate and cultivar combination, these phenotypes occurred either alone or segregated with one or two others. Such segregation occurred in *B. napus*, *B. juncea*, *B. oleracea* var. *capitata*, *B. rapa* var. *chinensis*, and *E. sativa*. In plants of *B. juncea*, *B. oleracea*, *B. rapa*, and *C. sativa*, growing plants at relatively high temperatures after inoculation decreased the delay between inoculation and symptom expression without altering overall phenotypic responses (Nyalugwe et al. 2015a). In contrast, this sometimes altered phenotype expression in *B. napus* and *B. carinata* (e.g., by altering phenotype O to other phenotypes and phenotype R to phenotype +), such that only higher temperatures gave a true picture of the spectrum of resistance types present (Nyalugwe et al. 2014). Therefore, our studies were done at 22 to 23°C to minimize the delay before systemic symptoms appeared and ensure that symptom expression was optimized. Under these conditions, systemic necrosis associated with phenotype +_N in *B. napus* was sometimes expressed so strongly that it caused plant

Fig. 1. Examples of symptoms induced by infection with two *Turnip mosaic virus* isolates in *Brassica napus* plants. **A**, Faint systemic chlorotic spots in young leaves caused by resistance-breaking isolates 12.1 or 12.5 at 4 days after inoculation, rapidly followed the following day by **B**, mosaic symptoms in the same young leaves. **C**, Obvious systemic mosaic and leaf deformation at 3 weeks after inoculation with isolates 12.1 or 12.5. **D**, Systemic necrotic spot lesions in uninoculated leaves 3 weeks after inoculation with isolate NSW-1. **E**, Systemic necrosis 3 weeks after inoculation with isolate NSW-1. **A** to **C**, Susceptibility phenotype + as it developed in plants of all cultivars and breeding lines. **D** and **E**, Resistance phenotype +_N as it developed in a Taroocool plant.

death. We did not classify this plant death as phenotype +_{ND} because plant death occurs more rapidly with this phenotype.

The relationship between TuMV pathotypes and *Brassica* resistance genes is complex. Moreover, in addition to being influenced by temperature (see above), resistance phenotype expression can be influenced by plant genetic background, *Brassica* cultivar or line infected, and individual isolates within a pathotype (Nyalugwe et al. 2014; Walsh and Jenner 2002, 2006). Extreme resistance (phenotype O) against pathotypes 1 and 3 is conferred by dominant resistance genes *TuRB01* and *TuRB03*, respectively. Dominant resistance gene *TuRB04* is epistatic to dominant resistance gene *TuRB05*. When both operate in conjunction, this confers extreme resistance to pathotype 3. On their own, *TuRB04* confers extreme resistance and *TuRB05* confers localized hypersensitivity (phenotype R_N) to some TuMV isolates (pathotypes not specified). Dominant resistance allele *TuRB01b* confers systemic necrotic resistance (phenotype +_N) to pathotype 3 (Hughes et al. 2002; Walsh and Jenner 2002, 2006). Dominant systemic hypersensitivity gene *TuRBju 01* is responsible for systemic necrosis phenotypes +_N and +_{ND} in its homozygous and heterozygous condition, respectively. It was effective against pathotypes 1, 7, and 8 but not against isolate NSW-3, which belongs to a new pathotype (Nyalugwe et al. 2015a, 2016c). Recessive resistance gene *retro01* and dominant resistance gene *conTR01* confer broad specificity against systemic spread (phenotype R) with pathotypes 1, 3, 4, 7 to 9, and 12 (Hughes et al. 2002; Walsh and Jenner 2002, 2006). Dominant resistance gene *TuRB02* confers partial resistance to two TuMV isolates (Walsh et al. 1999). No TuMV resistance genes have been identified that confer phenotype R_{N/+} which we found in one plant of *B. napus* Bravo TT inoculated with pathotype 1. Similarly, R_{N/+} was found only rarely in previous studies evaluating the responses of *B. napus* to inoculation with pathotype 8 isolates (Coutts et al. 20007; Nyalugwe et al. 2014) but occurred more commonly when isolate WA-Ap1 was inoculated to *B. juncea* and *B. rapa* (Kehoe et al. 2010; Nyalugwe et al. 2015a).

Based on the widespread occurrence of resistance phenotypes O, R_N, and +_N following inoculation of TuMV pathotype 8 isolate WA-Ap to 43 Australian *B. napus* cultivars and one breeding line, Coutts et al. (2007) suggested that resistance genes *TuRB01*, *TuRB03*, *TuRB04*, *TuRB05*, and a hitherto unidentified resistance gene may be present among them. Our inoculations to 19 more cultivars of *B. napus* and a breeding line using isolates belonging to pathotypes 1, 7, and 8 provide additional evidence of their likely widespread occurrence because resistance phenotypes O, R_N and +_N were again often found. Coutts et al. (2007) concluded that a good indication of TuMV resistance gene presence would be provided using variants of an infectious TuMV clone with single-nucleotide changes that alter phenotypes obtained with different virus-resistance gene interactions. However, ultimate proof would require cloning and sequencing of these genes. Such studies have not been attempted as yet with Australian *B. napus* cultivars or breeding lines. They are needed so that the resistance genes present can be deployed effectively against TuMV infection in Australian *B. napus* resistance breeding programs. However, they would be ineffective against the unpathotyped virulent resistance-breaking strain represented by isolates 12.1 and 12.5 because all *B. napus* cultivars and the breeding line inoculated with them developed susceptibility phenotype +.

Nyalugwe et al. (2016b) found that, although pathotype 8 isolate WA-Ap1 induced only phenotype R_N in plants of *B. carinata* TZ-SMN-44-6 in earlier research (Nyalugwe et al. 2014), after many cycles of serial subculture, it induced only phenotype R in this host. A similar alteration in phenotype induced by WA-Ap1 was also found in our study. Nyalugwe et al. (2014) found that it induced only phenotype + in *B. napus* Ag Muster but, here, it induced only phenotype O in this cultivar. Also, although Nyalugwe et al. (2014) found that isolates NSW-1 (pathotype 7) and NSW-2 (pathotype 1) both induced phenotype + alone in Ag Muster, in our study there was segregation for phenotypes + and O (NSW-1) or phenotypes + and +_N (NSW-2). Such changes after many cycles of serial TuMV subculture may reflect the presence of one or more mutations in these isolates influencing TuMV resistance expression

Table 2. Phenotypic responses induced by five isolates of Turnip mosaic virus (TuMV) in plants of 19 cultivars and one breeding line (06-63777) of *Brassica napus*^a

<i>B. napus</i>	Isolate ^b				
	NSW-1	NSW-2	WA-Ap1	12.1	12.5
Canola					
Ag Muster	+ (4), O (1)	+ (3), + _N (2)	O (5)	+ (5)	+ (5)
Ag Outback	+ (4), + _N (1)	R _N (3), + _N (1)	O (4), R _N (1)	+ (5)	+ (5)
Bonanza TT	O (5)	O (5)	O (5)	+ (5)	+ (5)
Bravo TT	O (5)	+ (4), R _{N/+} (1)	R (4), R _N (1)	+ (5)	+ (5)
CB Argyll	O (5)	R _N (5)	O (1), R _N (4)	+ (5)	+ (5)
CB Nitro HT	R _N (5)	O (2), R _N (3)	O (2), R _N (3)	+ (5)	+ (5)
CB Sturt TT	O (5)	+ (2), O (3)	O (2), R (3)	+ (5)	+ (5)
CB Tango	O (3), R _N (2)	O (3), R _N (2)	O (5)	+ (5)	+ (5)
Clancy	R _N (2), + _N (2)	R _N (4)	O (1), R (2), + _N (1)	+ (5)	+ (5)
Cobbler	O (4), R _N (1)	O (3), R _N (2)	O (4), R _N (1)	+ (5)	+ (5)
Crusher TT	O (5)	O (5)	O (5)	+ (5)	+ (5)
Fighter TT	+ (3), O (1)	+ (4)	O (5)	+ (5)	+ (5)
Flinders	R _N (4), + _N (3) ^c	R _N (7) ^c	R _N (7)	+ (7)	+ (7)
Jackpot TT	+ (3), O (2)	+ (3), O (1)	O (3), R (2)	+ (5)	+ (5)
AV Opal	O (5)	O (5)	O (5)	+ (5)	+ (5)
Nuseed GT 50	O (1), R _N (3), + _N (1)	R _N (5)	O (2), R _N (3)	+ (5)	+ (5)
Tarcoola	O (3), R _N (1), + _N (1)	+ (4), R (1)	O (4), R _N (1)	+ (5)	+ (5)
06-63777	O (5)	O (5)	O (5)	+ (5)	+ (5)
44C79	O (5)	O (3), R _N (2)	O (5)	+ (5)	+ (5)
Swede					
Invitation	+ (7) ^c	+ (6) ^c	R (7)	+ (5)	+ (5)

^a With each cultivar or breeding line, plants were inoculated with infective sap and two control plants were mock inoculated with sap from healthy plants. Symptoms were recorded weekly for 6 weeks after inoculation, and inoculated and leaf-tip samples were tested for TuMV by enzyme-linked immunosorbent assay 2 and 3 to 4 weeks after inoculation, respectively.

^b Coded phenotypic responses: + = systemic infection without necrosis, O = extreme resistance with no detectable infection, R = infection of inoculated leaves without necrosis but no detectable systemic infection, R_N = local necrotic spot lesions in inoculated leaves but no detectable systemic infection, R_{N/+} = local necrotic spot lesions in inoculated leaves and systemic infection without necrosis, and +_N = systemic infection with necrosis. Numbers in parentheses are numbers of plants with each type of phenotypic response.

^c Data for isolates NSW-1 and NSW-2 in cv. Flinders taken from a previous study (Nyalugwe et al. 2015b).

in *B. napus*. In contrast, there was no alteration in phenotype between these two studies when the same three isolates were inoculated to breeding line 06-6-3777, and isolate WA-Ap1 to AV Opal, because phenotype O developed in all instances. The lack of phenotypic stability

over time obtained with WA-Ap1 in *B. carinata* TZ-SMN-44-6 and all three isolates in *B. napus* Ag Muster suggest that infectious clones would be useful for TuMV resistance assessment in *Brassica* breeding programs. As mentioned above, they would also allow creation of chimeric

Table 3. Symptoms induced by five isolates of *Turnip mosaic virus* (TuMV) in nine species other Brassicaceae and four indicator species^a

Family, plant species	Common name	NSW-1 ^b	NSW-2 ^b	WA-Ap1	12.1	12.5
Amaranthaceae						
<i>Gomphrena globosa</i>	Globe amaranth	6/6, mLNS/–	6/6, mLNS/–	6/6, mLNS/–	7/7, LNS/–	7/7, LNS/–
Brassicaceae						
<i>Brassica carinata</i> PI 390133	Ethiopian mustard	7/7, SL/M	7/7, SL/M	7/7, SL/M	7/7, SL/M	7/7, SL/M
<i>B. juncea</i> JM 06006	Indian mustard	6/7, SL/M; 1/7, LNS/M	7/7, SL/mM	7/7, SL/mM	7/7, SL/mM	7/7, SL/mM
<i>B. oleracea</i> var. <i>botrytis</i> All year round	Cauliflower	3/3, –/–	3/3, –/–	7/7, –/–	3/3, –/–	2/2, –/–
<i>B. oleracea</i> var. <i>capitata</i> Sugarloaf	Cabbage	2/5, SL/–; 3/5, –/–	3/5, SL/–; 2/5, –/–	7/7, –/–	11/13, SL/mCRS, M; 2/2, –/–	15/31, SL/mCRS, M; 16/31, –/–
<i>B. oleracea</i> var. <i>italica</i> Romanesco	Broccoli	3/3, –/–	3/3, –/–	7/7, LCS/–	3/3, SL/M	3/3, SL/M
<i>B. rapa</i> var. <i>chinensis</i>	Bok choy	5/5, SL/M, LD	5/5, SL/M, LD	11/15, SL/M; 4/15, LNS/–	7/7, SL/M	7/7, SL/M
<i>B. rapa</i> Evigwe 2013	Field mustard	4/4, SL/M	3/3, SL/M	7/7, LCS/M	7/7, SL/M	7/7, SL/M
<i>Camelina sativa</i> China	False fax	5/5, SL/M	3/3, SL/M	7/7, LCS/M, SN, PD	7/7, SL/M, SN, PD	7/7, SL/M, SN, PD
<i>Eruca sativa</i>	Garden rocket	3/3, SL/VB, sM	3/4, SL/M; 1/4, –/–	8/10, LNS/–; 2/10, LNS/SN	6/13, –/–; 7/13, LNS/–	2/14, –/–; 3/14, LNS/SN; 9/14 LNS/–
<i>Raphanus sativus</i> Sparkler	Radish	7/7, –/–	5/5, –/–	7/7, –/–	7/7, –/–	7/7, –/–
<i>Sinapis alba</i>	White mustard	6/6, SL/M, LD	6/6, SL/M, LD	6/6, SL/M, LD	6/6, SL/M, LD	6/6, SL/M, LD
<i>S. arvensis</i>	Wild mustard	NT	NT	4/4, SL/M	10/10, SL/mM	13/13, SL/mM
Chenopodiaceae						
<i>Chenopodium amaranticolor</i>	Mercy goosefoot	5/5, sLNS/–	5/5, LNS/–	5/5, LNS/–	7/7, LNS/–	7/7, LNS/–
<i>C. quinoa</i>	Quinoa	5/5, sLNS/–	5/5, LNS/–	5/5, LNS/–	7/7, LNS/–	7/7, LNS/–
Solanaceae						
<i>Nicotiana benthamiana</i>	Wild tobacco	2/2, SL/mM	2/2, LNS/WK, PN, SN, PD	2/2, LNS/SN, PD	6/6, SL/PN, SN, PD	6/6, SL/PN, SN, PD
<i>N. glutinosa</i>	Wild tobacco	4/4, –/–	4/4, LCS/–	4/4, LCS/–	4/4, LCS/–	4/4, LCS/–

^a For each combination of isolate and species or line, plants were inoculated with infective sap and one or two were kept as healthy uninoculated controls for comparison. Symptoms were recorded weekly for 6 weeks after inoculation, and inoculated and leaf-tip samples were tested for TuMV by enzyme-linked immunosorbent assay 3 to 4 weeks after inoculation. Numbers indicate number of plants with each type of response/numbers of plants inoculated and letters indicate inoculated leaf symptoms/uninoculated leaf symptoms. Coded responses: – = no TuMV infection detected, LCS = local chlorotic spot lesions, LD = leaf deformation, LNS = local necrotic spot lesions, CRS = chlorotic rings, M = mosaic, PD = plant death, PN = petiole necrosis, SL = symptomless infection, SN = systemic necrosis, SNT = stem necrotic streaking, VB = vein banding, and WK = weak etiolated stem. Lowercase letters m and s in front of symptom symbols refer to symptom intensity: m = mild and s = severe. NT = not tested.

^b Results for isolates NSW-1 and NSW-2 are taken from a previous study (Nyalugwe et al. 2015b).

Table 4. Phenotypic responses induced by five isolates of *Turnip mosaic virus* in nine other Brassicaceae spp.^a

Plant species	Common name	NSW-1 ^b	NSW-2	WA-Ap1	12.1	12.5
<i>Brassica carinata</i> PI 390133	Ethiopian mustard	+	+	+	+	+
<i>B. juncea</i> JM 06006	Indian mustard	+	+	+	+	+
<i>B. oleracea</i> var. <i>botrytis</i> All year round	Cauliflower	O	O	O	O	O
<i>B. oleracea</i> var. <i>capitata</i> Sugarloaf	Cabbage	R	R	O	+	+
<i>B. oleracea</i> var. <i>italica</i> Romanesco	Broccoli	O	O	R	+	+
<i>B. rapa</i> var. <i>chinensis</i>	Bok choy	+	+	+	+	+
<i>B. rapa</i> Evigwe 2013	Field mustard	+	+	+	+	+
<i>Camelina sativa</i> China	False fax	+	+	+	+	+
<i>Eruca sativa</i>	Garden rocket	+	+	+	+	+
<i>Raphanus sativus</i> Sparkler	Radish	O	O	O	O	O
<i>Sinapis alba</i>	White mustard	+	+	+	+	+
<i>S. arvensis</i>	Wild mustard	NT	NT	+	+	+

^a For each combination of isolate and species or line, plants were inoculated with infective sap and one or two were kept as healthy uninoculated controls for comparison. Symptoms were recorded weekly for 6 weeks after inoculation, and inoculated and leaf-tip samples were tested for TuMV by enzyme-linked immunosorbent assay 3 to 4 weeks after inoculation.

^b Coded phenotypic responses: + = systemic infection without necrosis, O = extreme resistance with no detectable infection, R = infection of inoculated leaves without necrosis but no detectable systemic infection, R_N = local necrotic spot lesions in inoculated leaves but no detectable systemic infection, R_N/+ = local necrotic spot lesions in inoculated leaves and systemic infection without necrosis, +_N = systemic infection with necrosis, +_{ND} = systemic hypersensitivity and plant death, and NT = not tested. Numbers in parentheses are numbers of plants with each type of phenotypic response. Results for isolates NSW-1 and NSW-2 are taken from a previous study (Nyalugwe et al. 2015b).

constructs to identify the genomic determinants of particular resistance phenotypes.

When isolates belonging to *B. napus* resistance-breaking TuMV strain (12.1 and 12.5) pathotypes 1 (NSW-2), 7 (NSW-1), and 8 (WA-Ap1) were inoculated to plants of additional Brassicaceae spp., the two

resistance-breaking isolates were the only ones to cause susceptibility phenotype + in plants of *B. oleracea* var. *italica*. However, this was the sole instance in which this occurred. In plants of *C. sativa* China, the two resistance-breaking isolates and the pathotype 8 isolate induced resistance phenotype +_{ND} but phenotype + developed with pathotypes

Fig. 2. Neighbor-joining phylogram obtained from alignment of *Turnip mosaic virus* nucleotide sequences of 64 isolates of *Turnip mosaic virus* with complete genomes, including isolates 12.1, 12.5, WA-Ap1, and WA-EP1 sequenced in this study. The neighbor-joining tree was created with MEGA 7 with 1,000 replicates and bootstrap values are shown as percentages, with only values over 70% shown at the nodes. *Japanese yam mosaic virus* AB016500 (JYMV) was used as an outgroup. The symbol ● shows the four newly sequenced isolates from this study, whereas the symbol ▲ shows the 23 Australian isolates sequenced previously.

1 and 7. All five isolates caused phenotype + in plants of *B. carinata* PI 390133, *B. rapa* Evigwe 2013, and *S. alba*. This was also so in all plants of *B. juncea* JM 06006 and *B. rapa* var. *chinensis*, except that phenotype R_N developed in 1 of 7 plants inoculated with the pathotype 7 isolate and phenotype R_N in 4 of 15 plants inoculated with the pathotype 8 isolate, respectively. All isolates induced phenotype O in every plant inoculated of *B. oleracea* var. *botrytis* and *R. sativus*. *E. sativa* responded differently to each of the five isolates: three of the above phenotypes were found (+, O, and R_N) and, with phenotype +_N, one to three phenotypes developing per isolate. With all isolates, their responses to inoculation resembled those of biological type B isolates of Nguyen et al. (2013) because they infected most *Brassica* spp. but *R. sativus* was a nonhost. In the indicator hosts inoculated, the two *B. napus* resistance-breaking strain isolates resembled the other three isolates in inducing phenotype R_N in three species. They also resembled two other isolates (WA-Ap1 and NSW-2) in inducing phenotypes R in *N. glutinosa* and +_{ND} in *N. benthamiana*.

The pathotypes identified thus far among Australian TuMV isolates pathotyped in the United Kingdom using *B. napus* differentials are 1, 3, 7, 8, and a new pathotype (Coutts et al. 2007; Schwinghamer et al. 2014; Jenner and Walsh 1996). Pathotype 3 isolate AUS2 studied by Jenner and Walsh (1996) was unavailable and our study omitted the as-yet-unnamed new pathotype. In the future, evaluation of potential *B. napus* breeding parents for comprehensive TuMV resistance should include challenging them with the unpathotyped resistance-breaking strain, the unnamed new pathotype, and pathotypes 1, 3, 7, and 8.

This study highlights the emergence of a dangerous resistance-breaking strain able to overcome TuMV resistance genes that currently suppress its spread in *B. napus* crops. The likelihood of its further spread is cause for concern to global oilseed industries based on *B. napus*, because this could pose a significant threat to *B. napus* production, especially during the current era of increasing virus epidemic instability caused by climate change (Jones 2016). A strategy to delay its spread outside the area currently affected could include (i) attempting to contain it by killing (with herbicide) the alternative weed and volunteer crop hosts that would otherwise survive outside the annual growing season and (ii) preventing movement of vegetable and other crop host produce outside the affected area. Breeding *B. napus* cultivars with resistance to it is a critical priority for *B. napus* breeding programs in Australia and elsewhere where the virus may soon occur. This study not only highlights the opportunity to utilize the extreme resistance identified to it in *B. oleracea* but also the urgency to search a broad spectrum of diverse *B. napus* germplasm to identify other sources of TuMV resistance effective against it that could be deployed into areas suffering the most damage. Further, there is a need to investigate the histochemical and genetic basis of the extreme resistance to it in *B. oleracea*. Finally, to protect the oilseed industry in Australia and elsewhere from this new threat, there is an opportunity to explore the possibility of transferring both this extreme resistance and a resistance gene already found to be effective against it, *TuRBju 01* from *B. juncea* 'Oasis C1' (Nyalugwe et al. 2015a, 2016c), to *B. napus*.

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