Resistance Phenotypes in Diverse Accessions, Breeding Lines, and Cultivars of Three Mustard Species Inoculated with *Turnip mosaic virus*

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ABSTRACT

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The responses of 44 accessions, breeding lines, or cultivars of *Brassica juncea* (Indian mustard), 9 of *B. carinata* (Ethiopian mustard), 5 of *B. nigra* (black mustard), and 6 crosses between *B. juncea* and *B. napus* (canola) to sap inoculation with *Turnip mosaic virus* (TuMV) were investigated. Eleven different phenotypes were obtained, including six previously recognized in *B. napus* (+, O, R, R_N, $R_{N/+}$, and $+_N$) and five not recorded before ($+_{St}$, $R_{N/St}$, $R_{N/St/+}$, $+_N^1$, and $+_{ND}$). All but two (+ and $+_{St}$) were resistance phenotypes. The resistance phenotypes in *B. carinata* and *B. juncea* × *B. napus* crosses prevented systemic infection but those in *B. juncea* and *B. nigra* included systemic necrosis. Absence of systemic invasion associated with resistance phenotypes in *B. carinata* was confirmed by graft inoculations. The resistance phenotypes may reflect the presence of known TuMV resistance genes located in the A genome or unknown genes in the B genome in *B. juncea*, unknown resistance genes in the B or C genomes in *B. carinata*, and unknown resistance genes in the B genome in *B. nigra*. Further research to identify the resistance genes involved would establish the potential usefulness of these resistance phenotypes in breeding TuMV-resistant mustard cultivars for biofuel production.

Turnip mosaic virus (TuMV; family *Potyviridae*; genus *Potyvirus*) is transmitted nonpersistently by aphids. It occurs worldwide and has an extensive host range, including at least 300 plant species, with natural infections occurring in at least 135 of them (12,13,34). The first reports of TuMV were in 1921 infecting *Brassica pekinensis* (Chinese cabbage), *B. rapa* (turnip), and *B. juncea* (Indian mustard) (16,33). However, its natural host range now includes many additional crops: vegetables such as *B. oleracea* var. *botrytis* (cauliflower), *B. oleracea* var. *capitata* (white cabbage), *B. napobrassica* (swede), *Raphanus sativus* (radish), *Rheum rhabarbarum* (rhubarb); oilseed species such as *B. napus* (canola, oilseed rape), *B. carinata* (Ethiopian mustard), and *B. nigra* (black mustard); and ornamentals such as *Matthiola incana* (annual stock) and *Cheiranthus cheiri* (wallflower) (3,12,31,34,37–39). TuMV also infects many weed species, such as *R. raphanistrum* (wild radish) (9,25). After *Cucumber mosaic virus* (CMV; family *Bromoviridae*; genus *Cucumovirus*), it is considered the second most economically important virus affecting field-grown vegetable crops (38). Its economic importance varies depending on crop and location. It has a devastating effect on *Brassica* crops in China, where infection with it is widespread (26,46). Examples of quantified yield losses include 30% in *B. napobrassica* crops in Southern Ontario (35) and 70% in infected *B. napus* in England and Wales (17). Along with *Cauliflower mosaic virus* (CaMV; family *Caulimoviridae*; genus *Caulimovirus*), TuMV also causes quality defects such as internal necrotic disorders which account for around 10% of losses in stored *B. oleracea* var. *capitata* (18).

Among the different *Brassica* spp., three genomes called A, B, and C occur in different combinations: AA (*B. rapa*), BB (*B. nigra*), CC (*B. oleracea*), AABB (*B. juncea*), AACC (*B. napus*), and BBCC (*B. carinata*) (41). Since these genomes were first described, several genes and specificities for resistance to TuMV have been identified in *B. rapa* and *B. napus* (10,19,34,42–45). The dominant resistance genes in *B. napus* (TuRB01, TuRB03, TuRB04, and TuRB05) were mapped to the A genome (43), and most research with TuMV to date has concentrated on species of the *Brassica* family containing the A genome. Limited searches of the C genome did not find sources of TuMV resistance, while little attention has been paid to the B genome (43). This situation needs to be rectified because *B. juncea, B. nigra*, and *B. carinata* are oilseed plants that offer an economically and environmentally friendly alternative fuel source to other nonrenewable forms of oil. They are currently being evaluated for their biofuel potential (11,15,36). However, for a mustard species to be used in biofuel production, its oil needs to be deemed "canola-quality," which means that it must meet the same requirements as *B. napus* oil with regard to erucic acid (<2% in the oil) and glucosinolate content (<40 μmol/g in the meal) (4,5,32). As well as oil, mustard plants also generate other useful byproducts (e.g., glucosinolates which can suppress fungal pathogens and inhibit seed growth in weed species; 1,2). Mustard species are also suitable for crop rotations because they can provide an effective fungal disease break between successive cereal crops (36).

A survey of *B. napus* crops in the southwest Australian grain belt in 1998 and 1999 revealed only low incidences of TuMV infection, even when large numbers of its aphid vectors were present. Overall, 5% of *B. napus* crops sampled were TuMV infected but incidences in these crops were always low, only 1 to 2% of plants (9). In contrast, a substantial natural reservoir of TuMV infection was present in local populations of *R. raphanistrum* (9,25). The explanation for the low crop incidences was that virtually all Australian cultivars of *B. napus* have had TuMV resistances bred into them inadvertently (10). Whether the three mustard species have TuMV resistances similar to those in *B. napus* cultivars has not been studied. It is likely that TuMV infection will spread from *R. raphanistrum* weeds to nearby mustard crops, potentially causing damaging effects.

Because *B. juncea*, *B. carinata*, and *B. nigra* are out-crossing species, each of their accessions, breeding lines, and cultivars are likely to contain genetic variation. This article describes the reactions of different accessions, breeding lines, and cultivars of *B. juncea*, *B. carinata*, and *B.*

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nigra and of crosses between *B. juncea* and *B. napus* to inoculation with TuMV, and identifies frequent occurrence of resistance phenotypes, some of which were described previously (10,19) and others not recorded before.

MATERIALS AND METHODS

Growth and inoculation of plants. Seed of 37 accessions or breeding lines of *B. juncea*, 8 of *B. carinata*, 5 of *B. nigra*, 1 cultivar each of *B. juncea* and *B. carinata*, and 6 *B. napus* × *B. juncea* crosses was provided by M. Campbell from the Centre for Legumes in Mediterranean Agriculture, University of Western Australia. Seed of five breeding lines of *B. juncea* was provided by G. Walton, Department of Agriculture and Food Western Australia (DAFWA). Seed of *B. juncea* cv. Tendergreen was also from DAFWA. The plants were grown in steam-sterilized potting mix in insect-proofed, air-conditioned glasshouses maintained at approximately 18°C. For sap inoculations, infected leaves were ground in phosphate extraction buffer containing Na_2HPO_4 (11.5 g/liter) and NaH_2PO_4 (3 g/liter), and the infective sap mixed with celite (diatomaceous earth) before being rubbed onto leaves of healthy plants. *B. carinata* plants were also graft inoculated (side or top grafts) with scions from TuMV-infected *B. juncea* plants (29).

Virus isolates and antisera. TuMV isolate WA-Ap1 was from previous work (9). Isolates NSW-1 and NSW-2 were obtained from unidentified mustard species growing near Tamworth in New South Wales (NSW) and provided by M. Schwinghammer, NSW Department of Primary Industries. All three isolates were maintained by sap inoculation to *B. juncea* cv. Tendergreen plants. Polyclonal antiserum specific to TuMV was obtained from DSMZ, Braunschweig, Germany. Generic *Potyvirus* monoclonal antibodies were from Agdia Inc., USA. Except where indicated below, TuMV-specific polyclonal antibodies were always used.

Enzyme-linked immunosorbent assay. Leaf samples were extracted (1 g per 20 ml) in phosphate-buffered saline (10 mM potassium phosphate, 150 mM sodium chloride, pH 7.4, Tween 20 at 5 ml/liter, and polyvinyl pyrrolidone at 20 g/liter using a mixer mill (Retsch, Germany). Sample extracts were tested for the presence of TuMV antigen by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) as described by Clark and Adams (7). For generic *Potyvirus* testing, samples were extracted in 0.05 M sodium carbonate buffer, pH 9.6, and tested using the antigen-coated indirect ELISA protocol of Torrance and Pead (40). All samples were tested in duplicate wells in microtiter plates, and sap from TuMV-infected and healthy *B. juncea* cv. Tendergreen leaf samples was included in paired wells to provide controls. The substrate used was *p*-nitrophenyl phosphate at 0.6 mg/ml in diethanolamine, pH 9.8, at 100 ml/liter. Absorbance values at 405 nm (A_{405}) were measured in a Bio-Rad microplate reader (model 680; Bio-Sys, Australia). Absorbance values of positive samples were always more than three times those of the healthy sap control.

Responses to inoculation. In the first series of experiments, 10 plants of each accession, breeding line, or cultivar of the three mustard species (44 of *B. juncea,* 9 of *B. carinata*, and 5 of *B. nigra*) were inoculated with infective sap containing isolate WA-Ap1. At the time of inoculation, two plants of each were kept uninoculated as healthy controls to compare their appearance with that of symptomatic plants. Inoculations were at the two- to three-true leaf stage and, over the next 4 to 6 weeks, plants were inspected weekly for symptoms. Samples from inoculated and tip leaves were tested by ELISA 3 to 4 weeks after inoculation, or as early as 10 to 14 days after inoculation when symptoms were severe and developed rapidly. Uninoculated leaves recorded as uninfected were always resampled and tested again after 6 weeks. Additional inoculations with isolate WA-Ap1 were then done with nine accessions or breeding lines of *B. juncea* (5 to 10 plants inoculated each and two control plants).

In the second series of experiments, plants of selected accessions, breeding lines or cultivars of *B. juncea* (n = 13), *B. carinata* (n = 2), and *B. nigra* (n = 2) were inoculated with isolates WA-Ap1, NSW-1, and NSW-2, again using the same experimental design (10 plants inoculated each and 2 uninoculated healthy control plants).

In the third series of experiments, to determine whether systemic infection could be established in *B. carinata*, plants of three accessions that failed to develop systemic infection in the first series of experiments were graft inoculated with scions of *B. juncea* infected with isolate WA-Ap1. In all, 5 plants of 90255 and 10 plants each of 194242 and 196836 were used, and each plant had one to three scions grafted onto it. Two healthy plants of each accession were each graft-inoculated with two healthy *B. juncea* scions as controls. Observation for symptoms and testing of leaf samples was as described above, except that tip leaves from the main stem were sampled where side grafts were used whereas, with top grafts, tip leaf samples also came from axillary shoots that grew out just beneath the graft union.

In the fourth series of experiments, 10 plants from each of the six crosses (A to F) between *B. napus* and *B. juncea* were sap inoculated with isolate WA-Ap1 as in the first series of experiments (10 plants inoculated each and 2 uninoculated healthy control plants) to gauge whether the symptoms induced were similar to those of either parent. All seeds used in the fourth series of experiments were F1 generation,

Table 1. Classification of the phenotypes induced following sap inoculation of *Brassica juncea*, *B. carinata*, and *B. nigra* plants with *Turnip mosaic virus*

	Symptoms[a]		Detection by ELISA[b]		
Phenotype[c]	Inoculated leaves	Systemic	Inoculated leaves	Noninoculated leaves	Type of response
Original					
O	NI	NI	No	No	Extreme resistance with no infection detected
+	LCS	SCM, SLD	Yes	Yes	Susceptible
R	LCS	NI	Yes	No	Resistance to systemic movement without necrosis
R_N	LNS	NI	Yes	No	Resistance to systemic movement with necrosis
$+_N$	LNS	SN, SCM, SLD, S	Yes	Yes	Systemic infection with necrosis
$R_{N/+}$	LNS	SCM, SLD	Yes	Yes	Mixture of R_N and +
New					
$+_{St}$	LNS	N_{St}, SCM, SLD	Yes	Yes	Severe variant of +
$R_{N/St}$	LNS	N_{St}	Yes	No	Severe variant of R_N
$R_{N/St/+}$	LNS	N_{St}, SCM, SLD	Yes	Yes	Severe variant of $R_{N/+}$
$+_N^1$	LCS	SN, SCM, SLD	Yes	Yes	Mild variant of $+_N$
$+_{ND}$	LNS	SN, SND	Yes	Yes	Severe variant of $+_N$ (systemic hypersensitivity)

[a] Coded symptom descriptions: LCS, chlorotic spots or blotches in inoculated leaves; LNS, necrotic spots in inoculated leaves which often expanded rapidly, killing the leaves; SCM, systemic chlorotic mottle; SLD, leaf deformation; SN, systemic necrosis; N_{St}, necrotic stem streaking; S, stunting; SND, rapid plant death; NI, not infected.

[b] Detection by enzyme-linked immunosorbent assay (ELISA): Yes, positive detection; No, not detected.

[c] Original codes are those of Jenner and Walsh (19), except for $R_{N/+}$ first suggested by Coutts et al. (10).

except those of cross F, which were F2 generation.

The symptoms obtained in the first, second, and fourth series of experiments were classified according to the symptom codes of Jenner and Walsh (19), which are used as an international standard to identify resistance phenotypes caused by TuMV in *Brassica* spp. Code $R_{N/+}$ first suggested by Coutts et al. (10), was also used. These codes are shown in Table 1, along with additional codes we devised to describe the new symptom types found in mustard plants in this study, all of which were milder or more severe variants of the earlier codes.

RESULTS

Reactions to inoculation with isolate WA-Ap1. In the first series of experiments, the 44 accessions, breeding lines, or cultivars of *B. juncea* inoculated with WA-Ap1 developed varying combinations of eight phenotypes, four found previously in *B. napus* (+, $+_N$, R_N, and $R_{N/+}$) and four new phenotypes ($+_N^1$, $+_{ND}$, $R_{N/St}$, and $R_{N/St/+}$) (Table 2; Figs 1 and 2). Unlike the other phenotypes, $+_N^1$, R_N, and $R_{N/St}$ occurred only in single accessions or breeding lines. Moreover, the R_N phenotype only appeared once in a single plant of the affected accession (*B juncea* 4587), and *B. juncea* $R_{N/St}$ only in a single plant of *B. juncea* 39723233. The other five phenotypes occurred more frequently, especially $+_N$, $+_{ND}$, and $R_{N/+}$. In all, 17 accessions or breeding lines and cv. Tendergreen developed uniform reactions belonging to only one phenotype: $+_N$ (n = 8), $R_{N/+}$ (n = 6), $+_{ND}$ (n =

Table 2. Reactions of plants of *Brassica juncea*, *B. carinata*, and *B. nigra* accessions, breeding lines, and cultivars following sap inoculation with *Turnip mosaic virus* isolate WA-Ap1

Accession, breeding line, or cultivar	Geographical origin	No. plants infected/ no. inoculated	Phenotype[a]	Systemic symptoms	Detection in tip leaf
B. juncea 2529	India	10/10	$+_N$	Yes	Yes
B. juncea 3117	Kazakhstan	9/9	$+_{ND}$	Yes	Yes
B. juncea 4191	Krasnodar, Russia	10/10	$+_N$	Yes	Yes
B. juncea 4355	India	10/10	$+_N$	Yes	Yes
B. juncea 4361	India	10/10	$R_{N/+}$	Yes	Yes
B. juncea 82NO22-67	Victorian line, Australia	21/21	$R_{N/+}$	Yes	Yes
B. juncea 82NO22-98	Victorian line, Australia	20/20	$+_N$	Yes	Yes
B. juncea 82NO22-102	Victorian line, Australia	20/20	$+_N$	Yes	Yes
B. juncea ATC 93474	Former Soviet Union	10/10	$+_N$	Yes	Yes
B. juncea ATC 93948	CSIRO Breeders line, Australia	10/10	$R_{N/+}$	Yes	Yes
B. juncea JN22	Victorian line, Australia	10/10	$R_{N/St/+}$	Yes	Yes
B. juncea JN25	Victorian line, Australia	10/10	$R_{N/+}$	Yes	Yes
B. juncea original	NSW line, Australia	10/10	$+_N$	Yes	Yes
B. juncea PI 458928	Unknown	10/10	$R_{N/+}$	Yes	Yes
B. juncea PI 478333	Unknown	10/10	$+_N$	Yes	Yes
B. juncea sel4a	WA selection, Australia	10/10	$R_{N/+}$	Yes	Yes
B. juncea cv. Tendergreen	UK	10/10	+	Yes	Yes
B. juncea 2391	Siberia, Russia	8/10	$+_N$	Yes	Yes
		2/10	$+_{ND}$	Yes	Yes
B. juncea 2562	India	8/10	$+_N$	Yes	Yes
		2/10	$+_{ND}$	Yes	Yes
B. juncea 2569	India	6/10	$+_{ND}$	Yes	Yes
		4/10	$+_N$	Yes	Yes
B. juncea 3110	Kazakhstan	5/10	$+_N$	Yes	Yes
		5/10	$+_{ND}$	Yes	Yes
B. juncea 3119	Kazakhstan	8/10	$+_N$	Yes	Yes
		2/10	$+_{ND}$	Yes	Yes
B. juncea 3151	Volgograd, Russia	6/10	$R_{N/+}$	Yes	Yes
		4/10	$+_{ND}$	Yes	Yes
B. juncea 3155	Kazakhstan	12/14	$R_{N/+}$	Yes	Yes
		2/14	$+_{ND}$	Yes	Yes
B. juncea 4134	India	8/10	$R_{N/+}$	Yes	Yes
		2/10	$+_{ND}$	Yes	Yes
B. juncea 4135	India	10/14	+	Yes	Yes
		4/14	$R_{N/+}$	Yes	Yes
B. juncea 4136	India	7/10	$R_{N/St/+}$	Yes	Yes
		3/10	$+_{ND}$	Yes	Yes
B. juncea 4137	India	8/10	$+_N$	Yes	Yes
		2/10	$+_{ND}$	Yes	Yes
B. juncea 4321	Pakistan	6/10	+	Yes	Yes
		4/10	$R_{N/+}$	Yes	Yes
B. juncea 4352	India	6/10	$R_{N/+}$	Yes	Yes
		4/10	$+_{ND}$	Yes	Yes
B. juncea 4587	Vietnam	9/10	$+_N$	Yes	Yes
		1/10	R_N	No	No
B. juncea ATC 93429	Czech Republic	17/20	$+_{ND}$	Yes	Yes
		3/20	$R_{N/+}$	Yes	Yes
B. juncea ATC 93436	Germany	11/20	$R_{N/+}$	Yes	Yes
		9/20	$+_{ND}$	Yes	Yes
B. juncea ATC 93471	Former Soviet Union	7/10	$+_N$	Yes	Yes
		3/10	$R_{N/+}$	Yes	Yes

(*continued on next page*)

[a] Phenotype codes are explained in Table 1. NSW = New South Wales, WA = Western Australia, CSIRO = Commonwealth Scientific and Industrial Research Organization.

[b] Indicates that uninoculated leaves were all killed before time of sampling; therefore, enzyme-linked immunosorbent assay results could be obtained only with samples from inoculated leaves.

1), + (n = 1), and $R_{N/St/+}$ (n = 1). Twenty-five developed two different phenotypes and the remaining two developed three phenotypes each. Inoculation of eight accessions and one cultivar of *B. carinata* with WA-Ap1 produced only three phenotypes, all of which were found previously in *B. napus,* and prevented any systemic infection from occurring (R, R_N, and O). *B. carinata* 194252 developed the R reaction alone. The other eight developed two to three phenotypes each. When five accessions of *B. nigra* were inoculated with WA-Ap1, four phenotypes were obtained, two found previously in *B. napus* (+ and $R_{N/+}$) and two new phenotypes ($+_{ND}$ and $+_{St}$), one of which, $+_{St}$, only occurred in one plant of *B. nigra* 90255. All four phenotypes involved systemic infection. The five accessions developed two to four phenotypes each.

Reactions to inoculation with three different isolates. In the second series of experiments, plants of *B. juncea* infected with WA-Ap1 normally showed obvious symptoms on inoculated leaves within 10 to 14 days. In contrast, when plants of the same accessions, breeding lines, or cultivars were inoculated with NSW-1 or NSW-2, they developed symptoms of the same or greater severity on their inoculated leaves within just 7 days (Fig. 3). WA-Ap1 induced five phenotypes (+, $+_N$, $+_N{}^1$, $+_{ND}$, and $R_{N/+}$) (Table 3). It caused uniform phenotypes in 4 of 13 accessions, breeding lines, or cultivars, but 7 others developed two to four phenotypes each. With NSW-1, four of the phenotypes that developed were the same as for WA-Ap1 (+,$+_N$, $+_{ND}$, and $R_{N/+}$) but one additional phenotype, $R_{N/St/+}$, appeared in seven plants of 4137. Of the 13 accessions, breeding lines, or cultivars,

Table 2. (*continued from preceding page*)

Accession, breeding line, or cultivar	Geographical origin	No. plants infected/ no. inoculated	Phenotype[a]	Systemic symptoms	Detection in tip leaf
B. juncea ATC 93473	Former Soviet Union	8/10	$R_{N/+}$	Yes	Yes
		2/10	$+_N$	Yes	Yes
B. juncea ATC 93494	Unknown, breeders line	5/10	$+_N{}^1$	Yes	Yes
		5/10	$+_N$	Yes	Yes
B. juncea J0006	Victoria, Australia	9/10	$R_{N/St/+}$	Yes	Yes
		1/10	$+_{ND}$	Yes	Yes
B. juncea KHU4	Unknown	16/19	$R_{N/+}$	Yes	Yes
		3/19	$+_{ND}$	Yes	Yes
B. juncea cv. Manitoba	Canada	6/10	$R_{N/St/+}$	Yes	Yes
		4/10	$+_{ND}$	Yes	Yes
B. juncea PC9844	Unknown	8/10	$R_{N/+}$	Yes	Yes
		2/10	+	Yes	Yes
B. juncea PI458927	Unknown	5/10	$+_N$	Yes	Yes
		5/10	$R_{N/+}$	Yes	Yes
B. juncea PI458929	Unknown	6/10	+	Yes	Yes
		4/10	$R_{N/+}$	Yes	Yes
B. juncea 4133	India	6/10	$+_{ND}$	Yes	Yes
		2/10	$R_{N/+}$	Yes	Yes
		2/10	$+_N$	Yes	Yes
B. juncea 39723233	CSIRO, Australia	15/17	$R_{N/+}$	Yes	Yes
		1/17	$+_{ND}$	Yes	Yes
		1/17	$R_{N/St}$	No	No
B. carinata 194252	Kenta, Ethiopia	10/10	R	No	No
B. carinata 92476	India	8/10	R	No	No
		2/10	R_N	No	No
B. carinata 194904	Gonder, Ethiopia	9/10	R	No	No
		1/10	R_N	No	No
B. carinata 90255	Unknown	4/6	R	No	No
		2/6	O	No	No
B. carinata 194900	Gonder, Ethiopia	6/10	R	No	No
		4/10	R_N	No	No
B. carinata 196836	Welega, Ethiopia	6/10	R	No	No
		3/10	O	No	No
		1/10	R_N	No	No
B. carinata 273640	Shewa, Ethiopia	7/10	R_N	No	No
		1/10	O	No	No
		2/10	R	No	No
B. carinata cv. Gobhi Sarson	India	6/10	R_N	No	No
		3/10	O	No	No
		1/10	R	No	No
B. carinata 195921	Ethiopia	5/10	R	No	No
		3/10	R_N	No	No
		2/10	O	No	No
B. nigra 4318	Unknown	8/10	$R_{N/+}$	Yes	Yes
		2/10	+	Yes	Yes
B. nigra 90747	Unknown	7/10	+	Yes	Yes
		3/10	$R_{N/+}$	Yes	Yes
B. nigra 91182	Madrid, Spain	7/10	$R_{N/+}$	Yes	Yes
		2/10	+	Yes	Yes
		1/10	$+_{ND}$	Yes	Yes[b]
B. nigra 90745	Unknown	6/10	$+_{ND}$	Yes	Yes
		3/10	$R_{N/+}$	Yes	Yes
		1/10	+	Yes	Yes
B. nigra 90255	Ethiopia	3/10	$+_{ND}$	Yes	Yes
		3/10	$R_{N/+}$	Yes	Yes
		3/10	+	Yes	Yes
		1/10	$+_{St}$	Yes	Yes

4 gave uniform phenotypes but 9 developed two to four phenotypes each (e.g., in 3155; Fig. 3B). With NSW-2, the phenotypes that developed were the same as those obtained with WA-Ap1, except that one additional phenotype, R_N, developed in one plant of accession 4137. Of the 13 accessions, breeding lines, or cultivars, 5 gave uniform phenotypic responses and 8 developed two to four phenotypes each. WA-Ap1 infection resulted in three phenotypes in *B. carinata* 194252 (R, O, and R_N) and four in 196836 (R, O, R_N, and $R_{N/+}$) (Table 3). The single plant with $R_{N/+}$ represented the only occasion when systemic infection developed in this species (Fig. 2B). When inoculated with either NSW-1 or NSW-2, 194252 developed the same three phenotypes as with WA-Ap1 but with slightly different numbers of plants within each phenotypic category. With NSW-1 and NSW-2, 196836 only developed phenotypes R and O. In plants of *B. nigra,* WA-Ap1 infection resulted in only two phenotypes (+ and $+_{ND}$) in 90255 and 90747 (Table 3). With NSW-1 and NSW-2, 90255 developed the same two phenotypes (+ and $+_{ND}$). In 90747, NSW-1 infection resulted in four (+, $+_N^1$, $+_{ND}$, and $+_N$) and NSW-2 in three (+, $R_{N/+}$, and $+_{ND}$) phenotypes.

In the second series of experiments, the inoculations generally resulted in development of a similar number of phenotypes within each combination of isolate and accession, breeding line, or cultivar, although sometimes there was variation in the proportions of plants with each phenotype. However, with isolate WA-Ap1, although the plants developed symptoms at similar rates during the two experimental periods and the ELISA optical density (A_{405}) values remained similar, indicating no differences in virus titer, only 2 of 13 *B. juncea* accessions, breeding lines, or cultivars gave exactly the same results as in the first series of experiments (cv. Tendergreen and 4321). With the others, the numbers of phenotypes found varied by one to three per accession, breeding line, or cultivar. These differences reflected subtraction (i.e., absence) of individual phenotypes previously present, or addition of or substitution by more severe or milder variants of the original phenotypes described previously in *B. napus* (Table 1).

Reactions of *B. carinata* to graft inoculation. In five plants of *B. carinata* 90255 graft inoculated with isolate WA-Ap1, at 3 to 4 weeks, all 14 grafts were associated with stem necrosis just below the graft union, and the scion was dead at 4 weeks. After 6 weeks, when leaf samples from the stock were tested, no virus was detected. In the 10 graft-inoculated plants of 194252, at 3 to 4 weeks, 9 of 19 grafts were associated with yellowing and necrotic spotting of the stem just below the graft union and necrosis of the graft union itself. No symptoms developed on the stem with 10 of 19 grafts but, after 4 weeks, in four plants in which the scions had died, mild symptoms of chlorotic rings or mottle appeared in leaves of some axillary shoots close to the graft unions. When leaf samples were tested after 5 weeks, virus was detected in two samples of symptomatic leaves but not in samples from symptomless leaf tissue. However, these two positives were obtained only with generic *Potyvirus* monoclonal antibodies after initial

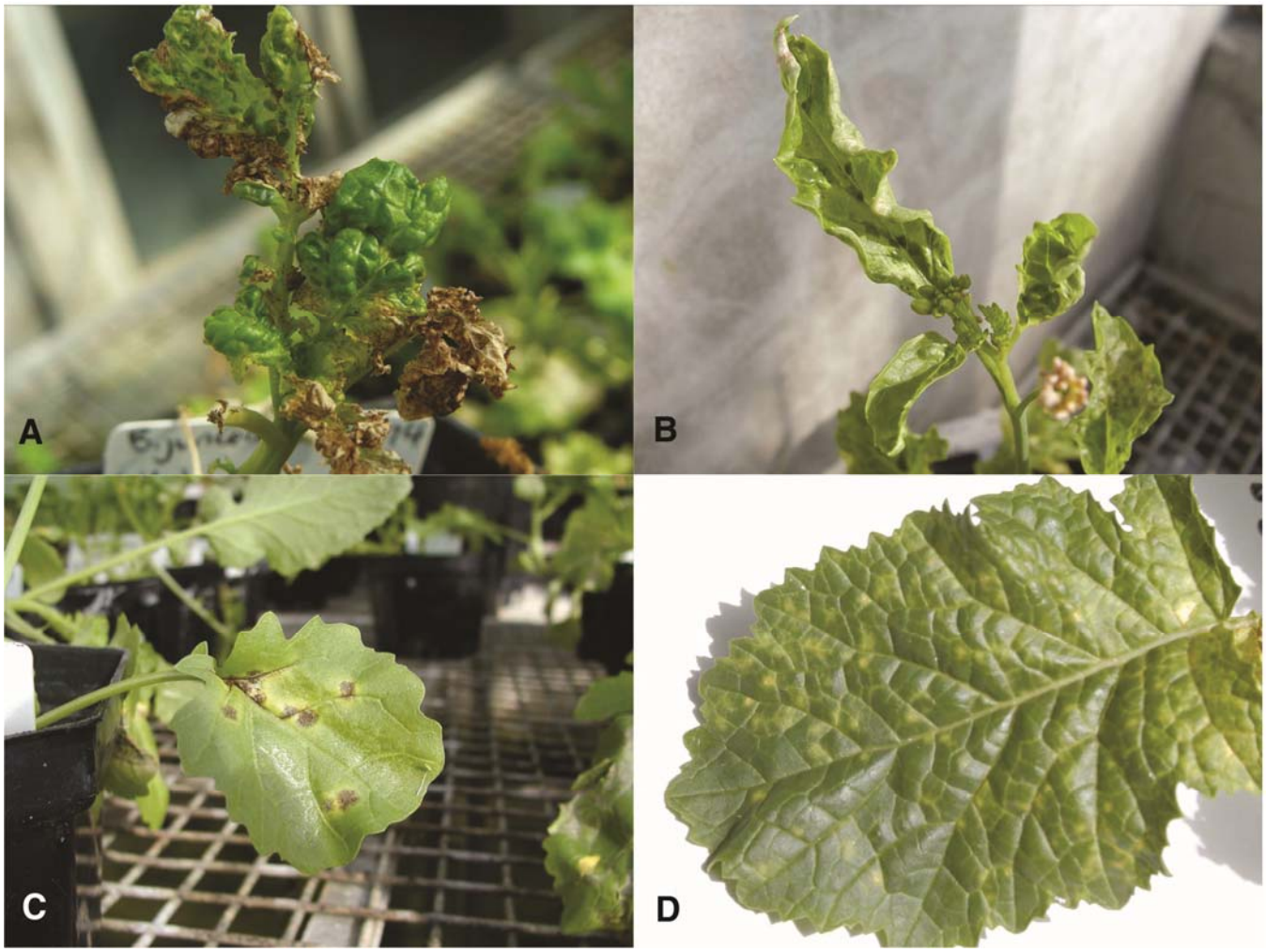

Fig. 1. Symptoms caused by infection of *Brassica juncea* plants following sap inoculation with *Turnip mosaic virus* isolate WA-Ap1. **A,** Severe systemic necrosis, chlorotic mottle, leaf deformation, and stunting in ATC93474 (phenotype $+_N$); **B,** systemic chlorotic mottle and leaf deformation in cv. Tendergreen (phenotype +); **C,** necrotic spot local lesions 13 days after leaf inoculation in 39723233 (shows R_N component of phenotype $R_{N/St}$); **D,** local chlorotic spot lesions about 2 weeks after leaf inoculation in 93429 (phenotype +).

negative results using TuMV-specific antibodies. At 6 weeks, when further samples were tested using generic *Potyvirus* antibodies, no virus was detected. In the 10 graft-inoculated plants of 196836, at 3 to 4 weeks 10 of 14 grafts were associated with yellowing or small necrotic spots on the stem just below the graft union. Two plants also showed additional symptoms of necrosis of the graft union itself, which led to scion death after 5 weeks. After 4 weeks, 3 of 10 plants had mild chlorotic rings or ringspots on leaves of axillary shoots close to the graft union but there were no other symptoms. At 5 weeks, samples of symptomatic leaf tissue all gave negative results when tested using specific TuMV antibodies. When the tests were repeated using generic *Potyvirus* antibodies, virus was detected in two of three symptomatic leaf samples. At 6 weeks, when further leaf samples from all plants were tested using the same antibodies, no virus was detected.

Reactions of crosses between *B. juncea* and *B. napus.* Ten days after sap inoculation with isolate WA-Ap1, plants of four of the six crosses (A to D) showed symptoms of necrotic or chlorotic spots in inoculated leaves (Table 4). Plants of the other two crosses (E and F) only developed these symptoms in some inoculated leaves after 31 days. Plants of crosses A and D developed phenotypes R_N or R, those of crosses B or C developed R_N or O, and those of crosses E and F developed R_N, R, or O. When samples from inoculated leaves were tested after 3 weeks, TuMV was detected in a few plants with necrotic or chlorotic spot local lesions (phenotypes R or R_N) but never in plants with phenotype O, indicating low titer that was difficult to detect resulting from the phenotypes R and R_N and lack of infection with O. At 7 weeks, when samples from tip leaves on all plants were tested, all were negative, indicating that infection had not spread out of inoculated leaves.

DISCUSSION

Although there are limitations to this study because additional replication is needed to help explain the phenotypic responses obtained, it nevertheless provides important new knowledge about TuMV resistance in mustard species. Diverse TuMV resistance phenotypes were found readily among different accessions, breeding lines, or cultivars of *B. juncea, B. carinata*, and *B. nigra.* Those in *B. carinata* limited spread of systemic infection successfully, resembling phenotypes O, R, and R_N of *B. napus*. By contrast, the resistance phenotypes in *B. juncea* and *B. nigra* included systemic necrosis, resembling systemic necrosis phenotype $+_N$ of *B. napus* (10,19,42–45). In addition, plants within individual mustard accessions, breeding lines, or cultivars often developed different resistance phenotypes, reflecting genetic variability arising from the outcrossing nature of these three species. Of the 11 resistance phenotypes observed, 5 were not recorded previously in studies with TuMV in *B. napus* (10,19,42–45). However, these additional phenotypes were milder or more severe variants of the six original phenotypes: $+_{St}$ of +, $R_{N/St}$ of R_N, $R_{N/St/+}$ of $R_{N/+}$, $+_N^1$ of $+_N$, and $+_{ND}$ of $+_N$ (Table 1). Three ($+_N^1$, $R_{N/St}$ and $+_{St}$) appeared to be of minor importance in this study because they rarely occurred. Although the mustard accessions used were from diverse geographical locations and collections, the phenotypic reactions obtained seemed unrelated to geographical origin of seed. When plants of 44 accessions, breeding lines, or cultivars of *B. juncea* were inoculated with TuMV isolate

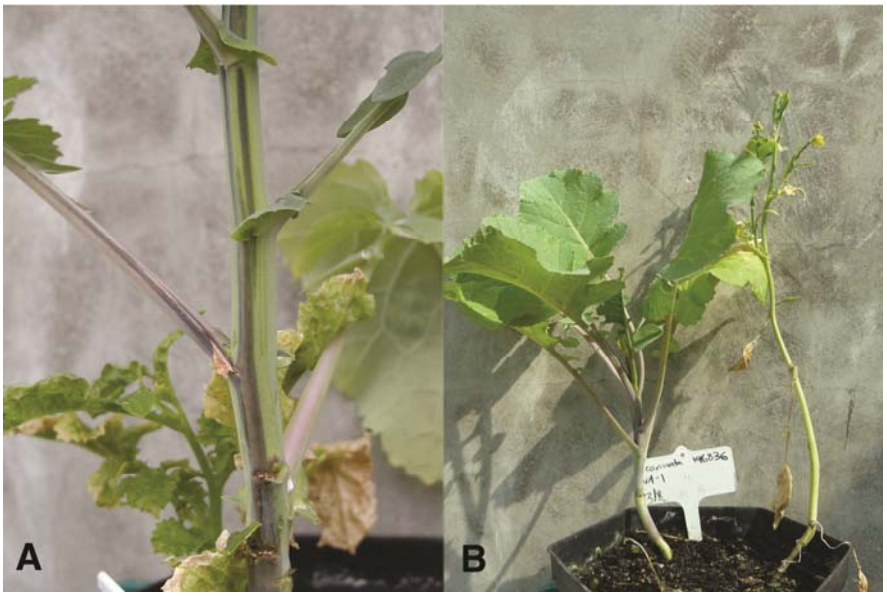

Fig. 2. Symptoms caused by infection following sap inoculation with *Turnip mosaic virus* isolate WA-Ap1. **A,** Necrotic stem streaking (which never reached the growing tip) caused by systemic movement in *Brassica juncea* 39723233 (phenotype $R_{N/St}$), same plant as that in Figure 1c; shoot at back shows a stunted, systemically infected plant of same accession (phenotype $R_{N/+}$); **B,** two plants of *B. carinata* 196836; on the right, the only plant that became infected systemically shows systemic chlorotic mottle, reduction in leaf size, and leaf deformation (phenotype $R_{N/+}$) and, on the left, a plant that became infected in its inoculated leaves showing healthy growth of upper leaves (phenotype R_N).

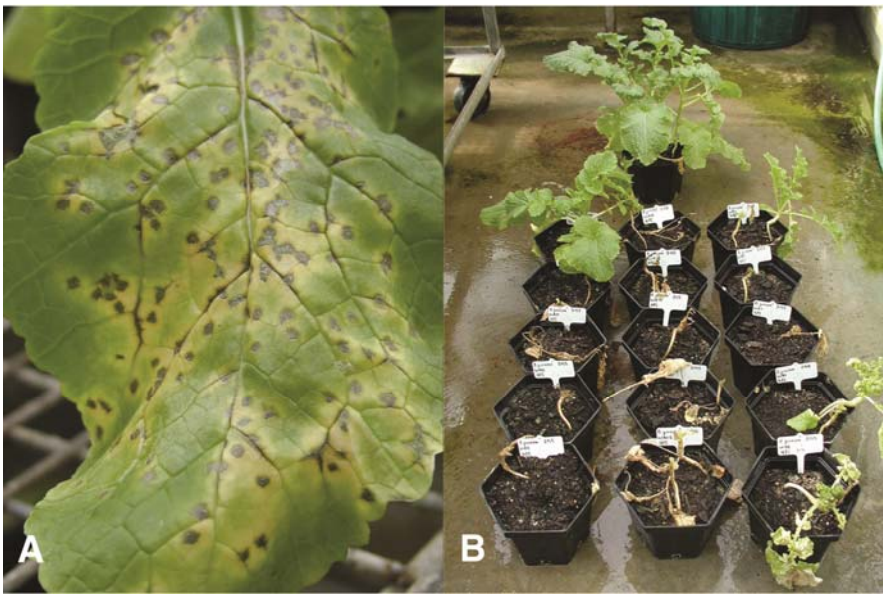

Fig. 3. Symptoms caused by infection of *Brassica juncea* plants following sap inoculation with *Turnip mosaic virus*. **A,** Necrotic spot local lesions 7 days after leaf inoculation with isolate NSW-2 in ATC 93471 (phenotype $+_{ND}$); **B,** segregation for systemic necrosis and death (phenotype $+_{ND}$), systemic necrosis (phenotype $+_N$), and susceptibility (phenotype +) in 3155; left row shows plants infected with isolate NSW-2 (phenotypes + in one plant and $+_{ND}$ in others), middle row shows plants infected with isolate NSW-1 (all plants, phenotype $+_{ND}$), and right row shows plants infected with isolate WA-Ap1 (phenotypes $+_N$ in three plants and $+_{ND}$ in two plants); plant at the back is a healthy control.

WA-Ap1, systemic infection always developed (except in two instances in one plant each), and the common occurrence of new resistance phenotype $+_{ND}$ (systemic death; the role of this phenotype in agronomic resistance is discussed below) was revealed. Earlier studies with *B. napus,* which has the A and C genomes, did not reveal such a reaction but found that TuMV resistance genes TuRB01 and TuRB03, which are located only in the A genome, control systemic necrosis phenotype $+_N$ (19,42–45). The $+_{ND}$ resistance phenotype may reflect more severe expression of the $+_N$ phenotype in response to the presence of TuRB01 or TuRB03 in the A genome of *B. juncea*. Phenotype R_N is induced in the presence of TuMV resistance genes TuRB04 and TuRB05 of *B. napus* (42–44). It was found only in *B. juncea* 4587, whereas phenotype $R_{N/St}$ was found only in 39723233. Both phenotypes might represent the presence of TuRB04 or TuRB05 in their A genomes, the latter merely reflecting a more severe expression of the R_N reaction. Phenotype $R_{N/+}$ (but not R) was found in *B. juncea*, as were the new phenotypes $+_N^1$ (mild variant of $+_N$), and $R_{N/St/+}$ (severe variant of $R_{N/+}$). No resistance genes controlling phenotypes R and $R_{N/+}$ have been identified as yet in *B. napus*. Thus, in *B. juncea*, $R_{N/+}$, $+_N^1$, and $R_{N/St/+}$ might be controlled by unknown resistance genes in its A or B genomes.

When 13 *B. juncea* accessions, breeding lines, or cultivars were inoculated with isolates NSW-1 and NSW-2, and a second time with isolate WA-Ap1, NSW-1 and NSW-2 were more virulent than WA-Ap1. WA-Ap1 was originally isolated from wild *R. raphanistrum* plants (9) whereas the other two came from mustards. These isolates were pathotyped at the University of Warwick, Wellesbourne, UK: WA-Ap1 belongs to pathotype 8 (10) while NSW-1 and NSW-2 belong to pathotypes 7 and 1, respectively (J. A. Walsh, *personal communication*). Often, across the three isolates, each accession, breeding line, or cultivar inoculated gave similar responses. In most instances, where a phenotype was different, it was just a variant of another present in the same accession, breeding line, or cultivar. The differences between the two repeat inoculations with WA-Ap1 presumably reflect genetic variability arising from the use of seed lots obtained from open-pollinated plants and the presence of phenotypes that are minor variants of the original phenotypes defined previously for TuMV in *B. napus* (19). It seems unlikely that the differences were due to environmental variation between successive experiments because the same air-conditioned glasshouse was used, in which the temperature settings remained unchanged and both humidity and light (intensity and photoperiod) remained similar. Also, the plants developed symptoms at similar rates during the two experimental times and the ELISA optical density (A_{405}) values did not indicate that differences in

Table 3. Reactions of plants of selected accessions, breeding lines, or cultivars of *Brassica juncea*, *B. carinata*, and *B. nigra* to sap inoculation with three isolates of *Turnip mosaic virus*

	Isolate[a]		
Accession, breeding line, or cultivar	**WA-Ap1**	**NSW-1**	**NSW-2**
B. juncea cv. Tendergreen	+ (10)	$+_N$ (9)	+ (9)
B. juncea cv. Manitoba	$+_N$ (8), $R_{N/+}$ (2)	$+_{ND}$ (8), $R_{N/+}$ (2)	$+_{ND}$ (7), $R_{N/+}$ (3)
B. juncea JN22	$R_{N/+}$ (8), $+_{ND}$ (1), $+_N$ (1)	$+_{ND}$ (4), + (4), $R_{N/+}$ (2)	$+_{ND}$ (7), + (2), $+_N$ (1)
B. juncea JN25	$R_{N/+}$ (7), $+_{ND}$ (3)	$R_{N/+}$ (7), $+_{ND}$ (3)	$+_{ND}$ (7), + (3)
B. juncea 2569	$+_{ND}$ (10)	$+_{ND}$ (5), $+_N$ (3)	$+_{ND}$ (10)
B. juncea 3151	$+_{ND}$ (10)	$+_{ND}$ (9), $+_N$ (1)	$+_{ND}$ (10)
B. juncea 3155	$+_{ND}$ (3), $+_N$ (2)	$+_{ND}$ (9)	$+_{ND}$ (9), + (1)
B. juncea 4136	$R_{N/+}$ (6), $+_{ND}$ (4)	$+_{ND}$ (6), $R_{N/+}$ (6)	$+_{ND}$ (5), $+_N$ (3), + (2)
B. juncea 4137	$+_N$ (7), $+_N^1$ (6), $R_{N/+}$ (2), + (2), $+_{ND}$ (1)	$+_{ND}$ (10), $R_{N/St/+}$ (7), $+_N$ (2), $R_{N/+}$ (1)	+ (10), $R_{N/+}$ (5), $+_{ND}$ (4), R_N (1)
B. juncea 4321	+ (6), $R_{N/+}$ (4)	+ (8), $R_{N/+}$ (2)	+ (9), $R_{N/+}$ (1)
B. juncea ATC 93429	$+_N$ (7), + (2)	$+_{ND}$ (5), $+_N$ (2)	$+_{ND}$ (8)
B. juncea ATC 93436	$+_N$ (7), + (1)	$+_{ND}$ (8)	$+_{ND}$ (9)
B. juncea ATC 93471	$+_N$ (10)	$+_{ND}$ (10)	$+_{ND}$ (9), $R_{N/+}$ (1)
B. carinata 194252	R (6), O (3), R_N (1)	R (8), R_N (1), O (1)	R (5), R_N (4), O (1)
B. carinata 196836	R (7), O (1), R_N (1), $R_{N/+}$ (1)	R (6), O (4)	R (6), O (4)
B. nigra 90747	+ (7), $+_{ND}$ (3)	+ (4), $+_N^1$ (4), $+_{ND}$ (1), $+_N$ (1)	+ (6), $R_{N/+}$ (3), $+_{ND}$ (1)
B. nigra 90255	+ (6), $+_{ND}$ (4)	$+_{ND}$ (6), + (4)	+ (9), $+_{ND}$ (1)

[a] Phenotype codes are explained in Table 1. Numbers in parentheses indicate number of plants showing that phenotype.

Table 4. Reactions of progeny plants from crosses between *Brassica napus* and *B. juncea* to inoculation with *Turnip mosaic virus* isolate WA-Ap1

					ELISA results[a]	
Cross	**Parents (*B. napus* × *B. juncea*)[b]**	**Total no. of plants inoculated**	**Phenotypes[c]**	**No. of plants with phenotype**	**Inoculated leaves**	**Tip leaves**
A	(Monty × JN25) × Monty	10	R_N	7	Yes (3)	No
	…	…	R	3	…	…
B	Karoo × (Karoo × JN22)	10	R_N	6	No	No
	…	…	O	4	…	…
C	(Karoo × JN22) × Karoo	10	R_N	9	Yes (3)	No
	…	…	O	1	…	…
D	(Karoo × JN25) × Karoo	10	R_N	5	Yes (4)	No
	…	…	R	5	…	…
E	Karoo × JN22	10	R_N	4	No	No
	…	…	R	3	…	…
	…	…	O	3	…	…
F	Karoo × JN22	10	R_N	4	No	No
	…	…	R	3	…	…
	…	…	O	3	…	…

[a] ELISA = enzyme-linked immunosorbent assay. Numbers in parentheses indicate the number of plants showing that phenotype.

[b] All seeds used were F1 generation, except those of cross F, which were F2 generation. Where a parent is represented in parenthesis by a cross between a cultivar and a breeding line, progeny of this cross was backcrossed to the other parent shown. In the *B. napus* parents used in these crosses, cv. Monty had previously segregated for phenotypes R, R_N, and $+_N$ and cv. Karoo for phenotypes R, R_N, and + (10).

[c] Phenotype codes are explained in Table 1.

TuMV titer could be responsible for variation in phenotypic responses. A noteworthy exception to the presence of different phenotypes was when cv. Tendergreen plants were inoculated with WA-Ap1 and NSW-2 versus NSW-1. Each isolate produced a uniform response with no segregation, and the phenotypes were + (susceptible) with WA-Ap1 and NSW-2, but $+_N$ (systemic necrosis) with NSW-1. The $+_N$ resistance phenotype obtained with isolate NSW-1 in cv. Tendergreen may represent a pathotype-specific response.

When five accessions of *B. nigra* were challenged by sap inoculation with isolate WA-Ap1, they developed three phenotypes that occur in *B. napus* (+, $+_N$, and $R_{N/+}$) (10,19,42) and two new phenotypes ($+_{ND}$ and $+_{St}$). Because the A and C genomes are absent in this species, the presence of phenotypes $+_N$, $R_{N/+}$, and $+_{ND}$ may reflect unknown resistance genes located in the B genome. Unknown resistance genes present in the B genome could also be responsible for the $+_N$ phenotypic reaction of some *B. juncea* accessions or breeding lines. When two *B. nigra* accessions were inoculated with all three isolates, phenotypes +, $+_N$, $+_{ND}$, and $R_{N/+}$ were again obtained; $+_N{}^1$ also developed but $+_{St}$ did not.

When inoculated with the three TuMV isolates, plants of *B. carinata* developed phenotypes (O, R, R_N, or $R_{N/+}$) similar to those recorded previously in *B. napus*. However, *B. carinata* contains only the B and C genomes whereas the TuMV resistance genes described so far are in the A genome (10,42). Therefore, phenotypes O, R, and R_N might reflect the presence of unknown resistance genes located in the B or C genomes. Phenotype $R_{N/+}$ was only found once in *B. carinata* and this was the only occasion when systemic infection was recorded on sap inoculation of this species. Because of the lack of systemic infection in all other plants of *B. carinata* following sap inoculation, graft inoculations were done with isolate WA-Ap1. Symptoms associated with the grafts included necrosis of the graft union, yellowing and necrotic spotting of the stem just below the graft union, and, when top grafts were used, chlorotic rings or mottle on leaves of axillary shoots formed just beneath the graft union, or no symptoms appeared. Virus was only detected occasionally in symptomatic leaves of axillary shoots, and then only with generic *Potyvirus* antibody rather than TuMV-specific antibodies. This sporadic detection of virus in axillary shoots might reflect low virus titer and greater sensitivity of the generic *Potyvirus* antibody than the TuMV-specific antibodies used. No virus was detected in tip leaves on the main stem, indicating that spread remained localized to the graft. Such localized reactions to graft inoculation in *B. carinata* reflect those found in other situations where plants with extreme resistance are graft-inoculated with a virus (e.g., when potato plants with gene *Ry* are graft inoculated with *Potato virus Y* [family *Potyviridae*; genus *Potyvirus*]; 21).

When F1 or F2 generation plants grown from crosses between different combinations of *B. napus* cultivars and *B. juncea* breeding lines were sap inoculated with TuMV, none became infected systemically. The phenotypes found in the progeny plants were R and R_N (crosses A and D), R_N and O (crosses B and C), and R, R_N, and O (crosses E and F). These resistances are likely to include those located in the A genome because the phenotypic reactions of these plants are similar to those seen in *B. napus*. In the *B. napus* parents used in these crosses, cv. Monty had previously segregated for phenotypes R, R_N, and $+_N$, and cv. Karoo for phenotypes R, R_N, and + (10).

Many resistance genes localize infection in the plant, thus preventing systemic movement (14), as occurred with the *B. carinata* TuMV resistance phenotypes. Examples of useful resistance genes that control systemic hypersensitive resistance phenotypes such as those in *B. juncea* and *B. nigra* include *Nbm-1* in lupin; *Nc, Nx, Nv*, and *Nytbr* in potato (*Solanum tuberosum*); *Nam-1* in button medic (*Medicago orbicularis*); and *Rsv1-s* in soybean (*Glycine max*) (6,8,20,21,24,27,30). Such genes have been used by plant breeders for more than 60 years (e.g., systemic hypersensitivity genes *Nc* and *Nx* in breeding for virus resistance in potato; 8). They are effective in the field because, by killing infected plants quickly, they are eliminated as internal within-crop sources of virus infection for acquisition and further spread by vectors or by contact. This greatly decreases rate of epidemic development and final infection incidence, resulting in much decreased yield losses (22,23,28).

In a separate study under the same conditions, when 55 Australian breeding lines of *B. juncea* were inoculated with isolate WA-Ap1, most developed fully susceptible phenotypes without necrosis (*data not shown*). This contrasts with the results for the *B. juncea* accessions, breeding lines, and cultivars evaluated in our study, in which almost all developed resistance phenotypes in some or all plants inoculated. Without breeding to incorporate TuMV resistance into future *B. juncea* cultivars, damaging epidemics of this virus are likely to occur. Our research suggests that useful sources of resistance to TuMV may be available in *B. juncea*, *B. nigra*, and *B. carinata* germplasm. Further research to identify the resistance genes involved would establish the potential usefulness of these resistance phenotypes in breeding TuMV-resistant mustard cultivars for biofuel production (4,5,11,15,32).

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