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Identification and genetic inheritance of a new source of broad-spectrum extreme resistance to turnip mosaic virus (TuMV) in *Brassica rapa*

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Abstract *Brassica rapa* line K185 was identified with broad-spectrum extreme resistance to turnip mosaic virus (TuMV). The resistance was effective against TuMV isolates UK 1, CZE 1, GBR 6, POL 1 and CDN 1 (representing major pathotypes 1, 3 and 4 and major resistance-breaking isolates) following mechanical inoculation. F_1 plants from a cross between K185 and the rapid-cycling *B. rapa* ssp. *trilocularis* line R-o-18 (uniformly susceptible to all the above TuMV isolates), were resistant following challenge with TuMV isolates CDN 1 and GBR 6, indicating the involvement of dominant gene(s). F_2 plants derived from a single F_1, CDN 1- and GBR 6-resistant plant segregated for resistance to TuMV isolate GBR 6. The segregation ratio of resistant to susceptible plants was consistent with at least two loci controlling resistance and with both loci having dominant alleles for resistance. Other sources of broad-spectrum resistance to TuMV have been shown to involve the recessive allele *retr01*. The broad-spectrum resistance in K185 clearly involves different alleles to *retr01* and provides the opportunity to introgress an alternative form of TuMV resistance into commercial *B. rapa* lines and thereby reduce the selection pressure for *retr01* resistance breaking.

Keywords Virus resistance · *Brassica rapa* · Turnip mosaic virus · Dominant resistance genes · Broad-spectrum resistance

Introduction

According to the FAO (FAO 2019), 70.2 million tonnes of 'cabbages and other brassicas' were produced worldwide in 2019. Of these, Chinese cabbage (*Brassica rapa*) had the greatest production and is one of the most important vegetable crops in the world, with a very large planting area and yield (Cao et al., 2006). It is a very important micronutrient (Fahey, 2016) and food source for many people, particularly in Asia.

Turnip mosaic virus (TuMV) is one of the major problems in the cultivation of Chinese cabbage and in some parts of the world is the biggest disease problem. It was ranked second to cucumber mosaic virus as the most important virus infecting field-grown vegetables in a survey of plant virologists from 28 countries and regions (Tomlinson, 1987).

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TuMV is classified in the *Potyvirus* genus, within the *Potyviridae* family (International Committee of Taxonomy of Viruses, 2019; Nellist et al. 2022). It has a very wide host range, at least 318 plant species, including most *Brassica* species (Walsh et al., 2002). TuMV affects the cultivation of oilseed rape in some parts of the world and the brassica vegetables grown widely in Europe, including broccoli, cauliflower and cabbage (*Brassica oleracea*) (Tomlinson, 1987). It has also been considered the most important virus of vegetable brassica crops cultivated in Asia, causing losses in Chinese cabbage, radish (*Raphanus raphanistrum*) and Indian mustard (*Brassica juncea*) for many years (Green & Deng, 1985).

TuMV is transmitted by at least 89 different species of aphids (Walsh & Jenner, 2002), however insecticides are not very effective at stopping aphid transmission of persistently transmitted viruses such as TuMV to plants (Walsh et al., 2002) and the most important vector of TuMV in Europe, *Myzus persicae* has evolved several resistance mechanisms to the most commonly used insecticides (Bass et al., 2014). The most effective insecticides, the neonicotinoids, were banned for use on field crops in the EU in 2013 (The European Commission, 2013). It has been suggested that the best way to stop outbreaks of TuMV would be through natural plant resistance to the virus (Walsh et al., 2002). A number of sources of dominant, strain-specific resistance to TuMV have been identified (Li et al., 2019; Nellist et al., 2022; Palukaitis & Kim, 2021; Walsh & Jenner, 2002), however, to date, the only broad-spectrum source of resistance that is undefeated is the recessive gene *retr01* (Nellist et al., 2014).

This paper describes the identification of the *B. rapa* line K185 with resistance to a range of TuMV isolates representing the most common and important pathotypes of the virus worldwide and some resistance breaking isolates. The line K185 was crossed to a rapid-cycling *B. rapa* ssp. *trilocularis* line that is uniformly susceptible to TuMV isolates to generate F_1 and F_2 generations. These were phenotyped to reveal the genetic inheritance of the resistance, clearly showing that it involves alleles different from *retr01*. This provides the opportunity to introgress an alternative form of TuMV resistance into commercial *B. rapa* lines and thereby reduce the selection pressure for resistance-breaking of both the extant and the future TuMV resistance sources.

Materials and methods

Plant material and cultivation

B. rapa line K185 is an inbred *B. rapa* ssp. *rapa* turnip line, selected for resistance to TuMV isolate CDN 1. Plant line R-o-18 is an inbred line of *B. rapa* ssp. *trilocularis*, known to be susceptible to a broad range of TuMV isolates (Rusholme et al., 2007). Line 165 is an inbred *Brassica napus* swede line with TuMV resistance genes *TuRB04* and *TuRB05*, which together confer resistance to pathotype 1 and 3 isolates of TuMV (Jenner & Walsh, 1996; Walsh, 1989). Line 22S is an inbred *B. napus* oilseed rape line with the TuMV resistance gene *TuRB03* conferring resistance to the CDN 1 isolate of TuMV and some pathotype 3 isolates (Hughes et al., 2003). A commercial variety of *Brassica rapa* ssp. *perviridis* susceptible to all TuMV isolates was deployed in this study as a susceptible control. Seeds were sown in 7cm plastic pots containing Levington M2 compost in a glasshouse and plants were subsequently maintained with air conditioning and heating at 18 ± 2°C.

A plant from line K185 was crossed with an R-o-18 plant to produce the F_1 generation (Fig. 1). Following challenge with TuMV isolates GBR 6 and subsequently CDN 1, a TuMV-resistant F_1 plant, was selfed to produce F_2 seed (Fig. 1).

Virus isolates

The origins and maintenance of TuMV isolates UK 1 (pathotype 1), CZE 1 (pathotype 3), GBR 6 (pathotype 4), CDN 1 (pathotype 4) and POL 1 (pathotype 4) have been described by Jenner and Walsh (1996).

Infection assays

In infection assays, the cotyledons and the first, second and third true-leaves of brassica plants were mechanically inoculated at the three true-leaf stage using the inoculation procedure described by Jenner and Walsh (1996). The symptoms of the plants were visually assessed on three occasions, 15 days post inoculation (dpi), 22 dpi and 34 dpi. Phenotypes

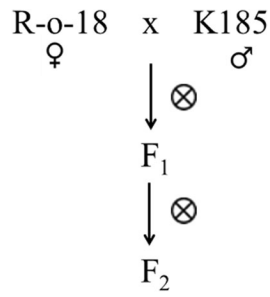

Fig. 1 Crossing strategy to develop the first (F_1) and second (F_2) filial generations used to assess the resistance/susceptibility to turnip mosaic virus (TuMV) in the inbred *B. rapa* line K185. ⊗, Self-pollination; ♀, female parent; ♂, male parent. K185 plants have resistance to a range of TuMV isolates and R-o-18 is susceptible to most TuMV isolates, including all those deployed in this study. The F_1 plant was challenged with TuMV isolates GBR 6, followed by CDN 1, prior to selfing to check that it was resistant

were classified as, susceptible with mosaic symptoms in leaves (+), susceptible with necrotic leaf symptoms ($+_N$), or extremely resistant (0) as described by Jenner and Walsh (1996). To verify the phenotypes, leaves from plants were tested 22 dpi by indirect plate-trapped antigen enzyme-linked immunosorbent assay (ELISA) as described by Walsh et al. (2002).

The *B. rapa* plant line K185 was mechanically inoculated with TuMV isolates UK 1, CZE 1, CDN 1, GBR 6 and POL 1, ten plants per virus isolate. Two plants were inoculated with sap from uninfected *B. rapa* ssp. *perviridis* plants as controls. The virus isolates were also inoculated to the susceptible control *B. rapa* ssp. *perviridis* line, two plants per isolate, to verify the viability of the inoculum.

TuMV isolate GBR 6, followed by isolate CDN 1 were inoculated to two F_1 plants from the cross R-o-18 x K185 and one F_1 plant was inoculated with sap from uninfected *B. perviridis* plants as a control.

TuMV isolate GBR 6 was inoculated to 100 F_2 plants derived from a GBR 6 and CDN 1-resistant F_1 plant. Ten plants each of *B. napus* lines 165 and 22S and *B. rapa* ssp. *perviridis* were inoculated with the same GBR 6 inoculum. Two plants of all the lines were inoculated with sap from uninfected *B. rapa* ssp. *perviridis* plants as mock-inoculated controls.

Resistance inheritance analysis

The study of inheritance was based on the phenotypic classes of extreme resistance (0) and susceptibility (+). The chi-square test (χ^2), 5% probability was used to test Mendelian models based on the action of one (3 resistant: 1 susceptible), two (9 resistant: 7 susceptible) and three (27 resistant: 37 susceptible) dominant alleles for TuMV resistance (Supplementary Table 1). Additional models to investigate the inheritance of the necrotic susceptibility phenotype ($+_N$) were tested based on the action of two dominant alleles, where both are required for resistance and the presence of only one (9 resistant: 3 necrotic: 4 susceptible without necrosis [mosaic]), or either one in the absence of the other allele (9 resistant: 6 necrotic: 1 susceptible without necrosis [mosaic]) results in necrosis. Models for the action of three dominant alleles where all three are required for resistance and the presence of any (27 resistant: 36 necrotic: 1 susceptible without necrosis [mosaic]), only one (27 resistant: 21 necrotic: 16 susceptible without necrosis [mosaic]), a combination of two specific (27 resistant: 9 necrotic: 28 susceptible without necrosis [mosaic]), or a combination of any two (27 resistant: 27 necrotic: 10 susceptible without necrosis [mosaic]) of the three dominant alleles results in necrosis, were tested (Supplementary Table 1).

Results

Resistance spectra of *B. rapa* line K185 plants following challenge with TuMV GBR 6

None of the K185 plants inoculated with the TuMV isolates UK 1, CZE 1, CDN 1, GBR 6, or POL 1 showed any symptoms of virus infection and ELISA failed to detect any TuMV in any of the plants

(Table 1). The susceptible control *B. rapa* ssp. *perviridis* plants inoculated with all five TuMV isolates showed clear mosaic symptoms, typical of TuMV infection and the presence of TuMV was confirmed by ELISA.

Resistance of F_1 and F_2 plants following challenge with TuMV

Both F_1 plants derived from the cross between the uniformly susceptible R-o-18 plant and the in-bred line K185 plant challenged with TuMV isolate GBR 6 followed by CDN 1 were resistant, showing no symptoms of virus infection and with no TuMV detected in either plant following ELISA, indicating that gene(s) controlling resistance to TuMV in K185 was/were dominant. As the already characterised dominant TuMV resistance gene *TuRB03* confers resistance to the CDN 1 isolate of TuMV, the GBR 6 isolate which overcomes *TuRB03* (Hughes et al., 2003) and other dominant genes (*TuRB01*, *TuRB02*, *TuRB04* + *TuRB05*) (Walsh & Jenner, 2002) was used to challenge the F_2 plants in order to determine whether K185 possessed any new resistance genes.

The 100 F2 plants challenged with the GBR 6 TuMV segregated for extreme resistance (0; 49 plants), systemic necrotic symptoms ($+_N$; 45 plants) and systemic mosaic symptoms (+; 6 plants) (Table 2). All *B. rapa* ssp. *perviridis* and *B. napus* lines 165 and 22S plants were infected, displaying systemic mosaic (+) symptoms, verifying the ability of GBR 6 to overcome *TuRB03* and *TuRB04* + *TuRB05*. All plants that were mock-inoculated with uninfected plant sap showed no symptoms. ELISA confirmed that those plants showing symptoms of virus infection were infected by TuMV and those not showing symptoms, were not infected by TuMV.

Table 2 Phenotypes of the F_2 generation from the *Brassica rapa*, R-o-18 × K185 cross, following challenge with turnip mosaic virus (TuMV) isolate GBR 6

Plant line	Number of plants in phenotypic class following			
	Inoculation with TuMV isolate GBR 6 (pathotype 4)			Mock inoculation
	0	$+_N$	+	0
F_2	49	45	6	2
Brassica napus 22S[a]	0	0	10	2
Brassica napus 165[b]	0	0	10	2
Brassica rapa ssp. *perviridis*[c]	0	0	10	2

0 = No visual symptoms of TuMV infection and no TuMV detected by ELISA

$+_N$ = Systemic necrotic symptoms and TuMV detected by ELISA

+ = Systemic mosaic symptoms and TuMV detected by ELISA

[a] *B. napus* line 22S possesses TuMV resistance gene *TuRB03*

[b] *B. napus* line 165 possesses TuMV resistance genes *TuRB04* and *TuRB05*

[c] Susceptible control line

Resistance inheritance

The segregation of resistance (0) versus susceptibility (+ and $+_N$) in the F_2 generation did not fit a dominant monogenic model. The numbers of resistant and susceptible plants observed were significantly different from the numbers expected ($\chi^2 > 3.84$; $P < 0.001$) (Supplementary Table 2).

Assuming balanced segregation ratios, the segregation fitted a two, unlinked, dominant-gene model; the numbers of resistant and susceptible plants observed, were not significantly different from the numbers expected ($\chi^2 < 3.84$; $P > 0.10$) (Supplementary

Table 1 Resistance spectra of *Brassica rapa* line K185 plants to a range of turnip mosaic virus (TuMV) isolates

Plant line	TuMV isolate (pathotype)					
	UK 1 (1)	CZE 1 (3)	CDN 1 (4)	GBR 6 (4)	POL 1 (4)	Mock
K185	0 (10)[a]	0 (10)	0 (10)	0 (10)	0 (10)	0 (2)
Brassica rapa ssp. *perviridis*	+(2)	+(2)	+(2)	+(2)	+(2)	-

0 = No visual symptoms of TuMV infection and no TuMV detected by ELISA

+ = Systemic mosaic symptoms and TuMV detected by ELISA

[a] Number of plants challenged

Table 2). A second two unlinked dominant-gene model was considered where, as with the first two gene model, both genes are required for the extreme resistance (0), but where infection occurs in the presence of either dominant allele, in the absence of the other, this results in necrosis ($+_N$). Again assuming balanced segregation ratios, the segregation of resistance, susceptibility with necrosis ($+_N$) and susceptibility without necrosis (+; mosaic symptoms) observed, was not significantly different from the numbers expected for this model ($\chi^2 < 5.99$; $P > 0.10$) (Supplementary Table 2). A two unlinked dominant-gene model where both genes are required for the extreme resistance (0), but where infection in the presence of only one of the dominant alleles results in necrosis ($+_N$) was tested. Again assuming balanced segregation ratios, the segregation of resistance, susceptibility with necrosis ($+_N$) and susceptibility without necrosis (+; mosaic symptoms) observed, was significantly different from that expected for this model ($\chi^2 > 5.99$; $P < 0.001$) (Supplementary Table 2).

The segregation of resistance versus susceptibility observed in the F_2 generation assuming balanced segregation ratios, where all of three dominant genes are unlinked and required for resistance fitted the expected outcome ($\chi^2 < 3.84$; $P > 0.10$). However, a three dominant-gene model where necrosis is seen in the presence of any of the dominant alleles did not fit the phenotypes observed ($\chi^2 > 5.99$; $P < 0.001$) (Supplementary Table 2). A three dominant-gene model where necrosis is seen in the presence of only one of the dominant alleles again did not fit the phenotypes observed ($\chi^2 > 5.99$; $P < 0.001$) (Supplementary Table 2). A further three dominant-gene model where necrosis is seen in the presence of two specific dominant alleles also did not fit the phenotypes observed ($\chi^2 > 5.99$; $P < 0.001$) (Supplementary Table 2). Although a three dominant-gene model where necrosis is seen in the presence of any two dominant alleles did not fit the phenotypes observed ($\chi^2 > 5.99$; $P < 0.05$), it was the three dominant-gene model closest to explaining the segregation of the necrotic phenotype ($P > 0.025$) (Supplementary Table 2).

Discussion

The *B. rapa* line K185 was found to possess extreme resistance to a range of TuMV isolates representing the major pathotypes and including known resistance-breaking isolates. Following challenge with these isolates, no discernible symptoms of infection were seen and no TuMV was detected by ELISA. Resistance in the limited number of F_1 plants tested indicated the resistance was dominant. Phenotyping of a greater number of F_2 plants confirmed the dominance and indicated the resistance was controlled by at least two dominant genes. The segregation of resistance and susceptibility in the F_2 population confirmed that the genes are unlinked and also suggested that the resistance may be controlled by three dominant genes.

Single dominant genes described to date conferring extreme resistance to TuMV have all been found in the A genome (U 1935) of *B. napus* or *B. rapa* and have narrow spectra of resistance (e.g. *TuRB01* [Walsh et al., 1999] and *TuRB03* [Hughes et al., 2003]). The resistances provided by the single dominant genes are readily overcome by many TuMV isolates. The *B. napus* line 165 possesses resistance to TuMV where two dominant genes are necessary (*TuRB04* and *TuRB05*) and together, these genes provide broader resistance than any of the single dominant genes. At least one of the resistance genes in the line K185 must be different to *TuRB04* or *TuRB05* as three of the TuMV isolates used in this study (CDN 1, GBR 6 and POL 1) are able to infect line 165 (Jenner & Walsh, 1996) and hence can overcome the *TuRB04* and *TuRB05* genes, whereas these isolates were not able to infect the line K185. Interestingly in the absence of *TuRB04*, *TuRB05* gives a necrotic response when challenged with TuMV (Jenner et al., 2002), in a similar manner to that seen in many of the susceptible F_2 plants derived from line K185. However, the segregation of the necrotic response in the F_2 plants derived from K185 did not fit the model for line 165; it appeared that both of the genes from the two gene model were capable of inducing necrosis in susceptible plants following challenge with the GBR 6 isolate of TuMV. The two dominant gene model appears to provide the simplest explanation of the resistance to TuMV in K185 plants and the inheritance of the necrotic phenotype. The three gene model should not be completely dismissed in terms of the resistance, however, there is currently no precedent for the action of two dominant genes together, being necessary for inducing necrosis in the TuMV / brassica pathosystem. It is clear that the inheritance of the resistance to TuMV in K185 plants is not simple, which is consistent with the broad-spectrum nature of the resistance.

The possibility that the K185 plants possess one, or more recessive genes in addition to dominant genes cannot be excluded. However, as F_1 plant were resistant, it is clear that the dominant genes alone are sufficient to provide resistance to TuMV, even if recessive gene(s) are present. Only one recessive resistance to TuMV is known in *B. rapa* and is due to a single gene (*retr01*; Nellist et al., 2014). It has been shown that line K185 does not possess *retr01* (Nellist, 2014). To investigate the possibility that K185 possesses recessive resistance gene(s), it would be necessary to produce a very large segregating F_2 population and challenge with TuMV to identify resistant plants. If all the resistant plants were then crossed to a susceptible plant, such as R-o-18 and F_1 plants from any of these crosses were susceptible to TuMV, that would identify the presence of any recessive gene(s) in the parents of susceptible F_1 families.

TuMV causes very significant diseases in a wide range of crop species as it dramatically reduces the weight (Hunter et al., 2002) and seed (Walsh & Tomlinson, 1985) yields of infected plants. Infected plants may die or are so severely damaged, they are not marketable (Hunter et al., 2002; Shattuck, 1992). All plant viruses are difficult to control. Many farmers have little awareness of the presence of viruses and when their crops are at risk of infection, until they are suffering losses. Viruses like TuMV that are transmitted in the non-persistent manner, where the insect vectors can acquire and transmit the viruses very rapidly in a matter of seconds, are even more difficult to control than semi-persistently and persistently-transmitted viruses. The neonicotinoids are the most effective insecticides in controlling the aphid vectors of TuMV, but are currently banned in the EU, as is growing GM and gene-edited crops. There is an urgent need for alternative virus control measures. Natural plant resistance offers an attractive option for virus control, but the single dominant gene resistances and some multigenic dominant resistances are rapidly defeated. Single nucleotide mutations in the TuMV genome have been shown to result in *TuRB01* and *TuRB03* resistance-breaking strains (Jenner et al., 2000, 2003) and the ability of TuMV isolates to overcome the resistance provided by the combination of *TuRB04* and *TuRB05* (Jenner et al., 2002). Hence, broader-spectrum resistances are needed to justify what can be long and expensive breeding programs to introgress resistances into commercial varieties and to promote the durability of TuMV resistance in the field.

The resistance in the *B. rapa* K185 plant line appears to be an extreme form of resistance, where no visual symptoms are seen following challenge with TuMV and no TuMV is detected by ELISA. As it was effective against the representatives of the major pathotypes 1 and 3 (all previously described resistance genes to these pathotypes have been overcome) and also pathotype 4 (where the only previously described dominant resistance has been defeated) and no TuMV isolates able to overcome the resistance have been identified yet, it has the potential to be durable. Another source of broad-spectrum, potentially durable TuMV resistance discovered in *B. rapa* ssp. *pekinensis* (Chinese cabbage), is controlled by a single recessive gene (*retr01*) (Nellist et al., 2014; Rusholme et al., 2007) and is currently being introgressed into commercial lines of Chinese cabbage. K185 plants do not possess *retr01* (Nellist, 2014) and must therefore possess, new, as yet unidentified TuMV resistance genes. This provides the opportunity to manage broad-spectrum TuMV resistance by exploiting K185-based resistance alongside *retr01*-based resistance in Chinese cabbage to reduce the selection pressure for the emergence of resistance-breaking TuMV isolates. Little is known about what makes some dominant virus resistances more durable than others, pointing to a need for more research on the nature and mechanisms of genes providing broad-spectrum resistance.

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Authors' contributions HMW conducted the experiments, lab work and analyses and wrote the manuscript. AR and JAW provided feedback on the analyses and the manuscript. All authors have read and approved the final manuscript.

Data Availability The data that support the findings of this study are available in the article and its online supplementary material.

Declarations

Competing interests The authors declare that they have no competing interests.

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