Theor Appl Genet (2003) 107:1169–1173
DOI 10.1007/s00122-003-1363-4

S. L. Hughes · P. J. Hunter · A. G. Sharpe · M. J. Kearsey · D. J. Lydiate · J. A. Walsh

Genetic mapping of the novel *Turnip mosaic virus* resistance gene *TuRB03* in *Brassica napus*

Received: 13 January 2003 / Accepted: 3 April 2003 / Published online: 2 August 2003

Abstract A new source of resistance to the pathotype 4 isolate of *Turnip mosaic virus* (TuMV) CDN 1 has been identified in *Brassica napus* (oilseed rape). Analysis of segregation of resistance to TuMV isolate CDN 1 in a backcross generation following a cross between a resistant and a susceptible *B. napus* line showed that the resistance was dominant and monogenic. Molecular markers linked to this dominant resistance were identified using amplified fragment length polymorphism (AFLP) and microsatellite bulk segregant analysis. Bulks consisted of individuals from a BC_1 population with the resistant or the susceptible phenotype following challenge with CDN 1. One AFLP and six microsatellite markers were associated with the resistance locus, named *TuRB03*, and these mapped to the same region on chromosome N6 as a previously mapped TuMV resistance gene *TuRB01*. Further testing of *TuRB03* with other TuMV isolates showed that it was not effective against all pathotype 4 isolates. It was effective against some, but not all pathotype 3 isolates tested. It provided further resolution of TuMV pathotypes by sub-dividing pathotypes 3 and 4. *TuRB03* also provides a new source of resistance for combining with other resistances in our attempts to generate durable resistance to this virus.

Keywords *Brassica napus* · TuMV resistance · Specificity · Pathotype · Genetic mapping

Communicated by H.C. Becker

S. L. Hughes · P. J. Hunter · J. A. Walsh (✉)
Horticulture Research International, Wellesbourne, Warwick, CV35 9EF, UK,
e-mail: john.walsh@hri.ac.uk
Fax: +44-1789-470552

M. J. Kearsey
University of Birmingham, School of Biosciences, Edgbaston, Birmingham, B15 2TT, UK,

A. G. Sharpe · D. J. Lydiate
Molecular Genetics Section, Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, Saskatchewan, S7N OX5, Canada

Introduction

Turnip mosaic virus (TuMV), a member of the *Potyvirus* genus, affects field vegetables throughout the world (Tomlinson 1987). It has the widest host range of any member of the *Potyvirus* genus (Shukla et al. 1994), infecting a wide range of plant species in temperate and tropical regions of the world, and causes severe reductions in yield and marketability, inducing systemic vein clearing, necrosis, stunting (Shattuck 1992) and, in severe cases, plant death (Walsh and Tomlinson 1985).

TuMV is difficult to control due to its wide host range and non-persistent, stylet-borne mode of transmission by aphids. The use of insecticides to control the disease has proven ineffective, therefore natural plant resistance is likely to be the most effective and environmentally friendly method of controlling TuMV. Recently identified resistances in *Brassica rapa* appear to be effective against a broad range of TuMV isolates (Suh et al. 1995; Hughes et al. 2002; Walsh et al. 2002). Several different modes of inheritance of TuMV resistance in *B. rapa* have been described (Yoon et al. 1993; Suh et al. 1995; Hughes et al. 2002; Walsh et al. 2002). In contrast, predominantly dominant resistance has been identified in *Brassica napus* (Shattuck and Stobbs 1987; Walsh 1989; Walsh et al. 1999; Jenner et al. 2002).

To breed TuMV-resistant *Brassica* varieties, it is important to identify individual or combinations of resistance genes effective against all pathotypes of the virus. Information on their modes of inheritance and the identification of markers for these genes will facilitate their transfer into elite cultivars using marker-assisted selection (MAS). Molecular markers have been used to position TuMV resistance genes on genetic linkage maps (Walsh et al. 1999; Rusholme 2000).

Four TuMV resistance genes and one quantitative trait locus (QTL) have already been mapped in *B. rapa* and *B. napus* (Walsh et al. 1999; Rusholme 2000) using restriction fragment length polymorphisms. The resistance genes mapped to the A genome and the QTL (which provides weak quantitative resistance against two Asian

isolates of TuMV) was located in the C genome of *B. napus*. TuMV resistance genes *TuRB01* and *TuRB01b* have been mapped in *B. napus* (Walsh et al. 1999) and *B. rapa* (Rusholme 2000), respectively.

This paper describes the identification of resistance to TuMV isolate CDN 1 (the type member of pathotype four; Jenner and Walsh 1996) in *B. napus*, characterisation of its inheritance and genetic mapping of the resistance using the bulk segregant analysis (BSA) approach described by Michelmore et al. (1991). Information on the specificity of the resistance to a range of TuMV isolates is provided, on its use in providing increased resolution for the pathotyping system of Jenner and Walsh (1996) and on its potential as part of a wider programme to create durable resistance to TuMV are discussed.

Materials and methods

Plant material

Line 22S was a selection of *B. napus* identified as susceptible to *Xanthomonas campestris* pv. *campestris* by J. Vicente, HRI, Wellesbourne. Selfed (S_1), F_1 and backcross (BC_1) generations were produced from 22S plants for further evaluation. F_1 seed was produced by bud-pollinating the CDN 1-susceptible parent N-o-1 (Walsh et al. 1999) with pollen from a CDN 1-resistant 22S individual. N-o-1 is a doubled haploid *B. napus* line derived from the spring oilseed rape cultivar Westar (Sharpe et al. 1995). For BC_1 seed production, N-o-1 was bud-pollinated with pollen from a CDN 1-resistant F_1 individual. Plants were grown in an insect-proof glasshouse at 18 ± 2 °C for the duration of the experiments.

Virus isolates and phenotypic assessments

S_1 individuals were tested with the virus isolates listed in Table 1. For CDN 1 (pathotype 4 type member; Jenner and Walsh 1996), 23 S_1 individuals were tested; for UK 1, CHN 5 and CZE 1, ten S_1 individuals were tested; for CZE 20, DEU 2, DEU 9, GBR 6, HUN 1 and POL 1, 14 S_1 individuals were tested; for GBR 18 to GBR 61, four S_1 individuals were tested. F_1 (30 individuals) and BC_1 (113 individuals) plants were tested with CDN 1. The virus isolates were maintained in the susceptible host *Brassica juncea* cv. Tendergreen (TGM) by mechanical inoculation (Jenner and Walsh 1996), in an insect-proof glasshouse at 18 ± 2 °C. Plants were inoculated as described by Jenner and Walsh (1996). Symptoms were recorded by visual assessment at weekly intervals up to 4 weeks after inoculation. At the end of the 4-week period, inoculated and uninoculated leaves were tested for the presence of TuMV using indirect plate-trapped antigen ELISA as described by Walsh et al. (2002). For each test, the stability and integrity of isolates were verified using differential lines as described by Jenner and Walsh (1996).

Statistical analysis

Data of susceptible and resistant reactions of the BC_1 generation were analysed by chi-square for goodness of fit to expected segregation ratios.

Table 1 Results from inoculation of *Brassica napus* 22S self-progeny with *Turnip mosaic virus* isolates from pathotypes 1, 3 and 4

Virus isolate[a]	Patho-type[b]	Pheno-type[c]
GBR 18 to GBR 51, UK 1	1	$+_N$
CHN 5, CZE 1	3	$+_N$
GBR 52 to GBR 61	3	0
CDN 1	4	0
CZE 20, DEU 2, DEU 9, GBR 6, HUN 1, POL 1	4	+

[a] GBR 18 to 44 were isolated from wild cabbage (*Brassica oleracea*) in Dorset, UK. GBR 45 to 51 were isolated from wild cabbage (*B. oleracea*) in Yorkshire, UK. GBR 52 to 61 were isolated from wild cabbage (*B. oleracea*) in Wales, UK. CZE 20 was isolated from oilseed rape in the Czech Republic. DEU 9 was isolated from oilseed rape in Germany. HUN 1 was isolated from *Alliaria petiolata* in Hungary. All other isolates were described by Jenner and Walsh (1996)

[b] Based on responses of the differential series of *Brassica napus* lines described by Jenner and Walsh (1996)

[c] 0, No symptoms observed in inoculated or uninoculated leaves, no virus detected by ELISA or inoculation to susceptible TGM plants; +, chlorotic symptoms observed and virus detected by ELISA in inoculated and uninoculated leaves following systemic spread of TuMV; $+_N$, necrotic symptoms observed in inoculated and uninoculated leaves (following systemic spread of TuMV) and virus detected by ELISA

Amplified fragment length polymorphism (AFLP) and microsatellite assay

Upon completion of disease assessments, leaves were collected from 20 resistant and 20 susceptible individuals. Each leaf sample was immediately frozen in liquid nitrogen and freeze-dried for 3 days. Leaf material was stored at –20 °C in a plastic bag containing silica gel dessicant, until required.

Genomic DNA was extracted from individuals using the Phytopure kit (Nucleon Biosciences) according to the manufacturer's instructions and stored at –20 °C. Two resistant and two susceptible DNA bulks were made (ten resistant or ten susceptible BC_1 individuals/bulk) after genomic DNA had been extracted from individual plants.

The AFLP Small Plant Mapping kit was used for AFLP analysis according to the manufacturer's instructions (AFLP plant mapping protocol; PE Applied Biosystems, Foster City, Calif.) with restriction enzymes *Eco*RI and *Mse*I (New England Biolabs, Beverly, Mass.). The Small Plant Mapping kit (*Eco*RI+2 nucleotides/*Mse*I+3 nucleotides) was used in preference to the Large Plant Mapping kit (*Eco*RI+3 nucleotides/*Mse*I+3 nucleotides) in order to increase the number of fragments amplified, thereby improving the chances of identifying polymorphisms. Primer pairs that produced polymorphisms between the parents and between resistant and susceptible bulks were used to screen the mapping parents N-o-1 and N-o-9 (Sharpe et al. 1995) in order to determine whether they could be positioned on a specific chromosome.

Primers for *B. napus* microsatellites were obtained from A.G. Sharpe and D.J. Lydiate, AAFC, Saskatoon (contact Dr. Derek Lydiate for information regarding primer pairs). The primers were labelled with one of three fluorescent dyes: HEX, 6-FAM or TET. Amplification reactions were performed in 10 μl reaction mixes containing forward primer (1 μM), reverse primer (1 μM), 40 ng DNA, 1× PCR buffer (PE Biosystems), $MgCl_2$ (2.5 mM), dNTP (0.8 mM) and 0.5 U AmpliTaq Gold (PE Biosystems). The amplification conditions were 10 min at 95 °C; eight cycles of 15 s at 95 °C, 15 s at 50 °C and 30 s at 72 °C; 27 cycles of 15 s at 89 °C, 55 s at 55 °C and 30 s at 72 °C; finally 10 min at 72 °C. Aliquots of 2.5 μl of the 6-FAM-labelled reaction, 2.5 μl of the TET-labelled

reaction and 10 μl of the HEX-labelled reaction were pooled with 15 μl of water so that the amplification products derived from the three primer pairs could be run in each lane of the gel. AFLP and microsatellite samples were run out on polyacrylamide gels on an ABI 377 using standard conditions including an internal lane standard (GeneScan Reference Guide 1997). GENESCAN 3.1 was used to analyse the results. GENOGRAPHER (http://hordeum.oscs.montana.edu/genographer/) was used to display the results for microsatellite analysis.

Linkage analysis

Linkage between DNA markers and the TuMV resistance locus was established using MAPMAKER 3.0 software (Lander et al. 1987) on the 20 resistant and 20 susceptible individuals. Markers were positioned with a LOD threshold of 4. The Kosambi (1944) function was applied to convert recombination fractions into map distances.

Fig. 1 *Brassica napus* line $22S_1$ 3 weeks after inoculation with *Turnip mosaic virus* isolate UK 1 (*left*) and CDN 1 (*right*)

Results

Specificity of resistance

S_1 progeny of 22S were resistant to the type member of pathotype 4, CDN 1 (Fig. 1) and a number of pathotype 3 isolates (GBR 52–GBR 61) (Table 1). However, they were susceptible to all the other pathotype 4 isolates, all pathotype 1 isolates and some of the pathotype 3 isolates tested (Table 1). All pathotype 1 isolates tested induced necrosis (Fig. 1), those pathotype 3 isolates that overcame

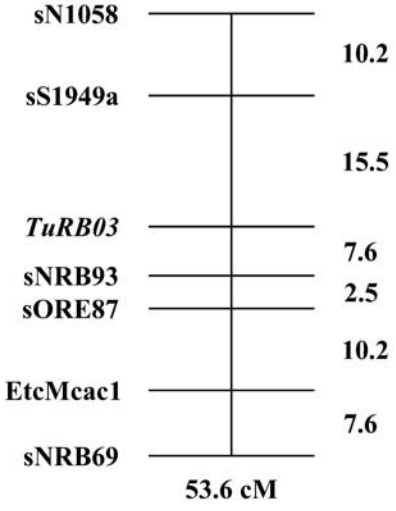

Fig. 2 Chromosome N6 with microsatellite-defined loci and one AFLP-defined locus (EtcMcac1). Map distances are in centiMorgans (cM) on the *right-hand side* of the linkage group, with the total length of the linkage group at the *bottom*. Scoring data allowing the positioning of *TuRB03* on linkage group N6 of *Brassica napus* is also shown. *Rows* Genetic loci, *columns* BC_1 individuals, – homozygous for the N-o-1 allele, + heterozygous, *S* susceptible to TuMV isolate CDN 1, *R* resistant to TuMV isolate CDN 1, *0* missing data point

sN1058	+ + - - + + + + + + + + + + + + - + + + - - + + - - - + - - - - - + - - - - - +
sS1949	+ + - - + 0 + + - + + + + + + + - + + + - - - - - - - + - - - - - - - - - - - +
TuRB03	R R R R R R R R R R R R R R R R R R R S S S S S S S S S S S S S S S S S S S S
sNRB93	+ + + + + + + + + + + + + + + + + + + - - - - - - - - - + - - + - + - - - - -
sORE87	+ + + + + + + + + + + + + + + + + + + - - - + - - - - - + - - + - + - - - - -
EtcMcac1	+ + + + + + + + + + + + + + - + + 0 - - + - - + - - - - - + - 0 0 - + - - - - -
sNRB69	+ + + + + + + + - - + + + + - + + + - - + - - + - - - - - + - - + - + - - - + -

the resistance induced necrosis and those pathotype 4 isolates that overcame the resistance caused mosaic symptoms (Table 1).

Genetic analysis of resistance

F_1 progeny were all resistant to CDN 1 (data not shown), indicating that TuMV resistance was dominant. The observed segregation data from virus testing the BC_1 generation with CDN 1 (57 resistant and 56 susceptible) fit the expected segregation for a Mendelian model based on the action of a single dominant allele ($\chi^2 = 0.009$, $P = 0.920$). This model is appropriate if the original resistant individual was either homozygous for the dominant allele (genotype RR) or heterozygous (genotype Rr). The data from testing the F_1 (30 individuals) and S_1 (23 individuals) generations with the CDN 1 isolate of TuMV confirmed that the original resistant individual was homozygous for the dominant allele since all individuals were resistant. Resistant S_1 (Fig. 1), F_1, and BC_1 plants were completely symptomless, and no virus was detected, indicating an extreme form of resistance. Susceptible BC_1 plants showed typical mosaic symptoms throughout the plant.

Bulk segregant analysis

Of the 52 AFLP primer pairs used to screen the resistant and susceptible bulks, 19 failed to produce a product and 27 produced monomorphic amplification band patterns. Six primer pairs produced polymorphic band patterns between 22S and N-o-1, however, five of these were monomorphic for mapping parents N-o-1 and N-o-9. The one primer pair that provided a clear polymorphism (*Eco*RI-TC, *Mse*I-CAC) amplified a 91-bp band in resistant bulks and N-o-9 that was less intense in the susceptible bulks and absent from N-o-1.

The AFLP marker, now called EtcMcac1, linked to TuMV CDN 1 resistance in line 22S was positioned on the *B. napus* genetic map on chromosome N6 by screening 30 individuals from the 72-8 mapping population (Sharpe et al. 1995) with primer pair combination *Eco*RI-TC, *Mse*I-CAC. These 30 lines were a subset of the 72-8 population (total of 92 individuals) which had been used for the original RFLP map (Sharpe et al. 1995) and gave a very robust set of segregation data which have been used to map new loci accurately. The likelihood of the AFLP locus being located elsewhere in the genome with matching flanking loci is tiny (2^{-30}).

Resistant and susceptible bulks from line 22S were screened with the 32 microsatellite primers available from chromosome N6 (Sharpe and Lydiate, unpublished). Five of the thirty-two primers produced polymorphic amplification products, seven did not give products and twenty gave products that were monomorphic.

Twenty resistant and twenty susceptible 22S BC_1 individuals were screened with the five polymorphic microsatellite primers. The scoring data (Fig. 2) allowed the dominant 22S TuMV resistance allele to be positioned on chromosome N6 between microsatellite markers sNRB93 (7.6 cM) and sS1949 (15.5 cM) (Fig. 2). The gene conferring resistance to CDN 1 has been named *TuRB03* (*TuMV RESISTANCE in BRASSICA 03*).

Discussion

The genetic and marker analysis of TuMV resistance in *B. napus* line 22S has shown that a single dominant allele confers resistance to the pathotype 4 isolate CDN 1. The dominant allele, *TuRB03*, has been successfully positioned in the lower region of chromosome N6 using AFLP and microsatellite bulk segregant analysis. At current resolution, *TuRB03* maps to the same interval as two previously mapped TuMV resistance genes, *TuRB01* and *TuRB01b* (Walsh et al. 1999; Rusholme 2000). It is conceivable that *TuRB01, TuRB01b* and *TuRB03* are members of a resistance gene cluster or even possibly allelic. *TuRB01* confers resistance to all pathotype 1 isolates of TuMV and plants possessing *TuRB03* were susceptible to all pathotype 1 isolates tested.

To date, only narrow-spectrum, mostly monogenic dominant, isolate- or pathotype-specific TuMV resistance genes have been identified in *B. napus* (Shattuck and Stobbs 1987; Walsh 1989; Walsh et al. 1999; Jenner et al. 2002). In contrast, broad-spectrum (mostly recessive), multigenic (in addition to monogenic) resistance has been identified in *B. rapa* (Rusholme, 2000; Hughes et al. 2002; Walsh et al. 2002). The predominance of monogenic resistance in *B. napus* could be due to an evolutionary bottleneck that occurred during the formation of *B. napus* from what may have been a limited number of interspecies hybridisation events between *B. rapa* and *B. oleracea*. The lack of broad-spectrum resistance in *B. napus* may be due to the presence of the C genome from *B. oleracea*, where no extreme forms of resistance have been identified (Walsh and Jenner 2002). Attempts are being made to introduce TuMV resistance genes from *B. rapa* into *B. napus* (Rusholme 2000).

The pathotype 4 isolate, CDN 1, has overcome all sources of resistance previously identified in *B. napus*. Line 22S was susceptible to all other pathotype 4 isolates tested. This shows that CDN 1 is different from all other pathotype 4 isolates in terms of the virulence determinant(s) for *TuRB03*. In this study, line 22S has successfully split pathotype 3 and pathotype 4 isolates into subgroups, hence it will provide further resolution in the pathotyping system described by Jenner and Walsh (1996).

TuRB03 and other TuMV resistance genes could be deployed through gene pyramiding to combine large numbers of genes. Information is already available on the incidence of TuMV pathotypes in different regions of the world, indicating which resistance gene combinations could be deployed in particular regions (Jenner and Walsh

1996). *TuRB01* (effective against pathotype 1 isolates) is present in commercial *B. napus* cultivars (Walsh et al. 1999), and TuMV isolates have been shown to mutate to defeat *TuRB01* quite frequently in the glasshouse (Jenner and Walsh, 1996; Jenner et al. 2000). *TuRB04* and *TuRB05* exist together in a selection from a commercial *B. napus* cultivar, and mutations to infect this line are far less frequent (Jenner and Walsh 1996; Jenner et al. 2002). This indicates that combining these TuMV resistance genes with further genes such as *TuRB03* would not only provide broader spectrum resistance, but also increased durability. If *TuRB01* and *TuRB03* were found to be allelic, it would still be possible to combine them in F_1 hybrids. The resistance genes could also be deployed through multi-lines, where the individuals of the crop are essentially the same as each other but have different combinations of resistance genes, thereby reducing the selection pressure for resistance breaking virus isolates.

Acknowledgements This research was funded by the European Commission (EC) grant number ERB 3514 PL96 1223 and the Department for Environment, Food and Rural Affairs (DEFRA). We thank Dr. R.L. Rusholme, J. Bambridge, E. Higgins, C. Wood and K. Bryce for advice and assistance and Professor H. C. Becker and colleagues for co-ordinating the EC project.

References

Hughes SL, Green SK, Lydiate DJ, Walsh JA (2002) Resistance to *Turnip mosaic virus* in *Brassica rapa* and *B. napus* and the analysis of genetic inheritance in selected lines. Plant Pathol 51:567–573

Jenner CE, Walsh JA (1996) Pathotypic variation in turnip mosaic virus with special reference to European isolates. Plant Pathol 45:848–856

Jenner CE, Sanchez F, Nettleship SB, Foster GD, Ponz F, Walsh JA (2000) The cylindrical inclusion gene of *Turnip mosaic virus* encodes a pathogenic determinant to the brassica resistance gene *TuRB01*. Mol Plant Microbe Interact 13:1102–1108

Jenner CE, Tomimura K, Oshima K, Hughes SL, Walsh JA (2002) Mutations in *Turnip mosaic virus* P3 and cylindrical inclusion proteins are required to overcome two *Brassica napus* resistance genes. Virology 300:50–59

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Rusholme RL (2000) The genetic control of resistance to turnip mosaic virus (Tumv) in *Brassica*. PhD thesis, University of East Anglia

Sharpe AG, Parkin IAP, Keith DJ, Lydiate DJ (1995) Frequent nonreciprocal translocations in the amphidiploid genome of oilseed rape (*Brassica napus*). Genome 38:1112–1121

Shattuck VI (1992) The biology, epidemiology and control of turnip mosaic virus. In: Janick J (ed) Plant breeding reviews 14. John Wiley and Sons, New York, pp 199–238

Shattuck VI, Stobbs LW (1987) Evaluation of rutabaga cultivars for turnip mosaic virus resistance and the inheritance of resistance. HortScience 22:935–937

Shukla DD, Ward CW, Brunt AA (1994) The Potyviridae. CAB Int, Wallingford, UK

Suh SK, Green SK, Park HG (1995) Genetics of resistance to five strains of turnip mosaic virus in Chinese cabbage. Euphytica 81:71–77

Tomlinson JA (1987) Epidemiology and control of virus diseases of vegetables. Ann Appl Biol 110:661–681

Walsh JA (1989) Genetic control of immunity to turnip mosaic virus in winter oilseed rape (*Brassica napus* ssp. *oleifera*) and the effect of foreign isolates of the virus. Ann Appl Biol 115:89–99

Walsh JA, Jenner CE (2002) *Turnip mosaic virus* and the quest for durable resistance. Mol Plant Pathol 3:289–300

Walsh JA, Tomlinson JA (1985) Viruses infecting winter oilseed rape (*Brassica napus* ssp.*oleifera*). Ann Appl Biol 107:485–495

Walsh JA, Sharpe AG, Jenner CE, Lydiate DJ (1999) Characterisation of resistance to turnip mosaic virus in oilseed rape (*Brassica napus*) and genetic mapping of *TuRB01*. Theor Appl Genet 99:1149–1154

Walsh JA, Rusholme RL, Hughes SL, Jenner CE, Bambridge JM, Lydiate DJ, Green SK (2002) Different classes of resistance to turnip mosaic virus in *Brassica rapa*. Eur J Plant Pathol 108:15–20

Yoon JY, Green SK, Opena RT (1993) Inheritance of resistance to turnip mosaic virus in Chinese cabbage. Euphytica 69:103–108