The Cylindrical Inclusion Gene of *Turnip mosaic virus* Encodes a Pathogenic Determinant to the Brassica Resistance Gene *TuRB01*

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The viral component of *Turnip mosaic virus* (TuMV) determining virulence to the *Brassica napus* *TuRB01* dominant resistance allele has been identified. Sequence comparisons of an infectious cDNA clone of the UK 1 isolate of TuMV (avirulent on *TuRB01*) and a spontaneous mutant capable of infecting plants possessing *TuRB01* suggested that a single nucleotide change in the cylindrical inclusion (CI) protein coding region (gene) of the virus was responsible for the altered phenotype. A second spontaneous mutation involved a different change in the CI gene. The construction of chimeric genomes and subsequent inoculations to plant lines segregating for *TuRB01* confirmed the involvement of the CI gene in this interaction. Site-directed mutagenesis of the viral coat protein (CP) gene at the ninth nucleotide was carried out to investigate its interaction with *TuRB01*. The identity of this nucleotide in the CP gene did not affect the outcome of the viral infection. Both mutations identified in the CI gene caused amino acid changes in the C terminal third of the protein, outside any of the conserved sequences reported to be associated with helicase or cell-to-cell transport activities. This is the first example of a potyvirus CI gene acting as a determinant for a genotype-specific resistance interaction.

Research on plant–virus interactions has contributed significantly to the understanding of genotype-specific disease resistance in plants. The study of infection, virus spread, and disease development in plants has been greatly aided by the recent availability of infectious genomic clones of several virus species. Viral components such as movement proteins, coat proteins (CPs), and replicases are essential for the establishment of infection and, in a few cases, the involvement of such proteins with specific plant resistance responses have been determined. Tobamoviruses have been best studied to date in this context, with all the viral genes implicated in various resistance responses. The CP is a direct elicitor of resistance in plants with the genes *N*, *L*¹, *L*², or *L*³ (Culver and

Dawson 1991; Berzal-Herranz et al. 1995; de la Cruz et al. 1997; Gilardi et al. 1998). The movement protein is an elicitor for resistance genes *Tm-2* and *Tm-2*² (e.g., Meshi et al. 1989; Weber and Pfitzner 1998). Similarly, the replicase protein is an elicitor in *N*-mediated resistance (Padgett and Beachy 1993; Erickson et al. 1999), and the replicase gene is implicated in *Tm-1* resistance (Meshi et al. 1988). The potyvirus *Potato virus X* (PVX) has also been well studied, with the CP implicated in *Rx1*, *Rx2*, and *Nx* resistances (Santa Cruz and Baulcombe 1993; Bendahmane et al. 1995; Querci et al. 1995), whereas the movement protein is the elicitor in *Nb*-mediated resistance (Malcuit et al. 1999).

The family *Potyviridae* is the largest family of plant viruses (Shukla et al. 1994) and members of this group cause many economically important diseases, but knowledge of the molecular basis of resistance breaking and elicitor identity is less advanced than for tobamoviruses and PVX. Domains of the *Soybean mosaic virus* HC-Pro and P3 genes are jointly associated with *Rsv1* resistance breaking (Eggenberger and Hill 1997). However, the VPg gene has so far been most commonly associated with resistance, e.g., with *va*-mediated resistance for *Potato virus Y* (PVY) and *Tobacco vein mottling virus* (Nicolas et al. 1997; Masuta et al. 1999), two other recessive genes acting against *Tobacco etch virus* (Schaad et al. 1997), and for *sbm-1* and *sbm-3* resistances against *Pea seed-borne mosaic virus* (Keller et al. 1998). The VPg gene has also been associated with the ability of *Potato virus A* to move systemically in *Nicandra physaloides* (together with the 6K2 gene) (Rajamäki and Valkonen 1999) and with strain-specific resistance in potato (together with the HC-Pro, CP, or both) (Hämäläinen et al. 2000).

Turnip mosaic virus (TuMV), a member of the family *Potyviridae*, is the most important virus infecting cruciferous crops and is found worldwide (Shattuck 1992). It has a wide host range, and sources of resistance have been identified in a number of *Brassica* spp. and other plant species (Shattuck 1992). However, the map positions of only three resistance genes have been published to date: *Tu* in lettuce (Robbins et al. 1994), and *TuRB01* and *TuRB02* in *B. napus* (Walsh et al. 1999). Further resistance sources are being characterized and the genes involved are being mapped (Rusholme et al. 1999). In this article, we report the use of an infectious cDNA clone of TuMV, p35Tunos (Sánchez et al. 1998), to examine viral

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Sequence data for TuMV UK 1 cDNA contained in p35Tu has been submitted to the GenBank data base as accession number AF169561.

components involved in *TuRB01* resistance, and demonstrate the first example of a potyvirus cylindrical inclusion (CI) gene as a determinant of virulence to a plant resistance gene.

RESULTS

Isolation of mutants.

The infectious cDNA clone p35Tunos (Sánchez et al. 1998) was constructed from the isolate TuMV UK 1, a typical pathotype 1 isolate that systemically infects *B. napus* differential lines S1 and S6 but is incapable of infecting lines R4 and 165 (Jenner and Walsh 1996). On inoculation to *B. napus* plants, the virus produced from p35Tunos DNA (denoted as v35Tunos) behaved like UK 1 except that, occasionally, a single R4 plant developed two or three small necrotic spots after several weeks. Virus was isolated from two such plants, from occasions separated by several months, and named v35TunosM1 and v35TunosM2. The isolates UK 1M and UK 1M2 were isolated from *B. napus* cv. Westar (Jenner and Walsh 1996) and line R4 plants (J. A. Walsh, *unpublished data*) respectively, following inoculation with UK 1. All of these mutants consistently infected *B. napus* line R4 and the doubled-haploid *B. napus* line N-o-1 (Sharpe et al. 1995) possessing *TuRB01* (Walsh et

al. 1999). Symptoms consisted of striking local necrotic lesions with systemic virus spread giving rise to smaller necrotic lesions on leaves, stems, and petioles. The altered phenotype was produced even after repeated subcultures of the virus in a plant host not possessing *TuRB01* (*B. juncea* cv. Tendergreen).

Analysis of sequences of mutants.

The sequence of TuMV UK 1 cDNA contained in p35Tu (Sánchez et al. 1998) has been submitted to GenBank as accession number AF169561. The plasmid p35Tunos was generated from p35Tu (Sánchez et al. 1998). It has a single nucleotide difference from p35Tu in the CP gene (at base 9195, where C replaces T). The cDNA sequence of the CP of virus derived from p35Tunos (v35Tunos) matched p35Tunos and UK 1.

The 5' end of v35Tunos and v35TunosM1 from Tendergreen mustard or line R4 plants consisted of four to six A residues, although six residues were used in the construction of p35Tunos (Sánchez et al. 1998). This variation in the TuMV genome has been reported previously (Nicolas and Lailiberté 1992). We have, therefore, numbered the nucleotides of mutations according to recent recommendations in which base +1 is the A of the start codon of the open reading frame (ORF) (Antonarakis and the Nomenclature Working Group 1998).

Fig. 1. Schematic representation of *Turnip mosaic virus* constructs. Point mutations are indicated using arrowheads. The affected nucleotide is numbered whereby +1 is the first base of the open reading frame. The fragment from the isolate CDN 2 introduced 22 nucleotide substitutions (not indicated). The mutations at positions +5056 and +5570 were derived from virus isolates, whereas the mutation at position +8637 was introduced using oligonucleotides and site-directed mutagenesis. The polyprotein is autocatalytically cleaved into P1, proteinase; HC Pro, helper component proteinase; P3, third protein; 6K1 and 6K2, 6-kDa proteins; CI, cylindrical inclusion (helicase); VPg, genome-linked protein; NIa, nuclear inclusion (proteinase); NIb, nuclear inclusion (replicase); and CP, coat protein.

The complete sequence of v35TunosM1 was determined from virus present in systemically infected *B. napus* line R4 plants. The sequences of p35Tunos and the mutant v35TunosM1 differed by a single nucleotide substitution at position +5056: A in the former, G in the latter. The mutation affected residue 1686 of the polyprotein, changing a predicted asparagine to aspartate in the CI protein.

The sequence of the CI region was determined similarly for the mutants UK 1M, UK 1M2, and v35TunosM2. The isolate UK 1M differed from p35Tunos at position +5570, where the mutant contained a G instead of A. This affected residue 1857 of the polyprotein, changing the predicted amino acid from histidine to arginine. Both UK 1M2 and v35TunosM2 contained the same mutation as v35TunosM1 at position +5056, but UK 1M2 differed additionally at position +4685 (changing from a G to an A and altering the predicted serine residue at position 1562 to asparagine).

Behavior of recombinant viruses containing point mutations in the CI gene on *B. napus* lines possessing *TuRB01*.

Recombinant viruses v35Tunos 5056A>G and v35Tunos 5570A>G were derived from the plasmids p35Tunos 5056A>G and p35Tunos 5570A>G (Fig. 1). Each was tested for its ability to infect *B. napus* lines S1, S6, 165, R4, N-o-1, and N-o-9 (Table 1). The viruses containing the point mutations in the CI gene infected the same lines as the parental mutants, breaking the resistance in lines N-o-1 and R4 and producing symptoms and virus content (as measured by enzyme-linked immunosorbent assay [ELISA]) (data not shown) indistinguishable from the original mutants. Symptoms induced by v35Tunos 5570A>G (and UK 1M) were more severe than those induced by v35Tunos 5056A>G (and v35TunosM1). Both mutants induced symptoms of greater severity on line R4 than on line N-o-1. The sequences of the exchanged sections of genome were confirmed in virus derived from both constructs following reverse transcription-polymerase chain reaction (RT-PCR) on infected N-o-1 plants. The sequence around the position of the other alternative mutation was also confirmed. None of the mutants or chimeras was able to overcome the resistance present in line 165.

Line N-o-1 possesses the resistance gene *TuRB01*; therefore, the recombinant viruses were also tested on 10 doubled-haploid lines, derived from a cross of N-o-1 with N-o-9 (Table

2), that are known to be segregating for the presence of *TuRB01* (Walsh et al. 1999). Recombinant viruses containing the mutations infected the lines lacking *TuRB01* in the same manner as the wild-type infectious clone, and overcame the resistance in the lines possessing *TuRB01* with the same symptoms seen on line N-o-1 (Fig. 2). This confirmed that either single nucleotide change was sufficient to enable the virus to overcome *TuRB01* resistance.

Experiments on the CP sequence of TuMV and *TuRB01* resistance.

Four additional viral constructs were produced to examine the potential effect of the CP sequence on resistance breaking (Fig. 1). In the first, the section of p35Tunos encompassing the CP coding region and 3' untranslated region of UK 1 was replaced by cDNA of this same region derived from TuMV CDN 2. The isolate CDN 2 is a typical pathotype 3 isolate (Jenner and Walsh 1996) and produced similar symptoms on lines N-o-1 and R4 to the UK 1 mutants. The exchanged section introduced 23 nucleotide substitutions, altering six amino acids of the CP (polyprotein positions 2915 Lys>Gln, 2919 Asp>Asn, 2926 Lys>Glu, 2958 Lys>Gln, 3057 Met>Ile, and 3162 Gln>Lys). The recombinant virus infected the same lines as UK 1, and did not infect lines N-o-1 or R4 (Table 1). Sequencing of the CP section confirmed the identity of the virus present in infected leaves. The presence of CDN 2 CP sequence and protein was not sufficient to enable v35Tunos to overcome *TuRB01* resistance, demonstrating that the virulence determinant for *TuRB01* lies outside this region of the viral genome.

Previous work (Lehmann et al. 1997) hypothesized a correlation between *TuRB01* resistance breaking and the nucleotide in the CP region of the ORF at position +8637 (G>A for virulent isolates). p35Tunos broke this correlation because it had an A at this position and virus derived from it could not infect lines possessing *TuRB01*. The CDN 2 sequence introduced above was also A at this position. Therefore, the plasmid p35Tunos was altered to introduce a G at position +8637. This base change was also introduced into the two other constructs that contained point mutations in the CI region (Fig. 1). Virus derived from the three 8637A>G constructs infected the same *B. napus* lines to the same degree as the three +8637A constructs (Table 1). The sequence around positions +5056, +5570, and +8637 of all three viruses was confirmed from

Table 1. Phenotypes of the interactions between *Turnip mosaic virus* isolates, mutants, and virus derived from cDNA constructs and *Brassica napus* lines

Virus isolate	<i>B. napus</i> line					
	N-o-1 ^a	R4	165	N-o-9 ^a	S1	S6
UK 1	0 ^b	0	0	+ ^c	+	+
UK 1M	+ _N ^d	+ _N	0	+	+	+
v35TunosM1	+ _N	+ _N	0	+	+	+
CDN 2	+ _N	+ _N	0	+	+	+
v35Tunos 5056A>G	+ _N	+ _N	0	+	+	+
v35Tunos 5570A>G	+ _N	+ _N	0	+	+	+
UK 1::CDN 2 CP+3' UTR	0	0	0	+	+	+
v35Tunos 8637A>G	0	0	0	+	+	+
v35Tunos 5056+8637A>G	+ _N	+ _N	0	+	+	+
v35Tunos 5570+8637A>G	+ _N	+ _N	0	+	+	+

^a Line N-o-1 possesses the resistance gene *TuRB01* and line N-o-9 lacks *TuRB01*.

^b 0 = No symptoms in any plants, and no virus was detected by enzyme-linked immunosorbent assay (ELISA).

^c + = All plants displayed systemic mosaic, and virus was detected by ELISA.

^d +_N = All plants displayed systemic necrosis, and virus was detected by ELISA.

infected N-o-1 leaves (constructs containing CI mutations) or from infected S6 leaves (v35Tunos 8637A>G, which did not infect line N-o-1). These experiments showed that the identity of the base at position +8637 was not the determining factor in the ability to overcome *TuRB01* resistance.

The correlation of resistance breaking with position +8637A that was noted by Lehmann et al. (1997) was based upon the CP sequences of a small number of isolates (three isolates had a G, including UK 1, and four isolates had an A, including UK 1M). The CP sections of seven further samples of UK 1 have now been sequenced, ranging from freeze-dried material from 1993 to virus in current culture. In all cases, the sequence matched that of p35Tunos, in which the nucleotide at position +8637 was A.

DISCUSSION

The identification of the TuMV CI coding region (gene) as a virulence determinant for the resistance gene *TuRB01* has been achieved through the characterization of v35TunosM1, a mutant derived from the infectious clone of TuMV UK 1. This has been confirmed by the discovery of a second mutation in the same gene (of TuMV UK 1M). The genotype specificity of this interaction was confirmed by inoculation of chimeric clones to plant lines segregating for *TuRB01*. This finding is novel for potyviruses, in that the CI gene has not previously been implicated in any genotype-specific resistance response.

The ability of virus containing an altered base in the CP gene at position +8637 (A>G) and either of the two CI mutations to infect plants possessing *TuRB01* showed that the CP sequence is not involved in resistance breaking. This, along with the identification of a number of virus isolates unable to break the resistance (P. J. Hunter and S. B. Nettleship, unpublished data) (and all isolates of UK 1 sequenced) with A at position +8637, has demonstrated that the hypothesis about a possible involvement of the CP sequence at this position in the interaction with *TuRB01* proposed by Lehmann et al. (1997) is not valid. It appears that the original finding of base +8637 G in UK 1 was either an error generated during PCR prior to cloning, or a subpopulation that has since been replaced by the A population.

The CI protein of potyviruses is a helicase (Laín et al. 1990) of the superfamily SF2 type (Kadaré and Haenni 1997) and has a role in viral cell-to-cell movement (Rodríguez-Cerezo et al. 1997; Carrington et al. 1998; Roberts et al. 1998). There are a few examples of the association of other viral movement proteins with resistance breaking. These include the movement proteins of *Tomato bushy stunt virus* (Chu et al. 1999), PVX (Malcuit et al. 1999), and tobamoviruses (Meshi et al. 1989; Weber and Pfitzner 1998). Infection of lines lacking *TuRB01* by the mutants or the chimeras appeared to be identical to infections by the wild-type virus, in that clear systemic symptoms were seen and the virus content as measured by ELISA was indistinguishable from that of wild-type virus. This suggested that the mutations were not deleterious to the ability to infect and spread through plants not possessing *TuRB01*. The altered phenotype was seen even after repeated subcultures in a plant host not possessing *TuRB01*; therefore, the mutations appeared to be stable. Both mutations (at positions +5056 and +5570) were outside the seven amino acid motifs associated with helicase action (Kadaré and Haenni 1997) and

the sections of sequence thought to have a role in viral movement (Carrington et al. 1998). The effect of the mutations on the tertiary and quaternary structure of the native protein and the structural position of the mutations relative to the above functional regions are not known. The Chou and Fasman algorithm (Protean program of DNASTAR, Chou 1990) predicted that the mutation at position +5056 would disrupt a very small region of β -sheet secondary structure. The CI protein accumulates around plasmodesmata in the early stages of infection before releasing into the cytoplasm as characteristic inclusion bodies that slowly degrade (Roberts et al. 1998). Electron microscopy revealed no obvious structural alteration in inclusion bodies produced by v35TunosM1 or v35Tunos 5570A>G (data not shown).

It is not known whether the mutation in v35TunosM1 arose in the original *Chenopodium quinoa* plant inoculated with plasmid DNA, in the mustard plant inoculated subsequently, or in the *B. napus* R4 plant under the selection pressure of a resistance gene. The R4 line possesses a single dominant allele for resistance to UK 1 (Walsh 1989) that has the same specificity to different TuMV isolates as *TuRB01* (Walsh et al. 1999), suggesting that it possesses *TuRB01*. This is also supported by the results described here, in that the single nucleotide changes in the TuMV genome gave the same phenotypic changes on line R4 plants as on plants possessing *TuRB01*. The virus mutation could only have arisen in the R4 plant if at least one round of viral replication had occurred before the resistance mechanism was triggered. Such a level of multiplication may be too low for our methods to detect.

CI gene involvement in specific interactions is not limited to *TuRB01* resistance. There is specificity in the association of the CI protein with the CP (Langenberg 1993). The CI protein may also possibly trigger a range of host responses associated with infection (Roberts et al. 1998). The mechanism of *TuRB01* resistance is not yet understood, particularly because it lacks an obvious hypersensitive reaction (HR). Some movement proteins in other virus–host interactions are direct elicitors of resistance (e.g., the 25-kDa movement protein of PVX is the elicitor of *Nb*-mediated resistance [Malcuit et al. 1999]

Table 2. Phenotypes of the interactions between *Turnip mosaic virus* isolates derived from cDNA constructs containing point mutations in the cylindrical inclusion gene and doubled-haploid *Brassica napus* lines derived from a cross of N-o-1 $\times$ N-o-9 used to map *TuRB01*

Plant line	Presence of <i>TuRB01</i>	Viral isolate/construct		
		v35Tunos	v35Tunos 5056 A>G	v35Tunos 5570A>G
N-72-8-1-48	Yes	0 ^a	+ _N ^b	+ _N
N-72-8-2-69	Yes	0	+ _N	+ _N
N-72-8-2-126	Yes	0	+ _N	+ _N
N-72-8-2-152	Yes	0	+ _N	+ _N
N-72-8-3-241	Yes	0	+ _N	+ _N
N-72-8-1-12	No	+ ^c	+	+
N-72-8-1-54	No	+	+	+
N-72-8-1-178	No	+	+	+
N-72-8-2-77	No	+	+	+
N-72-8-2-137	No	+	+	+

^a 0 = No symptoms in any plants, and no virus was detected by enzyme-linked immunosorbent assay (ELISA).

^b +_N = All plants displayed systemic necrosis, and virus was detected by ELISA.

^c + = All plants displayed systemic mosaic, and virus was detected by ELISA.

and the 30-kDa movement protein of *Tomato mosaic virus* elicits *Tm2²* resistance [Weber and Pfitzner 1998]. However, our data are not sufficient to infer whether the viral RNA or the CI protein itself is the elicitor or acts via intermediates. It is probable that the overall protein structure rather than individual amino acid residues is important in the interaction, because two distinct amino acid alterations altered the phenotype observed. It is possible that the mutations act as a suppressor of the resistance mechanism or that the CI protein is the elicitor and the mutations alter its structure. The production of

systemic necrotic lesions by the virulent mutants in lines possessing *TuRB01*, rather than the chlorotic mosaic seen in lines lacking *TuRB01*, might suggest that other (later and less-effective) resistance mechanisms are being triggered. If this is the case, *TuRB01* must be epistatic to host genes involved in the limited cell death (necrosis) observed. It is interesting to note that the non-HR resistance conferred by *Rx* (Kavanagh et al. 1992) has also been reported to be epistatic to a HR (Bendahmane et al. 1999). It is hoped that wild-type TuMV isolates (pathotype 4) (Jenner and Walsh 1996) and the identi-

Fig. 2. *Brassica napus* plants (doubled-haploid line N-72-8-2-152 possessing *TuRB01*) following inoculation with (on left) v35Tunos and (on right) v35Tunos 5056A>G. **A**, Leaves 7 days after inoculation. **B**, Plants 25 days after inoculation.

fication of further mutants able to overcome *TuRB01* without inducing necrosis, together with the use of transient expression assays, will enable us to gain a better understanding of the mechanism of *TuRB01*-mediated resistance.

MATERIALS AND METHODS

Plants and virus infections.

B. napus lines S1, S6, 165, R4 (Jenner and Walsh 1996), N-o-1, and N-o-9 (Sharpe et al. 1995) have been described previously. The 10 doubled-haploid lines (Table 2) were derived by microspore culture from an F_1 plant from N-o-1 $\times$ N-o-9 (Sharpe et al. 1995) and the presence of *TuRB01* determined previously (Walsh et al. 1999). Plants were grown in an insect-proof glasshouse, and viruses were transmitted by mechanical inoculation as described previously (Jenner and Walsh 1996). cDNA clones of TuMV were purified using plasmid midi preparations (Qiagen, Crawley, U.K.). Five micrograms of plasmid (1 mg ml^{-1}) was sufficient to infect *C. quinoa* and *B. juncea* (mustard) cv. Tendergreen. About half of the Tendergreen mustard plants and almost all of the *C. quinoa* plants became infected following inoculation with plasmid preparations. Interactions between virus isolates and plants were determined essentially as described by Jenner and Walsh (1996) using 10 plants per line, or four plants where the plants were doubled-haploid lines. Symptoms were assessed weekly for 4 weeks, after which the presence of TuMV was assessed by plate-trapped antigen ELISA using the monoclonal antibody EMA 67 (Jenner et al. 1999).

RT-PCR.

Oligonucleotide primers were based on the sequence of p35Tunos and designed using PrimerSelect (DNASTAR Inc., Madison, WI, U.S.A.) to produce overlapping products of approximately 1 kb. Leaf samples were ground in sample buffer (0.5 M Tris, pH 8.3; 0.15 M NaCl; 0.05% Tween 20; 2% polyvinylpyrrolidone; and 1% polyethylene glycol 6000) and diluted to 2 mg ml^{-1} . cDNA was prepared using SuperscriptII reverse transcriptase (Life Technologies, Paisley, U.K.) according to manufacturer's recommendations and with added ribonuclease inhibitor (2 U per reaction), using 2 μl of sap per 20- μl reaction. The cDNA was amplified by PCR using 2.5 U of *Taq* DNA polymerase (Life Technologies) and 5 pmol of each oligonucleotide primer, in 20 mM Tris pH 8.3, 50 mM KCl, 4 mM dithiothreitol, 0.2 mM dNTP, and 1.5 mM MgCl_2 . PCR was performed for 35 cycles at temperatures appropriate for the primer combinations in use. DNA products were separated on 1% agarose gels and were isolated from the gel using QIAquick gel extraction kits (Qiagen).

DNA sequencing.

The sequence of TuMV UK 1 cDNA contained in p35Tu (Sánchez et al. 1998) was determined from plasmid DNA at least three times per strand. The sequences of the mutant viruses were determined in both directions from RT-PCR products from virus present in systemically infected *B. napus* R4 plants. The complete sequence of v35TunosM1 was determined, but only the section encompassing the CI gene was determined for the other mutants. Sequencing reactions were prepared using the Big Dye Terminator cycle sequencing ready reaction mix (PE-Applied Biosystems, Foster City, CA,

U.S.A.) and run through 4.25% acrylamide gels on ABI 373A (Sequiseive, Vaterstetten, Germany) and ABI 377 Automated Sequencers. The sequences were assembled using SeqMan (DNASTAR). Sections of the mutants differing from p35Tunos were determined from three independent RT-PCR reactions. The sequence of p35Tunos in planta (i.e., v35Tunos) for these sections was also checked. The 5' nucleotides were determined using a 5' RACE kit (Life Technologies); the 3' nucleotides were obtained using 5'-GCGTCGACTAGTACTCGAGTT-TTTTTTTT-3' (P3S) as the reverse primer.

Construction of recombinant viruses.

A 1.2-kb RT-PCR product of v35TunosM1 encompassing the mutation at position +5056 was obtained from infected R4 leaves using the primers 5'-TGAACGCACAGGGTAT-3' and 5'-ATATTCCATCGGTTTTTGTA-3'. The mutation at position +5570 was similarly obtained in a 1.3-kb product from UK 1M using the primers 5'-GGTGGGACGTCCTTGGTA-AC-3' and 5'-CAGGTTTTGGTCGGCTTTCA-3'. The CP section of CDN 2 was obtained from infected TGM leaves using 5'-CTGGAACCTGAGCGAATAGTATCG-3' and P3S. Each product was cloned into pMOSBlue (Amersham, Little Chalfont, U.K.) according to manufacturer's recommendations and the sequences of the clones were checked. The mutation 5056A>G was introduced into p35Tunos in a 1.0-kb *MunI*-*BstEII* fragment, 5570A>G in a 0.2-kb *BstEII*-*SsrI* fragment, and the CDN 2 CP section in a 1.3-kb *MluI*-*SalI* fragment.

The nucleotide at position +8637 (CP nucleotide 9) was changed from A to G using subclones of p35Tunos. A 3.8-kb *PstI*-*SalI* fragment was cloned into pMOSBlue, and then a 14-bp *BspMI* fragment encompassing position +8637 was removed and replaced with a pair of annealed complementary 5'-phosphorylated oligonucleotides (5'-ATCACCAGGCAGGTGAGA-3' and 5'-AGCGTCTCACCTGCCTGG-3'). Similarly, a 1.6-kb *MluI*-*Apal* fragment was cloned into the plasmid pSL1180 (Amersham Pharmacia Biotech, St. Albans, U.K.). Mutagenesis of this clone was performed using the mutagenic oligonucleotide 5'-GCAGGTGAGACGCTTGAT-3' in the GeneEditor in vitro Site-Directed Mutagenesis System (Promega Corporation, Southampton, U.K.). Mutagenized fragments (1.3-kb *MluI*-*SalI* or 1.6-kb *MluI*-*Apal*, respectively) were replaced into p35Tunos, p35Tunos 5056A>G, and p35Tunos 5570A>G.

Recombinant clones were identified by standard methods prior to inoculation onto both *C. quinoa* and Tendergreen mustard plants for use as the source of inoculum for experiments using *B. napus*. The presence of the mutations was confirmed by sequencing the plasmids and RT-PCR products derived from virus present in infected *B. napus* line N-o-1 leaves (or line S6 where line N-o-1 was not infected) using the above primers.

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