The Dual Role of the Potyvirus P3 Protein of *Turnip mosaic virus* as a Symptom and Avirulence Determinant in Brassicas

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Two isolates of the potyvirus *Turnip mosaic virus* (TuMV), UK 1 and CDN 1, differ both in their general symptoms on the susceptible propagation host *Brassica juncea* and in their ability to infect *B. napus* lines possessing a variety of dominant resistance genes. The isolate CDN 1 produces a more extreme mosaic in infected brassica leaves than UK 1 and is able to overcome the resistance genes *TuRB01*, *TuRB04*, and *TuRB05*. The resistance gene *TuRB03*, in the *B. napus* line 22S, is effective against CDN 1 but not UK 1. The nucleic acid sequences of the UK 1 and CDN 1 isolates were 90% identical. The C-terminal half of the P3 protein was identified as being responsible for the differences in symptoms in *B. juncea*. A single amino acid in the P3 protein was found to be the avirulence determinant for *TuRB03*. Previous work already has identified the P3 as an avirulence determinant for *TuRB04*. Our results increase the understanding of the basis of plant–virus recognition, show the importance of the potyviral P3 gene as a symptom determinant, and provide a role in planta for the poorly understood P3 protein in a normal infection cycle.

The small size of viral genomes is of interest to scientists wishing to understand the genetic basis of pathogenicity, whether toward plants or animals. To this end, much effort has been expended in sequencing viral genomes and in the analysis of mutants. In the case of the family *Potyviridae*, the largest family of viral plant pathogens (Shukla et al. 1994), full-length sequences in databases are now available for at least 36 different species. Sequences from viruses such as *Potato virus Y* (PVY), *Potato virus A*, *Wheat streak mosaic virus*, and *Plum pox virus* (PPV) are particularly well represented. Data from such sources is useful for obtaining an understanding of the extent of variation within a species (Bateson et al. 1994; Glasa et al. 2002; Krause-Sakate et al. 2002). The identification of conserved motifs is useful in trying to understand common functions in viral life cycles (Shukla et al. 1991) (e.g., in the identi-

fication of the cytoplasmic inclusion [CI] protein as an RNA helicase [Lain et al. 1990]). However, many aspects of potyviral protein function and pathogenicity remain unclear (Revers et al. 1999). The functions of the highly variable P1 and P3 proteins in particular are still rather enigmatic (Urcuqui-Inchima et al. 2001).

Turnip mosaic virus (TuMV) is a potyvirus with an unusually wide host range and is of particular importance to brassica crops (Shattuck 1992). In addition to the economic importance of TuMV, the TuMV–crucifer pathosystem is proving to be an excellent tool for studying plant–virus interactions at many different levels (Walsh and Jenner 2002). The extent of genetic variation in the species has been studied using coat protein (CP) sequences (Lehmann et al. 1997) or P1 and CP sequences (Ohshima et al. 2002). The pathogenic variation has been assessed using a range of different hosts, such as *Brassica rapa* (Hughes et al. 2002; Provvidenti 1980; Walsh et al. 2002) or *B. napus* (Hughes et al. 2002; Jenner and Walsh 1996; Walsh 1989).

The nonpersistent mode of transmission of potyviruses by aphids makes control of the disease by insecticides difficult (Tomlinson 1987) and control through plant resistance more attractive. Several resistance genes to TuMV have been identified, and four have been mapped: *Tu* in lettuce (Robbins et al. 1994) and *TuRB01*, *TuRB02* (Walsh et al. 1999), and *TuRB03* (Hughes et al. in press) in *B. napus*. The TuMV determinants of avirulence for three *Brassica* resistance genes have been identified. These are the CI protein for *TuRB01/TuRB01b* (Jenner et al. 2000; Walsh et al. 2002) and the P3 and CI proteins for *TuRB04* and *TuRB05* respectively (Jenner et al. 2002a). These discoveries have helped researchers to understand the basis of the TuMV pathotyping system (Jenner and Walsh 1996). Other potyviral avirulence determinants include the *Pea seedborne mosaic virus* (PSbMV) genome-linked protein (VPg) and P3 (Johansen et al. 2001; Keller et al. 1998), the PVY VPg and nuclear inclusion proteins (NIa and NIb) (Fellers et al. 2002; Masuta et al. 1999; Mestre et al. 2000), the *Tobacco vein mottling virus* VPg (Nicolas et al. 1997), and the *Zucchini yellow mosaic virus* CP (Ullah and Grumet 2002).

The resistance gene *TuRB03* was identified in *B. napus* line 22S, a line not among the original *B. napus* lines used to pathotype TuMV isolates (Walsh 1989). It is not effective against the TuMV isolate UK 1, the type member of pathotype 1. However, it is the first dominant resistance gene found to be active against the pathogenic isolate CDN 1, the type member of pathotype 4 and, as such, is of potential interest for breeding lines.

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In this article, we report the construction of an infectious clone of the isolate CDN 1 and the identification of the viral avirulence determinant for *TuRB03*. Viral chimeras between isolates UK 1 and CDN 1 also identified the region of the genome responsible for the characteristic strong mosaic symptoms induced by CDN 1 on susceptible brassica hosts.

RESULTS

Natural sequence diversity.

The TuMV isolates UK 1 and CDN 1 originated from *B. napus* plants from different continents. The degree of genetic differences underlying their differing pathogenic abilities was assessed by genome sequence comparison. The UK 1 cDNA clone (p35Tunos) and CDN 1 cDNA clone (pVIR95) were 89.9% identical at the nucleic acid level and 95.4% identical at the amino acid level. The least conserved proteins were the 6K2, P1, and P3 proteins (83 to 90% identical). There were 10 nucleotide differences between the sequences of the CDN 1 cDNA clone (GenBank accession no. AY227024) and the CDN 1 virus (GenBank accession no. AB093610) (Tomimura et al. 2003). This level of difference is consistent with the accumu-

lation of mutations seen previously during subculture of UK 1 through *B. juncea* plants over several years in comparison to its infectious clone p35Tunos (Jenner et al. 2002a).

Identification of an avirulence determinant in P3.

In order to identify which viral gene was responsible for the differing phenotypes of UK 1 and CDN 1 on *B. napus* line 22S, reciprocal exchanges of all sections of the viral genome were made (Fig. 1A). Although UK 1 normally is able to infect line 22S, the introduction of the central 4.6-kb *XcmI*-*Bst*EII fragment from CDN 1 was sufficient to block this ability. Conversely, the introduction of the UK 1 version of this fragment into CDN 1 produced a construct able to infect line 22S (Fig. 1A). The smallest restriction fragment from CDN 1 conveying avirulence upon UK 1 was a 0.4-kb *Bbs*I-*Eco*47III fragment (in pVIR136; Fig. 1B). This introduced 68 nucleotide differences, but encoded only six amino acid differences, affecting P3 residues 65, 73, 118, 144, 148, and 153. Site-directed mutagenesis (SDM) was performed to introduce each amino acid change individually into the UK 1 background. Two amino acid changes affected the phenotype of UK 1 on *B. napus* line 22S (Table 1). The alteration of P3 D148N (using the nucleotide mutation +2902A>G) caused the severity of the necrotic infection to be lessened; the alteration of P3 I153F (using the nucleotide mutation +2917A>T) completely prevented any viral infection (Fig. 2).

The identification of the role of the P3 residue 153 in avirulence stimulated the construction via SDM of the reciprocal virus, a CDN 1 genome with the nucleotide mutation +2917T>A, thereby generating the UK 1 amino acid at this position, P3 F153I. Virus derived from this construct was virulent and infected line 22S with systemic mosaic symptoms (Fig. 2).

Confirmation that the P3 is the avirulence determinant for *TuRB03*.

Lines 22S and N-o-1 were crossed to produce the progenitors of the B₁S₁ families. The former possesses *TuRB03* and is resistant to CDN 1 (Hughes et al. in press). Line N-o-1 is susceptible to CDN 1 and is one of the parents of a well-characterized genetic mapping population (Sharpe et al. 1995). It possesses the resistance gene *TuRB01* and, therefore, is resistant to UK 1 (Walsh et al. 1999). However, the introduction of the mutation +5570A>G, causing the alteration *CI* H630R, into the UK 1 genome allows UK 1 to infect line N-o-1 (Jenner et al. 2000) and did not affect its ability to infect line 22S (Fig. 2). Consequently, 20 B₁S₁ families were inoculated separately with CDN 1 ± P3 F153I, and with UK 1 + *CI* H630R ± P3 I153F.

Six B₁S₁ families were uniformly susceptible to all of the viral constructs (Table 2) lacking any resistance to CDN 1. Fourteen families showed segregation for resistance to isolates possessing phenylalanine as residue 153 (CDN 1 or UK 1 *CI* H630R P3 I153F), but were uniformly susceptible to each of the viral constructs possessing isoleucine as P3 residue 153 (UK 1 *CI* H630R or CDN 1 P3 F153I). The ratio of families (14 with resistance: 6 totally susceptible) appears to be compatible with a hypothesis of two dominant resistance genes acting against CDN 1 (expected ratio 3:1). However, this may be an artifact of the small sample size. B₁ plants previously inoculated with CDN 1 segregated for resistance to susceptibility in a 1:1 ratio (57 and 56 plants, respectively; Hughes et al. in press), indicating the presence of not two but a single dominant resistance gene.

A χ^2 test of homogeneity confirmed that the data from the segregating families could be pooled. A total of 78 plants were resistant and 34 susceptible to UK 1 *CI*H630R P3 I153F, a ratio not significantly different from 3:1 ($P = 0.2$), as expected for a

Fig. 1. Chimeric constructs of *Turnip mosaic virus* isolates CDN 1 and UK 1 and their abilities to infect *Brassica napus* line 22S. **A**, Reciprocal exchanges of all genomic sections. **B**, Identification of region encoding avirulence determinant of CDN 1.

single resistance gene. The ratio of resistant to susceptible plants (94 to 18, respectively) inoculated with CDN 1 differed from 3:1 ($P < 0.05$, $P > 0.01$), and was compatible with a two-gene model (39:9, $P = 0.5$). If two genes are present, they both were overcome by the introduction of the single amino acid change P3 F153I into CDN 1. When the results of all inoculations using isolates with phenylalanine as residue 153 were combined, the segregation ratio was not significantly different from 3:1 (172 resistant, 52 susceptible, $P = 0.5$). The segregating families probably are derived from B₁ plants that were heterozygous for a single dominant allele of a resistance gene. Hughes and associates (in press) identified and mapped the resistance to CDN 1 in line 22S to a single locus, namely *TuRB03*.

The 20 B₁S₁ families also were inoculated with UK 1 ± P3 I153F. No B₁S₁ plant became infected with UK 1 P3 I153F, indicating that all plants possessed *TuRB01*, *TuRB03*, or both. Families identified above as segregating for the presence of *TuRB03* also showed segregation for resistance towards UK 1 (data not shown). UK 1 overcomes *TuRB03* resistance; therefore, the observed segregation was due to the presence of *TuRB01*. The results indicated that B₁ plants lacking *TuRB03* were homozygous for *TuRB01*, and that B₁ plants heterozygous for *TuRB03* also were heterozygous for *TuRB01*. No families with other combinations of alleles were identified. This is consistent with *TuRB01* and *TuRB03* residing on the same chromosome (Hughes et al. in press).

Natural mutations at P3 position 153 also overcome resistance.

On four occasions, individual plants predicted to be resistant to infection developed symptoms. In each case, the identity of the virus was examined by sequencing. A 22S plant inoculated with vVIR129, a UK 1 construct containing the CDN 1 P3 region (Fig. 1B), was found to be infected by a mutant (vVIR129M1). This virus possessed a single mutation at the nucleotide position +2917 (T > G) of the open reading frame, causing the alteration P3 F153V (Table 1). Two B₁S₁ plants inoculated with UK 1 P3 I153F and a single 22S plant inoculated with UK 1 CI H630R P3 I153F also became infected, and sequence analysis confirmed that each virus had gained the identical mutation. No instances of spontaneous reversion (P3 F153I) or mutation to other amino acids enabling virulence towards *TuRB03* were observed.

Other avirulence determinants.

Mutation of the P3 amino acid 153 did not affect the outcome of UK 1 or CDN 1 challenges against *B. napus* lines (R4 and 165) possessing other resistance genes (data not shown).

However, the introduction of CDN 1 CI C-terminus into UK 1 (on the 1.2-kb *Bst*EII-*Bst*EII fragment) caused *B. napus* line R4 to be systemically infected (i.e., consistent with the *TuRB01*-determinant being in this region as found previously for the isolate UK 1) (Jenner et al. 2000). Similarly, any construct containing the 0.5-kb 3' region of CDN 1 P3 in a UK 1 background caused *B. napus* line 165 to produce local necrotic lesions, consistent with the *TuRB04*-determinant being in this region as found previously for the isolates UK 1 and CHN 12 (Jenner et al. 2002a).

The introduction of P3 F153I into CDN 1 caused *B. napus* line 22S to be systemically infected without necrosis. Although UK 1 also possesses isoleucine at this position, the infection of line 22S was necrotic. Enzyme-linked immunosorbent assay (ELISA) data (not shown) indicated that extensive viral replication occurred in plants infected with UK 1 despite the extensive necrosis. Therefore, isolate UK 1 possesses a determinant of necrosis that is absent from CDN 1.

TuMV P3 is a symptom severity determinant.

The symptoms induced by CDN 1 on susceptible hosts such as *B. juncea* were much more pronounced and chlorotic than those induced by UK 1, particularly during periods of short daylight (Fig. 3). The introduction of the 0.9-kb *Eco*47III-*Btg*I fragment from CDN 1 into the UK 1 background (i.e., pVIR125; Fig. 1B) caused the typical CDN 1-like yellow mosaic symptoms. The 0.9-kb CDN 1 fragment encoded 24 amino acid differences in the P3 protein C-terminus, 2 differences in the 6K1 protein and 3 differences in the CI protein compared with the UK 1 sequence. Replacement of the section encoding the 6K1 and CI proteins with the original UK 1 sequence by overlap extension polymerase chain reaction (PCR) created pVIR156 (Fig. 1B). Virus derived from pVIR156 differed from UK 1 only in the P3 protein C-terminus. Virus derived from this construct induced the pronounced CDN 1-like symptoms on susceptible plants (Fig. 3).

DISCUSSION

We have shown that the TuMV P3 protein is the avirulence determinant for *TuRB03* and also affects symptom severity in susceptible plants. We also showed previously that P3 is the avirulence determinant for *TuRB04* (Jenner et al. 2002a). Just as it has been noted that bacterial avirulence genes have roles in pathogenicity (Gabriel 1999; van't Slot and Knogge 2002), so it seems to be the case for TuMV. The small size of viral genomes generally means that most, if not all, of the encoded proteins have indispensable and multiple roles in the viral life

Table 1. Six P3 amino acid residues differing between *Turnip mosaic virus* isolates UK 1 and CDN 1 in the 0.4-kb *Bbs*I-*Eco*47III fragment of pVIR136 and their effect on the phenotype of *Brassica napus* line 22S plants

Virus	Amino acid position in P3 ^a						Plant symptoms ^b
	65	73	118	144	148	153	
UK 1 (v35Tunos)	Y	V	V	W	D	I	+ _N
CDN 1 (vVIR95, 136)	*H	*A	*I	*Y	*N	*F	0
UK 1 P3 Y65H	*H	V	V	W	D	I	+ _N
UK 1 P3 V73A	Y	*A	V	W	D	I	+ _N
UK 1 P3 V118I	Y	V	*I	W	D	I	+ _N
UK 1 P3 W144Y	Y	V	V	*Y	D	I	+ _N
UK 1 P3 D148N	Y	V	V	W	*N	I	Mild + _N
UK 1 P3 I153F	Y	V	V	W	D	*F	0
CDN 1 P3 F153I	*H	*A	*I	*Y	*N	I	+
vVIR129	*H	*A	*I	*Y	*N	*F	0
vVIR129M1	*H	*A	*I	*Y	*N	V	Mild + _N

^a Letters preceded by an asterisk (*) indicate residues corresponding to CDN 1.

^b 0 = No symptoms observed, no viral replication detected by enzyme-linked immunosorbent assay; +_N = systemic necrotic infection; and + = systemic nonnecrotic infection.

cycle. The precise role of the P3 protein in the normal viral life cycle is not clear (Urcuqui-Inchima et al. 2001). Cleavage from the 6K1 protein occurs only slowly (Merits et al. 2002) and may be the method of viral control of its activity (Riechmann et al. 1995). An involvement in replication is suspected (Klein et al. 1994; Rodríguez-Cerezo et al. 1993) because P3 is found with nuclear inclusion bodies (Langenberg and Zhang 1997)

and is able to interact with proteins of the replication complex (Guo et al. 2001; Merits et al. 1999). Over-expression of P3 is known to produce deleterious effects on cell cycles (Rodríguez-Cerezo and Shaw 1991).

Segregation ratios in plants of the B₁ and B₁S₁ generations inoculated with TuMV CDN 1 suggested the presence of two dominant resistance genes. When a larger population of B₁

TuMV isolate/ construct

P3 amino acid 153

Ile

Phe

UK 1

CDN 1

UK 1
+ C/ H630R

Fig. 2. Effect of identity of *Turnip mosaic virus* P3 amino acid 153 on the symptoms produced by *Brassica napus* line 22S.

plants was tested, the segregation was consistent with a single dominant gene, *TuRB03* (Hughes et al. in press). The TuMV UK 1 construct that mimicked CDN 1 at P3 position 153 showed the same segregation for the B₁ generation as CDN 1 but at a segregation ratio consistent with one gene in the B₁S₁ generation. A single amino acid change in the CDN 1 P3 (F153I) resulted in all B₁S₁ individuals being infected; therefore, if there is a second gene interacting with CDN 1, this suggests that it has the same mechanism of action as *TuRB03*.

Our study has identified the C-terminal half (residues 182–355) of the P3 protein of TuMV as a symptom severity determinant. This domain contained not only most of the variation in P3 amino acids between the two isolates studied, but also encompassed the avirulence determinant for the *B. napus* resistance gene *TuRB04* (Jenner et al. 2002a). Therefore, we have demonstrated a dual role for the potyviral P3 protein in both pathogenicity and recognition by the host resistance machinery. The C-terminal third of the P3 protein of another potyvirus, *Tobacco etch virus*, previously has been identified as being one of two components needed for a wilting response in tabasco pepper (Chu et al. 1997). The P3+6K1 region has also been identified as involved in host range for PPV (Dallot et al. 2001; Sáenz et al. 2000).

The P3 gene and protein are among the most variable in potyvirus genomes (Shukla et al. 1991). The extent of its varia-

tion between potyvirus species, together with the examples of involvement of P3 in symptoms and host range within a species, may be an indication that this protein is critical to the host range and, hence, even the species designation or identity of any given potyvirus.

The P3+6K1 complex previously has been identified as critical to resistance controlled by the recessive gene *sbm2* for PSbMV (Johansen et al. 2001). We now have shown that the P3 gene is involved in avirulence for the *B. napus* resistance genes *TuRB03* and *TuRB04* (Jenner et al. 2002a). It is tempting to consider that the mechanisms for each mode of resistance may be very different depending on whether controlled by a recessive or dominant resistance gene. Recessive gene resistance, if based on the lack of a plant factor required for viral replication, may be broad spectrum due to the fundamental requirement for P3 for viral pathogenicity. Dominant gene resistance based on recognition of specific motifs could be quite isolate specific due to the high amounts of sequence variation.

Viral mutations to overcome the resistance genes *TuRB04* and *TuRB05* are unstable in the absence of selection pressure (Jenner et al. 2002a). In contrast, mutations that overcome *TuRB01* are more stable but nonetheless have a slight cost to viral fitness (Jenner et al. 2002b). The mutations affecting P3 position 153 appeared to be stable in the absence of *TuRB03*

Table 2. Ability of *Turnip mosaic virus* constructs to infect parental *Brassica napus* lines and families of selfed seed (B₁S₁) from the backcross N-o-1 × (F₁ from N-o-1 × 22S)

<i>Brassica napus</i> plant line	Virus construct ^a			
	CDN 1		UK 1 C1 H630R	
	(P3 153 = F)	P3 F153I	(P3 153 = I)	P3 I153F
22S	0 (8)	+ (8)	+ _N (8)	0 (8)
N-o-1	+ (8)	+ (8)	+ _N (8)	+ _N (8)
B ₁ S ₁ families 1,2,5,6,7,8	+ (48)	+ (48)	+ _N (48)	+ _N (48)
B ₁ S ₁ families 3,4,9,10,11,12,13,14,15,16,17,18,19,20	0 (94), + (18)	+ (112)	+ _N (112)	0 (78), + _N (34)

^a 0 = No symptoms observed, no viral replication detected by enzyme-linked immunosorbent assay; +_N = systemic necrotic infection; + = systemic nonnecrotic infection. Numbers of plants with phenotype in brackets.

Fig. 3. Symptoms induced by *Turnip mosaic virus* isolates CDN 1 and UK 1 and the chimeric construct vVIR156 (UK 1 possessing the C-terminal half of CDN 1 P3) on the susceptible propagation host *Brassica juncea* cv. Tendergreen. A, UK 1; B, vVIR156; C, CDN 1.

selection pressure, at least in the short term and in the different virus isolate backgrounds. The effect on viral fitness has not yet been examined.

We have now identified the viral avirulence factors involved in the interaction with four *B. napus* resistance genes. *TuRB03* is on the same chromosome as *TuRB01*, but the two genes interact with different parts of the viral genome (P3 and CI, respectively). Both *TuRB03* and *TuRB04* interact directly or indirectly in some way with the P3 protein, whereas *TuRB01* and *TuRB05* interact with the CI protein: the TuMV avirulence determinants for brassica resistance genes appear to be clustering in the P3/CI region of the viral genome. Our understanding of the basis of the *B. napus* pathotyping system (Walsh 1989) is increasing; however, as yet, we do not understand anything of the biochemical basis underlying the recognition processes.

MATERIALS AND METHODS

Virus isolates and plant lines.

The TuMV isolates UK 1 and CDN 1 have been described and characterized previously (Jenner and Walsh 1996). The isolate UK 1 was used to construct an infectious cDNA clone, p35Tunos (Sánchez et al. 1998), GenBank accession no. AF169561. All viruses were maintained by mechanical inoculation in *B. juncea* (mustard) cv. Tendergreen as described previously (Jenner and Walsh 1996).

The *B. napus* line 22S is resistant to TuMV CDN 1 and is susceptible to UK 1. *B. napus* line N-o-1 is a doubled-haploid line derived from cv. Westar (Sharpe et al. 1995) and is resistant to TuMV UK 1 but susceptible to CDN 1 (Walsh et al. 1999) and v35Tunos +5570 A>G (*CI* H630R) (Jenner et al. 2000). The *B. napus* lines R4 and 165 were described previously (Walsh 1989).

B. napus line N-o-1 was crossed with line 22S to generate F₁ plants, then a single F₁ plant was back-crossed to line N-o-1. In all, 20 B₁ plants were self-pollinated to create 20 B₁S₁ families. Each family was assessed for segregation of phenotypes by inoculation of eight plants with each viral construct as described previously (Jenner et al. 2002a).

Molecular genetic techniques.

Reverse-transcription (RT)-PCR was performed on virus in plants as described previously (Jenner et al. 2000). Sequences of plasmids and PCR products were obtained as described previously (Jenner et al. 2002a). Sequences of oligonucleotide primers used in this study are given in Table 3. Cloning and manipulation of plasmids were performed using standard molecular genetic techniques.

Production of infectious clone of TuMV CDN 1.

The *Cauliflower mosaic virus* 35S promoter region from p35Tunos was isolated by PCR using the primers T62 and T63. A cDNA copy of the 0.4 kb at the 5' end of the CDN 1 genome was obtained from infected mustard leaf by RT-PCR using the primers T64 and T65. The two products were combined as the template for overlap extension PCR (Horton et al. 1989) with the primers T62 and T65. The resultant 1.1-kb hybrid product was cloned into pMOSBlue (Amersham, Little Chalfont, U.K.). A cDNA copy of the majority of the CDN 1 genome was obtained by immunocapture RT-PCR (Romero et al. 1997) using the *TaqPlus Long* PCR System (Stratagene, Cambridge, U.K.) with the primers Tu240 and Tu9669. The resultant 9.4-kb fragment was cloned into pCR2.1-TOPO (Invitrogen, Paisley, U.K.). RT-PCR using the primers T1 and P3S produced a cDNA copy of the 3' 1.5 kb of the genome, which was cloned into pMOSBlue. The full-length infectious clone of CDN 1 (pVIR95) was produced by substitution of the three sections

Table 3. Oligonucleotide primers used in this study

Name	Sequence (5'-3') ^a	Sense
P3S	GCGTCGACTAGTACTCGAGTTT	-
Tu240	CCACCGTCACACAGTCG	+
Tu9669	TATTTCCATAAGCGAGAATACTAAC	-
T1	CTGGAACCTGAGCGAATAGTATCG	+
T9	GGTGGGACGTCCTTTGGTAAC	-
T50	GAGCTTGCGGATGGTGATACA	+
T51	GCGCCTGCGACAACGTCT	-
T62	GCGCGTTGGCCGATTTCAT	+
T63	CGTTTTGTGTATGTTGTGTTGAGTTTAT	-
T64	ATAAAACTCAACACAACATACAAAAAC	+
T65	GCATCCTCAACACGGCACTC	-
T78	TGCGTAACAACAGAGTCAGGAGAT	+
T79	ACTTCCGTGGCTGTTGCTCTGG	-
T80	ACAGCCACGGAAGTAGCGGGCAAG	+
T81	TTGAGGTTACCGTTTGAAGTACT	-
T82	AACGGTAACCTCAAATTGTGTTTC	+
T83	GTGGCTCTCTAGAGCACTTGATGAACCATTACATC	+
T84	GATGTAATGGTTCATCAAGTCTCTAGAGAGCCAC	-
T85	CCATTACATCAGGGCGGACAGCAACGTCG	+
T86	CGACGTTGCTGTCCGCCCTGATGTAATGG	-
T87	CGAAGCAAAGGCTAATATTAATGGGCCAGATGACG	+
T88	CGTCATCTGGCCCATTAATTAGCCTTTGCTTCG	-
T89	GGCAGAACCAAACTATGAGCTTGCGGATGGTGATAC	+
T90	GTATCCACCATCCGCAAGCTCATAGTTTGGTTCTGCC	-
T91	CCAAACTGGGAGCTTGCGAATGGTGATACAC	+
T92	GTGTATCCACCATTCGCAAGCTCCCAGTTTGG	-
T93	GGATGGTGATACACATTCTGAGGGATCATAGC	+
T94	GCTATGATCCCTCAGAAATGTGTATCCACCATCC	-
T95	GATTGTCTCTAGCTTGGTGGACT	+
T96	CAAGCTAAGAGACAATCCGAGCAAGAAC	-
T97	CGAACGGCGGATATACAATTCTGAGAGATCACAG	+
T98	CTGTGATCTCTCAGAAATGTATATCCGCCGTTTCG	-

^a Underlined base indicates introduction of mutation and italicized bases indicate introduction of a restriction enzyme site.

into p35Tunos: a 0.7-kb *Sma*I-*Ppu*MI fragment (35S promoter and 5' end of viral genome), a 8.2-kb *Ppu*MI-*Mlu*I fragment (main section), and a 1.3 kb *Mlu*I-*Sal*I fragment (3' end).

The CDN 1 virus and plasmid pVIR95 were sequenced (GenBank accession nos. AB093610 and AY227024, respectively) as above. Virus derived from the plasmid construct behaved in an identical manner to the original isolate CDN 1 on the *B. napus* lines.

Construction of chimeric viruses.

Each of the three segments used in the production of pVIR95 was exchanged individually with the corresponding region in p35Tunos. The main 8.2-kb section was divided using conserved restriction enzyme sites to make reciprocal exchanges between p35Tunos and pVIR95 of the 1.8-kb *Ppu*MI-*Xcm*I, 4.6-kb *Xcm*I-*Bst*EII, and 1.8-kb *Bst*EII-*Mlu*I fragments (Fig. 1A).

The 4.6-kb *Xcm*I-*Bst*EII section of pVIR95 (present in pVIR112; Fig. 1B) was divided further by the introduction of a *Btg*I and an internal *Bst*EII site corresponding to sites in the p35Tunos sequence. This was achieved by PCR using primers containing the novel sites (T78 with T79, T80 with T81, and T82 with T9), followed by cloning into pMOSBlue. Exchanges then were made using the resultant 1.9-kb *Xcm*I-*Btg*I, 1.5-kb *Btg*I-*Bst*EII, and 1.2-kb *Bst*EII-*Bst*EII fragments.

The 1.9-kb *Xcm*I-*Btg*I section of pVIR95 (present in pVIR117) was manipulated further in subclones before being returned to the full-length plasmid. A 0.4-kb *Xcm*I-*Bst*98I fragment was replaced by the corresponding UK 1 fragment without affecting the 22S phenotype (pVIR121). The 1.5-kb *Bst*98I-*Btg*I fragment of pVIR121 was dissected further by the replacement of either the 0.6-kb *Bst*98I-*Eco*47III or the 0.9-kb *Eco*47III-*Btg*I portions by the corresponding UK 1 fragments to create pVIR124 and pVIR125.

The 0.6-kb *Bst*98I-*Eco*47III fragment of pVIR124 was split by the replacement of the 0.2-kb *Bst*98I-*Bbs*I and 0.4-kb *Bbs*I-*Eco*47III segments by the equivalent UK 1 fragments, creating pVIR135 and pVIR136.

The 0.9-kb *Eco*47III-*Btg*I fragment of pVIR125 was subdivided by overlap extension PCR using the primers T50 with T95 on pVIR125, the primers T96 with T51 on p35Tunos, and then the primers T50 with T51 on a mix of the two previous products, to create pVIR156.

Site-directed mutagenesis was performed on subclones of the UK 1 and CDN 1 genomes using the Stratagene QuikChange mutagenesis kit according to the manufacturer's instructions. Each codon was altered with the minimum of nucleotide substitutions to achieve the desired amino acid. In the case of introducing CDN 1 amino acid residues into UK 1, this entailed three of the six codons not matching the original CDN 1 codon due to redundancy in the third position of the codons. The alterations Y65H, V73A, V118I, W144Y, D148N, and I153F were achieved using the oligonucleotide combinations T83-T84, T85-T86, T87-T88, T89-T90, T91-T92, and T93-T94, respectively. The only UK 1 amino acid change engineered into the CDN 1 P3 protein, F153I, was achieved using the oligonucleotide combination T97-T98.

Plasmid DNA was purified by alkaline lysis followed by passage through a QIAGEN-tip 100 (Qiagen, Crawley, U.K.). Mustard plants were dusted with Carborundum, and then 10 µg of plasmid was rubbed onto each plant to produce viral infection. Infectious plasmid constructs are denoted using the prefix p; virus derived from infection of plants with these plasmids is designated with the prefix v.

Assessment of infection of plants.

B. napus plants were mechanically inoculated as described previously (Jenner and Walsh 1996). Symptoms were assessed

visually for 4 weeks. Presence of TuMV was verified by plate-trapped antigen ELISA as described previously (Jenner et al. 2000) using the monoclonal antibody EMA67 (Jenner et al. 1999). The identity of virus present, and in particular the presence of introduced mutations, was verified by RT-PCR and sequencing of the appropriate part of the genome.

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