An important determinant of the ability of *Turnip mosaic virus* to infect *Brassica* spp. and/or *Raphanus sativus* is in its P3 protein

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### INTRODUCTION

Systemic infection of plants occurs when a virus establishes genome amplification, then movement cell-to-cell and over long distances via the plasmodesmata and phloem cells (Carrington *et al*., 1996; Lucas & Gilbertson, 1994). The factors that influence virus host range, symptomatology and/or pathogenicity have been studied using various virus mutants as well as recombinants constructed from closely related viruses (Rao, 1999). In many cases these factors involve one or more proteins involved in virus replication and/or transport, including coat protein (CP), movement protein and/or other proteins conferring the function necessary for virus movement.

The genus *Potyvirus* contains more than 200 members or possible members and belongs to the largest plant virus family, *Potyviridae* (Berger *et al*., 2000). Potyviruses have filovirus filamentous particles that contain an approximately 10 kb positive-sense single-stranded RNA that is covalently linked to a virus genome-linked protein (VPg) at the 5′ end and polyadenylated at the 3′ end (Revers *et al*., 1999). The RNA has a single open reading frame that is translated into a large polyprotein, which is proteolytically cleaved into mature proteins by three virus-encoded proteinases (Riechmann *et al*., 1992; Urcuqui-Inchima *et al*., 2001).

The potyvirus P3 protein is proteolytically cleaved from polyprotein by HC-Pro and Nla-Pro, resulting in either the P3 protein or its precursor P3-6K1 (Riechmann *et al*., 1992). Among potyviruses there is relatively little similarity in P3 proteins compared to other proteins (Urcuqui-Inchima *et al*., 2001). The P3 proteins are thought to be involved in virus replication (Merits *et al*., 1999), accumulation (Klein *et al*., 1994), symptomatology (Chu *et al*., 1997; Sáenz *et al*., 2000), resistance breaking (Hjulsager *et al*., 2002; Jenner *et al*., 2002, 2003; Johansen *et al*., 2001) and cell-to-cell movement (Dallot *et al*., 2001; Johansen *et al*., 2001).

*Turnip mosaic virus* (*TuMV*, genus *Potyvirus*, family *Potyviridae*) infects mainly cruciferous plants. Isolates Tu-3 and Tu-2R1 of TuMV exhibit different infection phenotypes in cabbage (*Brassica oleracea* L.) and Japanese radish (*Raphanus sativus* L.). Infectious full-length cDNA clones, pTuC and pTuR1, were constructed from isolates Tu-3 and Tu-2R1, respectively. Progeny virus derived from infections with pTuC induced systemic chlorotic and ringspot symptoms in infected cabbage, but no systemic infection in radish. Virus derived from plants infected with pTuR1 induced a mild chlorotic mottle in cabbage and infected radish systemically to induce mosaic symptoms. By exchanging genome fragments between the two virus isolates, the P3-coding region was shown to be responsible for systemic infection by *TuMV* and the symptoms it induces in cabbage and radish. Moreover, exchanges of smaller parts of the P3 region resulted in recombinants that induced complex infection phenotypes, especially the combination of pTuC-derived N-terminal sequence and pTuR1-derived C-terminal sequence. Analysis by tissue immunoblotting of the inoculated leaves showed that the distributions of P3-chimeric viruses differed from those of the parents, and that the origin of the P3 components affected not only virus accumulation, but also long-distance movement. These results suggest that the P3 protein is an important factor in the infection cycle of *TuMV* and in determining the host range of this and perhaps other potyviruses.
and the many TuMV isolates in the world have been classified into several strains or pathotypes (Fujisawa, 1990; Green & Deng, 1985; Jenner & Walsh, 1996; Provvidenti, 1980; Stavalone et al., 1998; Stobbs & Shattuck, 1989). Recently, many TuMV isolates have been collected around the world and grouped into four lineages by sequencing and phylogenetic analysis of sequences encoding P1 and CP (Ohshima et al., 2002), CP (Sánchez et al., 2003), or the entire polyprotein (Tomimura et al., 2003). These classifications were based on host range, but the factors affecting the virus host range in cruciferous plants are not clear.

In this study we used two Japanese TuMV isolates, Tu-2R1 and Tu-3. Tu-2R1 can systemically infect not only Japanese radish (Raphanus sativus L.) but also Brassica spp., although Tu-2R1 induces very mild symptoms in cabbage (Brassica oleracea L.). On the other hand, Tu-3 infects Brassica spp. but not radish. To identify the genetic determinant that plays a role in systemic infection and symptomatology of TuMV in cabbage and radish, we constructed two infectious full-length cDNA clones and a series of chimeric viruses in cabbage and radish showed that the determinant defining the differential infection phenotype is the P3 protein.

**METHODS**

**Virus isolates.** TuMV isolates Tu-2R1 and Tu-3 were used in this study. Tu-2R1 was isolated from field-grown Japanese radish (R. sativus) and was propagated in turnip (Brassica rapa subsp. rapa) cv. Fuyutoyo (Sakata Seed Co., Yokohama, Japan). Tu-3 was isolated from a diseased cabbage (B. oleracea var. capitata) and maintained in cabbage cv. Haruhikari No. 7 (Takii Seed Co. Ltd, Kyoto, Japan).

**Construction of TuMV infectious full-length cDNA clones.** Four cDNA fragments were amplified separately to construct full-length infectious clones of TuMV by immunocapture–reverse transcription–polymerase chain reaction (IC-RT-PCR) (Table 1, fragments 5'-S, S-B, B-X, X-3'). These cDNA fragments were cloned between a Cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthetase (NOS) terminator in a pUC-based plasmid vector modified from pBl121 (Clontech). First, 5'-S fragments were blunt-ended at the 5' terminus and ligated, without non-virus nucleotides, downstream of the CaMV 35S promoter. In a second step, B-X (position 6130–8435) and X-3' (position 8435–poly(A)) fragments were combined at a XhoI site, the resulting fragments were inserted upstream of the NOS terminator, and finally the S–B fragment was digested with Sall and BamHI, and inserted between the 5'-S and B-3' fragments. To confirm the nucleotide sequence of the clones, named pTuR1 and pTuC, plasmids were digested with a suitable restriction enzyme, subcloned and sequenced.

Almost all recombinant clones between pTuR1 and pTuC were prepared using unique corresponding restriction enzyme sites including Sall (position 2491), SmaI (position 3305) and BamHI (position 6130) in virus cDNA, KpnI upstream of the CaMV 35S promoter, and SmaI downstream of the NOS terminator. The Sall (position 2491) site in pTuR1 was created in oligonucleotide primers (Table 1, Tu-2460F2 and Tu-2480R) used in IC-RT-PCR and had no coding effect.

**Virus inoculation and detection.** TuMV cDNA constructs were inoculated to 3-week-old seedlings of turnip cv. Fuyutoyo by the Helios Gene Gun System (Bio-Rad). The bombardment conditions were: microcarrier (gold particles), 0–6, 1–30 μm in diameter; microcarrier loading quantity, 1 mg per shot; DNA loading ratio, 2–0 μg (mg gold)−1, 2–0 μg per shot; final concentration of polyvinylpyrrolidone (PVP), 0–5 mg (ml ethanol)−1; helium pressure 180–200 p.s.i.

At 3 weeks post-inoculation (p.i.) the upper leaves of individual turnip plants showing a systemic mosaic were ground in phosphate

**Table 1. Oligonucleotide primers used to amplify Tu-2R1 and Tu-3 cDNA**

<table>
<thead>
<tr>
<th>Fragments</th>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
<th>Positions</th>
<th>Sense*</th>
</tr>
</thead>
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<tr>
<td>5'-S</td>
<td>Tu-5'-1F</td>
<td>AAAATATAAAAACTACACAACACATA</td>
<td>1–27</td>
<td>Tu-2R1 (+)</td>
</tr>
<tr>
<td></td>
<td>TuMV5'-F</td>
<td>GGCAGCTTTAAAAATATAAAAACTC</td>
<td>1–16</td>
<td>Tu-3 (+)</td>
</tr>
<tr>
<td></td>
<td>Tu-2480R</td>
<td>GTCATGATCTCGAGTCATGCTCAGT</td>
<td>2483–2506</td>
<td>Tu-2R1, Tu-3 (−)</td>
</tr>
<tr>
<td></td>
<td>Tu-2460F2</td>
<td>GTTTAGATCTCGAGTCATGCTCAGT</td>
<td>2483–2509</td>
<td>Tu-2R1 (+)</td>
</tr>
<tr>
<td></td>
<td>Tu-6343R</td>
<td>CTGAAGGGATATGTTGTTGTTGTCAGATCCACC</td>
<td>6312–6343</td>
<td>Tu-2R1 (−)</td>
</tr>
<tr>
<td></td>
<td>Tu-2097F</td>
<td>GTCACACAGAATACGCCG</td>
<td>2097–2116</td>
<td>Tu-3 (+)</td>
</tr>
<tr>
<td></td>
<td>Tu-6137R</td>
<td>AGGCCATCCCAAAACGGAACAGCAAGAAATC</td>
<td>6106–6137</td>
<td>Tu-3 (−)</td>
</tr>
<tr>
<td></td>
<td>Tu-5824F</td>
<td>GTAGGGATATGTTGTTGTTGTCAGATCCACC</td>
<td>5824–5843</td>
<td>Tu-2R1, Tu-3 (+)</td>
</tr>
<tr>
<td></td>
<td>Tu-8822R</td>
<td>GCAGGTCTTCTTCTGAGCG</td>
<td>8803–8822</td>
<td>Tu-2R1, Tu-3 (−)</td>
</tr>
<tr>
<td>X-3'</td>
<td>Tu-8334F</td>
<td>GGGCCTGCAATCTGTCAGTCAGTCACGG</td>
<td>8334–8363</td>
<td>Tu-2R1 (+)</td>
</tr>
<tr>
<td></td>
<td>dT/Sac</td>
<td>TACATGACCTG(T)18</td>
<td>Poly(A) (−)</td>
<td></td>
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<tr>
<td></td>
<td>Tu-8207F</td>
<td>GTGGATACCTAGTGTGATCG</td>
<td>8207–8227</td>
<td>Tu-3 (+)</td>
</tr>
<tr>
<td></td>
<td>dT/SmaI</td>
<td>CTGGATATCCCGG(T)18</td>
<td>Poly(A) (−)</td>
<td></td>
</tr>
<tr>
<td>cDNA synthesis</td>
<td>Tu-6376R</td>
<td>GGAATCCACGAACTGTTGTC</td>
<td>6358–6376</td>
<td>Tu-2R1, Tu-3 (−)</td>
</tr>
</tbody>
</table>

*(+), Virus sense oligonucleotide; (−), complementary oligonucleotide.
buffer (0.1 M, pH 8.0) and mechanically inoculated simultaneously on cotyledons of both cabbage cv. Shikidori and Japanese radish cv. Awashinbansei. In each inoculation test four to six plants were used and the simultaneous inoculation experiments were repeated three or four times. Inoculated plants were grown separately to prevent cross-contamination. Systemic infection was determined by Western blotting analysis at 15 days p.i. The percentage infectivity values were compiled from separate inoculation tests (see Fig. 2). To confirm the validity of chimeric viruses used as inocula, restriction fragment-length polymorphism analysis of IC-RT-PCR products was carried out with restriction enzymes that could differentiate the sequences of parental viruses. Several progeny viruses in systemic leaves of inoculated cabbage and radish plants were also confirmed by sequence analysis.

Seeds of the cabbage and radish cultivars were obtained from Takii Seed Co. Ltd. All plants were grown under constant conditions in growth chambers maintained at 14 h per day photoperiod at 22 °C/19 °C (day/night).

IC-RT-PCR. PCR tubes (0.5 ml, polypropylene) were coated with 50 μl of an anti-TuMV rabbit antiserum diluted 1000-fold in phosphate-buffered saline (PBS), and were incubated at 37 °C for 1 h. After incubation the tubes were washed twice with PBS containing 0-05% Tween 20 (PBST) and 50 μl plant tissue extract ground at 1:3 (w/v) in PBST was added. Then the tubes were incubated at room temperature for 15 min in order to trap virus particles before washing twice with PBST, and 30 μl RNAse-free water was added. The tubes were incubated at 95 °C for 1 min, and cooled on ice immediately. From the resulting solution, 4 μl containing released virus RNA was used as the template for cDNA synthesis with M-MLV reverse transcriptase of the First-strand cDNA Synthesis Kit (Amersham Biosciences). cDNA was amplified by PCR using KOD-plus-DNA polymerase (Toyobo) according to the manufacturer’s protocol. Primers used in this study are listed in Table 1.

Protein analysis. Western blotting analysis of TuMV CP in the upper leaves of cabbage and radish was done using a modified protocol of Sambrook et al. (1989). Total protein extracts were tested for the presence and/or accumulation of TuMV CPs. Leaf tissue ground 1:6 (w/v) with 1×SDS-PAGE sample buffer [2% (w/v) SDS, 2% 2-mercaptoethanol, 0-05 M Tris/HCl pH 6-8] was boiled for 5 min, and centrifuged at 10,000 r.p.m. for 5 min. The supernatant fluid was mixed with 6× gel-loading dye [0-25% (w/v) bromophenol blue, 0-25% (w/v) xylene cyanol, 30% glycerol in H2O]. Samples of 8 μl were separated in a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes (Advantec) using a semi-dry trans-blotter (Nihon Eido). Membranes were then blocked for 30 min in TTBSPB [20 mM Tris/HCl pH 7-5, 500 mM NaCl (TBS) containing 0-05% Tween 20, 2% (w/v) PVP and 2% (w/v) bovine serum albumin]. The membranes were incubated for 1 h at room temperature with an anti-TuMV rabbit antiserum diluted 1:1000 in TTBSPB and washed twice before incubation with a goat anti-rabbit IgG-AP conjugate (Bio-Rad) diluted 1:3000 in TTBSPB for 1 h at room temperature. The protein–antibody complexes were visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Distributions of TuMV in cabbage and radish were analysed by tissue immunoblotting analysis (Srinivasan & Tolin, 1992). Briefly, inoculated leaves at 12 days p.i. were pressed between two pieces of 3 mm filter paper (Whatman). Residual green colour was removed from the filter by rinsing in 2% Triton X-100 prior to blocking with TTBSPB. Detection of virus CP was performed as described above.

RESULTS

Viral progeny of infectious full-length cDNA clones derived from TuMV isolates of Tu-2R1 and Tu-3 exhibit different pathogenicity in B. oleracea and R. sativus

Full-length cDNA clones of Tu-2R1 and Tu-3 (named pTuR1 and pTuC, respectively) were constructed by combining four cDNA fragments. Each clone comprised 9833 nt excluding the poly(A) tail of 18 nt, and contained a single large open reading frame encoding 3164 aa (first initiation codon positioned at 130–132 and terminal codon positioned at 9622–9624). The nucleotide and amino acid sequences of pTuR1 and pTuC were 96 and 97% identical, respectively. The nucleotide sequences of the 3’-UTRs and the amino acid sequences of 6K1 and 6K2 were 100% identical between the two clones.

Biologic inoculation with either pTuR1 or pTuC resulted in all inoculated turnip plants becoming infected. Symptoms appeared 9 to 10 days p.i. and were followed by severe mosaic symptoms.

The progeny viruses derived from pTuC or pTuR1 (hereafter denoted TuC and TuR1, respectively) in turnip plants were used as inoculum sources, and inoculated simultaneously onto cabbage (B. oleracea var. capitata) or Japanese radish (R. sativus). TuC caused systemic chlorosis and ringspots in cabbage plants (Fig. 1B1) but could not be detected in systemic tissue of radish plants (Fig. 1B2). In contrast, TuR1 infection induced mild chlorotic mottle in cabbage (Fig. 1A1) and mosaic symptoms in radish (Fig. 1A2). TuR1 caused a very mild symptom in cabbage, clearly distinguishable from the more severe symptoms provoked by TuC (Fig. 1A1 and B1). Results of Western blotting (Fig. 2) showed that the concentration of TuR1 in cabbage was approximately half that of TuC.

Exchanges of the P3 genes alter infection phenotype of TuMV

In order to map the determinants that define the host-specific infection phenotypes, a series of reciprocal chimeric clones between pTuR1 and pTuC was constructed (Fig. 2, chimeras 1 to 14). These chimeric cDNA clones were inoculated into turnip, which is a common susceptible host for the two isolates. All constructs were able to infect turnip systemically, inducing severe mosaic symptoms, and all accumulated to similar levels as determined by Western blotting (data not shown). The crude sap from turnip plants infected with either of the chimeric clones was used as an inoculum.

Initially we exchanged 6130-3’ regions of the two parental isolates containing most of N1a and all of N1b, CP and 3’-UTR (Fig. 2), because the sequences encoding host-specific virulence and/or systemic infection determinants have been shown to be in the central region of VPg in other
Chimeric virus 1 was able to infect both cabbage and radish. However, the symptoms induced in cabbage were the same as those of TuR1 infection. On the other hand, the reciprocal chimeric virus 2 exhibited a phenotype identical to that of TuC, and did not infect radish systemically. These results indicated that infectivity and symptomatology of the two isolates of TuMV in cabbage and radish did not involve the 6130-3' region.

When chimeras 3 and the reciprocal virus 4 were prepared by changing the 5'-2491 region containing all of the 5'-UTR and P1, and almost all of HC-Pro, and were then inoculated, this exchange did not alter the pathogenicity to cabbage and radish. These results indicated that the 5'-2491 regions did not play a significant role in the differential infection phenotype between TuC and TuR1, suggesting that the central region (positions 2491 to 6130) might affect host-specific infection and symptomatology of TuMV. The central region of potyvirus polyproteins has been reported to affect pathogenicity and symptomatology in specific hosts or one plant species (Chu et al., 1997; Dallot et al., 2001; Hjulsager et al., 2002; Jenner et al., 2000, 2002, 2003; Johansen et al., 2001; Sáenz et al., 2000). As expected, the progeny virus 5 caused the same host responses as TuC,

Fig. 1. Phenotypic comparison of upper leaves of cabbage (B. oleracea var. capitata, A1–G1) and radish (R. sativus, A2–G2) inoculated with the progeny viruses of TuMV infectious clones pTuR1, pTuC and relevant chimeric viruses, and mock-inoculated (G1 and G2). All leaves shown were photographed at 3–4 weeks p.i.
and the reciprocal recombinant 6 caused the same host responses as TuR1. These results showed that determinant(s) for host-specific virus infection phenotypes are located in the 2491–6130 region, presumably P3, CI and/or the N terminus of VPg. No difference in amino acid sequences was found between pTuR1 and pTuC for the C terminus of HC-Pro nor in all of 6K1 and 6K2.

Next, the 2491–6130 regions of pTuR1 C(2491–6130) and pTuC R(2491–6130) were divided by XhoI at nucleotide 3680 and four chimeric viruses TuR1C(2491–6130), TuR1C(3680–6130), TuC R(2491–3680) and TuC R(3680–6130) (Fig. 2, chimeras 7 to 10) were generated. Chimeric virus 7 had the same infection phenotype as TuR1. Similarly, chimera 8 induced a TuC infection phenotype in cabbage and radish. These

![Diagram](http://vir.sgmjournals.org)

### Fig. 2. (A) Schematic representation of parental TuMV and relevant chimeric viruses showing their systemic infectivities in cabbage and radish. Segments derived from TuR1 or TuC are depicted as solid or open boxes, respectively. Restriction sites used to generate the chimeric viruses and their positions are indicated below the map. Systemic symptoms were recorded and samples from upper leaves of each plant were collected at 15 days p.i. to be used in Western blotting with an antiserum to TuMV. CS, chlorosis and ringspots; cm, mild chlorotic mottle; M, mosaic; −, no symptoms. Inf. (%), mean percentage of plants with systemic infections detected by Western blotting. (B) Western blotting analysis of accumulation of TuMV CPs in upper leaves from cabbage and radish inoculated with parental viruses and the relevant chimeric viruses 9 to 14 at 15 days p.i.
results suggested that the 3680–6130 region, which encodes the CI protein and the N-terminal 76 amino acids of VPg, was unable to change TuMV infection phenotypes. The results of inoculation tests of chimeras 9 and 10 clearly indicated that a heterologous 2491–3680 genomic region conferred on TuC the ability of TuR1 to infect cabbage and radish (Fig. 1C1 and C2), and conferred on TuR1 the differential infection phenotype of TuC (Fig. 1D1 and D2). The 2491–3680 region encodes the C terminus of HC-Pro, all of P3 and the N terminal of 6K2, but the only differences in amino acid sequence between pTuR1 and pTuC were in the P3 gene. This suggests that the TuMV P3 gene encodes determinant(s) of host-specific infection phenotype and symptomatology in cabbage and radish.

Recombinant P3 genes cause infection different from those of either parent in Brassica and Raphanus

To locate more precisely the host-specific infection determinant in the P3 gene, the 2491–3680 region was analysed further. Chimeric virus 12, in which the C-terminal TuR1 P3 fragment of TuCR1(2491–3680) was exchanged with the corresponding fragment of TuC using the SmaI site, induced a host response of TuC-like symptoms in cabbage, but in radish infection could not be detected by Western blotting which could detect virus at concentrations about 2 to 3% of that reached in infected cabbage (data not shown). The reciprocal virus 11 induced mosaic symptoms in radish (Fig. 1E2) but no symptoms in cabbage (Fig. 1E1), in which no virus was detected by Western blotting (Fig. 2). This infection phenotype was completely different from that of either parental virus. However, a comparison of the results of two sets, chimera 9 with 11 and chimera 10 with 12, showed that the C terminus of the TuMV P3 gene has an important role in systemic infection and/or symptomatology in cabbage and radish. Chimeric virus 13, containing the TuC 3305–3680 fragment in a TuR1 background, induced the same infection phenotype as TuCR1(2491–3305) (Fig. 1F1 and F2). This result indicates that the C-terminal region of the TuC P3 gene was a crucial domain for TuC-like infection. Notably, the reciprocal chimera 14, containing TuR1 3305–3680 region in a TuC background, could not infect either cabbage or radish despite the high virus concentration reached in turnip (data not shown). The P3 genes of chimeras 11 and 14 were identical; however, neither virus was able to infect cabbage, in contrast to the parents TuR1 and TuC. It is possible that the C-terminal region of the TuR1 P3 gene alone is insufficient to induce TuR1-like infection in cabbage, and both the N and C termini of the TuR1 P3 gene are required. Although the C-terminal region of the TuR1 P3 gene played an important role in the infection and subsequent induction of symptoms by chimeras 11 and 14 in radish, it may depend on background genes other than P3. This behaviour of TuCR1(3305–3680) was different from that of other chimeric viruses (Fig. 2, chimeras 1 to 13).

Tissue immunoblotting analysis of the distribution of TuMV chimeric viruses

Because chimera viruses containing the recombinant P3 molecules can systemically infect turnip, but not cabbage and/or radish, we performed tissue immunoblotting analysis of inoculated cotyledons of cabbage and radish to visualize the extent of infection. Parental and chimeric TuMV (chimeras 9 to 14; constructs are shown in Fig. 2) were detected in both cabbage and radish cotyledons, but their distributions were different at 12 days p.i. (Fig. 3).

In cabbage, chimeric viruses 11 and 14 were restricted to smaller lesions than those induced by other viruses (Fig. 3B and J). These two viruses encoding the same P3 component were unable to infect systemically (Fig. 2), suggesting that in this chimera P3 might affect TuMV spread in inoculated cotyledons and/or long-distance movement in cabbage. The other viruses induced systemic symptoms in cabbage and did not differ in their distributions in inoculated leaves (Fig. 3A, C, D, I, K and L). These results indicated that symptom differences in systemic leaves did not result from differences in virus propagation in inoculated cotyledons.

In radish, chimeric virus 10 encoding intact TuR1 P3 was able to distribute throughout inoculated cotyledons, like TuR1 (Fig. 3H and Q). The other viruses containing TuC P3 or chimera P3 showed restricted distributions (Fig. 3E–G and N–P). However, there was a tendency for larger lesions to form when the N terminus of P3 was from TuR1 (Fig. 3G and P). On the other hand, chimeric virus 11 was able to infect radish systemically (Fig. 2) but in inoculated cotyledons localized along the line of lateral veins (Fig. 3F). When the same P3 component was in a TuC background, in chimera 14, the virus distribution was similar to those of TuC and chimera 9 (Fig. 3E, N and O). Taken together, the results suggest that the different parts of TuMV P3 protein affect systemic infection.

Amino acid sequence comparisons among TuMV P3 proteins

When amino acid residues of the P3 protein were compared between TuR1 and TuC, 37 positions were different in the 355 amino acid residues. In its C-terminal region, which is thought to contain all the information necessary for determining TuC-like infection phenotype, 15 amino acid residues were different. To narrow down the candidate sites for differential infection phenotypes, the P3 sequences of the 18 other isolates of TuMV recorded in the database were also aligned and compared using the CLUSTAL W program (Fig. 4). Information on TuMV isolates, original host, country and strain is listed in Table 2. Amino acid differences were found throughout P3, but the most variable region was located between amino acids 200 and 300, containing the site used to generate recombinant P3 (Fig. 4). In this region amino acids at positions 203, 231, 268, 279–280 and 286 (asterisks) were identical among strains.
able to infect radish (BR strain); positions 268, 279 and 280 were clearly different between BR and non-BR strains (B strain) (Fig. 4, grey shading).

DISCUSSION

The TuMV isolates TuR1 and TuC differ in host range and symptomatology in cruciferous plants belonging to different genera, *Brassica* and *Raphanus*. The results of our study show that the TuMV P3 protein is the factor determining these virus properties. Because the host ranges of most potyviruses are restricted (Hollings & Brunt, 1981), classifications of their strains or pathotypes are based on symptoms of different cultivars in inoculation tests.

In this study all chimeric viruses infected turnip and induced severe symptoms. However, as shown in Fig. 2, chimeric viruses 9 and 10, which contained heterologous *P3* genes, had the infection phenotypes of the cognate parental virus. Additional analysis of four chimeras, 11 to 14, containing *P3* genes recombined between those of TuC and TuR1, showed complicated infection phenotypes that differed from that of either parental virus. These results suggest that a component of the P3 molecule contains a key determinant of its ability to infect hosts in different genera.

Chimeric viruses 11 to 14 showed very low systemic infectivity in cabbage and/or radish (Fig. 2). Sequence analysis of virus in the systemically infected leaves did not show any amino acid mutations in the exchanged region that could have caused recovery (data not shown). The viruses incapable of full systemic infection were able to propagate in the inoculated leaves, and their distributions in cabbage and radish plants differed (Fig. 3). These facts could be explained by a model in which full systemic infection of a plant involves a race between the rates of virus replication and movement, and the rate of growth of the plant (Dawson & Hilf, 1992). An alternative explanation is the genetic non-uniformity of the cabbage and radish cultivars used in this study. The relative rate of virus spread in inoculated cotyledons was apparently unrelated to systemic infections in radish plants (Fig. 3F, G, O and P).
Fig. 4. Comparative sequences of 355 amino acids of P3 proteins from TuR1, TuC and other isolates of TuMV. Dots indicate amino acid residues identical to consensus sequence. Asterisked and grey-shaded positions indicate that amino acid residues are highly conserved among BR strains and divided into two strains (see Table 2).
Potyviruses do not encode a dedicated movement protein, but several proteins participate in virus movement functions: HC-Pro (Cronin et al., 1995; Kasschau et al., 1997; Rojas et al., 1997), CI (Carrington et al., 1998), VPg (Nicolas et al., 1997; Schaad et al., 1997) and CP (Dolja et al., 1994, 1995). It has been reported that these proteins are also involved in the ability of viruses to overcome host resistance (Hämäläinen et al., 2000; Jenner et al., 2000; Johansen et al., 2001; Nicolas et al., 1997; Schaad et al., 1997). In the yeast two-hybrid system, potyvirus proteins NLa of Pea seed-borne mosaic virus (PSbMV; Guo et al., 2001) and NLb of Potato virus A (PVA; Merits et al., 1999) have been shown to interact with P3, and in other in vitro assays other PVA proteins were found to interact with P3 (Merits et al., 1999).

Potyvirus P3 proteins play a role in virus replication (Merits et al., 1999) and accumulation (Klein et al., 1994), but although an involvement in movement has been proposed (Dallot et al., 2001; Johansen et al., 2001) it remains to be demonstrated. Our results clearly indicate that the P3 protein influences the efficiency of virus spread, especially in long-distance movement. When a combination of the N and C termini of the P3 protein was derived from TuC and TuR1, respectively, the efficiency of systemic invasion was dependent on the other parts as genomic background (Fig. 2, chimeras 11 and 14). This result suggests that the C terminus of TuR1 P3 may facilitate long-distance movement and interact with movement-associated virus proteins directly or indirectly. However, it is not possible to say whether the P3 protein affects either or both steps in replication and cell-to-cell movement, because cell-to-cell spread is the combined result of the level and rate of replication and the rate of cell-to-cell movement. To address this question, it will be necessary to examine virus replication at the single-cell level using protoplasts of cabbage and radish.

Establishment of systemic infection is a complex process, requiring a balance of the rates of replication, cell-to-cell movement and long-distance movement. In each of these phases there are interactions between virus proteins and host components. The P3 protein contains a putative integral transmembrane domain (Rodríguez-Cerezo & Shaw, 1991) and has no RNA-binding activity (Merits et al., 1998). Expression of P3 proteins can have detrimental effects on the growth of Escherichia coli (Merits et al., 1998) or plants (Moreno et al., 1998). Therefore it is reasonable to speculate that the P3 proteins of TuR1 and TuC also interact with host component(s) during the virus infection cycle. Among potyvirus–host relationships, the P3 genes have already been reported as pathogenicity or

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Original host</th>
<th>Country</th>
<th>Strain*</th>
<th>References and accession no.</th>
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<tbody>
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*Classification into two strains was according to Ohshima et al. (2002). BR strains have the ability to infect radish (R. sativus) and Brassica spp. B strains have an inability to infect radish.
resistance-breaking determinants. In Plum pox virus (PPV) the P3 gene with 6K1 influenced symptoms on systemic infection hosts *Pisum sativum* and *Nicotiana clevelandii* (Sænz et al., 2000), or infections of plum and peach, both *Prunus* species (Dallot et al., 2001). In TuMV (Jenner et al., 2002, 2003) and P5B MV (Hjulsager et al., 2002; Johansen et al., 2001) the P3 genes affected infections by virus pathotypes of *Brassica napus* and *P. sativum* expressing dominant or recessive resistance, respectively.

If our results were to be attributed to relationships between the P3 proteins of TuR1/TuC and resistance genes in cabbage/radish which mode, dominant or recessive resistance, is conceivable? Dominant resistance alleles are associated with strong mechanisms induced after a recognition event of virus infection, whereas incompletely dominant resistance alleles are associated with mechanisms to constitutively inhibit virus replication or movement (Fraser, 1992). In our study all chimeric viruses were able to multiply in inoculated cotyledons, and no hypersensitive responses were observed in inoculated and systemic leaves. The TuMV P3 protein is an avirulence determinant for an extreme form (immunity) of resistance in *B. napus* TuR803 and TuR804 (Hughes et al., 2003; Jenner et al., 2002). The amino acid positions at 153 and 312 in TuMV P3 are related to the overcoming of resistance in TuR803 and TuR804 (Jenner et al., 2002, 2003); however, in TuR1 and TuC both amino acids are the same, isoleucine and phenylalanine (Fig. 4). On the other hand, in *Arabidopsis thaliana*–potyvirus interactions, dominant genes for the blockage of virus long-distance movement have been identified for *Tobacco etch virus* (TEV) (Mahajan et al., 1998; Whitham et al., 1999; Chisholm et al., 2001) and for *Lettuce mosaic virus* (Revers et al., 2003).

Recessive resistance alleles are associated with negative effects, such as being resistant, or a non-host lacking functions essential for the virus infection cycle (e.g. replication and/or movement), or a dominant negative regulator of resistance in susceptible hosts (Fraser, 1992; Revers et al., 1999). Several avirulence determinants for recessive resistances, especially some affecting cell-to-cell or long-distance movement functions of potyviruses, have been identified in *P. sativum* as the P3 of PSbMV (Johansen et al., 2001), and in several solanaceous species as the VPg of TEV (Schaad et al., 1997), PVA (Hamalainen et al., 2000) and *Tobacco vein mottling virus* (Nicolas et al., 1997). Thus, although the potyvirus P3 has been reported to be involved in a gene-for-gene relationship with both dominant and recessive resistance genes, no genetic analysis is available to explain the interactions between TuR1/TuC P3 and host components (genes). However, this does not exclude the possibility that both dominant and recessive resistances are involved in controlling TuMV host range.

Comparisons among the amino acid sequences of P3 proteins of 20 TuMV isolates identified uniform amino acid residues in BR strains able to infect radish (Fig. 4). These are asterisked in Fig. 4 (positions 203, 231, 268, 279–280 and 286) within a most variable region encompassing a site used to construct recombinant P3. These conserved amino acids are not all markedly different between TuR1 and TuC. However, intact TuR1 P3 protein is required for the induction of a TuR1-like infection phenotype (Fig. 2, chimeras 9 to 14), suggesting that these six amino acids might be a core of a determinant for full systemic infection in radish. On the other hand, positions 268 and 279–280 in the C-terminal region of P3 (Fig. 4, grey shading) clearly divided the 20 viruses into two patterns, suggesting that these amino acids might play an important role in characterizing the TuC-like infection phenotype.

A few relevant analyses of potyvirus host-range limitation have been reported. Sænz et al. (2002) suggested that HC-Pro might be a factor for controlling the host range of PPV, based on the result that transgenic tobacco plants expressing HC-Pro of TEV were able to complement long-distance movement of PPV. In another study, Tóbias et al. (2001) constructed hybrid viruses by replacing the CP gene in an infectious PPV clone by that of *Zucchini yellow mosaic virus*, and indicated that CP genes had no effect on host range. It seems likely from our results that the P3 gene is one of the important determinants of potyvirus host range in different genera.

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