Turnip yellow mosaic virus isolates with experimentally produced recombinant virion proteins

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The consensus secondary structure of the virion proteins (VPs) of tymoviruses was predicted from their amino acid sequences using a combination of computer methods: profile alignment, hydrophobicity moment and ‘PredictProtein’. All methods predicted that they were eight-stranded anti-parallel β-barrels with two α-helical regions. The predicted structure was used to design recombinants of turnip yellow mosaic virus (TYMV) in which selected parts of its VP were replaced with homologous regions of belladonna mottle virus (BeMV) in a cDNA clone encoding the genome of TYMV. Six of ten such recombinants were fully viable and most gave symptoms in Chinese cabbage indistinguishable from those of TYMV, although they did not always infect plants systemically and none infected hosts of BeMV or of other tymoviruses. A TYMV recombinant with the N-terminal part of its VP replaced with the E71 epitope of Plasmodium falciparum was also viable, but others with the same region replaced with the V3 region of the lentivirus human immunodeficiency virus type 1 were not. Epitope analysis of antisera prepared against the virions of parental TYMV and some of the recombinants showed that, although the N terminus of the VP is immunogenically dominant, it is not exposed at the surface of the virion, a finding confirmed by comparing the electrophoretic mobilities of the virions. The recently published structure of the TYMV VP determined by X-ray crystallography confirms the accuracy of the predicted secondary structure of the VP, and hence the utility of the methods used.

Introduction

Turnip yellow mosaic virus (TYMV) is the type species of the tymoviruses (Gibbs, 1994), a genus of which at least 20 species are known. They infect a wide range of dicotyledonous plants, although most individual tymoviruses only infect the species of a single family or a few closely related families (Guy et al., 1984). For example, TYMV only infects species of the Brassicaceae and two closely related families, causing a bright yellow and green mosaic and mottling (Brunt et al., 1996). By contrast, belladonna mottle tymovirus (BeMV) mostly infects species of the Solanaceae, but not Brassicaceae.

TYMV has isometric virions approximately 30 nm in diameter. Their genomes are messenger-sense single-stranded RNA with three open reading frames (ORF) which encode the replicase protein (194–210 kDa), the movement protein (48.5–82 kDa) and the virion protein (19.6–215 kDa) (Fig. 1a). The virion protein (VP) gene is transcribed from a

![Fig. 1. (a) Genome map of TYMV-BL. ORF-1 encodes the replicase protein, ORF-2 encodes the movement protein and ORF-3 encodes the virion protein. (b) Plasmid map of pBL-16, the cDNA clone of TYMV-BL, used to construct the recombinant virion protein genes. A PstI–XbaI fragment containing the virion protein gene was subcloned for the construction of the recombinants.](https://example.com/fig1.png)
subgenomic messenger RNA (Morch et al., 1988). It was recently shown that the VP is dispensable for cell-to-cell spread, but is required for systemic movement in plants (Bransom et al., 1995).

Early immunochemical studies of TYMV virions and isolated VP showed that the protein had three antigenic determinants (Pratt et al., 1980; Quesniaux et al., 1983a; b) including the N terminus which, it was concluded, was exposed on the surface of the virions. Cross-linking methods also identified three regions of the VP in close contact with the viral RNA (Ehresmann et al., 1980).

Electron microscopy has shown the protein shell of the virion to be a \( T = 3 \) icosahedron of 180 molecules of the VP (Matthews, 1991), and reports of preliminary X-ray crystallographic studies of virions of TYMV (Canady et al., 1995; Klug et al., 1957; Klug & Finch, 1960) and erylsvirus latent tymovirus (ErLV; Colman et al., 1980; J. Varghese, personal communication) indicated they were \( \beta \)-barrel proteins. Furthermore, computer analyses of the TYMV VP amino acid sequence (Argos, 1981) also indicated that it was predominantly \( \beta \)-stranded. Thus, when the work reported in this paper was started, the structure of the TYMV VP was not known, but was predicted to be similar to that of the VP of southern bean mosaic, tomato bushy stunt and related viruses. These predictions were verified when the three-dimensional structure of the TYMV VP, determined by high-resolution X-ray crystallography, was reported by Canady et al. (1996).

In the studies reported in this paper, several computer methods and a large set of tymovirus VP sequences were used to predict a consensus secondary structure of tymovirus VPs. Based on the predicted structure, TYMV/BeMV VP recombinants were constructed \textit{in vitro} to test, in particular, whether the VP influences the host range of the virus. Selected parts of the VP gene of the cDNA clone of the Blue Lake isolate of TYMV (TYMV-BL) were replaced with the corresponding VP gene of the cDNA clone of the Blue Lake isolate of TYMV (TYMV-Blue Lake) and TYMV-Rothamsted isolates and kennedya yellow mosaic virus-Port Douglas isolate (all A. M. Mackenzie and others, unpublished data), erylsvirus latent tymovirus (Sniffa et al., 1990), eggplant mosaic virus (Osorio-Keese et al., 1989), kennedya yellow mosaic-ERVIS Bay isolate (Ding et al., 1990a), ononis yellow mosaic virus (Ding et al., 1989), and the Club Lake isolate of TYMV (Keese et al., 1989). Second, the ‘profile’ predictions were confirmed, in part, by calculating hydrophobicity moment plots (Eisenberg et al., 1984) for each of the VP sequences above, and additionally the VP sequences of BeMV (Ding et al., 1990b), kennedya yellow mosaic virus-Bawley Point isolate and wild cucumber mosaic virus (A. M. Mackenzie and others, unpublished data), using a program written for this purpose by John Armstrong and the hydrophobicity values of Kyte & Doolittle (1982). Finally, predictions of the secondary structure and accessibility to solvation were made using the PredictProtein internet server program (Predictprotein@emb-biHeidelberg.de) of Rost et al. (1994); all the tymovirus VP sequences listed above were analysed along with those of okra mosaic virus and TYMV cauliflower isolate (A. M. Mackenzie and others, unpublished data), physalis mottle virus (Jacob et al., 1992) and the type isolate of TYMV (Morch et al., 1988). The sequence encoding the E71 epitope of the major merozoite surface antigen of \textit{Plasmodium falciparum} was that reported by Peterson et al. (1988), and the sequence encoding part of the V3 loop of the gp120 protein of HIV-1 was the consensus of all nine subtypes (Korber, 1994). The isoelectric points of the parental and recombinant VP peptides were determined using the ‘Isoelectric’ program of the GCG package.

- **Primers.** Primers used for \textit{in vitro} mutagenesis, PCR and sequencing were synthesized in an Applied Biosystems DNA synthesizer at the Australian National University's Biomolecular Resource Facility.

- **Preparation and testing of recombinants.** The Promega ‘Altered Sites’ \textit{in vitro} Mutagenesis Kit was used to construct the TYMV/BL/BeMV VP recombinants and the TYMV/BL/E71 recombinant. A 900 bp \textit{PstI–Xbal} fragment containing the VP gene was excised from pBL-16 (Fig. 1b) and cloned into the pSELECT-1 vector and the manufacturer’s instructions were followed. Inverse PCR was used to insert the V3/1 or the V3/2 sequence at the 5’ end of the TYMV/BL VP gene using standard techniques (Sambrook et al., 1989). VP genes containing the altered sequences were sub-cloned back into pBL-16, and the resulting recombinants were checked by restriction enzyme analysis and sequencing before being transcribed into RNA for inoculation on to plants.

- **RNA transcription.** Five \( g \) of each plasmid containing the full-length cDNA clone of pBL-16 encoding the altered VP gene was linearized at the 3’ end of the viral cDNA with \textit{Ndel}. RNA was then transcribed at 37 °C using T7 RNA polymerase as described by Titus (1991). The RNA pellet was resuspended in sterile water for inoculation on to Chinese cabbage plants. Transcribed RNA was examined by electrophoresis through a 3:5% polyacrylamide–7 M urea gel to verify that it was of the correct size (6-3 kb) using TYMV/BL viral RNA as a control. Transcripts were inoculated manually to the first two true leaves of Chinese cabbage plants which had been kept in the dark for 24 h and then lightly dusted with carborundum powder before inoculation. RNA was also transcribed from 5 \( g \) linearized pBL-16 as a positive control.

- **Isolation of virions from infected leaves.** Virions were isolated as described by Keese et al. (1989) from fresh or frozen Chinese cabbage

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**Methods**

- **Virus isolates.** The recombinants were prepared as described below using a cDNA clone (pBL-16) encoding the genome of TYMV-BL (Skotnicki et al., 1992).

- **Computer analyses and gene sequences.** Sequences were recorded and analysed using the Staden programs (Staden, 1984). The secondary structures of the VPs of 11–18 different tymovirus species were predicted by three different methods, and consensus predictions were compared.

  First, the PROFILE and PROFILEGAP programs (Gribskov et al., 1987) of the GCG package (Wisconsin Package, Version 8; 1994) were used; the ‘profile’ used was the structure-based alignment of the VP sequences of turnip crinkle carmovirus, carnation mottle carmovirus, tomato bushy stunt tombusvirus and southern bean mosaic sobemovirus from Fig. 6 of Carrington et al. (1987). The tymovirus VP gene sequences examined were those of Andean potato latent virus, clitoria yellow vein virus, dulcamara mottle virus, TYMV-Blue Lake and TYMV-Rothamsted isolates and kennedya yellow mosaic virus-Port Douglas isolate (all A. M. Mackenzie and others, unpublished data), erylsvirus latent tymovirus (Sniffa et al., 1990), eggplant mosaic virus (Osorio-Keese et al., 1989), kennedya yellow mosaic-ERVIS Bay isolate (Ding et al., 1990a), ononis yellow mosaic virus (Ding et al., 1989), and the Club Lake isolate of TYMV (Keese et al., 1989). Second, the ‘profile’ predictions were confirmed, in part, by calculating hydrophobicity moment plots (Eisenberg et al., 1984) for each of the VP sequences above, and additionally the VP sequences of BeMV (Ding et al., 1990b), kennedya yellow mosaic virus-Bawley Point isolate and wild cucumber mosaic virus (A. M. Mackenzie and others, unpublished data), using a program written for this purpose by John Armstrong and the hydrophobicity values of Kyte & Doolittle (1982). Finally, predictions of the secondary structure and accessibility to solvation were made using the PredictProtein internet server program (Predictprotein@emb-biHeidelberg.de) of Rost et al. (1994); all the tymovirus VP sequences listed above were analysed along with those of okra mosaic virus and TYMV cauliflower isolate (A. M. Mackenzie and others, unpublished data), physalis mottle virus (Jacob et al., 1992) and the type isolate of TYMV (Morch et al., 1988). The sequence encoding the E71 epitope of the major merozoite surface antigen of \textit{Plasmodium falciparum} was that reported by Peterson et al. (1988), and the sequence encoding part of the V3 loop of the gp120 protein of HIV-1 was the consensus of all nine subtypes (Korber, 1994). The isoelectric points of the parental and recombinant VP peptides were determined using the ‘Isoelectric’ program of the GCG package.

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- **Isolation of virions from infected leaves.** Virions were isolated as described by Keese et al. (1989) from fresh or frozen Chinese cabbage
leaves 3 weeks post-inoculation. Purified virions were resuspended in TE pH 7.0 (10 mM Tris–HCl pH 7.0; 1 mM EDTA) with 1 mM sodium azide and stored at 4 °C. Virion preparations were examined in a Hitachi 6000 transmission electron microscope after negative staining with 3% ammonium molybdate.

Testing for infection using RT–PCR. RNA was extracted from leaf discs as described by Verwoerd et al. (1989). First-strand cDNA was synthesized using AMV reverse transcriptase (Promega) with an appropriate primer. DNA encoding the VP gene was then amplified using specific primers and sequenced to confirm the presence of the altered VP gene.

Preparation of polyclonal antisera and Western blotting. Polyclonal antisera were raised in New Zealand White rabbits by injection of purified virions. Total protein was extracted from infected and control plant leaves for analysis by Western blotting (Sambrook et al., 1989).

SPOTS epitope analysis. The SPOTS epitope analysis kit (Cambridge Research Biochemicals) was used to synthesize overlapping peptides covering the entire TYMV-BL virion protein. Each peptide was ten amino acids long and overlapped its preceding neighbour by eight. The peptides were synthesized as recommended by the manufacturer and then probed with several polyclonal rabbit antisera.

Electrophoretic mobility analysis. Virions were electrophoresed through a 1% agarose gel in 100 mM Tris–HCl, 25 mM EDTA pH 7.0, at 100 V for 2–3 h. The gel was stained with Coomassie blue and destained in a methanol–acetic acid solution to visualize the virions.

Results

Secondary structure prediction

When this study was started the structure of tymovirus VPs was unknown. Therefore, an attempt was made to define the positions of the main secondary structural elements of the tymovirus VPs using three different computer methods. First, a structural alignment of four viruses whose VP had been shown to be eight-stranded anti-parallel β-barrels by X-ray crystallography (Carrington et al., 1987) was used as a profile with which the tymovirus VPs were then aligned one at a time. It was found that the central two-thirds of the profile, where the profile sequences were aligned by the β-strands, consistently aligned all 11 tymovirus sequences in the same position. There were ambiguities only at the N and C termini of the profile in the alignment of different tymovirus sequences and then by three positions at most. The secondary structure predictions were then mapped on the aligned tymovirus VP sequences, and a consensus was generated (Fig. 2). The predicted consensus VP structure had all ten β-strand regions found in the cores of the profile proteins, but only two of their four surface α-helical regions. Second, using the ‘hydrophobicity moment’ method of Eisenberg et al. (1984), 14 VP sequences were examined. All VP sequences gave closely similar plots, verifying all the β-barrel structure elements identified by the ‘profile’ method, but there was only a single α-helix, just to the N-terminal side of the zB helix between βE and βF predicted by the ‘profile’ method. Finally, the sequences were examined by the PredictProtein program (PP; Rost et al., 1994). All 18 tymovirus VP sequences gave closely similar results, which were in agreement with the results of the other methods. In particular, they supported the likely position of the zB helix found by the hydrophobicity moment method, rather than that found by the ‘profile’ method. χ² tests showed that the predictions of Argos (1981), and those obtained using the ‘profile’ and PP methods were all significantly correlated (P < 0.001), although the last two sets of results correlated most closely.

Canady et al. (1996) have reported the structure of the TYMV VP resolved to 3.2 Å. The accuracy of the structure prediction methods that had been used could therefore be tested. The ‘profile’ method proved to be most accurate; it correctly assigned 15 of the 20 amino acids in the two α-helical regions compared with the next best, eight by PP, and similarly, correctly assigned 58 of the 71 amino acids in β-strands compared with 40 by Argos (1981) and 38 by PP.

Construction and characterization of recombinants

Selected regions of the TYMV VP gene sequence were replaced with corresponding parts of the BeMV VP gene sequence or ‘foreign’ epitopes. The resulting recombinants had the following modifications (for amino acid sequences see Table 1): R1 had the initiating methionine codon of its VP replaced with a stop codon, and would therefore be expected to produce no VP; R2, R3 and R4 produced VPs with BeMV sequences of different sizes and positions at their N termini. The N terminus has been shown to be a major antigenic determinant in TYMV (Pratt et al., 1980), and was predicted by PP to be accessible; R5 and R6 produced VPs with the predicted αA and αB helices, respectively, replaced with those of BeMV, although the hydrophobicity moment and PP analyses suggested that the αB helix was only partly within the region changed in the R6 recombinant; R7 produced a VP with the region of the TYMV VP that cross-linking studies had shown to interact with the viral RNA (Ehresmann et al., 1980) replaced with that of BeMV; ‘R8’, was a point mutant with a lysine residue found in all sequenced tymovirus VPs changed to arginine. R9 produced a VP with a surface loop region of the TYMV VP adjacent to the R8 lysine replaced with that of BeMV. This region was shown to have strong antigenic activity in inhibition tests (Pratt et al., 1980), and was predicted by the PP method to be accessible; R10 produced a VP with the BeMV C terminus. E71 produced a VP with the Plasmodium falciparum epitope replacing seven amino acids at the N terminus of TYMV-BL. V3 replaced the 16 N-terminal amino acids of the TYMV-BL VP with those of the HIV-1 V3 loop region. V3/1 inserted the V3 loop sequence at the N terminus of the TYMV-BL VP. Finally, V3/2 inserted a V3 loop sequence based on the alignment of the consensus sequence of the HIV-1 subtypes. The inserted sequence had an isoelectric
point closer to that of the corresponding TYMV sequence than those of V3 and V3/1.

Restriction enzyme analysis of the cloned recombinant genomes indicated that they had been assembled correctly.

**Infectivity of the recombinant transcripts for Chinese cabbage**

TYMV-BL/BeMV recombinants with changes at the N-terminal end of the VP (R2 and R3) consistently infected plants systemically. Plants inoculated with R2 showed systemic symptoms at the same time as those inoculated with the wild-type transcript, producing a systemic mosaic over the entire leaf, whereas the plants inoculated with R3 consistently showed symptoms later and, although chlorotic patches appeared along the leaf veins, these did not spread to the entire leaf. R4 and R5, which had VPs altered at the N-terminal end and αA respectively, systemically infected half the plants inoculated, with symptoms indistinguishable from those produced by pBL-16. R7 and R9 gave systemic symptoms in about 10% of the inoculated plants and, although the symptoms were similar to those shown by plants infected with R3, their appearance was retarded by at least 3 weeks. Leaf discs were taken from the inoculated and tip leaves of all the plants infected with each of these recombinants and total RNA was isolated and tested by RT–PCR. In every case a PCR product of the expected size was produced and sequence analysis verified that they were the expected recombinants; in all these experiments the recombinant virus inoculated was the only one recovered. R6 and R8 gave local lesions but these were probably artefacts as RNA from them did not yield a TYMV VP-specific PCR product. R1 and R10 never produced systemic symptoms or local lesions.

Western blotting of virions isolated from systemically infected leaves with VP-specific polyclonal antisera raised in rabbits showed that the recombinant VPs were the same size as those of the wild-type virus (data not shown). Virions purified from these plants were examined in a transmission electron microscope and found to be indistinguishable from those of the wild-type pBL-16 virus, and there was no discernable difference.
Table 1. The amino acids that were changed and their locations in the TYMV-BL VP in each of the ten recombinants and the E71 and V3 mutants.

<table>
<thead>
<tr>
<th>Recombinant name</th>
<th>Positions changed</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>(amino acid 1)</td>
<td>TYMV M * (Termination)</td>
</tr>
<tr>
<td>R2</td>
<td>(amino acids 1-12)</td>
<td>TYMV MEIDEKELAPQDR BeMV MDESKIVTVKQP</td>
</tr>
<tr>
<td>R3</td>
<td>(amino acids 13-20)</td>
<td>TYMV TVTVAT-V BeMV SISAPGFT</td>
</tr>
<tr>
<td>R4</td>
<td>(amino acids 1-20)</td>
<td>TYMV MEIDEKELAPQDTVTVAT-V BeMV MDESKIVTVKQPSISAPGFT</td>
</tr>
<tr>
<td>R5</td>
<td>(amino acids 57-67)</td>
<td>TYMV IDSSTTLTFY BeMV SDSLAKLTSGY</td>
</tr>
<tr>
<td>R6</td>
<td>(amino acids 106-114)</td>
<td>TYMV TQITKTYGG BeMV SKILSIYGG</td>
</tr>
<tr>
<td>R7</td>
<td>(amino acids 140-142)</td>
<td>TYMV PRV BeMV AMI</td>
</tr>
<tr>
<td>R8</td>
<td>(amino acid 143)</td>
<td>TYMV K N (point mutation)</td>
</tr>
<tr>
<td>R9</td>
<td>(amino acids 144-153)</td>
<td>TYMV DSISOYLDSPK BeMV DSTTITYDSDK</td>
</tr>
<tr>
<td>R10</td>
<td>(amino acids 181-189)</td>
<td>TYMV HSPLITDTST BeMV SAPLLQAL*</td>
</tr>
<tr>
<td>E71</td>
<td>(amino acids 1-9)</td>
<td>TYMV MEIDEKELAP E71 NOSTFNN</td>
</tr>
<tr>
<td>V3</td>
<td>(amino acids 1-16)</td>
<td>TYMV MEIDEKELAPQDTVTV V3 MNTRKSIHIHGPGRAFY</td>
</tr>
<tr>
<td>V3/1</td>
<td>(amino acids 1-9)</td>
<td>TYMV MEIDEKELAPQDTVTV V3/1 MNTRKSIHIHGPGRAFY</td>
</tr>
<tr>
<td>V3/2</td>
<td>(amino acids 1-16)</td>
<td>TYMV MEIDEKELAPQDTVTV V3/2 MNMTQTSITIGPAAA</td>
</tr>
</tbody>
</table>

in the ratio of virions and genome-free shells in the preparations. The erratic production of systemic infections by some recombinants was probably a consequence of changing environmental conditions, but these tests did not reveal which features of the environment were important.

Experiments were done to see if elevating the temperature at which TYMV-inoculated plants were held would induce recombinants that only gave lesions to spread systemically. However, at 30 °C none of the plants, including those inoculated with transcript of pBL-16, showed systemic symptoms. This result suggests that recombinants putatively restricted to lesions in inoculated leaves were probably movement-deficient and not restricted to lesions due to a hypersensitive response of the host.

**Ability of the recombinants to infect other tymovirus host plants**

BeMV infects a range of solanaceous plants including the tobacco cultivar SR1. However, none of the recombinants produced symptoms in inoculated SR1 and no TYMV VP gene could be amplified by RT–PCR, whereas control plants...
inoculated with BeMV RNA produced clear symptoms of infection. The recombinants were also inoculated to Arabidopsis thaliana (Brassicaceae), which is a host of TYMV. Those recombinants which gave systemic symptoms in Chinese cabbage gave similar symptoms in A. thaliana plants. All ten TYMV/BeMV VP recombinants were checked for their ability to infect a range of indicator plants, at least one of which is susceptible to all known tymoviruses (Brunt et al., 1996). Transcripts were manually inoculated direct to seedlings of Chenopodium quinoa, Cucumis sativus, Cucurbita pepo, Gomphrena globosa, Petunia hybrida, Phaseolus vulgaris, Pisum sativum, Nicotiana clevelandii and Nicotiana glutinosa. None of the plants developed symptoms, and no virus was detected by RT–PCR in the inoculated or tip leaves, except in the positive controls, which were Chinese cabbage plants inoculated with transcript of pBL-16.

**SPOTs epitope analysis**

The SPOTs membrane, on which overlapping peptides covering the entire TYMV-BL virion protein had been synthesized, was probed with several TYMV antisera in addition to antisera raised against virions of R2 and R3. The N-terminal peptides of the VP reacted with all the TYMV antisera used. When the SPOTs peptides were probed with R2 antiserum the most notable difference from the wild-type TYMV antiserum was that the antibodies did not react with peptides representing the N-terminal 14 amino acids (Fig. 3). The R2 VP contained the N-terminal 12 amino acids of BeMV in place of those of TYMV, indicating that antibodies raised against this recombinant did not recognize the wild-type TYMV N-terminal amino acids. When the peptides were probed with an antiserum raised against R3 virions (amino acids 13–20 replaced by those of BeMV) the N-terminal amino acids 2–28 were recognized by the antiserum. Other regions in the virion protein also reacted with the different antisera (Fig. 3).
Electrophoretic mobility of virus particles

The calculated isoelectric points of some of the peptides that were altered in the recombinants differed from the corresponding peptides of TYMV-BL. However, the electrophoretic mobilities of all the recombinant virions (Fig. 4), except that of R5, were the same as that of TYMV-BL. This indicates that the BeMV peptide inserted into the virion protein of R5 was exposed at the surface of recombinant virions and affected mobility, but that the peptides with changed isoelectric points inserted in other parts of the recombinant VPs were not exposed and mobility was unaltered.

‘Alien’ recombinants

As it had been shown that the N-terminal TYMV residues were immunodominant but could be replaced without affecting the infectivity of the recombinant, experiments were designed to test whether TYMV would form viable recombinants when the N-terminal amino acids of the VP were replaced with entirely unrelated coding sequences (Table 1). Those chosen were the E-71 major merozoite surface antigen of Plasmodium falciparum, and the V3 loop region of the gp120 protein of HIV-1, as TYMV virions expressing either of these antigens would have obvious uses (Jagadish et al., 1996; Porta et al., 1996).

The E71 recombinant transcripts consistently infected Chinese cabbage, but the appearance of symptoms was delayed by 2–3 days compared to infection with either pBL-16 or R3. Systemic infection was also delayed and the chlorotic mosaic spread along, rather than between, the leaf veins as in infections of TYMV-BL. RT–PCR and sequencing confirmed the presence of the E71 sequence (data not shown). The recombinant virus was readily passaged by sap inoculation and produced the same symptoms as the transscripts. An antiserum raised against the E71 virions was used in Western blots to test their immunogenicity. The antiserum reacted with the E71 virions used as immunogen, but not with a fusion protein containing the E71 epitope. An antiserum raised against the E71 fusion protein reacted only weakly with the E71 virions in Western blots. These results confirm that the E71 epitope was present in the E71 recombinant, but indicate that it was not detectably immunogenic in these limited tests. RNA transcripts from the recombinants with the gene for the HIV-V3 loop region at the 5’ terminus of the VP gene produced very small necrotic lesions in inoculated Chinese cabbage leaves 5–7 days after inoculation, but no systemic symptoms. RT–PCR tests did not detect infection 7–28 days after inoculation and sap from the inoculated leaves did not produce any visible signs of infection when inoculated to Chinese cabbage plants. Two other HIV-V1 recombinants, V3/1 and V3/2, were also tested. In V3/1 the full nucleotide sequence encoding the V3 loop sequence (18 amino acids) was placed 5’ of the N-terminal glutamine residue of the VP, a residue which is present in all tymovirus VPs. V3/2 was designed to avoid the charge difference between the V3 peptide (isoelectric point 11.7) and the TYMV peptide it replaced (TYMV-BL, 4.0). The V3/2 recombinant had its arginine, lysine and histidine residues replaced so that its isoelectric point was 6.1. Neither recombinant V3/1 nor V3/2 was infectious.

Discussion

The different methods used for predicting the secondary structures of tymoviral VPs gave unequivocally congruent results even though they assessed different features of the sequences. These results were confirmed with the recent publication of the tymovirus VP structure (Canady et al., 1996). The crystal structure was very similar to that predicted by the methods used, especially the ‘profile’ method. The virion protein consists of an eight-stranded antiparallel β-barrel and two β-helices with the β-helices located between βC/βD and βE/βF as predicted. In the crystal structure both β-helices are exposed and accessible in agreement with our experimental predictions based on the altered electrophoretic mobility of virions of R5 (β-helix A; amino acids 57–64 changed).

There are some discrepancies in the placement of amino acids in the β-helices in the actual compared to the predicted structures (αA: 56–66 predicted, 57–64 actual; αB: 98–113 predicted, 98–110 actual). The locations of amino acids in some of the β-sheets are also shifted in the actual compared to the predicted structures but by no more than one or two residues. These discrepancies may be explained by the fact that the virion proteins used to construct the profile were longer than the tymovirus sequences which were aligned with it to generate the predicted structure. Hence it is not surprising that there were minor differences between the actual and predicted structures.

The crystal structure of the VP shows that the N terminus of the virion protein is within the virions and the C terminus is exposed. Experiments with the N-terminal recombinants confirm this as, although these recombinants had N-terminal peptides with altered isoelectric points compared to the wild-type TYMV, their mobility was unaffected. However, the epitope analysis experiments showed that the N terminus was immunodominant despite being inside the virion. We conclude that the TYMV virions probably undergo conformational changes in animals immunized with them, as has been reported by Li et al. (1994) for poliovirus virions. The immunodominance of the N terminus also disproves the earlier assumption (Quesniaux et al., 1983a, b) that all immunogenic regions of the TYMV VP must lie at the surface of the virions.

Recombinants were constructed and used to test whether tymoviral VPs influenced the viability, host range and movement of the virus. The results of these experiments indicated that the VP was essential for TYMV infection in plants as four of the recombinants, (R1, R6, R8 and R10), did not produce lesions or systemic symptoms in plants. These results indicate
that the VP has an essential role in cell-to-cell movement of the virus. The role played by the tymovirus virion protein in cell-to-cell and long-distance movement of the virus was further demonstrated by recombinants 3, 7 and 9. R3 produced symptoms in plants which were different from those of the wild-type virus and the appearance of systemic infection was delayed by 3–4 days. Infection with R7 and R9 resulted in systemic symptoms which were identical to those produced by the wild-type virus but their appearance was delayed by up to 3 weeks. These results indicate that the tymovirus virion protein has an important role in the ability of the virus to spread locally and systemically in plants. A similar role for the virion proteins of other plant viruses in cell-to-cell and long-distance movement has been reported. These include potato virus X (Chapman et al., 1992), tobacco etch virus (Dolja et al., 1994, 1995) and cucumber mosaic virus (Suzuki et al., 1991).

Although six of the ten TYMV/BeMV recombinants (R2, R3, R4, R5, R7 and R9) were viable and spread systemically in plants to a greater or lesser extent, all had the same host range as TYMV and did not infect hosts of BeMV or other tymoviruses. Thus it appears that at least those parts of the VP that were changed in these experiments do not carry host range determinants, even though they affected virulence.

The results of our experiments have shown that the virion protein of TYMV-BL is involved in infection and movement of the virus, in contrast to earlier experiments reported by Bransom et al. (1995). In their experiments, several virion protein mutants of TYMV were constructed, all of which replicated in Chinese cabbage protoplasts and produced local lesions in inoculated Chinese cabbage plants. However, none of them, except a C-terminal mutant containing an extra five amino acids, was able to spread systemically. Particles produced by the C-terminal mutant were less stable than those of the parental virus. A role for the C terminus of the protein in viability of virions was also demonstrated by R10 which did not infect plants. The differences between the results reported here and those of Bransom et al. (1995) on the role of the tymovirus virion protein may be due to the different changes which were introduced into the virion protein in each case. Hence it is difficult to compare the two sets of results.

The N-terminal regions of the TYMV VP, which could be replaced with parts of the BeMV VP in R2 and R4 without loss of infectivity, seemed to be more amenable to change than the other regions tested. Therefore, recombinants were constructed with entirely foreign peptides in this region in order to test whether TYMV could be used as a surrogate for producing antigenic peptides in plants. Recombinants with the E71 peptide inserted immediately after the initiating methionine codon of the VP were viable in plants and spread systemically, although the appearance of symptoms was slightly delayed. However, limited tests indicated that the E71 peptide within the recombinant virions was probably not immunogenic, indicating that it may not undergo the same conformational changes as the corresponding region of wild-type TYMV virions when injected into rabbits. Three attempts to design viable TYMV recombinants containing the V3 region of HIV-1 gp120 were unsuccessful, although it has recently been reported that the same region of the HIV-1 gp120 was successfully attached to the C terminus of the tomato bushy stunt tombusvirus (TBSV) VP by gene fusion (Joelson et al., 1997). The TBSV chimera contained 13 amino acids of the V3 loop, all of which were included in the TYMV/V3 mutant. The TBSV/V3 chimera produced stable virions that reacted in Western blots with a monoclonal antibody raised against the V3 region. The chimeric virions also reacted with human sera from HIV-1-positive subjects in ELISA tests, demonstrating that the foreign epitope was exposed on the surface of the viral particles. When the chimeric virions were injected into mice, a primary antibody response to the foreign epitope was detected, but it was poor and the antibody titre was not boosted by an additional injection with the chimeric virions (Joelson et al., 1997). It is not clear from the experiments with the TYMV/V3 mutants why stable virions were not produced by using inverse PCR to place the V3 loop at the N terminus of the virion protein. With the publication of the crystal structure of the TYMV virion protein, regions in the protein which may be more amenable to change may be identified as possible candidates for the introduction of foreign epitopes which could then elicit an immune response in animals.

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References


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