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Expression of the tobacco mosaic virus movement protein using a baculovirus expression vector

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A cDNA clone of the tobacco mosaic virus 30K movement protein (MP) gene was constructed and introduced into an *Autographa californica* nuclear polyhedrosis baculovirus expression vector. Infection of *Spodoptera frugiperda* cells with the vector resulted in the synthesis of low levels of MP, which was detected by anti-MP serum as two closely related species of M_r approximately 34K and a third species of 32K. The authenticity of the recombinant MP was confirmed by comparison of the protein, on the basis of migration during SDS–PAGE, with authentic MP from several sources. It appeared that the recombinant MP was not modified by *N*-linked glycosylation, but was phosphorylated. The recombinant MP was produced in both a phosphorylated and an unphosphorylated state and the former species was shown to comigrate with plant-expressed MP during SDS–PAGE.

The 30K protein product of tobacco mosaic virus (TMV), the movement protein (MP), is thought to be involved in the facilitation of the cell-to-cell spread of the virus in the host plant (for review see Hull, 1989). It is unclear how the MP is involved in this process, but recent results suggest that MP interacts with both TMV RNA (Citovsky *et al.*, 1990) and the host plant plasmodesmata (Wolf *et al.*, 1989; Atkins *et al.*, 1991). One approach to elucidate the role of MP in cell-to-cell spread is to analyse it directly. Unfortunately, obtaining information on the structure, function or interactions of pure preparations of MP is hampered by the fact that the protein is produced in very small amounts in TMV-infected plants (Watanabe *et al.*, 1984). Furthermore, the MP produced following TMV infection, or in transgenic tobacco plants expressing the MP gene, becomes localized within the cell wall of the plant and can only be extracted by applying severe and denaturing techniques (Moser *et al.*, 1988; Deom *et al.*, 1990). Consequently, over-expression of the MP gene using heterologous expression systems is required to obtain large quantities of purified protein. Although the MP gene has been over-expressed in *Escherichia coli* (Citovsky *et al.*, 1990), studies of the MP isolated from plant cells suggest that it may be modified in some manner. The observation that the protein detected in extracts of transgenic plants expressing the MP gene, when analysed by SDS–PAGE, was shown to have an apparent M_r greater than the value of 30K calculated from the sequence information suggests that post-translational modification occurs (Deom *et al.*, 1990). In addition, MP has been reported to be phosphorylated (D. Zimmern, personal communication in Zaitlin & Hull, 1987). These observations suggest that to obtain correctly processed protein for analysis the MP gene should be expressed in a eukaryote.

The baculovirus expression system was chosen to express the MP gene because, in addition to the high level of expression of foreign genes frequently achieved, it has been shown to complete many eukaryotic post-translational modifications (Miller, 1988). This report describes the expression of the MP gene using an *Autographa californica* nuclear polyhedrosis virus (AcNPV) expression vector and the preliminary characterization of the post-translational modifications of the protein product.

A copy of the MP gene of the vulgare strain of TMV was constructed by joining the *Nco*I sites within an overlapping region of two TMV cDNA clones, pNMP2 and pCMP1, using techniques described by Maniatis *et al.* (1982). The N-terminal clone pNMP2 [TMV nucleotides (nt) 4654 to 5571 in the sequence of Goelet *et al.* (1982)] contained a *Bam*HI site 25 bp 5′ to the MP gene translation initiation codon. This site does not occur in wild-type TMV and was introduced by oligonucleotide site-directed mutagenesis (Kunkel, 1985), to facilitate cloning into the transfer vector and to minimize extraneous 5′ untranslated sequence. The C-terminal

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clone pCMPI (nt 5458 to 5766) was a gift from T. M. A. Wilson. The identity of the full-length cDNA copy of the MP gene constructed, pTK14, was confirmed by DNA sequence analysis.

pTK14 DNA was digested with *Bam*HI and the fragment containing the MP gene was ligated into the baculovirus transfer vector, pAcYM1 (Matsuura *et al.*, 1987), which had been digested previously with *Bam*HI and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). Putative recombinant plasmids were isolated, and the presence and orientation of inserts determined by restriction enzyme mapping. The integrity of the 5′ cloning junction and the translation initiation codon of the MP open reading frame of a recombinant, pAcYM1.TK14, was confirmed by DNA sequence analysis.

AcNPV was grown and assayed in confluent monolayers of *Spodoptera frugiperda* (IPLB-Sf21AE) cells (Brown & Faulkner, 1977), and genomic DNA was purified by the method of Smith & Summers (1978). AcNPV DNA (1 µg) and 25 µg CsCl gradient purified pAcYM1.TK14 DNA were used to cotransfect *S. frugipera* cells as described previously (Matsuura *et al.*, 1987). The cells were grown at 28 °C in TC100 medium (Gibco) supplemented with 10% foetal calf serum (Globepharm), with 50 units/ml each of penicillin, kanamycin and streptomycin being added before use. Suitable recombinants were identified by DNA dot blot hybridization and plaque assay. The putative recombinants were determined to contain the MP gene by Southern blot analysis (Southern, 1975) of *Bam*HI-digested viral DNA purified from infected *S. frugiperda* cells using a procedure described by Hirt (1967). Two recombinants, Ac.TK4 and Ac.TK6, were selected for analysis of protein production.

Recombinant MP was produced by infection of *S. frugiperda* cell monolayers with recombinant baculoviruses at a multiplicity of 5 to 10. Controls were produced by infecting monolayers with wild-type AcNPV and by mock inoculation with growth medium. Total cell extracts were prepared by resuspension of PBS-washed cells in RIPA buffer (1% v/v Triton X-100, 1% w/v sodium deoxycholate, 150 mM-NaCl, 10 mM-EDTA, 0·1% w/v SDS, 10 mM-Tris–HCl pH 8·0). The protease inhibitors PMSF (2 mM), aprotinin (10 µg/ml) and leupeptin (20 µg/ml) (all from Sigma) were added to the RIPA buffer immediately before use. *β*-Glycerophosphate (Sigma) was added to a final concentration of 100 mM to inhibit phosphatases. Radiolabelled proteins were prepared by starving the infected *S. frugiperda* cells for 1 h in either phosphate- or methionine-free medium, followed by incubation for 3 h in an appropriately deficient medium containing 100 µCi/ml of either $^{32}P_i$ (Amersham) or [^{35}S]methionine (Du Pont, NEN Research Products).

The extracted proteins were analysed by SDS–PAGE in 12% gels (Laemmli, 1970), followed by Western blotting (Towbin *et al.*, 1979) or autoradiography. Western blots were completed by transfer of electrophoresed proteins to Hybond-C+ membranes (Amersham) by semi-dry electroblotting. Immobilized proteins were probed with rabbit anti-MP serum (raised against an oligopeptide corresponding to amino acids 98 to 116 of MP) diluted 1/250 in blocking buffer (5% skimmed milk powder, 0·01% v/v Tween 20 in PBS) and bound antibodies were detected by incubation of the membrane with alkaline phosphatase-conjugated goat anti-rabbit IgG serum (Sigma) diluted 1/1000 in blocking buffer. The immobilized alkaline phosphatase was detected by incubation of the blot in a suitable chromogenic phosphatase substrate. In some cases the MP was immunoprecipitated before electrophoresis by incubation of the cell extracts with the anti-MP serum and Protein A–Sepharose CL-4B (Sigma). The resultant immune complexes were dissociated by boiling in Laemmli dissociation buffer for 5 min and subsequently analysed by SDS–PAGE. Radiolabelled proteins were detected by contact autoradiography using Fuji X-ray film.

The yield of MP from both Ac.TK4 and Ac.TK6 was low and an MP-specific band was not detectable by Coomassie blue staining of SDS–polyacrylamide gels. When MP was extracted in the presence of protease and phosphatase inhibitors, the protein was detected by probing with anti-MP serum as two closely migrating bands of M_r approximately 34K (designated 34u, the slower migrating and 34l, the faster migrating species), and a third rarer and occasionally undetected species of approximately 32K (Fig. 1*a*, lane 1). In some extracts one or more non-specific, high M_r species were detected by using the antiserum (see Fig. 1*b*, lane 1; and Fig. 3, lanes 3 and 4). In the absence of protease inhibitors several other protein bands were detected (Fig. 1*a*, lane 2; Fig. 2*b*, lanes 11 and 12). The level of expression of MP by the recombinant baculoviruses was lower than that of other foreign genes (Luckow & Summers, 1988). The fact that the relative levels of detectable MP were similar to those of MP RNA (data not shown) suggests that there may be a limitation in the transcription of the MP gene.

The procedure described by Citovsky *et al.* (1990) was used to attempt to purify recombinant MP. Approximately $1{\cdot}5 \times 10^6$ *S. frugiperda* cells infected with the recombinant baculovirus at multiplicity of 5 were washed in PBS and resuspended in 150 µl of lysis buffer (1% v/v NP40, 1 mM-DDT, 1 mM-EDTA, 10% v/v

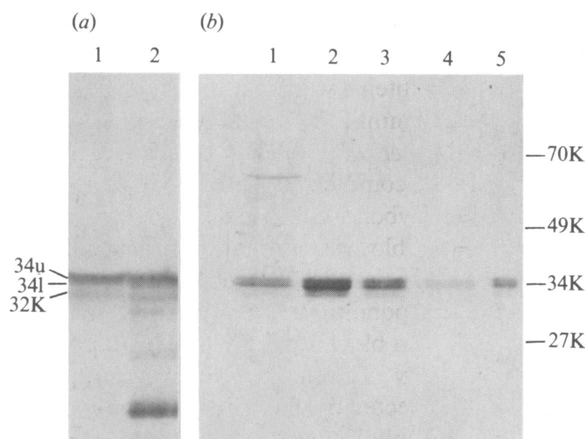

Fig. 1. (*a*) Proteins produced by infection of *S. frugiperda* cells with Ac.TK6 analysed by Western blotting and probed with anti-MP serum. Cells were extracted either with (lane 1) or without (lane 2) protease inhibitors. (*b*) Partial purification and analysis of the solubility of recombinant MP. The initial cell pellet was processed by sequential extraction, and the samples were analysed by SDS–PAGE and probing with the anti-MP antiserum. Each sample represents a part of the supernatant portion following the centrifugation of each fraction at 10000 *g* for 10 min at room temperature. MP soluble following cell lysis (lane 1), MP solubilized in 1 M-NaCl buffer (lane 2), MP solubilized in 1 M-NaCl, 4 M-urea buffer heated at 70 °C for 10 min (lane 3), MP solubilized in same buffer as in lane 3 but heated at 56 °C for 20 min (lane 4), final insoluble fraction (lane 5). The positions of M_r markers are indicated.

glycerol, 200 mM-NaCl, 20 mM-Tris–HCl pH 8·0 and protease and phosphatase inhibitors as described above). The cell suspension was frozen at −20 °C for 1 h and then thawed at room temperature with occasional vortexing. The soluble and insoluble fractions were separated into pellet and supernatant fractions following centrifugation at 10000 *g* for 10 min at room temperature. The insoluble fraction was then resuspended in solubilization buffer (lysis buffer containing 1 M-NaCl but without NP40), incubated at room temperature and centrifuged as before. The sequential treatment of the resultant insoluble fractions continued with incubation in solubilization buffer containing 4 M-urea at 70 °C for 10 min and at 56 °C for 20 min. The soluble supernatant fractions from each treatment were analysed by SDS–PAGE followed by immunoprobing of Western-blotted protein with the anti-MP serum as described above (Fig. 1*b*). A proportion of the MP was soluble following lysis of the infected *S. frugiperda* cells (Fig. 1*b*, lane 1); however, a majority of the MP sedimented as an insoluble aggregate (insoluble MP was then processed further and is represented by the cumulative total detected in lanes 2 to 5). Approximately 60% of the aggregated MP was solubilized following extraction in buffer containing 1 M-NaCl (lane 2). Subsequent treatment of insoluble MP by incubation at high temperature in 4 M-urea (lanes 3 and 4) solubilized more MP, but a proportion remained insoluble despite all these treatments (lane 5). Even after the partial purification an MP-specific band was not detectable by protein staining of samples resolved by SDS–PAGE.

The possibility of two forms of post-translational modification of the recombinant MP, glycosylation and phosphorylation, was investigated. Although most viral proteins are cytosolic and therefore would not be expected to be *N*-glycosylated, the targeting of MP to the plasmodesmata might be associated with glycosylation. There are two potential *N*-glycosylation sites in MP and two methods were used to determine whether either was modified. First, infected *S. frugiperda* cells were treated with a glycosylation inhibitor, tunicamycin (Matsuura *et al.*, 1989) (1 mg/ml stock solution prepared in 25 mM-NaOH), which was added to the medium to a final concentration of 10 µg/ml. After 1 h the cells were metabolically labelled with [^{35}S]methionine in the presence of tunicamycin for 3 h. Protein samples were extracted and analysed by immunoprecipitation with the anti-MP serum, followed by SDS–PAGE and autoradiography. Control samples were prepared from similarly infected and labelled, but untreated, *S. frugiperda* cells.

There was no detectable shift in the migration pattern of MP synthesized in tunicamycin-treated cells. Analysis of total *S. frugiperda* protein showed that tunicamycin treatment resulted in changes in the migration pattern of other proteins, confirming that the uptake and activity of the drug was effective (data not shown).

The second method for the detection of *N*-linked oligosaccharides exploited the fact that mannose is a constituent of the core structure of all *N*-linked glycans, and could be detected in proteins immobilized by SDS–PAGE and Western blotting with the lectin concanavalin A, which was conjugated to biotin (Dietz *et al.*, 1988). Duplicate sets of proteins extracted from recombinant baculovirus-infected *S. frugiperda* cells were subjected to SDS–PAGE and transferred to a membrane by Western blotting as described above. One set of samples was probed with the anti-MP serum to detect the position of the recombinant MP. The second set of blotted proteins was probed with biotin-conjugated concanavalin A (Dietz *et al.*, 1988). Recombinant MP, located on duplicate samples using the anti-MP serum, was not detected by concanavalin A probing, whereas baculovirus and *S. frugiperda* glycoproteins were detected by the probe, confirming its efficacy (data not shown). The results from both experimental approaches suggest that the MP expressed using the baculovirus vector was not modified by *N*-linked glycosylation.

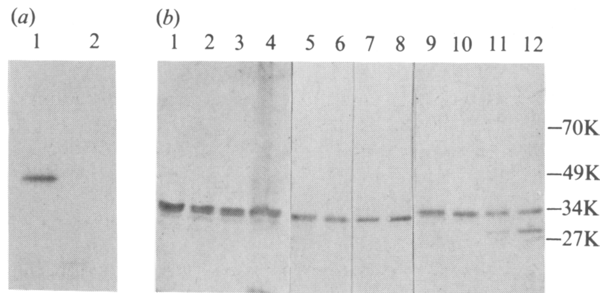

Fig. 2. (*a*) Autoradiograph of SDS–PAGE analysis of MP immunoprecipitated from extracts of Ac.TK6- (lane 1) and AcNPV-infected (lane 2) *S. frugiperda* cells metabolically labelled with $^{32}P_i$ for 4 h prior to harvesting and lysed in RIPA buffer containing protease inhibitors. (*b*) Analysis of the effect of the addition of protease and phosphatase inhibitors on the nature of the recombinant MP incubated in *S. frugiperda* cell extract. Aliquots of Ac.TK6-infected *S. frugiperda* extracts were incubated for 0 (lanes 1, 5 and 9), 1 (lanes 2, 6 and 10), 2 (lanes 3, 7 and 11) and 3 h (lanes 4, 8 and 12) in buffer containing protease and phosphatase inhibitors (lanes 1 to 4), protease inhibitors (lanes 5 to 8) and no inhibitors (lanes 9 to 12). Following incubation the samples were boiled in SDS–PAGE sample buffer, and analysed by SDS–PAGE and probing with the anti-MP serum. The positions of M_r markers refer to (*b*).

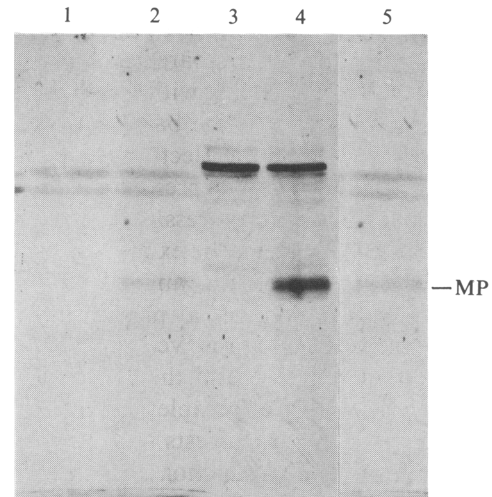

Fig. 3. Comparison of MP from transgenic plants with baculovirus-expressed MP. Proteins were extracted in the presence of protease and phosphatase inhibitors, and analysed by SDS–PAGE and probing with the anti-MP serum. Samples were from control transgenic tobacco plants (line 306) (lane 1), MP gene-expressing transgenic tobacco plants (line 277) (lanes 2 and 5), and AcNPV-infected (lane 3) and Ac.TK6-infected (lane 4) *S. frugiperda* cells. The position of the 34K MP is indicated.

To determine whether the recombinant MP was phosphorylated, MP was labelled in *S. frugiperda* cells with $^{32}P_i$, purified from protein extracts by immunoprecipitation with the anti-MP serum, and was analysed by SDS–PAGE and autoradiography. A single species of MP of approximately 34K was detected specifically in the sample prepared from Ac.TK6-infected *S. frugiperda* cells (Fig. 2*a*). No proteins were detected in samples prepared from AcNPV-infected *S. frugiperda* cells treated similarly. This result demonstrated that the recombinant MP is phosphorylated.

To examine the phosphorylation of the recombinant MP further, phosphatases present in the crude cell lysate were selectively inhibited. Ac.TK6-infected *S. frugiperda* cell extracts were prepared in RIPA buffer and samples were incubated at room temperature with either added protease and phosphatase inhibitors, added protease inhibitors or with no further addition. Aliquots of each sample were removed at hourly intervals and immediately boiled in SDS–PAGE sample buffer. After 3 h all the samples were analysed by SDS–PAGE and the MP was detected by probing proteins with the anti-MP serum following Western blotting. Fig. 2(*b*) (lanes 1 to 4) shows that incubation with both phosphatase and protease inhibitors resulted in the detection of the two 34K species and the 32K species of MP constantly during the 3 h period. Incubation in just protease inhibitors (Fig. 2*b*, lanes 5 to 8) resulted in the detection of all three species at time zero (lane 5), and after incubation only the 32K and 34l MP species were detected. Incubation in RIPA buffer containing neither inhibitor (Fig. 2*b*, lanes 9 to 12) resulted in the detection of all three species of MP initially, and the 34u species and degradation products of MP proteolysis after incubation.

These results suggest that the 34u species is a phosphorylated form of the 34l MP; the interconversion of the former to the latter is prevented by inhibiting phosphatases. The formation of the lower M_r MP polypeptides is a separate event and is preventable by the addition of protease inhibitors. Migration of phosphorylated species was anomalous in relation to the unphosphorylated species. This is similar to data reported for rabies virus M protein (Dietzschold *et al.*, 1979) and could be due to phosphorylation opening up the protein and allowing more binding of SDS.

No MP bioassays were available and so the authenticity of the recombinant MP was assessed by comparing its electrophoretic mobility with that of authentic MP from several sources. MP produced by *in vitro* translation of TMV RNA and of *in vitro* transcripts of the MP gene [subcloned downstream of the T7 RNA polymerase promoter in the Bluescript vector (Stratagene)] using an RNA-free rabbit reticulocyte lysate (Pelham & Jackson, 1976) comigrated with the 34l band of [^{35}S]methionine-labelled MP expressed by the baculovirus (data not shown). MP was extracted from plants transgenic for the TMV MP gene (line 277; Deom *et al.*, 1987) as described by Atkins *et al.* (1990). Control samples were prepared

from transgenic plants (line 306) that contained the transformation vector but not the MP gene. When MP from transgenic plants was compared with recombinant MP by SDS-PAGE and probing with the anti-MP serum (Fig. 3*b*), the 34u species of the baculovirus-expressed MP was shown to have an electrophoretic mobility identical to that of the plant-expressed MP.

This report describes the expression of the TMV MP gene in a heterologous eukaryotic expression system and is the first description of the analysis of the post-translational modification of a plant virus protein expressed using a baculovirus vector. Therefore, in addition to the ease of use and the potential for high levels of expression, the completion of apparently authentic phosphorylation suggests that the baculovirus vector is suitable for the production of easily accessible, authentic TMV MP, as compared to plant-expressed MP. Although the yields of MP are low, they are much better than those from other sources and do not prevent experimentation on the protein. These yields might be increased by alteration of the translation initiation ATG to a consensus sequence and by reducing the length of the extraneous TMV sequence 5′ to the MP gene. The vector is useful for the generation of radiolabelled MP, which could be used for the identification of the phosphorylated residue, and for the preparation of an MP affinity probe for the detection of putative plant plasmodesmatal MP receptor proteins.

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