Limitations on the use of fused green fluorescent protein to investigate structure–function relationships for the cauliflower mosaic virus movement protein

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To investigate the process of tubule formation for the cauliflower mosaic virus movement protein (CaMV MP), the green fluorescent protein (GFP) was fused to the MP to provide a vital marker for MP location after expression in insect cells. In contrast to the long tubular structures seen previously following baculovirus-based expression of the wild-type MP, the fusion protein produced only aggregates of fluorescing material in the cytoplasm. However, by co-expressing wild-type MP and GFP–MP, or by engineering their co-accumulation by introducing a foot-and-mouth disease virus 2A cleavage sequence between GFP and MP, long GFP-fluorescing tubules were formed. The experiments suggest that the presence of GFP at the N or C terminus of the tubule-forming domain of the CaMV MP places steric constraints upon the aggregation of the MP into a tubule but that this can be overcome by providing wild-type protein for inclusion in the aggregate.

The green fluorescent protein (GFP) is an important tool for studying the function of plant virus movement proteins (MPs) (reviewed in Oparka et al., 1996). When fused to the N or C terminus of the MP, GFP can act as an in vivo reporter for the location of the MP in infected tissues, in isolated cells and after transient expression in the absence of infection. In many cases, the fusion protein has been shown to function as a wild-type protein in gating plasmodesmata and even for supporting complete virus infections.

Viral MPs fall into several functional classes, of which the best characterized are the tobamovirus-like MPs and the comovirus-like MPs. Tobacco mosaic virus MP is functional as an N-terminal GFP fusion protein and mediates virus movement by directing the translocation of a viral RNA–MP complex through plasmodesmata. Comovirus MPs are one group in an increasing list of viral MPs that form tubular structures which displace the plasmodesmal desmotubule to provide a conduit for virus nucleocapsids to pass from cell to cell. Other virus groups present in this list are nepoviruses, bromoviruses, tospoviruses and plant pararetroviruses, including Cauliflower mosaic virus (CaMV).

To study CaMV MP function in isolation from the infection we have exploited the ability of the MP to form tubules at the surface of insect cells after expression in insect cells from baculovirus vectors (Kasteel et al., 1996; Thomas & Maule, 1999). With this expression system, we have shown that the MP tubule-forming domain comprises most (aa 1–282) of the MP (Thomas & Maule, 1999). The non-essential region consists of the C-terminal 45 amino acids (aa 283–327). By analogy with structure/function characteristics of the MP of Cucurbit mosaic virus (CPMV) (Lekkerkerker et al., 1996), with which CaMV MP has structural similarity in this region, the C-terminal part of the protein probably interacts with virus particles in the lumen of the tubule during virus translocation from cell to cell. We had proposed previously that the N terminus of the MP could be exposed on the outer surface of the tubule (Thomas & Maule, 1995 a) and also shown that a 6 × His addition to the N terminus of the protein did not abolish the movement function in an infection in plants (unpublished data). Using baculovirus expression in insect cells as a model system, we aimed to use GFP fused to the N or C terminus of the relevant domain of the CaMV MP to study the cellular components involved in tubule formation. This model system was selected to: (1) avoid the difficulties of expressing foreign sequences (e.g. GFP) from within the CaMV genome (Fütterer et al., 1990); (2) analyse tubule formation separate from other associated movement functions of the MP (e.g. RNA binding; Thomas & Maule, 1995 b) or functions associated with its nucleic acid sequence (e.g. splicing; Kiss-László et al., 1995); and (3) by-pass the inefficiency of tubule formation following transient expression of the CaMV MP in host plant cells (Perbal et al., 1993).

Translational fusions were made between the gfp gene and CaMV gene I (MP) from CaMV CM1841 (Gardner et al., 1981) using PCR and standard cloning techniques. The fusion was...
then cloned into recombinant baculoviruses (called bvMP) using the Gibco BRL Bac-to-Bac baculovirus expression system. The starting material was a CaMV gene I construct containing the c-myc epitope sequence within the C-proximal spacer region (SPmyc; Thomas & Maule, 1995a, 1999). This gene I sequence has been shown to be fully functional in planta when cloned into the CaMV genome (Thomas & Maule, 1995a).

To construct the MP C-terminal fusion with GFP, a gene I fragment (nt 364–1234; Gardner et al., 1981) was released from pFastbacSPmyc (Thomas & Maule, 1999b) by digestion with BamHI (immediately 5’ to the gene I ATG codon) and HindIII (within the c-myc epitope). This was ligated to gfp, generated by PCR from gfp4 (Haseloff et al., 1997) with primers containing HindIII (5’, but eliminating the ATG initiation codon) and XhoI (3’) adaptors. The resulting MP–GFP fragment was cloned into a BamHI/XhoI-digested vector (pFastbac). The resulting translational fusion had the junctional sequence CAG AAG CTT AGT (HindIII site in bold type), which inserted an additional two amino acids at the MP/GFP junction. To construct the MP N-terminal fusion with GFP, adaptor PCR primers were similarly used to generate gfp4 with a BamHI site and an EcoRV site outside of the ATG initiation codon and inside the TAA termination codon. The gene I fragment (CaMV nt 364–737; Gardner et al., 1981) was generated using primers that added an EcoRV site outside of the ATG start codon and included the Ncol site at nt 737. Gene I and gfp4 fragments were ligated with BamHI/Ncol-digested pFastbacSPmyc. The translational fusion had the sequence AAA GAT ATC ATG (EcoRV site in bold type) and similarly inserted an additional two amino acids (D, I) at the GFP/MP junction (Fig. 1). The control vector, pFastbacGFP, containing unfused gfp, was generated by ligating a PCR gfp fragment with primer-adapted sequences (5’ Byfl–XhoI 3’, including translational start and stop sequences) into pFastbac. MP–GFP and GFP–MP are illustrated in Fig. 1(a).

The maintenance and infection of Spodoptera frugiperda (Sf.iPLB-Sf21) followed standard baculovirus protocols (King & Possee, 1992). For the identification of bvMP-infected insect cells and MP tubule formation, 0–5 × 10⁶ Sf21 cells were grown on glass slides and infected at an m.o.i. of > 5. Under these conditions > 95% of cells became infected. MP expression was detected 48 h post-inoculation (p.i.) either by immunofluorescence microscopy with CaMV MP rabbit polyclonal serum (1/500; Harker et al., 1987) and fluorescein-conjugated goat anti-rabbit antibody (1/100; Sigma) visualized using an epifluorescence microscope, or as GFP-fluorescence (activation λ₄88nm/emission λ₃22nm) visualized using a confocal focal scanning microscope (CLSM; Leica TCS-NT system with Leica DMR module). Duplicate slides were analysed for each recombinant baculovirus and comparative analyses between baculoviruses were always done within experiments.

Fig. 1. (a) Junction details for the MP fusions. Construction of the MP–GFP fusion protein results in the addition of two amino acids (K, L) at the junction. Similarly two additional amino acids (D, I) occur at the junction in the GFP–MP fusion. Insertion of the FMDV 2A sequence, APVKQTLNDLLKLAGDVESNPGP (underlined), into GFP–2A–MP resulted in the addition of three junction amino acids (KIP) at the 2A C terminus, leaving four residues (PKIP) attached to the MP N-terminal methionine residue after cleavage. Additional amino acids are in bold type. (b)–(e) Fused GFP interferes with MP–tubule formation. Insect cells infected with either bvMP–GFP (b) or bvGFP–MP (c) and viewed by confocal microscopy to reveal GFP fluorescence show the accumulation of the expressed fusion protein aggregated in the cytoplasm. The morphology of the aggregates was different in each case. This pattern of fluorescence is different from that seen when free GFP (from bvGFP) is expressed (d) or when unfused MP (from bvSPmyc) is expressed (e). Unfused MP, visualized in this case by immunofluorescence and epifluorescence microscopy (e), showed typical surface thread structures interpreted as MP tubules. Bar, 5 µm.
When baculovirus bvMP–GFP or bvGFP–MP was used to infect Sf21 cells grown on glass slides (Thomas & Maule, 1999) aggregates of fluorescent material were visible without immunostaining (Fig. 1b, c) and corresponded to the pattern of distribution of the fusion protein detected using the anti-MP serum (not shown). In each case, the distribution differed from bvGFP expressing free GFP (Fig. 1d). It also differed from bvSPmyc, which showed the formation of surface tubules by immuno-staining (Fig. 1e), as seen previously (Thomas & Maule, 1999). This inability of the fusion protein to form tubules was contrary to results for similar experiments with MPs of Alfalfa mosaic virus (Zheng et al., 1997) and Olive latent virus 2 (Grieco et al., 1999) that were fused to GFP, which formed tubules at the surface of protoplasts. Until we know more about the three-dimensional structure of these tubule-forming MPs, we can only speculate that this contradiction represents differences in the positioning of the C and N termini relative to the core of the MP. For CaMV MP specifically, the failure to form tubules must be due to an inhibition of MP function attributable to incorrect folding of the MP when fused to GFP or to the interference of GFP in the MP aggregation into tubules. Analysis of the protein content of CPMV MP tubules (Kasteel et al., 1997) indicated that there were no other proteins involved in tubule formation.

To test whether GFP fusion proteins could result in steric hindrance at the N or C termini to the ordered aggregation of the MP into a tubule, bvMP–GFP or bvGFP–MP was co-expressed with bvSPmyc in insect cells in an m.o.i. ratio of 1:4 and 1:1 (Thomas & Maule, 1999). Immunoblot analysis of total insect cell proteins extracted (Thomas & Maule, 1999) at 48 h p.i., with anti-MP serum (Fig. 2a) and anti-GFP (Clontech) (Fig. 2b), showed that the GFP-tagged and untagged MP were both expressed in the insect cells and approximately in proportion to their relative m.o.i. In each case, when the co-infected cells were examined for GFP fluorescence, long thread-like structures, interpreted previously as tubules (Kasteel et al., 1996; Thomas & Maule, 1999), were seen extending from the cell surface (Fig. 2c, d). These structures were more abundant and more fluorescent with an m.o.i. ratio of 1:4 than with a ratio of 1:1. These structures were not seen when bvSPmyc
was absent (i.e. no unfused MP) or when bvGFP was co-infected with bvSPmyc (i.e. no MP–GFP or GFP–MP fusion present), although in the latter case, tubules could be detected by immuno-staining with anti-MP serum (data not shown).

Although we were confident that co-inoculation of insect cells at an m.o.i. of > 5 resulted in a large proportion of cells with double infection (Thomas & Maule, 1999), a more direct way to assess the importance of untagged MP in the formation of GFP-fluorescing tubules was to express a GFP–2A–MP fusion protein in which the GFP and MP sequences are separated by the foot-and-mouth disease virus (FMDV) 2A auto-proteolytic peptide sequence (Ryan et al., 1991). Peptide 2A cleaves close to the C terminus of the peptide, leaving one additional amino acid at the N terminus of the downstream protein. Although efficient, cleavage is less than 100% (Ryan & Drew, 1994), potentially resulting in a mixture of the pre- and post-cleavage products.

Construct pFastbacGFP–2A–MP was made by using PCR to add 72 nt of FMDV 2A sequence (Ryan et al., 1991) (with a BglIII adaptor) and a StuI site to the 3' and 5' ends, respectively, of gfp (Crameri et al., 1996). This was ligated with a CaMV gene I fragment [isolated from pFastbacSPmyc digested with BamHI (5') and XhoI (3')], into StuI/XhoI-digested pFastbac. This resulted in the insertion of 24 amino acids of FMDV with a GFP–2A–MP junction sequence of CCCAGATCCCTATG (BglIII/BamHI fusion in bold type) where auto-proteolysis would leave four amino acids (PKIP) at the N terminus of the MP (Fig. 1a). Immunoblot analysis of proteins extracted 48 h p.i. from insect cells infected with bvGFP–2A–MP, showed that the fusion protein (M, 66 kDa) cleavage by 2A was about 80% efficient, generating free MP (M, 46 kDa; Fig. 3a) and GFP with a C-terminal addition of 23 amino acids (M, 29 kDa; Fig. 3b). When insect cells were examined for GFP fluorescence, long tubules were seen (Fig. 3c). A similar number of tubules was seen when the insect cells were immunostained for MP distribution (Fig. 3d).

This work has shown that, in contrast to examples published for MPs from some other plant viruses (Oparka et al., 1996), MP fused to GFP cannot be used directly as a marker for CaMV MP function. The property of the CaMV MP to form tubules means that the ordered aggregation of the MP may be hindered by the presence of large additional peptides on the MP N or C termini. However, we have shown that this hindrance can be overcome by the addition of unfused MP into the same cell, either by co-expression of GFP–MP (or MP–GFP) and MP, or by proteolytic release of MP from a GFP–2A–MP fusion. This observation indicates that there are steric limitations in the self-association of the MP. This steric restriction could potentially occur at any point in the intracellular pathway leading to tubule formation (e.g. intracellular translocation, plasma membrane interactions etc.) which depends upon MP:MP interactions. However, it is perhaps most likely to occur during the organized aggregation of the MP into a tubule at the cell surface. In the insect cell model system, we will be able to use either strategy for the co-accumulation of GFP–MP fusion and MP to follow the cell biological processes underlying tubule formation. Considering the difficulty in expressing foreign sequences from the CaMV genome (Fütterer et al., 1990), it may be necessary to express GFP–MP or GFP–2A–GFP transgenically to study the function of the MP within the context of a full infection.

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References


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GFP reporter of virus movement proteins