A DNA-shuffled movement protein enhances virus transport by evasion of a host-mediated degradation pathway

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In the preceding report, we described a viral movement protein (MP) that was improved in its transport functions through the technique of DNA shuffling. *Tobacco mosaic virus* (TMV) vectors expressing the shuffled MP *(shuff3*MP*),* moved from cell to cell at rates that were substantially greater than the same vectors expressing a wild-type MP *(wt*MP*).* We were curious to determine the nature of the improved performance of *shuff3*MP clones, and designed a series of experiments to examine the subcellular basis of improved MP function. This work was carried out in collaboration with Biosource Genetics Corporation as part of an ongoing programme aimed at understanding and improving the functions of plant viral vectors.

The TMV movement process

TMV is a single-stranded RNA virus capable of local and long-distance movement. Local movement occurs through plasmodesmata, which become transiently enlarged ('gated') by the 30 kDa viral MP to allow the intercellular passage of the viral genome[1]. The TMV movement complex most likely exists in the form of an elongated ribonucleoprotein complex, the size and structure of which is compatible with the gated plasmodesmal pore. In order to reach the next cell, the MP-viral RNA complex must be transported from the subcellular site of replication to, and through, plasmodesmata. This movement is thought to occur on microtubules (MT), as MP shows a conspicuous association with MT during the viral replication cycle[2]. Once the viral genome has passed into new cells, the replication and movement cycles are repeated. Unlike other viral proteins, the MP of TMV is expressed transiently in infected cells and undergoes a cycle of synthesis and degradation at the leading edge of an infection site[3].

To study the subcellular behaviour of the TMV MP, we made translational fusions of the green fluorescent protein (GFP; from *Aequorea)* or the red fluorescent protein (DsRed; from *Discosoma)* to *wt*MP and *shuff3*MP. We then inoculated plants with viral vectors expressing these fluorescent proteins onto plants and studied their subcellular localisation using confocal laser scanning microscopy (CLSM). To aid in the identification of subcellular organelles, we inoculated viral vectors expressing DsRed fusions onto transgenic *Nicotiana benthamiana* plants expressing GFP either in the endoplasmic reticulum *(er*GFP*;* ex. Baulcombe) or fused to the cytoskeletal protein α-tubulin *(tua*GFP*;* constructed at SCRI by S. Mitchell).

wt MP is transferred from ER to MT during infection

During early infection of cells with vectors expressing *wt*MP-DsRed, we noticed that the *wt*MP became associated with punctate cortical bodies at the leading edge of expanding infection sites (Fig. l). On *er*GFP plants, the *wt*MP-DsRed fusion was seen to accumulate at the cisternal vertices of the cortical ER network (Fig, 1A). In cells immediately behind the infection

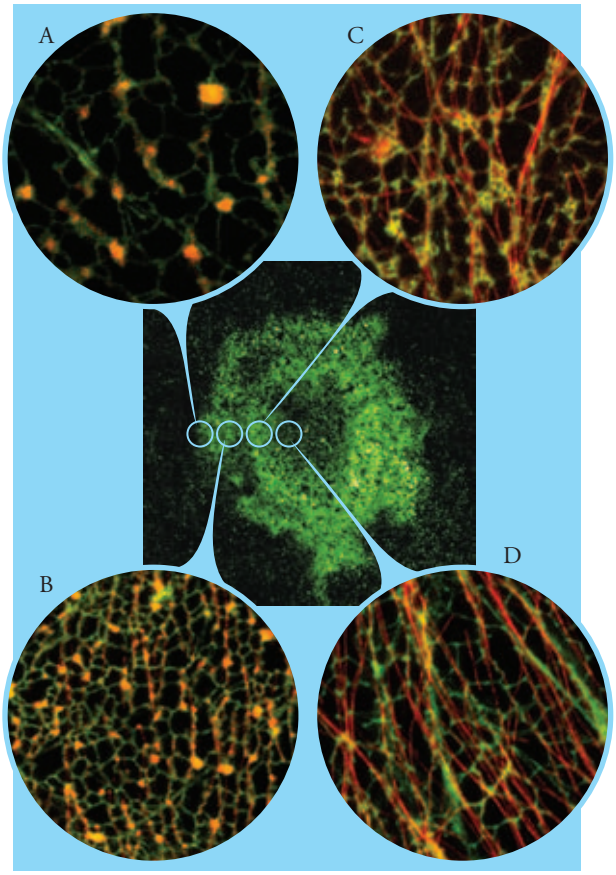

Figure 1 A *wt*MP infection site has a characteristic ring-like structure due to a cycle of MP synthesis and degradation (centre). At the leading edge of infection (A) *wt*MP (red) is localised to the vertices of the cortical ER network (green). Behind the infection front (B), the MP causes a distortion of the ER network. Subsequently, the *wt*MP is transferred onto microtubules (MT; red in C). After transfer, the ER resumes its polygonal shape (D).

front, these cortical bodies began to enlarge, and in older, infected cells the *wt*MP induced a transient deformation in the cortical ER network, causing ER tubules to become elongated in places, reminiscent of a fishing net being stretched between specific points (Fig. 1B). Subsequently, the fluorescent *wt*MP was passed onto underlying MT, causing these structures to fluoresce intensely (Fig. 1C). Confirmation that the underlying structures were MT was obtained by inoculating *tua*GFP plants with viral vectors expressing *wt*MP-DsRed (data not shown). In yet older infected cells (i.e., those towards the centre of an infection site), most of the *wt*MP was transferred from ER onto underlying cortical MT and, in these cells, the ER had once again resumed its normal, polygonal appearance (Fig. ID). In cells at the very centre of an infection site, most of the *wt*MP was degraded, giving the infec-

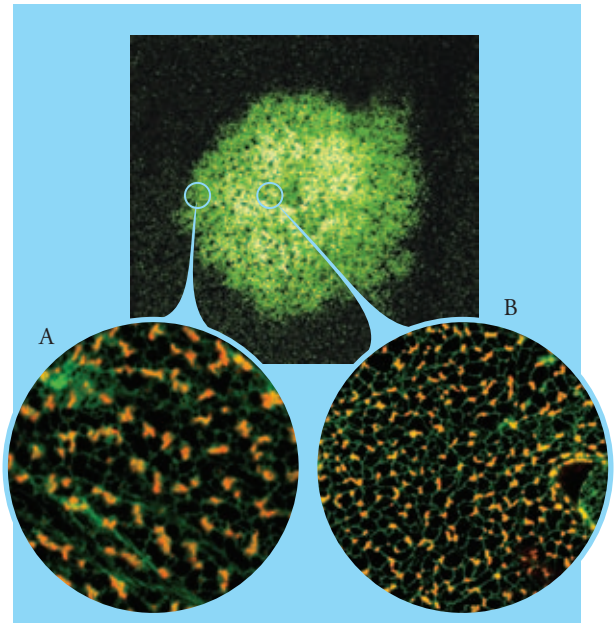

Figure 2 *shuff*MP infection sites are uniformly fluorescent throughout due to a lack of MP degradation (centre). At the infection front (A), *shuff*MP (red) localises to cortical ER (green). At the centre of infection (B), *shuff3*MP remains on the ER, without transfer to MT.

tion sites a 'ring'-like appearance (centre image, Fig.1). These observations suggest that during infection with TMV, *wt*MP is transferred from cortical ER to MT as part of the ongoing viral infection process.

*shuff*3MP fails to transfer from ER to MT

Next, we examined the behaviour of *shuff*MP-DsRed expressed from an identical TMV vector. The initial stages of infection were similar to those observed with *wt*MP, i.e., *shuff3*MP became attached to the vertices of the cortical ER network (Fig. 2A). However, in marked contrast to the *wt*MP, *shuff3*MP aggregates continued to enlarge at these sites without transfer to MT (Fig. 2B). Furthermore, the ER network did not undergo the distortion associated with *wt*M*P* expression, and retained its polygonal appearance throughout infection At the very centre of the infection site, *shuff3*MP was not degraded and remained associated with the cortical ER. These data suggest that successive rounds of DNA shuffling had induced changes in the *shuff3*MP that prevented the transfer of MP from ER to MT.

A single amino acid change prevents the transfer of *shuff*3MP from ER to MT

As the *shuff3*MP contains a number of coding (stuctural) and non-coding (silent) nucleotide changes that confer improved stability (see preceding report), we

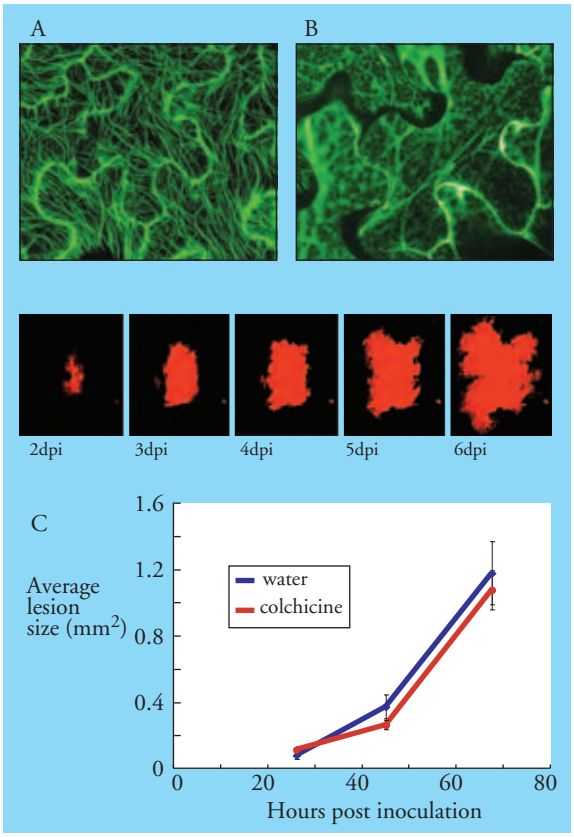

Figure 3 *Tua*-GFP transgenic plants before (A) and after (B) treatment with the MT-depolymerising drug, colchicine. In the presence of colchicine, infection sites continued to expand at the same rate as water-infiltrated controls (C). Inset shows growth of a single infection site in colchicine).

made fusions of GFP to clones containing the individual mutations. Clones expressing only the silent changes showed a MT-localisation phenotype identical to the *wt*MP (data not shown). However, the single amino acid coding change L-72-V was found to be responsible for the lack of MT association characteristic of the *shuff3*MP. This amino acid change, and its effects on the stability and function of the MP, are currently the subject of further studies in our laboratory.

MT are dispensable for TMV movement

MT have been suggested to play a central role in the TMV cell-cell movement process[2]. We were surprised that, given its ability to improve vector movement, *shuff3*MP did not strongly associate with MT at the leading edge of the infection. We therefore questioned whether MT are an absolute requirement for cell-cell transport of TMV. Leaves of transgenic plants expressing *tua*GFP (Fig.3A) were exposed to colchicine, a MT -depolymerising drug, to determine concentrations that would depolymerise the MT cytoskeleton, without affecting other cell functions. At O.5mM colchicine, the MT cytoskeleton was completely depolymerised while cytoplasmic streaming was unaffected (Fig. 3B). When viral vectors expressing either *wt*- or *shuff3*MP fusion proteins were inoculated onto colchicine-treated tissues, these vectors moved unimpeded from cell to cell (Fig. 3C), demonstrating that an intact MT cytoskelton is not required for normal TMV movement functions.

Accumulation of *shuff3*MP improves viral transport functions

The failure of *shuff3*MP to transfer from cortical ER to MT suggests that MT might function as part of a degradation pathway for viral MP. As inhibition of the ER-MT step led to accumulation of *shuff*MP on the ER, we examined the relative levels of MP accumulation in protoplasts transfected with either *wt*- or *shuff3*MP clones. As expected, increased levels of stable MP were detected in *shuff3*MP clones (Fig.4). Furthermore, the same amino acid change (L-72-V) responsible for inhibiting ER-MT transfer, was shown to be responsible for the increased accumulation of *shuff3*MP in protoplasts. These data suggest that blockage of part of the host degradation pathway for viral MP leads to accumulation of stable MP in infected cells.

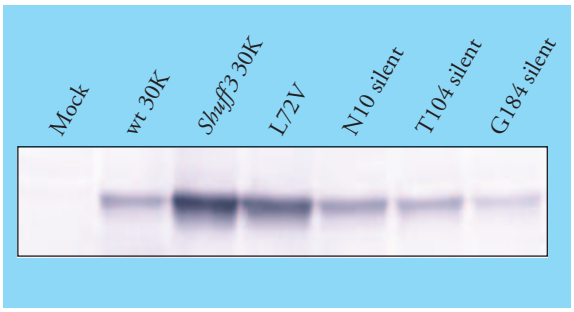

Figure 4 The L-72-V coding change, characteristic of all *shuff*MP clones, causes MP to accumulate in transfected protoplasts. Silent changes have no effect on MP accumulation.

We next examined whether two known functions of viral MPs, namely plasmodesmal gating and viral RNA trafficking, were improved in the *shuff3*MP clones. To examine plasmodesmal gating, we biolistically bombarded plasmids expressing GFP (M_r 27 kDa) under control of the 35S promoter into single leaf epidermal cells with and without plasmids expressing *wt*- or *shuff3*MP-DsRed. These data are shown in Figure 5. In control leaves (no MP expres-

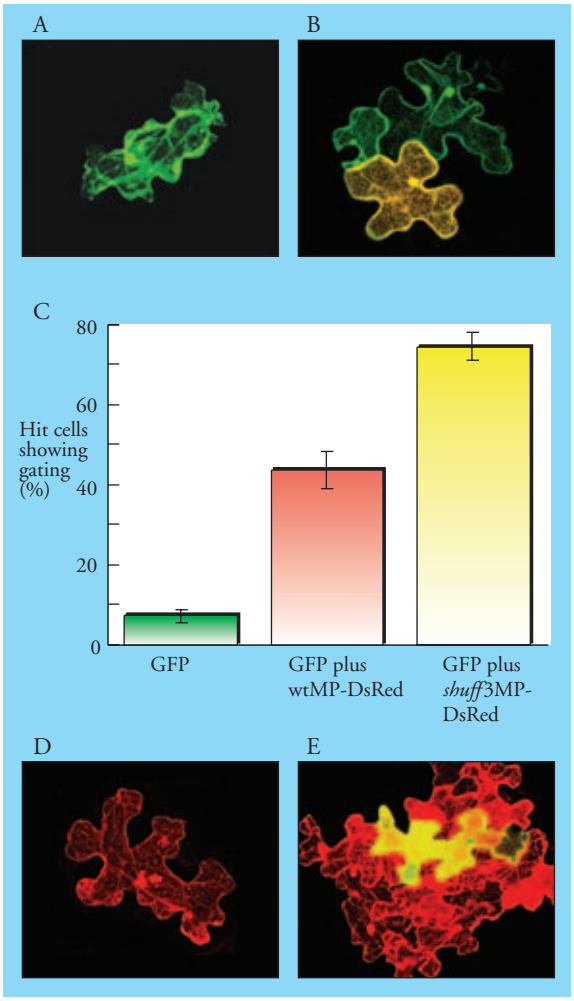

Figure 5 *shuff*MP improves both plasmodesmal gating (A-C) and viral RNA trafficking (D,E) compared to controls. To assess gating, MP was co-bombarded into single cells along with free GFP (27kDA; A-C)). To assess viral RNA trafficking, RNA transcript of a MP-defective virus was bombarded into single epidermal cells in the absence (D) or presence (E) of viral MP.

sion), GFP showed restricted trafficking to neighbouring cells (Fig. 5A), a result confirming the low size exclusion limit of source-leaf plasmodesmata (*Annual Report, 1999, 76-79*[4]). In the presence of *wt*MP, plasmodesmal gating was increased significantly above controls, as seen by the number of bombarded cells showing movement of GFP (Fig. 5C). However, values for plasmodesmal gating induced by *shuff3*MP were approximately double those of the *wt*MP, indicating substantial improvement in this viral MP function (Fig. 5B,C). To examine the capacity of *wt*- and *shuff3*MP to traffic viral RNA, we constructed a movement-defective viral vector expressing DsRed as a cytosolic protein (TMV. *FS*MP.DsRed). We then co-bombarded this vector into single cells with or without plasmids expressing *wt*- or *shuff3*MP*s* (expressed as GFP fusions). As expected, TMV.*FS*MP.DsRed failed to move from cell to cell (Fig. 5D). In the presence of the *wt*MP, viral RNA trafficking was rescued, and replicating virus was detected in epidermal cells outside the bombarded cell (data not shown). However, *shuff3*MP rescued viral RNA trafficking from a greater number of bombarded cells, and over a greater number of cell boundaries, than the *wt*MP (Fig. 5E).

Does *shuff3*MP evade a host degradation pathway?

The improved viral MP functions described above were consistent with the hypothesis that levels of functional *shuff3*MP were enhanced by evasion of a MP degradation pathway. The L-72-V coding change that leads to loss of MT association, and subsequent accumulation of *shuff3*MP, does not occur in regions of the MP known to be involved in plasmodesmal gating and viral RNA trafficking, suggesting that evasion of MP degradation led directly to increased MP levels, and indirectly to improved MP functions. Recently, it was shown that the TMV MP becomes ubiquinated prior to degradation by the 26S proteasome pathway[5]. The proteasome is a subcellular protein complex in animals and plants that is involved in the degradation of abnormal proteins, allowing amino acids to be recycled by the cell. Ubiquitin tagging is a prerequisite for this pathway, allowing the aberrant protein to be recognised and processed by the 26S proteasome. Our data are consistent with a model in which the *wt*MP is mobilised from the viral replication 'factories' established on the cortical ER prior to degradation. Instead of playing a role in cell-cell transport of the viral genome through plasmodesmata, we suggest that MT function as part of the host degradation pathway, perhaps in redistributing excess MP when the proteasome is saturated.

Epilogue

Three rounds of DNA shuffling led to the improvement of viral movement functions of TMV -based vectors. Intuitively, we expected the altered coding changes that conferred improved cell-cell movement to have occurred in regions of the viral MP known to have roles in viral transport, such as plasmodesmal gating and viral RNA trafficking. However, the most significant coding change induced by random DNA shuffling was associated with a stage of the MP 'life cycle' characterised by degradation rather than movement, an area of viral infection that has received

almost no attention. Our data emphasise that the viral replication cycle involves a complex interplay between viral and host factors, the balance of which determines the success of viral spread. The fact that genetically modified vectors based on TMV, but not *wt*TMV itself, are improved in their movement functions (see preceding report) suggests that *wt*TMV, in an evolutionary sense, is extremely 'fine tuned' to its host in terms of cell-cell movement. However, the burden of an increased genetic load on the viral vector leads to reduced stability and movement, facets of the infection process that apparently can be enhanced. We are therefore encouraged that viral vectors might be improved further in desirable traits through random DNA shuffling rather than by rational design.

References.

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