Long-distance movement of viral RNAs in the absence of coat protein

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Communication between cells is a fundamental process underlying many aspects of plant growth and development and the effects of viral pathogens on plants. It is believed that not only low molecular weight metabolites but also macromolecules, including mRNA, can move from cell to cell through plasmodesmata and systemically *via* the phloem system. Plant viruses move cell-to-cell and over long distances by exploiting and modifying these pre-existing endogenous pathways for macromolecular movement. For some time, it has been known that viruses produce specialized virus-encoded movement proteins (MP). Some viruses encode single MPs that modify plasmodesmata and facilitate transport of the viral nucleic acid, together with the MP itself, through the modified channel. Others contain a set of genes called the 'triple gene block', which encodes three proteins that, together with the viral coat protein (CP), are proposed to function coordinately to transport viral RNA through plasmodesmata. Less is known about the molecular details of long-distance movement, although there is evidence of the need for specific virus factors, different from those involved in cell-to-cell movement. With only a few exceptions, CP is essential for efficient long-distance transport of plant viruses.

Recent work has revealed some interesting information about the long-distance movement of two very different viruses, *Groundnut rosette virus* (GRV) and *Potato mop-top virus* (PMTV). GRV does not encode a CP, but nevertheless accumulates and spreads systemically within infected plants. PMTV encodes a triple gene block, and although it accumulates and moves systemically in *Nicotiana benthamiana*, its movement and systemic spread in potato seems poor. Often PMTV does not invade every stem on an individual potato plant and a proportion of progeny tubers produced on an infected plant are virus-free. Furthermore, the virus may be passed to only 20% of plants grown from infected tubers.

Characterizing PMTV behaviour in resistant transgenic plants The genome of PMTV comprises three single-stranded positive-sense RNA molecules; RNA 1 (6.5 kb) encodes the replicase functions, RNA 2 (3 kb) encodes the triple gene block proteins and a cysteine-rich protein of unknown function, and RNA 3 (2.3 kb) encodes the coat protein (CP) along with a possible readthrough protein (thought to be involved with vector transmission) which is expressed by leaky termination at the CP gene stop codon.

Challenge inoculum of PMTV does not multiply to produce symptoms or infective virus particles in transgenic *N. benthamiana* that express a translatable version of the PMTV CP gene[1]. In experiments made to understand the mechanisms underlying this resistance, RNA extracts were prepared from transgenic plants that had been inoculated with PMTV particles and analysed by Northern blotting using specific probes to PMTV RNAs 1, 2 and 3. Surprisingly, it was found that RNAs 1 and 2 accumulated in inoculated and systemically infected tissues to levels that were similar to those found in PMTV-infected wild-type plants[2]. However, neither genomic RNA 3 (approx. 2300 nucleotides) nor virus particles were found in the transgenic plants, although small amounts of CP transgene RNA transcript (650-700 nucleotides) were detected.

Accumulation and systemic movement of PMTV RNAs 1 and 2 in non-transgenic plants RNA extracts, prepared from transgenic plants containing RNAs 1 and 2, were inoculated to non-transgenic *N. benthamiana* and *N. clevelandii*. Total RNA extracts from systemically infected leaves of both species contained RNA 1 and RNA 2, which

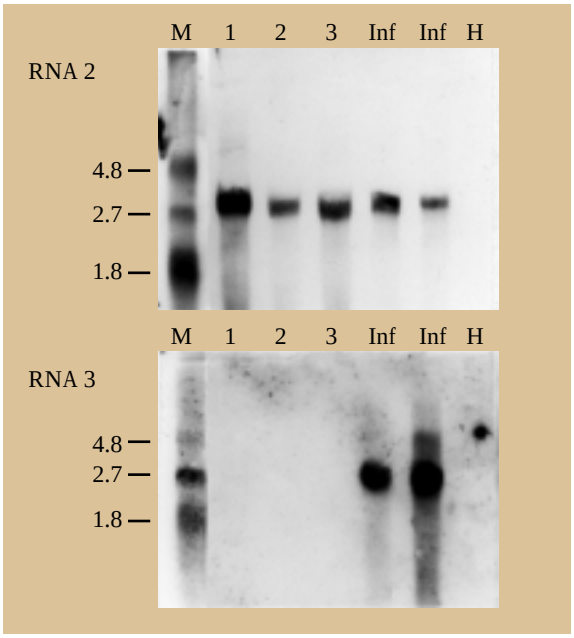

Figure 1 Northern blot analysis of systemic leaf RNA extracts from: three WT *N. benthamiana* plants (lanes marked 1-3) inoculated 22 days previously with RNA extracts from WT RNA 2 +/RNA 3 – plants; two infected WT *N. benthamiana* plants (lanes marked 'Inf'); a virus-free healthy plant of *N. benthamiana* (lane marked 'H'). RNA was run on two gels and blots were treated with specific probes to RNA 2 and RNA 3. The positions of genomic length PMTV RNA 2 and RNA 3 (3×10^3 and 2.3×10^3 respectively) are shown. Marker RNA is in lane 'M'.

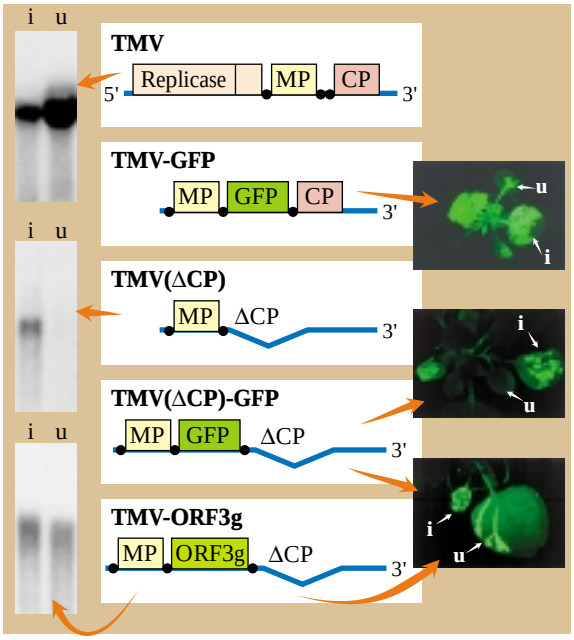

Figure 2 Long-distance movement in *Nicotiana benthamiana* of TMV derivatives. Left panel: Northern blot detection of viral RNA in inoculated (i) and upper uninoculated (u) leaves of plants inoculated with (left to right) unmodified TMV, TMV(ΔCP), and TMV(ORF3g). Right panels: fluorescence due to GFP expression in plants inoculated with (top to bottom) TMV-GFP, TMV(ΔCP)-GFP and TMV(ΔCP)-GFP+TMV(ORF3g).

had replicated in the absence of RNA 3. Because RNA 3 was absent from the infected plants, no coat protein or virus particles were produced. Symptoms did not develop. These data (illustrated in Figure 1) provide evidence that long-distance spread of infective PMTV RNA can occur in the absence of CP or virions.

Systemic spread of TMV(ORF3g), a hybrid TMV in which GRV ORF3 replaces the CP gene The RNA genome of GRV contains four open reading frames (ORFs), none of which encodes a CP. The two ORFs nearest the 3' end of the RNA (ORF3 and ORF4) overlap each other in different reading frames. ORF4 encodes a 28 K cell-to-cell MP which exhibits high similarity with MPs of several other viruses, which localises to plasmodesmata and which can functionally replace MPs of such unrelated viruses as *Cucumber mosaic virus* (CMV) or *Potato virus X*[3, 4]. Database searches with amino acid sequences of the 27 K ORF3 protein detected no significant similarities to any other known viral or non-viral proteins[5]. To analyze the function of the ORF3 protein, we employed a gene replacement strategy to generate hybrids between *Tobacco mosaic virus* (TMV) and GRV[6]. CP is not required for cell-to-cell movement of TMV but is essential for its long-distance movement. The CP gene of TMV was deleted and replaced by ORF3 of GRV to give the hybrid TMV(ORF3g) (Fig. 2). A TMV mutant with the CP gene deleted, TMV(ΔCP), was used as a control (Fig. 2).

Both viruses were inoculated to *N. benthamiana* plants, and total nucleic acids extracted from inoculated and upper uninoculated leaves. Tests for infectivity on the hypersensitive host, *N. tabacum* cv. Xanthi NN, and Northern blot analysis, both confirmed that TMV(ORF3g) RNA spread systemically in *N. benthamiana* plants. In contrast, TMV(ΔCP) spread from cell to cell in inoculated leaves but, as expected, did not move systemically. Dot blot hybridization experiments detected TMV(ORF3g) RNA in mesophyll protoplasts isolated from uninoculated systemically-infected leaves, demonstrating that it is able not only to move from inoculated to uninoculated leaves, but also to exit from the vascular system in uninoculated leaves.

Complementation of the long-distance movement defect of the TMV CP deletion mutant by TMV(ORF3g) Green fluorescent protein (GFP) is often used as a non-invasive reporter to monitor virus infections. However, attempts to generate infectious TMV derivatives expressing both GRV ORF3 protein and GFP were unsuccessful. Therefore, complementation of the long distance movement defect of TMV(ΔCP) by GRV ORF3 protein was tested using a TMV derivative in which the CP gene was replaced by the gene encoding GFP. As expected, this virus, TMV(ΔCP)-GFP (Fig. 2), was able to develop fluorescent foci 3 days post-inoculation in inoculated leaves but did not cause any systemic infection. However, when TMV(ΔCP)-GFP was co-inoculated with TMV(ORF3g) onto *N. benthamiana*, the majority of the doubly-infected plants showed systemic symptoms characteristic of TMV(ORF3g) and developed green fluorescent zones in both inoculated and uninoculated leaves, implying systemic spread of TMV(ΔCP)-GFP in the presence of TMV(ORF3g). The first indication of entry of TMV(ΔCP)-GFP into an uninoculated leaf in this case, was the appearance of fluorescent flecks along veins on the lamina, indicating that the virus was being unloaded at discrete points. Subsequently, some leaf veins became more clearly delineated by continuous fluorescence, and, with time, the mesophyll tissues neighbouring the flecks also became labelled. Confocal laser scanning microscopy confirmed these observations and showed that up to 90% of mesophyll cells in the fluorescent area were infected with TMV(ΔCP)-GFP. The time of appearance of GFP fluorescence (about 8 days post-inoculation) and the pattern of virus unloading in uninoculated leaves observed in mixed TMV(ΔCP)-GFP + TMV(ORF3g) infections were similar to those observed for TMV-GFP (i.e. 'normal' TMV with the GFP gene added (Fig. 2)) and corresponded to the usual manner of vascular-associated long-distance virus movement described for other viruses.

These results show that the GRV ORF3 protein can mediate the long-distance movement of RNA of the unrelated virus, TMV, both *in cis* in the form of TMV(ORF3g) and *in trans* in the form of TMV(ΔCP)-GFP. Thus, it seems likely that the role of the ORF3 protein in GRV is to mediate systemic movement of GRV RNA.

Conclusion CP is essential for efficient and rapid long-distance transport of most viruses. There are a few exceptions in which CP is dispensable for systemic infection. Members of the genus *Umbravirus*, such as GRV, represent a special situation because they do not encode a CP, but accumulate and spread systemically very efficiently within infected plants. Functional analysis of GRV ORF3 protein suggests that it is a long-distance RNA movement factor that can act *in cis* or *in trans*. In chimeric TMV, it can functionally replace CP which is critical for long-distance spread of TMV. PMTV is an example of a small group of viruses which possess a CP, but in which systemic movement is not dependent on CP expression or virion formation.

Thus, although PMTV and GRV are very different viruses, their RNA genomes share the ability to enter the vasculature and move long distances within the plant. The triple gene block proteins of PMTV and the ORF3 protein of GRV could represent a class of long-distance RNA movement factors.

Several other plant virus proteins, such as the 2b protein encoded by CMV or the HC-Pro protein encoded by potyviruses, have also been shown to be involved in systemic virus spread. These proteins can suppress post-transcriptional gene silencing and it is suggested that they act by blocking a potential host-defence mechanism (akin to gene silencing) that restricts systemic spread rather than by promoting the process of long-distance movement itself. In accordance with this suggestion, the CMV 2b or *Potato virus Y* (potyvirus) HC-Pro proteins were unable to replace functionally TMV CP[6]. Thus, these proteins are distinct from the class of long-distance movement factors represented by the GRV ORF3 protein and the PMTV triple gene block proteins.

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