Cell-to-cell Transport of Cowpea Mosaic Virus Requires Both the 58K/48K Proteins and the Capsid Proteins

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SUMMARY

Insertions and deletions have been introduced into an infectious cDNA clone of M RNA of cowpea mosaic virus (CPMV), in the coding regions of the 58K/48K and capsid proteins. Transcripts derived from these mutant clones appeared to be replicated in cowpea protoplasts as detected by immunofluorescent staining and Northern blotting. However in cowpea plants, mutations in either region restricted the replication of the viral RNAs to the inoculated cells and thus prevented a successful systemic infection of the plant. These results indicate that the M RNA-encoded 58K/48K proteins are involved in cell-to-cell transport of CPMV, and that the virus can spread only if the RNA is encapsidated in particles.

INTRODUCTION

Cowpea mosaic virus (CPMV) has a genome consisting of two positive-sense ssRNA molecules. Both RNAs (M, middle component; B, bottom component) are translated into polyproteins which are cleaved by a B RNA-encoded protease into functional proteins (Goldbach & van Kammen, 1985; Vos et al., 1988a). B RNA is able to replicate independently of M RNA in isolated protoplasts and encodes all the functions necessary for replication of the RNAs (Goldbach et al., 1980; Eggen & van Kammen, 1988). However for a successful infection of plants, M RNA is essential. Therefore it probably encodes proteins involved in transport of the virus (Rezelman et al., 1982).

M RNA is translated in vitro into two polyproteins, 105K and 95K (see Fig. 1), which have overlapping amino acid sequences. The translation of the smaller polyprotein is initiated at an internal AUG codon (Vos et al., 1984). Proteolytic processing of the polyproteins yields 58K and 48K proteins, and the 60K precursor to the capsid proteins (Fig. 1; Franssen et al., 1982). Because M RNA expression is necessary for plants to become infected, it was postulated that the 58K and/or 48K proteins were involved in cell-to-cell transport of the virus (Rezelman et al., 1982). The occurrence of the 48K protein in the membrane fraction of infected leaves supported this notion (Wellink et al., 1987). Furthermore, the 105K, 95K and 58K proteins have been detected in protoplasts inoculated with CPMV (G. Rezelman, A. van Kammen & J. Wellink, unpublished results) which shows that the same proteins are produced in vivo as in vitro.

A full-length cDNA clone of M RNA has been obtained which produced infectious RNA by in vitro transcription (Vos et al., 1988b; Eggen, 1989). To test the involvement of the M RNA-encoded proteins in cell-to-cell transport of the virus, this cDNA clone has been used to create insertion and deletion mutants in the coding regions of the 58K/48K proteins and the capsid proteins. The effects of these mutations on infectivity was studied in cowpea protoplasts and cowpea plants.

METHODS

Construction of mutant M cDNA clones. (See Fig. 2.) pTMABgl and pTMANco containing four nucleotide insertions in the M cDNA region were constructed by filling in the protruding ends left after digestion of pTMIG (Eggen, 1989) with BglII and NcoI respectively, followed by ligation of the blunt ends. pTMAXho and pTMΔEco

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Fig. 1. Model for the expression of CPMV M RNA. The ORF on the RNA is indicated by an open bar and the positions of the translational start and stop codons are indicated on this bar. VPg is represented by a black square and the other proteins by single lines below. Both capsid proteins are indicated by thick lines. Gln/Met and Gln/Gly cleavage sites are indicated by solid arrowheads and open arrowheads, respectively.

containing deletions in the M cDNA region were created by digestion of pTMIG with XhoI or EcoRV respectively, followed by ligation of the plasmids. This resulted in loss of the small XhoI or EcoRV fragments respectively. Two other plasmids containing deletions in the M cDNA region, pTMAP and pTMAB, were constructed by digestion of pTMIG with AccI and PstI, or BglII and BamHI respectively, filling in of the protruding ends and subsequent ligation of the blunt ends.

In vitro transcription and translation. In vitro transcription of the M cDNA clones with T7 RNA polymerase (New England Biolabs) was as described by Vos et al. (1988a). The transcripts were translated in rabbit reticulocyte lysates in the presence of [35S]methionine for 1 h at 30°C (Vos et al., 1988a). In addition, processing of
the translation products was carried out with 3 volumes of non-labelled translation products of CPMV RNA for 6 h at 30 °C. The proteins were separated in a 10% polyacrylamide gel (Richards et al., 1989).

**Inoculation of cowpea protoplasts and plants.** Cowpea protoplasts were isolated and inoculated with RNA using polyethylene glycol as described by Eggen (1989).

For inoculation of cowpea plants the upper epidermises of primary leaves of 9 day-old plants were dusted with carborundum powder. Five μg of the pTBIG transcript together with 5 μg of the pTMIG or mutant M transcript (in 30 μl H2O) were rubbed on the leaves with a gloved finger. The plants were grown at 28 °C under continuous illumination.

**Analysis of protoplasts.** At 42 h post-inoculation (p.i.) protoplasts were collected for immunofluorescent staining by centrifugation. Anti-24K serum was used to determine the amount of cells infected with B RNA, and anti-CPMV or anti-48K serum (Wellink et al., 1987) was used to detect cells infected with M RNA and producing M RNA-specific proteins as described by Vos et al. (1988b). For Northern blot analysis protoplasts were collected by centrifugation at 68 h p.i. and the pellets were frozen in liquid nitrogen. The RNA was extracted from the protoplasts with phenol and precipitated from 2 M-LiCl as described by de Vries et al. (1982). The RNAs were glyoxalated and separated in a 1% agarose gel, blotted onto GeneScreen and hybridized with an M RNA-specific 32P-labelled probe according to instructions supplied by the manufacturer (New England Nuclear). The probe comprised the SalI/BamHI fragment of pTMIG.

**Analysis of plants.** Six days p.i. the primary leaves were homogenized in 3 ml/g HB buffer (50 mM-Tris-acetate pH 7.4, 10 mM-potassium acetate, 1 mM-EDTA, 5 mM-2-mercaptoethanol, 0.5 mM-PMSF) and filtered through two layers of Miracloth (Calbiochem). The filtrate was subjected to a low-speed centrifugation to remove debris and the supernatant fraction was assayed for CPMV-specific proteins by immunoblot analysis using anti-24K serum and anti-VP23 (coat protein) serum (Richards et al., 1989).

### RESULTS

**Construction of mutant M RNAs**

We have used the full-length cDNA clone pTMIG to introduce deletions and small insertions into the open reading frame (ORF) of M RNA. In vitro transcription of these cDNA clones resulted in several mutant M RNAs. MANco RNA and MAEco RNA contained mutations in the coding region of the capsid proteins created by an insertion of four nucleotides into the coding region of VP23 and a deletion of most of the coding region of VP23 and part of VP37, respectively (Fig. 2 and 5). MAXho RNA lacks the coding region for the glutamine/methionine cleavage site between the 58K/48K proteins and the 60K precursor to the capsid proteins as a result of the deletion of a XhoI fragment. MAP, MAB and MABgl RNAs have mutations in the coding region of the 58K/48K proteins created respectively by a 486 nucleotide deletion in the 48K protein encoding region, a 1311 nucleotide deletion in the 58K protein encoding region and a four nucleotide insertion disrupting only the coding region of the N-terminal end of the 58K protein (Fig. 2 and 4).

To examine whether the ORFs in the deletion mutants were disrupted and frameshifts were present at the correct position in the insertion mutants, the mutant RNAs were first analysed by in vitro translation in rabbit reticulocyte lysates. As expected the mutant that lacked the 48K protein encoding region (MAB) produced only one polyprotein (Fig. 2 and 3). MABgl RNA, containing an insertion in the 58K protein encoding region, was expected to produce only the 95K polyprotein; however small amounts of a 110K protein were also generated (Fig. 3, lane 10). To explain the production of this protein it is necessary to assume that a frameshift occurs during translation, because the two AUG codons preceding the frameshift at positions 115 and 161 are out of frame with the MABgl RNA ORF (Van Wezenbeek et al., 1983). As expected, the other mutant RNAs produced two polyproteins.

All polyproteins could still be cleaved by the B RNA-encoded protease in an in vitro processing assay, except for the two polyproteins produced from MAXho RNA which lack the cleavage site (Fig. 3). The N-terminal cleavage product of the 88K polyprotein of MAP RNA was present in smaller amounts than expected (Fig. 3, lane 13). This truncated protein is probably very unstable and degraded rather rapidly.
Fig. 3. In vitro translations of the M RNA mutants. The translations were analysed in a 10% polyacrylamide gel. A plus sign (+) on top of each lane indicates that unlabelled translation products of CPMV RNA were added followed by incubation for 6 h at 30°C. Lane 1 contains the translation products of CPMV RNA.

**Infectivity of the mutant M RNA in cowpea protoplasts**

To determine whether the different M RNA mutants had lost their ability to be replicated, cowpea protoplasts were inoculated with transcripts from the full-length cDNA clone of B RNA, pTBIG, plus the M RNA mutant transcripts. The percentage of infected cells 42 h p.i. containing B and M RNA-encoded proteins was determined by immunofluorescent staining. The anti-24K serum was used to detect the protoplasts infected with B RNA; both anti-CPMV serum and anti-48K serum were used to detect protoplasts infected with M RNA. When 25 μg of the pTBIG transcript and 25 μg of the pTMIG transcript were used to inoculate 2.5 x 10⁶ protoplasts, about 20% of the living protoplasts became infected with B RNA and about 10% became infected also with M RNA. In Fig. 4 and 5 the percentage of infected cells found with the mutant M RNAs are shown, relative to the 10% found from infection with the non-mutagenized pTMIG transcripts. Almost all mutants were replicated as judged by the production of M RNA-encoded proteins in the protoplasts, with the exception of the MABgl mutant. Cells in which MAEco RNA was replicated could be identified only with anti-48K serum, and not with the anti-CPMV serum. The anti-CPMV serum probably cannot react with the truncated VP37 that is expressed in these cells. On the other hand, the severely truncated 58K protein that is expressed in MAB RNA-infected cells is still recognized by the anti-48K serum. In the case of the MABgl mutant, no fluorescent cells were detected in spite of the fact that in the previous in vitro translations the production of the 95K protein was not affected by the mutation.

For some mutants the replication of the M RNA was also examined by Northern blotting. At 68 h p.i., RNA was isolated from protoplasts inoculated with these mutants and analysed on a Northern blot using an M RNA-specific probe. This analysis showed that for these mutants, RNA of the expected size was found to hybridize with the M RNA-specific probe (Fig. 6). Also it was found that MABgl RNA was still replicated, although at a low level (Fig. 6, lane 2).
Proteins required for transport of CPMV

Fig. 4. Infectivity of M RNA mutated in the 58K/48K protein encoding region in cowpea protoplasts and plants. The infectivity of the pTMIG transcript in protoplasts is defined as being 100%. The position and the size of the deletion or insertion is shown for each mutant M RNA.

Fig. 5. Infectivity of M RNA mutated in the capsid proteins encoding region in cowpea protoplasts and plants. Details as for Fig. 4. ND, Not determined.

Infectivity of the mutant M RNAs in plants

Having established that the mutant M RNAs had not lost their capacity to be replicated in infected cells, the mutants were tested for their ability to spread in cowpea plants. Primary cowpea leaves were inoculated with 5 μg of the pTBIG transcript together with 5 μg of the pTMIG transcript or with a mutant M RNA. After 3 days, symptoms were visible on these leaves infected with pTMIG RNA; however, symptoms could not be detected with any of the mutant M RNAs, even after 6 days. To test whether virus-specific proteins were synthesized in
Fig. 6. Northern blot analysis of the M RNA mutants: ABgl, lane 2; AEco, lane 3; AP, lane 4; MIG, lane 5. Total RNA was extracted from protoplasts at 68 h p.i. and about 10 μg RNA was analysed on a glyoxal gel, except for lane 3 which contains about 5 μg RNA. Lane 1 contains RNA from non-infected protoplasts, and lane 6 contains RNA from protoplasts inoculated with 1 μg CPMV RNA. Lanes 1 to 4 were exposed for 16 h (with a screen) and lanes 5 and 6 for 3 h to a Kodak XAR film.

these plants crude extracts were prepared of the primary leaves 6 days p.i. and these extracts were analysed by Western blotting. In the leaves inoculated with the mutant M RNAs virus-species proteins were not detected (data not shown), whereas in leaves inoculated with non-mutant pTMIG RNA, virus-specific proteins were detected, and virus could be recovered from these leaves, as described by Eggen (1989).

DISCUSSION

The B RNA of CPMV is able to replicate independently in isolated protoplasts without M RNA being present, but for a successful infection of a whole cowpea plant, M RNA is indispensable (Goldbach et al., 1980; Rezelman et al., 1982). The availability of both the protoplast system and the plant to study the molecular biology of CPMV makes it possible to separate functions such as replication and transport. In this paper we have directed our attention to the latter function.

Although M RNA contains only one large ORF, two sets of proteins can be easily distinguished. These are the 58K and 48K putative transport proteins at the N terminus and both capsid proteins VP23 and VP37 at the C terminus of the 105K and 95K polyproteins. In this paper we have shown that both sets of proteins are necessary for a successful infection of a whole cowpea plant. With respect to the 58K and 48K proteins, this result was not unexpected. These proteins show a limited homology with the 30K transport protein of tobamoviruses (Meyer et al., 1986; Deom et al., 1987; Meshi et al., 1987). Furthermore the 48K protein is found in the membrane fraction of infected leaves (Wellink et al., 1987) and recent results with immunogold labelling of sections of infected leaves with anti-48K serum have revealed an association of this protein with tubular structures protruding from the cell wall (J. van Lent, unpublished results). These structures are found only in infected cells and are probably involved in cell-to-cell transport of the virus. The need for an intact coding region of the capsid proteins for successful infection of a plant is a strong indication that during a normal infection CPMV is transported as particles and not as naked RNA. This is supported by the observation that until now no coatless mutants of CPMV have been identified.
Proteins required for transport of CPMV

Malyshenko et al. (1988) have reported that it is possible to complement the transport of the B RNA of red clover mottle comovirus (RCMV) with sunn-hemp mosaic tobamovirus (SHMV). At first sight this result seems contradictory with our results because the B RNA of RCMV will probably not be encapsidated by the SHMV coat protein and therefore must be transported as naked RNA in this mixed infection. However a simple explanation would be to assume that the CPMV transport protein(s) transports (or facilitates the transportation of) encapsidated RNA only, whereas the tobamovirus transport protein can transport (or perhaps can transport only) naked RNA; tobacco mosaic tobamovirus coat protein is indeed dispensable for transport from cell to cell (Takamatsu et al., 1987).

The results with the deletion mutants of M RNA show that substantial parts of the coding region are dispensable for replication in cowpea protoplasts. The numbers of fluorescent protoplasts obtained by inoculation with the mutants cannot be taken as an exact measure of the replicability of the RNAs. The reason for this is that the truncated proteins may be less stable, or less reactive than wild-type proteins are with the antisera. Also with Northern blot analysis, differences between the mutants are caused not only by the differences in replicability of the RNAs but also by differences in stability of the RNAs and whether they are still encapsidated. Ideally, differences in replicability of the RNAs should be determined in an in vitro replication system, but so far all attempts to develop such a system for CPMV have failed (Eggen & van Kammen, 1988).

In CPMV-infected protoplasts, the 48K protein was found to be present in the membrane fraction (Wellink et al., 1987). Recently the 105K, 95K and 58K proteins have also been detected in protoplasts (G. Rezelman, A. van Kammen & J. Wellink, unpublished results). If M RNA is translated into the same polypeptides in vitro and in vivo, it is very likely that, in vivo, separate AUG codons are used to produce the 105K and 95K polypeptides as was shown to occur in vitro by Vos et al. (1984). In this respect the result with the MAbgl mutant in protoplasts is difficult to explain. In in vitro translations the 105K protein is no longer produced because of a four nucleotide insertion behind the start codon of this protein. As expected, the production of the 95K protein was not affected in vitro. In spite of this, translation products could not be detected in protoplasts inoculated with this mutant, although the RNA was replicated as revealed by Northern blotting.

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REFERENCES


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