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Engineering Cowpea Mosaic Virus RNA-2 into a Vector to Express Heterologous Proteins in Plants

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A series of new cowpea mosaic virus (CPMV) RNA-2-based expression vectors were designed. The jellyfish green fluorescent protein (GFP) was introduced between the movement protein (MP) and the large (L) coat protein or downstream of the small (S) coat protein. Release of the GFP inserted between the MP and L proteins was achieved by creating artificial processing sites each side of the insert, either by duplicating the MP-L cleavage site or by introducing a sequence encoding the foot-and-mouth disease virus (FMDV) 2A catalytic peptide. Eight amino acids derived from the C-terminus of the MP and 14–19 amino acids from the N-terminus of the L coat protein were necessary for efficient processing of the artificial Gln/Met sites. Insertion of the FMDV 2A sequence at the C-terminus of the GFP resulted in a genetically stable construct, which produced particles containing about 10 GFP-2A-L fusion proteins. Immunocapture experiments indicated that some of the GFP is present on the virion surface. Direct fusion of GFP to the C-terminus of the S coat protein resulted in a virus which was barely viable. However, when the sequence of GFP was linked to the C-terminus by an active FMDV 2A sequence, a highly infectious construct was obtained.

INTRODUCTION

As an alternative approach to stable genetic transformation, autonomously replicating plant viruses have been modified into vectors to express heterologous proteins in plants. The speed with which a virus infection becomes established throughout the plant and the high yield of virus-encoded proteins getting accumulated in plants make virus-based vector systems, and in particular those based on RNA viruses, an attractive and cost-effective route for the overproduction of valuable proteins in the plants (reviewed in Porta and Lomonossoff, 1996; Scholthof *et al.,* 1996). Infection with a virus-based vector is simpler and faster than transformation and subsequent regeneration.

The genome of cowpea mosaic virus (CPMV) is distributed over two different RNA molecules which are separately encapsidated. RNA-1 (5889 nt) and RNA-2 (3481 nt) are translated into polyproteins which are cleaved into 15 intermediate and final cleavage products at specific Gln-Gly, Gln-Ser, and Gln-Met sites by the 24-kDa viral proteinase encoded by RNA-1. All the proteins encoded by RNA-1 are involved in replication. RNA-2 is translated into two carboxy coterminal proteins (Fig. 1A) which are processed into large (L) and small (S) coat proteins, a 58-kDa protein which is probably involved in replication, and a 48-kDa movement protein (MP) (reviewed in Goldbach and Wellink, 1996). MP induces tubular structures in modified plasmodesmata, through which virus particles move from cell to cell (van Lent *et al.,* 1990, 1991). Similar tubules are also observed in the protoplasts infected with CPMV (van Lent *et al.,* 1991; Kasteel *et al.,* 1993). The capsids of CPMV contain 60 copies each of L and S coat proteins arranged with icosahedral symmetry (Lomonossoff and Johnson, 1991) and the structure of CPMV has been resolved to atomic resolution (Stauffacher *et al.,* 1987; Chen *et al.,* 1990).

The potential advantages that make CPMV an attractive vector system are that the virus grows extremely well in the host plants, attaining yields up to 1–2 g/kg of plant tissue, virus particles are thermostable, and their purification is straightforward. Until now, the exploitation of CPMV as a vector has concentrated on the expression of peptides on the surface of virus particles (Usha *et al.,* 1993; Porta *et al.,* 1994, 1996). In an earlier report, we have demonstrated that the green fluorescent protein (GFP) gene can be expressed from CPMV RNA-2, when it is introduced between the MP and L coat protein genes (Verver *et al.,* 1998). This was achieved by creating two artificial proteolytic cleavage sites by duplicating a coding region of 29 amino acids from the N-terminus of the L coat protein and 11 amino acids from the C-terminus of the MP. This construct was found to be as infectious as

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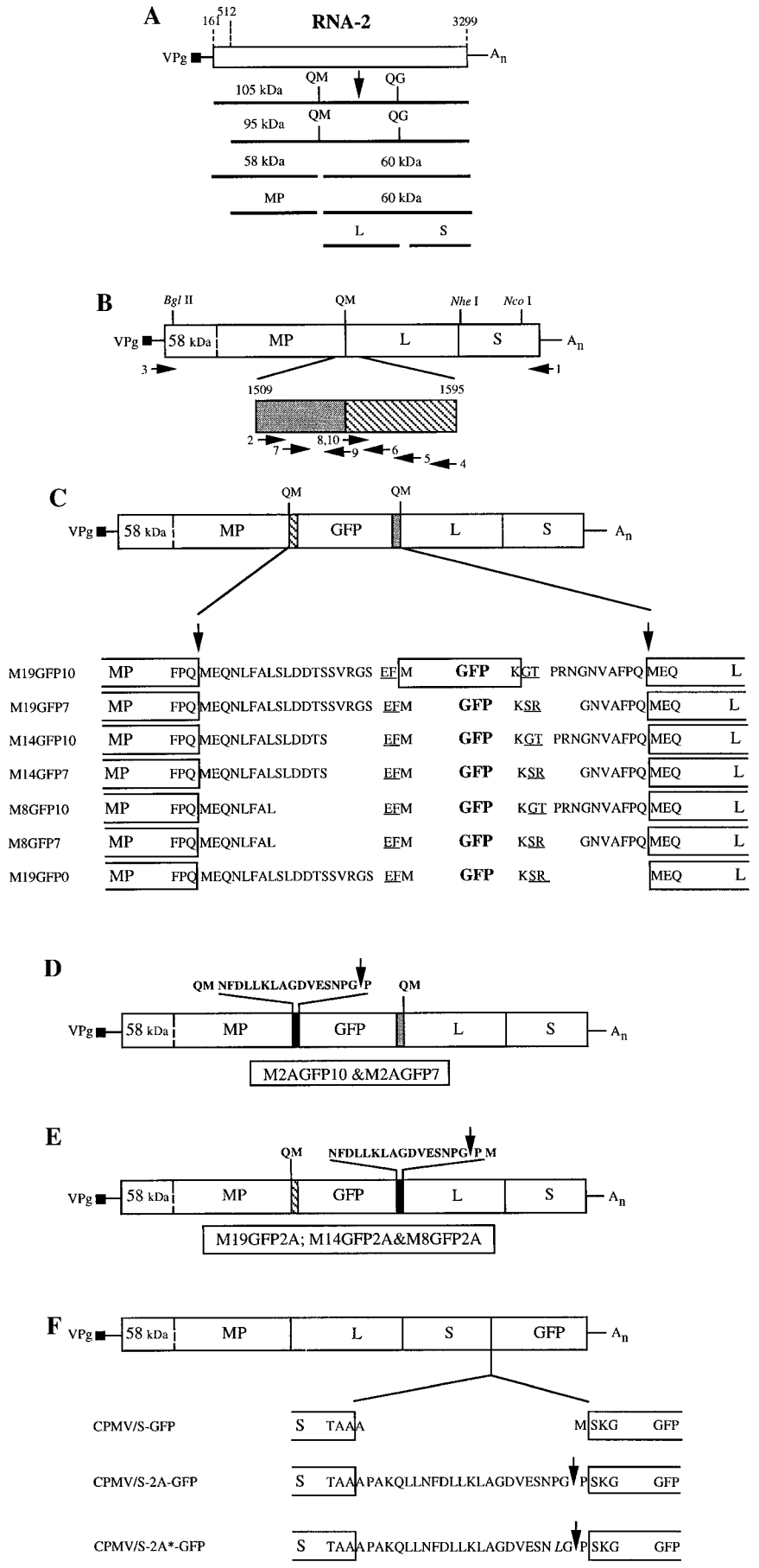

A
RNA-2
161
512
3299
VPg
An
105 kDa
95 kDa
58 kDa
60 kDa
MP
L
S
QM
QG
B
Bgl II
Nhe I
Nco I
58 kDa
MP
L
S
1509
1595
C
GFP
M19GFP10
M19GFP7
M14GFP10
M14GFP7
M8GFP10
M8GFP7
M19GFP0
D
M2AGFP10 &M2AGFP7
E
M19GFP2A; M14GFP2A&M8GFP2A
F
CPMV/S-GFP
CPMV/S-2A-GFP
CPMV/S-2A*-GFP

wild-type CPMV and processing of the artificial cleavage sites was highly efficient.

To decrease the number of virus-derived amino acids linked to the expressed foreign protein and to identify the minimum number of amino acids needed for efficient processing, several new constructs were engineered. In addition, we have used the sequence encoding the foot-and-mouth disease virus (FMDV) 2A catalytic peptide to achieve cleavage at defined sites in the RNA-2-encoded polyprotein. The results obtained with these new constructs demonstrate that CPMV can be used as an efficient and stable vector for the expression of whole proteins in plants.

RESULTS

RNA-2 vectors with duplicated Q/M sites

In an effort to minimize the number of virus-derived amino acids linked to the foreign proteins expressed between the MP and L proteins and to decrease the chances of homologous recombinations, we have constructed seven new RNA-2-based vectors (Fig. 1C and Table 1). In these, the size of the duplications used to create the artificial cleavage sites has been varied. To analyze the suitability of these vectors for the expression of foreign genes, the GFP coding region was introduced as a reporter gene (Fig. 1C). Some of the vector constructs were made in pCP2 and plasmid DNA used directly to infect protoplasts; the remaining constructs were made in pTM1G, in which case *in vitro* transcripts were used for infection (Table 1). The transcripts or DNA of these vector constructs when coinoculated with CPMV RNA-1 initiated infections efficiently. Green fluorescence was readily detected in an equivalent percentage of protoplasts, in each case indicating that the insert had little effect on replication (van Bokhoven *et al.,* 1993; Verver *et al.,* 1998). Extracts of these protoplasts were subsequently used as a source of inoculum for cowpea primary leaves.

With constructs in which the processing of the two artificial proteolytic sites occurs efficiently, green fluorescence is expected to be present throughout the cytoplasm and in the nucleus of the protoplasts (Verver *et al.,* 1998). Such a pattern of fluorescence was observed for constructs M19GFP10, M19GFP7, M14GFP10, M14GFP7, and M19GFP0 (throughout this work, the prefix "p" in front of a construct designation denotes a bacterial plasmid; this prefix is omitted when progeny virus is being referred to). With M14GFP10 and M14GFP7, small green fluorescent tubular structures were observed on some of the protoplasts, indicating that the processing between the GFP and the MP is incomplete (data not shown). With M8GFP10 and M8GFP7, green fluorescent tubular structures were observed on the surface of almost all of the infected protoplasts, indicating the presence of substan-

TABLE 1

Expression Characteristics of CPMV RNA-2 Vectors with the Coding Region of GFP between the MP and L Genes

Vector[a]	Inoculum[b]	Inoculated leaf[c]	Systemic infection
M19GFP10	DNA	Normal	5 days
M14GFP10	DNA	Normal	5/6 days
M8GFP10	DNA	10–12 cells	No
M19GFP7	RNA	Normal	5/6 days
M14GFP7	RNA	Normal	6/7 days
M8GFP7	RNA	10–12 cells	No
M19GFP0	RNA	Single cell	No
M2AGFP10	DNA	80–100 cells	No
M2AGFP7	RNA	30–40 cells	No
M19GFP2A	RNA	Normal	5/6 days
M14GFP2A	RNA	Normal	6/7 days
M8GFP2A	RNA	30–35 cells	No

[a] The numbers in the names of the vectors indicate the number of amino acids that have been duplicated around the artificial proteolytic cleavage sites. 2A represents 16-amino-acids length FMDV 2A protease.

[b] With constructs made in the pCP2 background, DNA is used to inoculate the protoplasts and with constructs made in the pTM1G background, RNA transcripts are used.

[c] Normal movement in the inoculated leaf indicates that the speed of the cell-to-cell movement is similar to the M19GFP10 construct; in the other cases, the number of green fluorescent cells at an infection site is shown, indicating limited cell-to-cell movement.

FIG. 1. Schematic representation of CPMV RNA-2 and the vector constructs used in this study. (A) Genetic organization and expression of CPMV RNA-2. The RNA contains a long open reading frame shown by the open bar. The positions of the start and stop codons are indicated on the RNA. The RNA is translated into two carboxy coterminal proteins, which are subsequently cleaved into a 58-kDa protein, a 48-kDa MP, and two capsid proteins L and S by the 24-kDa viral proteinase from RNA-1. The position of the Gln/Met and Gln/Gly cleavage sites is indicated on the polyproteins with QM and QG. VPg is represented by a black box and the other proteins by single lines. (B) Schematic representation of CPMV RNA-2 with the position of the restriction enzyme sites used in the constructions indicated above. The region corresponding to nt 1509 to 1595 has been enlarged. The positions of the primers used to amplify different PCR fragments from pTM1G (as described under Materials and Methods) are indicated with arrows. (C) Schematic representation of CPMV RNA-2 constructs with GFP placed between two artificial proteolytic cleavage sites. The numbers in the vector names denote the number of amino acids from the N-terminus of the L coat protein and the C-terminus of the MP that are duplicated for the Gln/Met cleavage sites. A 0 indicates a direct fusion of the GFP to the viral capsid protein. The underlined amino acids EF, KG, and SR codes for *Eco*RI, *Kpn*I, and *Xba*I restriction sites respectively. D–F: Schematic representation of CPMV RNA-2 vectors with FMDV 2A protease sequence. (D) FMDV 2A protease sequence on the N-terminal side of the GFP. (E) FMDV 2A protease sequence at the C-terminal side of the GFP. (F) FMDV 2A protease sequence at the C-terminus of S coat protein. Two forms of the FMDV 2A protease sequence of 16 and 19 amino acids, respectively, along with a proline residue that will remain fused to the N-terminus of the protein downstream of the 2A sequence after cleavage have been used.

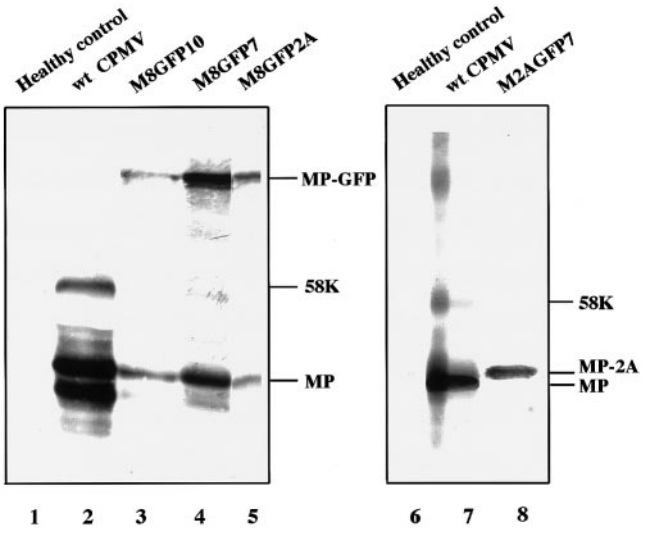


FIG. 2. Western blot analysis of membrane fractions isolated from the protoplasts (lanes 1–8) infected with different constructs as indicated above each lane. Proteins were separated by SDS–PAGE, transferred onto nitrocellulose membranes, and immunostained with MP antibody at 1:10,000 dilution.

tial amounts of MP-GFP fusion protein. Western blot analysis of extracts of these protoplasts showed the presence of both a fusion product of MP and GFP (Fig. 2, lanes 3 and 4) and free MP, in a ratio estimated to be about 1:1 based on visual observation; thus, the processing efficiency of the artificial proteolytic site with 8 amino acids from the N-terminus of the L coat protein is around 50%. The transcripts of pM19GFP0 (Fig. 1C), in which the C-terminus of the GFP was directly fused to the N-terminus of the L coat protein, replicated well in protoplasts, but attempts to reinfect protoplasts with extracts of these protoplasts failed. Since functional virions are required for such reinfections, this experiment suggested that no such virions were formed by M19GFP0. Western blot analysis of the protoplast extracts supported this interpretation, as only a 70-kDa GFP-L fusion protein and S coat protein, but no free L coat protein, could be detected (data not shown).

On plants, M19GFP10 and M19GFP7 induced the development of green fluorescent spots visible with a handheld UV lamp within 36 h postinoculation (hpi), whereas with M14GFP10 and M14GFP7, fluorescent spots could be detected 48 hpi. With all four constructs, systemic green fluorescence was visible on the first compound leaf 5–6 days postinoculation (dpi; Table 1) and subsequently spread over the leaf and later, the entire plant. Western blot analysis of soluble proteins from the M19GFP7-infected leaf material revealed the presence of a small amount of a 70-kDa GFP-L fusion product estimated to be less than 1% of the amount of free L protein (data not shown). This indicated that the processing efficiency of the artificial proteolytic site containing 7 amino acids derived from the C-terminus of the MP is around 99%. Probably with 8 amino acids, cleavage efficiency will be close to the wild-type efficiency, though this has not been demonstrated. In contrast to these observations, with M8GFP10 no green fluorescent symptoms were visible with a handheld UV lamp. Rather, wild-type symptoms appeared after a period, probably as a result of reversion. No wild-type symptoms were observed with M8GFP7, but the foci of infection consisted of a maximum of 10–12 green fluorescent epidermal cells (Table 1). Probably, by decreasing the number of duplicated nucleotides from 30 to 21 downstream of GFP, the rate of reversions to wild-type as well as the efficiency of processing has been decreased. With M19GFP0, GFP fluorescence was confined to single epidermal cells (Fig. 3D; Table 1), a result consistent with the previous conclusion that direct fusion of GFP to the L protein abolished the formation of functional virions.

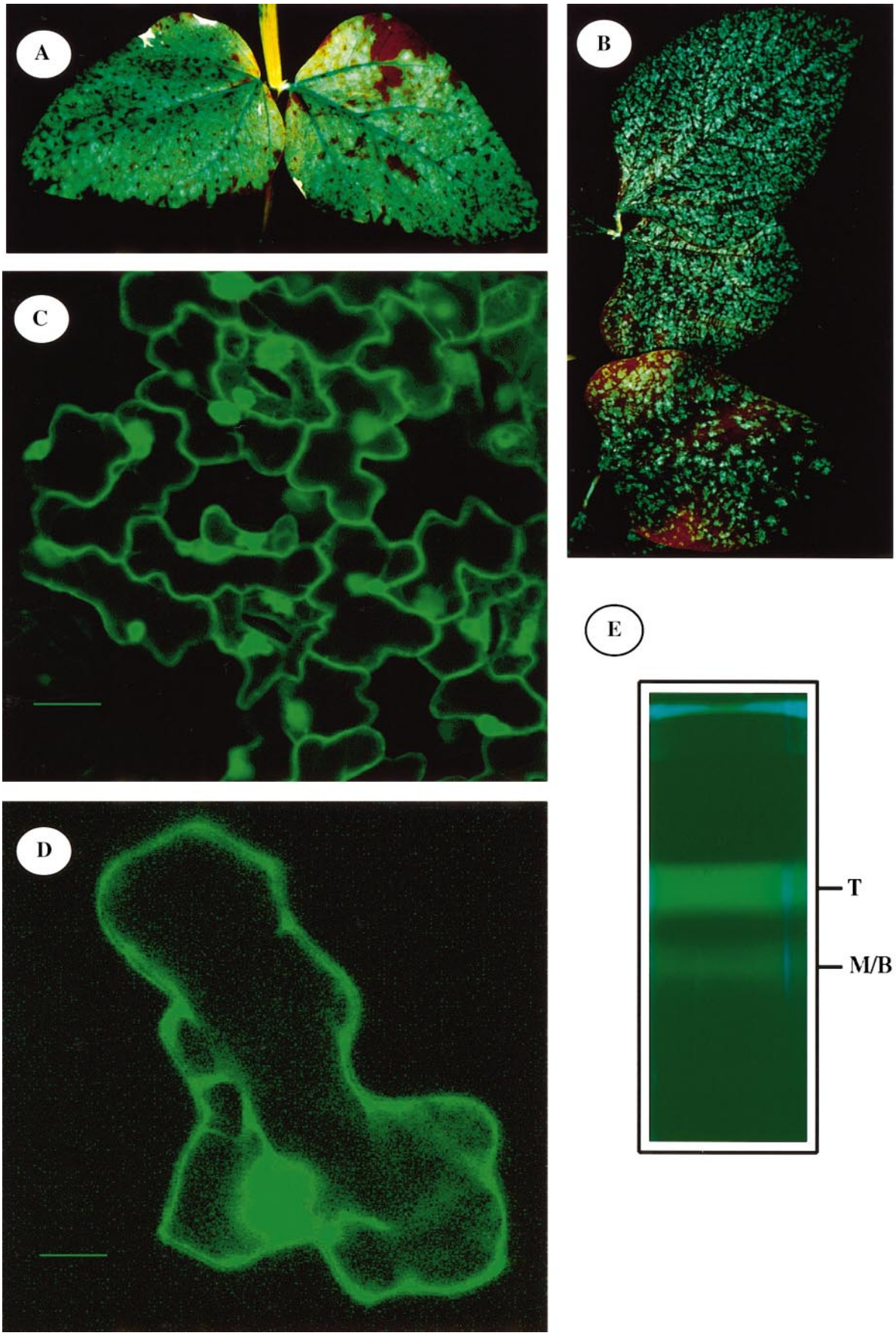
Overall, analysis of the properties of the seven constructs described earlier in both protoplasts and whole plants indicates that 8 amino acids from the C-terminus of the MP and 14–19 amino acids from the N-terminus of the L protein are required to achieve efficient processing at the two artificial Q/M cleavage sites.

RNA-2 vectors with FMDV 2A catalytic peptide sequence

As an alternative to the duplication of natural CPMV cleavage sites the FMDV 2A catalytic peptide sequence (NFDLLKLAGDVESNPG) (Ryan *et al.,* 1991) was tested on both sides of the GFP insert (Figs. 1D and 1E). The 2A peptide mediates a processing event between the 2A and 2B regions of the FMDV polyprotein by cleaving between its C-terminal glycine residue and the N-terminal proline residue of the 2B protein (Ryan *et al.,* 1991). Hence, 2A-mediated processing requires a proline to follow the 2A sequence; 2A-mediated processing has been shown to function in plants in the potato virus X (PVX) system (Santa Cruz *et al.,* 1996).

In pM2AGFP10 and pM2AGFP7, the 2A peptide sequence along with a proline residue was introduced next

FIG. 3. Green fluorescent symptoms on the inoculated cowpea primary leaves (A) and emergence of green fluorescent symptoms in the first and second compound leaves (B) as a result of infection with CPMV/S-2A-GFP. Confocal laser scanning microscopic image of a cowpea leaf infected with M2AGFP7, showing GFP fluorescence in 30–35 epidermal cells (C), and single fluorescent epidermal cell infected with M19GFP0 (D). The bar represents 20 μm in C and 10 μm in D. Sucrose density gradient, onto which virus particles isolated from leaves infected with M19GFP2A had been loaded, illuminated by a handheld UV lamp, showing a bottom zone which is comprised of middle and bottom components and a top zone containing the empty capsids (E).


A
B
C
D
E
T
M/B

to the Q/M dipeptide at the N-terminal side of the GFP (Fig. 1D). As a result of processing by the 2A peptide, a 16-amino-acids tag will remain fused to the MP and the expressed protein will contain only a single additional proline. Western blot analysis of protoplasts infected with these constructs revealed that cleavage did not occur between the Q and M; however, efficient processing was observed at the C-terminus of the 2A peptide (Fig. 2, lane 8). M2AGFP10 induced minute green fluorescent spots just visible to the naked eye on inoculated cowpea leaves (~80–100 cells under the fluorescence microscope; Table 1) but failed to move systemically. Wild-type symptoms were also observed, probably as a result of reversion. M2AGFP7 spread to about 30–40 epidermal cells (Fig. 3C; Table 1) and also failed to give systemic symptoms; in this case, however, no wild-type symptoms were visible on the inoculated leaf. Probably the 16-amino-acids tag, which is fused to the C-terminus of the MP, has deleterious effects on the cell-to-cell movement.

In pM8GFP2A, pM14GFP2A, and pM19GFP2A, the 2A peptide was placed at the C-terminal side of the GFP (Fig. 1E). As a result of 2A-mediated processing, a single proline residue will remain fused to the N-terminus of the L protein. Protoplasts transfected with M8GFP2A showed a 1:1 ratio of MP:MP-GFP fusion product as determined by Western blot analysis (Fig. 2, lane 5). Extracts of these protoplasts failed to give fluorescent spots visible with the naked eye on inoculated cowpea leaves, a result similar to that observed with M8GFP10 and M8GFP7. A maximum of 30–35 green fluorescent epidermal cells could be found using a fluorescence microscope (Table 1). M14GFP2A induced green fluorescent lesions on the inoculated leaf 3 days dpi and on systemic leaves, 7 dpi (Table 1). With pM19GFP2A, bright fluorescent spots were visible with a handheld UV lamp on the inoculated leaf within 48 hpi. The spread of symptoms on the inoculated leaf was slightly slower compared with that of M19GFP10 and M19GFP7 and systemic green fluorescence could be seen on the first compound leaf 6 dpi (Table 1). The systemic green fluorescence emerged from the veinal ends and subsequently spread all along the leaf. Fluorescent systemic symptoms were visible throughout the life of the plant.

RNA-2 constructs with GFP downstream of S coat protein

In pCP2/S-GFP, GFP was fused to the last amino acid of S protein via a single alanine residue (Fig. 1F). The rationale of this construct was to take advantage of the natural cleavage event which occurs between Leu 189 and Leu 190, resulting in the loss of 24 amino acids from the C-terminus of S protein (Taylor *et al.,* 1999). This results in the conversion of the slow-migrating form of the S protein (Ss) into the fast-migrating form (Sf). Fusion of GFP after Ala 213 could therefore be expected to result in a sufficient proportion of Sf and free GFP (linked to amino acids derived from the C-terminal region of Ss) to allow assembly of virus particles. Coinoculation of pCP1 with pCP2/S-GFP onto cowpea leaves gave rise to minute fluorescent symptoms which could be detected only between 3 and 4 weeks postinoculation. Moreover, sap prepared from leaf tissue comprising these fluorescent spots did not induce an infection when passaged onto new plants. These results suggest that cleavage between the S protein and GFP was not efficient enough to produce sufficient free S protein at an appropriate time to permit virus assembly.

To alleviate the problem of inefficient cleavage, a FMDV 2A sequence was inserted between Ala 213 of the S protein and Ser 2 of GFP. A 25-amino-acid sequence APAKQLLNFDLLKLAGDVESNPGP (Fig. 1F), consisting of 5 amino acids from the FMDV capsid protein 1D, a 19-amino-acid 2A sequence, and a single proline, was chosen because it has previously been shown to maximize cleavage (Donnelly *et al.,* 1997). The FMDV-derived sequences remain linked to the C-terminus of the S protein, while GFP will contain only an extra N-terminal proline. Coinoculation of the primary leaves of cowpea plants with pCP1 and pCP2/S-2A-GFP produced fluorescent symptoms after 1–2 weeks. Spread of the infection to the upper leaves followed 2 to 3 weeks later. After sap transmission, the time for the appearance of fluorescent spots on the inoculated leaves (Fig. 3A) was reduced to less than 72 h and systemic spread (Fig. 3B) of the virus occurred after about 10 days.

To ascertain whether the phenotype of CPMV/S-2A-GFP was due to the cleavage activity of the 2A peptide or merely resulted from the introduction of a spacer sequence between S protein and GFP, the proline in the conserved motif NPG, required for the processing activity of 2A (Palmenberg *et al.,* 1992), was mutated to leucine. The resulting construct, pCP2-S-2A*-GFP, induced small fluorescent symptoms on inoculated cowpea primary leaves with a delay of 1 week compared to inoculations involving pCP2-S-2A-GFP. Although passaging of the infection using plant sap produced new fluorescent lesions, they remained small. Systemic spread of the infection was never observed and after three passages, the vast majority of symptoms no longer fluoresced under UV light. Fusion of GFP to the S protein via the inactive 2A sequence, 2A*, therefore appears to generate a virus with debilitated growth and movement properties and which rapidly loses the GFP sequence.

Genetic stability of the most efficient constructs

In view of their potential utility as vectors, M19GFP10, M19GFP7, M19GFP2A, and CP2/S-2A-GFP were analyzed for their stability and maintenance of the insert during serial plant passages, by monitoring the fluorescence visually and by RT-PCR analysis. With M19GFP10,

green fluorescence was very bright in the inoculated leaf as well as in the first compound leaf and subsequently decreased in the second and third compound leaves. RT-PCR analysis showed traces of wild-type virus already in the inoculated leaf and increased amounts of wild-type virus in the second and third compound leaves, probably as a result of homologous recombination. With M19GFP7, green fluorescence was maintained throughout the plant during the first passage and RT-PCR analysis showed wild-type virus only after a second passage (data not shown). When the infected first compound leaf was used as a source of inoculum to infect fresh plants, M19GFP10 produced green fluorescent symptoms for two to three passages, whereas with M19GFP7, green fluorescence was maintained for three to four passages before being reduced or lost in subsequent passages. With M19GFP2A, the green fluorescence was usually maintained for six to seven passages. RT-PCR analysis showed that deletions were present in the GFP sequence in the sixth passage, probably as a result of heterologous recombination (data not shown); however, reversion to wild-type has so far not been detected with this construct. With CP2/S-2A-GFP, GFP fluorescence was maintained during five passages and RT-PCR showed the presence of the intact insert.

Expression levels of GFP from different constructs

The expression levels of GFP in plants infected with the constructs (whose genetic stability was determined earlier) were compared by SDS–PAGE analysis of soluble proteins extracted from the primary leaves of infected plants. On a Coomassie-stained gel, GFP expression could readily be detected in M19GFP10-infected leaf material (Fig. 4A, lane 3), and was visually estimated to comprise around 1–2% of total soluble proteins. With M19GFP7, GFP expression was slightly lower (Fig. 4A, lane 4). In the case of M19GFP2A, no clear band representing GFP was visible (Fig. 4A, lane 5). In the case of the extract from a plant infected with CPMV/S-2A-GFP, a diffuse band migrating at the position expected for GFP, which was not present in the extract from wild-type-infected leaves, could be seen (Fig. 4B; compare lanes 3 and 4). The identity of this band was confirmed in the immunoblot probed with anti-GFP serum (Fig. 4B′, lane 4) and the level of GFP produced is estimated to be about 1% of soluble protein.

Western blot analysis of soluble proteins isolated from M19GFP2A-infected leaf material revealed both a 70-kDa fusion protein (GFP-2A-L) and free GFP (Fig. 4A′, lane 5) in about a 1:1 ratio. The combined expression levels of these two forms of GFP was estimated to be around 50% of the level of free GFP produced by M19GFP10 and M19GFP7 (Fig. 4A′; compare lane 5 with lanes 3 and 4). As a result of the presence of the 16-amino-acids 2A tag, a slight shift in the mobility of GFP was observed in M19GFP2A-infected leaf material (Fig. 4A′, lane 5). Western blot analysis of a CPMV/S-2A-GFP-infected leaf extract revealed that the level of fusion protein (S-2A-GFP) was about 10% of unfused GFP (Fig. 4B′, lane 4).

Analysis of virus particles

To investigate whether uncleaved GFP-2A-L is actually incorporated into virus particles, M19GFP2A virus was purified from infected leaf material. The purified virus particles resolved into two distinct fluorescent zones on sucrose density gradients (Fig. 3E), indicating the presence of GFP associated with the virus particles. The bottom zone consisted of two barely distinguishable layers, which probably contain the middle and the bottom components, and a very thick top fluorescent zone, probably containing empty capsids. These assignments were supported by electron microscopy (Fig. 5, panels A and B) and confirmed by the observation that almost no RNA could be isolated from the top fluorescent band, whereas the lower fluorescent band contained RNA-1 and a larger than wild-type RNA-2 (data not shown). The increased size of RNA-2 was a consequence of the presence of the inserted GFP gene.

SDS–PAGE analysis of the particles from the two sucrose gradient zones obtained with M19GFP2A virus showed a 70-kDa fusion protein (GFP-2A-L) in addition to the L and S coat proteins (Fig. 6A, lanes 2 and 3). Western blot analysis, using CPMV-specific and GFP-specific antisera, confirmed the identity of the proteins (data not shown). The ratio between the fusion protein (GFP-2A-L) and the free coat proteins was visually estimated to be around 1:6, indicating that each virus particle of M19GFP2A contained, on average, approximately 10 fusion proteins.

To determine the location of the GFP in the virus particles, M19GFP2A virus was subjected to immunocapture electron microscopy. Large amounts of wild-type CPMV and M19GFP2A (M/B) particles were trapped with antibodies raised against CPMV (Figs. 7A and 7C, respectively). No wild-type CPMV particles (Fig. 7B) and a substantial number of M19GFP2A (M/B) particles were trapped with the GFP-specific antiserum (Fig. 7D), strongly suggesting that GFP is present on the surface of M19GFP2A virus particles. Immunotrapping studies with M19GFP2A empty capsids (T) were unsuccessful, as the particles broke up during the phosphotungstic acid staining procedure.

When purified CPMV/S-2A-GFP was analyzed on a CsCl gradient, the resulting three bands did not fluoresce, indicating that the fusion protein (S-2A-GFP) was not incorporated into virus particles. Unlike the observation made with M19GFP2A, the top component band of CPMV/S-2A-GFP was thin and comparable in size to that of wild-type CPMV. The band corresponding to the middle component of CPMV/S-2A-GFP had a greater buoyant density than that of wild-type CPMV, as a result of the presence of the additional sequence encoding GFP on RNA-2 (data not shown).

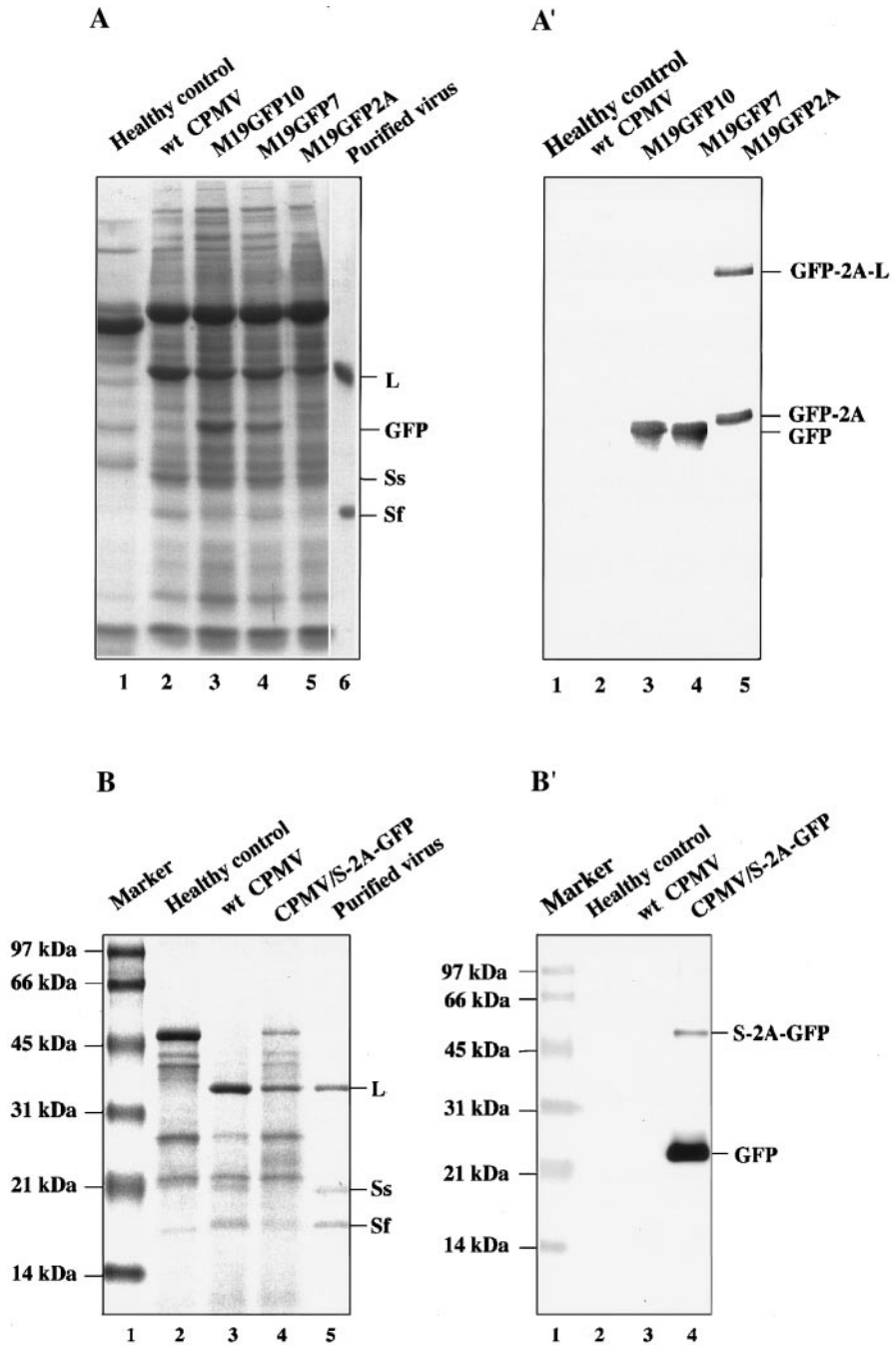


FIG. 4. SDS–PAGE analysis of leaf soluble proteins extracted from leaves infected with constructs as indicated above the lanes. The gels were either stained with Coomassie brilliant blue R-250 (A and B) or the proteins were transferred to nitrocellulose and immunostained with anti-GFP antibody (A′ and B′). Equal amounts of soluble proteins were loaded in each lane. Ss is the uncleaved slow form of S, Sf is the cleaved fast form. We have no explanation for the faster migration of the large subunit of rubisco in the healthy control sample in lane A1.

SDS–PAGE analysis of the viral components isolated from CPMV/S-2A-GFP revealed the presence of L and Sf protein only. No Ss-2A-GFP protein could be detected in any of the three components on a Coomassie-stained gel (Fig. 6B, lanes 2, 3, and 4). The expression of an additional sequence at the carboxyl-terminus of S seems to lead the natural cleavage of S at position 198 toward completion. Only by Western blotting of purified virus using GFP-specific antiserum could a faint band of about 58 kDa corresponding to a Ss-2A-GFP fusion protein be identified (data not shown).

DISCUSSION

In a previous study, the GFP gene was inserted between the MP and L protein genes and duplications of

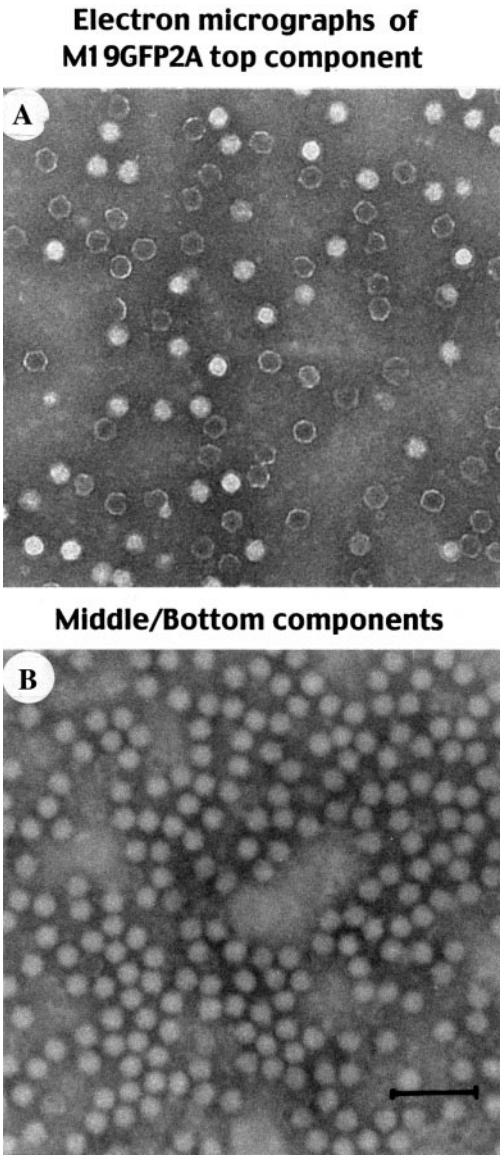


FIG. 5. Electron microscopic images of purified M19GFP2A top (A) and bottom components (B), negatively stained with 0.1% PTA. The bar represents 100 nm.

the coding regions from the L coat protein (29 amino acids) and from MP (11 amino acids) to allow protein processing to occur (Verver *et al.,* 1998). In this report, we have shown that it is possible to reduce the duplications to 14–19 and 7 amino acids, respectively, without affecting efficient processing at these sites. Furthermore, we have shown that it is also possible to use the FMDV 2A catalytic peptide sequence as an alternative to the duplications to achieve processing. Using this strategy we have also been able to express GFP at the C-terminus of the S protein.

With M19GFP10 and M19GFP7, the expression levels of free GFP reached approximately 1–2% of the total soluble proteins (Fig. 4A, lanes 3 and 4), which is comparable to those reported for TMV (10 μg/g tissue) (Hamamoto *et al.,* 1993), and PVX (Chapman *et al.,* 1992). The main disadvantage of these CPMV vectors, when compared to viral vectors which express foreign genes using duplicated promoters for subgenomic RNA production, is that the expressed proteins will contain additional virus-derived amino acids. This has been largely overcome with CPMV/S-2A-GFP, in which the expressed protein will contain only a single additional proline residue. A further advantage of the FMDV 2A-containing vectors is that foreign genes can be expressed as either free proteins (CPMV/S-2A-GFP) or as fusion proteins incorporated into virus particles (M19GFP2A). Previous descriptions of assembly-competent CPMV constructs carrying coat-protein fusions involved the expression of only small oligopeptide sequences (Usha *et al.,* 1993; Porta *et al.,* 1994, 1996; Lin *et al.,* 1996; Dalsgaard *et al.,* 1997). With M19GFP2A construct we have demonstrated the ability of the virus to assemble with a protein of 238 amino acids (GFP) fused to one of the capsid proteins.

An unexpected finding is that some GFP-2A-L fusion proteins are readily incorporated into the virus particles, resulting in a "green virus" (Fig. 3E). A majority of the particles in a M19GFP2A virus preparation were empty. SDS–PAGE analysis showed that both the empty and RNA-containing particles incorporate about 10 copies of the fusion protein. Since leaf material contained about 50% of the L protein in a fused form (GFP-2A-L), these results indicate that not all the GFP-2A-L fusion protein molecules present in the cytosol can be incorporated into virus capsids, probably due to structural limitations; however, this has not been analyzed further. For PVX, it has been shown that rod-shaped particles containing up to 50% of fusion proteins can be formed (Santa Cruz *et al.,* 1996), while for TMV it has been shown that coat protein and fusion proteins can be incorporated into the virus capsids at a ratio of 20:1 (Sugiyama *et al.,* 1995; Turpen *et al.,* 1995). The experiments with M19GFP0 indicated that in the presence of GFP-L fusion only, no functional virions are formed. Assembly of CP fusion proteins into virions is probably facilitated by the presence of a pool of free CP, as has been demonstrated for modified forms of the TMV and PVX CP (Hamamoto *et al.,* 1993; Santa Cruz *et al.,* 1996).

It is evident from the crystallographic data of CPMV that the N-terminus of the L coat protein is buried inside the virus particles (Lomonossoff and Johnson, 1991). Data obtained with bean pod mottle virus (BPMV) middle component revealed that the N-terminus of the L protein interacts with the RNA (Chen *et al.,* 1989). This might explain the large amounts of empty capsids found in plants infected with M19GFP2A. The presence of GFP-2A-L fusion protein molecules could disturb efficient encapsidation of the viral RNA. The immunotrapping studies revealed that at least some of the GFP in the fusion protein is present on the surface of the virus particles. More detailed structural analysis of the green virus will

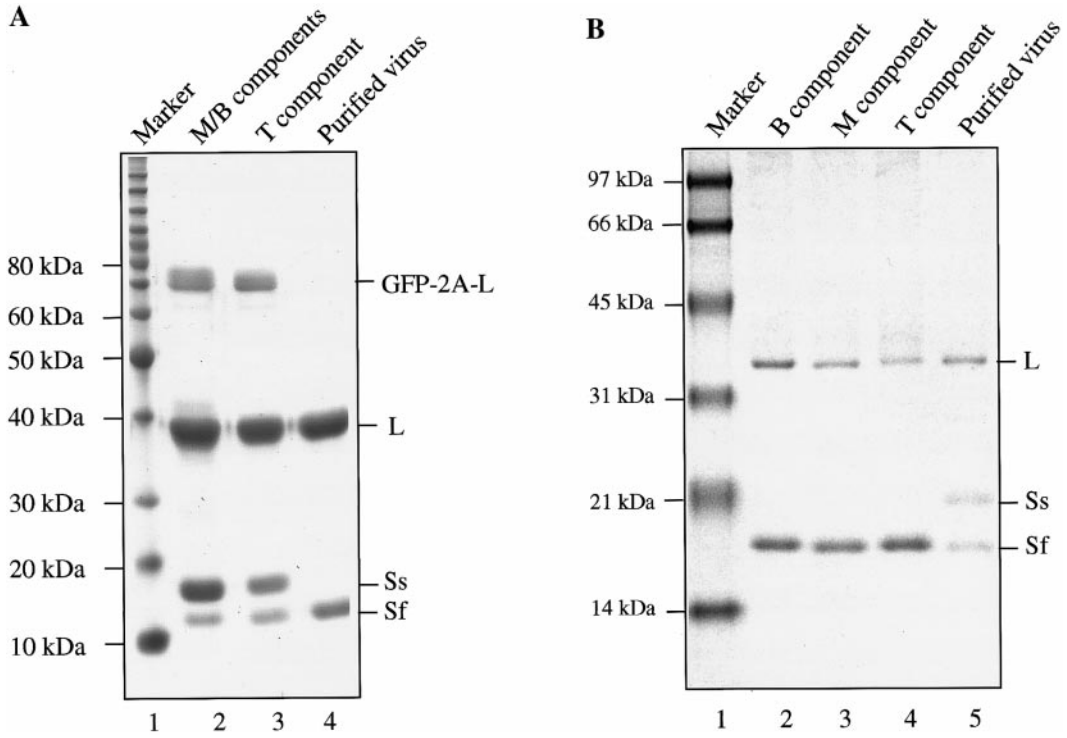


FIG. 6. SDS–PAGE analysis of (A) purified M19GFP2A top (T) and middle + bottom (M/B) components and (B) CPMV/S-2A-GFP top (T), middle (M), and bottom (B) components, stained with Coomassie brilliant blue R 250. Lanes A4 and B5 contain purified wild-type CPMV particles.

be needed to determine whether all GFP molecules are surface exposed. Nevertheless, for the first time we have demonstrated the incorporation of GFP into spherical plant RNA virus capsids. Other reports of incorporation of GFP into spherical capsids have involved herpes simplex virus (Desai and Person, 1998) and hepatitis B virus (Kratz *et al.,* 1999).

In the constructs with the duplicated Q/M sites, the reduction of the number of repeated nucleotides at the 3′ side of the inserted gene from 30 to 21 greatly improved the stability of the vector, an observation that is consistent with previous findings. In TMV vectors, direct duplications have been avoided using the coat protein gene and promoter sequence from heterologous tobamoviruses in a TMV genomic background (Donson *et al.,* 1991). In poliovirus-based vectors, a 37% reduction of the homology of the direct repeats surrounding the foreign sequences greatly increased the stability (Tang *et al.,* 1997), and the FMDV 2A protease sequence also proved to be successful in overcoming the problem of homologous recombinations (Mattion *et al.,* 1996).

In potyviruses, all known processing sites in the polyprotein cleaved by the 3C-like proteinases are flanked by conserved sequence motifs, which in the case of tobacco etch virus (TEV) is E-X-X-Y-X-Q-S/G, with the scissile bond located between the Gln-Ser or Gly dipeptide (Carrington and Dougherty, 1988; Carrington *et al.,* 1988). In TEV, the conserved motif of 7 amino acids upstream of the cleavage site is sufficient for recognition and cleavage by the 49-kDa proteinase (Carrington *et al.,* 1988). Only a very limited sequence homology is found around the processing sites cleaved by the 3C-like proteinases of the comoviruses (A/P-X-A-Q-M/G/S) and polioviruses (A-X-X-Q-G) (Nicklin *et al.,* 1986; Goldbach and Wellink, 1996). Our findings with the CPMV vectors with the duplicated Q/M sites show that at least for the Q/M site between the MP and L protein, a significant number of amino acids (14–19) downstream of the scissile bond and 7 upstream amino acids are required for efficient processing. The requirement for an extended sequence to achieve processing of the RNA-2-encoded polyprotein has also been found for the L-S site in studies using hybrid comoviruses (Clark *et al.,* 1999). Both that study and another study on the processing of nepovirus polyproteins (Carrier *et al.,* 1999) indicate that the requirements for processing in *trans* are stricter than those required for *cis* cleavage. In the case of comoviruses, it is tempting to speculate that these stricter requirements are attributable to the involvement of the 32-kDa polypeptide encoded by RNA-1 as a cofactor for the 24-kDa viral proteinase, in the *trans* processing of the RNA-2-encoded polyproteins (Vos *et al.,* 1988; Peters *et al.,* 1992).

Overall, the results presented in this report demonstrate that CPMV has the potential to serve as a versatile vector for the expression of high levels of heterologous proteins in plants. So far we have introduced two GFP genes (~1.5 kb; data not shown) and the cowpea aphid

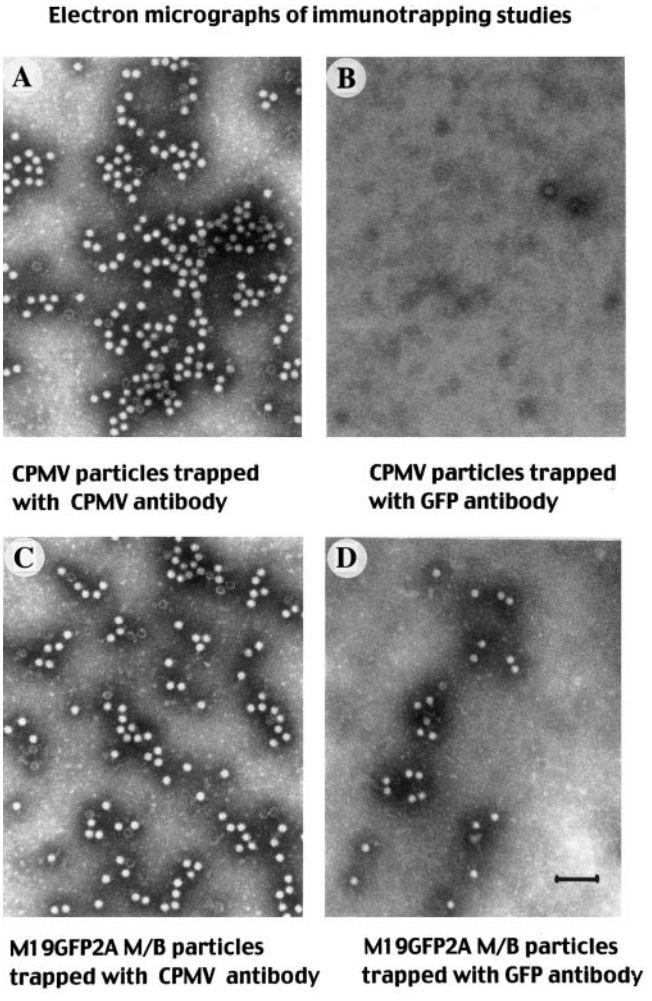


FIG. 7. Immunotrapping electron microscopy of M19GFP2A middle and bottom components and wild-type CPMV particles. The grids were coated with either anti-CPMV (1:1000) or anti-GFP (1:500) antibodies and virus particles (1 mg/ml) were trapped onto the grids, stained with 0.1% PTA, and examined by the electron microscope. The bar represents 100 nm.

born mosaic virus HC-PRO coding sequence (~1.3 kb; S. Mlotshwa, unpublished data) in the M19–7- and M19–2A-based vectors, and these constructs are as infectious as the M19GFP10.

MATERIALS AND METHODS

Design and construction of RNA-2 vectors

Two sets of plasmids containing full-length cDNA copies of CPMV RNAs were used throughout this study. In the first set, cDNA copies of RNA-1 (pTB1G) and RNA-2 (pTM1G) are linked to the T7 RNA polymerase promoter sequence, from which the infectious transcripts can be obtained by *in vitro* transcription (Eggen *et al.,* 1989). In the second set, the full-length cDNA copies of both RNA-1 (pCP-1) and RNA-2 (pCP-2) are inserted immediately downstream of the cauliflower mosaic virus 35S promoter (Dessens and Lomonossoff, 1993). Both pTM1G and pCP2 are used in the constructions described below. Standard DNA and RNA techniques were essentially carried out as described by Sambrook *et al.* (1989). PCR fragments were amplified with Vent DNA polymerase (NEB) from pTM1G, pBINm-GFP5-ER (Siemering *et al.,* 1996), and pTXS.GFP-CP (Santa Cruz *et al.,* 1996) and with Pfu DNA polymerase (Stratagene) from pPVX208 (a gift from E. Mueller) and pCP2/S-GFP.

pM19PL10, pM14PL10, and pM8PL10. These vectors were constructed by a three-piece ligation with the 2.92-kb *Bgl*II (nt. 189 in the RNA-2 cDNA) and *Nco*I (nt. 3068) plasmid vector portion of pTM1G and two PCR products (Fig. 1B). The first PCR fragment was amplified from pTM1G with 5′ oligo CCGGAATTCCCGGGTACCCCACGAAATGGGAATGTGGCT (Primer 2; *Eco*RI, *Sma*I, and *Kpn*I sites are underlined) and 3′ oligo GAAGGGACGACCTGCTAAAC (Primer 1), and digested with *Eco*RI and *Nco*I. The second PCR fragment was amplified from pTM1G with 5′ oligo AGAAAACCATGCAGTTCATGC (Primer 3) and 3′ oligo CCGGAATTCAGAACCACGAACTGAGCTTGTATC (Primer 4; *Eco*RI site underlined) for pM19PL10 (Fig. 1B), and 3′ oligo CCGGAATTCGCTTGTATCATCCAAAGAAAGGGC (Primer 5; *Eco*RI site underlined) for pM14PL10 (Fig. 1B), and 3′ oligo CCGGAATTCAAGGGCAAACAAGTTTTGCTC (Primer 6; *Eco*RI site underlined) for pM8PL10 (Fig. 1B), and digested with *Bgl*II and *Eco*RI.

pM19PL7, pM14PL7, and pM8PL7. To construct these vectors, the 4.3-kb *Eco*RI and *Nco*I plasmid vector portion of pM19PL10, pM14PL10, and pM8PL10, respectively, were ligated with a PCR fragment amplified from pTM1G with 3′ oligo (Primer 1) and a 5′ oligo CCGGAATTCCCCGGGTCTAGAGGGAATGTGGCTTTTCCACAAATG (Primer 7; recognition sequences for *Eco*RI, *Sma*I, and *Xba*I are underlined; Fig. 1B) that has been digested with *Eco*RI and *Nco*I.

pM19GFP10, pM14GFP10, and pM8GFP10. The 5.8-kb *Eco*RI and *Kpn*I plasmid vector portions of pM19PL10, pM14PL10, and pM8PL10, respectively, were ligated with a PCR fragment amplified from pBINm-GFP5-ER with 5′ oligo (Primer 11) and 3′ oligo CGGGGTACCTTTGTATAGTTCATCCATGCCATG (Primer 15; *Kpn*I site underlined, which removes the stop codon of GFP) that has been digested with *Eco*RI and *Kpn*I. Subsequently, the 3.2-kb *Bgl*II and *Nhe*I (at nt. 2709) vector fragments of the resulting clones were introduced into pCP-2 digested with the same enzymes.

pM2AGFP10. The 5.0-kb *Bgl*II and *Eco*RI plasmid vector portion of pM19GFP10 was used in a three-piece ligation with two PCR fragments. The first PCR fragment was amplified from pTM1G with 5′ oligo (Primer 3) and 3′ oligo TGCTCTAGACATTTGTGGAAAAGCCACATTCC (Primer 9; *Xba*I site underlined) and digested with *Bgl*II and *Xba*I. The second PCR fragment (70 nt) was amplified from pTXS.GFP-CP with 5′ oligo (Primer

11) and 3′ oligo CCGGAATTCGGGCCCAGGGTTG-GACTCGACGTC (*Xba*I site underlined) and digested with *Eco*RI and *Xba*I. The 3.2-kb *Bgl*II and *Nhe*I fragment of the resulting clone was introduced into pCP-2 digested with the same enzymes.

pM2AGFP7. The 1.86-kb *Bgl*II and *Afl*II plasmid vector portion of pM2AGFP10 was ligated with the 5.3-kb *Bgl*II and *Afl*II plasmid vector portion of pM19GFP7 described below.

pM19GFP7, pM14GFP7, and pM8GFP7. The GFP 5 gene contains a *Cla*I restriction site, which is also used to linearize plasmid pTM1G for making runoff transcripts. To allow its insertion into pTM1G-based vectors, the *Cla*I site of GFP 5 was eliminated by primer-derived mutation. A PCR fragment was amplified from the pBINm-GFP5-ER with a 5′ oligo CGGGAATTCATGAGTAAAGGAGAACTTT-TACAT (Primer 11; *Eco*RI site underlined) and a 3′ oligo CGGTCTAGATTTGTATAGTTCATCCATGCCATG (Primer 12, *Xba*I site underlined), digested with *Eco*RI and *Xba*I and ligated into pUC 19 cut with the same restriction enzymes that resulted in pUC-GFP. Subsequently, the 3.48-kb *Afl*II and *Xba*I fragment from pUC-GFP was ligated with a PCR fragment amplified from pUC-GFP with 5′ oligo GAGCTTAAGGGAAT**A**GATTTCAAGGAG (Primer 13; *Afl*II site underlined and mutated nucleotide is shown in bold) and 3′ oligo CGGTCTAGATTTGTATAGTTCATC-CATGCCATG (Primer 14; *Xba*I site underlined) and digested with *Afl*II and *Xba*I, resulting in pUC-GFPΔC. The 5.89-kb *Eco*RI and *Xba*I plasmid vector portions of pM19PL7, pM14PL7, and pM8PL7, respectively, were ligated with a PCR fragment amplified from pUC-GFPΔC by 5′ oligo (Primer 11) and 3′ oligo (Primer 12) and digested with *Eco*RI and *Xba*I.

pM19GFP0. The 5.3-kb *Xba*I and *Nhe*I plasmid vector portion of pM19GFP7 was ligated with a PCR fragment amplified from pTM1G with 5′ oligo CCGTCTAGAATG-GAGCAAAACTTGTTTGCCCTT (Primer 10) and 3′ oligo (Primer 1) and digested with *Xba*I and *Nhe*I (Fig. 1B).

pM19GFP2A, pM14GFP2A, and p8GFP2A. The 5.09-kb *Xba*I and *Nco*I plasmid vector portions of pM19GFP7, pM14GFP7, and pM8GFP7, respectively, were used in a three-piece ligation with two PCR fragments. The first PCR fragment was amplified from pTM1G with 5′ oligo AACAACCCGGGGCCCATGGAGCAAAACTTGTTTGCCC (Primer 8; *Apa*I site underlined; Fig. 1B) and 3′ oligo (Primer 1) and digested with *Apa*I and *Nco*I. The second PCR fragment (63 nt) was amplified from pTXS.GFP-CP (Santa Cruz *et al.,* 1996) with a 5′ oligo (Primer 11) and a 3′ oligo TGTGCTCGCGGGCCCCAGGGTTGGACTCGAC (*Apa*I site underlined) and digested with *Xba*I and *Apa*I.

pCP2/S-GFP. To allow expression of GFP as a fusion protein to the C-terminus of S, vector pCP2-CVW was generated. A 2-kb *Bam*HI–*Eco*RI fragment from pCP2 was subcloned into M13mp18, resulting in M13-CP1/*Aat*II (after an *Aat*II site had been engineered by a silent mutation at position 2740). Using the method of Kunkel *et al.* (1987) and primer CACACTGCTACTGCTGCAGAAG-GCCTTTAACTCTGGTTTCATTAAATTTTC (*Pst*I site underlined, *Stu*I site double-underlined) a mutagenesis cassette was introduced at position 3298, i.e., overlapping the codon for the last amino acid of S. From pPVX208 (a PVX clone with a GFP insert), a PCR fragment was amplified using 5′ sense primer, which comprises GFP nucleotides 1 to 19 (CCCCTGCAGCGATGAGTAAAG-GAGAAGAAC; *Pst*I site underlined) and 3′ antisense primer, which anneals to GFP nucleotides 694 to 714 (GGGAGGCCTTTATTTGTATAGTTCATCCATGCC; *Stu*I site underlined). The PCR product harboring the sequence for wild-type GFP (Prasher *et al.,* 1992) was digested with *Pst*I and *Stu*I and ligated to the large *Pst*I–*Stu*I fragment of pCP2-CVW, to form pCP2/S-GFP.

pCP2/S-2A-GFP.* A PCR fragment was amplified from pCP2/S-GFP. The 5′ sense primer comprised the coding sequence for 24 amino acids from FMDV serotype A10, encompassing a mutated 2A region, and GFP nucleotides 3 to 23 (CCCTGCAGCGCCTGCAAAACAGCTCTTA-AACTTTGACCTACTTAAGTTAGCAGGTGACGTTGAGTC-CAACC*T*TGGGCCCAGTAAAGGAGAAGAACTTTTC; *Pst*I site underlined, *Apa*I site double-underlined, mutation shown in italics). The 3′ antisense primer was complementary to GFP nucleotides 478 to 497 (TTGAAGTTA-ACTTTGATTCC; *Hpa*I site underlined). The resulting 0.57-kb product was digested with *Pst*I and *Hpa*I and ligated to the large fragment of similarly digested pCP2/S-GFP.

pCP2/S-2A-GFP. The small *Pst*I–*Apa*I fragment from pCP2/S-2A*-GFP was excised and replaced by a pair of complementary oligonucleotides encoding the native 2A sequence from FMDV serotype A10 + strand primer GCGCCTGCAAAACAGCTCTTAAACTTTGACCTACTTAA-GTTAGCAGGTGACGTTGAGTCCAACC*C*TGGGCC-strand primer CA*G*GGTTGGACTCAACGTCACCTGCTAACTT-AAGTAGGTCAAAGTTTAAGAGCTGTTTTGCAGGCGCTG-CA (*Pst*I-compatible ends underlined; *Apa*I-compatible ends double-underlined; reverse mutation shown in italics).

In vitro transcription and protoplast transfections

Cowpea protoplasts were isolated as described by Rezelman *et al.* (1989). Prior to protoplast infections, plasmids were purified by Quiagen miniprep spin columns (Quiagen). Viral RNAs were transcribed from different vector constructs as described previously (Vos *et al.,* 1988) and the quality and yield of the transcripts was assessed by agarose gel analysis. In general, 1–2 μg of RNA-1 and 1–2 μg of mutant RNA-2 transcripts or 8–10 μg of mutant pCP-2 DNA in 50–70 μl of sterile distilled water were used to transfect 10^6 protoplasts by the polyethylene glycol method as developed by van Bokhoven *et al.* (1993). Protoplasts were incubated under continuous illumination at 25°C. After 36 h, an aliquot of

the protoplast sample was used for immunofluorescence analysis, while the reminder was harvested by centrifugation at 3000 rpm and stored at −80°C until further use. Immunofluorescence was used to determine the percentage of infected protoplasts for every construct using 110-kDa antibody to detect RNA-1-infected protoplasts (van Bokhoven *et al.,* 1993) and anti CPMV or anti 48/58-kDa MP antibodies to detect protoplasts in which RNA-2 has been replicated (Wellink *et al.,* 1993).

Plant infections

For plant infections, extracts of different protoplast samples transfected with DNA and runoff transcripts were prepared by adding 200 μl of PBS (100 mM sodium phosphate; 1.5 M NaCl, pH 7.2) to the pellet of 10^6 protoplasts and disrupting the protoplasts by repeated uptake in a syringe through a small needle. These extracts were used for both protoplast reinfections as well as for cowpea primary leaf infections as described by Wellink and van Kammen (1989).

For pCP2/S-GFP, pCP2/S-2A-GFP, and pCP2/S-2A*-GFP, plasmid DNA was directly used to inoculate the cowpea primary leaves. For this, 10 μg of pCP1 linearized with *Mlu*I and 10 μg of mutant pCP2 linearized with *Eco*RI were coinoculated onto one primary leaf. Following symptom development, infected plant tissue was macerated in the presence of 0.1 M sodium phosphate buffer, pH 7.0. After centrifugation at 10,000*g*, the supernatant (crude plant sap) was used for passaging of the infection onto new plants.

Detection of fluorescence in the protoplasts and plants

Protoplasts transfected with GFP constructs were analyzed by fluorescence microscope equipped with FITC/UV filters (Nikon Optiphot 2). Infection process and green fluorescence of different GFP constructs on the inoculated leaves and whole plants were analyzed daily by a handheld UV lamp (B100AB; Ultraviolet Products) and the leaves were analyzed by fluorescence microscopy as well as by confocal laser scanning microscopy as described by Baulcombe *et al.* (1995).

Virus purification

Virus particles from infected cowpea leaf material were partially purified by the polyethylene glycol method as described by van Kammen (1967). In the case of M19GFP2A, the partially purified virus preparations were layered onto preformed 10–40% sucrose density gradients buffered with 10 mM sodium phosphate buffer, pH 7.0, and subjected to ultracentrifugation for 3 h at 26,000 rpm in SW28 rotor at 4°C. Viral bands corresponding to top, middle, and bottom components were visualized by passing through a monochromatic light beam from top to bottom of the gradient tube or by illumination with a handheld UV lamp. Top and Bottom/Middle components were collected separately by a syringe with a long needle. Separation of the components of CPMV/S-2A-GFP virus was achieved by centrifugation on 43–65% (w/v) CsCl gradients buffered with 100 mM Tris–Cl, pH 8.3, for 20 h at 35,000 rpm at 15°C using a Sorvall TH641 rotor. The three individual viral components were visualized under visible light and collected as described earlier.

RNA extraction and RT-PCR analysis

RNA was isolated from purified virus particles or from leaf material by phenol/chloroform extraction and ethanol precipitation and either analyzed directly on agarose gels or by RT-PCR. For amplication of sequences around the N-terminus of L the method of Sijen *et al.* (1995) was followed. First-strand cDNA was synthesized with Superscript reverse-transcriptase (Gibco) and an oligo (dT) 15 primer. The cDNA was amplified by using 5′ oligo AGAAAACCATGCAGTTCATGC (1414+) and 3′ oligo GCCTTGGACAACAAAACTCG (1641−). The PCR reaction contained 250 μM of each dNTPs, 1 unit *Taq* polymerase (Boehringer), the accompanying enzyme buffer, and 2 μl cDNA reaction mixture.

For amplification of sequences at the C-terminus of the S, synthesis of first-strand cDNA was performed using Superscript reverse-transcriptase (Gibco) and a primer complementary to nucleotides 3436–3456 of wt RNA2 (CTTGCTGAAGGGACGACCTGC). PCR amplification was carried out using a 5′ sense primer corresponding to nucleotides 3101–3120 (TGTGTTGCTACCAATCCCAG) and the same 3′ antisense primer as that used for the cDNA synthesis. The PCR reactions contained dNTPs at 200 μM each, 2.5 units of Amplitaq DNA Polymerase (Perkin–Elmer), Gene Amp PCR buffer, and varying amounts of 1:100 dilutions of cDNA.

Protein analysis and Western blotting

For M19GFP10, M19GFP7, and M19GFP2A, tissue from first-passage plants was analyzed, while for CP2S-2A-GFP material from a fifth-passage plant was used. Leaf tissue was frozen in liquid nitrogen and macerated into a fine powder before extraction of the proteins in TB buffer containing 50 mM Tris–acetate (pH 7.4), 10 mM potassium acetate, 1 mM EDTA, 5 mM DTT, and 0.5 mM PMSF (12 ml TB/g tissue). The homogenate was pressed through miracloth (Calbiochem), centrifuged at 3000*g* for 15 min and the supernatant subjected to a further centrifugation of 30,000*g* for 30 min. The final supernatant fraction contains the soluble proteins and the pellet fraction contains the membrane-bound proteins. Equivalent amounts of protein samples from the supernatant and membrane fractions were boiled with sample buffer (Laemmli, 1970). Proteins were separated on 12.5 or 15% polyacrylamide/SDS gels and either stained with Coomassie brilliant blue R 250 or blotted onto nitrocellulose

membranes (Koenig and Burgermeister, 1986). The membranes were blocked with 5% (w/v) nonfat milk powder and probed with either polyclonal antibodies raised against the CPMV, the 48/58-kDa movement protein, or GFP and antirabbit igG coupled with alkaline phosphatase. Frozen protoplast samples resuspended in TB buffer and purified virus particles were analyzed on polyacrylamide/SDS gels as described earlier.

Electron microscopy and immunotrapping studies

The purified virus particles were coated onto the carbon-coated electron microscopic grids and stained with 0.1% phosphotungstic acid (PTA), pH 5.5, and examined by electron microscopy using a Philips CM12 microscope. For immunotrapping studies, the EM grids were initially coated with coat protein (1:1000) or GFP (1:500) antibodies in PBS containing 1% BSA and incubated at room temperature for 45 min. The grids were washed with 20 drops of PBS and purified virus particles (1 mg/ml) were applied and incubated for 45 min. Subsequently, the grids were rinsed with 20 drops of PBS followed by 5–10 drops of water, stained with 0.1% PTA, and examined as described earlier.

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REFERENCES

Baulcombe, D. C., Chapman, S., and Santa Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J.* **7,** 1045–1053.

Carrier, K., Hans, F., and Sanfaon, H. (1999). Mutagenesis of amino acids at two tomato ringspot nepovirus cleavage sites: effect on proteolytic processing in *cis* and *trans* by the 3C-like protease. *Virology* **256,** 161–175.

Carrington, J. C., and Dougherty, W. G. (1988). A viral cleavage site cassette: identification of amino acid sequence required for tobacco etch virus polyprotein processing. *Proc. Natl. Acad. Sci. USA* **85,** 3391–3395.

Carrington, J. C., Cary, S. M., and Dougherty, W. G. (1988). Mutational analysis of tobacco etch virus polyprotein processing: cis and trans proteolytic activities of poly proteins containing the 49-kilodalton proteinase. *J. Virol.* **62,** 2313–2320.

Chapman, S., Kavanagh, T., and Baulcombe, D. (1992). Potato virus X as a vector for gene expression in plants. *Plant J.* **2,** 549–557.

Chen, Z., Stauffacher, C., Li, Y., Schimidt, T., Bomu, W., Kamer, G., Shanks, M., Lomonossoff, G., and Johnson, J. E. (1989). Protein-RNA interactions in an icosahedral virus at 3.0 Å resolution. *Science* **245,** 154–159.

Chen, Z., Stauffacher, C. V., and Johnson, J. E. (1990). Capsid structure and RNA packaging in comovirus. *Semin. Virol.* **1,** 453–466.

Clark, A. J., Bertens, P., Wellink, J., Shanks, M., and Lomonossoff, G. P. (1999). Studies using hybrid comoviruses reveal the importance of three-dimensional structure for processing of the viral coat proteins and show that the specificity of cleavage is greater in *trans* than in *cis*. *Virology* **263,** 184–194.

Dalsgaard, K., Uttenthal, A., Jones, T. D., Xu, F., Merryweather, A., Hamilton. W. D., Langeveld, J. P., Bochuizen, R. S., Kamstrup, S., Lomonossoff, G. P., Porta, C., Vela, C., Casal, J. I., Meloen, R. H., and Rodgers, P. B. (1997). Plant-derived vaccine protects target animals against a viral disease. *Nat. Biotechniques* **15,** 248–252.

Desai, P., and Person, S. (1998). Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. *J. Virol.* **72,** 7563–7568.

Dessens, J. T., and Lomonossoff, G. P. (1993). Cauliflower mosaic virus 35S promoter-controlled DNA copies of cowpea mosaic virus are infectious on plants. *J. Gen. Virol.* **74,** 889–892.

Donelly, M. L. L., Gani, D., Flint, M., Monaghan, S., and Ryan, M. D. (1997). The cleavage activities of aphthovirus and cardiovirus 2A proteins. *J. Gen. Virol.* **78,** 13–21.

Donson, J., Kearney, C. M., Hilf, M. E., and Dawson, W. O. (1991). Systemic expression of bacterial gene by tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. USA* **88,** 7204–7208.

Eggen, R., Verver, J., Wellink, J., De Jong, A., Goldbach, R., and van Kammen, A. (1989). Improvements of the infectivity of in vitro transcripts from cloned cowpea mosaic virus cDNA: impact of terminal nucleotide sequences. *Virology* **173,** 447–455.

Goldbach, R. W., and Wellink, J. (1996). Comoviruses : molecular biology and replication. *In* "The Plant Viruses: Polyhedral Virions and Bipartite RNA Genomes" (B. D. Harrison and A. F. Murant, Eds.), Vol. 5, pp. 35–76. Plenum, New York.

Hamamoto, H., Sugiyama, Y., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y., and Okada, Y. (1993). A new tobacco mosaic virus vector and its use for the systemic production of Angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. *Biotechnology* **11,** 930–932.

Kasteel, D., Wellink, J., Verver, J., van Lent, J., Goldbach, R., and van Kammen, A. (1993). The involvement of cowpea mosaic virus M RNA-encoded proteins in tubule formation. *J. Gen. Virol.* **74,** 1721–1724.

Koenig, R., and Burgermeister, W. (1986). Applications of immunoblotting in plant virus diagnosis. *In* "Developments and Applications in Virus Testing" (R. A. C. Jones and L. Torrance, Eds.), pp. 121–137. Association of Applied Biologists, Warwick, United Kingdom.

Kratz, A. P., Bottcher, B., and Nassal, M. (1999). Native display of complete foreign protein domains on the surface of hepatitis B virus capsids. *Proc. Natl. Acad. Sci. USA* **96,** 1915–1920.

Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and effective site-specific mutagenesis without phenotypic selection. *In* "Methods in Enzymology" (R. Wu and L. Grossman, Eds.), pp. 154, 367–382. Academic Press, San Diego.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lin, T., Porta, C., Lomonossoff, G. P., and Johnson, J. E. (1996). Structure-based design of peptide presentation on a viral surface: the crystal structure of a plant/animal virus chimera at 2.8 Å resolution. *Folding Design* **1,** 179–187.

Lomonossoff, G. P., and Johnson, J. E. (1991). The synthesis and structure of comovirus capsids. *Prog. Biophys. Mol. Biol.* **55,** 107–137.

Mattion, N. M., Harnish, E. C., Crowley, J. C., and Reilly, P. A. (1996). Foot-and-mouth disease virus 2A protease mediates cleavage in attenuated sabin 3 poliovirus vectors engineered for delivery of foreign antigens. *J. Virol.* **70,** 8124–8127.

Nicklin, M. J. H., Yoyoda, H., Murray, M. G., and Wimmer, E. (1986). Proteolytic processing in the replication of polio and related viruses. *Biotechnology* **4,** 33–42.

Palmenberg, A. C., Parks, G. D., Hall, D. J., Ingraham, R. H., Seng, T. W., and Pallai, P. V. (1992). Proteolytic processing of the cardioviral P2

region: primary 2A/2B cleavage in clone-derived precursors. *Virology* **190,** 754–762.

Peters, S. A., Voorhorst, G. B. W., Wery, J., Wellink, J., and van Kammen, A. (1992). A regulatory role for the 32K protein in proteolytic processing of cowpea mosaic virus polyproteins. *Virology* **191,** 81–89.

Porta, C., Spall, V. E., Loveland, J., Johnson, J. E., Baker, P. J., and Lomonosoff, G. P. (1994). Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides. *Virology* **202,** 949–955.

Porta, C., and Lomonossoff, G. P. (1996). Use of viral replicons for the expression of genes in plants. *Mol. Biotechnol.* **5,** 209–221.

Porta, C., Spall, V. E., Lin, T., Johnson, J. E., and Lomonossoff, G. P. (1996). The development of cowpea mosaic virus as a potential source of novel vaccines. *Intervirology* **39,** 79–84.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992). Primary structure of *Aequorea victoria* green fluorescent protein. *Gene* **111,** 229–233.

Rezelman, G., van Kammen, A., and Wellink, J. (1989). Expression of cowpea mosaic virus M RNA in cowpea protoplasts. *J. Gen. Virol.* **70,** 3043–3050.

Ryan, M. D., King, A. M. Q., and Thomas, G. P. (1991). Cleavage of foot-and-mouth disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. *J. Gen. Virol.* **72,** 2727–2732.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual, 2nd ed." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Santa Cruz, S., Chapman, S., Roberts, A. G., Roberts, I. M., Prior, D. A. M., and Oparka, K. J. (1996). Assembly and movement of a plant virus carrying green fluorescent overcoat. *Proc. Natl. Acad. Sci. USA* **93,** 6286–6290.

Scholthof, H. B., Scholthof, K. B. G., and Jackson, A. O. (1996). Plant virus gene vectors for transient expression of foreign proteins in plants. *Annu. Rev. Phytopathol.* **34,** 299–323.

Siemering, K. R., Golbik, R., Sever, R., and Haselloff, J. (1996). Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.* **1,** 1653–1663.

Sijen, T., Wellink, J., Hendriks, J., Verver, J., and van Kammen, A. (1995). Replication of cowpea mosaic virus RNA-1 or RNA-2 is specifically blocked in transgenic Nicotiana benthamiana plants expressing the full length replicase or movement protein genes. *Mol. Plant Microbe Interact.* **8,** 340–347.

Stauffacher, C. V., Usha, R., Harrington, M., Schimidt, T., Hosur, M., and Johnson, J. E. (1987). The structure of cowpea mosaic virus at 3.5 Å. *In* "Crystallography in Molecular Biology" (D. Moras, J. Drenth, B. Strandberg, D. Suck, and K. Wilson, Eds.), pp. 293–308. Plenum, New York.

Sugiyama, Y., Hamamoto, H., Takemoto, S., Watanabe, Y., and Okada, Y. (1995). Systemic production of foreign peptides on the particle surface of tobacco mosaic virus. *FEBS Lett.* **359,** 247–250.

Tang, S., van Rij, R., Silvera, D., and Andino, R. (1997). Towards a poliovirus-based simian immunodeficiency virus vaccine: correlation between genetic stability and immunogenicity. *J. Virol.* **71,** 7841–7850.

Taylor, K. M., Spall, V. E., Butler, P. J. G., and Lomonossoff, G. P. (1999). The cleavable carboxyl-terminus of the small coat protein of cowpea mosaic virus is involved in RNA encapsidation. *Virology* **255,** 129–137.

Turpen, T. H., Reinl, S. J., Charoenvit, Y., Hoffman, S. L., Fallarme, V., and Gill, L. K. (1995). Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Biotechnology* **13,** 53–57.

Usha, R., Rohll, J. B., Spall, V. E., Shanks, M., Maule, A. J., Johnson, J. E., and Lomonossoff, G. P. (1993). Expression of an animal virus antigenic site on the surface of a plant virus particle. *Virology* **197,** 366–374.

van Bokhoven, H., Hendriks, J., Verver, J., and van Kammen, A. (1993). Protoplasts transiently expressing the 200 kD coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA. *J. Gen. Virol.* **74,** 2233–2241.

van Kammen, A. (1967). Purification and properties of the components of cowpea mosaic virus. *Virology* **31,** 633–642.

van Lent, J., Wellink, J., and Goldbach, R. (1990). Evidence for the involvement of the 58K and 48K proteins in the intercellular movement of cowpea mosaic virus. *Virology* **71,** 633–642.

van Lent, J., Stroms, M., van der Meer, F., Wellink, J., and Goldbach, R. (1991). Tubular structures involved in movement of cowpea mosaic virus are also formed in infected cowpea protoplasts. *J. Gen. Virol.* **72,** 2615–2623.

Verver, J., Wellink, J., Lent, J. V., Gopinath, K., and van Kammen, A. (1998). Studies on the movement of cowpea mosaic virus using green fluorescent protein. *Virology* **242,** 22–27.

Vos, P., Jaegle, M., Wellink, J., van Kammen, A., and Goldbach, R. (1988). Two viral proteins involved in the proteolytic processing of the cowpea mosaic virus polyproteins. *Nucleic Acids Res.* **16,** 1967–1985.

Wellink, J., and van Kammen, A. (1989). Cell-to-cell transport of cowpea mosaic virus requires both the 58/48K proteins and the capsid proteins. *J. Gen. Virol.* **70,** 2279–2286.

Wellink, J., van Lent, J. W. M., Verver, J., Sijen, T., Goldbach, R. W., and van Kammen, A. (1993). The cowpea mosaic virus M RNA-encoded 48-kilodalton protein is responsible for induction of tubular structures in protoplasts. *J. Virol.* **67,** 3660–3664.