Expression of Cowpea Mosaic Virus M RNA in Cowpea Protoplasts

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SUMMARY

Cowpea mosaic virus (CPMV) M RNA is translated in vitro into two polyproteins of Mr values 105000 (105K) and 95K. Using antiserum against the small capsid protein VP23, these proteins have now been detected in cowpea protoplasts, a few hours after inoculation with CPMV. These proteins could also be detected at later stages of infection, but only when proteolytic processing was inhibited by the addition of ZnCl₂. Using antiserum against a synthetic peptide, corresponding to a part of the overlapping C-terminal ends of the 58K and 48K proteins, the 58K protein, which is the amino-terminal cleavage product of the 105K protein, was found in the cytoplasmic fraction of infected protoplasts. The 48K protein, derived from the 95K protein, was detected in both the cytoplasmic and membrane fractions of protoplasts. The presence of the 105K, 95K, 58K and 48K proteins in CPMV-infected protoplasts indicates that separate initiation codons on the M RNA are used in vivo to produce the 105K and 95K polyproteins, as demonstrated in vitro.

INTRODUCTION

The expression of the two positive-stranded RNAs of cowpea mosaic virus (CPMV) has been studied extensively both in vitro and in vivo (Goldbach & van Kammen, 1985; Wellink et al., 1987; Vos et al., 1988). Both RNAs are translated into polyproteins that are cleaved by a B RNA-encoded protease into functional proteins (Vos et al., 1988). Remarkably, M RNA is translated in vitro into two polyproteins (Mr 105K and 95K) with overlapping amino acid sequences, as a result of translation initiating at different AUG codons (Vos et al., 1984). These polyproteins can be cleaved in vitro into 58K and 48K proteins and a 60K precursor to the capsid proteins by a B RNA-encoded protease (Fig. 1; Franssen et al., 1982). In studies with CPMV-infected cowpea protoplasts, the two capsid proteins, the 60K precursor of the capsid proteins and the 48K protein have been the only M RNA-encoded proteins detected so far (Wellink et al., 1987); the 58K, 95K and 105K polyproteins have not been found. That leaves the possibility open that M RNA is translated in vivo into only the smaller 95K polyprotein. In this paper we report the detection of both the 105K and 95K polyproteins, as well as the 58K N-terminal cleavage product of the 105K protein, in CPMV-infected cowpea protoplasts. The presence of these proteins in protoplasts shows that CPMV M RNA is translated into the same set of proteins in vivo as it is in vitro.

METHODS

Inoculation of protoplasts with RNA by electroporation. Mesophyll protoplasts were prepared from 10-day-old primary leaves of Vigna unguiculata L., California Blackeye. The lower epidermis of the leaves was peeled off with forceps and three leaves were floated on a 15 ml enzyme solution (0.5% purified cellulase onozuka R10, 0.05% macerozyme R10 in 0.6 M-mannitol, pH 5.5) (Rottier et al., 1979) for 3.5 h at 25°C with gentle shaking. Electroporation was done in a chamber with gold-coated glass panel electrodes, as described by Hibi et al. (1986). A platinum thread attached to the glass panels was used to connect the electrodes with the capacitor. The latter had a capacitance of 0.47 µF and was loaded by a power supply (0 to 300 V). In our standard procedure the distance between the electrodes was 5 mm and the volume of the chamber was 1 ml. Protoplasts (0.5 x 10⁶/ml) were resuspended in 0.6 M-mannitol and cooled on ice after which viral RNA (final concentration 2.5 µg/ml) was added. This suspension was immediately pipetted into the precooled chamber and electroporated.
The power supply was set at 225 V (approx. 450 V/cm) and the capacitor was discharged three times with intervals of about 1 s. After the pulses, protoplasts were transferred to a vial and allowed to stand at room temperature to recover. After 30 min, they were collected by centrifugation, washed with 0.6 M-mannitol and incubated (1 x 10^6/ml) at 25°C under continuous illumination as described by Rottier et al. (1979). The percentage of protoplasts infected with CPMV was determined by fluorescent staining with anti-CPMV serum and fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (Nordic) 24 h after inoculation (Hibi et al., 1975; Maule et al., 1980). This inoculation method was very efficient; using only 0.5 to 1 μg CPMV RNA, approximately 80% of the protoplasts became infected (data not shown).

**Labelling of proteins and subcellular fractionation.** Protoplasts (1 x 10^6/ml) were labelled with about 80 μCi/ml [35S] methionine (1000 Ci/mmol; New England Nuclear). In pulse-chase experiments protoplasts were centrifuged down after the pulse-labelling, resuspended in culture medium with 1 mM-methionine and chased for 1 to 8 h. Protoplasts (5 x 10^6) were collected by centrifugation and the pellets were frozen. For subcellular fractionation, the pellets were resuspended in 30 μl of HB buffer (50 mM-Tris-acetate pH 7.4, 10 mM-potassium acetate, 1 mM-EDTA, 1 mM-dithioerythritol, 1 mM-PMSF, 0.5 mM-ZnCl2 and 10% sucrose) and centrifuged at 4°C for 30 min at 30000 g yielding a supernatant (S30) fraction and a pellet fraction.

**Immunological methods.** Samples were boiled for 3 min in 1 x SB (2% SDS, 5% 2-mercaptoethanol, 10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 10% glycerol, 0.001% bromophenol blue), diluted at least 10-fold, adjusted to 1 x PBSTDS (10 mM-sodium phosphate pH 7.2, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and incubated in a final volume of 300 μl with 5 to 10 μl of antiserum at 4°C. After 17 h a 10% (w/v) suspension of *Staphylococcus aureus* cells (five times the volume of antiserum used; Pansorbin, Calbiochem) in PBSTDS (containing 10 mg of bovine serum albumin per ml) was added and the incubation was continued for 1 h at 4°C. The *S. aureus* cells were collected by centrifugation and the precipitate was washed five times with PBSTDS, dissolved in 1 x SB, heated for 3 min at 100°C and centrifuged. The supernatants were analysed by SDS-PAGE (Richards et al., 1989). The gels were blotted onto nitrocellulose filters (Van der Meer et al., 1984), which were exposed to Kodak XAR films.

The preparations of antisera directed against a synthetic peptide of 30 residues at the carboxy terminus of the 48K protein of M RNA and against the coat protein VP23 have been described by Wellink et al. (1987) and Franssen et al. (1982) respectively.

**RESULTS**

**Detection of the M RNA-encoded 105K and 95K proteins in CPMV-infected protoplasts**

Protoplasts were inoculated with 2.5 μg/ml CPMV M RNA. They were labelled with [35S] methionine from 30 min before inoculation until 4 h after inoculation and extracts were analysed by immunoprecipitation with anti-VP23 serum. No virus-specific proteins were detected (data not shown). In parallel experiments, protoplasts were inoculated with CPMV
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Fig. 2. Detection of the 105K and 95K polyproteins in CPMV RNA-infected cowpea protoplasts. Mock- (−) and CPMV-inoculated (+) protoplasts were labelled with [35S]methionine either from 0-5 to 4.5 h after inoculation (lanes 2 to 5) or from 21 to 21.75 h (lanes 7 to 10). In some cases 2 mM-ZnCl₂ was added to the protoplasts during the last 45 min of the labelling period (lanes 4, 5, 9 and 10). Immunoprecipitations were carried out with anti-VP23 serum on the S30 fractions of protoplasts. The precipitates were analysed in a 7.5% acrylamide gel. Proteins immunoprecipitated with anti-VP23 serum (lane 1) or anti-VP23 serum (lanes 6 and 11) from an in vitro translation of CPMV RNA were used as electrophoretic markers.

(B + M) RNA and labelled from 30 min until 4.5 h after inoculation. In an attempt to accumulate the polyproteins in larger amounts, ZnCl₂ was added to the protoplasts during the last hour of the labelling period. ZnCl₂ inhibits the processing of the polyproteins by the viral protease and has been successfully used before (Wellink et al., 1987). Upon analysis of these protoplasts with anti-VP23 serum, two proteins that comigrated with the 105K and 95K proteins obtained after in vitro translation of CPMV RNA were precipitated (Fig. 2, lane 5). Many other proteins are visible in this lane; however, these proteins were also precipitated when mock-inoculated protoplasts were used (Fig. 2, lane 4). This background of non-specifically precipitated proteins is high because the film had to be overexposed to make the 105K and 95K proteins visible. For this reason the 60K precursor to the capsid proteins, which is also recognized by the anti-VP23 serum (Wellink et al., 1987) is visible as a big black spot (Fig. 2, lane 5). Also visible in the lanes containing extracts of infected protoplasts are small amounts of the B RNA-encoded 170K protein, but it is not specifically recognized by the anti-VP23 serum and is part of the background (compare with the specifically precipitated 60K protein). In samples of protoplasts not treated with Zn²⁺ ions, it was possible to detect the 105K protein; however, the 95K protein was barely visible among the background host proteins (Fig. 2, lanes 2 and 3).

When protoplasts were labelled later in infection, it was not possible to detect the 105K and 95K polyproteins (Fig. 2, lanes 7 and 8) nor were they detectable in protoplasts labelled for
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Supernatant Total

+Zn\textsuperscript{2+}

- - - - - - +

Anti-VP23 Anti-48K

18-19 43-44 18-19 43M.4

Fig. 3

Fig. 4

Fig. 3. Detection of 60K and 59K proteins in CPMV RNA-infected cowpea protoplasts. Mock- (−) and CPMV-inoculated (+) protoplasts were labelled with \([35S]\)methionine from 18 to 19 h after inoculation in the absence (lanes 1, 2, 5 and 6) or presence of 2 mM-ZnCl\textsubscript{2} (lanes 3 and 4). Immunoprecipitations were carried out with anti-VP23 serum on S30 fractions of protoplasts (lanes 1 to 4) or extracts of total protoplasts (lanes 5 and 6). Proteins immunoprecipitated with anti-VP23 serum from an in vitro translation of CPMV RNA were used as electrophoretic markers (lane 7). These precipitates were analysed in a 7.5\% acrylamide gel.

Fig. 4. Detection of the 58K and 48K proteins in CPMV RNA-infected cowpea protoplasts. Mock- (−) and CPMV-inoculated (+) protoplasts were labelled with \([35S]\)methionine either from 18 to 19 h after inoculation (lanes 2, 3, 6 and 7) or from 43 to 44 h (lanes 4, 5, 8 and 9). Immunoprecipitations were carried out on S30 fractions of protoplasts with anti-VP23 serum (lanes 2 to 5) or anti-48K serum (lanes 6 to 9). The precipitates were analysed in a 7.5\% acrylamide gel. Proteins immunoprecipitated with anti-VP23 serum (lane 1) or anti-48K serum (lane 10) from an in vitro translation of CPMV RNA were used as electrophoretic markers.

shorter periods of time (data not shown). However, when ZnCl\textsubscript{2} was added during the labelling the two polyproteins were again visible (Fig. 2, lanes 9 and 10).

Short exposures of acrylamide gels containing similar samples as shown in Fig. 2 revealed that at the position of the 60K precursor to the capsid proteins, two bands were visible. When ZnCl\textsubscript{2} was used, the relative amount of the faster migrating 59K protein decreased in favour of the 60K protein (Fig. 3, lanes 2 and 4), suggesting that the 60K protein is the precursor of the 59K protein. When immunoprecipitations were carried out on non-fractionated protoplasts, the 59K protein was not detectable (Fig. 3, lane 6). The 59K protein seems to be formed during fractionation of the protoplasts. It is possible that the 60K and 59K proteins are related to the VP23 and VP20 forms of the small capsid protein (Franssen et al., 1986). The significance, if any, of this conversion is not known.
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**Fig. 5.** Detection of the 58K and 48K proteins in the supernatant and pellet fractions of CPMV RNA-infected protoplasts. Mock- (−) and CPMV-inoculated (+) protoplasts were labelled with [35S]methionine from 18 to 19 h after inoculation in the absence (lanes 1, 2, 5, 6, 7 and 8) or presence of 2 mM-ZnCl₂ (lanes 3, 4, 9 and 10). Immunoprecipitations were carried out with anti-48K serum on supernatant (lanes 1 to 4) and pellet (lanes 7 to 10) fractions of protoplasts and on extracts of total protoplasts (lanes 5 and 6). The precipitates were analysed in a 7.5% acrylamide gel. Proteins immunoprecipitated with anti-48K serum from an in vitro translation of CPMV RNA were used as electrophoretic markers (lane 11).

_Detection of the M RNA-encoded 58K and 48K proteins in CPMV-infected protoplasts_

Infected and control protoplasts were labelled for 1 h with [35S]methionine 18 or 43 h after inoculation. Immunoprecipitations were performed on the S30 fraction of these protoplasts with anti-48K serum and with anti-VP23 serum as a control. At both labelling times the proteins comigrating with the 58K and 48K proteins obtained after in vitro translation (Fig. 4, lane 10) were precipitated with the anti-48K serum (Fig. 4, lanes 7 and 9). A 47K protein was also precipitated with this antiserum. With anti-VP23 serum the 60K precursor of the capsid proteins is again easily visible (Fig. 4, lanes 3 and 5).

We note that the difference in migration of the 60K and 58K proteins in this gel system is much greater than that expected on the basis of their designated size. However, to avoid confusion we will use the conventional names of these proteins throughout this paper.

Immunoprecipitations with anti-48K serum were also carried out on pellet fractions of protoplasts (Fig. 5). These precipitations revealed that after 1 h of labelling the 48K and 47K proteins are found in both the S30 and pellet fractions, whereas the 58K protein is present mainly in the S30 fraction (Fig. 5).
Supernatant Pulse Chase
- + - + - + - + - +

Pellet Pulse Chase
- + - + - + - + - +

Fig. 6. Pulse-chase experiment on CPMV RNA-infected cowpea protoplasts. Mock (–) and CPMV-infected (+) protoplasts were labelled with [35S]methionine from 18 to 19 h after inoculation. The label was chased for 1, 2, 4 and 8 h. Immunoprecipitations were carried out with anti-48K serum on supernatant (lanes 1 to 10) and pellet fractions (lanes 12 to 21) of protoplasts. The precipitates were analysed in a 7.5% acrylamide gel. Proteins immunoprecipitated with anti-48K serum from an in vitro translation of CPMV RNA were used as electrophoretic markers (lanes 11 and 22).

To examine the possible relationship between the 48K and 47K proteins, immunoprecipitations were carried out on fractions of protoplasts incubated with ZnCl₂. In the presence of Zn²⁺ ions the ratio of the 48K and 47K proteins in the pellet fraction is changed in favour of the 48K protein (Fig. 5, lanes 8 and 10). When immunoprecipitations were performed on non-fractionated protoplasts, only 48K could be detected (Fig. 5, lane 6). This suggests that the 47K protein is a cleavage product of the 48K protein and that, similar to the conversion of the 60K into the 59K protein, this cleavage occurred during fractionation of the protoplasts.

Stability of the 58K and 48K proteins in protoplasts

A pulse–chase experiment was carried out to determine the stability of the 58K and 48K proteins in protoplasts and to determine whether a precursor–product relationship exists between these proteins. Therefore infected and control protoplasts were labelled with [35S]methionine between 18 and 19 h after inoculation, followed by a chase of up to 8 h. Immunoprecipitations with anti-48K serum were performed on S30 and pellet fractions of these protoplasts (Fig. 6).

From this analysis it can be concluded that the 48K and 47K proteins disappear rather quickly from the S30 fraction. However only a small amount of the 48K and 47K proteins is detectable in the pellet fraction after 8 h of chase. The 58K protein appeared to be more stable than the 48K protein and was found only in the S30 fraction.

To determine whether the 58K and or 48K proteins were excreted into the medium, immunoprecipitations were also performed on the culture medium. No substantial amounts of these proteins could be detected at any time point of the pulse–chase experiment. From these
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In vivo experiments it can be concluded that the 58K and 48K proteins are less stable than the other viral proteins in infected protoplasts, as demonstrated by the invariable amounts of the pulse-labelled 170K protein and VP37, which are non-specifically precipitated by the anti-48K serum during the chase (Fig. 6). Furthermore it is unlikely that the 48K protein is a cleavage product of the 58K protein.

DISCUSSION

The detection of the 105K, 95K and 58K proteins in the CPMV-infected cowpea protoplasts fills a space that existed between the results obtained with *in vitro* translation experiments with M RNA (105K, 95K, 58K, 48K and 60K proteins) and the results of studies on the *in vivo* expression of M RNA (48K and 60K proteins; Wellink *et al.*, 1987).

Our initial experiments to detect the M RNA-encoded 105K and 95K polyproteins from transiently expressed M RNA were not successful. However, when protoplasts were labelled with [35S]methionine immediately after inoculation with CPMV RNA, it was possible to detect the polyproteins. Therefore M RNA multiplication seems to be essential for the detection of primary translation products. It is probably also important that the viral protease responsible for processing the polyproteins is present at much lower amounts than at later stages of infection (Vos *et al.*, 1988). At these later times it was possible to detect the polyproteins only when ZnCl₂ was used to inhibit the viral protease (Wellink *et al.*, 1987).

*In vitro* experiments have shown that initiation of translation of the smaller (95K) polyprotein occurs at an internal AUG codon at position 512 or 524 (Vos *et al.*, 1984). The detection of a 48K and a 47K protein might indicate that both these start codons were used. On the other hand, the effect of ZnCl₂ and the failure to detect the 47K protein in unfractionated protoplasts clearly demonstrated that the 47K protein is a cleavage product of the 48K protein. Recent *in vitro* results from M RNA with mutations in these start codons show that the one at position 512 is used for initiation of translation of the 95K protein (J. Verver, T. Schotman, A. van Kammen & J. Wellink, unpublished results). Because the same set of proteins are found *in vitro* and in CPMV-infected protoplasts it is highly likely that *in vivo* the initiation of the translation of the 95K protein also takes place at position 512. Furthermore, results of the pulse-chase experiment seem to indicate that the 58K protein (derived from the 105K protein) is not a precursor of the 48K protein (derived from the 95K protein).

The location of the 58K and 48K proteins in the protoplasts is clearly different. The 58K protein is found mainly in the cytoplasm, whereas the 48K protein is present in both the cytoplasm and the membrane fraction. The pulse-chase experiment further revealed that the 58K and 48K proteins are not very stable in protoplasts. The instability of both proteins may be a natural characteristic, but may also be intrinsic to the protoplast system. The 58K and/or 48K proteins probably are involved in cell-to-cell transport of the virus (Rezelman *et al.*, 1982; Wellink & van Kammen, 1989). Therefore it is possible that the behaviour of the 48K and 58K proteins in protoplasts (i.e. stability, location) do not reflect the situation of these proteins in whole plants.

Previously we have reported the presence of the 48K protein in the culture medium of protoplasts at 40 h after inoculation, using the immunoblot technique (Wellink *et al.*, 1987). In the experiments reported here we were not able to detect the 48K protein in the medium by immunoprecipitation of radiolabelled proteins at either early or late times in infection. At present we have no explanation for this discrepancy other than the possibility that it is caused by differences between the techniques used to detect these proteins. Because the 58K/48K proteins seem to pursue their main function in plants, experiments are now being done to localize and study these proteins in whole cowpea plants.

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