Defective cell-to-cell movement of cowpea mosaic virus mutant N123 is efficiently complemented by sunn-hemp mosaic virus


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During an infection with cowpea mosaic virus (CPMV) both virion assembly and formation of tubules associated with plasmodesmata are required for cell-to-cell movement. These functions are encoded by the M-RNA of CPMV. To study the mechanism of CPMV movement, mutant N123 was used in complementation studies with sunn-hemp mosaic virus (SHMV), a legume-infecting tobamovirus. Previous studies have shown that N123 fails to spread in cowpea plants because of mutation(s) in its M-RNA. However, the mutant was efficiently replicated in cowpea protoplasts, in which virions were formed and tubular transport structures were induced. After high-dose inoculation of cowpeas with N123, only a few infected protoplasts could be isolated, indicating that cell-to-cell transport of N123 was greatly impaired, if not completely abolished. Upon coinoculation with SHMV, mutant N123 infected cowpea plants systemically and accumulated to levels which were comparable to those of wild-type CPMV. In contrast, separate B-RNA of CPMV and a CPMV deletion mutant lacking the tubule-inducing function, were complemented by SHMV to only low levels. It is concluded that SHMV-facilitated spread of CPMV in the non-virion tobamovirus mode is inefficient and that spread of mutant N123 is probably in the CPMV mode, SHMV providing an as yet unidentified helper function.

Introduction

Cowpea mosaic virus (CPMV; comovirus group) has a bipartite positive RNA genome consisting of bottom component (B) and middle component (M) RNAs. Both RNAs are translated into polyproteins which are cleaved by a B-RNA-encoded protease into functional proteins (Goldbach & van Kammen, 1985). 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; Eggens & van Kammen, 1988). However, for a successful infection of plants, M-RNA is essential. This RNA encodes two overlapping 58K and 48K proteins and the two capsid proteins (Franssen et al., 1982). Using deletion and insertion mutants derived from an infectious cDNA clone of M-RNA, Wellink & van Kammen (1989) showed that all of these M-encoded proteins are required for cell-to-cell movement of CPMV. Van Lent et al. (1990, 1991) reported that CPMV induces the formation of special tubular structures extending from the plasmodesmata of infected cells. Immunogold analysis has revealed that they contain the 58K and/or 48K proteins. The tubules are formed in cells in situ and also on the surface of isolated and inoculated protoplasts. It is assumed that they pave the way for cell-to-cell spread of CPMV. They are formed early in infection and contain mature virions (van Lent et al., 1990, 1991). This finding, together with the fact that the capsid proteins are necessary for virus movement, suggests that CPMV is transported from cell to cell through the tubules in the form of virions. Very recently it has been shown that only the 48K protein (and not the 58K protein) is responsible for the induction of the tubular structures on protoplasts (J. Wellink, J. Verver & J. van Lent, unpublished).

A different mechanism for viral translocation appears to be used by tobacco mosaic virus (TMV). It has been suggested that the 30K transport protein of TMV interacts with the plasmodesmata, thereby increasing their permeability and facilitating the passage of the virus in a non-virion form (Wolf et al., 1989; Atkins et al., 1991). In addition, it has been suggested that RNA-binding properties of the 30K protein play a role in cell-to-cell movement of TMV RNA (Citovsky et al., 1992).

Despite the differences between the transport mechanisms of tobamoviruses and comoviruses, the former have been shown to complement the transport of B-RNA of red clover mottle comovirus (Malyshenko et al., 1988, 1989). The processes involved in the two types of transport are largely unknown. Detailed
description of the intergroup complementation phenomenon at cellular and molecular levels may shed light on either transport mechanism.

We initiated a study of CPMV transport using the nitrous acid-induced CPMV mutant N123. This mutant is defective for multiplication in cowpea plants. In Pinto bean plants the mutant induces necrotic local lesions that are much smaller than those of the wild-type virus. In supplementation tests one mutation, or more, was located in the M-RNA of the mutant (de Jager, 1976). As almost all the genetic information of M-RNA is essential for transport, this N123 mutant seemed to be a good candidate for investigating the CPMV transport mechanism. Here we show that the cell-to-cell transport of N123 is defective and can be complemented by the tobamovirus sunn-hemp mosaic virus (SHMV), which itself readily infects cowpea plants.

Methods

Viruses and plants. The wild-type CPMV used was a laboratory culture of the SB strain. The nitrous acid-induced CPMV mutant N123 was derived from this strain (de Jager, 1976). The SHMV isolate used was described by Rees & Short (1975) as the cowpea strain of TMV. CPMV-SB and SHMV were maintained and multiplied in cowpea [Vigna unguiculata (L.) Walp., cv. California Blackeye], purchased from Aylee Burpee Co., Warminster, U.S.A. This cultivar was also used for infection and complementation experiments. Mutant N123 was maintained by local lesion transfer in beans [Phaseolus vulgaris (L.), cv. Pinto UI114] developed by the University of Idaho. These beans were also used for quantitative local lesion assays and for discriminating between wild-type SB and mutant N123 by local lesion type.

Plants were raised in a greenhouse at 22 to 25 °C, with supplementary light from Philips HPIT lamps to give a daylight of 16 h. Inoculation of plants was on primary leaves, 9 days after sowing, using glass rods with a roughened flat end and Carborundum 500-mesh as an abrasive.

SHMV was purified from cowpea by the method of Goording & Hebert (1967) and CPMV by the method of van Kammen (1967).

Protoplasts. Cowpea protoplasts were isolated (Rezelman et al., 1989) and inoculated with extracts of local lesions and transcripts using polyethylene glycol (Eggen et al., 1989). Extract containing N123 was prepared by homogenizing 10 local lesions in 75 μl of 0.1 M-sodium phosphate buffer pH 7.5 in an Eppendorf tube using a tightly fitting pestle.

Protoplasts were incubated under continuous illumination as described by Rottier et al. (1979). The percentage of infected cells was determined by immunofluorescence staining of the protoplasts with anti-CPMV serum as described by van Lent et al. (1991). Extracts of protoplasts were prepared by sonicating 106 protoplasts in 100 μl of 0.01 M-sodium phosphate containing 0.15 M-NaCl pH 7.2 (PBS). Negative staining of tubular structures generated in infected protoplasts was carried out as described by van Lent et al. (1991).

ELISA procedures. Leaf discs with a diameter of 5 mm were collected from inoculated primary and non-inoculated trifoliolate leaves of cowpea plants at different intervals after inoculation, using a cork borer. Composite samples of eight discs taken from at least four or five plants were homogenized in 1 ml of PBS, and analysed by ELISA. Control samples of mock (buffer)-inoculated plants were also included.

Double antibody sandwich (DAS) or indirect ELISA procedures (Clark et al., 1986) were used to detect CPMV coat proteins or the CPMV B-RNA-encoded 24K protein (viral protease), respectively. For DAS-ELISA, polyclonal antisera to CPMV was raised in rabbits. Antibodies were purified and conjugated with alkaline phosphatase. Microtiter plate wells were coated with 200 μl of the anti-CPMV antibodies (5 μg/ml in 50 mM-sodium carbonate, pH 9.6) overnight at 4 °C. Wells were rinsed with water and incubated for 2 h at 37 °C with 200 μl samples of tissue homogenate in serial twofold dilutions in PBS, containing 0.05% Tween-20 (PBS-Tween). After rinsing, wells were incubated for 1 h at 37 °C with 200 μl alkaline phosphatase-conjugated rabbit anti-CPMV antibodies (1:1000) in PBS-Tween and subsequently, after rinsing again, with p-nitrophenyl phosphate substrate (1 mg/ml in 10% diethanolamine, pH 9.8). Readings were in a EL 312 microplate reader (Biotek Instruments). Concentrations of CPMV in the samples were estimated by comparing the readings of the sample dilution series with those of a twofold dilution series of purified CPMV.

For indirect ELISA, the microtiter plates were coated with 200 μl volumes of serially diluted tissue homogenate in blocking buffer (PBS containing 1% BSA) overnight at 4 °C. Wells were rinsed and incubated sequentially with blocking buffer (1 h at 37 °C), rabbit anti-CPMV antibodies (5 μg/ml in PBS-Tween, 1 h at 37 °C), goat anti-rabbit IgG conjugated with alkaline phosphatase (1:1000, 1 h at 37 °C), and p-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine, pH 9.8). The anti-24K protein antibodies were made in rabbits against a synthetic peptide (Wellink et al., 1987). Results were read as described for DAS-ELISA. Concentrations of 24K protein were expressed as relative units. The amount of 24K protein accumulated in CPMV-inoculated primary leaves of California Blackeye cowpeas during 4 days after inoculation was taken as 1 unit. Sap from such leaves was stored in aliquots at −70 °C and twofold dilution series were used as standard throughout the work.

In vitro transcription. In vitro transcription of pTM1G and pTBIG, which contain full-length cDNAs of CPMV M- and B-RNA respectively (Eggen et al., 1989) and pTMAP, which contains a deletion of 486 nucleotides in the 58K/48K protein-coding region (Wellink & van Kammen, 1989), were carried out as described previously (Vos et al., 1988). The yield and integrity of the RNA were checked on agarose gels.

Results

Infectivity of N123 in cowpea protoplasts

To determine whether the mutation(s) induced in the N123 genome affected viral replication in single cowpea cells, the ability of this mutant to replicate was examined using the cowpea protoplast system.

Since N123 is defective for infection in cowpea leaves and thus could not be purified in significant quantities, cowpea protoplasts were inoculated with extracts of local lesions, induced in Pinto bean primary leaves. Infection of protoplasts was scored by immunofluorescence with anti-CPMV serum 48 h post-inoculation (p.i.). About 70% of the protoplasts inoculated with N123 were fluorescing, as compared with approximately 80% of the protoplasts inoculated with wild-type SB. Extracts of protoplasts were analysed by Western blotting using antiserum against the 110K protein and found to contain similar amounts of B-RNA-encoded proteins (data not shown). Although the exact inoculum dose was unknown owing to the use of lesion extracts as inoculum, these
results indicate that the mutant readily infects cowpea protoplasts and can multiply in single cowpea cells.

Protoplasts were also inoculated with extracts of mutant lesions from bean plants coinoculated with SHMV. In these mixedly infected bean plants, lesions were slightly larger than lesions of N123 alone, but still much smaller than wild-type lesions. Since SHMV infects Pinto beans systemically, the protoplast inoculum obtained from these lesions must have contained both viruses, and protoplasts were assumed to be infected by both the CPMV mutant and SHMV.

This double infection, however, did not change appreciably either the percentage of fluorescing protoplasts (approx. 80%) or the intensity of staining, indicating that SHMV did not affect the replication of mutant N123 in single cells.

The nature and quantity of virus progeny in the protoplasts were checked by inoculation of protoplast extracts onto primary leaves of Pinto beans. Extracts containing wild-type CPMV or mutant N123, either with SHMV or without, gave similar numbers of lesions. The small size of the lesions induced by mutant progeny indicated that it was N123 that multiplied in the protoplasts and not wild-type revertant. Lesions resulting from extracts of protoplasts inoculated with N123 in the presence of SHMV were, on the average, slightly larger than N123 lesions, indicating that at least some of the protoplasts were indeed infected by both viruses.

Previously it was shown that virus-containing tubular structures were formed on protoplasts infected with CPMV. These tubules extended into the medium (van Lent et al., 1991). Although mutant N123 did not spread in cowpea plants, protoplasts infected with the mutant exhibited the presence of similar tubular structures, whether or not SHMV had been co-inoculated. Virions were also found in the N123-induced tubules. It should be noted that SHMV particles were never observed within the tubules.

**Infectivity of N123 in cowpea plants**

To ascertain that the N123 isolate used was still incapable of infecting cowpea plants, regular checks on infectivity were incorporated into the experiments. In contrast to the results with isolated protoplasts, there was no or hardly any detectable infection by mutant N123 of intact cowpea plants, which were otherwise fully susceptible to wild-type SB. As found earlier (de Jager, 1976), N123 failed to give symptoms of infection in inoculated or non-inoculated leaves. Only when the mutant inoculum dose was relatively high (Pinto bean lesions homogenized in one drop of buffer per lesion), a superficial etching was seen in inoculated primary leaves of some plants. In contrast, SB caused many chlorotic local lesions with diffuse borders in inoculated leaves and a mild, light green mosaic with slight malformation of leaflets in upper non-inoculated leaves.

Failure of N123 to spread in cowpea plants was shown by three types of tests: (i) back inoculations on Pinto beans, (ii) isolation and immunofluorescence screening of protoplasts from inoculated and non-inoculated leaves and (iii) ELISA with leaf samples, using antisera against the coat proteins (encoded by M-RNA) or against the 24K viral protease (encoded by B-RNA). For results of these tests see the data given in the next section for control inoculations of separate mutant and wild-type viruses.

After a high dose inoculation of N123 few infected protoplasts (1-4%) could be isolated from inoculated cowpea leaves, which showed superficial etching. In contrast, 93% of protoplasts from SB-inoculated leaves stained. ELISA values for N123-inoculated leaves were above background but low in comparison with those for wild-type infection. In back tests on Pinto beans only a few small lesions were induced. Non-inoculated leaves of N123-inoculated plants gave negative results with any test method used (Table 1).

In another experiment, in which a lower inoculum dose was used for N123, all tests were negative, for both inoculated and non-inoculated leaves. Data for SB inoculations showed a high virus content in all leaves tested (Table 2, Fig. 1).

These results and those of protoplast inoculations support the suggestion that the mutational defect in mutant N123 greatly impairs or even inhibits the translocation from cell to cell in cowpea rather than its replication in single cells.

**Effective complementation of N123 spread by SHMV in cowpea**

To test whether spread of mutant N123 in cowpea could be complemented by unrelated SHMV, primary leaves of cowpea plants were inoculated with a mixture of a high-dose N123 lesion extract (one drop of buffer per lesion) and purified SHMV. Single inoculations of N123, wild-type SB (lesion extract), SHMV, and inoculation of a mixture of the SB lesion extract and purified SHMV, served as controls.

The N123 + SHMV mixture induced many lesions in inoculated leaves which were of the same type as lesions of the SB + SHMV mixture. These lesions were more sharply defined than the lesions induced by SB alone.

Seven days after inoculation, the non-inoculated upper leaves of SB + SHMV-inoculated plants showed strongly reduced development, severe malformation and some veinal necrosis. Two weeks after inoculation growth had stopped and almost all leaflets had dropped. At that time
Table 1. Complementation by SHMV of mutant N123 spread in cowpea as determined by three different test methods

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Primary/secondary leaves tested</th>
<th>Back test on Pinto bean</th>
<th>Isolation of protoplasts</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sampling (days p.i.)*</td>
<td>No. of lesions</td>
<td>Sampling (days p.i.)</td>
<td>Protoplasts fluorescing (%)†</td>
</tr>
<tr>
<td>SB</td>
<td>Prim 7 &gt; 100</td>
<td>8</td>
<td>93</td>
<td>5</td>
</tr>
<tr>
<td>N123</td>
<td>Sec NT</td>
<td>11(1)</td>
<td>66</td>
<td>5</td>
</tr>
<tr>
<td>SB + SHMV</td>
<td>Prim 7 9</td>
<td>8</td>
<td>1:4</td>
<td>5</td>
</tr>
<tr>
<td>N123 + SHMV</td>
<td>Sec NT</td>
<td>7(1)</td>
<td>79</td>
<td>5</td>
</tr>
<tr>
<td>SHMV</td>
<td>Prim NT</td>
<td>7(1)</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Sec NT</td>
<td>11(1)</td>
<td>20</td>
<td>5</td>
</tr>
</tbody>
</table>

* In parentheses: position of the secondary leaf sampled, where 1 refers to the oldest trifoliolate leaf and 2 to the younger leaf.
† Percentages for fluorescing protoplasts are of one test plant per treatment.
‡ ELISA absorbance values are averages of three or four test plants per treatment.
§ NT, Not tested.

Table 2. Analysis on Pinto beans of wild-type CPMV and mutant N123 progeny in cowpea plants in the presence or absence of SHMV

<table>
<thead>
<tr>
<th>Type of leaf tested</th>
<th>Inoculum</th>
<th>No. of lesions*</th>
<th>Lesion size†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>SB</td>
<td>95 Large</td>
<td></td>
</tr>
<tr>
<td>N123</td>
<td>0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>N123 + SHMV</td>
<td>49 Small</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHMV</td>
<td>0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>N123</td>
<td>38 Small</td>
<td></td>
</tr>
<tr>
<td>N123 + SHMV</td>
<td>38 Small</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHMV</td>
<td>0</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers of lesions are averages for six half-leaves.
† Large wild-type lesions have a diameter of 2 to 3 mm. Small lesions typical of mutant N123 have a diameter of 0.5 to 1 mm. Lesions induced by the N123 + SHMV extract were, on average, slightly larger than N123 lesions. However they were still much smaller than wild-type lesions.

In another experiment ELISA was used to quantify the accumulation of virus with time. Back tests on Pinto were done from the primary and secondary leaves. The results (Table 2) show that the presence of SHMV facilitated spread and multiplication of N123 in cowpea. However, considering the sampling dates for the three test types and the position of the secondary leaves sampled, it appears that the accumulation of mutant virus in upper leaves is somewhat delayed. This is in accordance with the delayed development of symptoms.

Fig. 1 shows results of ELISA, with both antisera against coat proteins and 24K viral protease. In primary inoculated leaves N123 accumulated in the presence of SHMV to almost the level of wild-type CPMV (Fig. 1 a). SHMV was also able to stimulate spread to and multiplication of mutant N123 in upper non-inoculated leaves of the plant, although with some delay, as found in the previous experiment (Fig. 1 b).

When 24K protease antiserum is used in ELISA the accumulation of B-RNA-encoded proteins is monitored.
Limited complementation of spread of B-RNA and a transport protein mutant of CPMV by SHMV

Although the mutation of N123 was located in the M-RNA of CPMV (de Jager, 1976), it was not clear which gene was affected. However since virus particles could be formed in N123-infected protoplasts and in plants co-infected with N123 and SHMV, one could hypothesize that the mutation was in the 58K/48K protein gene rather than the coat protein genes. If so, spread of N123, as facilitated by SHMV, could be in the form of particles or of viral RNA. To distinguish between the two modes, complementation of N123 was compared to complementation of CPMV B-RNA and complementation of mutant MAP, which has a deletion in the 58K/48K gene of M-RNA (Wellink & van Kammen, 1989). B-RNA alone accumulates in primary inoculated cells in a non-virion form and fails to spread. The MAP deletion mutant can accumulate as virus particles, but cannot spread and fails to induce tubules on protoplasts (Kasteel et al., 1993).

As it was impossible to obtain B-RNA of wild-type CPMV completely free of contaminating M-RNA, an infectious transcript derived from a full-length B-cDNA clone (pTB1G; Eggen et al., 1989) was used. As a positive control, this transcript B-RNA was also applied in combination with transcripts of the full length M-cDNA clone pTM1G (Eggen et al., 1989).

Upon inoculation with the B-RNA transcript alone the infection did not spread, neither locally nor systemically, as determined by ELISA using the anti-24K serum. However, a mixture of B- and M-RNA transcripts behaved like wild-type CPMV, showing a high level of infection of both inoculated and non-inoculated leaves and acceleration of systemic spread upon coinoculation with SHMV (Fig. 2). Symptoms induced were also comparable to those after CPMV infection. Thus, the B-RNA transcript used was fully infectious.

Coinoculation of the B-RNA transcript with SHMV resulted in some accumulation of the 24K protein in both inoculated and non-inoculated leaves but, despite full infectivity of the B-RNA transcript, to only a low level as compared with infection by N123 complemented by SHMV (Fig. 2, c, d).

The 58K/48K deletion mutant MAP was propagated by infecting cowpea protoplasts with MAP RNA in the presence of the B-RNA transcript and inoculum was prepared by sonicating 10^6 protoplasts 72 h p.i. in 100 μl of PBS.

As seen from Fig. 2(a, b), SHMV could promote some accumulation of capsid proteins of MAP, both in primary leaves and in secondary leaves, but only to a very low level. Fig. 2(c, d) shows that accumulation of 24K protein of MAP also occurred in the presence of SHMV.
but only temporarily and to only about half the level of N123 (Fig. 1 c).

In conclusion, with both CPMV B-RNA (incapable of forming virus particles) and a CPMV mutant with a non-functional tubule-inducing transport protein, complementation by SHMV did occur, but only to a low level. This contrasted with the very efficient complementation of the nitrous acid-induced mutant N123 by SHMV.

**Discussion**

In this paper we show that the nitrous acid-induced CPMV mutant N123 is able to infect and replicate in cowpea protoplasts and to form virus particles. In contrast, in cowpea plants, the mutant cannot develop symptoms of infection and no virus, or only very little, can be recovered from inoculated leaves. Hence, infection of cowpea by N123 is strongly localized by impairment or full blockage of translocation from cell to cell.

For CPMV, both assembly of RNA and proteins into virus particles and the induction of tubular structures in the host cells are required for virus transport. Both functions are encoded by the M-RNA of CPMV. Although the movement mutant N123 has mutation(s) in this RNA, this mutant forms particles and induces tubular structures in infected protoplasts. Thus it is not immediately clear which transport function is affected in N123.

As explained in the Introduction, tobamoviruses and comoviruses have different modes of spread. Yet there is an efficient complementation of N123 by SHMV. Since B-RNA alone was inefficiently complemented as compared to N123, unencapsidated CPMV RNA seems not to be efficiently transported in the tobamovirus mode, possibly due to inefficient competition with SHMV RNA for the ‘services’ of the 30K transport protein. This then should also apply to the RNA of MAP, and this mutant is, indeed, complemented only at a low level. The presence of a functional coat protein in this case is not helpful and indeed encapsidation of the MAP RNA in its coat proteins may even hamper transport of this RNA in the tobamovirus mode. This may explain the ephemeral nature of the complementation of MAP.

Fig. 2. Accumulation of CPMV capsid proteins (a, b) and 24K protein (c, d) during infection of California Blackeye plants with infectious CPMV B-RNA transcript or MAP mutant in the absence or presence of SHMV helper virus. For further details see Fig. 1 legend. Amount of CPMV capsid antigen (μg/ml) in inoculated primary leaves is shown in (a), and in systemically infected trifoliolate leaves in (b). Amount of CPMV 24K protein (relative units) in inoculated primary leaves is shown in (c) and in systemically infected trifoliolate leaves in (d).
As N123, with functional coat proteins as well as tubule-inducing function(s), is complemented efficiently, it can be hypothesized that spread of this mutant in the presence of SHMV is in the CPMV mode, requiring both tubular structures and viral particles. In that case the mutation(s) in N123 is such that the effect can be compensated by an as yet unidentified SHMV function. The helper function of SHMV could be, for instance, the suppression of a defensive host response or the increased mobilization of a host factor required for transport. If so, comparative sequence studies with N123 and revertants of this mutant showing the wild phenotype could possibly lead to identification of domains in the M-RNA (coding sequence) involved in interaction with cellular proteins.

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References


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