Expression of alfalfa mosaic virus coat protein in tobacco mosaic virus (TMV) deficient in the production of its native coat protein supports long-distance movement of a chimeric TMV


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ABSTRACT Alfalfa mosaic virus (AlMV) coat protein is involved in systemic infection of host plants, and a specific mutation in this gene prevents the virus from moving into the upper uninoculated leaves. The coat protein also is required for different viral functions during early and late infection. To study the role of the coat protein in long-distance movement of AlMV independent of other vital functions during virus infection, we cloned the gene encoding the coat protein of AlMV into a tobacco mosaic virus (TMV)-based vector Av. This vector is deficient in long-distance movement and is limited to locally inoculated leaves because of the lack of native TMV coat protein. Expression of AlMV coat protein, directed by the subgenomic promoter of TMV coat protein in Av, supported systemic infection with the chimeric virus in Nicotiana benthamiana, Nicotiana tabacum MD609, and Spinacia oleracea. The host range of TMV was extended to include spinach as a permissive host. Here we report the alteration of a host range by incorporating genetic determinants from another virus.

The interaction between virus and plant proteins determines the capability of the virus to multiply and systemically infect the host plant (1). Systemic infection with plant viruses requires cell-to-cell and long-distance movement of viral genomic RNA (2, 3). Many plant viruses find access into cells through wounds. Upon initial entry into a plant cell, the virus multiplies and moves locally from cell to cell (local infection). In most cases, the transfer of viral RNA between cells is required for the encapsidation and long-distance movement of the virus in an infected host (16, 17, 24). Movement and coat proteins are translated from subgenomic mRNAs (30–32).

Alfalfa mosaic virus is a member of the Bromoviridae family. The genome of this virus consists of three plus-sense RNAs (RNAs 1, 2, and 3), which are encapsidated by a single CP (24 kDa) that results in bacilliform or spherical particles depending on the size of RNA encapsidated. A fourth RNA (subgenomic RNA4) of AlMV is the messenger for the CP and is synthesized from genomic RNA3. The CP plays a key role in early and late infection (33–36). The CP is involved in symptom formation (37, 38), virus assembly (39, 40), stability of viral RNA (41, 42), and long-distance movement of viral RNA (18). Moreover, the AlMV CP is involved in symptom formation (37, 38).

The multiple functions of the AlMV CP has made it difficult to analyze any single function without interfering with others. To address the role of AlMV CP in long-distance movement, we used the TMV-based expression vector Av, which is deficient in TMV CP production and, therefore, limited to inoculated leaves. Expression of the AlMV CP supported the long-distance movement of Av in Nicotiana benthamiana, Nicotiana tabacum MD609, and Spinacia oleracea.

MATERIALS AND METHODS

DNA Constructs. All cloning and cell transformations were performed according to Sambrook et al. (44). Escherichia coli DH5α-competent cells (Life Technologies, Gaithersburg, MD) were used for transformation. The TMV-derived vector was constructed such that the translation start codon ATG of TMV CP was replaced with AGA, and multiple cloning sites PacI, Pmel, AgeI, and XhoI were introduced 42 nt downstream of the mutated ATG codon. Av (Fig. 1) contains the full-length TMV but is defective in CP production and, therefore, limited to inoculated leaves. Expression of the AlMV CP supported the long-distance movement of Av in Nicotiana benthamiana, Nicotiana tabacum MD609, and Spinacia oleracea.

Abbreviations: AlMV, alfalfa mosaic virus; TMV, tobacco mosaic virus; CP, coat protein; RT-PCR, reverse transcription–PCR.

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and processed according to the manufacturer's guidelines. Northern blot analysis of RNA was performed as described (45) using 5 μg of total RNA. The RT reaction was performed using Moloney murine leukemia virus reverse transcriptase (Promega) and 5'-TTTTTCCGGAAACCTTTTCCG-3' as primer, which was also used in PCR with 5'-GGGCCCCATG-GAACCTTACAGAAGAA-3' Taq DNA polymerase (Promega).

**Western Blot Analysis.** AIMV CP produced in virus-infected plants was analyzed by Western immunoblotting (46) using antibodies from Agdia (Elkhart, IN). Proteins from crude plant extracts or from purified virus particles were separated electrophoretically on SDS-polyacrylamide gels and electroblotted onto a nylon membrane overnight at 33 mA. After blocking with milk (Kirkegaard & Perry Laboratories), proteins were allowed to react with appropriate antibodies and detected by using the Vectastain ABC kit (Vector Laboratories).

**RESULTS**

**Infection of Plants with AIMV, Av/A4, and TMV.** Leaves of *Nicotiana benthamiana*, *Nicotiana tabacum* MD609, and *Solanum oleracea* were inoculated with AIMV, TMV, or in vitro synthesized transcripts of Av/A4. *N. benthamiana* and *N. tabacum* MD609 are systemic hosts for both AIMV and TMV, whereas *S. oleracea* is systemically infected only with AIMV but not with TMV (47).

*N. benthamiana.* Inoculation of *N. benthamiana* with either TMV, Av/A4, or AIMV resulted in systemic infection with symptoms developing in the upper un inoculated leaves within 7–10 days. Systemic infection of *N. benthamiana* with either AIMV, Av/A4, or TMV resulted in mild curling and yellowing of leaves, with no significant differences between the viruses in the symptoms induced early in infection. Although very similar amounts (0.6–0.8 mg/g fresh tissue) of AIMV, Av/A4, and TMV were recovered from systemically infected *N. benthamiana* leaves, the TMV-infected plants developed stem necrosis and died within 12–15 days postinoculation, whereas AIMV- or Av/A4-infected plants remained nonnecrotic and alive. Even after 30 days of infection, AIMV- or Av/A4-infected plants never developed the necrotic reactions observed with TMV.

*N. tabacum* MD609. Movement of TMV into the upper un inoculated leaves of *N. tabacum* MD609 (9–12 days postinoculation; dpi) was indicated clearly by mild mosaic symptoms and yellowing along leaf veins, leaving occasional green islands of uninfected tissue. Whereas TMV caused no symptoms on a locally inoculated leaf, the infection with either AIMV or Av/A4 resulted in the formation of necrotic lesions (5–6 dpi), which led to the death of the inoculated leaf 15–17 dpi. This necrotic death of the inoculated leaf, however, did not prevent the systemic infection with AIMV or Av/A4 from proceeding. Systemic invasion (8–10 dpi) of *N. tabacum* MD609 with AIMV or Av/A4 resulted in distinct mosaic symptoms with occasional necrotic spots. Systemic infection of TMV began at the apex, whereas AIMV or Av/A4 never reached the apex, leaving the uppermost two to three leaves symptomless. These results indicate that AIMV and TMV differ in the mechanisms of systemic movement.

*S. oleracea.* Inoculation of *S. oleracea* plants with either AIMV or Av/A4 resulted in mild mosaic yellowing of systemically infected leaves, whereas inoculation with TMV induced no symptoms and did not result in infection of uninoculated leaves as determined by Western and Northern blot analyses (not shown). In addition, we inoculated *N. tabacum* cv. Xanthi-nc plants with the sap from inoculated and the upper uninoculated leaves of *S. oleracea* exposed either to TMV, AIMV, or Av/A4. Inoculation of *N. tabacum* cv. Xanthi-nc with the sap from both inoculated and the upper uninoculated leaves of *S. oleracea* infected with AIMV resulted in...
infection. Similarly, sap from both inoculated and the upper unoinoculated leaves of *S. oleracea* infected with *Av/A4* resulted in infection, inducing local lesions on *N. tabacum* cv. Xanthi-nc. However, when we inoculated *N. tabacum* cv. Xanthi-nc with the sap from both the lower inoculated and the upper unoinoculated leaves of *S. oleracea* exposed to TMV, only sap from inoculated leaves resulted in lesion formation. As it was reported by Holmes (47), *S. oleracea* is a local infection host for TMV. These results indicate that incorporation of AlMV CP into the TMV genome extended the host range of TMV, enabling it to systemically infect the upper unoinoculated leaves of *S. oleracea*.

**Western Analysis of Systemic Infection of Plants with Av/A4.** Leaves of *N. benthamiana*, *N. tabacum* MD609, and *S. oleracea* were inoculated with *in vitro* synthesized transcripts of *Av* or *Av/A4*. Western immunoblot analysis of tissue samples taken 10 dpi demonstrated the presence of AlMV CP in both locally and systemically infected leaves (Fig. 2A). *N. benthamiana* and *N. tabacum* MD609 plants inoculated with *in vitro* transcripts of *Av* developed symptoms only on locally inoculated leaves, and the virus did not move into the upper unoinoculated leaves. *S. oleracea* infected with *Av* transcripts showed no signs of infection, and no viral RNA was detected by Northern blot analysis in the upper unoinoculated leaves (Fig. 3A). TMV CP also was undetected by Western analysis (data not shown) in tissue samples from plants infected with *Av* or *Av/A4*. Plants inoculated with *Av/CP/+P* did not develop systemic symptoms, and virus could not be purified from locally infected leaves, although the 24.0-kDa protein was detected in inoculated leaves using AlMV CP-specific antibodies (Fig. 2B).

**Northern Blot Analysis and RT-PCR of Viral RNA from Systemically Infected Leaves.** *N. benthamiana* and *N. tabacum* MD609 plants were inoculated with sap from leaves systemically infected with chimeric *Av/A4*. RNA was isolated from leaves that were systemically infected 7–10 dpi. Northern blot analysis of the viral RNA using a minus-strand RNA probe (corresponding to the 250 5′ nt of TMV) revealed no difference between the migration of RNA from *in vitro* synthesized *Av/A4* transcripts (Fig. 3A, lane 5) used as a positive control and RNA purified from systemically infected leaves of *N. benthamiana* (Fig. 3A, lane 1) or *N. tabacum* MD609 (Fig. 3A, lane 2), indicating the stability of construct during systemic infection. Total RNA purified from upper leaves of *N. benthamiana* (Fig. 3A, lane 3) and *N. tabacum* MD609 (Fig. 3A, lane 4) infected with *Av/CP/+P* served as a control. RT-PCR of total RNA from systemically infected tissue confirmed the presence of the correct-sized insert of AlMV RNA4 in recombinant *Av/A4* (Fig. 3A). *In vitro* synthesized transcripts of *Av* (lane 2) and total RNA purified from upper unoinoculated leaves of *N. benthamiana* infected with *Av/CP/+P* were used as control (Fig. 3B, lane 1). These results demonstrate that the AlMV CP supported long-distance movement of *Av/A4*, the chimeric TMV.

**Infection of N. tabacum cv. Xanthi-nc with Av/A4.** *N. tabacum* cv. Xanthi-nc is a systemic host for AlMV, whereas TMV infection results only in local lesion formation. However, TMV can systemically invade *N. tabacum* cv. Xanthi-nc at 30°C, a temperature at which the plant cannot trigger a hypersensitive reaction (HR) to virus infection. Infection of *N. tabacum* cv. Xanthi-nc with *Av/A4* at 27°C resulted in local lesions (Fig. 4A, *Av/A4*), and the virus did not move into upper unoinoculated leaves. Local lesions in *Av/A4*-infected leaves appeared within 5–6 days after inoculation compared with 2–3 days in TMV-infected plants (Fig. 4A, TMV). Moreover, the local lesions in *Av/A4*-infected leaves were smaller (1–2 mm) than those of TMV-infected leaves (4–5 mm), perhaps reflecting the slower cell-to-cell movement in the *Av/A4* infection. Inoculation of *N. tabacum* cv. Xanthi-nc with *Av/A4*, AlMV, or TMV at 30°C resulted in systemic spread of virus, which was phloem-mediated for TMV but not for *Av/A4* or AlMV (Fig. 4B, *Av/A4*, AlMV, and TMV). Because the HR does not take place at 30°C, both TMV and *Av/A4* moved into the upper unoinoculated leaves (Fig. 4B, TMV and *Av/A4*). However, during the systemic infection, TMV reached the apex of the plant via the phloem and then spread to upper unoinoculated leaves (Fig. 4B, TMV), whereas *Av/A4* did not reach the apex and systemic infection started from the continuous movement of virus into the upper unoinoculated leaves (Fig. 4B, *Av/A4*). Thus, inoculation of *N. tabacum* cv. Xanthi-nc with *Av/A4* and incubation at 30°C resulted in systemic infection with symptoms similar to those of AlMV (Fig. 4B,
AlMV). This similarity in the pattern of systemic infection of Av/A4 and AlMV suggests the key role of the AlMV CP in determining the systemic movement of virus. When N. tabacum cv. Xanthi-nc plants infected with Av/A4 were transferred into a 27°C room and maintained 3 days after inoculation at 30°C, local lesions and necrotic death of whole leaves, where the virus had moved, were observed, indicating the HR of the plant to Av/A4 infection.

**DISCUSSION**

Using a TMV-based expression vector, Av, we have addressed the role of AlMV CP in the long-distance movement of viral RNA without involving genome activation and replication, because TMV does not require the CP to initiate the infection or, unlike AlMV, to replicate. This study demonstrates that the AlMV CP is capable of encapsidating the TMV genomic RNA in vivo. Thus, Av provided an excellent system with which to study the role of AlMV CP in long-distance movement and systemic infection. Inoculation of N. benthamiana, N. tabacum MD609, and S. oleracea with Av/A4 resulted in systemic infection of plants that displayed symptoms similar to those caused by AlMV. Whereas N. benthamiana and N. tabacum MD609 are systemic hosts for both TMV and AlMV, S. oleracea is systemically infected only by AlMV. Expression of AlMV CP in Av supported the systemic infection of S. oleracea with chimeric TMV, thereby extending the host range of TMV. Another study, using p30BRzCPg24 (48), which produces AlMV CP and TM GMV U5 CP, suggested the key role of the AlMV CP in virus spread in this plant as well as the importance of the specificity of the interaction between AlMV CP and host molecules to enable virus spread. This report concerns the extension of the host range of a virus by the expression of heterologous coat protein. A number of hybrid viruses were
designed by replacing the coat proteins (1, 49–51). None, however, resulted in the extension of the host range.

*N. benthamiana* plants inoculated with *in vitro* transcripts of *Av/CP*+P evidenced no virus in uninoculated upper leaves, and infection was limited to inoculated leaves. No virus, however, could be recovered from locally infected leaves of *N. benthamiana* expressing CP+P (Fig. 2B). Because no virus expressing CP+P could be recovered, it was evident that just the binding of CP+P to viral RNA may not be sufficient for systemic infection of the virus. From earlier experiments, it is expected that CP+P might bind viral RNA in *vivo* during infection.

The systemic invasion of the host plant with *Av/A4* and the similarity of long-distance movement and symptoms in the *Av/A4* and AIMV infections suggest a key role of the AIMV CP in systemic infection and symptom development. The experiments with CP+P suggest that particle formation is essential for the long-distance movement of AIMV in the host plant. Moreover, expression of AIMV CP in Av extends the host range of TMV to include spinach. The latter may have an advantage in the development of plant virus-based vectors for functional studies of genes as well as the production of biomedicals in targeted host species.

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