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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 supported by a virus-encoded movement protein(s) (4–7), whereas in some viruses the capsid protein is a primary determinant of cell-to-cell movement (8–10). The movement protein interacts with the plasmodesmata and transfers the viral RNA into a neighboring uninfected cell (11–13). During systemic infection, the virus moves through the vascular system and multiplies in upper uninoculated leaves. Movement into the upper uninoculated tissue is a critical step in the infection by many plant viruses, and prevention of this step can result in significant protection of the host(s) from virus invasion (14). Coat proteins (CPs) of several plant viruses are essential for long-distance movement (15–23). Moreover, some RNA plant viruses, such as tobacco mosaic virus (TMV) (16, 17, 24), cowpea mosaic virus (25), alfalfa mosaic virus (AlMV) (18), tobacco etch potyvirus (26), and red clover necrotic mosaic virus (27) require functional CP for long-distance movement.

Here, to study the role of AlMV CP in long distance movement, we designed a hybrid virus consisting of TMV, the type member of the tobamovirus group, and the CP of AlMV. The TMV genome consists of a single plus-sense RNA (6,395 nt) encapsidated with a 17.5-kDa CP, which results in rod-shaped particles (300 nm in length). In addition to CP, TMV has three nonstructural proteins. Proteins (183 and 126 kDa) are translated from genomic RNA and are required for virus replication (28). The 30-kDa protein is a movement protein and provides the transfer of viral RNA from cell to cell (29). The only structural protein of TMV is a CP (17.5 kDa), which 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).

AIMV 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 AlMV infection, functioning in genome activation (33–36), RNA replication (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 (43).

The multiple functions of the AlMV CP has made it difficult to analyze any single function without interfering with others. Thus, 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 *Pac*I, *Pme*I, *Age*I, and *Xho*I were introduced 42 nt downstream of the mutated ATG codon. Av (Fig. 1) contains the full-length TMV but is defective in CP production. In addition, Av contains a ribozyme at the 3′ end for self-cleavage of *in vitro* RNA transcripts. A chimeric Av containing the AlMV CP was constructed using pSP65A4 (41), which contains the full-length cDNA of AlMV RNA4 (Fig. 1). The *Eco*RI–*Sma*I fragment of pSP65A4 containing the 5′ and 3′ noncoding regions in addition to the ORF of AlMV CP was cloned into Av (Fig. 1), which was linearized by *Xho*I using blunt-end

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

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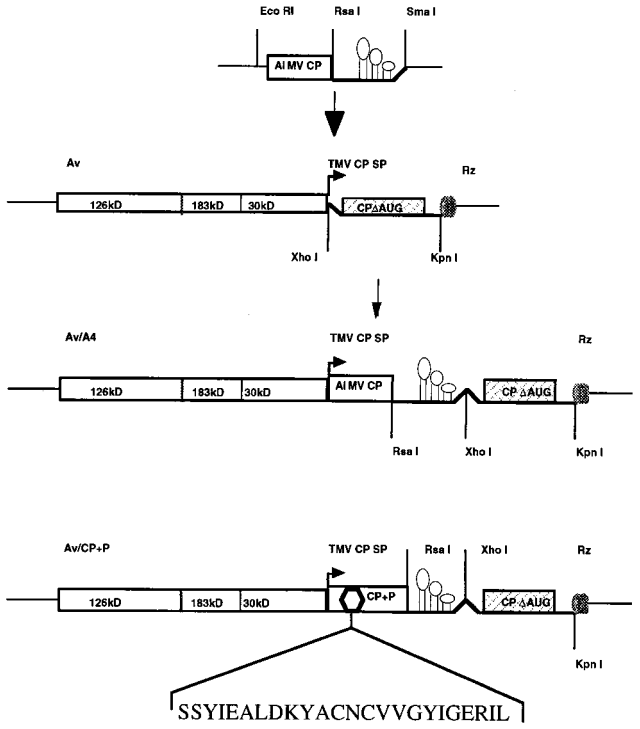

FIG. 1. Schematic representation of the genome of TMV-derived Av and construction of Av/A4. The 126- and 183-kDa proteins are required for TMV replication, the 30-kDa protein is the viral movement protein, and CPΔAUG is the translation-deficient gene of the viral CP. Right arrow under TMV CP SP indicates the subgenomic promoter of TMV CP. Rz indicates ribozyme for self-cleavage. Av/A4 and Av/CP+P are the hybrid viruses engineered to express AlMV CP using Av. Amino acids used to replace the AlMV CP sequences to create CP+P are shown under Av/CP+P.

ligation to create Av/A4 (Fig. 1). An additional construct, Av/CP+P (Fig. 1), containing a mutated AlMV CP was engineered to test whether particle formation is required for long-distance movement. The mutation was introduced by replacing coding sequences between amino acids 66 and 86 of AlMV CP with a synthetic peptide of equal size (Fig. 1), by using PCR cloning. Although the mutant protein CP+P is functional in genome activation, binds viral RNA, and forms dimers *in vitro,* no particle assembly was observed in infected protoplasts (V.Y., *et al.*, unpublished results). Thus, mutant CP+P was digested with *Eco*RI and *Sma*I and cloned into Av linearized with *Xho*I to create Av/CP+P (Fig. 1).

***In Vitro* Transcription.** *In vitro* transcripts of recombinant TMV were synthesized by using T7 RNA polymerase (Promega) and CsCl-purified plasmid DNA, according to the manufacturer's guidelines. Transcripts were capped by using the RNA cap-structure analog m7G(5)ppp(5)G (New England Biolabs).

Plant Inoculation. Three upper leaves of each plant were inoculated with *in vitro* transcription products of recombinant Av constructs after adding 1 vol (vol/vol) of FES buffer [1% sodium pyrophosphate (wt/vol)/1% macaloid (wt/vol)/1% celite (wt/vol)/0.5 M glycine/0.3 M K_2HPO_4, pH 8.5, with phosphoric acid]. Inoculum was applied by gentle rubbing on leaves after abrading the leaf surface with carborundum (320 grit; Fisher). In experiments to confirm the stability of chimeric Av/A4, new plants were inoculated with sap from leaves systemically infected with this chimera.

Northern Blot Hybridization and Reverse Transcription–PCR (RT-PCR). Total RNA was extracted from locally and systemically infected leaf tissue. Briefly, 100–200 mg of leaf tissue was homogenized in 10 vol of TRIzol reagent (GIBCO) 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′-TTTTCCGGAACCTTTTCG-3′ as primer, which also was used in PCR with 5′-GGGCCCATG-GAACTT ACAGAAGAA-3′ *Taq* DNA polymerase (Promega).

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

N. benthamiana. Inoculation of *N. benthamiana* with either TMV, Av/A4, or AlMV resulted in systemic infection with symptoms developing in the upper uninoculated leaves within 7–10 days. Systemic infection of *N. benthamiana* with either AlMV, 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 AlMV, 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 AlMV- or Av/A4-infected plants remained nonnecrotic and alive. Even after 30 days of infection, AlMV- or Av/A4-infected plants never developed the necrotic reactions observed with TMV.

N. tabacum MD609. Movement of TMV into the upper uninoculated 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 AlMV 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 AlMV or Av/A4 from proceeding. Systemic invasion (8–10 dpi) of *N. tabacum* MD609 with AlMV or Av/A4 resulted in distinct mosaic symptoms with occasional necrotic spots. Systemic infection of TMV began at the apex, whereas AlMV or Av/A4 never reached the apex, leaving the uppermost two to three leaves symptomless. These results indicate that AlMV and TMV differ in the mechanisms of systemic movement.

S. oleracea. Inoculation of *S. oleracea* plants with either AlMV 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, AlMV, 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 AlMV resulted in

infection. Similarly, sap from both inoculated and the upper uninoculated 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 uninoculated 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 uninoculated 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. 2*A*). *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 uninoculated 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 uninoculated leaves (Fig. 3*A*). 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. 2*B*).

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. 3*A*, lane 5) used as a positive control and RNA purified from systemically infected leaves of *N.*

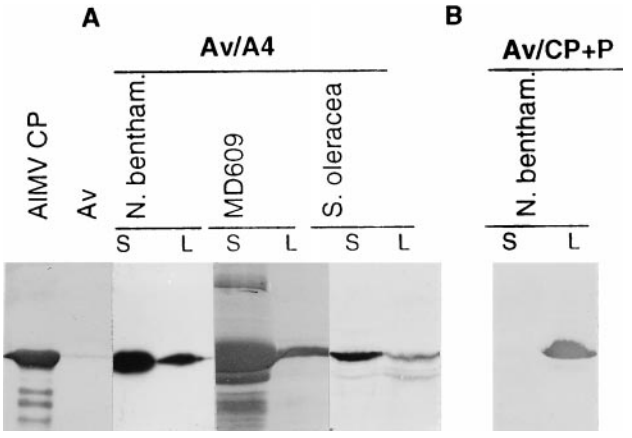

FIG. 2. Western immunoblot analysis of AlMV CP expression in plants infected with Av/A4. Proteins were separated electrophoretically on a 13% SDS-polyacrylamide gel, transferred to a membrane, and reacted with mAbs specific for AIMV CP. (*A*) Antibodies recognized a 24-kDa protein in samples from locally (L) and systemically (S) infected tissue collected from *N. benthamiana, N. tabacum* MD609, and *S. oleracea*. (*B*) Antibodies also reacted with the 24-kDa protein in extracts from tissue locally infected (lane L) with Av/CP+P. Av/CP+P was not detected in upper uninoculated leaves (lane S). Left lanes show AlMV CP from wild-type virus (positive control) and the absence of CP in an Av-infected plant sample (negative control), respectively.

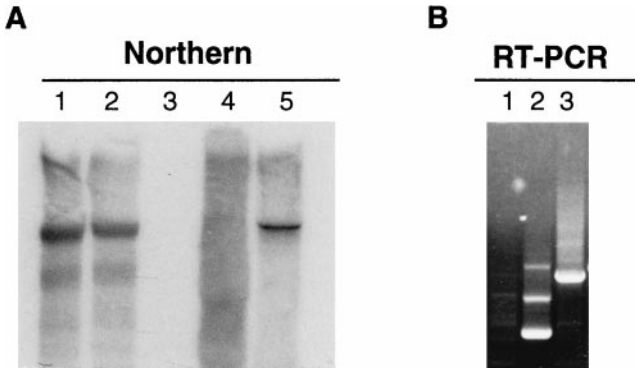

FIG. 3. Northern analysis (*A*) and RT-PCR (*B*) of viral RNA isolated from leaves of plants systemically infected with Av/A4. RNA molecules were separated by electrophoresis in denaturing conditions, transferred to a membrane, and probed with minus-strand RNA resembling 250 5′ nt of the TMV genome. *In vitro* synthesized transcripts of Av/A4 were used as a positive control (lane 5). Lanes 1 and 2 show RNA purified from systemically infected leaves of *N. benthamiana* and *N. tabacum* MD609, respectively. Total RNA purified from the upper uninoculated leaves of *N. benthamiana* (lane 3) and *N. tabacum* MD609 (lane 4) infected with Av/CP+P was used as a negative control. (*B*) RT-PCR of total RNA confirmed the presence of the full-size AlMV RNA4 insert in recombinant Av/A4 (lane 3). *In vitro* synthesized transcripts of Av (lane 2) and total RNA purified from upper uninoculated leaves of *N. benthamiana* infected with Av/CP+P were used as negative controls (lane 1).

benthamiana (Fig. 3*A*, lane 1) or *N. tabacum* MD609 (Fig. 3*A*, lane 2), indicating the stability of construct during systemic infection. Total RNA purified from upper leaves of *N. benthamiana* (Fig. 3*A*, lane 3) and *N. tabacum* MD609 (Fig. 3*A*, 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. 3*B*, lane 3). *In vitro* synthesized transcripts of Av (Fig. 3*B*, lane 2) and total RNA from upper uninoculated leaves of *N. benthamiana* infected with Av/CP+P were used as controls (Fig. 3*B*, lane 1). These results demonstrate that the AIMV 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. 4*A*, Av/A4), and the virus did not move into upper uninoculated leaves. Local lesions in Av/A4-infected leaves appeared within 5–6 days after inoculation compared with 2–3 days in TMV-inoculated plants (Fig. 4*A*, TMV). Moreover, the local lesions in Av/A4-infected leaves were smaller (1–2 mm) than those of TMV-inoculated 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. 4*B*, Av/A4, AlMV, and TMV). Because the HR does not take place at 30°C, both TMV and Av/A4 moved into the upper uninoculated leaves (Fig. 4*B*, TMV and Av/A4). However, during the systemic infection, TMV reached the apex of the plant via the phloem and then spread to upper uninoculated leaves (Fig. 4*B*, TMV), whereas Av/A4 did not reach the apex and systemic infection started from the continuous movement of virus into the upper uninoculated leaves (Fig. 4*B*, 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. 4*B*,

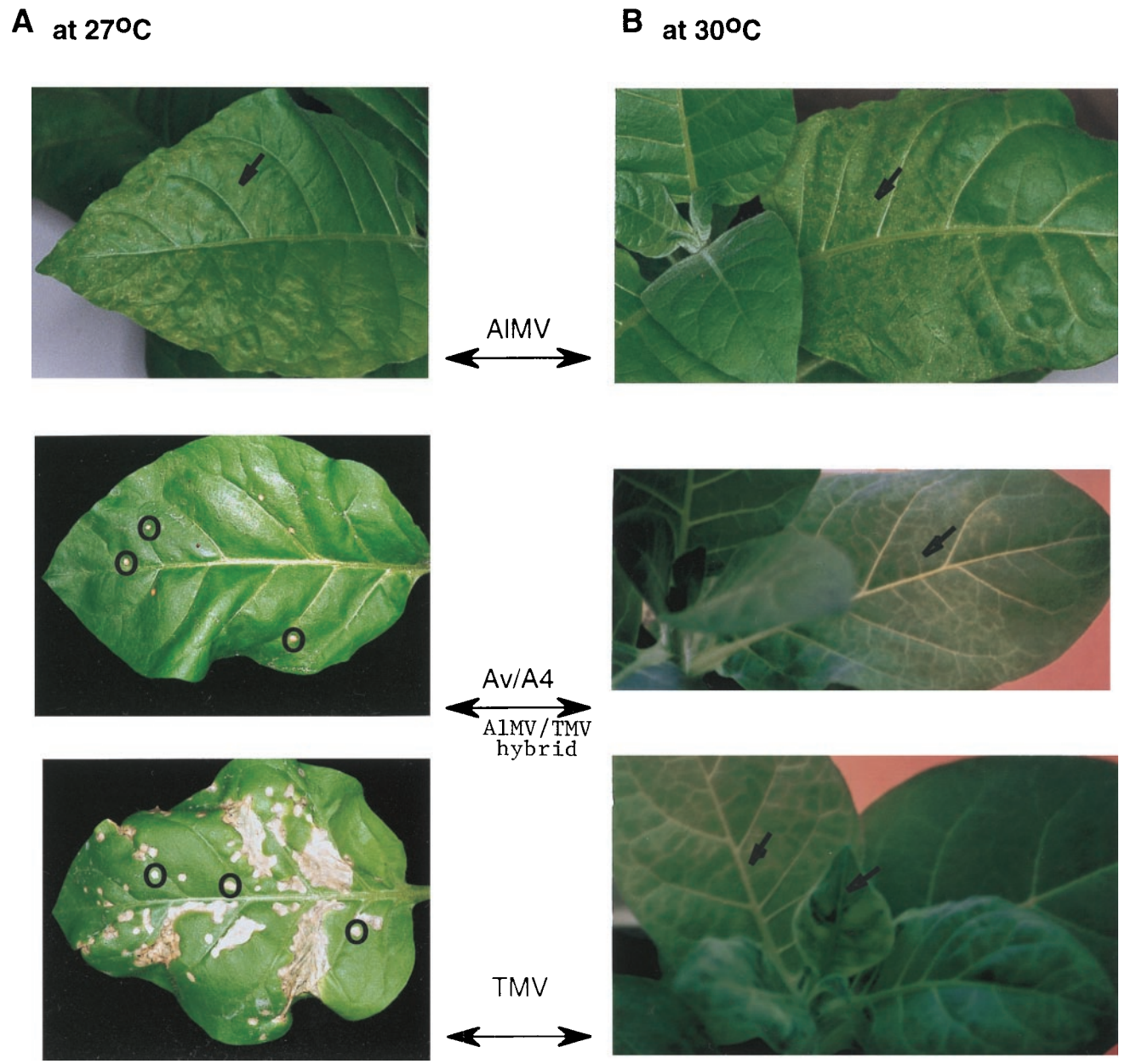

FIG. 4. Comparison of symptoms induced by infection with TMV, AlMV, and Av/A4 on *N. tabacum* cv. Xanthi-nc at 27°C (*A*) and at 30°C (*B*). (*A*) TMV and Av/A4 caused necrotic local lesion formation, limiting the infection to the inoculated leaf, while AlMV moved into the upper uninoculated leaves. (*B*) Inoculation of *N. tabacum* cv. Xanthi-nc with Av/A4, AlMV, or TMV at 30°C resulted in the systemic spread of the virus. Local lesions and systemically infected leaves are indicated by circles and arrows, respectively.

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. 2*B*). 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 AlMV infections suggest a key role of the AlMV CP in systemic infection and symptom development. The experiments with Av/CP+P suggest that particle formation is essential for the long-distance movement of AlMV in the host plant. Moreover, expression of AlMV 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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