Complementation and Recombination between Alfalfa Mosaic Virus RNA3 Mutants in Tobacco Plants

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Deletions were made in an infectious cDNA clone of alfalfa mosaic virus (AIMV) RNA3 and the replication of RNA transcripts of these cDNAs was studied in tobacco plants transformed with AIMV replicase genes (P12 plants). Previously, we found that deletions in the P3 gene did not affect accumulation of RNA3 in P12 protoplasts whereas deletions in the coat protein (CP) gene reduced accumulation 100-fold (A. C. van der Kuyl, L. Neeleman, and J. F. Bol, 1991, *Virology* 183, 687–694). In P12 plants deletions in the P3 gene reduced accumulation by about 200-fold and accumulation of CP deletion mutants was not detectable. When P12 plants were inoculated with a mixture of P3- and CP-deletion mutants, both mutants replicated efficiently and various amounts of full-length RNA3 molecules were formed by recombination. The observation that some P3 and CP mutants did not recombine at a detectable level after several passages in P12 plants demonstrated that mutations in the AIMV P3 and CP genes can be complemented *in trans*. © 1991 Academic Press, Inc.

INTRODUCTION

The genome of alfalfa mosaic virus (AIMV) consists of RNAs 1, 2, and 3. The observation that RNAs 1 and 2 are able to replicate in protoplasts but do not accumulate detectably in plants indicates that RNA3 encoded functions are required for movement of the virus in plants. RNA3 encodes a 32-kDa protein called P3 and the viral coat protein (CP) which is translated from a subgenomic messenger, RNA4. AIMV P3 is believed to be functionally equivalent to a tobacco mosaic virus (TMV) encoded 30-kDa protein which plays a role in cell-to-cell movement of the virus (Deom *et al.*, 1987; Meshi *et al.*, 1987; Stussi-Garaud *et al.*, 1987). CP of TMV is not required for cell-to-cell transport but is involved in the long distance movement of the virus (Dawson *et al.*, 1988; Saito *et al.*, 1990). Another well-known example of CP-independent cell-to-cell movement is the replication of tobacco rattle virus RNA1 in the absence of RNA2 (Harrison and Robinson, 1978). CP of brome mosaic virus (BMV) and of cowpea chlorotic mottle virus (CCMV), which resemble AIMV in genome structure, is required for systemic movement of these viruses (Sacher and Ahlquist, 1989; Allison *et al.*, 1990). For cowpea mosaic virus (CPMV) a functional CP gene appears to be required already at the level of cell-to-cell movement (Wellink and van Kammen, 1989).

In addition to its structural role, AIMV CP has an early function in the viral replication cycle: a mixture of the three genomic RNAs becomes infectious only after addition of a few CP molecules per RNA molecule to the inoculum (Bol *et al.*, 1971). This phenomenon was called "genome activation." Tobacco plants transformed with a DNA copy of the AIMV CP gene were shown to be highly resistant to infection with AIMV particles but could be infected with a mixture of the three genomic RNAs (van Dun *et al.*, 1987). Apparently, the endogenous CP in these transgenic plants was functional in genome activation. The finding that these plants did not support the accumulation of inoculum RNAs when a deletion was introduced in the CP gene, suggested that CP was required for a step in the replication cycle other than genome activation (Dore *et al.*, 1991). Recently, we described the transformation of tobacco with DNA copies of both AIMV RNAs 1 and 2 which encode proteins P1 and P2, respectively (Taschner *et al.*, 1991). When these plants (P12 plants) were inoculated with RNA3 the inoculum RNA was replicated but not the transcripts of the nuclear viral genes. This replication of RNA3 could be initiated by an inoculum without CP, indicating that in non-transgenic plants the early function of CP is related to expression of the inoculum RNAs 1 and 2 (Taschner *et al.*, 1991). A study of AIMV RNA3 mutants transcribed *in vitro* from cDNA clones, revealed that deletions in the P3 gene did not interfere with accumulation of the transcripts in P12 protoplasts whereas deletions in the CP gene caused a 100-fold reduction of RNA3 accumulation in this system (van der Kuyl *et al.*, 1991). Apparently, P3 is not required for virus multiplication at the protoplast level but CP has a regulatory role in viral RNA synthe-

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sis. In the present study we analyzed the replication of RNA3 mutants in P12 plants to identify RNA3 encoded functions in cell-to-cell movement. In addition, we investigated the ability of mutants with P3 and CP deletions to complement each other *in trans*.

MATERIALS AND METHODS

Construction of mutant cDNAs

cDNA clones yielding wild type RNA3 transcripts were pAL3 (strain 425 Leiden) and pYSMV3 (strain YSMV) (Neeleman *et al.*, 1991). A schematic representation of the wild-type and mutant clones used in this study is shown in Fig. 1. Details of the construction of frameshift mutants pY5, pY6, and pY7 are described elsewhere (van der Kuyl *et al.*, 1991). Constructs pY8, pY9, and pA16 containing deletions in the P3 gene, were made by removing the indicated restriction fragments, blunting ends with T4 DNA polymerase, and religating. pY9 and pA16 both possess the same *XhoI–NdeI* deletion, introduced in the two different strains. pA2 was constructed as described (van der Kuyl *et al.*, 1991). Mutants pY10 and pY11 were generated by deleting the *ScaI–ApaI* or *SstI–ApaI* fragment of the coat protein gene, respectively. In pYSMV3, a *ScaI*-site is present in the intercistronic region. Also, the *HindIII*-site (nucleotide 725) is unique to pYSMV3.

In vitro transcription and infection of plants

Clones were transcribed with T7 RNA polymerase after linearization with *PstI* and generating blunt ends with T4 DNA polymerase. This gives rise to uncapped transcripts with 5′- and 3′-terminal sequences identical to the corresponding wild type RNA. Transcripts were used to inoculate transgenic P12 plants, expressing DNA copies of RNAs 1 and 2 from AlMV strain 425 (Taschner *et al.*, 1991). The P12 plants were grown and inoculated as described by van Dun *et al.* (1988). Inoculation was done on three half-leaves per plant with 500 ng per half-leaf of each of the indicated transcripts. From four to six plants were inoculated with each transcript or mixture of transcripts. Symptoms were scored 3 to 5 days after inoculation. Systemic symptoms induced by transcripts containing the YSMV CP gene generally appeared 4 to 8 days after inoculation.

All enzymes used were from GIBCO/BRL and enzymatic incubations were performed under conditions recommended by the supplier.

Analysis of RNA and protein

In all experiments only the inoculated leaves were used. Leaves from infected plants were collected 5
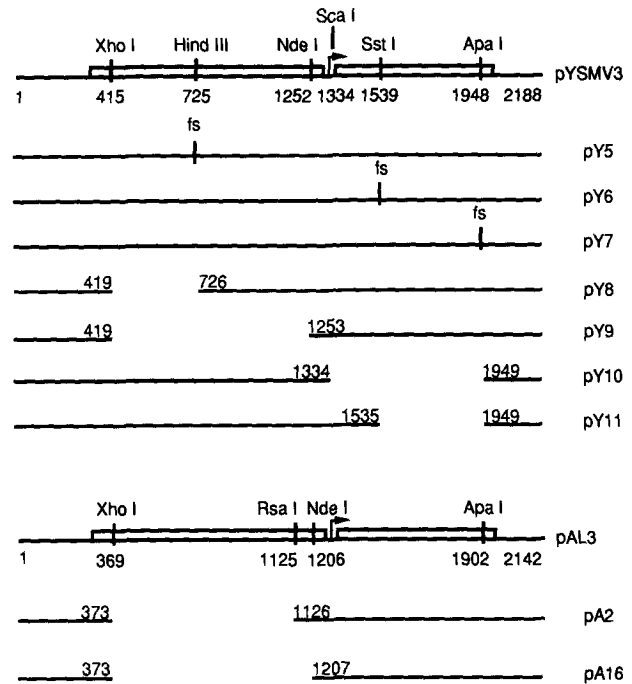

FIG. 1. Schematic representation of mutant AlMV cDNAs derived from pYSMV3 (pY-series) and pAL3 (pA-series). Frameshifts are indicated with a vertical bar denoted "fs." In the deletion mutants, numbering indicates the bordering nucleotides of the restriction fragment removed. In AlMV RNA3, the P3 protein is encoded by the 5′-proximal gene, while the downstream gene encodes the CP. Restriction sites are shown, and an arrow indicates the RNA4 transcription start site. Clones pYSMV3 and pAL3 correspond to wild type RNA3 of AlMV strains YSMV and 425, respectively.

days after inoculation. Total RNA was extracted by grinding leaf material, frozen in liquid nitrogen, in a buffer containing 0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, and 4% SDS, the slurry was extracted with phenol/chloroform (1/1), and the RNA was collected from the aqueous phase by ethanol precipitation. Virus particles were isolated from leaves as described by van Vloten-Doting and Jaspars (1972). RNA extracted from 0.01 g leaf material or 20 ng of purified virus was analyzed, after glyoxylation and electrophoresis in 1% agarose gels, by Northern blot hybridization using random primed cDNA3 as probe (Feinberg and Vogelstein, 1984). Scanning of autoradiograms was done with a LKB 2222-020 Ultroscan XL Laser Densitometer. Protein from 0.15 mg leaf material was analyzed by the Western blot technique (Towbin *et al.*, 1979) using antiserum against AlMV CP.

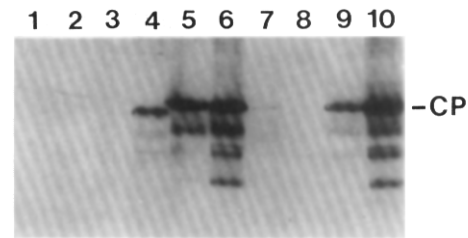## RESULTS

Replication of RNA3 frameshift mutants

The schematic representation of mutants in Fig. 1 shows the location of a frameshift in the P3 gene of
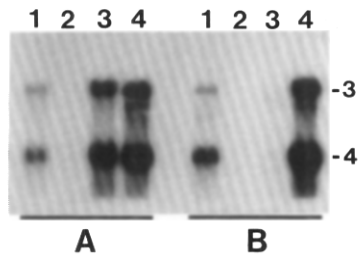
FIG. 2. Replication of AIMV RNA3 frameshift mutants in P12 plants. P12 plants were inoculated with transcripts of pY5 (lanes 1), pY6 (lanes 2), pY7 (lanes 3), or pY5 + pY6 (lanes 4). Total RNA (A) and RNA extracted from purified virus particles (B) was analyzed by Northern blot hybridization. Lanes 1 and 4 were loaded with the standard amount of RNA; lanes 2 and 3 were loaded with 50 times this amount. The position of RNAs 3 and 4 is indicated in the right margin.

FIG. 3. Western blot analysis of total protein isolated from P12 plants inoculated with AIMV RNA3 frameshift or deletion mutants. CP produced by these mutants was detected with antiserum against AIMV CP. Protein was extracted from P12 plants mock-inoculated with buffer (lane 1), or inoculated with transcripts of pY5 (lane 2), pY6 (lane 3), pY7 (lane 4), pY5 + pY6 (lane 5), pYSMV3 (lanes 6 and 10), pY8 (lane 7), pY11 (lane 8), and pY8 + pY11 (lane 9). The position of AIMV CP is indicated in the right margin.

mutant pY5 and frameshifts in two different positions of the CP genes of mutants pY6 and pY7. Figure 2 shows a Northern blot analysis of the accumulation of these mutant transcripts in P12 plants. RNA was isolated either directly from the inoculated leaves (Fig. 2A) or from purified virus particles isolated from these leaves (Fig. 2B). The P3 mutant pY5 accumulated at a low but detectable level and was encapsidated (Fig. 2, lanes 1). A comparison with wild-type RNA3 (not shown) revealed that the accumulation of mutant pY5 was reduced by about 200-fold. Mutant pY6 with a frameshift in the 5'-terminal half of the CP gene did not accumulate at a detectable level (Fig. 2, lanes 2). Accumulation of mutant pY7 with the frameshift near the 3'-end of the CP gene was detectable (Fig. 2A, lane 3), but this mutant was not efficiently encapsidated (Fig. 2B, lane 3). Compared to the wild-type, the accumulation of pY7 was reduced about 50-fold (comparison not shown). When P12 plants were inoculated with a mixture of mutants pY5 and pY6, accumulation of RNA molecules of the size of RNA3 and synthesis of subgenomic RNA4 occurred at wild-type levels (Fig. 2, lanes 4). It should be noted that in Fig. 2 the standard amount of RNA was applied to lanes 1 and 4 of the gel, whereas in lanes 2 and 3 this amount was increased 50-fold to reveal possible low levels of the accumulation of mutant pY6 (Fig. 2A, lane 2) or encapsidation of mutant pY7 (Fig. 2B, lane 3). A faint signal in lane 3 of Fig. 2B could not be quantified by densitometry but may suggest that mutant pY7 is encapsidated at a very low level.

The production of CP in P12 plants inoculated with the frameshift mutants was assayed by Western blot analysis (Fig. 3). As a control, plants inoculated with a transcript from the wild type clone pYSMV3 were included (Fig. 3, lane 6). P3 mutant pY5 produced a relatively low amount of CP that is barely visible in lane 2 of Fig. 3. Mutant pY6 did not produce immunoreactive

material (Fig. 3, lane 3) but mutant pY7 produced a significant amount of truncated CP molecules (Fig. 3, lane 4). The frameshift in mutant pY7 results in a reading frame for a CP molecule of which the 21 C-terminal amino acids are replaced by 4 nonviral amino acids. Apparently, such a truncated protein is translated from the subgenomic RNA produced by mutant pY7. Plants inoculated with a mixture of mutants pY5 and pY6 produced CP at wild-type levels (Fig. 3, lane 5).

Complementation between RNA3 deletion mutants

The efficient accumulation of viral RNAs and particles observed after inoculation of P12 plants with a mixture of pY5 and pY6 could be due either to complementation between the mutants or to recombination to the wild-type sequence. To permit a discrimination between mutant and wild-type RNA3 molecules the mixed infections were repeated with deletion mutants. Figure 4 shows a Northern blot analysis of P12 plants inoculated with mutants pY8 and/or pY11 which are
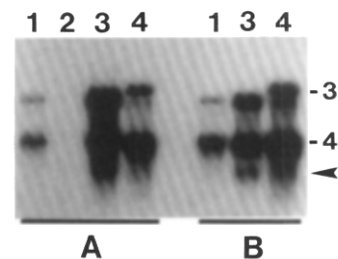
FIG. 4. Replication of AIMV RNA3 deletion mutants pY8 and pY11 in P12 plants. P12 plants were inoculated with transcripts of pY8 (lanes 1), pY11 (lane 2), pY8 + pY11 (lanes 3), or pYSMV3 (lanes 4). Total RNA (A) and RNA extracted from purified virus particles (B) was analyzed by Northern blot hybridization. The position of RNAs 3 and 4 is indicated in the right margin; subgenomic RNA produced by mutant pY11 is indicated by an arrowhead.

schematically shown in Fig. 1. RNA was extracted either directly from the inoculated leaves (Fig. 4A) or from virus particles purified from these leaves (Fig. 4B). The accumulation and encapsidation of transcripts from the wild-type clone pYSMV3 is shown in Fig. 4, lanes 4.

Mutant pY8 has a deletion of 307 nucleotides in the P3 gene and RNA and particles of this mutant accumulated at the same low level as was observed previously for the frameshift mutant pY5 (Fig. 4, lanes 1). Mutant pY11 has a deletion of 414 nucleotides in the CP gene and did not accumulate at a detectable level in P12 plants (Fig. 4A, lane 2); encapsidation of this mutant was not tested. When P12 plants were inoculated with a mixture of the two mutants, both mutants accumulated and were encapsidated at wild-type levels (Fig. 4, lanes 3). A shorter exposure of the blot of Fig. 4 revealed two bands of RNA molecules corresponding to the size of mutants pY8 and pY11 (not shown). In addition to full-length RNA4, a truncated subgenomic RNA is produced which is probably derived from mutant pY11 (arrowhead in Fig. 4). The ratio of full-length and subgenomic RNAs was comparable for both mutants (not shown). However, no full-length RNA3 molecules were detectable in lanes 3 of Fig. 4, indicating that mutants pY8 and pY11 were able to complement each other *in trans* without giving rise to recombination.

CP production directed by mutants pY8 and pY11 is shown in Fig. 3. Mutant pY8 produced a very low level of CP (lane 7) but CP is not detectable in plants inoculated with pY11 (lane 8). A mixture of the two mutants induced CP synthesis (Fig. 3, lane 9) at a level that was slightly reduced compared to that in plants infected with the wild-type transcript (Fig. 3, lane 10).

Recombination between RNA deletion mutants

To further investigate the stability of mutants in mixed infections, several additional deletions were made in the infectious clones of RNA3 of AIMV strains YSMV (pYSMV3) and 425 (pAL3). These mutants (pY9, pY10, pA2, and pA16) are schematically shown in Fig. 1. Different mixtures of P3 and CP deletion mutants were inoculated on P12 plants and 5 days after inoculation homogenates of equal amounts of tissue of the inoculated leaves of these plants were inoculated to healthy P12 plants. RNA from virus particles purified 5 days after inoculation from the inoculated leaves of the first and second group of plants was analyzed in Figs. 5A and 5B, respectively. Table 1 lists the symptoms that were visible on the first and second group of plants. The inoculum numbers given in Table 1 correspond to the lane numbers used in Fig. 5. When the mutants listed in Fig. 1 were inoculated separately on P12 plants, none of them induced visible symptoms.
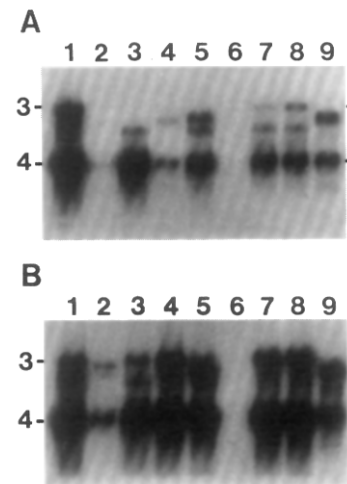
FIG. 5. Complementation and recombination between AlMV RNA3 mutants. P12 plants were inoculated with transcripts from pYSMV3 (lane 1), pA2 + pY11 (lane 2), pA2 + pY10 (lane 3), pY8 + pY11 (lane 4), pY8 + pY10 (lane 5), pY9 + pY11 (lane 6), pA16 + pY10 (lane 7), pY9 + pY10 (lane 8), and pY8 + pY11 (lane 9). Five days after inoculation homogenates of these plants were inoculated to a second group of P12 plants. RNA from virus particles purified from the first group of plants (A) and the second group of plants (B) was analyzed by Northern blot hybridization. Inocula used in lanes 4 and 9 are from different transcription mixtures; inoculum 9 consists of transcripts of the same batch used in the experiment of Fig. 4. Lane numbers correspond to the inocula listed in Table 1. The positions of RNAs 3 and 4 are indicated in the left and right margin.

Inoculation of P12 plants with wild type RNA3 of AIMV strains 425 and YSMV results in a development of mild chlorosis and large yellow necrotic lesions, respectively, on the inoculated leaves. On the systemically infected leaves strain YSMV causes severe yellowing whereas strain 425 gives no visible symptoms. When the codon for Gln-29 in the CP of strain 425 was mutated into the Arg-codon present at this position in strain YSMV, the symptoms induced by RNA3 on the inoculated leaves changed from chlorosis to necrosis (Neeleman *et al.*, 1991). In YSMV RNA3 the codon for Arg-29 of the CP is present upstream of the *SstI* site at nucleotide position 1539 (Fig. 1).

Lane 1 of Fig. 5 shows the accumulation of wild-type RNA3 of strain YSMV. The level of accumulation was similar in the first and second group of plants. Lane 2 of Fig. 5 shows the RNA synthesis induced by a mixture of mutants pA2 and pY11. On the first group of plants this inoculum caused the formation of small brown necrotic lesions, 1 to 3 days after inoculation, which further developed into severe necrosis of the leaf. This may be responsible for the low level of RNA synthesis seen in Fig. 5A, lane 2. On the second group of plants this inoculum caused chlorosis characteristic of strain 425 (Table 1) and a low level of RNA synthesis (Fig. 5B,

TABLE 1

SYMPTOMS ON P12 PLANTS INDUCED BY WILD-TYPE AND MUTANT AIMV TRANSCRIPTS[a]

Inoculum[b]	First passage		Second passage	
	Local	Systemic	Local	Systemic
425 (pAL3)	Chlorosis	None	Chlorosis	None
YSMV (pYSMV3)	Large yellow necrotic lesions	Systemic yellowing	Large yellow necrotic lesions	Systemic yellowing
2. pA2 + pY11	Small brown lesions	None	425	None
3. pA2 + pY10	425	None	425	None
4. pY8 + pY11	YSMV	None	YSMV	YSMV
5. pY8 + pY10	YSMV	None	YSMV	YSMV
6. pY9 + pY11	Small brown lesions	None	None	None
7. pA16 + pY10	425	None	425	None
8. pY9 + pY10	YSMV	None	YSMV	YSMV
9. pY8 + pY11	YSMV	None	YSMV	None

[a] Transcripts were inoculated to a first group of P12 plants (first passage); 5 days after inoculation homogenates were inoculated to a second group of P12 plants (second passage). The symptoms induced by wild-type RNA3 or AIMV strains 425 and YSMV are described. Local symptoms were scored on the inoculated leaves, while systemic symptoms are those on the upper noninoculated leaves. Mutant symptoms indistinguishable from wild-type are referred to as "425" or "YSMV."

[b] Inoculum numbers correspond to lane numbers in Fig. 5. Inoculum 9 was the inoculum used in Fig. 4, lane 3; inoculum 4 consisted of a new batch of transcripts of mutants pY8 and pY11.

lane 2). Inoculum 6, containing mutants pY9 and pY11, caused a severe necrosis on the plants of group 1 similar to that induced by inoculum 2. RNA synthesis induced by inoculum 6 was below the level of detection (Fig. 5, lanes 6) and no symptoms were detectable on the second group of plants, indicating that the mutants had not survived the first passage.

The mutant RNAs present in inocula 3, 4, and 5 were detectable in the group 1 plants without a clear accumulation of full-length recombinants (Fig. 5A, lanes 3, 4 and 5). However, after passage of these inocula to the second group of plants, the plants all contained full-length RNA3 molecules in addition to various levels of the mutant RNAs. Inoculum 4 consisted of a new batch of transcripts of mutants pY8 and pY11, similar to the transcripts tested in the experiment shown in Fig. 4. In the experiment of Fig. 4 no recombination was detectable but in the experiment of Fig. 5 these mutants clearly recombined. A mixture of the same batch of transcripts analyzed in Fig. 4 was again used as inoculum 9 in the experiment of Fig. 5 but no recombination after two passages was observed (Fig. 5, lanes 9).

Inocula 7 and 8 gave rise to the accumulation of full-length recombinant RNA3 molecules already in the first group of plants, although some mutant RNAs were still detectable (Fig. 5A, lanes 7 and 8). After passage to the second group of plants, only genomic RNAs of the size of wild type RNA3 were detectable (Fig. 5B, lanes 7 and 8).

Inocula 3 and 7 caused chlorosis characteristic of strain 425, whereas inocula 4, 5, 8, and 9 all induced

the large yellow necrotic lesions of strain YSMV on the inoculated leaves of both the first and second group of plants (Table 1). However, inocula 4, 5, and 8 caused systemic yellowing only on the second group of plants but not on the first group. This supports the evidence that recombination between the mutants in these inocula had occurred. On the other hand, inoculum 9 did not induce systemic symptoms on any of the plants, corroborating the absence of recombination.

DISCUSSION

Function of AIMV RNA3 encoded proteins

At present, three potential families of viral transport proteins have been identified which are related to those of TMV, AIMV, and CPMV (Hull, 1989). The TMV 30-kDa protein is associated with plasmodesmata and has been shown to modify the size exclusion limit of this structure (Wolf et al., 1989). The AIMV P3 protein was visualized immunocytochemically in the middle lamella of the walls of those cells which had just been reached by the infection front (Stussi-Garaud et al., 1987). Infection with CPMV is accompanied by the appearance of tubular structures which protrude from the cell wall and contain virus-like particles. The putative CPMV 48-kDa/58-kDa transport proteins are associated with these structures (van Lent et al., 1990). Also, the 48-kDa protein of the comovirus red clover mottle virus (RCMV) has been detected in the plasmodesmata of infected peas (Shanks et al., 1989). Deletions in the TMV 30-kDa gene do not affect replication

of the virus in protoplasts but interfere with replication in plants (Meshi et al., 1987). Deletions in the TMV CP gene affect neither the replication in protoplasts nor the cell-to-cell spread of viral RNA in inoculated leaves (Meshi et al., 1987; Dawson et al., 1988). Recent studies on barley stripe mosaic virus (BSMV) showed that the CP gene of this virus was dispensable for systemic spread of the infection in barley plants (Petty and Jackson, 1990). On the other hand, cell-to-cell movement of CPMV requires both functional 48-kDa/58-kDa and CP genes (Wellink and van Kammen, 1989). CP also plays a role in the movement of bromoviruses. Deletions in the 32-kDa and CP genes of BMV and CCMV do not interfere with the replication of RNA3 in protoplasts although in the absence of functional CP the stability of the RNA is reduced (French and Ahlquist, 1987; Pacha et al., 1990). However, for systemic infection of plants with these viruses the 32-kDa and CP gene are both essential (Sacher and Ahlquist, 1989; Allison et al., 1990).

Similar to the results with TMV and bromoviruses movement genes, frameshifts or deletions in the AIMV P3 gene do not affect replication of RNA3 in protoplasts (van der Kuyl et al., 1991) but strongly reduce virus production in plants. The low level of accumulation of mutants pY5 and pY8 in P12 plants may reflect a limited replication at the site of infection. To confirm this histochemically we will replace the P3 gene by the GUS reporter gene. Anyhow, the 200-fold reduction in the accumulation of pY5 and pY8 clearly supports a role of the P3 gene in cell-to-cell spread.

Frameshifts and deletions in the AIMV CP gene reduced accumulation of RNA3 in P12 protoplasts by about 100-fold (van der Kuyl et al., 1991) and accumulation in P12 plants was below the detection limit. The defect in accumulation of CP mutants in plants could not be complemented by CP produced in plants transformed with the CP gene (Dore et al., 1991) but in the present study we showed that the CP mutants could be complemented in trans by P3 mutants. After coinoculation of P12 plants with P3 and CP deletion mutants, a level of CP accumulation is obtained (Fig. 3, lane 9) that is much higher than the CP accumulation in transgenic CP plants (van Dun et al., 1987). This indicates that replication and spread of AIMV in plants requires a relatively high level of CP synthesis. The severe reduction of the accumulation of CP mutants in protoplasts indicates that CP has a role in viral RNA synthesis (van der Kuyl et al., 1991). Such a role is further supported by the presence of CP in the RNA-dependent RNA polymerase that was purified from AIMV infected tobacco (Quadt et al., 1991). Therefore, it is difficult to decide whether the failure of CP mutants to accumulate in plants is due to a defect in a function

of CP in RNA synthesis, in cell-to-cell movement or in both.

An indication that CP has a role in cell-to-cell transport comes from the difference in the behavior of mutants pY6 and pY7. Compared to wild-type RNA3, the accumulation in P12 protoplasts of both these mutants is reduced by about 100-fold, indicating that the truncated CP molecules encoded by these mutants are equally defective in RNA synthesis (van der Kuyl et al., 1991). However, in P12 plants pY7 accumulates at a detectable level but pY6 does not (Fig. 2). It could be that the truncated CP of pY7 (Fig. 3, lane 4) is still functional in cell-to-cell spread allowing the mutant to move from the site of infection. As the RNA of pY7 is not efficiently encapsidated, a function of CP in cell-to-cell spread would not be related to its structural role.

Complementation and recombination between RNA3 mutants

Attempts to obtain complementation in cowpea plants between CCMV mutants with deletions in the 32-kDa and CP genes yielded negative results (Allison et al., 1990). Either a combination of the two mutants did not replicate or they recombined to wild type RNA3 molecules. The observation that one of the mixtures of mutants pY8 and pY11 survived several passages in P12 plants without formation of a detectable level of full-length recombinants, demonstrates that complementation between AIMV P3 and CP mutants is possible. However, with most combinations of P3 and CP mutants wild type recombinants appeared after one or two passages in tobacco and the mutants were gradually lost. There was no indication that the deletion mutants interfered with the replication of wild-type RNA.

Recombination between viral RNAs is well documented for the animal picornaviruses and coronaviruses (King et al., 1982; Lai et al., 1985; Keck et al., 1988) and for a number of plant viruses. The demonstration that a small deletion in the 3'-noncoding sequence of a BMV RNA3 mutant was restored by recombination with the conserved 3'-sequences of wild type RNA1 or RNA2 was the first example of recombination in a plant virus RNA genome (Bujarski and Kaesberg, 1986). Also, the recombinational restoration of 3'-deleted sequences of BMV RNA2 has been reported (Rao and Hall, 1990). Intramolecular recombination in infected plants has been described for TMV mutants expressing a foreign gene or double copies of the 30-kDa gene or CP gene (Dawson et al., 1989; Beck and Dawson, 1990; Lehto and Dawson, 1990). Recombination has been observed between homologous sequences but also between nonhomologous sequences and its mechanism is not known. In our AIMV mutants recom-

bination between a P3 and CP mutant must have taken place in the common region between the deletions in the two mutants. This common region varies in size between 809 nucleotides in the combination of mutants pY8 and pY11 (inocula 4 and 9 in Fig. 5) and 81 nucleotides in the combination of mutants pA16 and pY10 (inoculum 7). Apparently, the size of this overlap does not affect the frequency of recombination. Possibly, encapsidation may be one of the factors in the selection between mutant and wild type progeny from a mixed infection. Several of our recombinants will have the P3 gene of strain YSMV and the CP gene of strain 425. Because the sequence similarity between the RNA3 molecules of the two strains is 97% (Neeleman et al., 1991) it is not possible to identify the exact site of recombination.

Symptom formation

Previously, we showed that the differences in symptoms caused by AIMV strains YSMV and 425 on inoculated leaves are due to an amino acid substitution at position 29 of the CP, but that genetic determinants for the difference in systemic symptoms are more complex (Neeleman et al., 1991). In complementation experiments mutant pY11 produced a subgenomic messenger (Fig. 4) which could be potentially translated into a polypeptide consisting of the N-terminal 64 amino acids of the CP fused to a non-viral sequence of 13 amino acids. Possibly, this polypeptide is responsible for the formation of small brown lesions followed by severe necrosis by the mutant mixtures pA2 + pY11 and pY9 + pY11. In mutant pY9 the P3 deletion removed the enhancer element of the subgenomic promoter, thereby reducing RNA4 synthesis (van der Kuyl et al., 1991). Under these conditions the pY11 encoded truncated CP may be dominant over the wild type CP of strain YSMV encoded by pY9. We are currently investigating this hypothesis by introducing a mutation in the AUG initiation codon of the CP gene in pY11. In mutant pY8 the subgenomic promoter is fully active and the mixture of pY8 and pY11 induced wild-type YSMV symptoms on the inoculated leaves. In mutant pY10 the 5'-terminal region of the CP gene is deleted and it can not produce the CP related polypeptide of mutant pY11. Combinations of pY10 with mutants containing the YSMV CP gene (pY8, pY9) induced YSMV-type symptoms on inoculated leaves, whereas combinations of pY10 with mutants containing the 425 CP gene (pA2, pA16) induced 425-type symptoms. Table 1 shows that none of the mixtures of deletion mutants induced systemic symptoms on the primary infected plants. After passage to a second group of plants the development of systemic symptoms was

correlated with the occurrence of recombination to wild-type RNA3. After several inoculations on P12 plants the mixture of transcripts pY8 and pY11 present in inoculum 9 did not show a detectable level of recombination and did not induce the systemic symptoms characteristic of strain YSMV. We are currently investigating whether this lack of symptom formation is due to an inability of these mutants to complement each other in functions required for long distance transport in the plant.

ACKNOWLEDGMENTS

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