Alfalfa mosaic virus RNA3 mutants do not replicate in transgenic plants expressing RNA3-specific genes

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The RNA3 of alfalfa mosaic virus (AIMV) encodes the P3 protein and the viral coat protein (CP). RNA3 molecules transcribed in vitro replicated in protoplasts and plants when inoculated in mixtures with AIMV RNA1, RNA2 and CP. Transcripts with a deletion or inversion in the P3 gene replicated well in protoplasts but not in transgenic plants transformed with the P3 gene. Transgenic plants expressing the CP gene became infected after inoculation with a mixture of RNA1, RNA2 and wild-type RNA3 transcripts without addition of CP to the inoculum. Transcripts with a deletion in the CP gene replicated at a reduced level in protoplasts but not in CP-transformed plants. This suggests that P3 and CP are both required for cell-to-cell spread of AIMV and that mutations in the inoculum RNA could not be complemented in trans by the wild-type chimeric nuclear genes.

Introduction

Alfalfa mosaic virus (AIMV) has a genome composed of three positive-sense RNA molecules. RNA1 and RNA2 are monocistronic (Cornelissen et al., 1983a, b). RNA3 is bicistronic (Barker et al., 1983; Ravelonandro et al., 1984; Langereis et al., 1986). The 5'-proximal cistron in RNA3 encodes the P3 protein and the 3'-proximal cistron encodes the viral coat protein (CP). CP is translated from a subgenomic messenger, RNA4. A mixture of the three genomic RNAs is not infectious unless a few CP molecules are added per RNA molecule (Bol et al., 1971). This phenomenon is called genome activation. RNA1 and RNA2 are able to replicate in protoplasts in the absence of RNA3 provided that CP is present in the inoculum (Nassuth & Bol, 1983). The same inoculum is not infectious to plants. This indicates that proteins encoded by RNA1 and -2 have a role in viral RNA replication and that one or both RNA3-encoded products is required for cell-to-cell transport of the virus.

DNA copies of RNA3 of the S strain (RNA3-S) of AIMV have been successfully transcribed in vitro into biologically active RNA molecules (Dore et al., 1990). This permits a further analysis of the function of RNA3-encoded proteins. In the work reported here, we studied the replication of RNA3 transcripts with a deletion in the CP cistron in transgenic tobacco plants transformed with a wild-type CP gene (CP plants). Previously, we have shown that such CP plants are resistant to infection with AIMV particles but susceptible to infection with AIMV RNAs (van Dun et al., 1987). Moreover, these plants can be infected with a mixture of the three genomic RNAs without CP. Because genome activation is performed by the CP encoded by the chimeric nuclear gene, these transgenic plants can be used to study other functions of the mutant CP encoded by the inoculum RNA3.

To permit a similar study of the P3 gene, tobacco plants were transformed with a DNA copy of this gene (P3 plants). The ability of these P3 plants to support the replication of transcripts with mutations in the P3 gene was investigated. As a control we assessed the ability of the replicase activity induced by RNA1 and -2 to replicate mutant transcripts in protoplasts.

Methods

Plants and viruses. The CP plants used in this study were the tobacco plants S40.7 described by van Dun et al. (1987). RNA3-S transcripts were combined with RNA1, RNA2 and CP of the L strain of AIMV, also called strain 425. The sequence similarity of strains L and S is greater than 90% (Langereis et al., 1986).

Construction of mutants. Restriction sites in the wild-type cDNA clone of RNA3-S (clone 3BS-(1,0); Dore et al., 1990) that were used to construct mutants (Maniatis et al., 1982) are shown in Fig. 1. To construct mutants delP3 and invP3, the wild-type clone was digested with XhoI and with HincII, the XhoI protruding ends were filled in with Klenow DNA polymerase and the resulting mixture of fragments was subjected to blunt-end ligation. Intramolecular ligation of the vector-containing fragment resulted in the delP3 mutant and insertion of the small DNA fragment in the reverse orientation in the vector-containing fragment gave the invP3 mutant. The inversion introduced in invP3 results in frameshifts in all three reading frames. The deletion
in the CP gene was made by digestion with BamHI and ApeI, making blunt ends with T4 DNA polymerase and religation. This deletion was engineered in the wild-type and delP3 clones to obtain mutants delCP and delP3CP, respectively. The deletions in the P3 and CP cistrons lead to frameshifts in the corresponding reading frames.

**Purification of viral RNAs and in vitro transcription.** Viral RNAs were separated on 1% agarose gels and extracted from the gel by a modified freeze–squeeze method (Tautz & Renz, 1983). Transcription of cDNA clones with T7 RNA polymerase was done as previously described (Dore et al., 1990).

**Protoplast infection.** Cowpea protoplasts were isolated as described by Albias & Bol (1977). The protoplasts (10⁶) were inoculated by the polyethylene glycol method (Loesch-Fries et al., 1985) with 1-5 μg of RNA1, 0-3 μg of RNA2 and 7-5 μg of CP with or without transcripts corresponding to the transcription of 0-5 μg of DNA (approximately 50 ng of transcript). DNA was not removed prior to inoculation. The protoplasts were collected by centrifugation after a 24 h incubation at 25 °C.

**Plant transformation.** cDNA of the P3 gene extending from nucleotides 186 to 1258 of the RNA3 of the L strain of AIMV (RNA3-L) was inserted into a binary transformation vector derived from pAGSHB (van Dun et al., 1987) such that its expression was controlled by the cauliflower mosaic virus 35S promoter and the nos polyadenylation signal. The plasmid pCA30 was used to transform Agrobacterium tumefaciens strain LBA4404 by means of triparental mating (Hoekema et al., 1983) and transformation of leaf disks of Nicotiana tabacum cv. Samsun was carried out (Horsch et al., 1985). Transformed cells were selected on shooting medium containing kanamycin. Kanamycin-resistant shoots were transferred to rooting medium containing kanamycin. After development of the root system (2 to 3 weeks) plantlets were cultured in a greenhouse. The resulting transformed plants were designated S30. Progeny plants of transformant S30.5 were used in this study (P3 plants).

**Plant inoculation.** For inoculations, young plants at the three leaf stage were used. Inoculation of P3 plants was done with 20 μl per leaf of a mixture containing 1 μg of RNA1, 0.5 μg of RNA2 and 7.5 μg of CP supplemented with the amount of transcript transcribed from 0.5 μg of cDNA (approximately 50 ng of transcript). For inoculation of the CP plants, CP was omitted from the inoculum.

**Nucleic acid analysis.** Transformed plants were analysed for the presence of integrated cDNA by alkaline DNA blotting as described previously (van Dun et al., 1988). Accumulation of polyadenylated transcripts derived from the chimeric gene was analysed by Northern blotting as described previously (van Dun et al., 1987). Total RNA extracted from inoculated protoplasts and plants (Jackson et al., 1976) was analysed by Northern blotting. Detection of the complete set of viral RNAs was done with a cDNA probe obtained by priming on AIMV RNAs (Dore et al., 1989) with random hexamers. Selective detection of RNA3 and -4 was done with a 32P-labelled oligodeoxynucleotide complementary to nucleotides 1980 to 2032 of RNA3-L (Ranger et al., 1986). Discrimination between the RNA3s of AIMV strains S and L was as described by Dore et al. (1989).

**Results**

**Replication of RNA3 mutants in protoplasts**

The wild-type RNA3-S was used to create the mutants shown in Fig. 1. Using unique restriction sites, a deletion or inversion was made in the P3 gene (delP3 and invP3, respectively) and deletions were made in the CP gene (delCP) or in both the P3 and CP genes (delP3CP). RNA3 molecules transcribed in vitro with the T7 RNA polymerase from the wild-type and mutant clones were mixed with native RNA1, RNA2 and CP of the L strain of AIMV, and the mixtures were used to inoculate cowpea protoplasts. Replication of viral RNAs in these protoplasts was monitored by Northern blot analysis as shown in Fig. 2. Panel (a) was probed with random cDNA detecting all AIMV RNAs, and panel (b) was probed with a synthetic oligodeoxynucleotide that selectively hybridized to RNA3 and -4. In addition, this synthetic oligodeoxynucleotide hybridized to a host RNA also seen in mock-inoculated protoplasts (lane 8). When protoplasts were inoculated with RNA1-L, RNA2-L and CP, the replication of RNA1 and 2 was detectable (Fig. 2a, lane 1) and a very low amount of RNA4 was detected (Fig. 2b, lane 1). This demonstrates that contamination of the RNA1 and -2 preparation with RNA3 is low. When the mixture of RNA1, RNA2 and CP was supplemented with RNA3-S synthesized in vitro (lane 2) or when protoplasts were inoculated with all viral RNAs (lane 7) a relatively high level of synthesis of RNA3 and -4 was detectable. This demonstrates that the replicase activity induced by RNA1 and -2 of strain L is able to replicate RNA3-S (Dore et al., 1989).

Lanes 3 to 6 of Fig. 2 show the replication of mutant RNA3-S molecules in protoplasts. A deletion in the P3 cistron (delP3) had no effect on the replication of the genomic RNA3 and the synthesis of subgenomic RNA4 (lane 3). Similarly, an inversion of part of the P3 cistron (invP3) did not affect the template activity of this genome segment (lane 5). In contrast, a deletion in the CP cistron (delCP) considerably reduced the accumulation of the mutant (lane 4). The mutant RNA itself (the same size as delP3 in lane 3) was barely detectable but some subgenomic RNA of the size expected for the delCP mutant was detectable. In addition, a small

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**Fig. 1.** Schematic representation of mutations made in a wild-type cDNA clone of RNA3-S. Deletions are shown by gaps; the arrow in invP3 represents an inversion of the XhoI/HincII fragment and the arrow in wild-type RNA3-S indicates the subgenomic RNA start site.
AIMV RNA3 mutants

Fig. 2. Northern blot analysis of the replication of mutants in cowpea protoplasts. Protoplasts were inoculated with a mixture of RNA1, RNA2 and CP, supplemented with water (lane 1) or RNA3 synthesized in vitro from clones of the wild-type (lane 2), delP3 (lane 3), delCP (lane 4), invP3 (lane 5) or delP3CP (lane 6). As controls, the protoplasts were inoculated with native RNA1 to -4 (lane 7) or mock-inoculated (lane 8). The blots were hybridized with a probe detecting all AIMV RNAs (a) or with a probe detecting only RNA3 and RNA4 (b). The positions of the viral RNAs are indicated in the left margin.

amount of wild-type RNA4 is visible in lane 4, indicating that the inoculum RNAs were contaminated with a minor amount of RNA3-L. The replication of a mutant with deletions in both the P3 and the CP cistrons (delP3CP) was undetectable and no subgenomic RNA of mutant size was seen (lane 6).

Construction of transgenic P3 plants

Transgenic CP plants expressing the CP gene of AIMV strain L were found to be susceptible to infection with AIMV RNA1 to -3 indicating that the endogenously produced CP was able to activate the AIMV genome (van Dun et al., 1987). We wanted to investigate whether this endogenously produced CP was also able to complement the defect in the CP gene of the mutant delCP. Similarly, it was of interest to see whether transgenic P3 plants would be able to complement the defective P3 cistron in mutants delP3 and invP3. To obtain transgenic P3 plants, Samsun NN tobacco was initially transformed with a complete DNA copy of RNA3-L fused to the 35S promoter and nos termination signal. In all transformants the transcript of the chimeric gene was specifically processed by removal of an internal sequence containing the 3' end of the P3 gene and the 5' end region of the CP gene (van Dun, 1988). To prevent processing of the viral transcripts, tobacco plants were subsequently transformed with cDNA corresponding to nucleotides 186 to 1258 of RNA3-L, i.e. the region that contains the P3 gene. Fig. 3 shows that transformants so obtained accumulated various levels of viral transcripts of the expected size (1400 nucleotides) composed of a vector-derived 5'-terminal sequence of 20 nucleotides, a viral sequence of 1072 nucleotides, a vector-derived 3'-terminal sequence of 150 nucleotides and a poly(A) tract.

We were unable to detect the P3 protein in the transgenic plants or in AIMV-infected control plants by Western blot analysis with an antiserum raised against a synthetic peptide corresponding to the C-terminal 23 amino acids of the P3 protein. Thus, expression of the transgene in the P3 plants at the protein level could not be confirmed. The P3 plant with the highest expression at the RNA level (plant S30.5; Fig. 3, lane 4) was used in further studies. These transformants were as susceptible to AIMV infection as non-transformed control plants (results not shown).
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Fig. 4. Northern blot analysis of the replication of mutants in transgenic plants. (a) Transgenic P3 plants were inoculated with a mixture of RNA1, RNA2 and CP, supplemented with water (lane 1) or with RNA synthesized in vitro from delP3 (lane 2), invP3 (lane 3) or wild-type cDNA3 (lane 4). Total RNAs were extracted from the inoculated leaves. (b) Transgenic CP plants were inoculated with a mixture of RNA1 and RNA2, supplemented with RNA synthesized in vitro from delCP (lanes 1 and 2) or wild-type (lanes 3 and 4) cDNA3. Total RNAs were extracted from the inoculated leaves (lanes 1 and 3) or non-inoculated upper leaves (lanes 2 and 4). The positions of RNA1 to -4 are indicated in the right margin.

Inoculation of transgenic plants with mutant transcripts

P3 plants were inoculated with a mixture of RNA1, RNA2 and CP of the L strain, supplemented with water or transcripts synthesized in vitro. Total RNAs were extracted from the inoculated leaves and analysed by Northern blot hybridization (Fig. 4a). The inoculum produced a low level of viral RNA synthesis without added transcript (Fig. 4a, lane 1), indicating a minor contamination with RNA3-L. After addition of wild-type RNA3-S transcript, the inoculum induced a relatively high level of viral RNA synthesis (Fig. 4a, lane 4). Owing to the difference in length of the leader sequences of RNA3-S and RNA3-L, these two RNAs could be readily distinguished by primer extension experiments (Dore et al., 1989). Moreover, an oligonucleotide complementary to nucleotides 171 to 187 of RNA3-S did not hybridize to RNA3-L. By using this oligonucleotide for primer extension analysis, it was confirmed that the RNA3 seen in lane 4 of Fig. 4a was from the S strain (Fig. 5, lane 4).

When the inoculum was supplemented with a delP3 transcript, a low level of viral RNA synthesis was detected in the inoculated P3 plants (Fig. 4a, lane 2). However, it was shown by primer extension that RNA3 produced in this infection originated from contaminating RNA3-L (results not shown) and no RNA3-S derived from the transcript was detectable (Fig. 5, lane 3). An inoculum supplemented with the invP3 transcript was not infectious (Fig. 4a, lane 3). This demonstrates that the P3 plants do not support the replication of RNA3 molecules with mutations in the P3 cistron.

To test the ability of the CP plants to support the replication of RNA3 molecules with a mutated CP gene, these plants were inoculated with a mixture of RNA1 and RNA2 supplemented with either wild-type or delCP transcripts. The production of viral RNA in the primary inoculated and systemically infected leaves was monitored by Northern blot hybridization (Fig. 4b). The inoculum supplemented with wild-type transcript induced a relatively high level of primary and systemic infection (Fig. 4b, lanes 3 and 4). The endogenously produced CP in these plants appeared able to activate the mixture of genomic RNAs, as shown earlier (van Dun et al., 1987). It was confirmed by primer extension that the RNA3 in the progeny of this infection was from the S strain (Fig. 5, lane 1). When the CP plants were inoculated with an inoculum to which the delCP transcript had been added, no viral RNA synthesis was detected in the inoculated or upper leaves of the plant (Fig. 4b, lanes 1 and 2). This shows that the endogenous CP encoded by the chimeric nuclear gene is not able to complement the mutation in the CP gene of the inoculum RNA.

Discussion

The observation that RNA3 molecules with a mutation in the P3 gene efficiently replicate in protoplasts but not in plants supports the notion that the product encoded by
the AIMV P3 cistron is involved in cell-to-cell spread of the virus. The P3 protein was found in the middle lamellar region of the cell wall when infected cells were immunogold-labeled, suggesting that it performs its function at this location (Stussi-Garaud et al., 1987). Transgenic plants expressing the corresponding P3 gene of tobacco mosaic virus (TMV) were able to complement a temperature-sensitive defect in P3 on infection with the TMV mutant Ls1 (Deom et al., 1987). Our recent finding that transcripts of full length cDNA3-L synthesized in vitro are infectious to plants (A. C. van der Kuyl & J. F. Bol, unpublished results) demonstrates that the cloned P3 gene is functional. It could be that expression at the protein level of this gene is too low in P3 plants to permit complementation of the delP3 and invP3 mutants. The possibility exists that mutants in the P3 cistron cannot be complemented in trans. Evidence for this possibility was recently obtained for cowpea chlorotic mottle virus (Allison et al., 1990). Moreover, the antisense fragment in the invP3 mutant may block the translation of the truncated viral messenger RNA produced by the P3 plant.

Deletions in the CP cistron in mutants delCP and delP3CP did not reduce the replication in protoplasts of the wild-type RNA1 and RNA2 molecules that were present in the inoculum (Fig. 2a, lanes 4 and 6). The reduced accumulation of the mutant RNA3 molecules could indicate that cis-acting elements required for in vitro replication of RNA3 are more extensive than those required in vitro (van der Kuyl et al., 1990). Alternatively the deletion in the CP gene could affect the stability of the mutant RNA molecules. Nevertheless, mutant delCP replicated at a detectable level in protoplasts but failed to replicate in CP plants. This indicates that in addition to genome activation, the AIMV CP is required for cell-to-cell spread of the virus. At least two different mechanisms seems to exist for the movement of plant viruses. For viruses such as TMV (Takamatsu et al., 1987) and tobacco rattle virus (Harrison & Robinson, 1986), the transport in the inoculated leaf occurs efficiently in the absence of functional CP, while for viruses like bromoviruses (Sacher & Ahlquist, 1989; Allison et al., 1990) and cowpea mosaic virus (Wellink & van Kammen, 1989) CP is required for this process. AIMV seems to belong to this latter category of viruses. The level of accumulation of CP in transgenic CP plants was 10- to 100-fold lower than that obtained during a productive infection (van Dun et al., 1987). The level of endogenous CP in the transgenic plants may be sufficient for activation of the wild-type viral genome but too low to permit cell-to-cell spread of a mutant with a defective CP cistron.

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References


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