

Replication of alfalfa mosaic virus RNA 3 with movement and coat protein genes replaced by corresponding genes of Prunus necrotic ringspot ilarvirus

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Alfalfa mosaic virus (AMV) and Prunus necrotic ringspot virus (PNRSV) are tripartite positive-strand RNA plant viruses that encode functionally similar translation products. Although the two viruses are phylogenetically closely related, they infect a very different range of natural hosts. The coat protein (CP) gene, the movement protein (MP) gene or both genes in AMV RNA 3 were replaced by the corresponding genes of PNRSV. The chimeric viruses were tested for heterologous encapsidation, replication in protoplasts from plants transformed with AMV replicase genes P1 and P2 (P12 plants) and for cell-to-cell transport in P12 plants. The chimeric viruses exhibited basic competence for encapsidation and replication in P12 protoplasts and for a low level of cell-to-cell movement in P12 plants. The potential involvement of the MP gene in determining host specificity in ilarviruses is discussed.

Alfalfa mosaic virus (AMV) and ilarviruses are positive-strand viruses with similar tripartite genomes. They differ from other plant viruses in that genomic RNAs become infectious only upon addition of a few CP molecules, a phenomenon known as 'genome activation' (Bol *et al.*, 1971; Jaspars, 1985). The CPs of several ilarviruses are interchangeable in that they can activate each others' genome (van Vloten-Doting, 1975; Gonsalves & Garnsey, 1975; Gonsalves & Fulton, 1977). In addition to this early function, the CP of AMV has late functions in asymmetric positive-strand RNA accumulation, cell-to-cell transport and encapsidation of viral RNA. Early and late functions can be mutated separately (van der Vossen *et al.*, 1994).

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Recent phylogenetic analysis has shown that AMV and Prunus necrotic ringspot ilarvirus (PNRSV) are closely related (Sánchez-Navarro & Pallás, 1997). In spite of this high degree of similarity, AMV and PNRSV differ greatly in their range of

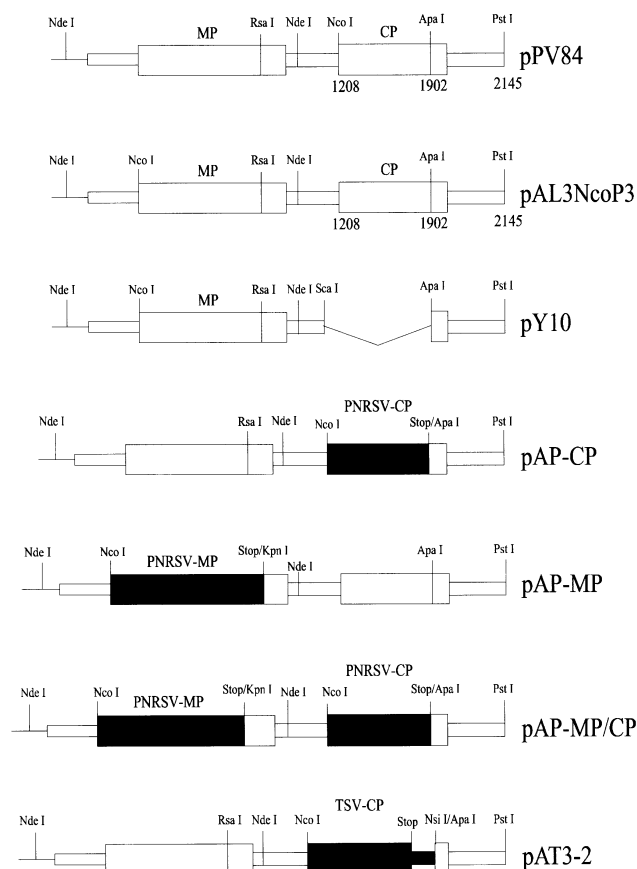


Fig. 1. Schematic representation of AMV cDNA 3 and AMV/PNRSV chimeric constructs, and the previously described pY10 (van der Kuyl *et al.*, 1991) and pAT3-2 (Reusken *et al.*, 1995) constructs. Wide bars represent coding regions for MP and CP of AMV and PNRSV as indicated. Open bars represent AMV-derived sequences and filled bars represent PNRSV-derived sequences. The numbers shown on the representations of pPV84 and pAL3NcoP3 refer to the nucleotide sequence in RNA 3 of AMV.

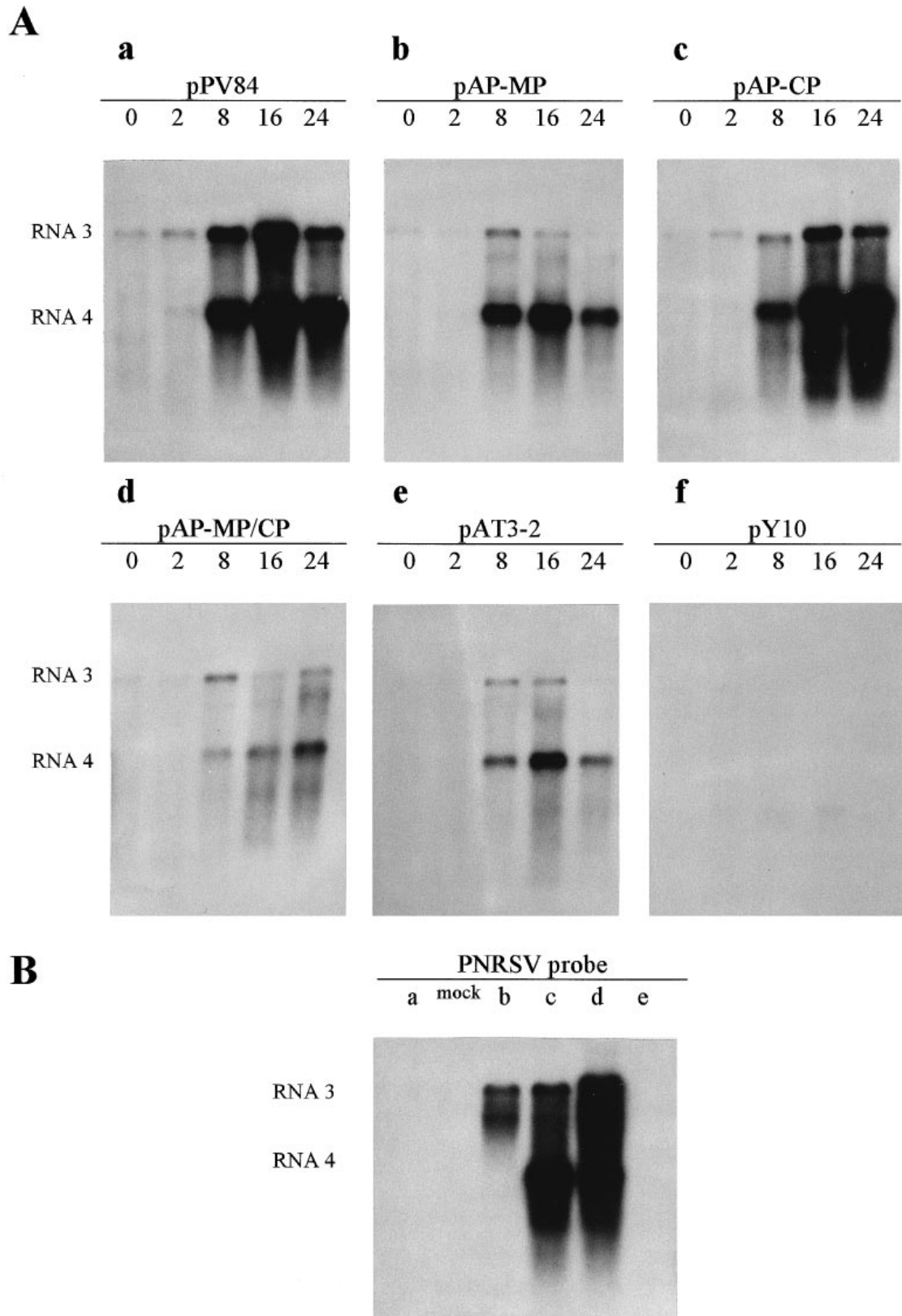


Fig. 2. For legend see facing page.

natural hosts. PNRSV mostly infects species of the genus *Prunus* whereas AMV mainly infects herbaceous hosts, including many leguminous plants. Tobacco is the best experimental host for AMV, whereas it is a non-permissive host for PNRSV. Previously, it has been shown that tobacco plants transformed with AMV replicase genes P1 and P2 (P12 plants) support replication of AMV RNA 3 without the requirement for CP in the inoculum (Taschner *et al.*, 1991; van Dun *et al.*, 1988).

Although it has now been established that the host specificity phenomenon has a multigenic basis (Bancroft & Lane, 1973; Allison *et al.*, 1988), there is evidence indicating that the movement protein (MP) gene plays an important role (Mise *et al.*, 1993; Fujita *et al.*, 1996).

To contribute to our knowledge of the different steps in the life cycle of ilarviruses, we exchanged the CP gene, the MP gene or both genes in AMV RNA 3 for the corresponding genes of PNRSV and studied their influence on encapsidation and replication in P12 protoplasts and in cell-to-cell transport in P12 plants.

To investigate whether the ability of AMV to infect protoplasts was affected by substitution of the MP and CP genes of RNA 3 for the corresponding PNRSV genes, different hybrid constructs were made (Fig. 1). The entire PNRSV-PV96 movement protein (P3 or MP) and coat protein (CP) genes were amplified by RT-PCR with a proofreading DNA polymerase (Vent polymerase, New England Biolabs) using total RNA extracted from PNRSV-inoculated cucumber plants. First-strand cDNA synthesis of the PNRSV MP gene was done using avian myeloblastosis virus reverse transcriptase (Promega) and primer A (5' ACCTCAAGCACTTCCAGAACT-ACCG 3'; stop codon in italics) in which the 22 nt at the 3' end were complementary to those at the 3' end of the P3 gene. Second-strand synthesis was done using Vent DNA polymerase and primer B (5' CATGCCATGGCCGGTGTGAC3'), containing an *NcoI* site (underlined) at the 5' end and a 3' end sequence identical to nt 174–183 of PNRSV RNA 3. Amplification of the PNRSV CP gene was done using the antisense primer C (5' CATGGGGCCCTAGATCTCAAGCAGGTC 3'), containing a sequence of 18 nt at the 3' end complementary to the PNRSV CP end gene (nt 1757–1774 of RNA 3) plus an *ApaI* restriction site at the 5' end, and the sense primer D (5' GTTGCCATGGTTTGCCGAATTTGC 3'), containing a *NcoI* restriction site at the 5' end and a 3' sequence identical to the first 18 nt of the CP gene (nt 1100–1117 of RNA 3).

pAL3NcoP3 and pPV84 are two infectious clones of AMV RNA 3 (strain 425) with *NcoI* sites engineered over the

initiation codon of the MP and CP genes, respectively (van der Vossen *et al.*, 1993; Taschner *et al.*, 1994). Three constructs were made by replacing the MP, CP or both genes of AMV with the corresponding genes of PNRSV (hybrids pAP-MP, pAP-CP and pAP-MP/CP, respectively; Fig. 1). Hybrid pAP-MP was made by replacing the *NcoI*–*RsaI* fragment of pAL3NcoP3 by the fragment obtained after RT-PCR from a PNRSV preparation using primers A and B described above and *NcoI* digestion. After ligation, a new *KpnI* restriction site was generated at the end of the PNRSV MP gene. This new site was used to screen the new recombinant clones. Hybrid pAP-CP was made by replacing the *NcoI*–*ApaI* fragment of pPV84 by the cDNA obtained after RT-PCR from a PNRSV preparation using primers C and D described above and *NcoI* and *ApaI* digestions. To obtain the pAP-MP/CP hybrid, the *NdeI* sites located in the intercistronic region and the plasmid 5' polylinker region were used. The *NdeI* fragment of pAP-CP (containing the wt AMV P3 gene) was replaced by the equivalent fragment of pAP-MP (containing the PNRSV MP gene) to yield pAP-MP/CP. In addition to the hybrid constructs, two control constructs were used: construct pY10 had a deletion in the CP gene that reduced RNA 3 and 4 accumulation in P12 protoplasts 100-fold (van der Kuyl *et al.*, 1991), and in construct pAT3-2 the AMV CP gene in AMV RNA 3 was replaced by the corresponding tobacco streak virus (TSV) CP gene (Reusken *et al.*, 1995). The synthesis of wt and hybrid RNA 3 transcripts (van der Kuyl *et al.*, 1991), inoculation of protoplasts from P12 plants (van Dun *et al.*, 1988), extraction of total RNA and Northern blot analysis of viral RNA accumulation (van der Vossen *et al.*, 1994) were performed as described. Duplicate gels were blotted and probed with either AMV or PNRSV probes. The AMV 3' UTR cDNA probe was randomly ³²P-labelled to detect all the different constructs. At the same time, RNAs corresponding to hybrids containing MP and/or CP PNRSV genes were detected with a mixture of probes of randomly ³²P-labelled PNRSV MP and CP cDNA genes. The PNRSV probe produced a stronger signal than the AMV 3' UTR probe for the same sample. Northern blot hybridization signals were quantified with a Shimadzu digital radioactive image system.

To study the kinetics of accumulation and stability of the different hybrids, total RNA was extracted at different time intervals post-inoculation (p.i.) of P12 protoplasts. Northern analysis with an AMV probe (Fig. 2A) showed that accumulation of wt RNA 3 and 4 was already detectable 8 h p.i. and increased until 16 h p.i. The reduction in viral RNA accumulation observed at 24 h p.i. was probably due to the fact

Fig. 2. Time course analysis of the accumulation of wt (pPV84) and AMV/PNRSV-TSV chimeric transcripts (see Fig. 1). (A) Protoplasts were inoculated with the RNA 3 transcript from plasmids pPV84 (a), pAP-MP (b), pAP-CP (c), pAP-MP/CP (d), pAT3-2 (e) or pY10 (f). At the times indicated (in h p.i.), RNA was extracted from the protoplasts, glyoxylated, loaded onto 1.5% agarose gels and analysed by Northern blot hybridization using a randomly labelled AMV 3' UTR cDNA probe. (B) RNAs extracted from the same constructs at 16 h p.i. were probed with a randomly labelled mixture of PNRSV MP and CP cDNA coding regions. The positions of AMV RNAs 3 and 4 are indicated on the left. Lanes: a, pPV84; mock, mock inoculation; b, pAP-MP; c, pAP-CP; d, pAP-Mp/CP; e, pAT3-2.

that the number of viable protoplasts at this time is reduced. Quantification of positive-strand RNA accumulation revealed that all of the different hybrid constructs produced lower levels of RNAs 3 and 4 than wt RNAs. The highest RNA accumulation was with the pAP-CP construct (twofold reduction for RNA 3 and no reduction for RNA 4; Fig. 2A, panel c), indicating that PNRSV CP could replace the AMV CP for upregulation of positive-strand RNA accumulation. Inoculation with the other hybrid constructs resulted in 7- to 11-fold reduction in the levels of RNA 3 and 2- to 4-fold reduction in RNA 4 accumulation. The pY10 clone with the deleted CP did not induce the accumulation of detectable amounts of viral RNAs 3 and 4, indicating the crucial role of the CP (Fig. 2A, panel f; van der Vossen *et al.*, 1994). Compared to pAP-CP, the pAT3-2 construct (in which the AMV CP gene was replaced by the TSV CP gene) showed a fivefold reduction (Fig. 2A, panels e and c, respectively). This differential RNA accumulation may be due to the fact that AMV and PNRSV CPs are phylogenetically closer to each other than the TSV and AMV CPs (Sánchez-Navarro & Pallás, 1997). The highly basic motif found at the N-terminal region of all ilarviruses sequenced to date has been shown to bind to the 3' UTR region in AMV (Baer *et al.*, 1994; Reusken *et al.*, 1994; Houser-Scott *et al.*, 1994; Reusken & Bol, 1996; Ansel-McKinney *et al.*, 1996). This N-terminal sequence shows greater similarity between PNRSV and AMV than between TSV and AMV (Sánchez-Navarro & Pallás, 1997). The accumulation of subgenomic RNAs of the expected sizes demonstrated that the subgenomic promoters in the chimeric RNAs were functional and that (–) RNA 3 was synthesized.

The expression and identity of the progeny chimeras were also analysed with a PNRSV probe. For this purpose, total RNA was extracted at 18 h p.i. from P12 protoplasts transfected with the different hybrid constructs (Fig. 2B). RNA 4 produced by pAP-MP was not detected by the PNRSV probe because it contains AMV sequences only (Fig. 2B, lane b).

To investigate whether PNRSV CP and AMV CP genes are able to encapsidate their chimeric viral RNAs, virus particles were purified from transfected protoplasts as previously described (van der Vossen *et al.*, 1994; Reusken *et al.*, 1995). As shown in Fig. 3 (A–B), electrophoretic mobilities characteristic of virus particles were observed, indicating the ability of PNRSV CP and AMV CP genes to encapsidate the different hybrid RNAs. Note that the pAP-MP construct signal, after being probed with the PNRSV probe (Fig. 3B, lane 3), was significantly lower than the other constructs because only RNA 3 is detected in this case. The observation that pAP-CP and pAP-MP/CP are encapsidated could indicate that the AMV origin of assembly is recognized by PNRSV CP. Alternatively, this origin could be located in the exchanged CP sequence.

At the P12 plant level, cell-to-cell movement is a prerequisite for detecting the accumulation of AMV mutants by

Northern blot hybridization since a construct in which the MP gene was deleted (construct pY9 described by van der Vossen *et al.*, 1994) can replicate in P12 protoplasts but cannot produce any signal after Northern analysis in P12 plants. Viral RNA accumulation was studied by inoculating P12 plants with chimeric RNA 3 transcripts. Fig. 3 (C–D) shows a Northern blot analysis of the accumulation of pAP-MP, pAP-CP, pAP-MP/CP and pAT3-2 mutants hybridized with a 3' UTR AMV cDNA probe (Fig. 3C) or a PNRSV-MP/CP cDNA probe (Fig. 3D).

Unlike the protoplast results, the *in vivo* RNA accumulation of all the different mutants was significantly lower than that of wt AMV RNA. The absence of a hybridization signal corresponding to RNA 3 in all hybrids except pAP-MP/CP is believed to be due to a low accumulation of this RNA versus RNA 4, which is close to the hybridization detection limit. In fact, as can be observed for wt RNA accumulation (Fig. 3C, lane 1), RNA 4 is at substantially higher levels than RNA 3. In the two different experiments carried out, the highest viral RNA accumulation corresponded to the construct harbouring both CP and MP genes (pAP-MP/CP), clearly observed after using the PNRSV probe (Fig. 3D, lane 5). RNA 3 of the pAP-MP/CP hybrid was not detected by the AMV probe due to the fact that this probe consistently gave a weaker hybridization signal than the PNRSV probe. The band corresponding to RNA 4 in the construct pAP-MP was observed when probed against AMV (Fig. 3C, lane 3), indicating functionality of the subgenomic promoter, synthesis of (–) RNA 3 and at least a low level of cell-to-cell movement. This RNA 4 band cannot be recognized by the PNRSV probe (Fig. 3D, lane 3). No signal was observed with the pAT3-2 hybrid when using either of the two probes (Fig. 3C–D, lanes 6). These results show that all of the hybrid constructs are able to permit viral RNA accumulation and at least a low level of cell-to-cell movement in P12 plants. The low viral RNA accumulation observed could be due to low cell-to-cell movement or to deficient stability of viral RNA. As stated above, tobacco is a non-host for PNRSV. We have shown that PNRSV MP is functional in tobacco plants, allowing a low level of cell-to-cell movement. This suggests that the MP gene would not be involved in host specificity. However, as in the case of bromoviruses (Mise & Ahlquist, 1995), host restriction by the MP could occur after initial cell-to-cell spread of infection, which would explain the low level of cell-to-cell movement observed in the pAP-MP chimera.

AMV seems to require CP molecules for short-distance movement, apparently in a non-virion form (van der Vossen *et al.*, 1994). This requirement for the CP gene in cell-to-cell movement could explain why the construct pAP-CP, in which the CP gene was replaced, provided a lower signal in infected plants than the one in which the MP gene was replaced (pAP-MP; Fig. 3C). Viral RNA accumulation of the pAP-MP/CP clone was always higher than that of the pAP-MP clone at the P12 plant level. This observation can be explained by assuming

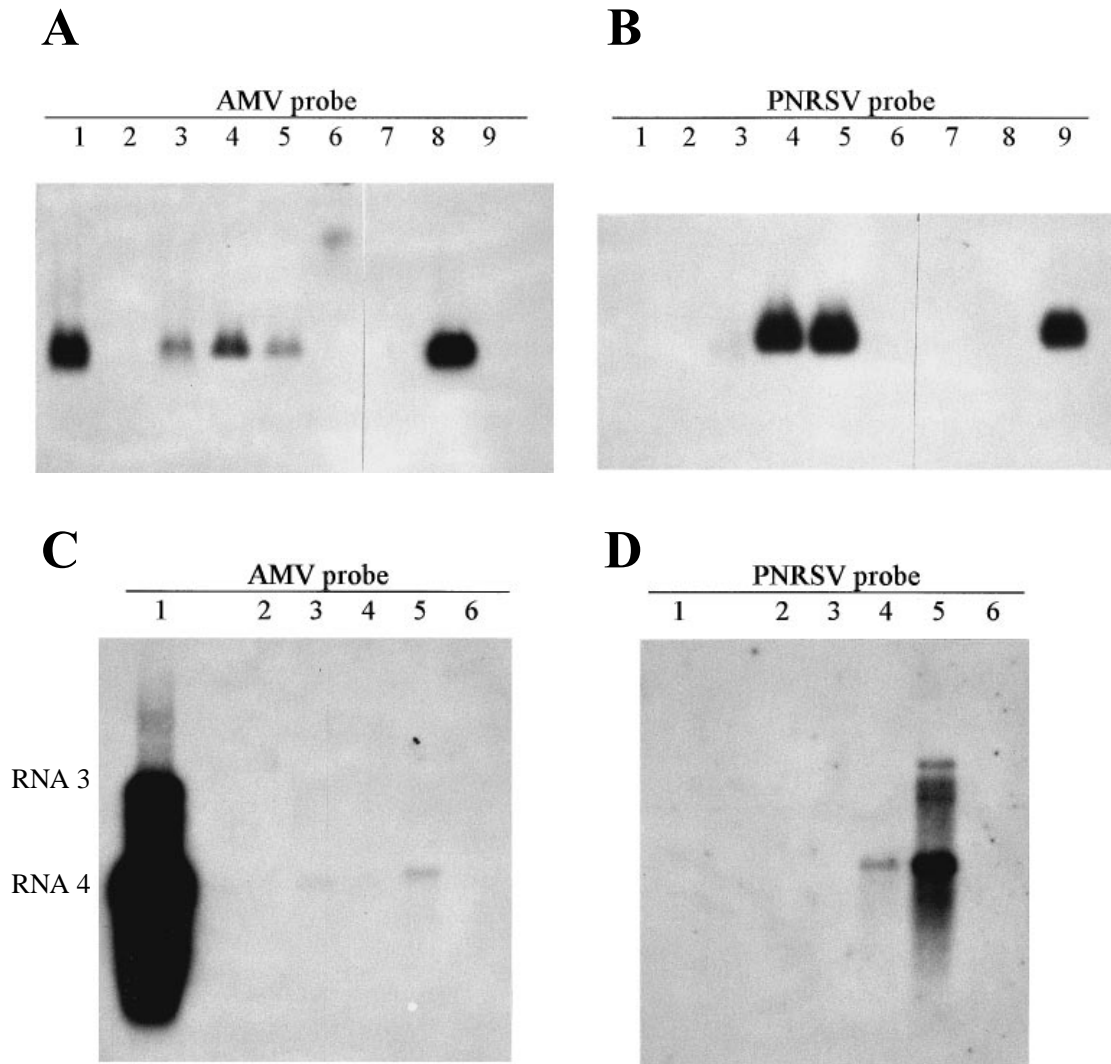


Fig. 3. Northern blot analysis of the accumulation of viral particles in P12 protoplasts (A, B) or viral RNAs in P12 plants (C, D) inoculated with mutant AMV RNA 3 transcripts. Duplicate 0.8% (A, B) or 1.5% (C, D) agarose gels were loaded with homogenates of protoplasts containing viral particles (A, B) or with total glyoxylated RNAs extracted from P12 plants (C, D) and probed with either randomly labelled AMV 3' UTR cDNA (A, C) or a mixture of PNRSV MP and CP cDNA coding regions (B, D). Protoplasts or P12 plants were inoculated with the RNA 3 transcript from plasmids pPV-84 (lanes 1), pAP-MP (lanes 3), pAP-CP (lanes 4), pAP-MP/CP (lanes 5) or pAT3-2 (lanes 6). Lanes 2 were loaded with homogenates from healthy P12 protoplasts (A, B) or RNA extracted from healthy P12 plants (C, D). Lanes 7 were loaded with homogenates of protoplasts to which AMV RNAs 3 and 4 had been added. Lanes 8 and 9 were loaded with homogenates of healthy protoplasts to which purified AMV and PNRSV particles, respectively, had been added. The positions of AMV RNAs 3 and 4 are indicated on the left. Lanes 6 and 7 in A and B were not adjacent on the gel.

the requirement of an interaction between MPs and CPs of the same virus or to putative interactions between the two proteins with *cis*-elements found in the RNA sequence. Protein-protein interactions essential for virus movement have been postulated between MP and CP in the case of cowpea chlorotic mottle virus (Allison *et al.*, 1990) and between HC-Pro and CP of tobacco etch virus (Cronin *et al.*, 1995).

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