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Recognition of *cis*-acting sequences in RNA 3 of *Prunus necrotic ringspot virus* by the replicase of *Alfalfa mosaic virus*

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***Alfalfa mosaic virus* (AMV) and *Prunus necrotic ringspot virus* (PNRSV) belong to the genera *Alfamovirus* and *Ilarvirus*, respectively, of the family *Bromoviridae*. Initiation of infection by AMV and PNRSV requires binding of a few molecules of coat protein (CP) to the 3′ termini of the inoculum RNAs and the CPs of the two viruses are interchangeable in this early step of the replication cycle. *Cis*-acting sequences in PNRSV RNA 3 that are recognized by the AMV replicase were studied in *in vitro* replicase assays and by inoculation of AMV–PNRSV RNA 3 chimeras to tobacco plants and protoplasts transformed with the AMV replicase genes (P12 plants). The results showed that the AMV replicase recognized the promoter for minus-strand RNA synthesis in PNRSV RNA 3 but not the promoter for plus-strand RNA synthesis. A chimeric RNA with PNRSV movement protein and CP genes accumulated in tobacco, which is a non-host for PNRSV.**

Prunus necrotic ringspot virus (PNRSV) and *Alfalfa mosaic virus* (AMV) are viruses with tripartite RNA genomes belonging to the genera *Ilarvirus* and *Alfamovirus*, respectively. RNAs 1 and 2 encode the polymerase proteins P1 and P2. RNA 3 is translated into the movement protein (MP) whereas the encoded coat protein (CP) is translated from a subgenomic RNA 4. In contrast to other genera in the family *Bromoviridae*, initiation of infection by alfamo- and ilarviruses requires the binding of a few molecules of CP to the 3′ termini of the inoculum RNAs (reviewed in Bol, 1999; Jaspars, 1999). Although the overall sequence similarity between AMV and ilarviruses is relatively low, the 3′ termini of the viral RNAs contain similar stem–loop structures flanked by AUGC sequences that represent specific binding sites for homologous as well as heterologous CPs of AMV and ilarviruses (Zuidema & Jaspars, 1985; Houser-Scott *et al.*, 1994; Reusken & Bol, 1996; Reusken *et al.*, 1994). CPs of AMV and ilarviruses are interchangeable in initiation of infection by these viruses (van Vloten-Doting, 1975; Gonsalves & Garnsey, 1975; Gonsalves & Fulton, 1977). In addition to its role in the initiation of infection and encapsidation of viral RNA, AMV CP is required for plus-strand RNA accumulation and cell-to-cell movement of the virus (van der Vossen *et al.*, 1994; de Graaff *et al.*, 1995; Neeleman & Bol, 1999; Tenllado & Bol, 2000).

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AMV and PNRSV are phylogenetically closely related (Sánchez-Navarro & Pallás, 1997). Previously, we have replaced the MP and CP genes in AMV RNA 3 by the corresponding PNRSV genes and studied the replication of the chimeric RNAs in transgenic P12 tobacco plants and protoplasts (Sánchez-Navarro *et al.*, 1997). P12 plants express the AMV P1 and P2 proteins and can be infected with AMV RNA 3 without a requirement for CP in the inoculum (Taschner *et al.*, 1991). PNRSV CP could substitute for all functions of AMV CP in the replication cycle, and PNRSV MP and CP mediated a reduced level of cell-to-cell transport of chimeric RNAs in plants, although tobacco is a non-permissive host for PNRSV (Sánchez-Navarro *et al.*, 1997). In the present study, we analysed the *cis*-acting sequences in PNRSV RNA 3 that are recognized by the AMV RNA-dependent RNA polymerase (RdRp) by exchanging the 5′-untranslated regions (UTRs) and 3′-UTRs of RNA 3 of the two viruses. The 5′-UTRs of AMV and PNRSV RNA 3 are 345 and 176 nucleotides (nt) long, respectively, and do not show significant sequence identity. The 3′-UTRs of AMV and PNRSV RNA 3 are 183 and 171 nt long, respectively, and show an overall similarity of 42%. The chimeric RNAs, shown in Fig. 1(A), were used as templates in *in vitro* polymerase assays with purified AMV RdRp, and the replication of the chimeras was analysed in P12 protoplasts and plants.

Plasmid pAL3NcoP3 is a infectious clone of AMV RNA 3 with a *Nco*I site engineered over the initiation codon of MP (van der Vossen *et al.*, 1993). Plasmid pUC3m1 contains a cDNA clone of PNRSV RNA 3 (NcM1 isolate; Aparicio *et al.*, 1999). To engineer plasmid pUC3m1 (Fig. 1A), total RNAs extracted from PNRSV-infected cucumber plants were subjected to reverse transcription and subsequent PCR amplification (RT–PCR) using an antisense primer complementary

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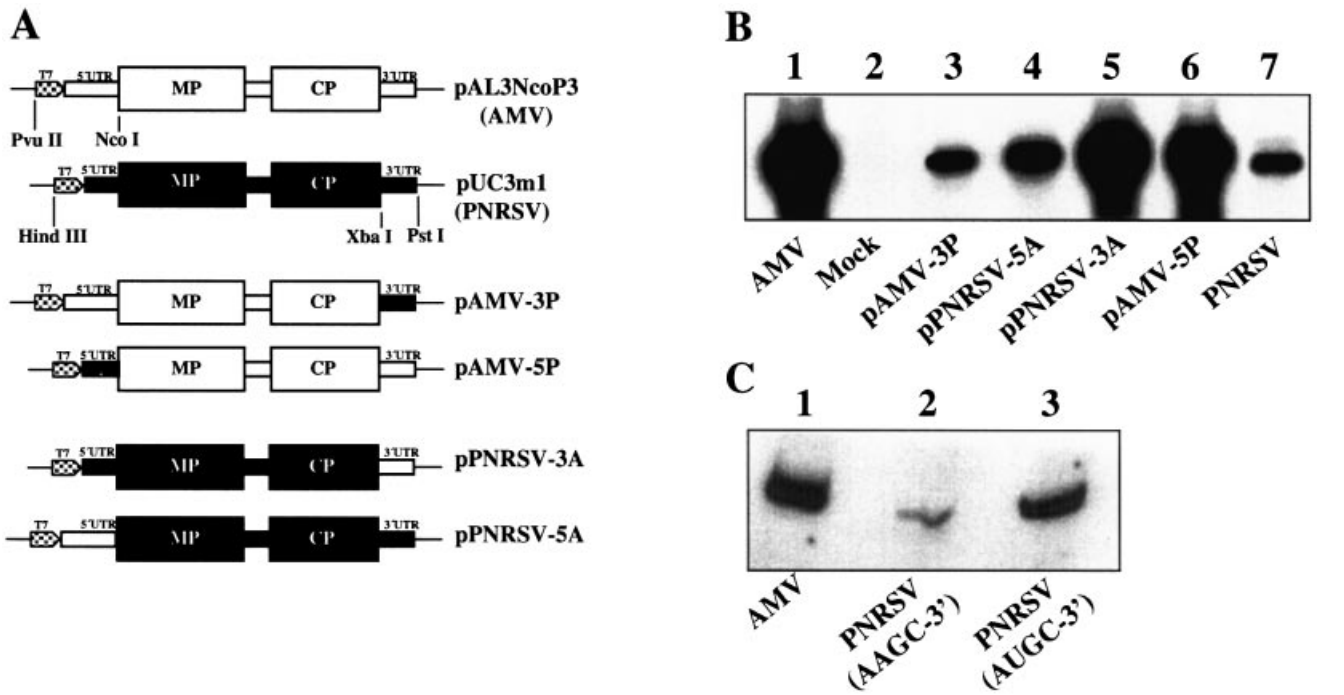

Fig. 1. (A) Schematic representation of AMV and PNRSV cDNA 3 and AMV–PNRSV chimeric constructs. Restriction sites used to construct cDNA 3 hybrids are indicated. Open bars represent AMV-derived sequences and filled bars represent PNRSV-derived sequences. Untranslated regions (UTRs) and open reading frames (MP and CP) are represented by narrow and wide bars, respectively. Crosshatched arrowheads represent the T7 RNA polymerase promoter. (B) *In vitro* RdRp assay with plus-strand RNA templates transcribed from the constructs shown in (A): pAL3NcoP3 (lane 1), no template added (lane 2), pAMV-3P (lane 3), pPNRSV-5A (lane 4), pPNRSV-3A (lane 5), pAMV-5P (lane 6), pUC3m1 (lane 7). (C) *In vitro* RdRp assay with plus-strand RNA templates corresponding to the 3′-UTR of AMV RNA 3 (lane 1), the 3′-UTR of PNRSV RNA 3 (lane 2) or the 3′-UTR of PNRSV RNA 3 with the 3′-terminal AAUG sequence mutated into AUGC (lane 3). ^{32}P-labelled minus-strand products were synthesized *in vitro* with purified AMV RdRp as described (de Graaff *et al*., 1995). The labelled products were run in agarose gels; autoradiograms of the gels are shown.

to the terminal 18 nt of viral RNA 3 plus an extra *Pst*I site (Sánchez-Navarro & Pallás, 1997). PCR was carried out with the antisense primer described above and a sense primer containing a *Hin*dIII site, the T7 RNA polymerase sequence promoter and the first 18 nt of PNRSV RNA 3 (PV96 isolate). Construct pAMV-5P was made by amplification of the PNRSV 5′-UTR sequence from plasmid pUC3m1 using specific primers flanked with *Pvu*II and *Nco*I sites. The resulting fragment with the T7 promoter and the PNRSV 5′-UTR was then inserted in plasmid pAL3NcoP3 previously digested with *Pvu*II and *Nco*I. To engineer construct pPNRSV-5A, a PNRSV cDNA 3 fragment lacking the 5′-UTR region was amplified from plasmid pUC3m1 using appropriate primers bordered with *Nco*I and *Pst*I sites. The PCR product was digested with the corresponding restriction enzymes and introduced in the pAL3NcoP3 construct. Construct pPNRSV-3A was created by amplification of the AMV 3′-UTR from pAL3NcoP3 using specific primers flanked with *Xba*I and *Pst*I sites. The fragment was introduced in the pUC3m1 construct using an *Xba*I site after the CP stop-codon and the *Pst*I site located at the end of the cDNA clone. In the same way, construct pAMV-3P was engineered by amplification of the PNRSV 3′-UTR and introduction of the PCR fragment in plasmid pAL3NcoP3 using *Kpn*I and *Pst*I sites that flank the AMV 3′-UTR. Plasmids containing wt AMV cDNA 3, PNRSV cDNA 3 and the chimeric AMV–PNRSV constructs were linearized with *Pst*I and transcribed with T7 RNA polymerase as described previously (van der Kuyl *et al*., 1991). Inoculation of plants and protoplasts with the transcripts was done as described by Neeleman & Bol (1999).

Fig. 1(B) shows the autoradiogram of an agarose gel run with ^{32}P-labelled minus-strand RNA 3 products that were synthesized by the purified AMV RdRp (de Graaff *et al*., 1995) in an *in vitro* polymerase assay when the chimeric RNAs were used as templates. The template activity of PNRSV RNA 3 (Fig. 1B, lane 7) was 18% of the activity of AMV RNA 3 (Fig. 1B, lane 1). In AMV RNA 3, the promoter for *in vitro* minus-strand RNA synthesis is located in the 3′-terminal 166 nt (van Rossum *et al*., 1997). The two chimeras with the AMV 3′-UTR showed a 100% level of template activity (Fig. 1B, lanes 5 and 6) whereas the chimeras with the PNRSV 3′-UTR, i.e. AMV-3P and PNRSV-5A, showed a template activity of 16 and 35%, respectively (Fig. 1B, lanes 3 and 4). When the 3′-UTR sequences were used as templates instead of full-length RNA 3,

template activity of the PNRSV 3′-UTR (Fig. 1C, lane 2) was 20% of that of AMV (Fig. 1C, lane 1). AMV RNA 3 ends with the sequence AUGC whereas PNRSV RNA 3 ends with AAGC. When this AAGC sequence was changed to AUGC, the template activity of the 3′-UTR of PNRSV (Fig. 1C, lane 3) increased to 83% of that of AMV.

Fig. 2 shows the accumulation of viral RNAs in P12 protoplasts inoculated with the chimeric RNAs. For the detection of plus-strand RNAs, the Northern blots were hybridized to DIG-labelled riboprobes (Pallás *et al.*, 1999) complementary to the 3′-UTR of AMV (Fig. 2A) or PNRSV (Fig. 2B). Panels (A) and (B) of Fig. 2 were developed for the same time to permit a comparison of the signals. The 42% sequence similarity between the two 3′-UTRs was too low to permit cross-hybridization of the probes under the conditions used. Minus-strand RNAs in the protoplasts were detected by using a mixture of plus-strand probes corresponding to the MP genes of AMV and PNRSV (Fig. 2C). The accumulation of AMV RNA 3 is shown as a control in Fig. 2(A), lane 1. (The minor band at the top of the gel may represent an aggregate or incompletely melted double-stranded RNA.) Only chimeras AMV-3P and PNRSV-5A (Fig. 2B, lanes 3 and 4) induced accumulation of plus-strand RNAs at levels similar to the control. The relatively low amounts of plus-strand RNA detectable in protoplasts inoculated with PNRSV-3A (Fig. 2A, lane 5), AMV-5P (Fig. 2A, lane 6) and PNRSV RNA 3 (Fig. 2B, lane 7) probably represent fragments of inoculum RNAs as these RNAs are shorter than full-length wild-type or chimeric RNA 3. The signals observed in lanes 1, 3 and 4 of Fig. 2(A, B) may have to be corrected for similar background levels. Fig. 2(C) shows that plus-strand AMV RNA 3 (2142 nt), PNRSV RNA 3 (1951 nt) and chimeric inoculum RNAs are all transcribed into complementary minus-strand RNAs by the transgenic AMV RdRp. Particularly, the results with chimera AMV-5P demonstrate that minus-strand RNA 3 synthesis (Fig. 2C, lane 6) is independent of *de novo* plus-strand RNA 3 synthesis (Fig. 2A, lane 6). Previously, we have shown that minus-strand RNA 3 transcribed in P12 protoplasts from inoculum RNA 3 is sufficient to direct wild-type levels of asymmetric plus-strand RNA 3 synthesis (Neeleman & Bol, 1999). Apparently, the AMV RdRp recognized the 3′-UTR of PNRSV RNA 3 both *in vitro* (Fig. 1B, C) and *in vivo* (Fig. 2C). However, only chimeras that contained the 5′-UTR of AMV RNA 3 (AMP-3P, PNRSV-5A) were able to direct *de novo* plus-strand RNA synthesis *in vivo* (Fig. 2B). This indicates that the AMV RdRp does not recognize promoter sequences for plus-strand RNA synthesis in the 5′-UTR of PNRSV RNA 3.

Fig. 3 shows the accumulation of viral RNAs in P12 plants. Seven days after inoculation of plants with the chimeric RNAs, RNA was extracted from inoculated leaves and analysed by Northern blot hybridization using a mixed probe, detecting both AMV and PNRSV sequences. Subliminal infections confined to single cells are not detectable in this assay (van der Vossen *et al.*, 1994). In addition to the control with AMV RNA 3 (Fig. 3, lane 1), only the chimera consisting of PNRSV RNA 3 with the 5′-UTR replaced by the 5′-UTR of AMV was able to accumulate in plants (Fig. 3, lane 4). This chimera (PNRSV-5A) encodes the MP and CP of PNRSV, which were previously shown to mediate cell-to-cell transport in tobacco (Sánchez-

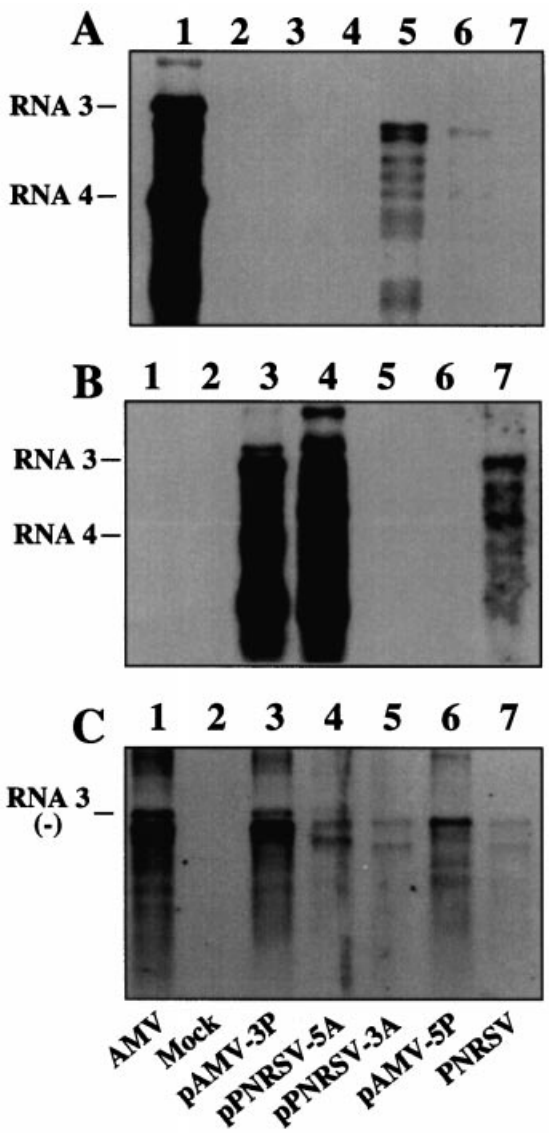

Fig. 2. Northern blot analysis of the accumulation of RNAs 3 and 4 in P12 protoplasts inoculated with chimeric AMV–PNRSV transcripts. Glyoxylated RNAs extracted from inoculated P12 protoplasts were loaded in triplicate on 1% agarose gels and hybridized with different riboprobes. (A) Minus-strand probe complementary to the 3′-UTR of AMV RNA 3 to detect plus-strand RNAs containing this 3′-UTR. (B) Minus-strand probe complementary to the 3′-UTR of PNRSV RNA 3 to detect plus-strand RNAs containing this 3′-UTR. (C) A mixture of plus-strand probes corresponding to the MP genes of AMV and PNRSV to detect minus-strand RNA synthesis directed by all inoculum RNAs. Protoplasts were inoculated with RNA 3 transcripts from plasmids pAL3NcoP3 (lane 1), pAMV-3P (lane 3), pPNRSV-5A (lane 4), pPNRSV-3A (lane 5), pAMV-5P (lane 6), pUC3m1 (lane 7). Lane 2, mock inoculation. The positions of plus-strand AMV RNAs 3 and 4 (A, B) and minus-strand AMV RNA 3 (C) are indicated in the left margin.

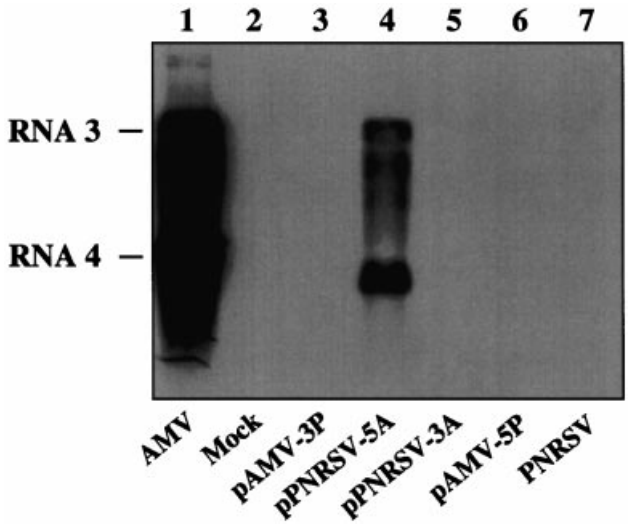

Fig. 3. Northern blot analysis of the accumulation of RNAs 3 and 4 in P12 plants inoculated with chimeric AMV–PNRSV transcripts. Total RNAs extracted from P12 plants were glyoxylated, loaded on 1% agarose gels and hybridized with a mixture of riboprobes complementary to the 3′-UTRs of AMV and PNRSV. Each lane was loaded with 0·2 μg RNA extracted from 0·1 mg of leaf material. P12 plants were inoculated with RNA 3 transcripts from plasmids pAL3NcoP3 (lane 1), pAMV-3P (lane 3), pPNRSV-5A (lane 4), pPNRSV-3A (lane 5), pAMV-5P (lane 6) and pUC3m1 (lane 7). Lane 2, mock inoculation. The positions of AMV RNAs 3 and 4 are indicated in the left margin.

Navarro *et al.*, 1997). It remains to be determined why these two proteins do not permit cell-to-cell movement of PNRSV in tobacco. One possibility is that PNRSV triggers a host response that prevents the virus from infecting tobacco. Surprisingly, the chimera consisting of AMV RNA 3 with the 3′-UTR replaced by the 3′-UTR of PNRSV (pAMV-3P) accumulated in protoplasts but not in plants (Fig. 3, lane 3). Previously, we showed that various AMV–PNRSV chimeras were encapsidated by CPs of either virus but these chimeras all contained the 3′-UTR of AMV (Sánchez-Navarro *et al.*, 1997). We have not yet analysed the possibility that a defect in encapsidation of pAMV-3P affects cell-to-cell movement. However, AMV CP mutants have been described that do not form stable virions but do move from cell to cell (Tenllado & Bol, 2000; J. A. Sánchez-Navarro & J. F. Bol, unpublished). Alternatively, an interaction of the AMV CP with the 3′-UTR of PNRSV could be incompatible with a putative role in cell-to-cell transport or the chimera could activate a host response that blocks movement.

In summary, we conclude that AMV RdRp recognizes the minus-strand promoter in PNRSV RNA 3 but not the plus-strand promoter. Apparently, the single nucleotide difference at position 3 from the 3′-end that results in the reduced recognition of the PNRSV minus-strand promoter *in vitro* has little effect on replication *in vivo*. Currently, we cannot exclude the possibility that the transcripts corresponding to PNRSV RNA 3 or chimeras with the 5′-UTR of this virus are not replicated by the AMV RdRp due to a mutation in the 5′-UTR that renders clone pUC3m1 non-infectious. However, the 5′-UTR sequence in this clone is highly conserved in various PNRSV isolates (Aparicio *et al.*, 1999). Rather, our data support the notion that between virus species from one genus or species from different genera of one family of plant viruses, minus-strand promoter sequences are more conserved than plus-strand promoters. In the genus *Tobravirus* the RdRp encoded by RNA 1 of *Tobacco rattle virus* (TRV) replicates RNA 2 of *Pea early browning virus* only when a 5′ non-coding sequence of this RNA is replaced by the corresponding non-coding sequence of TRV RNA 2 (Mueller *et al.*, 1997). In the family *Bromoviridae*, replacement of the 3′-UTR of RNA 3 of *Brome mosaic virus* (BMV) by the corresponding 3′-UTR of *Cucumber mosaic virus* (CMV) did not affect replication of the chimeric RNA by the BMV RdRp (Rao & Grantham, 1994). The 3′-UTR of CMV is also recognized by the RdRp of *Tomato aspermy virus* (Teycheney *et al.*, 2000). In the family *Potyviridae*, the 3′-UTR of *Lettuce mosaic virus* is recognized by the RdRp of several potyviruses (Teycheney *et al.*, 2000). *Cis*-acting elements in the 5′-UTR of AMV RNA 3 have been partially characterized (van der Vossen & Bol, 1996). A comparison of the 5′-UTRs of AMV and PNRSV may provide further insight in *cis*-acting elements that are essential for plus-strand promoter activity.

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