Change in phenotype and encapsidated RNA segments of an isolate of alfalfa mosaic virus: an influence of host passage

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A local lesion isolate of alfalfa mosaic virus (AMV-N20) from lucerne was found to encapsidate two extra RNAs in addition to the four major RNAs (RNA1, -2, -3 and -4). These were resolved by gel electrophoresis both under native conditions and after glyoxal denaturation. The RNA with an electrophoretic mobility between that of RNAs 2 and 3 was designated RNA31, that between RNAs 3 and 4 was designated RNA3s. Sucrose density gradient centrifugation analysis of AMV-N20 showed six instead of the normal four nucleoprotein components, the additional two presumably representing encapsidated RNAs 31 and 3s. RNAs 31 and 3s were both shown by Northern blot hybridization to be unrelated to host plant RNA and to contain the AMV coat protein gene sequence, which resides in RNA3. Primer extension of the RNAs 31 and 3s using a primer complementary to the 3' common terminus of all genomic AMV RNAs provided further evidence that they contained AMV sequences. RNA31 represents an addition of about 255 nucleotides, compared with RNA3, and RNA3s represents a loss of about 308 nucleotides. The coat proteins of variants encapsidating either RNA31, -3 or -3s had the same M, indicating that the addition or deletion of the nucleotides was outside the coat protein gene. Serial mechanical passage of AMV-N20 over 5 years in four host species led both to changes in the composition of the RNA3 mixture, and to changes in symptom severity. For example, following passage in Nicotiana clevelandii, RNA3 was lost whereas passage in either N. glutinosa, Chenopodium quinoa or C. amaranticolor resulted in the loss of RNA31. No association was found between the changes in RNA3 and phenotypic changes that resulted from continuous passage for 5 years. Phenotypic changes with passage are thus presumably determined by mutations elsewhere in the virus genome.

Introduction

Alfalfa mosaic virus (AMV) is one of the most biologically variable plant viruses and numerous natural variants with different pathogenicity have been reported (Crill et al., 1971; Paliwal, 1982; Walter et al., 1985; Hiruki & Miczynski, 1987; Hajimorad & Francki, 1988). Although AMV encapsidates four major ssRNAs (RNA1, -2, -3 and -4) of positive sense, some isolates have been reported which encapsidate additional RNA species. For example, additional RNAs with a mobility between that of RNA3 and -4 (X-RNAs) or faster than RNA4 (Z-RNAs) have been reported in the AMV-425 strain (Bol & Lak-Kaashoek, 1974). Also an additional RNA with an electrophoretic mobility faster than RNA3 has been detected in extracts of virions of AMV strain S (Walter et al., 1985; Dore & Pinck, 1988). AMVRNA is encapsidated with a single species of coat protein of M, about 24525 (Van Beynum et al., 1977) to form bacilliform particles of the same width but differing in length depending on the RNA encapsidated (Hull et al., 1969). Although bacilliform particles longer than the B component, which encapsidates RNA1, have been found in AMV strains VRU and 15/64, the encapsidated RNA was shown to be no longer than RNA1 (Hull, 1970; Heijtink & Jaspars, 1974).

Repeated passage of some viruses in single host species can lead to the appearance of new variants (Yarwood, 1979) or the de novo generation of defective interfering RNA (Knorr et al., 1991; Burgyan et al., 1991). However continuous culture of two isolates of AMV over a period of 4 years in tobacco did not induce any change in their symptom type (Hull, 1968). In this paper we report changes in both the phenotype and pattern of encapsidated RNA segments of a lucerne isolate of AMV following its continuous passage in each of four different host species.
Methods

Virus isolate, maintenance and purification. Isolate AMV-N20 (Hajimorad & Francki, 1988) was mechanically transferred from lucerne to *Nicotiana clevelandii* Gray. A biologically uniform local lesion isolate was selected by five successive transfers of single local lesions at limiting dilution through *Phaseolus vulgaris* cv. Hawkesbury Wonder. The resulting isolate was propagated in *N. clevelandii*. It was then transferred to two *N. clevelandii*, one *N. glutinosa* and one *Chenopodium amaranticolor*. After 15 passages in *C. amaranticolor*, the virus was also subcultured in *C. quinoa*. Unless otherwise indicated each line was mechanically passaged in the same host plant species over a period of 5 years and maintained in an insect-proof greenhouse. The scheme used for isolation and passage is summarized in Fig. 1. The RNA content of virus was monitored immediately after extraction at specific times by purifying virus from 5 to 10 g samples of systemically infected leaf 10 to 12 days post-inoculation. The procedure used was as described previously (Hajimorad & Francki, 1988) except that high speed centrifugation was done in a Beckman TL100 centrifuge for 30 min at 95000 r.p.m. Purified virus in 10 mM-phosphate buffer pH 7.0 was mixed with an equal volume of glycerol and kept at -20 °C. Virus preparations were stained for electron microscopy with 1% uranyl acetate (Hatta & Francki, 1984). Analysis of nucleoprotein component ratios was done by sucrose density gradient centrifugation as described by Hajimorad & Francki (1991a).

Isolation of RNAs from virus and host plant. RNA was isolated from purified virus by three phenol extractions in the presence of 1% SDS (Peden & Symons, 1973). After concentration by ethanol precipitation, the RNA was resuspended in sterile TE buffer (10 mM-Tris–HCl, 1 mM-EDTA, pH 8-3) and kept at -20 °C until required. Total nucleic acids from an uninoculated *N. clevelandii* were prepared by two phenol–chloroform extractions as described by Carrington & Morris (1984).

Analysis of RNA under native and denaturing gel conditions. The analysis of RNA under native gel conditions was done in submerged 1.5% agarose gels buffered in TAE (40 mM-Tris-HCl pH 8.0, 20 mM-sodium acetate, 2 mM-sodium EDTA) containing 0.5 μg/ml ethidium bromide (Francki et al., 1986). Samples of 2 μg of each RNA in 8 μl sterilized TE buffer were mixed with an equal volume of glycerol containing 0.2% bromophenol blue, incubated for 5 min at 70 °C, ice-cooled and then subjected to electrophoresis. Bands were detected under u.v. illumination. Glyoxylation of RNA and analysis under denaturing conditions were done according to McMaster & Carmichael (1977), except that TAE electrophoresis buffer was used. Silver staining of gels was done according to Gottlieb & Chavko (1987).

**Primer extension.** A primer complementary to 15 nucleotides at the common 3′ terminus of all AMV RNAs (GCATCCCTTAGGGC) (Langereis et al., 1986) was synthesized. This was used in primer extension reactions which were carried out in 1.5 ml microcentrifuge tubes using an Amersham cDNA synthesis kit [4 μl of 5 x first-strand synthesis reaction buffer, 1 μl of sodium pyrophosphate solution, 1 μl (20 units) of human placental ribonuclease inhibitor, 2 μl of the deoxynucleotide triphosphate mixture] with 2 μl (2 μg) of the above primer, 1 μl (10 μCi) of [α-32p]dCTP (3000 Ci/mmol, Amersham), 2 μl (2 μg) AMV RNA and 7 μl of sterile double-distilled water (SDDW).

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Fig. 1. Programme of passage of AMV-N20, over 5 years, with its effect on composition of RNAs 31, 3 and 3s. All RNA3 variants are present except where indicated on the right of the vertical lines with a subtraction symbol. Passage numbers are shown on the left.
After addition of 2 μl (40 units) of the reverse transcriptase the reaction was incubated for 60 min at 42 °C. Aliquots (2 μl) were taken, mixed with 8 μl alkaline gel loading buffer and cDNA was analysed in a 1% alkaline agarose gel (Sambrook et al., 1989) or in a native gel system. Electrophoresis, processing of the gel, autoradiography and end-labelling of HindIII fragments of λ DNA M, markers with T4 DNA polymerase were done as described by Sambrook et al. (1989).

Synthesis of 32P-labelled cDNA by reverse transcription or nick translation. RNA was reverse-transcribed to cDNA using random hexanucleotide primers (Pharmacia) and Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories) (Palukaitis & Symons, 1980). For nick translation a plasmid with an insert of the full-length coat protein gene of AMV strain 425 (Van Dun et al., 1987) provided by J. Bol, Leiden University, The Netherlands, was transferred into Escherichia coli HB101 cells and purified by CsCl-ethidium bromide gradient centrifugation (Sambrook & Symons, 1980), was removed by digestion with Apal. The reaction mixture contained 40 μl of insert, 5 μl SDDW, 5 μl 10 × Apal buffer and 5 μl (50 units) of Apal restriction endonuclease and was incubated at 30 °C for 2 h. Apal cleaved the AMV coat protein gene at 601 nucleotides from the AUG start codon. The fragments were gel-purified and nick-translated using a nick translation kit (Bresatec) as recommended in a 25 μl reaction mixture containing 2 μl (2 μg) DNA fragment, 5 μl (50 μCi) [α-32P]dCTP (3000 Ci/mmol, Amersham), 4 μl nucleotide/buffer mix, 2 μl dCTP, 7 μl SDDW and 5 μl (5 units) of E. coli DNA polymerase. Incubation was at 14 °C for 90 min, and cDNA was purified by gel filtration on Sephadex G50.

Northern blot hybridization. RNAs separated by electrophoresis under native conditions were blotted overnight between two sheets of nitrocellulose (Schleicher & Schuell) as described by Palukaitis (1984). The nitrocellulose sheets were baked at 80 °C for 2 h under vacuum and prehybridized in hybridization buffer [3 × SSC (450 mM-sodium chloride, 4.5 mM-sodium citrate) with 0.005% w/v each of BSA, Ficoll 400 and polyvinylpyrrolidone (M, 40000), 1 mM-disodium EDTA and 250 μg/ml phenol-extracted yeast RNA] for 20 to 24 h at 65 °C. Hybridization was done under the same conditions for 20 to 24 h after addition of an appropriate volume of [32P]cDNA. Filters were washed and autoradiographed (Palukaitis et al., 1985).

Immunoblotting. Proteins were separated by SDS discontinuous buffer PAGE (Mini-PROTEAN II dual slab gel apparatus, Bio-Rad) according to Laemmli (1970). Proteins were stained with silver nitrate (Wray et al., 1981). Immunoblotting was as described by Dietzgen & Francki (1988), except that ethanol was substituted for methanol during the electrophoretic transfer. Polyclonal antibodies raised against native particles of AMV-N20 (Hajimorad & Francki, 1991b) or monoclonal antibody (Mab) 1 raised against AMV (Hajimorad et al., 1990) were used to detect AMV coat protein on blots. Goat anti-rabbit IgG or goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) and diluted to 1:2000 were used for detection of bound antibodies. Phosphatase activity was detected with the nitroblue tetrazolium-phenazine methosulphate-5-bromo-4-chloro-3-indoly phosphate substrate (Ey & Ashman, 1986).

Results

Properties of AMV-N20

The primary AMV-N20 isolate (Hajimorad & Francki, 1988, 1991b) caused a severe bright systemic mosaic and severe distortion of N. glutinosa (Fig. 2a) and severe stunting and mottling with accelerated death of inoculated leaves in N. clevelandii (Fig. 2e). In C. quinoa it caused chlorotic local lesions and systemic distortion, whereas in C. amaranthicolor it caused necrotic local lesions and systemic chlorotic mottling (not shown). Sucrose density gradient centrifugation analysis of virus preparations identified six nucleoprotein components and electron microscopy showed bacilliform particles with a range of lengths up to 100 nm (not shown).

Effect of host passage on phenotype

Each of two lines of AMV-N20 which had been maintained separately for 5 years in N. clevelandii expressed different phenotypes. Fig. 2 (a and e) shows the primary N20 phenotype. Fig. 2 (g) shows the mild variant which resulted from one set of passages (designated NcM), whereas Fig. 2 (h) shows the severe rugose phenotype (designated NcS) which resembles the primary N20 phenotype. NcM and NcS induced the same symptoms irrespective of the inoculum used, that is whether it was sap, purified virus or extracted viral RNA (not shown) indicating that the changed symptom severity was associated with AMV, and not possibly contaminating virus or viral RNA. The phenotypes of both variants remained stable at different seasons of the year (data not shown). A line of N20 maintained as above in N. glutinosa (variant Ng) did not show symptom attenuation (Fig. 2b) and remained as virulent as the primary isolate in this host. However its pattern of symptoms varied slightly from that of the primary N20 (Fig. 2a) compared with Fig. 2 (b). This phenotype also remained stable irrespective of the time of year and the inoculum used (data not shown).

When after 30 passages in N. clevelandii variant NcS was subcultured in N. glutinosa, a delay in symptom expression occurred. Moreover, some of the inoculated plants escaped infection whereas those which were infected showed a phenotype different from that of Ng (Fig. 2d) or of the primary N20 (Fig. 2a) in N. glutinosa. This phenotype then remained stable for a further 23 successive passages in N. glutinosa (data not shown) and the use of sap, purified virus or isolated viral RNA as inocula did not change the type of symptoms expressed (data not shown). When Ng was subcultured in N. clevelandii it caused severe mosaic and stunting (Fig. 2f).

This phenotype again remained stable for 17 successive passages in this host. Continuous culturing of N20 in C. quinoa resulted in a significant reduction in the number of necrotic local lesions on inoculated leaves and an attenuation of systemic symptoms (data not shown). This variant is referred to as Cq. However, after 55 passages in C. amaranthicolor, no change in symptom type occurred (data not shown). This variant is referred to as Ca.
Effect of host passage on encapsidated RNA species

RNA isolated from purified primary N20 separated into six species (Fig. 3, lane 2) including the four major RNAs (RNAs 1 to 4) and two additional species designated RNA3l (intermediate between RNA2 and -3) and RNA3s (between RNA3 and -4). The RNA of NcS (Fig. 3, lane 5) and NcM (not shown) both differed from primary N20 (Fig. 3, lane 2) in that they lacked RNA3. In contrast, Ng lost RNA3l (Fig. 3, lane 3). Ng then retained the same pattern after 17 further passages in N. clevelandii, but the relative amount of RNA3 increased (Fig. 3, lane 4).

When NcS (Fig. 3, lane 5) was passaged nine times in N. glutinosa, RNA3l was lost as for Ng, whereas RNA3 was regained (Fig. 3, lane 6). N20 maintained in C. quinoa and C. amaranticolor also lost RNA3l (Fig. 3, lane 7) as shown for Ng. Fig. 1 summarizes these changes in RNA content.

Fig. 2. Phenotypes of the primary AMV-N20 and its variants arising from continuous passage in N. glutinosa (a to d) and N. clevelandii (e to h). (a, e) Primary AMV-N20; (b, f) Ng; (c, g) NcM and (d, h) NcS.

Fig. 3. Analysis of the host RNAs, the RNA species encapsidated in the primary AMV-N20 and its variants NcS, NcM, Ng and Cq. Lane 1, total RNA from uninfected N. clevelandii; lane 2, RNA from purified virus of the primary AMV-N20 propagated in N. clevelandii; lane 3, Ng RNA; lane 4, Ng RNA, after 17 further passages in N. clevelandii; lane 5, NcS; lane 6, NcS after 15 further passages in N. glutinosa; lane 7, Cq RNA.
Effect of host passage on an isolate of AMV

Fig. 4. Analysis of undenatured (a) and glyoxal-denatured (b) RNA of the primary AMV-N20 and its variants. Lane 1, RNA from uninfected N. clevelandii; lane 2, RNA from purified virus of the primary AMV-N20 isolate propagated in N. clevelandii; lane 3, NcS RNA, after 15 passages in N. glutinosa; lane 4, NcS. The gel was silver-stained according to Gottlieb & Chavko (1987).

After glyoxal denaturation the RNAs showed the same relative electrophoretic mobilities (Fig. 4). The sizes of RNAs 3l, 3 and 3s were estimated to be 2205, 1950 and 1642 nucleotides respectively.

A comparison of coat protein Mr values from variants

The coat protein gene of AMV is situated in the 3'-terminal 881 nucleotides of RNA3 (Brederode et al., 1980). To determine whether RNAs 3l and 3s arose from addition or deletion of nucleotides in this gene, the coat protein Mr values of primary N20, NcS and Ng were compared. As shown in Fig. 5 (a), all were the same size, and reacted uniformly with specific AMV polyclonal serum (Fig. 5 b) and the MAb (Fig. 5 c), indicating that insertions or deletions occurred outside this gene.

Identification of coat protein sequences in RNA3l, -3 and -3s

Reverse transcription of RNA from NcS and Ng, using a primer complementary to the 3' common region of AMV RNA, yielded cDNA species which corresponded to the sizes of the RNA species of each variant (Fig. 6).
Northern blot analysis was done using cDNA probes complementary to either N. clevelandii total RNA, Ng RNA or the AMV coat protein gene. As shown in Fig. 7 (b), none of the RNAs purified from the primary N20, Ng or NcS showed homology with host nucleic acids. When RNA from Ng was used as the template for cDNA synthesis, all six RNAs in primary N20 were detected, with an additional minor band between RNA3s and -4 which is likely to be a degradation product (Fig. 7c). Ng was shown to lack RNA31, whereas NcS had RNA31. The coat protein gene probe binds to RNA31, -3, -3s and -4. In the experiment shown in Fig. 7 (d), N20 contained detectable levels of RNA31 and -3, Ng contained RNA3, and NcS contained RNA31 and -3s but not -3. N20 was shown to contain RNA3s in a subsequent experiment (not shown). These hybridization data, along with the results of primer extension using a 3' AMV-specific primer, confirm that both RNA31 and RNA3s are derived from AMV sequences.

Discussion

The two additional RNAs (RNA31 and -3s) identified in this study were both present in the primary AMV-N20 after its first transfer from lucerne to N. clevelandii. The loss of RNA31 during passage in N. glutinosa and Chenopodium spp. and the loss of RNA3 during passage in N. clevelandii indicate that the host species can select for different RNA segments. The host factor in the viral RNA polymerase (Quadt & Jaspars, 1989) can play a role in this selection process. The presence of RNA31 molecules in preparations from NcS and NcM but not the Ng, Cq and Ca variants was shown not to be an artefact of purification because of the reversibility of the shift from one RNA composition to another during passage. Thus, transferring NcS after 30 passages in N. clevelandii to N. glutinosa led to the regaining of RNA3 and the loss of RNA31 between passages 5 and 9 (Fig. 1). The reappearance of RNA3 could be due either to its de novo formation from RNA31 or to amounts of RNA3 in NcS preparation which co-replicated with RNA31 but which were below the level of detection on gels. Conversely, however, subcloning of Ng in N. clevelandii did not restore RNA31 after 17 passages (Fig. 1) and this may be taken as evidence that RNA31 was not formed de novo from RNA3 and that no trace of RNA31 had been left in Ng preparations prior to their transfer to N. clevelandii. However, we can not rule out the possibility that RNA31 could have been restored if more passages had been carried out in this host. Nevertheless, the failure to restore RNA31 shows that it was not derived from either RNA1 or RNA2 and this is confirmed by its sequence homology with the AMV coat protein gene.

Evidence that the two additional RNA species are not contaminants from other viruses comes from the following observations. (i) Protein derived from the purified particles encapsidating these RNAs was shown to be AMV coat protein by its electrophoretic mobility and its antigenic reactivity with both anti-AMV polyclonal serum and the MAb. (ii) AMV-specific sequences were generated by primer extension of encapsidated
RNA species with an AMV-specific oligodeoxynucleotide primer. (iii) cDNA to total RNA of Ng devoid of RNA31 hybridized to this RNA. (iv) No hybridization was observed between the additional RNA species and cDNA made to total RNA from uninoculated *N. clevelandii*.

The observation that both a mild (NcM) and severe (NcS) variant could be derived after passage in *N. clevelandii* and that they had the same encapsidated RNA patterns suggests that passaging of the virus in this host has induced and selected some mutations in genomic sites associated with virus pathogenicity. Attenuation of viruses by continuous culturing has been reported for other viruses (Yarwood, 1979) and this practice has been recommended as a means of generating mutants suitable for cross-protection (Gonsalves & Garney, 1989). One implication of our study is that if such a practice is to be pursued, then it will be important to passage the desired virus in different host species. It seems that the mutational events leading to attenuation may not occur at an equal rate in all host plant species. The molecular basis for attenuation of viruses is poorly understood. However, for tobacco mosaic virus (TMV), mutations in the putative RNA polymerase gene (Watanabe et al., 1987) and for the Sabin type 3 poliovirus mutation in the 5' non-coding region as well as in viral structural protein VP3 (Almond et al., 1987) have been shown to be responsible for attenuation.

Despite the apparent loss of RNA31 from variant Ng, its phenotype remained as severe as that of primary N20-AMV isolate. Therefore RNA31 has no apparent role in the severity of AMV-N20 symptoms in *Nicotiana*. Nevertheless, differences in the symptom type of this variant were observed. It is more likely that a mutation in another part of the genome is responsible for such phenotypic variation. The observation that subculturing of NcS in *N. glutinosa* after nine passages restored the RNA pattern, but not its phenotype, to that typical of the Ng variant supports this conclusion. Further passage did not result in any change in phenotype or encapsidated RNA pattern of this variant.

The variation observed may originate from molecular impurities. Although a phenotypically pure line of AMV-N20 was selected by five successive local lesion transfers, it is possible that it remained as a mixture of two or more variants. The failure of this procedure to produce homogeneous virus populations of TMV after five local lesion transfers has been reported previously (Garcia-Arenal et al., 1984). On the other hand, the AMV-N20 strain may have contained a mixture of RNA3 species in a balanced proportion which competed with each other with different efficiency depending on their host species. For example, it has been shown that the 5' leader sequences of RNA3 of AMV strains vary from 240 nucleotides for strain B (Ravelonandro et al., 1983) to 314 for strain S, 345 for strain 425 L (Langereis et al., 1986) and 391 for strain YSMV (Neeleman et al., 1991). Our study also shows that the variation in size of the RNA3 species extends over approximately 550 nucleotides. If this variation is to be attributed to variation in leader size it means that the leader size varies over a much longer range than was known before. The leader sequence of RNA3 could vary in length because of the accumulation of extensive direct repeats (Langereis et al., 1986), the replacement of 5'-terminal RNA3 sequences for (longer) RNA1-derived sequences (Ravelonandro et al., 1983; Huisman et al., 1989) or the presence or absence of internal poly(A)-rich sequences (Langereis et al., 1986; Neeleman et al., 1991). Although no clear insight into the functional aspects of this structural variation exists, differential competition between different RNA3s of AMV has been related to the structure of their 5' leader sequences (Dore et al., 1989).

RNA3s was consistently detected from virion extracts of all the AMV variants studied here irrespective of the host species from which the virions were purified. By Northern blot analysis, RNA3s was shown to contain a coat protein gene-specific sequence. Hence this RNA segment may have features similar to that of the RNA3' detected in an extract of virions of strain S (Walter et al., 1985) and which originate from in vitro cleavage of RNA3 at position 154 from the 5' terminus (Ravelonandro et al., 1983; Dore & Pinck, 1988). It is possible that a non-specific host contaminant of the virion is responsible for a specific cleavage event in the leader sequence of RNA3 during or after the isolation of the virions. Alternatively, RNA3 could be a newly recognized defective RNA which is encapsidated in vivo. Such an origin has been suggested for X-RNAs as well as for Z-RNAs (Bol & Lak-Kaashoek, 1974). However, both of these possibilities remain to be investigated.

The significance of the RNA31 and RNA3s for AMV-N20 is not known. The observation that RNA31 was lost in *C. quinoa*, *C. amaranticolor* as well as *N. glutinosa* without affecting the ability of the virus to establish the infection, suggests that it is not essential for virus replication. On the other hand, compensation for the loss of RNA3 in both NcM and NcS was achieved by RNA31 and/or RNA3s. This suggests that they can provide the essential functions of RNA3. It was noted that no variant lost both RNA3 and RNA31, which indicates that RNA3s alone is not sufficient to supply the essential functions of RNA3. Hence, both RNA3 and RNA31 have functional 32K protein and coat protein genes which further supports the hypothesis that their length difference is due to variation in their leader sequences. Further work is necessary to reveal the properties of RNA31 and RNA3s. Presumably a knowledge of their
nucleotide sequences would be most informative for revealing their origin. The availability of their full-length infectious clones is also necessary to investigate their possible de novo generation. Work is currently in progress to clone and characterize these RNA species in detail and obtain the required constructs.

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