Identification of artichoke mottled crinkle virus (AMCV) proteins required for virus replication: complementation of AMCV p33 and p92 replication-defective mutants

Paola Molinari, Carla Marusic, Alessandra Lucioli, Raffaela Tavazza and Mario Tavazza

Biotechnology and Agriculture Division, Dip. INN., ENEA C. R. Casaccia, S. Maria di Galeria, via Anguillarese 301 (Roma) CP 2400, Italy

Introduction

Artichoke mottled crinkle virus (AMCV) is a member of the genus Tombusvirus (Russo et al., 1994). The genome has been cloned and sequenced, and infectious transcripts have been obtained (Tavazza et al., 1994). The genome is a single-stranded monopartite RNA molecule of positive polarity, 4790 nucleotides (nt) in length containing five open reading frames (ORFs), the expression of which involves both translational readthrough and production of subgenomic (sg) RNAs (Tavazza et al., 1994). ORF 1 encodes a 33 kDa protein (p33) while ORF 2 encodes a 92 kDa protein (p92), which is expressed by readthrough of the amber stop codon of ORF 1. The readthrough portion of p92 contains the eight conserved motifs characteristic of viral RNA-dependent RNA polymerases (RdRp) belonging to supergroup II as defined by Koonin (1991). It has been suggested that one of these motifs, the amino acid motif Gly-Asp-Asp (GDD), is involved in the catalytic activity and/or in metal ion coordination of the enzyme (Argos, 1988). ORF 3 encodes the 41 kDa coat protein (CP; Tavazza et al., 1989) and is expressed by sg 1 RNA. The two nested ORFs 4 and 5 code for 21 kDa (p21) and 19 kDa (p19) proteins, respectively, and they are both expressed by sg 2 RNA. A sixth ORF close to the 3′ end of the tombusvirus genome has also been identified (Boyko & Karasev, 1992). In AMCV, ORF 6 extends from base 4506 to base 4712 and has the potential to encode a 7 kDa protein (p7) (Tavazza et al., 1994). Recently, the presence of an additional short RNA, which could be the sg RNA involved in ORF 6 expression, has been reported for cucumber necrosis tombusvirus (CNV) (Johnston & Rochon, 1995).

Previous studies have indicated the involvement of sequences within ORFs 1, 2 and 6 in tombusvirus genome replication. In particular, it has been shown that mutations of ORFs 1 or 2 of cymbidium ringspot (CymRSV) and tomato
busky stunt virus (TBSV) abolish genome replication (Dalmay et al., 1993; K. B. Scholthof et al., 1995) and that sequences within ORF 1 are involved in the origin of multivesicular bodies which are thought to be the site of tombusvirus replication (Burgyan et al., 1996). Kollar & Burgyan (1994) used a different approach to show that transgenic N. benthamiana protoplasts expressing CymRSV ORFs 1 and 2 support the replication of a CymRSV defective interfering (DI) RNA, thus indicating that, at least for DI RNA amplification, only ORF 1 and/or ORF 2 products are required. However, since ORF 2 is expressed by readthrough of the ORF 1 stop codon, every mutation in ORF 1 also implies a mutation in ORF 2, preventing definitive conclusions on the requirement of p33 in replication. With regard to the involvement of ORF 6 in tombusvirus replication, the only data available are those on CymRSV (Dalmay et al., 1993). Although some mutations in ORF 6 affected genome replication, the putative gene product does not seem to be required for this process.

In this paper, the viral proteins essential for AMCV genome replication were identified by combining replication analysis of the AMCV mutants at the single-cell level with the molecular characterization of revertants generated in planta and complementation tests between replication-defective mutants.

**Methods**

**Plasmids.** The AMCV mutants used in this work were derived from the infectious AMCV cDNA clone pA, which differs from pAM-G (Tavazza et al., 1994) by a few single nucleotide substitutions. The reason for the higher infectivity of pA compared to pAM-G remains to be investigated. All the mutants with the exception of pA41(–) were obtained by PCR mutagenesis using Pfu DNA polymerase (Stratagene); the mutated nucleotides of primers are indicated below in bold. A schematic representation of the AMCV mutants is shown in Fig. 1. The ORF 1 mutant pA33(–) was generated as follows: the PCR fragment obtained using the oligonucleotides a (GGATTGGTTGTTGGTGATT, nt 717–735) and R-Mut33 (CAGTGTAGCATCTAGGGCTCATATTTCAT, nt 1093–1064) was cloned into HindIII-digested pUC9 and the SalI–Accl fragment (nt 745–1085) from this subclone was introduced into pA. The same procedure was used to obtain the ORF 2 mutant pA92(–) except that oligonucleotide R-Mut92 (AGGTAGACTGGCTCA, nt 2185–2164) was cloned into pBlueScript®MI site. Finally, the missing 5’ fragment was reinserted to obtain pA21(–). To obtain pA19(–), the same procedure was followed except that oligonucleotides Mut19 (AACAACAAAGGACCGGTGATTATTTGTCGTT, nt 3889–3919) and R-Mut19 (GTATAACTGCTCCAGCTTTGATTTAGCTTG, nt 3919–3889) were used instead of Mut19 and R-Mut21. Mutagenesis of the first two ORF 6 codons (AUGAUG) was achieved using the degenerate oligonucleotide Mut7 (GGTTCTAGATGTTTAAC/GG) together with R-f (ggg-cgccgacgtcatctgctgaatg, nt 4790–4772) which contains seven additional pA-derived nucleotides (lower case) and a KspI site (underlined). The PCR products were digested with KspI and XhoI (nt 4496) and used to replace the corresponding fragment of pA, an AMCV subclone lacking the 5’ region up to the EcoRI site (nt 2185) and thus containing a unique KspI site. Finally, the missing 5’ fragment was reinserted to obtain pA21(–). To obtain pA19(–), the same procedure was followed except that oligonucleotides Mut19 (AACAACAAAGGACCGGTGATTATTTGTCGTT, nt 3889–3919) and R-Mut19 (GTATAACTGCTCCAGCTTTGATTTAGCTTG, nt 3919–3889) were used instead of Mut19 and R-Mut21. Mutagenesis of the first two ORF 6 codons (AUGAUG) was achieved using the degenerate oligonucleotide Mut7 (GGTTCTAGATGTTTAAC/GG) together with R-f (ggg-cgccgacgtcatctgctgaatg, nt 4790–4772) which contains seven additional pA-derived nucleotides (lower case) and a KspI site (underlined). The PCR products were digested with KspI and XhoI (nt 4496) and used to replace the corresponding fragment of pA. The mutant clones pA7(–)1 and pA7(–)2 were chosen on the basis of sequence analysis.

**In vitro transcription and plant inoculation.** RNA transcripts were synthesized from KspI-linearized templates using phage T7 RNA polymerase (BioLabs) according to the supplier’s instructions. Transcripts are referred to by the name of the plasmid from which they derive, preceded by the letter ‘t’ instead of ‘p’. Reaction transcription mixtures were diluted to the desired RNA concentration; 1 vol. of inoculation buffer (Heaton et al., 1989) was added and 20 µl of the resulting mixture was rubbed onto one N. benthamiana leaf. Two leaves of each plant were inoculated with 1–2 µg of transcripts for tA33(–), tA92(–) and tA92GED and with 200 ng of all the other transcripts. Inoculation with 50/100 ng of wt AMCV tA RNA transcripts resulted in 100% infected plants. After inoculation, plants were maintained under greenhouse conditions (23 °C, 14 h light/10 h dark) and monitored daily for symptom development.

**Protoplast isolation and infection.** N. benthamiana protoplasts were isolated from 4-week-old greenhouse plants. Small leaf strips were plasmolysed in K3 medium containing 0.4 M sucrose (Nagy & Maliga, 1976) for 1 h in the dark at 26 °C and then digested in the same medium supplemented with 1/10 vol. of concentrated enzyme solution (Crepy et al., 1986). Protoplasts were purified by flotation on K3 medium and then pelleted in washing medium (Ordas et al., 1991). The protoplasts (5 × 10^6) were inoculated (Tavazza et al., 1994) with 1 µg of in vitro synthesized RNA that had been previously treated with RQ1 RNase-free DNase (Promega) for 20 min at 37 °C (followed by phenol–chloroform extraction and ethanol precipitation). The inoculated protoplasts were suspended at 5 × 10^7/ml in K3 medium.

**RNA extraction and Northern blot analysis.** RNA from leaf tissue was isolated as described by Iardi et al. (1995). The same procedure was used for RNA protoplast extraction, with minor modification of the first extraction step: 800 µl of pre-warmed (65 °C) EB buffer–acid phenol (1:1, v/v) was added to 100 µl of frozen protoplasts and mixed by vortexing before adding chloroform. RNAs were electrophoresed through 1% agarose gel containing formaldehyde and blotted to
positively charged nylon membranes (Boehringer). Probes were digoxigenin-labelled RNA transcripts corresponding or complementary to the 3′-terminal 1000 nt of the AMCV genome. Hybridization, washes and chemiluminescent detection using CSPD (Tropix) were all performed according to the supplier (Boehringer), with the addition of an RNase treatment (10 μg/ml, 15 min at 37 °C in 2 × SSPE) of hybridized filters prior to washes.

**Isolation and analysis of revertants.** RNAs extracted from tA92(-)-, tA7(-)-1- and tA7(-)-2-infected plants were reverse-transcribed using MuLV reverse transcriptase (Pharmacia). PCR-amplified with β′′ DNA polymerase (Stratagene), and a fragment containing the original mutation was used to substitute the corresponding wt sequences of pA. For isolating tA92(-) revertants, reverse transcription was primed with random hexanucleotide primers and PCR with oligonucleotides a and R-g (GGTCTTGACGAACTC, nt 1172–1156) were performed and the SacI–Accl fragment (nt 745–1085) was reintroduced in pA. For tA7(-)-1 and tA7(-)-2 revertants, reverse transcription was primed with oligonucleotide R-f and the fragment obtained by PCR amplification with oligonucleotides Mut19 and R-f was digested with XbaI (nt 4496) and KspI (nt 4790) and reintroduced in pA. Revertants pA92amber, pA92ochre, pA7(-)-1/C and pA7(-)-2Rev were identified by sequence analysis. The stability in planta of tA92ochre and tA7(-)-1/C was evaluated by sequencing the regions of interest (nt 930–1160 and 4300–4790, respectively).

**Analysis of complementation between tA33(N) and tA92(N).** RNA extraction from doubly infected protoplasts was as described above except for the addition of an RQ1 RNase-free DNase (Promega) treatment. RNA was reverse transcribed using MuLV reverse

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**Fig. 1.** Schematic representation of wild-type (pA) and mutant AMCV cDNA clones. The ORFs are shown as boxes. Restriction enzymes used in the construction of mutant clones are indicated. The arrows below the ORFs represent the putative encoded proteins p33, p92, p41, p21, p19 and p7, referred to by their predicted molecular masses (in kDa). Elimination of an ORF or part of it in the drawing indicates that the translation of the ORF was disrupted. GDD indicates the Gly-Asp-Asp amino acid motif present in p92.
transcriptase and random hexanucleotide primers. PCR was then performed on the resulting first strand using Pfu DNA polymerase (Stratagene), oligonucleotides a and R-g. The fragment obtained was cloned in pBlueScript (Stratagene) and sequenced.

**Computer analysis.** Computer analyses were carried out with the software developed by the Genetics Computer Group (Devereux et al., 1984). The predicted RNA secondary structure of the 3′ terminus of the AMCV genome was obtained using the Fold program. The sequences used in the comparison of the region surrounding the AMCV readthrough stop codon were from tomato bushy stunt virus (Hearne et al., 1990), cymbidium ringspot virus (Grieco et al., 1989), cucumber necrosis virus (Rochon & Tremaine, 1989), potos latent virus (Rubino et al., 1990), carnation Italian ringspot virus (Rubino et al., 1995b), tobacco necrosis virus D (Coutts et al., 1991), oat chlorotic stunt virus (Boonham et al., 1995), tobacco necrosis virus A (Meulewaeter et al., 1990), carnation mottle virus (Guilley et al., 1992), turnip crinkle virus (Carrington et al., 1989), cowpea mottle virus (You et al., 1995), cardamine chlorotic fleck carnivirus (Skotnicki et al., 1993), melon necrotic spot virus (Riviere & Rochon, 1990) and lettuce white stripe virus (Lot et al., 1996).

**Results**

**Construction of AMCV mutants and analysis of their replication in N. benthamiana protoplasts.**

The AMCV mutants used in this study are schematically represented in Fig. 1. Except for the CP gene frameshift mutant pA41(−), all were obtained by point mutations, thus minimizing the alteration of the RNA sequence. In pA33(−), the amber stop codon (UAG) of ORF 1, which undergoes readthrough to yield p92, was mutated to a tyrosine (UAU) to abolish p33 expression, while in pA92(−) it was replaced together with the following codon with the ochre-opal ‘double stop’ codon (UAAUGA). pA92GED was designed to test if a single conservative amino acid substitution in the p92 GDD motif (mutated to GED) impaired AMCV replication. In pA41(−), a four nucleotide insertion in the CP gene resulted in the expression of the first 28 amino acids fused to 37 frameshift-derived amino acids. The expression of ORFs 4 and 5 was impaired in pA21(−) and pA19(−) by mutagenesis of their respective start codons. Finally, the first two codons of ORF 6 (AUGAUG) were mutated to AUGCGU and AUAGCG in pA7(−)1 and pA7(−)2, respectively.

Several independent inoculation experiments with in vitro-synthesized RNAs from the wt and the mutant clones shown in Fig. 1 were performed on N. benthamiana protoplasts. Infectivity of mutant viral genomes was evaluated by Northern blot analysis of RNA extracted from the inoculated protoplasts 24 h post-inoculation (p.i.) using probes complementary (Fig. 2 A) or corresponding (Fig. 2 B) to the 3′ terminus of the AMCV genome. Protoplasts inoculated with mutants tA33(−), tA21(−) and tA19(−) accumulated the genomic and the two sg RNAs of both polarities to levels comparable with that observed for the wt tA. The plus-sense RNA probe employed in these Northern blot analyses did not cross-react with virion-purified AMCV genomic RNA (data not shown). Conversely, protoplasts inoculated with mutant transcripts tA33(−), tA92(−), tA92GED, tA7(−)1 and tA7(−)2 generally did not accumulate detectable amounts of genomic or sg RNAs, although in one experiment amounts of viral RNAs close to the background level of Northern blot analysis were observed in tA7(−)1- and tA7(−)2-inoculated protoplasts (data not shown).

**Analysis of the replication-impaired mutants and their pseudorevertants in N. benthamiana plants and protoplasts.**

The AMCV mutants which were replication-impaired in protoplasts were inoculated on N. benthamiana plants to allow the amplification and identification of possible slow replicating viral genomes. Plants challenged with tA33(−) or tA92GED remained completely symptomless and virus-specific RNAs were not detected in the upper non-inoculated leaves at 30–40 days p.i. (data not shown). On the other hand, one out of eight plants inoculated with tA92(−) was systemically infected at 17 days p.i. RNA was extracted from one inoculated leaf of this tA92(−)-infected plant and was reverse-transcribed; a DNA fragment encompassing the mutagenized viral sequences was PCR-amplified, back-cloned into pA and four clones were analysed. In three out of these clones (referred to as pA92amber), the first stop codon (ochre) was replaced with the original amber codon while in the other clone (referred to as pA92ochre) it was preserved. In both pA92amber and
pA92ochre, the second stop codon (opal) present in pA92(−) was mutated to GGG, which contains a silent mutation with respect to the wt codon GGA (Fig. 3 A). Both tA92amber and tA92ochre replicated in protoplasts, but at different levels: the first behaved similarly to wt, while the second replicated with a slightly reduced efficiency (Fig. 3 C). N. benthamiana plants challenged with tA92ochre showed milder systemic symptoms characterized by lesions which expanded and merged more slowly than for the wt. RNA was extracted from an upper non-inoculated leaf of a tA92ochre-infected plant and the region surrounding the ORF 1 stop codon was subjected to RT–PCR and sequenced. Six clones were analysed: two of them were identical to the input inoculum (UAAGGG), while four and sequenced. Six clones were analysed: two of them were identical to the input inoculum (UAAGGG), while four

Inoculation of N. benthamiana plants with the ORF 6 mutants resulted in the infection of two of 12 tA7(−)-1-inoculated plants and six of 11 tA7(−)-2-inoculated plants. RNAs were extracted from the upper leaves of one tA7(−)-1- and one tA7(−)-2-infected plant, reverse-transcribed and a PCR DNA fragment encompassing the mutagenized sequences was back-cloned into pA. Four clones were sequenced for each mutant. In all the clones isolated from the tA7(−)-2-infected plant, the first AUG of putative ORF 6 (Fig. 3 B, pA7(−)2Rev) had been restored, while in the clones derived from the tA7(−)-1 infection, the introduced mutations in the two AUG codons were conserved but an additional single nucleotide substitution was present 125 nt downstream to the ORF 6 start codon (position 4631) [Fig. 3 B, pA7(−)1/C]. Computer-assisted analysis of the secondary structure of the 3′ terminal region of AMCV RNA revealed possible base-pairing between nt 4502–4510 (UGUUAGUAU), which contain the first ORF 6 AUG (in italics), and nt 4630–4638 (GUCAGAGCA), indicating that nucleotide 4631 could be involved in base-pairing with the A of the second AUG codon of the ORF 6 (Fig. 3 B). Replication of tA7(−)1/C in N. benthamiana protoplasts was only slightly reduced compared to wt (Fig. 3 C), and infectivity on plants of this revertant was very similar to wt. Moreover, sequence analysis of six clones obtained by RT–PCR on RNA extracted from the upper leaves of a tA7(−)-1/C-infected plant showed that all clones were identical to the input inoculum and that no other compensating mutations had occurred in the ORF 6 region.

Complementation between the replication-defective mutants tA33(−) and tA92(−) in N. benthamiana protoplasts

The mutation in tA33(−) is designed to abolish synthesis of p33 by replacing the ORF 1 amber termination codon by a sense codon (UAU) so that the amino acid sequence corresponding to p33 is only expressed as part of p92. This modification, however, may also alter the amino acid sequence of p92 since it is not known what amino acid is inserted in this position during translation readthrough programmed by the wt sequence. To demonstrate that the nonreplicability of tA33(−) was not due to the putative alteration of the p92 sequence, we tested if tA33(−) and tA92(−) could complement each other when coinfected into protoplasts, thus rescuing their respective replication defects.

Coinoculation of equimolar amounts of tA33(−) and tA92(−) to N. benthamiana protoplasts resulted in the accumulation, albeit at low levels, of both genomic and sg RNAs (Fig. 4, lanes 2 and 4) which were not detected when the two mutant transcripts were inoculated separately (Fig. 4, lanes 1, 3 and 5). Moreover, when analysed, genomic and sg RNAs of negative polarity were also detected in coinfected protoplasts (data not shown). The AMCV RNAs accumulating in the doubly infected protoplasts could be the result of complementation, or of the production of a viable recombinant genome. To distinguish between these two possibilities, RNA was extracted from coinoculated protoplasts (sample shown in Fig. 4,
analogue of AMCV p19, could function as a helicase. However, inoculated with these mutant transcripts. Previously, it has RNAs of negative polarity was observed in protoplasts evident alteration in the accumulation of genomic and sg et al. 1993; H. B. Scholthof genomes. Mutant transcripts of ORF 3, 4 and 5, 14 of them were identical to tA92(g), and one contained the new sequence UUAUGA. PCR and sequence analyses of RNA extracted from these infected protoplasts showed that 15 out of 16 clones analysed were identical to tA92(—) and one was identical to tA33(—), thus confirming both the absence of recombination between the mutated genomes and the preferential accumulation of tA92(—) over tA33(—).

Discussion

In a first series of experiments, the viral genes essential for genome replication were identified by studying the viability, in N. benthamiana protoplasts, of mutants for each of the six AMCV ORFs. Mutant transcripts of ORF 3, 4 and 5 accumulated in protoplasts to a similar amount as the wt transcript tA, indicating that CP, p21 and p19 are dispensable for AMCV replication as previously shown for other tombus-viruses (Rochon, 1991; Dalmay et al., 1993; McLean et al., 1993; H. B. Scholthof et al., 1993, 1995). Furthermore, no evident alteration in the accumulation of genomic and sg RNAs of negative polarity was observed in protoplasts inoculated with these mutant transcripts. Previously, it has been speculated (Rochon, 1991) that CNV p20 protein, the analogue of AMCV p19, could function as a helicase. However, the absence of any modification in the accumulation of AMCV RNAs of either polarity in tA19(—)-infected protoplasts (Fig. 2 A, B), together with the absence of known helicase motifs in the tombusvirus p19/p20 proteins, does not support a helicase role for AMCV p19. On the other hand, the absence of detectable RNA amplification in protoplasts inoculated with tA33(—), tA92(—), tA92GED or (in three out of the four experiments) with tA7(—)1 or tA7(—)2 suggests that the products of ORF 1, 2 and 6 and/or the sequences within these ORFs are involved in virus replication.

Additional evidence for the requirement of the AMCV ORF 1, 2 and 6 in replication came from analysis of the infectivity of mutant transcripts on N. benthamiana plants. Neither tA33(—) nor tA92GED were able to infect N. benthamiana plants while tA92(—), tA7(—)1 and tA7(—)2 gave rise to viable pseudorevertants. The isolation and analysis of the pseudorevertants in the tA92(—)-inoculated plants led to the following observations: (i) the double stop-codon UAUAUA introduced at the end of ORF 1 in tA92(—) can apparently undergo sporadic readthrough, leading to a minimal production of functional p92; the lack of replication of mutant tA92GED, together with the absence of revertants from this clone, tends to rule out the possibility that p33 by itself was responsible for the replication necessary for selection of the viable tA92(amber) and tA92(ochre) genomes; (ii) the recovery of a mixture of viral genomes with ochre or opal stop codons from the tA92(ochre)-infected plant together with the viability of the tA92(amber) mutant indicates that all the three stop codons can undergo significant readthrough in vivo, thus supporting the view that the signals which cause the suppression are not specific to UAG suppression. Previous mutagenesis studies on tobacco mosaic virus (TMV) (Ishikawa et al., 1988) and TBSV (K. B. Scholthof et al., 1995) genomes showed that the leaky UAG stop codon of ORF 1, which is readthrough in vivo to express the RNA polymerase domain, could be replaced with an ochre codon giving rise to viable genomes. However, the evidence that plant viruses can use all three stop codons in the readthrough mechanism came from in vivo experiments using the TMV readthrough region fused to a reporter gene (Skuzeski et al., 1991). Similarly to AMCV, the infectivity of Moloney murine leukaemia virus was not substantially affected when the UAG whose readthrough yields the Gag–Pol fusion protein was changed to UAA or UGA (Feng et al., 1989); (iii) tA92(amber), tA92(ochre) and t92(opal) all contain a silent nucleotide substitution, A to G, in the third nucleotide after the amber stop codon. Alignment of the sequences surrounding the amber stop codon of tombus-, necro- and carmoviruses ORF 1 shows that the third nucleotide after the UAG codon could be either A or G [consensus sequence UAG G(G/A) (T/G)GC]. Further study will be necessary to determine if the two codons downstream of the ORF 1 UAG of tombus-, necro- and carmoviruses form part of the signal governing readthrough as in TMV (Skuzeski et al., 1991; Valle et al., 1992) or if an RNA secondary structure at the 3′ end of the UAG is required, as in several gag–pol retroviral

![Fig. 4. Northern blot of 33% of the RNA extracted from 5 × 10⁵ N. benthamiana protoplasts inoculated with the following transcripts: 1 µg of tA92(—) (lane 1), 1 µg of tA92(—) plus 1 µg of tA33(—) (lane 2), 1 µg of tA33(—) (lane 3), 3 µg of tA92(—) plus 3 µg of tA33(—) (lane 4), 3 µg of tA33(—) (lane 5) and 5 µg of tA92(—) plus 0-7 µg of tA33(—) (lane 6). The probe was a digoxigenin-labelled transcript complementary to the ca. 1000 3′-terminal nucleotides of the AMCV genome. Arrows indicate the position of genomic (g) and subgenomic (sg) RNAs.](image-url)
genes (Wills et al., 1994). Interestingly, the first nucleotide after the leaky AUG in the tombus-, necro- and carmoviruses is a G, as is the nucleotide found at the junction of several gag–pol retroviral genes, whereas a pyrimidine is found in this position in the TMV and in some eukaryotic leaky stop codons (Skuzeski et al., 1991; McCaughan et al., 1995).

With regard to the involvement of ORF 6 in virus replication, we have shown that the replication-competent revertant pA7(−1)/C, which was not able to express the putative p7 protein, replicated efficiently in protoplasts and, more importantly, no other compensatory mutations arose after its multiplication in planta. This analysis suggests that p7, if expressed during wt infection, is not required for AMCV replication. This view is in accordance with the infectivity analysis of CymRSV ORF 6 mutants (Dalmay et al., 1993). Moreover, computer-assisted analysis of the RNA secondary structure at the 3′ end of the AMCV genome suggests that the viability of pA7(−1)/C correlates with the restored base-pairing in an RNA stem structure (Fig. 3 B). The ORF 6 data thus suggest that a region containing the mutagenized sequence either acts itself as a cis-acting sequence involved in virus replication (Duggal et al., 1994) or serves to stabilize such a cis-acting sequence.

The nonviability of tA92GED, in which a single conservative amino acid substitution (GDD → GED) was introduced in p92, supports a role as polymerase for this protein and underlines the importance of the preservation of the GDD motif for the replication activity of p92, as previously shown for other plant viral RdRps belonging to bromo-, tobamo- and potexviruses (Buck, 1996). Moreover, the inability of tA33(−) to give rise to viable revertants suggests that the expression of AMCV p92 alone was not sufficient for virus replication. Similar results have been obtained in CymRSV (Dalmay et al., 1993) and TBSV (K. B. Scholthof et al., 1995). However, the lack of viral amplification of CymRSV and TBSV p33 mutants in these experiments was not rigorous proof of the requirement of p33 because it was not demonstrated that the p92s produced by these mutants were functional.

Our complementation data show that RNA transcripts derived from pA33(−) and pA92(−) did not accumulate in protoplasts to detectable levels unless they were mixed before inoculation, indicating that both p33 and p92 were functional and required for virus replication. Moreover, sequence analysis of the reverse-transcribed RNA extracted from these co-infection experiments showed that out of the 33 clones analysed, 29 were identical to tA92(−), three to tA33(−) and one had the sequence UUAUGA which can be derived by a single nucleotide substitution in tA92(−). This indicates both the preferential accumulation of tA92(−) over tA33(−) and the absence of recombinant genome(s). In fact, site-specific recombination between the mutated regions of tA92(−) (UAUUGA) and tA33(−) (UAUGGA) would be expected to generate a new mutant (UAAGGA), which should efficiently replicate, based on its similarity to tA92ochre.

Recently, White et al. (1995) used a similar experimental approach to show the involvement of the turnip crinkle carmovirus (TCV) ORF 1 and ORF 2 products (p28 and p88) in TCV replication. TCV, which belongs to the Tombusviridae, shares with AMCV a similar organization of the 5′ portion of the genome encompassing the first two ORFs, although no significant similarity was found between AMCV p33 and TCV p28 (Tavazza et al., 1994). We do not know at the moment if the low level of AMCV RNA amplification found in the doubly inoculated protoplasts and the preferential accumulation of tA92(−) over tA33(−) reflects a genuine cis-preferential replication of the tombusvirus genome with p33 interacting preferentially with the RNA genome from which it is translated. The evidence that tombusvirus DI RNAs and satellite RNAs can efficiently utilize the tombusvirus replication complex in trans does not exclude the possibility that the full-length genome could be replicated in a cis-preferential fashion. In this regard, Weiland & Dreher (1993) have shown that the cis-preferential replication of the turnip yellow mosaic virus (TYMV) genome could be overcome by some TYMV deletion mutants. Similarly to AMCV, the replication of both TYMV (Weiland & Dreher, 1993) and TCV (White et al., 1995) required the expression of two viral encoded proteins, one of which has the characteristic signatures of the RdRps (i.e. GDD motif). Interestingly, and similarly to AMCV, in the complementation experiments with both TYMV (Weiland & Dreher, 1993) and TCV (White et al., 1995), there was a preferential accumulation of the replication-defective genome encoding the replication protein which does not contain the GDD motif. However, the low level of RNA detected in tA33(−)/tA92(−) co-inoculated protoplasts and/or the preferential accumulation of tA92(−) over tA33(−) may be due to the fact that: (i) the replacement of the amber stop codon with a tyrosine codon in tA33(−) yields a p92 with a diminished activity; (ii) the nucleotide substitutions introduced could have altered a sequence required in cis for efficient virus replication in tA33(−) or in both mutated viral genomes [but note that hypotheses (i) and (ii) are difficult to reconcile with the observed ability of the amber, ochre and opal stop codons to be read-through giving viable mutants]; or (iii) RNA amplification could require defined relative amounts of p33 and p92. In this regard, it should be noted that a 20-fold molar excess of p33 over p92 was detected in TBSV infection (K. B. Scholthof et al., 1995). However, we show here that a 7-fold molar excess of tA92(−) over tA33(−) does not substantially increase RNA accumulation, suggesting that additional factor(s) should be taken into consideration to explain the low level of complementation observed.

In conclusion, we have provided here the first definitive evidence that the AMCV ORF 1 and ORF 2 gene products p33 and p92 are the only viral proteins absolutely required for tombusvirus replication and that trans-complementation between replication-defective full-length tombusvirus genomes can be achieved albeit at a low level. Moreover, we provide evidence
that the amber termination codon of ORF 1, which is suppressed \textit{in vivo} to yield p92, can be replaced by an ochre or opal codon without substantially impairing virus viability, thus suggesting that the signal(s) governing the leakiness of the ORF 1 stop codon of the tombusviruses are not amber-specific.

We thank H. B. Scholthof and M. Russo for suggestions during the work and G. P. Accotto for critical reading of the manuscript.

References


Received 1 September 1997; Accepted 4 November 1997