RNAs 1 and 2 of *Alfalfa mosaic virus*, expressed in transgenic plants, start to replicate only after infection of the plants with RNA 3

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RNAs 1 and 2 of the tripartite genome of *Alfalfa mosaic virus* (AMV) encode the two viral replicase subunits. Full-length DNA copies of RNAs 1 and 2 were used to transform tobacco plants (R12 lines). None of the transgenic lines showed resistance to AMV infection. In healthy R12 plants, the transcripts of the viral cDNAs were copied by the transgenic viral replicase into minus-strand RNAs but subsequent steps in replication were blocked. When the R12 plants were inoculated with AMV RNA 3, this block was lifted and the transgenic RNAs 1 and 2 were amplified by the transgenic replicase together with RNA 3. The transgenic expression of RNAs 1 and 2 largely circumvented the role of coat protein (CP) in the inoculum that is required for infection of nontransgenic plants. The results for the first time demonstrate the role of CP in AMV plus-strand RNA synthesis at the whole plant level.

The genome of *Alfalfa mosaic virus* (AMV) is divided among three plus-strand RNAs which are separately encapsidated. The RNA 1-encoded P1 protein with putative methyltransferase and helicase activity and the RNA 2-encoded P2 protein containing the GDD motif of viral plus-strand RNA polymerases have been identified as subunits of the purified AMV RNA-dependent RNA polymerase complex (RdRp; Quadt et al., 1991). The P3 protein encoded by RNA 3 is involved in virus movement, and the subgenomic RNA 4, which is synthesized from the 3’ half of minus-strand RNA 3, serves as messenger RNA for the multifunctional coat protein (CP). Initiation of infection by the three genomic RNAs requires CP (or RNA 4) in the inoculum (Bol et al., 1971). In infected protoplasts, CP in the inoculum appears to be required in a step of the replication cycle prior to viral minus-strand RNA synthesis whereas CP expressed from RNA 3 is required for plus-strand RNA accumulation (Neeleman & Bol, 1999). Also in vitro, plus-strand RNA synthesis by the purified AMV RdRp is stimulated by CP (de Graaff et al., 1995). Moreover, CP is required for cell-to-cell movement of the virus (van der Kuyl et al., 1991a; van der Vossen et al., 1994).

Previously, we transformed tobacco plants with incomplete DNA copies of AMV RNAs 1 and 2 (Taschner et al., 1991). In these P12 plants, the integrated cDNAs lack the 5’-terminal 36 nucleotides of RNA 1 and the 3’-terminal 10 nucleotides of RNA 2. The P12 plants can be infected with RNA 3 without the requirement for CP in the inoculum. The truncated RNAs 1 and 2 are not replicated by the transgenic RdRp in healthy or RNA 3-infected P12 plants. In the present work, we have engineered transgenic plants that express full-length RNAs 1 and 2 and viral RdRp. An analysis of steps in the replication cycle that occurred in the absence and presence of RNA 3 provided further insight in the role of CP in viral RNA replication at the whole plant level.

The AMV cDNAs 1 and 2, each surrounded by the 35S promoter of *Cauliflower mosaic virus* and the transcriptional terminator of the nopaline synthase gene (Neeleman et al., 1991), were inserted in two steps as KpnI–PvuII and SstI–PvuII fragments, respectively, in a tandem arrangement into the binary vector pMOG800. The resulting plasmid, pMOG-AMV1 + 2, was mobilized into *Agrobacterium tumefaciens* strain LBA4404. Following leaf disc transformation of *Nicotiana tabacum* cv. Samsun NN (Horsch et al., 1985), 19 independent plant lines carrying AMV cDNAs 1 and 2 were regenerated on kanamycin-containing MS medium (R12 lines: R12-1, 3 to 8, 10, 11, 14, 15 to 25). To determine the levels of viral RNA in the 19 individual transformants (T0), total RNA was extracted from healthy leaves of the primary transformants and analysed by Northern blot hybridization using 32P-labelled, random primed probes of cDNA 1 and 2 (van der Kuyl et al., 1991a;
In subsequent experiments, kanamycin-resistant T₁ plants grown from seeds of self-pollinated transformed plants were used. Accumulation of transgenic RNA in the T₁ progeny was similar to that in the corresponding T₀ plants. Isolation of poly(A) RNA from total RNA extracts of the T₁ plants using the PolyAtract mRNA isolation kit (Pharmacia) revealed that most of the transgenic RNAs 1 and 2 contained a poly(A) tail (result not shown).

To investigate whether an active replication complex is assembled in R12 plants, the T₁ generation of 13 R12 lines (R12-1, 4 to 8, 11, 17 to 19, 21, 24, 25) was infected with (in)complete mixtures of AMV virions and RNA 3 transcripts in the presence or absence of CP. The inoculum RNA 3 was in vitro transcribed with T7 RNA polymerase from clone pAL3 (Neelamn et al., 1991) and used either on its own or supplemented with CP molecules in an RNA 3:CP molecule ratio of 1:40. P12 virus particles (AMV virions containing RNAs 3 and 4) were isolated from RNA 3-infected P12 plants and a complete set of AMV virions was purified from AMV-infected nontransgenic tobacco plants (van Volten-Doting & Jaspars, 1972). Three leaf halves of two plants per line were inoculated with one of the different inocula (Neelamn et al., 1993). Total nucleic acids were extracted from inoculated leaves 5 days post-inoculation (p.i.) and analysed by Northern blot hybridization using a 32P-labelled, random-primed probe mixture containing AMV cDNAs 1 to 3 (Fig. 2 and data not shown for lines R12-1, 7, 11, 18, 19, 21, 24). The replication patterns of viral and transgene-derived RNAs from six R12 lines displayed in Fig. 2 represent an overall picture for the population of R12 plants tested and demonstrate the interclonal variability. The exposure time of the blots did not permit detection of the transgenic viral RNAs in mock-inoculated plants (Fig. 2, lanes 5). Inoculation with the complete AMV genome revealed that none of the R12 lines showed resistance to AMV infection (Fig. 2, lanes 4). When R12 plants were inoculated with RNA 3 (Fig. 2, lanes 1), RNA 3 plus CP (Fig. 2, lanes 2) or P12 virus particles containing RNAs 3 and 4 (Fig. 2, lanes 3), the transgene-derived RNAs 1 and 2 accumulated together with RNAs 3 and 4 although the level of replication varied between different transgenic lines.

In all R12 lines analysed, replication of inoculum RNA 3 and transgenically expressed RNAs 1 and 2 could be initiated in the absence of CP, although for lines R12-8 and R12-25 (Fig. 2, lane 1) a longer exposure of the blot was required to reveal RNA accumulation. Initiation of infection of line R12-5 was fully independent of the presence of CP in the inoculum, but addition of CP to inocula containing RNA 3 stimulated infection of the other lines shown in Fig. 2 (compare lanes 1 and 2). Although the CP dependency of the initiation of infection did not correlate with the level of transgenic RNAs 1 and 2, a correlation with the level of functional replicase activity in different R12 lines cannot be ruled out. Infection with RNA 3 of P12 plants was not stimulated by CP in the inoculum but a possible variation in CP dependency of the...
infection between different P12 lines was not investigated (Taschner et al., 1991).

To determine whether the transgenically expressed RNAs 1 and 2 were replicating in R12 plants prior to RNA 3 inoculation, the presence of minus-strand RNAs 1 and 2 in healthy and RNA 3-infected R12 plants. Total nucleic acid extracts from healthy R12 plants (R12 lines 4, 5, 6, 8, 17, 18, 19 and 25; lanes 5 to 12) and RNA 3-infected R12 lines 5 and 6 (lanes 13 and 14) were analysed by Northern blot hybridization in amounts corresponding to 50 µg of fresh leaf material. As controls, the blot was loaded with minus-strand transcripts from cDNA 1 (–1; lane 1), minus-strand transcripts from cDNA 2 (–2; lane 2), plus-strand virion RNAs (V; lane 3) and total RNA from a healthy nontransgenic tobacco plant (H; lane 4). The blots were probed with DIG-labelled minus-strand RNAs 1 and 2 transcribed with T7 RNA polymerase from BamHI-linearized antisense cDNAs 1 or 2 (panel A) or plus-strand RNAs 1 and 2 transcribed from EcoRI/BglII-linearized sense cDNAs 1 and 2 (panel B), respectively. The blots shown in this figure were exposed for the same time. The position of AMV RNAs 1 and 2 is indicated on the left of the autoradiograms. The numbers on top of panel (A) represent lane numbers; numbers at the bottom of panel (B) denote the numbers of transgenic lines.

steps in the replication cycle were blocked. When healthy plants of R12 lines 5 and 6 (Fig. 3, lanes 6 and 7) were inoculated with RNA 3, a massive accumulation of plus-strand RNAs 1 and 2 was observed whereas little or no increase in the accumulation of minus-strand RNAs 1 and 2 occurred (Fig. 3, lanes 13 and 14). Possibly, the amount of minus-strand RNA synthesized in healthy R12 plants is largely sufficient to act as template for the synthesis of plus-strand RNAs 1 and 2 that is induced by the RNA 3-encoded CP.

When nontransgenic protoplasts are inoculated with AMV genomic RNAs, minus-strand RNA accumulation is detectable only when CP is added to the inoculum. However, when P12 protoplasts are inoculated with RNA 3, minus-strand RNA 3 accumulation requires neither CP in the inoculum nor expression of CP from RNA 3 (van der Vossen et al., 1994; Neelame & Bol, 1999). This demonstrates that CP is not required for minus-strand RNA synthesis per se, and we have proposed that in nontransgenic protoplasts CP is required in a step prior to the initiation of minus-strand RNA synthesis, possibly translation of inoculum RNAs (Neelame & Bol, 1993; Neelame & Bol, 1999; Olsthoorn et al., 1999). Apparently, this step is circumvented in R12 plants as minus-strand RNAs 1 and 2 are synthesized in the absence of the RNA 3-encoded CP. Translation of cellular mRNAs is synergistically enhanced by an interaction between the 3′ poly(A) tail and 5′ cap structure that is mediated by the poly(A)-binding protein (PABP) and translation initiation factors eIF4E and eIF4G (Dever, 1999; Gallie, 1991; Imataka et al., 1998). The rotavirus NSP3 protein has been shown to interact with the 3′ end of the non-polyadenylated viral mRNAs and eIF4G to substitute for PABP in the enhancement of translation (Vende et al., 2000). Possibly, the binding of PABP to the poly(A) tail of the transgenic RNA 1 and 2 transcripts in R12 plants may compensate for the putative role of CP in translation of these RNAs in nontransgenic plants. It should be noted that other models for the early function of CP have been proposed (de Graaff et al., 1995; Houser-Scott et al., 1997; Houwing & Jaspars, 1993).

When AMV minus-strand RNA accumulation is successfully initiated in protoplasts from nontransgenic plants or P12 plants, CP expressed from RNA 3 is required for asymmetric plus-strand RNA accumulation in both protoplast systems (van der Kuyl et al., 1991a, b; Neelame & Bol, 1999). Our results with the R12 plants demonstrate for the first time the requirement of CP for plus-strand RNA accumulation at the whole plant level.

Viruses from the genus Bromovirus neither require CP in the inoculum to initiate infection nor the RNA 3-encoded CP for plus-strand RNA synthesis (Pacha et al., 1990). This may explain the observation of Mori et al. (1992) that in protoplasts from plants transformed with full-length copies of RNAs 1 and 2 of Brome mosaic virus (BMV), replication of the transgenic RNAs 1 and 2 is largely independent on infection of the protoplasts with RNA 3. In contrast to the susceptibility of
R12 plants to AMV infection, tobacco plants expressing multiplying RNAs 1 and 2 of Cucumber mosaic virus (CMV) exhibit resistance to challenge inoculation with CMV (Suzuki et al., 1996). Similarly, protoplasts from plants transformed with replicable BMV RNAs 1 and 2 show resistance to BMV (Kaido et al., 1995). Expression of replicating RNA of Potato virus X in transgenic plants consistently resulted in the activation of a gene silencing mechanism (Angell & Baulcombe, 1997). It has been proposed that the inability of BMV P2 transgenic plants to replicate BMV RNA 2 is due to RNA 2-specific gene silencing (Iyer & Hall, 2000). In R12 plants, the transgene transcription levels and the replication events that occur in the absence of AMV RNA 3 might be insufficient to activate a gene silencing mechanism.

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