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Cis-Preferential Stimulation of Alfalfa Mosaic Virus RNA 3 Accumulation

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RNA 3 of alfalfa mosaic virus (AlMV) encodes the movement protein P3 and the viral coat protein (CP) which is translated from the subgenomic RNA 4. RNA 3 is able to replicate in tobacco plants transformed with the AlMV replicase genes P1 and P2 (P12 plants). Frameshifts or deletions in the P3 gene have little effect on RNA 3 accumulation in P12 protoplasts whereas such mutations in the CP gene result in a 100-fold reduction of plus-strand RNA 3 accumulation. When P12 protoplasts were inoculated with a mixture of a RNA 3 mutant with a deletion in the P3 gene and a mutant with a deletion in the CP gene, CP expressed by the P3 mutant was unable to upregulate plus-strand RNA accumulation of the CP mutant. However, when a wild-type CP gene and subgenomic promoter were inserted in a RNA 3 mutant with a defective CP gene, the mutant accumulated at wild-type levels. It is concluded that the function of CP in plus-strand RNA 3 accumulation acts *in cis* and cannot be complemented *in trans.* In P12 plants, P3 and CP mutants were able to complement each other at low and variable levels. This complementation in plants appeared to be correlated with the occurrence of recombination to wild-type RNA 3.

INTRODUCTION

Alfalfa mosaic virus has a tripartite RNA genome of messenger-sense polarity. RNAs 1 and 2 encode the replicase proteins P1 and P2, respectively. RNA 3 encodes the viral movement protein P3 and coat protein (CP). Expression of the CP gene involves the synthesis of a subgenomic mRNA, RNA 4, that is colinear with the 3′-terminal 881 nucleotides of RNA 3. The phenomenon that a mixture of AlMV RNAs 1, 2, and 3 is able to initiate infection only after addition of CP or RNA 4 to the inoculum has been termed "genome activation" or the "early" function of CP (Bol *et al.,* 1971). When the genomic RNAs in the inoculum are replaced by corresponding cDNAs fused to the 35S promoter, the initiation of infection becomes largely independent of addition of CP to the inoculum (Neeleman *et al.,* 1993). Tobacco plants transformed with DNA copies of the P1 and P2 genes (P12 plants) can be infected with RNA 3 only and no CP is required to initiate RNA 3 replication in these transgenic plants (Taschner *et al.,* 1991). It has been suggested that in nontransgenic plants the early function of CP is to protect the 3′-end of the viral RNAs from degradation until a replicase activity has been assembled (Neeleman *et al.,* 1993).

Although CP is dispensable for the initiation of RNA 3 replication in P12 plants, CP is still required in later steps of the AlMV replication cycle. These later steps include asymmetric accumulation of positive-strand RNA (Nassuth and Bol, 1983; van der Kuyl *et al.,* 1991a,b; Houwing and Jaspars, 1993; de Graaff *et al.,* 1995), encapsidation of viral RNA, and cell-to-cell spread of the virus (van der Kuyl *et al.,* 1991c; van der Vossen *et al.,* 1994). A mutational analysis showed that the early and three late functions of CP could be mutated separately (van der Vossen *et al.,* 1994). This conclusion was supported by the evidence provided by Reusken *et al.* (1995) that CP of tobacco streak ilarvirus (TSV) could not upregulate AlMV plus-strand RNA accumulation, although this CP was able to activate the AlMV genome and to encapsidate AlMV RNAs.

CP expressed in transgenic plants was able to activate the AlMV genome (van Dun *et al.,* 1987) but could not complement a defective CP gene in RNA 3 (Dore *et al.,* 1991). On the other hand, preliminary evidence indicated that in P12 plants a RNA 3 mutant with a defective CP gene could be complemented *in trans* by a mutant with a defective P3 gene (van der Kuyl *et al.,* 1991c). Here, we investigated the ability of CP expressed by a P3 mutant to upregulate plus-strand RNA accumulation of a CP mutant in P12 protoplasts. Because the results showed that the function of AlMV CP in plus-strand RNA accumulation is required *in cis* and could not be complemented *in trans,* we reinvestigated the complementation observed between RNA 3 mutants in P12 plants.

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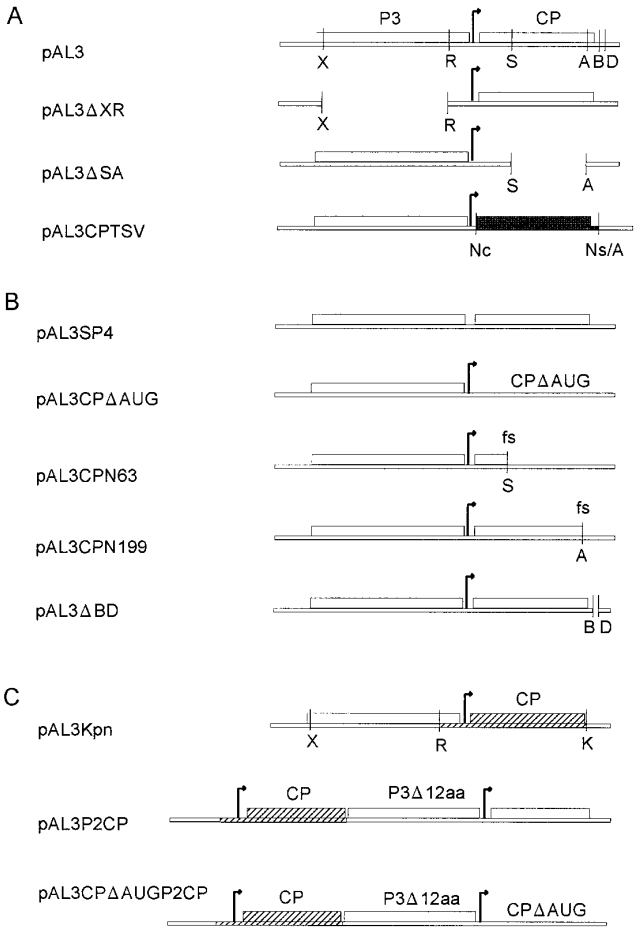

FIG. 1. Schematic representation of mutant AlMV cDNAs derived from pAL3, a wt clone of RNA 3 from AlMV strain 425. (A) P3 deletion mutant (ΔXR) and CP deletion and chimeric mutants (ΔSA and CPTSV, respectively). Open bars represent AlMV-derived sequences and the black bar represents a TSV-derived sequence. ORFs encoding proteins are indicated as rectangles above the bars. (B) RNA 3 mutants defective in plus-strand RNA accumulation. In mutant SP4 the transcription start site of RNA 4 was changed from G to A. In mutant CPΔAUG the initiation codon of the CP gene was changed to AAG, respectively. Mutants CPN63 and CP N199 contain frameshifts (fs) at positions 1493 and 1902 in the CP cistron, respectively. Mutant δBD contains a deletion of 38 nucleotides (nucleotides 1978–1977). (C) Double CP gene mutants P2CP and CPΔAUGP2CP. Cross-hatched bars represent the restriction fragment containing the AlMV CP gene and subgenomic promoter sequence derived from a pAL3 clone containing a *Kpn*I restriction site at nucleotide position 1965, pAL3Kpn. An arrowhead indicates the RNA 4 transcription start site (X, *Xho*I; R, *Rsa*I; S, *Sst*I; A, *Apa*I; Nc, *Nco*I; Ns, *Nsi*I; K, *Kpn*I; B, *Bst*XI; D, *Dra*III).

MATERIALS AND METHODS

Plasmid constructions

Schematic representations of the wild-type (wt) and mutant clones used in this study are shown in Fig. 1. Plasmid pAL3 contains a full-length DNA copy of AlMV RNA 3 downstream of the T7 promoter (Neeleman *et al.,* 1991). Mutants with deletions in the P3 gene (pAL3ΔXR) or CP gene (pAL3ΔSA) were made by removing the indicated restriction fragments, blunting the ends with T4 DNA polymerase (Promega) and religating (van der Kuyl *et al.,* 1991a,b).

The construction of plasmids pAL3CPTSV (pAT3-2; Reusken *et al.,* 1995), pAL3SP4 (van der Vossen *et al.,* 1995), and pAL3CPΔAUG pAL3CPN63, pAL3CPN199, and pAL3ΔBD (van der Vossen *et al.,* 1994) has been described previously. In pAL3CPTSV, the AlMV CP gene is replaced by the TSV CP gene. Mutant pAL3SP4 contains a mutation of the subgenomic transcription start site from G to A which does not allow for synthesis of any subgenomic RNA 4. In pAL3CPΔAUG, a mutation of the initiation codon of the CP gene interferes with CP expression. Mutants pAL3CPN63 and pAL3N199 are frameshift mutants that encode the N-terminal 63 and 199 amino acids of the CP gene, respectively. Mutant pAL3ΔBD contains a deletion of 38 nucleotides (nucleotides 1978–1977) in the 3′-untranslated region of RNA 3.

The double CP gene construct pAL3P2CP was made by inserting the *Rsa*I–*Kpn*I restriction fragment containing the CP gene of pAL3Kpn in the *Xho*I site (position 369) of pAL3 (van der Vossen *et al.,* 1994). As a result, the inserted CP gene will be expressed from a novel subgenomic RNA (van der Vossen *et al.,* 1995), and the P3 protein is truncated by 12 amino acids at the amino terminus. Insertion of the *Rsa*I–*Kpn*I restriction fragment of pAL3Kpn in the *Xho*I site of pAL3CPΔAUG resulted in plasmid pAL3CPΔAUGP2CP.

Inoculation of P12 protoplasts and plants

Wt and mutant RNA 3 transcripts were synthesized with T7 RNA polymerase on plasmids that were digested with *Pst*I prior to transcription (van der Kuyl *et al.,* 1991a).

Isolation and inoculation of P12 protoplasts with wt and mutant transcripts were performed essentially as described previously (van Dun *et al.,* 1987; Loesch-Fries *et al.,* 1985). For each protoplast experiment 2.5×10^5 protoplasts were inoculated by the PEG method with either 3 μg of transcript or a mixture of two transcripts containing 3 μg of each transcript. Inoculated protoplasts were incubated at 25° under constant illumination. Transgenic P12 tobacco plants were grown and inoculated as described previously (Taschner *et al.,* 1991) using 0.5 μg per half leaf of each of the indicated transcripts. Inoculation was done on three half leaves per plant using two plants per sample.

RNA and protein analysis

Total RNA was extracted from protoplast samples (2.0×10^5 protoplasts) using TRIzol reagent (GIBCO BRL) as described previously (van der Vossen *et al.,* 1994). Inoculated leaves were collected 5 days after inoculation (d.p.i.) and total RNA was isolated according to van der Kuyl *et al.* (1991b). Total RNA from protoplasts and plants

was subsequently analyzed by Northern blot hybridization using indicated random primed cDNA probes (van der Vossen *et al.,* 1994; Feinberg and Vogelstein, 1984).

For protein analysis, 0.5×10^5 protoplasts were pelleted by centrifugation and resuspended in 50 μl of Laemmli loading buffer (Laemmli, 1970). CP accumulation was measured by Western (immuno)blotting using AlMV CP specific antiserum. The protein bands were visualized using the phosphatase conjugate–nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system according to Leary *et al.* (1983).

RESULTS

A defective CP gene is not complemented *in trans* in protoplasts

Figure 1A shows schematic representations of RNA 3 mutants with a deletion in the P3 gene (ΔXR) or the CP gene (ΔSA). To study a possible complementation between these mutants in P12 protoplasts, the protoplasts were inoculated with RNA transcripts of either ΔXR or ΔSA or a mixture of transcripts ΔXR and ΔSA. RNA accumulation was analyzed 24 hr p.i. by Northern blot hybridization using probes corresponding to the deletions in the P3 and CP genes (Fig. 2A). As was shown earlier (van der Kuyl *et al.,* 1991b), accumulation of RNA 3 with a deletion in the P3 gene was only slightly reduced (ΔXR, Fig. 2A, lane 2; see also Fig. 3A, lane 2), whereas a deletion in the CP gene reduced RNA accumulation approximately 100-fold (ΔSA, Fig. 2A, lane 8). When P12 protoplasts were inoculated with a mixture of the P3 and CP mutant transcripts, the CP mutant ΔSA accumulated at the same 1% level as in protoplasts singly inoculated with this mutant (Fig. 2A, lane 9). RNA accumulation of the P3 mutant ΔXR remained largely unaffected (Fig. 2A, lane 4). CP production in the inoculated P12 protoplasts was assayed by Western blot analysis (Fig. 2B). CP accumulation directed by the P3 deletion mutant ΔXR (Fig. 2B, lane 2) was about 50% of the wt level (Fig. 2B, lane 1), whereas the CP mutant ΔSA did not induce accumulation of CP (Fig. 2B, lane 3). The presence of mutant ΔSA had little effect on CP accumulation directed by mutant ΔXR (Fig. 2B, lane 4).

When the CP gene in AlMV RNA 3 was replaced by the CP gene of TSV, the mutant was defective in asymmetric plus-strand RNA accumulation (Reusken *et al.,* 1995). To see if this defect could be complemented *in trans* by wt AlMV CP, P12 protoplasts were inoculated with a mixture of mutants CPTSV and ΔXR. RNA accumulation was monitored via Northern blot analysis using AlMV- and TSV-specific probes (Fig. 2C). Accumulation of wt RNA 3 is shown in Fig. 2C, lane 1. Similar to the previous study, replacement of the AlMV CP gene in RNA 3 with the TSV CP gene resulted in an approximately 50-fold reduction in the level of RNA accumulation (Fig. 2C, lane 3). Addition of the P3 deletion mutant ΔXR to the inoculum had no effect on the accumulation CPTSV RNA (Fig. 2C, lanes 4 and 9). In this mixed infection, the accumulation of AlMV CP was similar to that in protoplasts singly inoculated with mutant ΔXR (Fig. 2D, lanes 2 and 4). Apparently, the CP expressed by mutant ΔXR was unable to complement the defect in plus-strand RNA accumulation of mutant CPTSV *in trans.*

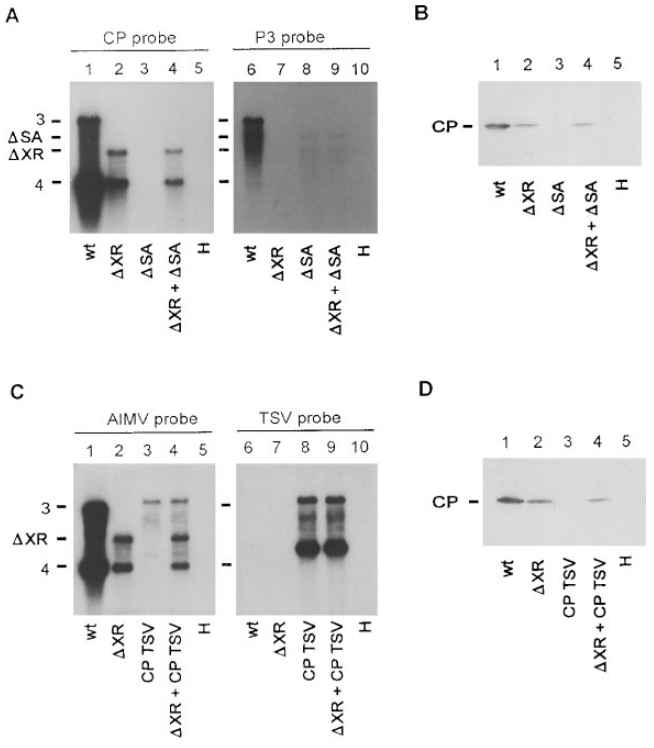

FIG. 2. Accumulation of P3 and CP mutants or a combination thereof in P12 protoplasts. (A) RNA accumulation in P12 protoplasts inoculated with wt (lanes 1 and 6) and mutant transcripts ΔXR (lanes 2 and 7), ΔSA (lanes 3 and 8), and a mixture of ΔXR and ΔSA (lanes 4 and 9). Total RNA was extracted 24 hr after inoculation and analyzed by Northern blot hybridization using randomly primed cDNA probes corresponding to the deletions in the CP and P3 genes. The positions of RNA 3, ΔXR, ΔSA, and RNA 4 are indicated in the margin. (B) CP accumulation in P12 protoplasts inoculated with the same inocula as in (A). Total protein was extracted 24 hr after inoculation and analyzed by Western blotting using antiserum prepared against AlMV CP. The bands were visualized using the phosphatase conjugate–nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system. The position of CP is indicated in the margin. (C) RNA accumulation in P12 protoplasts inoculated with wt (lanes 1 and 6) and mutant transcripts ΔXR (lanes 2 and 7), CPTSV (lanes 3 and 8), and a mixture of ΔXR and CPTSV (lanes 4 and 9). Total RNA was extracted 24 hr after inoculation and analyzed by Northern blot hybridization using randomly primed cDNA probes corresponding to the AlMV and TSV CP genes. The positions of RNA 3, ΔXR, CPTSV, and RNA 4 are indicated in the margin. (D) CP accumulation in P12 protoplasts inoculated with the same inocula as used in (C). Total protein was extracted 24 hr after inoculation and analyzed by Western blotting using antiserum prepared against AlMV CP. The bands were visualized using the phosphatase conjugate–nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system. H, total RNA or protein extracted from healthy P12 protoplasts.

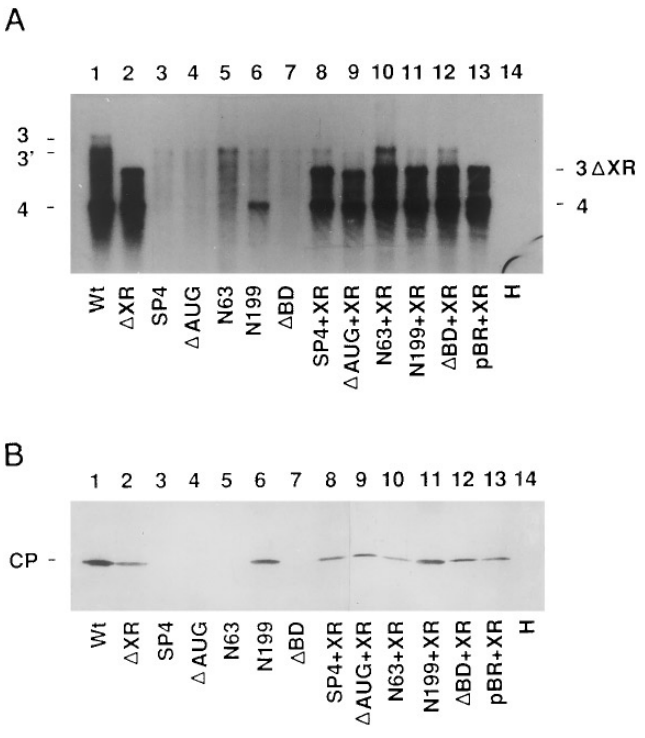

FIG. 3. Analysis of the complementation of RNA 3 mutants SP4, CPΔAUG, CPN63, CPN199, and ΔBD by the P3 mutant ΔXR. (A) RNA accumulation in P12 protoplasts inoculated with wt (lane 1) and mutant transcripts ΔXR (lane 2), SP4 (lane 3), CPΔAUG (lane 4), CPN63 (lane 5), CPN199 (lane 6), ΔBD (lane 7), or a mixture of the indicated mutant transcripts (lanes 8–13). Total RNA was extracted 24 hr after inoculation and analyzed by Northern blot hybridization using a randomly primed cDNA 3 probe. The positions of RNA 3, RNA 3′, and RNA 4 are indicated in the margin. RNA 3′ is a degradation product of RNA 3. The length of RNA 3′ is estimated to be approximately 1720 nucleotides and this molecule is believed to lack the 5′-terminal 420 nucleotides of RNA 3. (B) CP accumulation in P12 protoplasts inoculated with the same inocula as was used in (A). Total protein was extracted 24 hr after inoculation and analyzed by Western blotting using antiserum prepared against AlMV CP. The bands were visualized using the phosphatase conjugate–nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system. The position of CP is indicated in the margin. H, total RNA or protein extracted from healthy P12 protoplasts.

The inability of CP from mutant ΔXR to restore plus-strand RNA accumulation of mutants ΔSA and CPTSV could be due to a deletion of *cis*-acting elements from the CP gene of these two mutants. To investigate this possibility, the complementation of mutants with minor base substitutions in the CP gene (Fig. 1B) was studied. Figure 3A shows the RNA accumulation in P12 protoplasts inoculated with mutants SP4, CPΔAUG, CPN63, CPN199, and ΔBD alone, or with these mutants each mixed with mutant ΔXR. In mutants SP4 and CPΔAUG, expression of CP was eliminated by changing the RNA 4 transcription start site from G to A and the initiation codon of the CP ORF to AAG, respectively. Mutants CPN63 and CPN199 contain frameshifts at positions 1493 and 1902 in the CP cistron and encode the N-terminal 63 and 199 amino acids of the CP followed by 13 and 4 nonviral amino acids, respectively (van der Vossen *et al.,* 1994). Mutant ΔBD contains a deletion of 38 nucleotides in the 3′-untranslated region of RNA 3. RNA accumulation in P12 protoplasts singly inoculated with these mutants was approximately 100-fold reduced (Fig. 3A, lanes 3–7, respectively) compared to accumulation of the wt (Fig. 3A, lane 1). When the P3 mutant ΔXR was added to the inocula, no increase in the accumulation of mutant RNAs was observed (Fig. 3A, lanes 8–12). RNA accumulation of mutant ΔXR remained unaffected in the mixed infections (Fig. 3A, lanes 8–12). Similarly, inoculation of P12 protoplasts with a mixture of mutant ΔXR and a 783-nucleotide-long pBR322-derived transcript (Reusken *et al.,* 1995) had no effect on the accumulation of the P3 mutant (Fig. 3A, lane 13). In these mixed infections, the accumulation of AlMV CP was similar to that in protoplasts singly inoculated with mutant ΔXR (compare lane 2 with lanes 8–13 in Fig. 3B). These results indicate that the inability of CP synthesized by the P3 mutant ΔXR to rescue *in trans* the defective plus-strand RNA accumulation of CP mutants is not due to a deletion of *cis*-acting RNA sequences in these mutants.

A defective CP gene is complemented *in cis* by a wild-type CP gene

The results presented in Figs. 2 and 3 indicated that mutations in the CP gene of AlMV RNA 3 could not be complemented *in trans* by a wt CP gene present in another RNA 3 molecule. To see if a wt CP gene could complement when present *in cis,* a RNA 3 molecule with two CP genes was constructed. Mutant P2CP contains two wt CP genes each under the control of a subgenomic (sg) promoter (Fig. 1C). We have shown previously that the upstream sg promoter in such a construct is functional (van der Vossen *et al.,* 1995). In mutant CPΔAUGP2CP the AUG initiation codon of the downstream CP gene is changed into AAG. This mutation blocks the expression of the original CP gene (van der Vossen *et al.,* 1994). Figure 4A shows the accumulation of mutant RNAs in P12 protoplasts. The accumulation level of genomic RNA of mutant P2CP (Fig. 4A, lane 2) is similar to that of wt RNA 3 (Fig. 4A, lane 1). In addition, mutant P2CP directs the synthesis of RNA 4 and a sg RNA derived from the upstream sg promoter, designated sg3 RNA. As reported earlier (van der Vossen *et al.,* 1995), the presence of the upstream sg promoter downregulated the activity of the downstream sg promoter. Lane 3 of Fig. 4A illustrates that RNA accumulation by mutant CPΔAUG is reduced about 100-fold (van der Vossen *et al.,* 1994). However, in mutant CPΔAUGP2CP the upstream wt CP gene complemented the defect in the downstream CP gene and the RNA accumulation of this mutant (Fig. 4A, lane 4) is approximately 50-fold in-

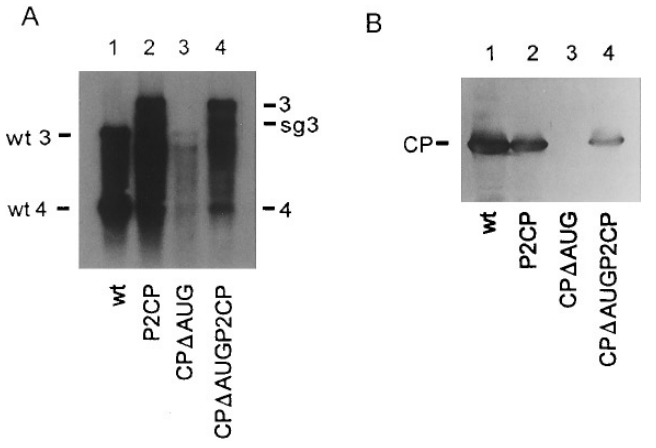

FIG. 4. Accumulation of double CP gene mutants in P12 protoplasts. (A) RNA accumulation in P12 protoplasts inoculated with wt (lane 1) and mutant transcripts P2CP (lane 2), CPΔAUG (lane 3), and CPΔAUGP2CP (lane 4). Total RNA was extracted 24 hr after inoculation and analyzed by Northern blot hybridization using randomly primed cDNA 3 as a probe. The positions of wt RNA 3 and wt RNA 4 are indicated to the left and the positions of full-length mutant RNA 3, subgenomic (sg) RNA 3, and mutant RNA 4 are indicated to the right. (B) CP accumulation in P12 protoplasts inoculated with the same inocula as in (A). Total protein was extracted 24 hr after inoculation and analyzed by Western blotting using antiserum prepared against AlMV CP. The bands were visualized using the phosphatase conjugate–nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system. The position of CP is indicated to the left.

creased when compared to that of mutant CPΔAUG (Fig. 4A, lane 3). Accumulation of CP in the mutant infected protoplasts is shown in Fig. 4B. Mutants P2CP and CPΔAUGP2CP directed CP accumulation at 50 and 20% of wt levels, respectively, whereas mutant CPΔAUG did not induce detectable CP accumulation.

Complementation and recombination in P12 plants

Previously, we reported that mutants with deletions in the P3 and CP gene were able to coreplicate in P12 plants but in those experiments the mutants rapidly recombined to full-length RNA 3 molecules (van der Kuyl *et al.,* 1991c). To reinvestigate these results, we inoculated P12 plants with P3 and CP mutants and combinations thereof. Total RNA was extracted from the inoculated leaves 5 d.p.i. and analyzed by Northern blot hybridization. Six independent experiments with the same set of mutants were done and the results of two of these experiments are shown in Figs. 5A and 5B. When plants were inoculated with mutants ΔXR, ΔSA, or CPTSV alone, no accumulation of mutant RNA was detected when an AlMV RNA 3 specific probe was used (Fig. 5, lanes 1, 3, and 5, respectively). When a probe corresponding to the TSV CP gene was used, a low level of accumulation of CPTSV RNA was detectable in the experiment shown in Fig. 5B (lane 12); this low level was also detectable in lane 12 of Fig. 5A after a longer exposure of the blot (result not shown). In plants inoculated with a mixture of mutants ΔXR and ΔSA, the accumulation of both mutants and their subgenomic RNAs was detectable (Figs. 5A and 5B, lane 4). In agreement with previous results (van der Kuyl *et al.,* 1991c), in all six experiments this accumulation was accompanied by the formation of various levels of full-length RNA 3 recombinants.

Studies on the ability of mutants CPTSV and ΔXR to complement each other's defects in the infection of P12 plants yielded variable results. In three experiments results as shown in Fig. 5A were obtained, whereas Fig. 5B represents results observed in three other experiments. In the first three experiments, a clear complementation between the two mutants was observed. RNA of the size of mutant ΔXR accumulated at a much higher level in the mixed infection (Fig. 5A, lane 6) than in leaves inoculated with mutant ΔXR alone (Fig. 5A, lane 2). The use of a TSV specific probe revealed a similar increase in the level of mutant CPTSV in the mixed infection (compare lanes 12 and 13 of Fig. 5A). In all three experiments represented by Fig. 5A, complementation between the mutants was accompanied by accumulation of detectable levels of recombinant full-length RNA 3. In the exper-

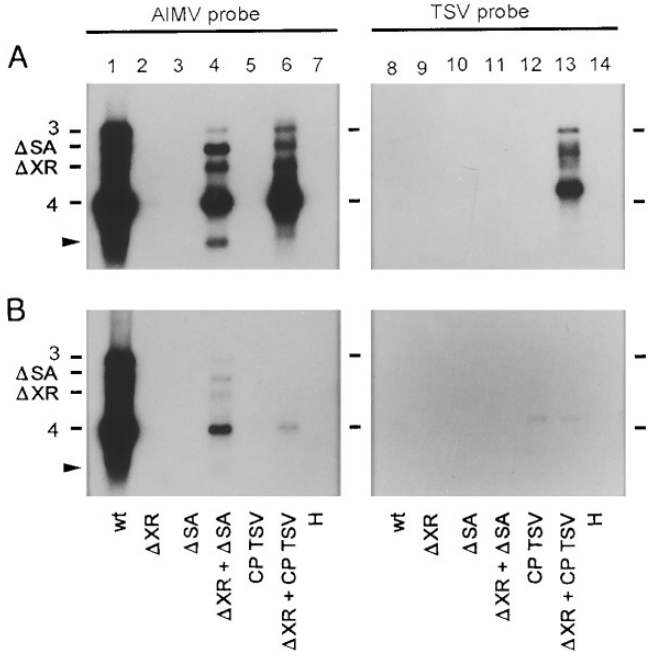

FIG. 5. RNA accumulation of P3 and CP mutants in P12 plants. P12 plants were inoculated with the indicated mutants or a combination thereof. Total RNA was extracted from the inoculated leaves 5 days after inoculation and analyzed by Northern blot hybridization using randomly primed cDNA probes corresponding to the AlMV and TSV CP genes. The results of two separate experiments are shown, (A) and (B). The positions of RNA 3, ΔXR, ΔSA, and RNA 4 are indicated in the margin. The arrowhead indicates the position of subgenomic RNA 4 produced by mutant ΔSA. H, total RNA extracted from healthy P12 plants.

iments represented by Fig. 5B, no complementation of mutant CPTSV was detectable (Fig. 5B, lanes 12 and 13) but a relatively low level of complementation of mutant ΔXR could be observed (Fig. 5B, lanes 2 and 6). Moreover, no recombination to full-length RNA 3 was detectable in these three experiments. A possible correlation between recombination and complementation is discussed below.

DISCUSSION

Previously, we reported that AlMV RNA 3 mutants with deletions in the P3 and CP gene showed significant levels of complementation in P12 plants (van der Kuyl *et al.,* 1991c). Therefore, the finding that no complementation between such mutants was detectable in P12 protoplasts was unexpected. When tobacco protoplasts are inoculated with a mixture of AlMV RNAs 1–4 or RNAs 1, 2, 3, and CP by the method developed by Loesch-Fries *et al.,* about 80% of the protoplasts become infected (Loesch-Fries *et al.,* 1985; Yusibov and Loesch-Fries, 1995). In the RNA inoculum, RNA 4 is required to initiate infection (Bol *et al.,* 1971), indicating that a large portion of the protoplasts is hit by four different inoculum RNAs. When RNA 3 is omitted from the inoculum, accumulation of plus-strand RNAs 1 and 2 drops to low levels (Nassuth and Bol, 1983; Taschner *et al.,* 1991). Similarly, in protoplasts of transgenic P1 or P2 plants the replication of RNAs 2 and 1, respectively, is dependent on RNA 3 in the inoculum (Taschner *et al.,* 1991). This demonstrates that *trans*-acting functions encoded by RNA 3 can be measured in the protoplast system. We have confirmed that 60% of P12 tobacco protoplasts become infected after inoculation with an RNA 3 transcript (van der Vossen, unpublished results). This indicates that in a mixed infection with two RNA 3 mutants a significant portion of the protoplasts will become infected with both mutants.

Several frameshifts and deletions in the CP gene or a point mutation in its initiation codon resulted in a 100-fold reduction of plus-strand RNA 3 accumulation (van der Kuyl *et al.,* 1991b; van der Vossen *et al.,* 1994). Also, results from *in vitro* experiments support a role of CP in plus-strand AlMV RNA synthesis (Houwing and Jaspars, 1993; de Graaff *et al.,* 1995). The inability of CP synthesized by mutant ΔXR to rescue *in trans* the defective plus-strand accumulation of mutant ΔSA is not due to a deletion of *cis*-acting RNA sequences in mutant ΔSA. Also CP mutants with minor nucleotide substitutions (SP4, CPΔAUG, CPN63, CPN199) could not be rescued *in trans* by mutant ΔXR. It has been shown by De Graaff *et al.* (1995) that CP stimulates AlMV RNA 4 synthesis in an *in vitro* system. We propose that in an early step of the replication cycle CP is required *in cis* to stimulate RNA 4 synthesis and that once this stimulation has occurred, CP can be used *in trans* for a function in the replication of the genomic RNAs. Recently, we obtained evidence that the P1 and P2 genes are required *in cis* for the accumulation of RNAs 1 and 2, respectively (C. M. A. van Rossum, M.-L. García, and J. F. Bol, submitted for publication). This supports the notion that each AlMV genomic RNA encodes a *cis*-acting protein that can be used *in trans* for the accumulation of other genomic RNAs. Only the P3 protein may exclusively function *in trans.*

Cis-preferential replication of viral RNA has been demonstrated for several plus-strand RNA viruses and DI RNAs (de Groot *et al.,* 1992; White *et al.,* 1992; Weiland and Dreher, 1993; Wellink *et al.,* 1994; Novak and Kirkegaard, 1994). Several models to explain this phenomenon have been proposed (Novak and Kirkegaard, 1994). It is more difficult to envisage how a protein translated from a subgenomic mRNA could be required *in cis* for RNA replication. This would suggest a coupling between translation of a viral subgenomic RNA and replication of the genome segment from which the mRNA is transcribed. The P14 protein encoded by RNA 2 of the beet necrotic yellow vein furovirus is translated from a subgenomic mRNA. Recently, this protein was reported to be required *in cis* for the accumulation of BNYVV RNA 2 and *in trans* for the accumulation of CP (Hehn *et al.,* 1995). AlMV CP appears to be a second example of a *cis*-acting viral protein translated from a subgenomic mRNA.

RNA synthesis of many plus-strand RNA viruses has been found to be membrane-bound (for a review see Dreher and Hall, 1988). AlMV replication complexes, consisting of an RNA-dependent RNA polymerase (RdRp) and template RNA, are associated with the outer chloroplast membrane (de Graaff *et al.,* 1993; for a review see de Graaff and Jaspars, 1994). When P12 protoplasts become doubly infected with mutants ΔXR and ΔSA, each mutant could establish its own replication complex at the chloroplast membrane, using the transgenic replicase. Our results indicate that CP translated from RNA 4 of mutant ΔXR is able to modify RNA synthesis in the replication complex of this mutant but not in a distantly located replication complex of mutant ΔSA. However, if a mutant with a defective CP gene (CPΔAUG) was provided *in cis* with a wild-type CP gene (mutant CPΔAUGP2CP), asymmetric plus-strand accumulation was restored. Currently, we are investigating the ability of mutant ΔXR to upregulate plus-strand RNA synthesis of RNAs 1 and 2. If this is the case, the proposed *cis*-acting function of CP in RNA 4 synthesis is an obligatory step in RNA 3 replication or a difference in the replication strategy of the two larger and the smallest AlMV genome segment has to be envisaged.

Previously, we reported that plants transformed with the AlMV CP gene could be infected with a mixture of AlMV RNAs 1, 2, and 3 (van Dun *et al.,* 1987) but not with

a mutant with a deletion in the CP gene in RNA 3 (Dore *et al.,* 1991). Apparently, the transgenic CP could complement the function of CP in genome activation but not a function later in the replication cycle. Either the level of CP in the transgenic plant is too low to allow replication to proceed or this later step reflects a *cis*-acting function of CP that cannot be complemented *in trans.*

In agreement with previous results (van der Kuyl *et al.,* 1991c), the experiments shown in Fig. 5 indicated that mutants ΔXR and ΔSA could complement each other to some extent when co-inoculated on P12 plants. P3 and CP are both believed to have a role in cell-to-cell transport of AlMV (van der Vossen *et al.,* 1994) and the increased accumulation in plants could reflect complementation of transport rather than complementation of a defect in plus-strand RNA accumulation. However, recombination between the two mutants to full-length RNA 3 was consistently observed in these experiments and we cannot rule out the possibility that this recombination influenced the apparent complementation. Recombination is most probably the result of template switching of the RdRp. It could be that a nascent RNA chain with associated RdRp dissociates from an RNA 3 template in one replication complex and associates with a template in another replication complex. Recent results with BMV, however, support the notion that two templates must interact by base pairing to permit template switching of the RdRp (Nagy and Bujarski, 1995). This would imply a direct communication between different replication complexes. In protoplasts we have not observed recombination between AlMV RNAs, possibly because the time span of virus replication that is analyzed is too short. Multiple rounds of replication in P12 plants during a 5-day infection period could be required for the interaction between different replication complexes and such an interaction could also permit a complementation of the accumulation of mutant ΔSA by CP of mutant ΔXR. Although this model is rather speculative, the correlation between the occurrence of recombination and complementation seen in the plants co-inoculated with mutants ΔXR and CPTSV could be taken as support.

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