The second amino acid of alfalfa mosaic virus coat protein is critical for coat protein-mediated protection

(coat protein-mediated resistance/transgenic plants/crop protection)

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ABSTRACT Transgenic plants expressing the coat protein (CP) of alfalfa mosaic virus (AIMV) are resistant to infection by AIMV. A mutation was introduced into the second amino acid of the cDNA for the CP of AIMV. Three different transgenic tobacco lines expressing the mutant CP and two different transgenic tobacco lines expressing the wild-type CP at similar levels were challenged with AIMV virions and viral RNA. Whereas the lines expressing the wild-type CP were highly resistant to infection by AIMV virions and viral RNA, the lines expressing the mutant CP were susceptible to infection by both. The binding affinity of the mutant and the wild-type CPs for the 3' terminal protein binding site on AIMV RNAs was similar, as determined by electrophoretic mobility shift assay. A mixture of AIMV genomic RNAs 1–3 was infectious on the plants expressing the mutant CP but not on vector control plants or plants expressing the wild-type CP, indicating that the mutant CP can activate the AIMV genomic RNAs for infection. These results demonstrate that the second amino acid of the AIMV CP is critical for protection from AIMV but not for the initial interaction between the AIMV RNA and CP, suggesting that this initial interaction does not play a major role in CP-mediated protection.

Alfalfa mosaic virus (AIMV) is a tripartite plus-strand RNA virus that contains three genomic RNAs designated RNAs 1–3. RNA 4 is a subgenomic RNA derived from RNA 3 and encodes the coat protein (CP). Ilarviruses and AIMV differ from other plant viruses in that they form nonisometric, nonhelical nucleoprotein particles of size proportional to the RNA encapsidated and require CP to initiate infection in susceptible plants (1). If the three genomic RNAs of these viruses are inoculated onto leaves of host plants or on leaf protoplasts, no infection occurs unless RNA 4 or a few copies of the CP are added. The AIMV CP binds to specific sites near the 3' termini of AIMV RNAs and nonspecifically to other parts of the RNAs. It is postulated that binding of a few CP molecules to the 3' ends of viral RNAs is necessary for template recognition by the viral replicase (2). At later stages of infection, the interaction between the AIMV RNAs and CP is thought to act as a nucleation site for the encapsidation of AIMV RNAs. The specific binding of the AIMV CP to RNA and the ability of the protein to activate the RNA for infection are dependent on the presence of 25 basic amino acids at the N terminus of the CP. This region of the protein could be removed by treating the protein with trypsin (3). If AIMV CP has lost its N terminus, it can no longer bind to RNA at the specific sites.

It is thought that at the later stages of assembly there is an interaction between the AIMV RNAs and the CP. AIMV is

stabilized by RNA–protein interactions. Degradation of the RNAs *in situ* by RNase or mild digestion of the virion by trypsin leads to degradation of the virion structure. An important role has also been postulated for CP in regulating the balance between plus-strand and minus-strand synthesis during RNA replication (4, 5).

Recently, our group and several other groups have shown that transgenic plants expressing the CP of AIMV are resistant to infection by AIMV (6–8). Loesch-Fries *et al.* (7) and van Dun *et al.* (8) have reported that AIMV RNA is infectious on plants that expressed the CP. In addition, van Dun *et al.* (8) have shown that a mixture of AIMV genomic RNAs 1–3 are infectious on transgenic plants, indicating that the CP accumulating in the transgenic plants is biologically active and is able to associate with the incoming RNAs.

The CP cDNA used by the three groups included the untranslated leader from the CP of AIMV (RNA 4 leader), which has been shown to enhance the translational efficiency of chimeric mRNAs *in vitro* (9). To examine the effect of AIMV RNA 4 leader on translation *in vivo*, we deleted this sequence by engineering a *Nco* I site at the N terminus of the CP. Since the *Nco* I site changed the second amino acid of the CP, we made a CP vector that addressed solely the effect of this mutation. We studied the effect of this mutation *in vivo* by examining the susceptibility of transgenic plants expressing the mutant CP to infection by AIMV virions or viral RNA and *in vitro* by examining the interaction between the mutant CP and the AIMV RNA.

We demonstrate here that the second amino acid residue of the AIMV CP is critical for protection from AIMV virions and RNA. The mutant CP can still bind to AIMV RNA 4 *in vitro* and activate replication of the AIMV RNAs *in vivo*, indicating that the second amino acid is not critical for this interaction.

MATERIALS AND METHODS

Construction of Expression Vectors. The 881-base-pair *Eco*RI fragment encoding AIMV RNA 4 was previously cloned into the expression vector pMON316 between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase 3' end (pMON9800) (6). We have constructed another plasmid (pMON9822) by cloning the AIMV cDNA fragment into the vector pMON9818 between the CaMV 35S promoter and the pea ribulose 1,5-bisphosphate carboxylase (*rbcS*) E9 3' polyadenylation site (10). Plasmid pMON9874 was constructed by introducing an *Nco* I site at the N terminus of the AIMV CP in pMON9822. Plasmid

Abbreviations: CP, coat protein; AIMV, alfalfa mosaic virus; EMSA, electrophoretic mobility bandshift analysis; TMV, tobacco mosaic virus; PVX, potato virus X; CMV, cucumber mosaic virus; CaMV, cauliflower mosaic virus.

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pMON9862 was constructed by deleting the RNA 4 leader (36 nucleotides) and cloning the CP cDNA downstream of the CaMV 35S leader by using the *Nco* I site. The CP gene was flanked by the *rbcs* E9 polyadenylation site at the 3' end.

Oligonucleotide Site-Directed Mutagenesis. Site-directed mutagenesis was performed by using the method of Kunkel (11). Mutant plasmid was checked for the presence of the *Nco* I site, and the DNA sequence of the entire mutant CP cDNA was determined (12).

Plant Transformation. *Agrobacterium tumefaciens*, carrying the wild-type and modified AIMV CP vectors, was used to transform *Nicotiana tabacum* cv. Samsun leaf disks (13).

Detection of AIMV CP Transcripts. RNA was isolated from tobacco leaves and analyzed by electrophoresis on 1.3% agarose containing formaldehyde (14). The RNA was transferred to GeneScreen (New England Nuclear) and hybridized with a AIMV CP cDNA probe.

Detection of AIMV CP by ELISA. Tobacco leaf extracts from kanamycin-resistant plants were assayed for AIMV CP by a two-step ELISA (15). AIMV virion standards or purified CP standards were included on the plates at 0.2–50 ng per well.

Purification of Virus, Separation of Viral Components, and Preparation of Viral RNA. AIMV was propagated on *N. tabacum* cv. Samsun and purified 7–10 days postinoculation according to the procedure of van Vloten-Doting (16). Ta component was separated from other components by MgSO_4 precipitation (17). Final separation of the Ta component and the B, M, and Tb components was performed by 10–40% sucrose density gradient centrifugation. Gradients were centrifuged for 3 hr at 25,000 rpm in an SW28 rotor (Beckman).

AIMV coat protein was isolated from purified virus in a solution of 0.5 M MgCl_2 (18). RNA was prepared by phenol extraction and ethanol precipitation (19).

Plant Protection Assays. CP-expressing plants and non-transgenic control plants were inoculated mechanically with AIMV or AIMV RNAs in several cross-protection experiments (6). In each experiment, local lesions were counted on inoculated leaves, and systemic symptoms were scored. Virus levels in the plants were determined by double-antibody sandwich ELISA (20) after 1000-fold dilution of plant extracts. At this dilution, no reaction due to CP expressed in the transgenic plant was observed.

Electrophoretic Mobility Shift Assay Reactions. AIMV RNA 4 cDNA was subcloned downstream of the bacteriophage SP6 promoter, and mRNA transcripts were prepared by *in vitro* transcription (9) after linearizing the plasmid DNA at a unique *Bst*XI site to prevent transcription of the protein binding site of AIMV RNA 4. Radioactive proteins were generated by translating mRNA transcripts in an *in vitro* rabbit reticulocyte lysate translation system in the presence of [^{35}S]methionine. The CP translation products were separated from hemoglobin and other reticulocyte proteins by batch ion-exchange chromatography before incubation with AIMV RNA 4 fragments in the electrophoretic mobility bandshift analysis (EMSA) (21). The eluted protein solution was diluted with 50 mM Tris-HCl (pH 7.6) to a final NaCl concentration of 150 mM and then concentrated using a Centricon-10 microconcentrator (Amicon).

Plasmid DNA containing the AIMV RNA 4 cDNA (6) was digested with *Bst*XI, which cleaves the DNA in the 3' untranslated region immediately upstream of the CP binding site. The 3' fragment was excised and subcloned into a modified SP6 plasmid (22). Following the *in vitro* transcription, 5 pmol of the RNA was incubated with 200,000 cpm of wild-type CP or mutant CP (serine to glycine mutation) translation products in a 10- μl reaction containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 10 units of RNasin (Promega), 1 mM EDTA, 1.5 μg of yeast phenylalanine tRNA, and 5% glycerol. After a 10-min incubation at

22°C, the reaction mixture was loaded onto a 7.5% nondeaturing polyacrylamide gel (Mini-Protean, Bio-Rad).

RESULTS

AIMV Coat Protein Vectors. To determine the effect of the point mutation created by the *Nco* I site on CP-mediated protection, this mutation was introduced into the AIMV CP cDNA by site-directed mutagenesis, and the mutant CP cDNA was cloned into a plant expression vector. As shown in Fig. 1, plasmids pMON9800 and pMON9822 contain the wild-type AIMV CP cDNA; they only differ in their 3' polyadenylation sequences. Plasmid pMON9874 is identical to pMON9822 except that it contains the *Nco* I site, which changes the second amino acid of the CP from serine to glycine. Plasmid pMON9862 contains the same sequence change; in addition, the AIMV RNA 4 leader is deleted in this plasmid. The CP coding regions of pMON9822 and pMON9874 were sequenced and found to be identical except for the expected change at the *Nco* I site. Leaf disks of tobacco (*N. tabacum* cv. Samsun) were transformed with *A. tumefaciens* containing these vectors, selected for kanamycin resistance, and regenerated into plants.

Detection of Wild-Type and Mutant AIMV CP Transcripts. The presence of AIMV CP transcripts was examined by Northern blot analysis. As shown in Fig. 2, transcripts of the expected size, 1.2 kilobases, were present in two plants from R1 progeny of line 6309, containing pMON9800. Two plants from R1 progeny of lines 8542, 8755, 9735, and 9740 contained transcripts of the expected size (1.4 kilobases). Total RNA from control line 6918, transformed with the vector without the CP gene, did not show any transcripts homologous to the AIMV probe. The lower molecular weight transcripts observed in lines 6309 and 8542 were much less abundant in lines 8755, 9735, and 9740.

The predicted N-terminal nucleotide sequences of the CP transcripts in plants that contain the wild-type CP cDNA (pMON9800 and pMON9822) or the mutant CP cDNA (pMON9862 and pMON9874) are compared in Fig. 2. Interestingly, two nucleotide changes near the N terminus of the wild-type CP cDNA can significantly reduce the levels of the lower molecular weight transcripts in transgenic plants. These transcripts have been detected in transgenic plants (6, 7), and it has been shown that they are not polyadenylated (6). Their origin, as yet, remains undetermined.

Detection of Wild-Type and Mutant AIMV CP in Transgenic Plants. The R1 progeny from self-fertilized transgenic lines

FIG. 1. AIMV CP vectors. The N-terminal sequence of wild-type AIMV CP, the amino acids that are modified by site-directed mutagenesis, and the restriction sites that are created are indicated.

FIG. 2. (Upper) Analysis of AIMV CP transcripts in transgenic tobacco plants. Total RNA (40 μ g) isolated from tobacco leaves was fractionated on a 1.3% agarose gel containing formaldehyde, transferred to GeneScreen, and hybridized with an AIMV CP cDNA probe. RNA from two plants from the R1 progeny of lines 6309 (pMON9800), 8542 (pMON9822), 8755 (pMON9862), 9735 and 9740 (pMON9874), and 6918 (which contains the vector without the CP gene) was analyzed in each lane. Size markers (in kilobases) are indicated at right. STD, standards. (Lower) Comparison of the predicted N-terminal nucleotide sequences of the CP transcripts in plants that contain the wild-type CP cDNA (pMON9800 and pMON9822) or the mutant CP cDNA (pMON9862 and pMON9874).

6309, 8542, 8755, 9735, and 9740 were planted and maintained under greenhouse conditions. The levels of CP in all the different transgenic lines examined were similar by ELISA analysis (Table 1). Immunoblot analysis of the CP from progeny plants confirmed that the CP is expressed at similar levels among the different lines and indicated that the mutant CP was the same size as the wild-type CP (data not shown). The CP levels in the R1 progeny of different transgenic lines estimated by immunoblot analysis varied from 0.04% to 0.1% of total protein. Similarly, with other CP genes such as potato virus X (PVX) and cucumber mosaic virus (CMV), the level of CP expression estimated by ELISA was lower than the level estimated by immunoblot analysis (N.E.T. and W.K., unpublished data). The source of this variation is, as yet, unknown. It could be because the ELISA reaction is more specific than the immunoblot analysis. Nevertheless, both methods indicated that the CP levels were similar in the different transgenic lines analyzed.

Susceptibility of Plants Containing the Wild-Type and the Mutant CP to Infection by AIMV Virions and Viral RNA. Ten progeny from the transgenic lines 6309, 8542, 8755, 9735, and 9740 and the vector control line 6918, which was transformed with the vector without the CP gene, were inoculated with AIMV at 50 μ g/ml on two young leaves per plant. Ten days

postinoculation, the inoculated leaves and the second leaf above the inoculated leaves (systemic leaf) were sampled from each plant, and virus levels were quantitated by ELISA. As shown in Table 2, at 10 days postinoculation, vector control plants contained high levels of virus in their inoculated and systemic leaves. Progeny of 6309 and 8542, both of which contained the wild-type CP, were completely resistant to infection by AIMV. Progeny of 8755, 9735, and 9740, which contain the mutant CP, did not show any protection from viral infection. The differences observed in virus titers between the infected lines 8755, 9735, and 9740 and the control plants could be due to the position of the disk sampled from the leaves.

To determine the susceptibility of the transgenic plants to inoculation by AIMV RNA, we inoculated five progeny plants from each line with AIMV RNA at 50 μ g/ml. Previous titration of the inocula on a local lesion host for AIMV, *Phaseolus vulgaris*, indicated that RNA at 50 μ g/ml and purified virus at 50 μ g/ml had similar infectivity.

Ten days postinoculation with AIMV RNA, high levels of virus accumulated in the inoculated and systemic leaves of the control plants. Progeny of 6309 and 8542, which express the wild-type CP, were completely resistant to infection by AIMV RNA, whereas 8755, 9735, and 9740, which express the mutant CP, were completely susceptible to infection by the AIMV RNAs. ELISA values correlated very well with symptoms. Plants that contained viral antigen by ELISA showed symptoms, and plants that did not contain any viral antigen did not show symptoms. These results indicated that transgenic plants expressing the wild-type AIMV CP are resistant to infection by AIMV RNA.

Plants Expressing the Mutant CP Are Susceptible to Infection by AIMV RNAs 1–3. To determine if binding of both CPs to the AIMV RNAs will lead to the replication of genomic RNAs, we have fractionated the AIMV components, extracted RNA, and determined the infectivity of different RNA fractions on the transgenic plants. RNAs 1–4 or RNAs 1–3 were inoculated on vector control (6918), wild-type CP (8542), and mutant CP (9735 and 8755) plants.

After inoculation with AIMV RNAs 1–4, the control lines and the lines containing the mutant CP developed lesions on their inoculated leaves, whereas the CP⁺ plants did not develop any lesions. In general, more plants from lines containing the mutant CP developed lesions, and the numbers of lesions were higher on the inoculated leaves of these plants compared to the control plants. After inoculation with RNAs

Table 2. Susceptibility of transgenic tobacco plants to infection by AIMV virions and AIMV viral RNA

Vector/line	Level of AIMV in inoculated plants,* ng of virus per mg of tissue			
	AIMV virus inoculum [†]		AIMV RNA inoculum [‡]	
	Inoc. leaves	Syst. leaves	Inoc. leaves	Syst. leaves
pMON6918	402 $\pm$ 148	256 $\pm$ 104	193 $\pm$ 113	91 $\pm$ 124
pMON9800/6309	0	0	0	0
pMON9822/8542	0	0	0	0
pMON9862/8755	263 $\pm$ 87	110 $\pm$ 100	383 $\pm$ 96	140 $\pm$ 82
pMON9874/9735	1039 $\pm$ 490	444 $\pm$ 382	864 $\pm$ 587	558 $\pm$ 539
pMON9874/9740	324 $\pm$ 154	82 $\pm$ 83	392 $\pm$ 84	230 $\pm$ 96

Inoc., inoculated.

*The AIMV concentration was determined by ELISA 10 days postinoculation. Mean values $\pm$ SD (σ_{n-1}) are shown. Syst., systemic. The leaves were inoculated with AIMV virions or AIMV viral RNA at 50 μ g/ml.

[†]Data represent the average virus levels in 10 plants.

[‡]Data represent the average virus levels in 5 plants.

Table 1. Expression of AIMV CP in different transgenic tobacco lines

Vector/line	CP, ng/mg of protein
pMON9800/6309	4.4 $\pm$ 1.9
pMON9822/8542	4.6 $\pm$ 2.9
pMON9862/8755	5.1 $\pm$ 1.4
pMON9874/9735	4.8 $\pm$ 2.7
pMON9874/9740	5.4 $\pm$ 1.9

Data are expressed as the mean $\pm$ SD (σ_{n-1}) for 20–50 progeny assayed from each line. The amount of CP was determined by ELISA 2 weeks after transplanting.

1–3, control plants and transgenic plants containing the wild-type CP did not show any lesions, whereas the plants containing the mutant CP showed lesions on their inoculated leaves. Plants that showed lesions contained virus by ELISA (data not shown).

Systemic symptoms correlated very well with the local lesions in the lines analyzed (Table 3). The plants that had lesions on their inoculated leaves developed systemic symptoms. After inoculation with AIMV RNAs 1–4, a small percentage of vector control plants developed systemic symptoms, indicating that the RNAs purified from the isolated components of AIMV in this experiment were not very infectious. A higher percentage of plants expressing the mutant CP developed systemic symptoms after inoculation with the same RNAs. In contrast, vector control plants did not develop systemic symptoms after inoculation with AIMV RNAs 1–3, whereas the mutant CP plants did develop systemic symptoms. These results indicated that AIMV RNAs 1–3 are infectious on plants expressing the mutant CP. Since this requires the interaction between the AIMV RNAs and CP, the mutant CP is able to interact with the AIMV RNAs.

We confirmed these results by coinoculating the mutant and wild-type CPs made *in vitro* in a rabbit reticulocyte lysate system with AIMV RNAs 1–3 to control tobacco plants. RNAs 1–3 alone were not infectious on control plants. When they were combined with wild-type or mutant CP, they did infect the control tobacco plants as determined by symptom development and quantitation of virus levels by ELISA (data not shown). These results confirm that the mutant CP can activate AIMV genomic RNAs for infection.

Both Wild-Type AIMV CP and Mutant CP Recognize the CP Binding Site. The interaction of the wild-type and mutant AIMV CPs and the 3' terminal protein binding site on AIMV RNA 4 was characterized by EMSA. Four specific binding sites for the CP can be identified by EMSA by using a 3' terminal 180-nucleotide fragment of AIMV RNA 4 (L.G., K. Liem, and M. Baer, unpublished results). The results shown in Fig. 3 demonstrate that the RNA binding properties of the wild-type AIMV CP and the mutant AIMV CP are indistinguishable. CP synthesized *in vitro* was incubated with the CP binding site of AIMV RNA 4 before electrophoresis into a nondenaturing polyacrylamide gel. Lanes 1–4 of Fig. 3 represent addition of water-control translation reaction mixtures (without addition of exogenous mRNA template) to the mobility bandshift reactions. The absence of bands in lanes 1–4 of Fig. 3 demonstrates that the mobility bandshift is dependent upon proteins encoded by added AIMV CP templates and not upon nonspecific reticulocyte translation products. EMSA reactions analyzing the binding of wild-type and mutant CPs are presented in lanes 5–8 and 9–12, respectively, of Fig. 3. Lanes 5 and 9 of Fig. 3 are binding reactions containing radioactive wild-type CP (lane 5) or mutant CP (lane 9), but no RNA. If RNA is not added, the labeled translation products fail to enter the gel, as has been reported

FIG. 3. Binding of wild-type (wt) and mutant (mut.) CP molecules to the AIMV RNA 4 fragment. Nonradioactive RNA fragment was incubated with 35 S-labeled wild-type or mutant CP translation products. Lanes 1, 5, and 9, EMSA reactions were incubated in the presence of *in vitro* translation reaction products but without any RNA. Lane 1, water-control translation; lane 5, wild-type CP; lane 9, mutant CP. Lanes 2, 6, and 10, EMSA reactions containing water-control translation products, wild-type translation products, and mutant CP translation products, respectively. Lanes 3, 7, and 11, same as lanes 2, 6, and 10 except that 20 pmol of unlabeled viral CP was added. Lanes 4, 8, and 12, same as lanes 2, 6, and 10 except that 20 pmol of unlabeled human interleukin 1 β protein was added.

for other nucleic acid binding proteins (23). Lanes 6 and 10 of Fig. 3 show the mobility bandshift pattern resulting from addition of identical amounts of wild-type CP or mutant CP to the RNA fragment. At this concentration of CP, two specific bands are observed, with an intense rapidly migrating band and a fainter band with decreased mobility. Addition of purified interleukin 1 β protein (Fig. 3, lanes 8 and 12) has no effect upon the bandshift pattern, suggesting that the RNA-protein complexes observed are specific.

The appearance of the multiple bands in this assay is dependent upon both the molar excess of protein to RNA and the concentration of macromolecules (L.G. and M. Baer, unpublished results). Increasing the CP concentration by addition of virion CP (Fig. 3, lanes 7 and 11) results in a further shift of ribonucleoprotein complexes from the fast migrating band to the slower migrating band, which is also present in lanes 6 and 10 of Fig. 3. The bands present in lanes 7 (wild-type CP) and 11 (mutant CP) of Fig. 3 comigrate; moreover, the increased intensity of the slower migrating bands suggests that both CP translation products are capable of interacting with virion protein to form protein dimers (2).

DISCUSSION

We tested several different transgenic tobacco lines, expressing similar levels of wild-type or a mutant AIMV CP with a serine to glycine change at its second amino acid, for susceptibility to AIMV. We demonstrate here that plants expressing the mutant AIMV CP are susceptible to infection, whereas plants expressing the wild-type CP are nearly immune to infection by AIMV virions and viral RNA. To confirm that this mutation is responsible for the loss of protection, we determined the entire nucleotide sequence of the mutant CP in the plant expression vector and analyzed several independent lines containing pMON9862 and pMON9874 in protection tests. In each case, plants expressing the mutant CP were susceptible to infection by AIMV. These results indicate that the susceptibility of the lines containing the mutant CP is due to the *Nco* I mutation alone and not to any other mutation.

The dramatic difference in the susceptibility of these lines as a result of a single amino acid change is highly unexpected. A serine to alanine change in a similar position of the CP gene of soybean mosaic virus still confers protection in transgenic plants (24). In addition, plants expressing the CP of one virus

Table 3. Effects of inoculation of transgenic tobacco with AIMV RNAs

Inoculum	% plants showing systemic symptoms			
	pMON6918	pMON9822/ 8542	pMON9874/ 9735	pMON9862/ 8755
RNAs 1–4	12.5	0	75.0	50.0
RNAs 1–3	0	0	87.5	37.5

Progeny of tobacco plants from line 8542 (wild-type CP) and lines 9735 and 8755 (mutant CP) and vector control plants (pMON6918) were inoculated with AIMV RNAs 1–4 or AIMV RNAs 1–3 extracted from isolated AIMV components. Each treatment consisted of eight plants, and inoculum (50 μ g/ml) was used on two leaves per plant. The percentage of plants showing systemic symptoms was determined 14 days postinoculation.

can show protection against different strains of the same virus (25) or against different but related viruses (24, 26). One such example is the protection observed in plants expressing the tobacco mosaic virus (TMV) CP to tomato mosaic virus, which has a CP only 80–85% homologous in its amino acid sequence to TMV CP (26).

In contrast to previous reports, which indicate that the protection in CP⁺ plants is overcome when the inoculum is AIMV RNA (7, 8), our CP⁺ plants were protected to the same extent whether virus or viral RNA was used as inoculum. The CP concentrations in the transgenic lines estimated by ELISA analysis were significantly lower than the concentration estimated by immunoblot analysis, suggesting that these values are highly dependent on the particular assay and the conditions used. Thus, it is not clear if the difference between our results and those published is due to the levels of CP expressed in the transgenic plants or to other factors such as the different strains of AIMV used in the experiments (7, 8).

Similarly, it has been shown that inoculation with PVX RNA (19) or with CMV RNA (27) does not overcome CP-mediated resistance. In contrast, it has been reported that TMV RNA and preswelled TMV particles partially overcame the resistance in CP⁺ plants and protoplasts (28). These results indicated that the TMV CP might be blocking viral uncoating. Although in the CP⁺ plants expressing the AIMV or PVX CP there may be a block in viral uncoating, the consistent resistance against viral RNA implies another mechanism of resistance that acts after the uncoating event.

We have shown that both wild-type and the mutant CPs can bind the 3' untranslated region of AIMV RNA 4. Furthermore, plants expressing the mutant CP can be infected by a mixture of AIMV RNAs 1–3, whereas these RNAs are not infectious on control plants. Therefore, it appears that the CP accumulating in the transgenic plants is biologically active and is able to associate with the incoming RNAs. Although it is still possible that the interaction at the 3' untranslated region of the RNAs might be necessary for protection, this interaction alone is not sufficient to confer resistance.

It is possible that an interaction between the CP and the 5' untranslated region of RNAs could be responsible for resistance. AIMV CP may be binding to the 5' ends of the AIMV RNAs and regulating the translation of the replicase genes and/or replication of the RNAs. The CP of several RNA bacteriophage can repress translation of their RNAs (29). It has been shown that AIMV CP blocks the *in vitro* transcription of the virion RNAs of AIMV (30). If this involves an interaction with the RNA, the mutant CP might not undergo this interaction.

Alternatively, it is possible that protein–protein interactions involved in encapsidation of viral RNAs could play a role in AIMV resistance. The results of the binding experiments indicate that the mutant CP can dimerize, since each one of the bands in the bandshift reaction represents binding of a dimer to the CP (L.G., unpublished results). However, if partial assembly of the CP molecules expressed in the plant is required for resistance, the mutant CP might not confer resistance if it is unable to undergo partial reassembly in transgenic plants. The CP isolated from the virion starts with acetylated serine (31). This modification could play a role in CP assembly. Although glycine can undergo acetylation, this modification is not very common (32). Therefore, the mutant CP might not be conferring protection because it is not acetylated.

Availability of transgenic plants expressing mutant CPs allowed us to identify a specific residue in CP that is critical for protection. Availability of infectious transcripts of AIMV RNAs will allow further investigation of the effects of this mutation on virus replication and assembly and will help elucidate the mechanism of CP-mediated resistance against AIMV in transgenic plants.

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