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Transgenic Tobacco Plants Expressing a Truncated Form of the PAMV Capsid Protein (CP) Gene Show CP-Mediated Resistance to Potato Aucuba Mosaic Virus

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Four constructs of the potato aucuba mosaic virus (PAMV) coat protein (CP) gene were engineered for expression in tobacco plants: The full-length CP sequence (FL CP), two truncated forms—one at the N terminus (46 amino acid residues are deleted) (NT138), one in the conserved core portion (86 amino acids deleted) (CT258) of the gene—and an antisense RNA construct. These constructs were introduced into tobacco plants (*Nicotiana tabacum*) via *Agrobacterium tumefaciens*-mediated transformation. The plants transformed with the NT138 and FL CP constructs produced the mRNAs and proteins from the respective transgene. Transformants with the CT258 construct produced the transgenic mRNA, but the modified CP was not detected in the 20 different transformants tested. Transgenic R_0 and R_1 tobacco plants expressing the full-length, CT258, and the antisense constructs exhibited protection to PAMV infection and a delay in symptom development when inoculated with 0.1 and 0.5 μg/ml of purified PAMV. Transgenic plants expressing the NT138 construct did not confer any detectable protection to PAMV infection. These results suggest that an engineered coat protein mediated resistance (CPMR) can be obtained from a CP gene truncated in its core region. The role of the N-terminal domain of the CP in the CPMR of PAMV and the implication of either the RNA or the protein in the protection is discussed.

Additional keywords: genetically engineered cross-protection.

Potexviruses are flexuous, rod-shaped plant viruses characterized by a single-stranded positive sense RNA genome (approximately 6,000–7,000 nt), a m^7GpppG cap structure at the 5′ terminus (AbouHaidar and Bancroft 1978), and a poly(A) tail at the 3′ end (AbouHaidar 1988). Members of this group have RNA genomes with five open reading frames (ORFs). ORF1 encodes the viral replicase (Sit *et al.* 1989); ORF2, 3, and 4 are involved in viral movement (Beck *et al.* 1991); and ORF5 encodes the structural viral coat protein (AbouHaidar 1988).

Genetically engineered resistance to a plant virus was first reported by Powell *et al.* (1986), who observed a delay in symptom development when transgenic tobacco plants expressing the tobacco mosaic virus (TMV) coat protein gene were inoculated with TMV. This type of resistance is now known as coat protein-mediated resistance (CPMR) and has been demonstrated to be effective for the tobamo-, tobra-, carla-, poty-, luteo-, and alfalfa mosaic virus groups (for reviews see Beachy *et al.* 1990; Hull and Davies 1992; Kawchuk *et al.* 1990, 1991). A nucleoprotein-mediated protection has also been described for a minus-strand RNA virus (tomato spotted wilt) (Gielen *et al.* 1991; MacKenzie and Ellis 1992).

In the potexvirus group there are reports of CPMR against potato virus X (PVX) (Hemenway *et al.* 1988), cassava common mosaic virus (CCMV) (Fauquet *et al.* 1991), and cymbidium mosaic virus (CyMV) (Chia *et al.* 1992). It was suggested that the PVX CP expressed in transgenic plants can interfere with viral replication (Hemenway *et al.* 1988) by interacting with the PVX origin of assembly (OAS) which is presumably located at the 5′ end of the RNA as was shown for papaya mosaic virus (PMV) assembly (Sit *et al.* 1994). The mechanism of CP protection is not well understood. However, it remains possible that other mechanisms for CPMR to potexviruses are involved. Wilson (1989) suggested that the reassembly of the virus might not be the only mechanism of CPMR. Here we describe the effect of large deletions in the CP gene of PAMV on its capacity to provide protection. We report that a deletion of 258 nt (86 amino acid residues) in the "core" of the CP did not affect its ability to protect against viral infection in transgenic tobacco plants and that resistance was conferred by an antisense construct. However, a deletion of 138 nt (46 amino acids residues) which was generated at the N terminus of the CP seems to affect its capacity to protect against viral infection. These data illustrate the potential of both RNA and protein-mediated resistance mechanisms in transgenic plants.

RESULTS

Analysis of transgenic plants.

Several constructs were produced containing the gene for the PAMV CP coupled to the CaMV 35S promoter. These constructs included a full-length gene for two PAMV CP in both sense (FL CP) and antisense orientation (Fig. 1). There

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were also two sense orientation constructs with in-frame deletions at the N terminus (NT138) or C terminus (CT258) of the CP gene (Fig. 1).

Transgenic plants expressing constructs with the PAMV CP sense orientation were assayed by Western blot analysis using polyclonal antisera raised against SDS-denatured PAMV CP. The FL CP was detected in 10 different lines of transgenic tobacco plants and the NT138 protein was detected in nine different lines. Representative data from two lines expressing the FL CP and NT138 protein are shown in Figure 2A. A strong band which reacted with the antibody was also visible in all samples, including the controls (Fig. 2A). The nature of this band is assumed to be of cellular origin.

The CT258 protein was not detectable by Western blot analysis in the 20 different transformants analyzed. To determine if the polyclonal antibody raised against PAMV coat protein recognizes the CT258 protein, this protein was expressed in *E. coli*. Two expression vectors derived from the expression plasmid pP_1R_9 (Ivanov 1990) were constructed. One vector contains the CT258 (pP_1R_9CT258) and the other contains the full capsid protein of PAMV (pP_1R_9PAMV CP). After 12 hr of growth at 37° C, *E. coli* cells (strain LE392) were lysed and total bacterial proteins analyzed by 12% SDS-PAGE followed by Western blotting.

The results show that the polyclonal antibody recognizes the CT258 protein and, as expected, the PAMV coat protein (Fig. 2B). Bands corresponding to the expected molecular weights (18.5 and 24 kDa, respectively) for CT258 and capsid protein were obtained (Fig. 2B). The CT258 protein seems to be stable in *E. coli* for at least 24 hr of bacterial growth (data not shown). Thus the failure to detect the CT258 protein in transgenic plants is not because the C-terminal deletion has removed the immunoreactive part of the protein. It is believed that the CT258 protein does not accumulate in transgenic plants.

To determine whether the failure to accumulate transgenic CT258 protein is due to a low level of the respective mRNA, the RNA of transgenic plants was analyzed by Northern blotting with PAMV-specific probes. The RNA samples tested were total RNAs from two lines expressing each of the antisense, CT258, NT138, and full-length PAMV CP constructs. The transgenic RNA was detected at similar levels in each of the lines (Figs. 2C and D). It is therefore ruled out that the low level of CT258 protein in the plants is due to low level mRNA accumulation.

Protection assays.

The virus protection assay was carried out with two or more lines for each construct. These lines were selected as high-level transgene expression and were propagated either by seeds or cuttings. When the plants were propagated by seeds, the expression of the transgene was verified by Western blotting for the lines expressing the full-length PAMV CP and the NT138 constructs. In all cases, the level of transgene expression was comparable to the mother plant from which the seeds originated. Plants propagated by seeds and used for the protection assays with the CT258 construct were verified by a neomycin phospho-transferase II (NPTII) test and by Western blotting using polyclonal antibodies raised against SDS denatured PAMV CP. The variations in the level of expression of the transgene in the CT258 plants are unknown.

Plants in the tests of PAMV resistance were inoculated with the same preparation of purified virus. To determine the level of the viral infection, we performed a dot blot hybridization on leaf disks taken from inoculated and uninoculated upper leaves and hybridized with α-[^{32}P]dATP-labeled random primer probe specific to PAMV CP gene. The results obtained are detailed in Table 1 and plotted in summary form in Figure 3. These data showed that several lines were resistant to PAMV. The level of resistance of plants originating from cuttings was more consistent between the different experiments than plants grown from seeds.

The resistance was most pronounced with an inoculum of 0.1 µg/ml of PAMV virions. Thirty days after inoculation with this dilute inoculum in the experiment C^1 and 31 days for the experiment S^1, the plant lines expressing the FL CP showed a high degree of resistance. However, the resistance was not complete: Three plants accumulated the virus at the same level as control plants. Similar results were obtained

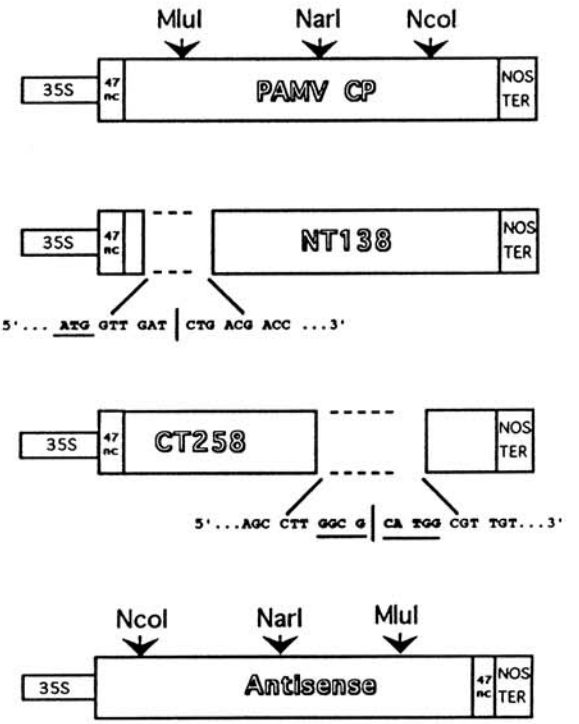

Fig. 1. Forms of the potato aucuba mosaic virus (PAMV) coat protein (CP) gene inserted into *Nicotiana tabacum* (cultivar Samsun). All constructs were cloned by insertion of the PAMV cDNA between the *Sma*I and *Sac*I sites located between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthetase (NOS) polyadenylation site in the shuttle vector POLYROK. The resulting plasmids were used to generate transgenic plants that could express a PAMV FL CP, two shortened forms: one in the N terminus where 138 nucleotides have been deleted after the first nine nucleotides of the PAMV CP open reading frame (NT138), and one in the core of the CP between nucleotides 6586 and 6844 (CT258), and an antisense construct identical to the PAMV CP but cloned in the opposite orientation. The nucleotide sequence in the region of mutation is shown. The junction of the fusions is shown by a vertical bar. The underlined nucleotides are those left from the *Nar*I and *Nco*I sites after ligation or the ATG start codon of the PAMV CP gene. 35S, CaMV 35S promoter; 47 nc, represents the 47 nucleotides found before the ATG start codon of the PAMV CP containing the ribosome binding site and a part of the 8 kDa protein. Arrows indicate the location of the restriction sites along the PAMV CP open reading frame and dotted lines represent deleted areas.

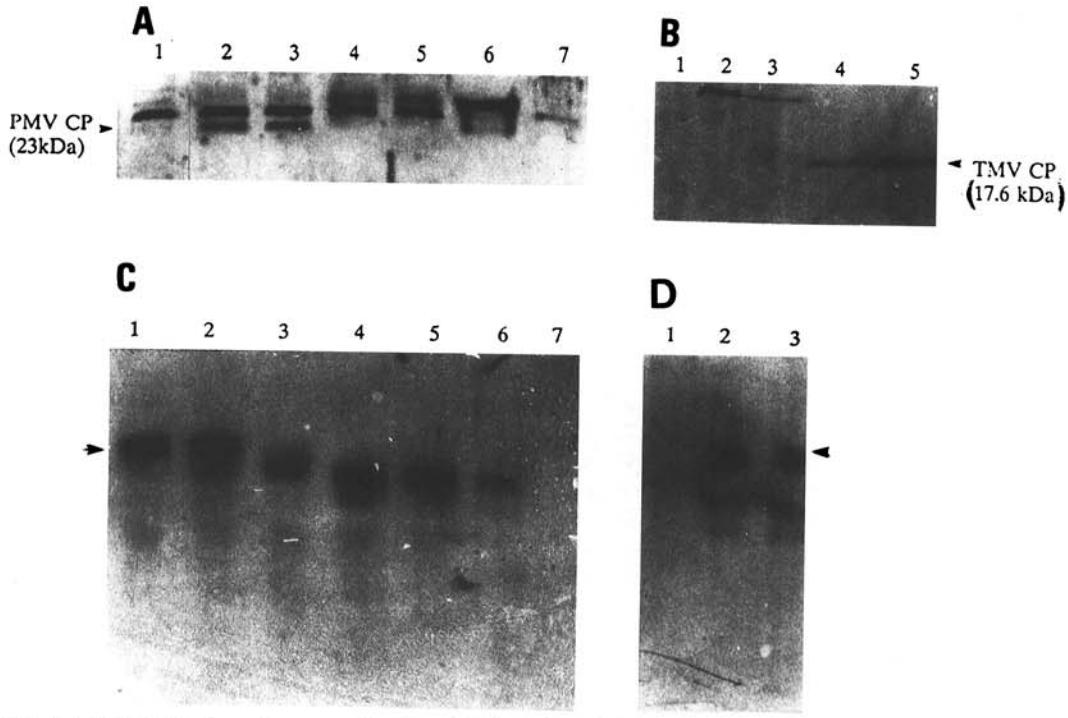

Fig. 2. Expression of the full-length, the antisense, and the two deleted versions of the potato aucuba mosaic virus (PAMV) coat protein (CP) gene in transgenic tobacco plants and expression of the full-length (FL) PAMV CP gene and the deleted version CT258 in *Escherichia coli*. **A,** Western blot analysis of PAMV CP and the NT138 construct. Samples of the transgenic and control plants (20 µg of proteins) were subjected to SDS-PAGE, and immunoblots were labeled with a polyclonal anti-PAMV CP antibody followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Lanes 1 and 7 contain 20 and 10 µg of proteins from nontransformed tobacco plants; lanes 2 and 3 contain proteins from the plants 2.4–55 and 2.4–36 expressing the NT138 protein; lanes 4 and 5 contain proteins from the plants 9.14 and 9.5 expressing the PAMV FL CP; lane 6 contains 10 ng of purified PAMV. Two micrograms of the purified CP of papaya mosaic virus (PMV) was used as a protein size marker (23 kDa) (indicated by the arrow) and comigrated with NT138 of an estimated size of 22.8 kDa. **B,** Western blot analysis of PAMV CP and the CT258 construct. Bacterial samples (5 µg of total proteins) were subjected to SDS-PAGE and processed as before. Lane 1 contains the proteins from bacteria with the P_1R_9 expression vector without the CP; lane 2 contains 3 ng of purified PAMV; lane 3 contains the PAMV FL CP overexpressed in *E. coli*; lanes 4 and 5 contain 5 and 15 µg of total proteins extracted from *E. coli* overexpressing the CT258 truncated protein. Two micrograms of tobacco mosaic virus (TMV) were used as a protein size marker (17.6 kDa) (indicated by an arrow) and comigrated with CT258 of an estimated size of 18.2 kDa. **C,** Accumulation of mRNA of PAMV CP gene constructs in transgenic tobacco plants. RNA gel was loaded with 20 µg of total RNA isolated from leaves of transgenic and non-transformed plants and blotted on a nylon membrane followed by hybridization with a ^{32}P-labeled riboprobe specific for plus-sense CP transcripts. After washing under stringent conditions (65° C in 0.1× SSC, 1 hr), filters were exposed overnight to X-ray films. Lanes 1 and 2 contain RNA from plants 9.14 and 9.5 expressing the PAMV FL CP; lane 3 contains RNA from plants 2.4–55 expressing the truncated CP NT138; lanes 4 and 5 contain RNA from plants NN.4-1 and NN.E expressing the shortened CP CT258; lane 6 contains 5 µg of total RNA of plant NN.4-1; lane 7 contains RNA from a nontransformed tobacco plant. Arrow indicates position of an RNA transcript of PAMV CP gene. **D,** Same as C, except that a riboprobe specific for antisense transcripts was used. Lane 1 RNA from a non-transformed tobacco plant; lanes 2 and 3 RNA from plants 10.B and 10.5 expressing the antisense transcript of the CP. Arrow indicates the position of an RNA transcript of PAMV CP gene.

with plants expressing the CT258 construct inoculated with 0.1 µg /ml PAMV (Table 1). Transgenic plants expressing the NT138 protein responded to this dilute inoculum in much the same way as the non-transgenic control plants. There was complete resistance to the dilute inoculum in eight plants representing two lines transformed with the antisense construct (Table 1 and Fig. 3). More than 90% of the infected plants expressing either CT258, antisense, or FL CP did not show symptoms, even those plants which contained as much virus as the non-transgenic control plants. Twenty eight days after inoculation with a more concentrated inoculum of 0.5 µg/ml of PAMV virions (experiments C^3,C^4, and S^2, see Table 1), plant lines expressing the FL CP, CT258, and antisense constructs were showing moderate protection to viral infection. As with the more dilute inoculum (0.1 µg/ml), no resistance was observed in transgenic plants expressing the NT138 protein (Table 1 and Fig. 3). All non-transgenic control plants showed typical mild symptoms under the same conditions.

Twenty one days after inoculation with 1 µg/ml of virions, most (all but two) transgenic plants expressing either NT138, CT258, or the full-length CP (experiments C^5 and S^3, Table 1) were accumulating PAMV at the same level as control plants. However, a delay of 7 days in appearance of symptoms was observed with the lines expressing the full-length PAMV CP and the CT258 protein. Plants expressing the antisense construct still showed resistance at this inoculum concentration.

The level of infection in transgenic plants was variable. In general, inoculated transgenic plants were either as heavily infected as the controls or entirely free of virus. We observed only few plants (indicated as low infection in Table 1) accumulating a lower level of virus in comparison with control plants. However, infection of the controls was always uniform from one plant to another, implying that variation in transgenic plants was not due to inconsistencies in the techniques of inoculation and the detection of PAMV.

Table 1. Susceptibility of transgenic plant lines to potato aucuba mosaic virus (PAMV) infection[a]

Plant line	PAMV concentration		
	0.1 µg/ml	0.5 µg/ml	1 µg/ml
Full-length PAMV CP		Infectivity[b]	
9.14	$C^1(0/6) + S^1(3/9) = 3/15$	$C^3(1/3) + C^4(0/2) + S^2(6/12) = 7/17$; C^3(1/3 L.I.)[c]; S^2(1/12 L.I.)	$C^5(5/6) + S^3(10/10) = 15/16$
9.5	ND	3/6	ND
NT138 protein			
2.4-55	$C^1(5/6) + S^1(4/5) = 9/11$	$C^3(4/5) + C^4(5/5) = 9/10$	$C^5(3/3)$
2.4-36	ND	$C^3(4/4)$	$C^5(2/2)$
2.4-24	ND	$C^3(2/2)$	ND
2.4-25	ND	$C^3(2/2)$	ND
CT258 protein			
NN.4-1	$C^1(0/4) + S^1(5/14) = 5/18$	$C^4(4/8 + S^2(4/8) = 8/16$; S^2(1/8 L.I.)	$C^5(3/4) + S^3(6/6) = 9/10$
NN.E	$C^2(0/2)$	$C^3(1/5)$	ND
Antisense			
10.B	$C^1(0/2) + C^2(0/4) = 0/6$	$C^3(4/8) + C^4(2/6) = 6/14$	$C^5(2/4) + S^3(5/10) = 7/14$
10.5	C2(0/2)	C3(3/4)	ND
Samsun (–) control	$C^1(4/4) + C^2(5/6) + S^1(5/5) = 14/15$	$C^4(2/2) + C^3(4/4 + S^2(4/4) = 10/10$	$C^5(4/4) + S^3(6/6) = 10/10$

[a] Transgenic and nontransgenic plants were mechanically inoculated with approximately 200 µl of pure virus diluted at different concentrations.
[b] Number of plants infected with PAMV over total number of inoculated plants. Infectivity was determined by dot blot hybridization using ^{32}P-labeled PAMV specific probes.
[c] Low infection (L.I.) is noted when hybridization signal is negative at plant sap dilution of 1/128 and 1/512 (fully infected plants showed signals at all dilutions. ND = not determined. C^n = Plants grown from cuttings. S^n = Plants grown from seeds. n = Number of repeated experiments.

DISCUSSION

Our study shows that resistance to PAMV infection was observed with inocula of 0.1 and 0.5 µg/ml of PAMV virions in transgenic tobacco plants expressing the FL CP, antisense, and CT258 constructs. Expression of the NT138 protein in transgenic plants did not confer resistance under the same conditions. The strongest resistance was observed in a line expressing an antisense construct. This resistance was partly effective at an inoculum density of 1 µg/ml, which was sufficient to overcome the resistance in lines expressing FL CP or CT258.

Observed CPMR for the FL and CT258 constructs was efficient only at relatively low inocula (0.1 and 0.5 µg/ml). Even at a low inoculum concentration, the virus occasionally infected plants expressing the FL CP and CT258 construct. Once the infection started, these plants accumulated the virus to the same level as the controls. This confirms that an early event in viral infection is critical for the mechanism of protection as proposed by Beachy *et al.* (1990).

The CPMR observed with the constructs FL CP and CT258 may be due to the capsid protein which could reassemble with the viral RNA as was suggested by Hemenway *et al.* (1988) for PVX. However, the model for reassembly poses some problems in cases where the capsid protein is not detectable in transgenic plants. Protection was observed when the CP was not detectable by Western blotting for potato leafroll (Kawchuk *et al.* 1991; van der Wilk *et al.* 1991), PVY (Farinelli and Malnoe 1993), and with the TMV 54-kDa gene (Golemboski *et al.* 1990). Carr *et al.* (1992) demonstrated that the untranslatable sequence of the 54-kDa protein failed to provide resistance, indicating that the protein, and not the RNA, was involved in protection. Using a similar strategy, Powell *et al.* (1990) showed that the TMV CP, and not RNA, was necessary for resistance to TMV in transgenic plants. Farinelli and Malnoe (1993) described protection to PVY^n infection with a construct expressing the CP of PVY^n which was fused to a part of the NIb gene (putative replicase of

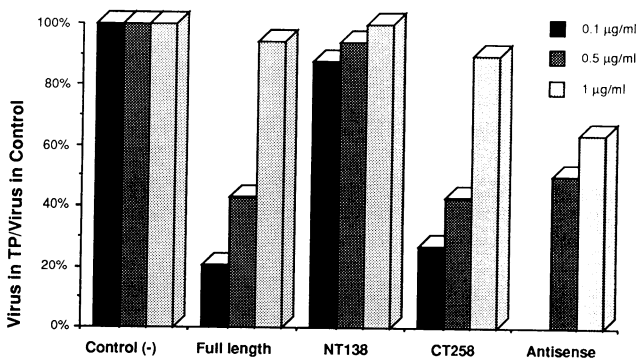

Fig. 3. Accumulation of PAMV in transgenic and control plants inoculated with different concentrations of virus (0.1, 0.5, and 1 µg/ml). The accumulation of virus was assayed 28–30 days after inoculation. The average virus levels in transgenic plants are presented relative to those in the control plants (see Table 1). For easy comparison, the value for each construct is depicted as a percentage of the control (100%). Results from two lines tested for each construct were pooled. Control (–): non-transformed *Nicotiana tabacum* (cultivar Samsun); full length: transgenic plants expressing the full-length PAMV CP; NT138: transgenic plants expressing the PAMV CP truncated of 46 amino acids at the N terminus; CT258: transgenic plants expressing the PAMV CP truncated of 86 amino acids in the core of the CP; the antisense: transgenic plants expressing the antisense of the PAMV CP which includes sequences corresponding to the 3′ noncoding region of viral RNA. TP = Transgenic plants.

PVY) even if the CP could not be detected. In some susceptible lines of transgenic plants, the CP became detectable when inoculated with a serologically different PVY^o strain. They suggested that the assembly of the invading virus was possibly stabilizing the CP produced in the transgenic plants.

The CT258 protein was not detected in transgenic plants because the protein may be unstable in plants, or the amount of CT258 produced was below the detection level of Western blotting. The possibility that the polyclonal antibodies could not recognize the truncated protein (CT258) was eliminated because the CT258 protein was easily detectable by Western

blotting in protein extracts of bacteria containing pP_1R_9CT258. Northern blots (Fig. 2C) indicated that the number of transcripts found in CT258 transgenic plants was comparable to that found in either the full-length CP or the NT138 transgenic plants. The non-coding region found upstream of the start codon and the sequence around the first methionine of the CT258 ORF were identical to that of the PAMV FL CP and NT138, which were detected in transgenic tobacco plants. If the FL CP or the NT138 protein could be translated in transgenic plants, the CT258 should also be translated and similar amounts of protein should have been detected. It is likely that the CT258 protein was unstable in the transgenic plants, making its detection by Western blotting difficult. The deletion of a region in the "core" of the coat protein may have perturbed the folding into the shell domain as suggested by Bendena *et al.* (1987).

The mechanism of protection for the CT258 construct is still unclear. The CT258 protein, its mRNA, or a combination of both could be responsible for protection. If the protein is responsible for protection, in this case reassembly of the virus by the expressed CP is unlikely to be the mechanism of protection, because the CT258 protein has a deletion in a region believed to interact with the viral RNA (Baratova *et al.* 1992) and known to be essential for the infectivity of PVX (Chapman *et al.* 1992) or PMV (Sit and AbouHaidar 1993). It is unlikely that a CP missing 86 amino acids from the core, can self-assemble with the viral RNA. Furthermore, this deleted region of the capsid protein may also be responsible for the protein subunit interactions required for the formation of disks which are required not only for assembly, but also for the stability of the protein. The possibility that the resistance obtained from CT258 resulted from an affinity of the CT258 protein to the 5′ end of the viral RNA inhibiting the translation of the polymerase gene can not be excluded.

If the CT258 protein was responsible for protection, these results suggest that the N terminus of the PAMV CP is the active domain of the protein which is involved in the CPMR, as the removal of this region in the NT138 construct seems to abolish protection in the transgenic plants tested. The failure to see resistance in transgenic lines expressing the NT138 protein may possibly be due either to the small number of transgenic lines analyzed in our study or to the fact that the N terminus of the CP plays an important role in the CPMR. Similar conclusions related to the role of the N terminus in the CPMR were drawn with alfalfa mosaic virus (AlMV) when the second amino acid of the CP was mutated from serine to glycine. Plants that expressed the mutant CP were susceptible to infection by AlMV (Tumer *et al.* 1991). These data concur with our conclusion related to the role of the N terminus of the CP in the protection process.

The N terminus of potato virus X (PVX) CP is known to be exposed outside the virion (Baratova *et al.* 1992; Chapman *et al.* 1992). A PVX mutant missing eight amino acids at the N-terminal domain showed a delayed infection and milder symptoms on tobacco (Chapman *et al.* 1992). We suggested that the interaction of this region with a plant component may help facilitate the virion production, or the stabilization of the virion in the wild-type CP has an effect on the development of symptoms. This experiment implicates a plant component in an interaction with the viral CP as has been proposed by van Dun and Bol (1988) with tobacco rattle virus (TRV). Similar type(s) of interaction may be involved in CPMR with PAMV. We suggest that such interaction could be blocked by an expressed protein in transgenic plants containing the N terminus of the viral CP leading to the attenuation of the viral infection. However, when the inoculum is increased, the virions compete for the available sites, leading to a successful infection of such plants. However, the nature of such a plant component is unknown.

If CT258 RNA is involved in protection, the sequence involved in this protection should be located in the 138 nucleotides deleted in the NT138 construct. Sense RNA sequences are usually believed to interfere with viral replication by hybridization with the minus-strand template (Lindbo and Dougherty 1992b). It is difficult to conceive this mechanism in our case with the CT258 construct because the NT138 construct should have also been efficient in providing protection, since it contains a larger portion of the PAMV CP RNA than the CT258 construct. However, as mentioned before, a large number of transgenic lines should be tested to confirm the total absence of resistance in the NT138 construct.

Lindbo and Dougherty (1992a, 1992b) demonstrated that the untranslatable transcripts of tobacco etch virus (TEV) CP gene and its antisense were more efficient in protection than the FL or the truncated versions of the TEV CP. It is, however, unclear why the RNA of the translatable TEV CP could not be as efficient as the untranslatable TEV CP in protection. Van der Vlugt *et al.* (1992) showed that the (+) sense nontranslatable RNA of the PVY CP was as efficient as the translatable construct in protecting against PVY. However, the PVY CP could not be detected in the translatable construct. The CP of PVY with a frameshift mutation that generated a stop codon 14 amino acids downstream of the first amino acid, still provided resistance but to a smaller extent than the FL CP construct (CP was undetectable; Farinelli and Malnoe 1993). This was in contradiction to the results of Lindbo and Dougherty (1992b) and van der Vlugt *et al.* (1992). For the potyvirus group, it seems that the (+) sense RNA of the CP is involved in the protection observed in the transgenic plants. To verify this phenomenon in the potexvirus group, we are currently generating transgenic plants expressing an untranslatable PAMV CP where the ATG start codon has been mutated to an amber stop codon to determine if the CP RNA or protein are involved in CPMR.

Transgenic plants expressing the antisense of the CP showed similar resistance to 0.1 and 0.5 µg/ml of virions as the FL CP or the CT258 constructs. However, the protection observed at 1 µg/ml was better than the FL CP or CT258 constructs. Protection using antisense construction has already been observed for several plant viruses. The protection observed with the antisense construct is usually weaker than the CP-mediated protection. Our results show that protection with the antisense was at least as good as PAMV CP. The mechanism of this protection is unknown, but two models may be suggested. First, the insertion of the antisense sequence in the vicinity of a plant promoter may induce the production of a translatable (+) sense transcript. The production of FL CP could then be responsible for the observed protection. However, Western and Northern blot analyses were unable to detect the presence of PAMV CP or (+) sense transcript in the plant lines 10.B and 10.5 expressing the antisense RNA (data not shown). Second, the antisense RNA

may bind to the 3′ end of the incoming virus, preventing negative-strand synthesis as suggested by Powell *et al.* (1989).

In summary, we have developed a series of transgenic plant lines that express various forms of the PAMV CP sequence. The FL CP, the antisense, and the CT258 constructs show genetically engineered protection to PAMV. However, the NT138 construct does not show protection, suggesting that the active element in CPMR is either at the N terminus of the CP or at the 5′ end of the CP open reading frame. This is the first report showing that a CP gene truncated in the core of its sequence can still provide resistance. This work lays the ground for future studies to determine more precisely which amino acid(s) or RNA sequence(s) of the PAMV CP are involved in the CPMR. Since the mechanism of the two forms of protection are presumably different, it may be possible to increase resistance by expressing both the antisense RNA for the 3′ non-coding region as well as the CP mRNA lacking these sequences in plants.

MATERIALS AND METHODS

All restriction enzymes were purchased from New England Biolabs, unless noted otherwise. Plasmids were maintained in *Escherichia coli* strains LE392 or DH5α. Radioactive isotopes and plant tissue culture media and chemicals were purchased from ICN Biomedicals.

Mutagenesis of PAMV CP.

The plasmid pBSPAMVCP contains a cDNA copy of the genomic sequence of PAMV CP corresponding to nt 6185 to 7052 (*Dra*I fragment). This fragment was cloned in two opposite orientations in the PBS *Hin*cII site. The start and stop codons are at nt 6232 and 6979, respectively. The CP open reading frame is flanked by 47 nt at the 5′ end (corresponding to a non-coding region and a part of the 8-kDa protein) and the complete 3′ non-coding region of PAMV without the poly A tail.

Two in-frame deletions were generated in the PAMV CP gene, with the first deletion made in the N terminus of the PAMV CP. The pBSPAMVCP plasmid was digested first with *Mlu*I followed by exonuclease III and mung bean nuclease treatment. The resulting plasmid was then ligated with T4 DNA ligase and transformed in the *Escherichia coli* strain LE392. Many clones (with different deletions) were sequenced and the clone NT138 with an in-frame deletion of 138 nt from nt 6241 to 6379 was selected (Fig. 1). NT138 encodes a protein of an estimated size of 22.8 kDa. This deletion of 46 amino acid residues at the N terminus of the protein may be located in a region exposed outside of the virion as was shown for potato virus X (PVX) (Baratova *et al.* 1992).

pBSPAMVCP was digested with *Nar*I (nt 6582) and *Nco*I (nt 6843) followed by T4 DNA polymerase treatment and ligation. A deletion of 258 nucleotides (86 amino acid residues) was made. This deletion maintained the open reading frame and encoded a protein of an estimated size of 18.2 kDa (CT258) (Fig. 1).

The PAMV CP, the antisense, and the NT138 and CT258 were subsequently cloned in the shuttle vector Polyrok between the *Sma*I and *Sac*I sites located between the cauliflower mosaic virus (CaMV) 35S promoter and the NOS terminator.

Plant transformation.

Agrobacterium tumefaciens strain LBA4404 was transformed with Polyrok using the freeze-thaw method (Holster *et al.* 1978).

Leaf disks of *Nicotiana tabacum* L. (cultivar Samsun) were transformed and plants were regenerated according to Horsch *et al.* (1985). Transformed tissues were selected by culturing callus on MS plates (Murashige and Skoog 1962) containing (per milliliter) 1 μg of 6-benzylaminopurine, 0.25 μg of naphthaleneacetic acid, 500 μg of carbenicillin, and 100 μg of kanamycin sulfate. Shoots were rooted on MS plates containing 3% (w/v) sucrose and 200 μg/ml kanamycin sulfate, and plantlets were transplanted into sterile soil and transferred to a growth chamber at 24° C with a 16-hr photoperiod.

Transgenic plants expressing the PAMV FL CP and the NT138 were analyzed by Northern and Western blotting. Transgenic plants expressing the antisense and the CT258 protein were analyzed by Northern blotting. Plant lines with the highest expression were selected and propagated by cuttings in moist sterile soil. Also F_1 seeds were screened by NPTII assays (McDonnell *et al.* 1987) and Western blotting.

Western blot analysis.

Protein extraction was done according to Hurkman and Tanaka (1986) with minor modifications. Total plant proteins were precipitated with 5 volumes of 0.1 M ammonium acetate in methanol at –20° C. Protein samples were subsequently heated at 75° C for 5 min and 25 μg of total proteins were separated on a 12.5% polyacrylamide gel containing SDS and subjected to the immunoblot transfer procedure described by Towbin *et al.* (1979). Anti-PAMV rabbit polyclonal primary antibodies (raised against the SDS-denatured PAMV CP), alkaline phosphatase-conjugated secondary antibodies, chromogenic substrates NBT (para-nitro blue tetrazolium chloride), and BCIP (5-bromo-4-chloro-3-indolyl phosphate paratoluidine salt) were used to detect bound antigen.

Northern blot analysis.

Total nucleic acids were isolated from young leaves and RNA precipitated with LiCl as described by Majeau and Coleman (1991). RNAs were separated by electrophoresis on a 1.5% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane. Prehybridization, hybridization, and washing conditions were as suggested by Promega Corporation, Madison, WI. Strand-specific riboprobes were generated from T_3 or T_7 DNA transcription of pBSPAMVCP, linearized with *Xba*I or *Hin*dIII respectively, using α-labeled [^{32}P]CTP.

Inoculation of transgenic plants.

Purification of PAMV was according to Leclerc *et al.* (1992). Approximately 12- to 16-cm tall tobacco plants produced from cuttings or seeds (F_2 generation) were inoculated with various concentrations (0.1, 0.5, and 1 μg/ml) of purified virus preparations. Inocula (200 μl in 50 mM sodium phosphate buffer, pH 7.0) were applied on two mature leaves per plant, dusted lightly with Carborundum. Five different experiments were conducted with transgenic plants grown from cuttings and three from seeds (see Table 1).

Detection of virus.

Samples of inoculated and upper uninoculated leaves of infected plants were taken, processed, diluted two times with extraction buffer, and spotted onto nitrocellulose filters. Successive dilutions (1/8, 1/32, 1/128, and 1/512) were made and spotted. Successive dilutions of known quantities of pure PAMV were spotted on the same nitrocellulose filter. The amount of virus in infected plants was compared to that of the pure virus. The resulting nitrocellulose membranes were prehybridized and hybridized according to Leclerc *et al.* (1992). Random primer DNA probes were prepared according to Feinberg and Vogelstein (1983) using α-[^{32}P]dATP and an 850-bp fragment (containing the PAMV CP gene) eluted from agarose gel.

Expression of PAMV CP and CT258 proteins in *Escherichia coli*.

A *Hin*dIII site was generated upstream of the start codon of the PAMV CP using the polymerase chain reaction (PCR). The synthetic primer 5′ GCG CCA AGC TTT ATG GTT GAT TCT AAG AAA ACT GAA ACT 3′ (the *Hin*dIII site and the start codon are underlined) and the M13 reverse primer (Pharmacia) were used to amplify the mutagenized fragment from pBSPAMVCP. One nanogram of template was amplified for 30 cycles in the following conditions: 1 min at 48° C for annealing, 1 min at 72° C for elongation, and 30 sec at 94° C for denaturation in presence of 1 mM Mg^{++}. Amplified DNA fragments were cloned in the *Hin*dIII site of pJP_1R_9, a bacterial expression vector in which transcription is driven by the strong T_5P_{25} promoter of the phage T_5 (Ivanov 1990). pJP1R9PAMVCP and pJP1R9CT258 were then transformed in *Escherichia coli* strain LE392 and grown for 7 hr at 37° C under constant shaking. One milliliter of 1 OD_{600} unit was collected. The bacteria were pelleted and resuspended in a solution of 0.1 M sodium carbonate and 0.1 M dithiothreitol (DTT). The bacterial suspension was then subjected to lysis by adding 1/2 volume of the loading buffer (30% sucrose, 5% sodium dodecyl sulfate [SDS], and 0.1% bromophenol blue) and heated to 75° C for 5 min. Ten microliters of the extract was subjected to SDS-PAGE and Western blotting.

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