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Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance

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***Agrobacterium*-mediated transfer from a binary vector was used to produce transgenic *Nicotiana tabacum* plants that expressed coat protein of the plant virus, alfalfa mosaic virus (AMV). Expression levels of the chimeric gene, which was under the control of the cauliflower mosaic virus 19S promoter, were determined in primary transformed plants, in the progeny from self-fertilization and in the progeny from crosses to normal tobacco. RNA transcripts that were of the expected size as well as a protein of the M_r and antigenicity of AMV coat protein accumulated in the transgenic plants. Plants that expressed the highest levels of coat protein developed fewer primary infections following inoculation with two strains of AMV and developed systemic infection slower than did plants that did not express coat protein. Resistance was specifically against virions of the AMV strains. AMV RNA and the unrelated virus, tobacco mosaic virus, were as infectious on progeny that expressed coat protein as they were on progeny that did not. The relationship between the virus resistance expressed by these transgenic plants and that observed in virus cross-protection is discussed.**

Key words: alfalfa mosaic virus/cross-protection/transgenic tobacco/virus coat protein/virus resistance

Introduction

The practical control of plant viruses relies on methods that prevent or restrict virus infection. Currently, the most effective method to prevent infection is the use of resistant plant varieties. However, breeding cultivars for resistance is often limited by a lack of sources of resistance within the crop species. Another approach to virus control is to exploit the phenomenon of cross-protection, whereby plants that are infected with a mild strain of virus do not develop severe symptoms when challenged with a severe strain of the same virus. Cross-protection has been successfully used for control of citrus tristeza virus (Cohen, 1976; Muller and Costa, 1977), papaya ringspot virus (Yeh and Gonsalves, 1984), and tobacco mosaic virus (TMV) (Rast, 1972; Fletcher, 1978). However, this procedure can be labor-intensive and carries a risk of the mild strain becoming more virulent or acting synergistically with other viruses. If the molecular basis for cross-protection were known, it might be possible to mimic the resistance of cross-protection without the need to infect plants with a protecting strain of virus.

Several studies have indicated that the coat protein of the protecting virus has an important role in systemic cross-protection. For example, from studies of TMV cross-protection, Sherwood and Fulton (1982) suggested that the protection results from an inhibition of uncoating of the challenge virus thereby preventing the release of infectious RNA. They found that leaf areas containing high concentrations of virus in tobacco plants infected with a strain of TMV causing mosaic symptoms were not susceptible to infection by virions of a TMV strain causing necrotic lesions, whereas these areas were susceptible to infection by unencapsidated TMV RNA of the necrotic strain. Dodds *et al.* (1985) reported similar results from studies of cross-protection of tomato plants infected with cucumber mosaic virus (CMV). They found that CMV RNA could superinfect leaves infected with strains of CMV. Although the challenge virus RNA could infect healthy plants systemically, the infection resulting from the challenge RNA in previously infected leaves remained localized.

Experiments with heterologously encapsidated virions, in which the RNA of one strain is encapsidated in the coat protein of another, reinforce the role of coat protein in cross-protection. Virus-infected plants are not easily superinfected by viruses for which they are normally hosts when the RNA of the challenging virus is encapsidated in the coat protein of the resident virus (Sherwood and Fulton, 1982; Zinnen and Fulton, 1986). Thus, studies of systemic cross-protection indicate that protection occurs when the resident virus and challenge virus have the same or closely related coat proteins. However, coat protein may be only part of the explanation; additional factors are probably involved (Sherwood and Fulton, 1982; Palukaitis and Zaitlin, 1984; Zinnen and Fulton, 1986).

Recently developed techniques of gene manipulation and plant genetic engineering now provide a method for determining whether the expression of individual viral genes can mimic the cross-protection reaction. For example, Abel *et al.* (1986) have developed transgenic tobacco plants that express the TMV coat protein gene. The plants expressing coat protein were slower to develop systemic symptoms following inoculation with TMV than were sibling plants that did not contain TMV coat protein. Also, 10–60% of the expressing plants never developed symptoms. It was suggested that the plants expressing coat protein have fewer sites susceptible to infection than do the non-expressing plants.

Alfalfa mosaic virus (AMV) is another plant virus which has been shown to exhibit cross-protection in experiments with whole plants (Schmelzer, 1963). The biology of this virus (Jaspars and Bos, 1980) is substantially different from TMV (Zaitlin and Israel, 1975), however. First, AMV is a multipartite virus with a genome consisting of three messenger-sense RNAs (RNA 1, 2, 3) separately encapsidated into bacilliform particles of different lengths. In contrast, the genome of TMV is a single messenger-sense RNA. Second, individual genomic AMV RNAs are not infectious alone or in combination, whereas TMV RNA is infectious. Infection results only when AMV genomic RNAs are co-infected with either coat protein or its messenger RNA (RNA 4) which is a subgenomic, non-self-replicating RNA, derived from genomic RNA 3. Thus, the coat protein of AMV has at least two functions in infection: encapsidation of RNA and initiation of infection. Third, AMV is transmitted in a non-persistent manner by aphids and is commonly transmitted through seed. TMV is not normally transmitted by insects or through seed.

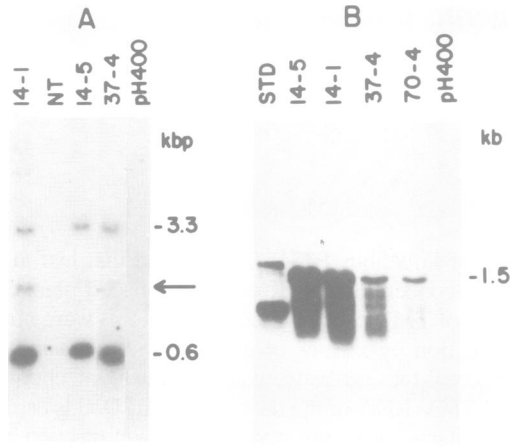

Fig. 1. Analysis of DNA and RNA from regenerated transformants. (**A**) Autoradiograph of a DNA blot hybridized to a nick-translated AMV RNA 4 cDNA fragment. The lanes contain 10 μg DNA, cut with *Bam*HI, from the indicated transformants, from normal tobacco (NT), or from plants transformed with pH400. The arrow indicates the position of an ~2-kb band. (**B**) Autoradiograph of an RNA blot hybridized to nick-translated AMV RNA 4 cDNA fragment. The lanes contain equal amounts of total RNA from plants transformed with pH400A4 or pH400. The STD lane shows the position of AMV RNA 3 and RNA 4.

Here we report the production of transgenic plants which express the coat protein gene of AMV from a nuclear chimeric gene. These plants are resistant to local infection by AMV and they exhibit a delay in the onset of symptoms of systemic infection.

Results

Transformation

From 378 leaf pieces originally inoculated with *Agrobacterium tumefaciens* containing pH400A4 (Figure 5), 117 morphologically normal shoots were excised and placed under kanamycin selection. Of the 51 shoots that rooted, 32 (63%) expressed coat protein. Only about half of the plants that expressed coat protein contained detectable levels of octopine; plants that did not express coat protein contained no detectable octopine. Most of the transformants were morphologically indistinguishable from Xanthi-nc, although some plants showed variations such as internode shortness, sterility or poor seed set. Transformants that expressed high levels of coat protein were selected for further analysis of DNA, RNA and coat protein expression.

DNA analysis

Blots of DNA fragments generated by *Bam*HI digestions of DNA from transgenic plants hybridized to labeled AMV cDNA are shown in Figure 1A. Hybridization of the fragment whose migration corresponds to a size of 3.3 kb indicates the presence of a full-length octopine synthase gene. This fragment hybridizes to AMV cDNA because the first 293 nucleotides of AMV 4 cDNA are part of the fragment (Figure 5). The band at a position of 0.6 kb indicates the presence of a fragment containing the last 588 nucleotides of AMV 4 cDNA. Therefore, the presence of the 3.3-kb and 0.6-kb bands in DNA digested with *Bam*HI from 14-1, 14-5 and 37-4 regenerants indicates the presence of a full-length octopine and AMV coat protein gene. Analysis of DNA from untransformed plants or DNA from regenerants transformed with the pH400 vector alone revealed no hybridization to the AMV probe (Figure 1A).

In addition, a band corresponding to ~2 kb in plant 14-1 was detected (Figure 1A). This band also hybridized to an octopine synthase cDNA probe. Dhaese *et al.* (1983) have reported that an internal T-DNA sequence upstream from the major octopine synthase polyadenylation site can serve as an internal border. If this site was used in transformation, the fragments containing the AMV cDNA plus a portion of the octopine synthase gene would be ~2 kb. Therefore, the presence of this band in the DNA of 14-1 transformant suggests that the internal T-DNA border was used. The 2-kb band in plant 14-1, along with the expected full-length band at 3.3 kb, suggests that 14-1 contains more than one T-DNA copy.

Table I. Genetic analysis of transgenic progeny

Transformant	Number of seedlings[a] Kanamycin resistant	Kanamycin sensitive	Segregation ratio	Predicted NPT II loci	χ^2	P^b
14-1	484	37	15:1	2	0.64[c]	0.42
14-5	903	63	15:1	2	0.12	0.73
37-4	436	41	15:1	2	4.48	0.03
70-4	306	110	3:1	1	0.46	0.50

[a]Seedlings were the progeny from self-fertilization.
[b]Probability that the observed deviation from the indicated segregation ratio occurs by chance if the segregation ratio is correct.
[c]χ^2 computed for the indicated segregation ratio.

An estimation was made of T-DNA copy number based on the expression of NPTII in the progeny population. Seeds from self-fertilization were germinated and grown on media containing 300 mg/l kanamycin. The numbers of resistant and sensitive seedlings were determined 4–5 weeks after germination when the sensitive seedlings were chlorotic and unable to produce true leaves. The data were used to compute χ^2 values for segregation ratios of 3:1, 15:1 and 63:1 which predict 1, 2 or 3 gene loci respectively. The predicted copy number of several transformants is shown in Table I. The *P* values for transformants 14-1, 14-5 and 70-4 indicated that the observed distributions do not deviate from expectations. The indicated segregation ratios for these plants are likely to be correct. However, the observed deviation from a 15:1 ratio for 37-4 is greater than expected; thus, a 15:1 ratio may not be correct for this plant. It is likely that NPTII and AMV 4 cDNA segregate together; we are not aware of any reports of the segregation of genes co-transferred within a single pair of T-DNA border sequences. Thus, we assume that the copy number of AMV 4 cDNA equals that of NPTII.

RNA analysis

RNA from pH400A4 transformants that expressed coat protein showed distinct and prominent species that hybridized to the AMV RNA 4 probe (Figure 1B). These RNAs were not detectable in transformants that did not express coat protein (unpublished data), pH400-transformed plants or normal tobacco plants. The RNA band that showed the strongest hybridization corresponded to a size of ~1.5 kb. This is the expected size of the AMV RNA 4-CaMV transcript which includes 0.88 kb of AMV RNA 4 followed by 0.6 kb of CaMV-derived sequence. The nature of the faster migrating RNA species in Figure 1B that hybridized to the AMV probe is, as yet, unknown. Comparison of the hybridization intensity of known quantities of AMV RNA 4 to the hybridization intensity of the 1.5-kb transcript suggests that the transgenic plants contain up to 10 pg of AMV sequences per μg of total RNA.

Expression of coat protein

Coat protein expression in leaves of several transformants is shown in Table II. The amount of coat protein expression varied

Table II. Expression of AMV coat protein in transgenic tobacco

Transformant	Amount of coat protein[a] (ng/mg protein)	
	Leaf 1	Leaf 2
14-1	482 ± 11	800 ± 83
	518 ± 39	482 ± 52
	685 ± 30	468 ± 19
	416 ± 28	453 ± 103
14-5	348 ± 66	422 ± 85
37-4	326 ± 5	248 ± 72
	195 ± 32	320 ± 23
70-4	66 ± 0	40 ± 4

[a]The level of expression of coat protein is shown for two young leaves, 3–4 cm in size, from vegetatively propagated plants of the indicated transformant. The coat protein concentration of half leaves was determined by ELISA and the soluble protein concentration was determined by the Bradford dye-binding assay (Bradford, 1976). Data are expressed as the average of both halves of leaf 1 or leaf 2.

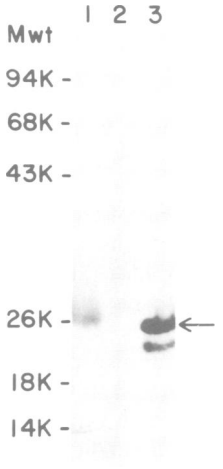

Fig. 2. Detection of AMV coat protein in a transgenic plant by immunoblotting. A young leaf of 14-1 or of normal tobacco was homogenized in 0.06 M Tris–HCl, pH 6.8 containing 2% SDS, 30% sucrose, and 5% β-mercaptoethanol (Halk, 1986) and boiled for 2 min. Fifteen microliters of each extract were subjected to electrophoresis in a 13% polyacrylamide gel (Laemmli, 1970). The separated proteins were blotted to nitrocellulose. AMV coat protein was detected on the blot with alkaline phosphatase-labeled monoclonal antibodies specific for AMV coat protein (Halk, 1986) followed by goat anti-mouse antibodies labeled with alkaline phosphatase. The lanes contain: (**1**) proteins from 14-1; (**2**) proteins from a normal tobacco; (**3**) 100 ng AMV coat protein. The position to which mol. wt markers migrated is indicated at the left and the position of coat protein is indicated by an arrow at the right. The band that migrated faster than coat protein in **lane 3** is a fragment of coat protein (Bol *et al.*, 1974).

from 40 ng/mg total protein for plant 70-4 to 800 ng/mg total protein for plant 14-1. Electrophoretic analysis of proteins from young leaves showed that the size of coat protein in transformants was identical to that of full-length virion coat protein (Figure 2). The level of coat protein depended on leaf age (Figure 3). The highest levels of coat protein were in young expanding leaves; older leaves further than 10 nodes from the apical meristem often did not contain detectable amounts. In addition to leaves, coat protein was detected in roots and stems of six plants that were tested, but the levels were < 10% of the levels in leaves.

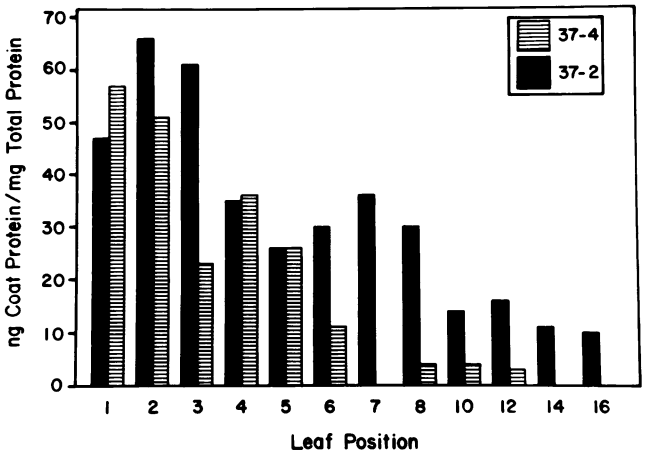

Fig. 3. Coat protein levels relative to leaf position. The amount of coat protein was determined for individual leaves from plant 37-4 and 37-2. Leaves were numbered down the 45-cm plants beginning with a young leaf ~ 4 cm long. Leaves 5–16 were fully expanded. Coat protein concentration was determined by ELISA and soluble leaf protein was determined by the Bradford dye-binding procedure (Bradford, 1976).

Susceptibility of transgenic plants to primary virus infection by AMV virions

Susceptibility to virus infection was determined for young transgenic progeny of transformants 14-1 and 70-4. Groups of ~50 progeny were tested by ELISA to identify 4–9 plants that did not express coat protein (non-expressers) and an equal number that expressed the highest levels (expressers). Three young leaves of each plant were inoculated with 2–15 μg/ml AMV. Virus preparations varied in specific infectivity; therefore, the inoculum concentration was adjusted so that discrete chlorotic primary lesions developed following inoculation. In Table III, experiments 1 and 2 show that the progeny of 14-1 that expressed coat protein developed fewer primary chlorotic lesions than did the progeny that did not contain coat protein. The expressers developed 14% of the lesions that developed on non-expressers. To determine if the level of coat protein in the transgenic plants affected infection, progeny of transformant 70-4 were inoculated with AMV strain 425-Madison (AMV 425). Plant 70-4 expressed nearly 8-fold less coat protein than did 14-1 (Table II); 70-4 progeny were weaker expressers than were 14-1 progeny. Experiments 3 and 4 (Table III) show that the susceptibility of 70-4 expressers and non-expressers was nearly equal. The presence of a low level of coat protein in the 70-4 expressers, therefore, did not alter their susceptibility to AMV. This suggests that coat protein must accumulate to levels higher than in 70-4 progeny to effect resistance.

Expressers in the progeny of 14-1 developed fewer lesions than did the non-expressers. These expressers, therefore, can be described as resistant to AMV because invasion of the virus was restricted (Cooper and Jones, 1983). To determine if the resistance was effective against other strains of AMV, 14-1 progeny were inoculated with AMV strain McKinney (AMV McKinney). This isolate of AMV differs from AMV 425 in host range and symptomology (our observations). Generally, the McKinney strain causes severe necrotic mosaic on Xanthi-nc, whereas strain 425 causes milder chlorotic mosaic. Experiments 5 and 6 (Table III) show that expressers in 14-1 progeny were also resistant to infection by AMV McKinney. Thus, transgenic progeny of 14-1 that expressed coat protein were resistant to infection by two different strains of AMV.

Table III. Relative susceptibility of transgenic progeny to infection by virions of AMV and TMV or AMV RNA

Experiment	Pedigree[a]	Inoculum	Average number of lesions per half leaf		Relative susceptibility[b]
			Expressers	Non-expressers	
1	14-1 ∅	AMV 425	3 ± 5(9)[c]	22 ± 9(8)	14
2	14-1 ∅	AMV 425	2 ± 1(5)	14 ± 8(5)	14
3	70-4 ∅	AMV 425	34 ± 20(8)	27 ± 17(8)	126
4	Xnc × 70-4	AMV 425	37 ± 16(7)	43 ± 16(8)	86
5	Xnc × 14-1	AMV McKinney	4 ± 7(5)	219 ± 154(5)	2
6	Xnc × 14-1	AMV McKinney	56 ± 58(7)	166 ± 54(7)	34
7[d]	14-1 ∅	AMV 425	2 ± 2(6)	13 ± 12(4)	15
		AMV 425 RNA	38 ± 26(6)	60 ± 42(4)	63
8[d]	14-1 ∅	AMV 425	1 ± 2(6)	20 ± 29(5)	5
		AMV 425 RNA	14 ± 16(6)	26 ± 25(5)	54
9[d]	Xnc × 14-1	AMV 425	6 ± 7(5)	46 ± 24(4)[e]	13
		AMV 425 RNA	38 ± 10(5)	38 ± 39(5)	100
10	Xnc × 14-1	AMV McKinney RNA	23 ± 14(9)	8 ± 10(7)	288
11[d]	14-1 ∅	AMV 425	4 ± 12(9)	33 ± 32(7)	12
		TMV U_5	157 ± 106(7)[e]	149 ± 71(7)	105
12[d]	Xnc × 14-1	AMV 425	2 ± 5(5)	24 ± 12(6)	8
		TMV U_5	48 ± 42(5)	24 ± 8(6)	200
13[d]	Xnc × 14-1	AMV 425	22 ± 33(9)	60 ± 23(5)	37
		TMV U_5	141 ± 38(6)[e]	110 ± 13(4)[e]	128

[a] ∅ denotes progeny from self-fertilization; Xnc denotes normal Xanthi-nc.
[b]Relative susceptibility is defined as (average number of lesions per youngest inoculated half leaf on expressers ÷ average number of lesions per youngest inoculated half leaf on non-expressers) × 100.
[c]Number in parentheses denotes the number of inoculated plants.
[d]Inoculation on one half of the leaf was with AMV 425 and on the opposite half with AMV 425 RNA for experiments 7–9 or with TMV for experiments 11–13.
[e]On one or more plants, the lesions on the youngest inoculated leaf were not discrete and therefore could not be counted.

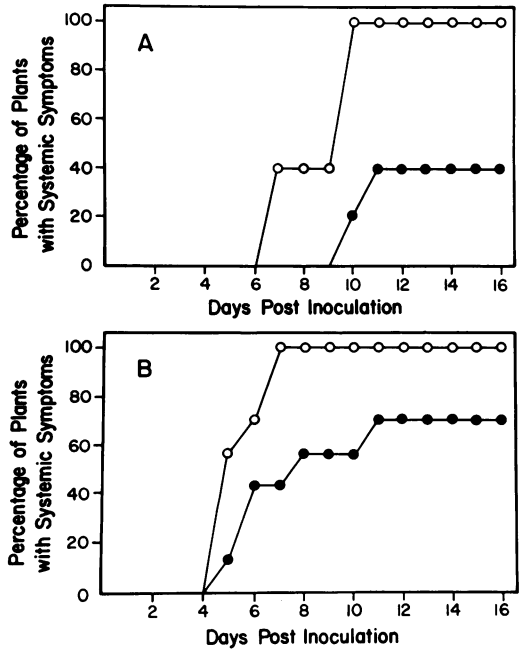

Fig. 4. Development of systemic symptoms following inoculation of progeny with AMV. Progeny that expressed (●) or did not express (○) coat protein were inoculated on three young leaves with 6 μg/ml AMV 425 (**A**) or with 6 μg/ml AMV McKinney (**B**). Local infection of the expressers and non-expressers is shown in Table III, experiment 2 for the plants in (A) and in Table III, experiment 6 for the plants in (B). Each day following inoculation, the plants were observed for the development of systemic symptoms on young uninoculated leaves. The percentage of plants that displayed systemic symptoms is shown each day following inoculation.

Expressers are resistant to AMV virions but not to AMV RNA

Sherwood and Fulton (1982) and Dodds *et al.* (1985) found that virus-induced cross-protection could be overcome by challenging the protected plants with virus RNA. This observation led to the coat protein hypothesis of cross-protection, which predicts that the presence of coat protein decreases infection by inhibiting the uncoating of viral particles (Sherwood and Fulton, 1982). If coat protein in the transgenic plants functions in such a manner, there would be little or no resistance to infection by AMV RNA. To test whether the transgenic plants were susceptible to RNA, leaves were inoculated with virions on one half of the leaf and with 30 μg/ml AMV RNA on the opposite half of the leaf. Experiments 7–9 (Table III) indicate that, in all cases, the expressers were more susceptible to AMV 425 RNA than they were to virions. Susceptibility of the expressers to RNA varied from 54 to 100% of the non-expressers. A similar result was found when AMV McKinney RNA was used as the inoculum (experiment 10); expressers were not resistant to infection by RNA. These experiments show that plants that are resistant to AMV virions are not similarly resistant to infection by AMV RNA.

Expressers are not resistant to an unrelated virus

To determine if the virus resistance of the transgenic plants was specific for AMV, plants were inoculated with AMV on one half of the leaf and with 10 μg/ml TMV on the opposite half of the leaf. TMV causes primary necrotic lesions with no secondary spread in Xanthi-nc plants. The results of experiments 11–13 (Table III) indicate that, unlike AMV, TMV was highly infectious to both expressers and non-expressers. Thus, the resistance is specific for strains of AMV; the transgenic plants are susceptible to infection by another unrelated virus.

Development of systemic infection in the transgenic plants is delayed

Most of the studies of cross-protection with virus-infected plants have focused on the development of local or primary infections by the challenging virus in systemically infected leaves. However, a study of CMV-infected plants found that the challenging strain of CMV or CMV RNA did not cause systemic infection (Dodds *et al.*, 1985). Recently, Abel *et al.* (1986) reported that the development of systemic symptoms was delayed in transgenic

plants expressing TMV coat protein. To monitor the development of systemic symptoms in our transgenic plants, three leaves of each plant were inoculated with AMV. Plants were scored as positive for systemic symptoms if systemic vein clearing, chlorotic mottle, or chlorotic lesions appeared. Figure 4 shows that there was a delay in the appearance of systemic symptoms following inoculation with either AMV 425 or AMV McKinney in plants expressing coat protein compared to the non-expressers. Some of the expressers never developed systemic symptoms following inoculation. Assay of these plants by ELISA indicated that AMV had not invaded the uninoculated leaves; the plants which did not show symptoms did not contain AMV. In another experiment, however, all of the expressers eventually developed symptoms (unpublished data).

Discussion

More than 50 transformants selected for kanamycin resistance were evaluated for the expression of octopine and for AMV coat protein. Octopine expression was not predictive for the strong expression of coat protein; many strong expressers had no detectable octopine. Transformants expressing coat protein contained the expected viral transcript and a full-length coat protein. No change in plant morphology, vigor or fertility could be attributed to the expression of coat protein or to the copy number of T-DNA inserts. However, we cannot rule out a role for coat protein in disease symptom production following viral infection because the transformants contained much less coat protein than is present in AMV-infected plants.

Coat protein made up nearly 0.1% of total protein in the highest expressers; infected plants contain >100 times this amount. Expression of the AMV coat protein gene appeared to provide virus resistance to the transgenic plants similar to that observed in two virus–host systems. Previous studies of cross-protection with virus-infected plants by Sherwood and Fulton (1982) and by Dodds *et al.* (1985) showed: (i) the virus-infected plant was resistant to strains of the infecting virus; (ii) the protected plant was much more susceptible to RNA of the infecting virus than to virions; and (iii) the protected plant was still a host to other viruses not related to the infecting virus. Progeny of transformant 14-1 that expressed the highest levels of coat protein were resistant to virions of two strains of AMV. The expressers often developed only ~10% of the primary virus lesions that developed on the non-expressers. The expressers were much more susceptible to infection by AMV RNA than they were to infection by AMV virions. In some cases, the RNA caused as much infection in expressers as in non-expressers. And lastly, the expressers were highly susceptible to infection by the unrelated virus, TMV. Therefore, the transgenic plants behaved as if they had been protected from infection by AMV.

Sherwood and Fulton (1982) suggested that the presence of coat protein in the infected plants inhibited the uncoating of related challenging virions and resulted in a virus-resistant plant. Our results are consistent with this hypothesis. Our transgenic plants contained AMV coat protein in the absence of other viral proteins and were resistant to infection by virions but susceptible to RNA. This hypothesis is further supported by the recent *in vitro* translation experiments of Brisco *et al.* (1986) which suggest that the presence of extraneous AMV coat protein has an inhibitory effect on AMV uncoating. Susceptibility of the transgenic plants to infection by all four AMV RNAs suggests that these plants are also susceptible to infection by AMV RNAs 1, 2 and 3. These three RNAs, which are not infectious to Xanthinc, would be expected to infect the transgenic plants because endogenous coat protein obviates the requirement for RNA 4.

Although the characteristics of the resistance in transgenic plants were similar to those of cross-protection, the protection was not complete. This may be due to the involvement of other virus components present in virus-infected cells. However, our data suggest it may be due to the lower concentration of coat protein in the transgenic plants than in virus-infected plants. Progeny of transformant 14-1, that expressed high levels of coat protein, were resistant to local infection by AMV, while progeny of 70-4, which were weak expressers, were quite susceptible to infection. Analysis of transformant 70-4 predicted that it contained only one functional T-DNA locus while transformant 14-1 contained two (Table I). This suggests that expression of a single copy of virus cDNA in 70-4 progeny did not provide the level of coat protein needed for AMV resistance. In contrast, Abel *et al.* (1986) found that the expression of a single locus of TMV coat protein gene under control of the CaMV 35S promoter was sufficient to protect plants against TMV infection. This difference may be due to the relative strengths of the CaMV 19S and 35S promoters, and/or to the choice of polyadenylation sites used for the virus RNA. Additionally, sites of T-DNA insertion in each transformant may account for differences in levels of coat protein expression among transformants. Greater resistance to AMV infection may be achieved by higher levels of coat protein expression by the use of stronger promoters or higher gene copy numbers.

The inoculated transgenic progeny that expressed high levels of coat protein developed systemic symptoms at a slower rate than did the non-expressers. In a number of cases, infection was completely confined to the inoculated leaves. Thus, high level expressers were resistant to local and, in some cases, to systemic infection by AMV. Several mechanisms may account for the delayed systemic infection. First, a reduction in cell-to-cell and long-distance transport in the vascular tissue may be responsible. Second, coat protein may inhibit AMV RNA transcription in plants as observed *in vitro* by Houwing and Jaspars (1986). Such an inhibition would slow initial virus multiplication following inoculation, decrease virus replication in neighboring cells and thereby result in delayed virus spread throughout the plant. Third, the delay in systemic spread of virus in the expressers may have been simply a result of milder primary infection. Some viruses require a minimum level of initial infection for the development of systemic infection (Matthews, 1981). If the first or second mechanism is responsible, a delay in systemic symptom development may also occur following inoculation with AMV RNA, without resistance to local infection. Such a result would suggest two separate mechanisms for resistance: one responsible for local resistance and a second for systemic resistance.

This study shows that the expression of the AMV coat protein gene in transgenic plants inhibited local infection by two AMV strains and prevented or delayed the development of systemic virus symptoms. Abel *et al.* (1986) reported that the expression of TMV coat protein gene prevented or delayed the development of systemic symptoms following inoculation with TMV. Thus, for two very different viruses, the presence of virus coat protein interfered with disease development. This result is especially intriguing for AMV because, paradoxically, coat protein is required for infection by this virus. The mechanisms for virus inhibition by the coat protein gene may be similar for AMV and TMV, or the mechanisms may be markedly different and depend upon the unique functions of each coat protein. Nevertheless, virus

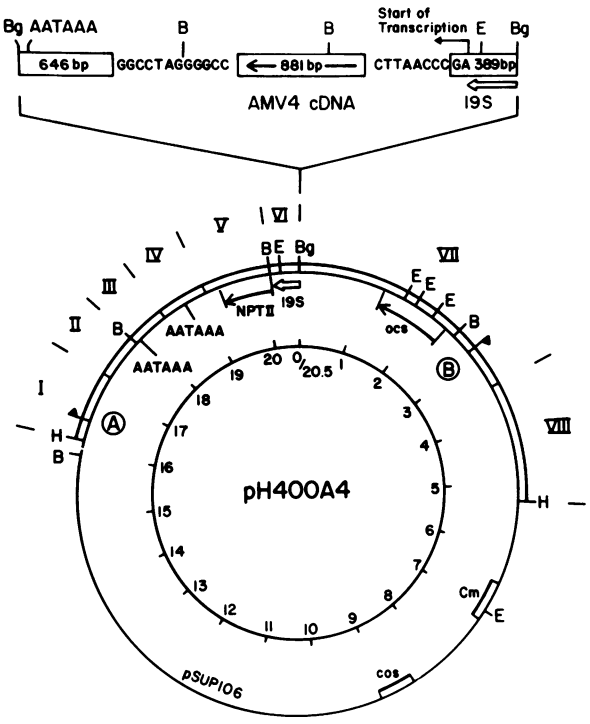

Fig. 5. Map of pH400A4. pH400 is a 'micro Ti' binary vector based on the broad-host-range cosmid pSUP106 (Priefer *et al.*, 1985) and the T_L region of the octopine-type plasmid pTi-15955. Resistance to kanamycin is conferred to transformed plant cells by the neomycin phosphotransferase II (NPTII) gene from bacterial transposon Tn*5*, under control of the cauliflower mosaic virus (CaMV) 19S promoter (Balázs *et al.*, 1985). The 5′ region of the NPTII coding region was modified by oligonucleotide site-directed mutagenesis to introduce a *Bam*HI recognition site between the 'false' ATG at Tn*5* base pair (bp) 1535 and the NPTII start codon at Tn*5* bp 1551 (Beck *et al.*, 1982). The various regions (I–VIII) of the pH400 T-DNA are comprised of the following nucleotide sequences, oriented clockwise. T-DNA bases are numbered according to Barker *et al.* (1983), CaMV nucleotides are according to Franck *et al.* (1980), and Tn*5* nucleotides are according to Beck *et al.* (1982) and Mazodier *et al.* (1985). **I**, T-DNA bp 603–1617; **II**, CaMV bp 7667–7018; **III**, Tn*5* bp 2936–2399; **IV**, T-DNA bp 22 440–21 727; **V**, Tn*5* bp 2517–1540 (bp 1544 'G' was mutated to 'C'); **VI**, CaMV bp 5765–5376; **VII**, T-DNA bp 11 208–14 710; **VIII**, T-DNA bp 1618–3390. A and B (circled) indicate the 25-bp border repeats, which specify T-DNA ends; 'AATAAA' indicates positions of RNA polyadenylation signals; 19S indicates the CaMV 19S promoter sequences; ocs indicates the octopine synthase-coding region. A *Bgl*II fragment containing a cDNA copy of AMV RNA 4, under control of the CaMV 19S promoter, and terminated by the CaMV 19S RNA polyadenylation signals, was ligated into the unique *Bgl*II site of pH400 to generate plasmid pH400A4. Additional details of the construction are in Materials and methods. Other abbreviations: Cm, the bacterially expressed gene encoding chloramphenicol resistance; cos, sequences which allow packaging into lambda phage; B, Bg, E and H indicate positions of recognition sequences for restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII respectively.

coat protein genes will likely have broad applicability in the development of virus-resistant plants.

Materials and methods

DNA constructions

An AMV RNA 4 cDNA fragment was recovered from pSP65A4 (Loesch-Fries *et al.*, 1985) by restriction digest using *Eco*RI and *Sma*I according to the suppliers' instructions. This fragment (AMV 4 cDNA, Figure 5) contained the 881 nucleotides of AMV RNA 4 cDNA with an *Eco*RI recognition site at the 5′ end and an *Sma*I site at the 3′ end. To provide plant transcription initiation and termination signals, we constructed pDOB513, which is a derivative of plasmid pDOB512 described by Balázs *et al.* (1985). The unique *Hin*dIII site of pDOB512 was converted to a *Bgl*II site by digesting pDOB512 with *Hin*dIII, followed by ligating *Bgl*II linkers to the blunt ends generated by T4 polymerase treatment. Plasmid pDOB513, therefore, contains the cauliflower mosaic virus (CaMV) 19S promoter and polyadenylation sites separated by an *Sma*I restriction site and flanked by *Bgl*II recognition sites. The RNA 4 cDNA fragment was treated with T4 polymerase to create a 5′ blunt end and was ligated into the *Sma*I site in the sense orientation with respect to the CaMV promoter. A fragment containing the promoter/gene/polyadenylation region was isolated following *Bgl*II digestion and inserted into pH400, a micro Ti plasmid binary vector (Merlo *et al.*, 1985). The plasmid with the AMV cDNA coding region in the same orientation as that of octopine synthase was designated pH400A4 (Figure 5). This plasmid contained the left and right borders of T-DNA, an octopine synthase gene under its own promoter, and a neomycin phosphotransferase II (NPTII) gene, which confers kanamycin resistance in plants, under control of the CaMV 19S promoter, pH400A4 was mobilized into *A. tumefaciens* strain LBA4404 via triparental matings with *E. coli* strain MM294 (pRK2013) as described by Ditta *et al.* (1980), and transconjugants were selected by resistance to streptomycin and chloramphenicol. Strain LBA4404 contains a deleted Ti plasmid which is able to promote T-DNA transfer *in trans* (Hoekema *et al.*, 1983).

Plant transformation

Leaves of axenic *Nicotiana tabacum* L. cv. Xanthi-nc plants growing in Magenta GA-7 vessels (Magenta Corporation, Chicago, IL) were cut into pieces ~3 × 7 mm. Transformation was essentially as described by Horsch *et al.* (1985). The leaf pieces were exposed to a suspension of *A. tumefaciens* LBA4404 (10^8–10^9/ml) containing pH400 or pH400A4 for 5 min, blotted on sterile filter paper and placed on a filter paper over a tobacco cell feeder layer on regeneration medium (Firoozabady, 1986). After 4–6 days, the leaf pieces were placed on regeneration medium containing 500 mg/l carbenicillin, 50 mg/l cloxacillin and 100 mg/l kanamycin for selection. Morphologically normal shoots were excised, placed on an MS medium (Murashige and Skoog, 1962) containing 200 mg/l kanamycin, then potted into soil mix and placed in the greenhouse where they were fertilized weekly. A clone of each plant was also kept axenically growing in Magenta vessels in a medium containing kanamycin.

Nucleic acid analysis

DNA was isolated from leaves of plants growing in the greenhouse essentially as described by Murray and Thompson (1980), treated with restriction endonucleases, electrophoretically separated in 0.8% agarose gels, blotted to Genetran nylon membrane (AMF, Inc., Meriden, CT) according to supplier's instructions and hybridized to nick-translated DNA fragments (Rigby *et al.*, 1977). RNA was isolated from leaves of greenhouse plants (Murray *et al.*, 1983), separated electrophoretically in 1.0% agarose gels, blotted to Genetran in distilled water and hybridized to nick-translated DNA fragments.

Protein analysis

Polyclonal rabbit anti-AMV serum was produced in a New Zealand white rabbit following an immunization schedule outlined by Fulton (1968). Anti-AMV monoclonal antibodies were prepared essentially as described by Halk *et al.* (1984) and Halk (1986). The monoclonal antibodies were conjugated to alkaline phosphatase by the method of Avrameas (1969).

Immunoblots of AMV coat protein were as described by Halk (1986). Coat protein was isolated from purified AMV by the method of Bol *et al.* (1974). Samples from transformed or untransformed plants were prepared by homogenizing leaf tissue in two volumes (w/v) of 0.06 M Tris–HCl, pH 6.8 containing 2% SDS, 30% sucrose, and 5% β-mercaptoethanol. The samples were boiled for 2 min and 15 μl of each was subjected to electrophoresis in a 13% polyacrylamide gel (Laemmli, 1970). The separated proteins were blotted to nitrocellulose. AMV coat protein was detected with alkaline phosphatase-labeled specific monoclonal antibodies (Halk, 1986) followed by goat anti-mouse antibodies labeled with alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersberg, MD).

Coat protein in transgenic plants was quantitated by an enzyme-linked immunosorbant assay (ELISA). ELISA buffers and protocol are essentially as described by Halk (1986). Polyvinyl chloride ELISA plates (Dynatech, Alexandria, VA) were coated with rabbit anti-AMV serum at 1:2500 in 0.1 M carbonate buffer, pH 9.6, rinsed in 127 mM NaCl, 2.6 mM KCl, 8.5 mM Na_2HPO_4, 1.1 mM KH_2PO_4, pH 7.4 (PBS) containing 0.05% Tween 20, and blocked for 30 min in PBS containing 1% BSA. Plant samples were homogenised in ice-cold PBS containing 1% polyvinylpyrrolidone 40 000 (PVP-40), or in PBS containing 0.05% Tween 20, 1% PVP-40 and 0.1% BSA. The detecting antibody was a 0.5 μg/ml mixture of alkaline phosphatase conjugates of AMV coat protein-specific monoclonal antibodies. A solution of 500 ng coat protein/ml was prepared in a 1:20 dilution of Xanthi-nc extract and 3-fold dilutions were used as standard for calculating AMV coat protein concentrations in extracts from transformed plants.

Octopine analysis

Expression of octopine in the transformants was analyzed on t.l.c. plates essentially as described by Christou *et al.* (1986). Samples of leaf tissue of regenerants growing in Magenta vessels were placed in media containing 2 mg/l arginine

overnight to enhance octopine synthesis. The samples were homogenized without added buffer and briefly centrifuged in a microfuge to pellet debris. Samples of 3 μl were spotted onto Whatman silica gel 150A K5 plates followed by chromatography as described. The plates were sprayed with a 1:1 mixture of 10% NaOH in 60% ethanol:0.04% phenanthrenequinone in ethanol for detection of octopine under u.v. illumination. By this method, 3 ng octopine/mg tissue could be detected.

Virus

Alfalfa mosaic virus strains 425 and McKinney (obtained from R.W.Fulton) were purified from infected Xanthi-nc, as described by van Vloten-Doting and Jaspars (1972). Virus RNA was isolated by phenol extraction and ethanol precipitation (Pinck and Hirth, 1972).

Infectivity assays

Transgenic plants were allowed to flower and were either self-pollinated or crossed as pollen parents to Xanthi-nc plants. Groups of 40–50 progeny were tested by ELISA for expression of coat protein to identify 4–9 plants that did not express coat protein and 4–9 plants that expressed high levels of coat protein. The selected plants were inoculated with 2–15 μg/ml of AMV 425, AMV McKinney, TMV-U5 or with 30 μg/ml of AMV RNA. Inocula in 0.03 M sodium phosphate buffer, pH 7.5, were applied with sterile cheesecloth pads to leaves or half-leaves. Following inoculation, plants were placed in a growth chamber under 18 h light at 23°C and 6 h darkness at 16°C. Chlorotic or necrotic lesions that developed in 3–4 days on the inoculated leaves were counted as a measure of local infection and the development of systemic symptoms on each plant was monitored.

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