Transformation of *Nicotiana benthamiana* with the Potato Mop-Top Virus Coat Protein Gene Produces a Novel Resistance Phenotype Mediated by the Coat Protein

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Transformed lines of *Nicotiana benthamiana* expressing a translatable transgene (CP_{trans}) encoding the coat protein (CP) gene of potato mop-top virus (PMTV) differed considerably in steady-state levels of transgene RNA transcript and CP that were positively correlated. A mean of 88% of transgenic T₁ plants from seven transgenic lines were resistant to manual PMTV inoculation. Resistance was identified by failure to develop symptoms in plants and inability to recover infectious virus with a sensitive infectivity assay: many of the 12% of transgenic plants that gave a positive infectivity assay did not produce symptoms and virus was not detected in repeat assays on the same plants. Resistant plants did not express a “recovery” phenotype and resistance was not overcome by use of RNA inoculum. Strong resistance was expressed in all CP_{trans} lines irrespective of the amount of transcript RNA or CP detected, and there was no evidence that resistance was mediated by a “transgene silencing” mechanism. Lines transformed with PMTV CP gene sequence in a nontranslatable form (CP_{nontrans}) also contained differing steady-state levels of RNA transcript, but were not resistant to PMTV, although in some lines symptoms were slightly milder and were slower to develop than those in nontransgenic control plants. These results suggest that CP_{trans} is unique because, although it depends on CP translation to be effective, it mediates very strong resistance in 10 independent transformed lines (tested here and in other work).

Additional keywords: coat protein-mediated resistance, *Pomovirus*, transformation, transgenic resistance.

Viruses that cause damaging symptoms and diseases in potato tubers can have an immediate and important economic impact on production of potatoes for the processing market. Infection with potato mop-top virus (PMTV) can cause damage known as “spraing” that occurs as brown arcs and circles in the tuber flesh of susceptible cultivars (Harrison and Jones 1971). Some potato cultivars are particularly sensitive, with PMTV-infected plants producing tubers exhibiting severe

spraing symptoms. For example, cv. Saturna, widely used in the Scandinavian potato-processing industry, is a particularly sensitive cultivar, and incidences of spraing in 25% of tubers are commonly found (Sandgren 1995). High incidences of spraing can lead to whole batches of tubers being rejected for processing, with consequential loss of income for farmer and processor.

PMTV was until recently classified in the genus *Furovirus*. However, reclassification of furoviruses has placed PMTV in the proposed new genus *Pomovirus* (Torrance and Mayo 1997). The virus is prevalent in potato crops grown in areas with cool climates and has been identified in Northern Europe, Canada, China, Japan, and the Andean region of South America. PMTV is transmitted by the motile zoospores of the plasmodiophoromycete fungus *Spongospora subterranea* (Wallr.) Lagerh. (Jones and Harrison 1969; Arif et al. 1995), which causes powdery scab on tubers. Effective and environmentally acceptable chemical control of the fungal vector is not commercially available, and there are no sources of resistance or tolerance to PMTV that have been deliberately used in breeding programs.

The advantages of transgenic resistance to plant viruses with pathogen-derived sequences are well established and there are many examples of this approach being successfully applied against potato viruses (reviewed by Acosta et al. 1995). The most common pathogen-derived transgene used for resistance is that encoding the coat protein (CP), from which is derived coat protein-mediated resistance (CP-MR) (reviewed by Lomonossoff 1995). *Nicotiana benthamiana* was transformed to express a translatable version of the CP gene from a Scottish isolate of PMTV. Plants of these lines exhibit very strong resistance to infection with that isolate, a second Scottish isolate, and four Scandinavian isolates of PMTV (Reavy et al. 1995, 1997). This resistance was effective following manual, graft, or fungal inoculation (Reavy et al. 1995).

Important prerequisites to developing this transgene for use in commercial potato cultivars are identification of the characteristics that enable it to function effectively, and understanding of the fundamental mechanisms by which resistance operates. In some cases of CP-MR, resistance is expressed as a delay in symptom development and/or a reduction in virus titer and is thought to be mediated by the CP. In other cases, a very strong resistance or immunity appears to be mediated by

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the presence of translatable (CP_{trans}) or untranslatable ($CP_{nontrans}$) CP gene RNA transcripts. This latter resistance is thought to be due to selective targeting of virus CP gene sequences for degradation by mechanisms involved in gene silencing (for review see Baulcombe 1996). We describe experiments to assess characteristics of the PMTV CP transgene and the resistance it mediates in transformed lines of *N. benthamiana*, and attempt to determine what features of the transgene are required for expression of the resistant phenotype.

RESULTS

Number of loci and expression of a translatable PMTV CP transgene (CP_{trans}) in T_1 plants.

Plants of lines transformed with the CP_{trans} transgene displayed no abnormalities and were indistinguishable from nontransformed control plants. Presence of the transgene in individual plants within the T_1 progenies was determined by polymerase chain reaction (PCR). The number of transgene loci was estimated genetically from the segregation ratios of transgenic to nontransgenic plants. Such analyses indicated that lines contained one, two, or more than two transgene loci (Table 1). It is possible that some insertion events contained several copies of the T-DNA in tandem arrays. The segregation ratio obtained for line W1 was approximately 7:1 transgenic:nontransgenic, which suggested that W1 contained two transgene loci integrated on the same chromosome, leading to linkage.

Amounts of transgene RNA transcript in the CP_{trans} lines (determined by Northern [RNA] blotting with a digoxigenin-labeled cDNA probe and quantification by densitometry) were found to vary over a 13-fold range, with line W16 containing the most and line W8 the least (Fig. 1 and Table 1). The amount of transcript RNA detected was not correlated with the number of transgene loci (Table 1).

Table 1. Assessment of transgene RNA transcript and coat protein, resistance to potato mop-top virus (PMTV) inoculation, and number of transgene loci in T_1 CP_{trans} lines of *Nicotiana benthamiana*

T_1 line	Transcript	Steady-state levels of coat protein ^a and RNA transcript ^b in T_1 progeny plants		Resistance in T_1 progeny plants ^c	Transgene loci (no.) ^d
		Test 1	Test 2		
W1	72.6	1.72	1.55	81.4%	2
W2	64.8	1.48	1.06	81.2%	1
W5	14.9	0.16	0.02	99.3%	2
W8	7.0	0.56	0.13	90.4%	2
W9	32.0	0.50	0.13	87.3%	2
W16	88.2	0.78	0.48	98.5%	1
W20	38.7	0.30	0.05	81.0%	>2

^a Data given are enzyme-linked immunosorbent assay A_{405} values after deducting signal given by a sample from nontransformed plants (0.2).

^b Arbitrary units.

^c Resistance assessed by infectivity assay 18 days post inoculation; 88% of control nontransgenic plants became infected.

^d Number of transgene loci determined by polymerase chain reaction of 60 unselected plants of each line and analysis of segregation ratios by a χ^2 test. Numbers of plants tested are insufficient to allow a distinction to be made between lines with more than two loci. In W1 the two loci are linked, in other lines loci are unlinked. Number of loci relate to the T_0 parental plant, or a pool of T_1 plants.

The amount of transgene-expressed PMTV CP in non-inoculated plants of the CP_{trans} lines was determined by enzyme-linked immunosorbent assay (ELISA) of tissue extracts obtained by combining leaf samples from between 15 and 20 transgenic plants (selected by PCR) from each line. Similar relative amounts of CP were detected in the different lines, although there was variation in A_{405} values between tests (Table 1). Most importantly, the amount of CP detected varied substantially between lines: lines W1, W2, and W16 always contained large amounts of CP, and line W5 always gave the lowest A_{405} value. This reflected the amounts of transgene RNA transcript detected in plants and indicated that there was, in general, an association between steady-state levels of CP and RNA transcript.

When individual plants from the T_1 populations were analyzed by ELISA, it was found that the A_{405} values obtained differed greatly in some lines (Fig. 2). All plants of lines W1, W2, and W16 produced readily detectable amounts of CP. However, with lines W5, W8, W9, and W20, a few plants accumulated large amounts of CP but expression in the majority of plants was negligible, with ELISA values being little different from those given by nontransformed control plants. Individual T_1 plants of line W8 were examined for steady-state levels of RNA transcript and large differences (up to 100-fold) were found (Table 2). Similar results were obtained when individual T_1 plants of lines W9 and W20 were examined (results not shown). Because of the variation between individual plants of a line, amounts of CP and transcript were more strongly positively correlated in individual plants than from tests on pooled leaf samples.

Tests for resistance in T_1 plants expressing the CP_{trans} transgene.

Many tests for resistance to PMTV were made in the T_1 generation of CP_{trans} lines with different PMTV isolates. Reavy et al. (1997) found that resistance in the transformed lines was expressed equally to several isolates of PMTV, and estimates of resistance in Table 1 are based on data from tests with five isolates. Seedlings of nontransgenic control *N. benthamiana* plants were inoculated in each test, in addition to

Fig. 1. Northern (RNA) blot analysis of total RNA extracts from pooled leaf samples obtained from 24 plants of each of seven transgenic lines and one nontransformed, wild-type (WT) line, 10 μ g of RNA per lane. Lines BG1 to BG6 contain $CP_{nontrans}$ (a nontranslatable transgene), lines W8 and W16 contain CP_{trans} (a translatable transgene). Probe (approximately 600 bp) used was a digoxigenin-labeled cDNA that hybridizes with sequence on RNA3 encoding the coat protein (CP) gene. Bands detected in extracts from the various lines are of expected mobility for a polyadenylated transcript with an estimated length of approximately 650 to 700 nucleotides.

seedlings from transformed lines, and a mean of 88.1% of 463 inoculated control plants became infected. We found that the infected plants developed symptoms, although in some tests (possibly related to environment) these were dif-

ficult to detect and could not always be used as a definitive indication of infection. All infection data in the resistance tests were based on the results of infectivity assay. The infection data for seedlings of the transformed lines were

Fig. 2. Steady-state levels of coat protein in individual T_1 seedlings of transgenic lines of *Nicotiana benthamiana* detected by enzyme-linked immunosorbent assay. Each bar represents the A_{405} value (vertical axis) given by a leaf extract from an individual transgenic plant. Each panel is labeled with the number of the T_1 transgenic line tested.

normalized to take into account the efficiency of infection in the control plants. An allowance was also made in estimating the resistance of transformed lines, for the characteristic proportion of nontransgenic plants that should be susceptible to infection due to transgene segregation (determined by PCR as described above). Thus, we estimate that a mean of 11.9% of 851 inoculated transgenic plants became infected, with the resistance of individual lines varying from 81 to 99% (Table 1). These results are consistent with others (Reavy et al. 1995, 1997), which indicates that in some tests a few transgenic seedlings do become infected with PMTV although the majority are immune. There did not appear to be any correlation between resistance and amounts of transgene RNA transcript or CP (Table 1).

For some experiments, individual DNA extracts were made of all plants in a batch and were tested by PCR to detect the transgene prior to the resistance tests. These tests confirmed that infectious virus could be detected in a small proportion of transgenic plants after inoculation, although the majority of these plants did not produce symptoms. Some tests were made to determine how readily infectious virus could be detected, with repeated infectivity assays on the same plants at approximately 7-day intervals. Results of these tests suggested that many transgenic plants that gave a positive infectivity assay result did so in only one of either two or three assays, whereas assays on extracts from infected nontransgenic plants always gave positive results.

Tests were made to determine if the CP_{trans} transgene conferred nonspecific resistance to other viruses. Batches of 24 plants of line W8 were each inoculated with isolates of pea early browning tobnavirus, cucumber mosaic cucumovirus, and potato Y potyvirus. The three viruses multiplied in all inoculated transgenic plants and produced readily identifiable symptoms that were indistinguishable from those produced in similarly inoculated, nontransgenic control plants.

Table 2. Assessment of transgene transcript RNA, coat protein, and number of transgene loci in selected T₁ and T₂ generations of CP_{trans} lines of *Nicotiana benthamiana* and resistance of T₂ lines to infection with potato mop-top virus (PMTV)

T ₂ line	Steady-state levels of coat protein ^a and RNA transcript ^b					
	Parental T ₁ plant		T ₂ progeny plants			Transgene loci (no.) ^d
	Coat protein	Transcript	Coat protein	Transcript	Resistance ^c	
W8-27	1.63	98.9	1.64	98.7	100%	>2
W8-29	0.06	1.4	0.42	45.0	100%	>2
W8-30	0.66	56.8	0.27	44.9	100%	1
W8-43	0.46	33.5	0.36	53.5	100%	1
W8-44	1.61	85.5	1.0	94.1	100%	>2
W8-56	0.03	1.0	0.02	27.3	100%	2

^a Data given are enzyme-linked immunosorbent assay A₄₀₅ values after deducting signal given by a sample from nontransformed plants (0.2).

^b Arbitrary units.

^c Resistance assessed by infectivity assay 18 days post inoculation; 100% of control nontransgenic plants became infected.

^d Number of transgene loci determined by polymerase chain reaction of about 40 unselected plants of each line and analysis of segregation ratios by a χ^2 test. Numbers of plants tested are insufficient to allow a distinction to be made between lines with more than two loci. Number of loci relate to the T₁ parental plant, or a pool of T₂ plants.

Number of transgene loci, expression, and resistance to PMTV in T₂ generation plants containing the CP_{trans} transgene.

Six T₁ plants of CP_{trans} line W8 were selected as representing two high-, two intermediate- and two low-CP-expressing plants. These plants were allowed to self-fertilize and set seed, and T₂ seedling progenies were grown. The numbers of nontransgenic seedlings, determined by PCR and analysis of segregation ratios (transgenic:nontransgenic), indicated that the T₂ lines contained one, two, or more than two transgene loci (Table 2). The T₂ generation seedlings were assessed for steady-state levels of RNA transcript and CP in samples combined from 24 transgenic plants of each line. There was considerable variation in the steady-state levels of RNA transcript and CP accumulation between T₂ lines (Table 2), as was found with the T₁ plants. Steady-state levels of RNA transcript and CP tended to be greatest in T₂ progeny that came from high-expressing T₁ plants, and vice versa. Thus, two T₂ lines (W8-27 and W8-44) that contained the greatest amounts of CP were derived from the two T₁ plants that had the highest A₄₀₅ values in ELISAs. ELISA signals differed considerably between individual T₂ plants of W8 (Fig. 3). Thus, plants of W8-27 gave A₄₀₅ values that were all considerably greater than those given by nontransformed control plants (Fig. 3). By contrast, plants of line W8-30 gave A₄₀₅ values that were considerably less than those from plants of line W8-27, and values from many plants were only 2 to 3 times those given by control nontransformed plants (Fig. 3). The amounts of transgene RNA transcript or CP in the T₂ progenies were not related to the number of transgene loci in the T₁ parent plant. As with the T₁ generation, the correlation between CP and RNA transcript in T₂ progenies was greatest with individual plants rather than pooled samples because of the variation between individual plants.

The six lines of T₂ seedlings obtained from individual T₁ plants of line W8 were tested for resistance to PMTV. All these transgenic T₂ plants were immune to inoculation with PMTV (Table 2), indicating that, as with the T₁ plants, there was no correlation between resistance and levels of RNA transcript or CP.

Resistance tests on inoculated and systemic leaves at early time points after inoculation.

Further resistance tests on inoculated and systemic leaves of plants of CP_{trans} line W5 showed that leaf tissue was as resistant to infection 7 and 14 days post inoculation (DPI) as it was at 21 DPI (Table 3). Systemic leaves tested at 7 DPI were those immediately above the inoculated leaves; at 14 and 21 DPI they were separated from the inoculated leaves by 1 to 2 and 3 to 4 untested leaves, respectively. Thus, there was no indication that a "recovery" phenotype was manifested in the inoculated transgenic plants. If "recovery" were a factor in the resistance mechanism, lower leaves would show greater susceptibility to infection and the upper "recovered" leaves would be resistant. However, excluding the very small proportion of transgenic plants that do become infected, all leaves of the inoculated plants expressing the CP_{trans} transgene were resistant.

Characteristics of transformed lines containing an untranslatable PMTV CP transgene (CP_{nontrans}).

The steady-state level of transgene RNA transcript varied substantially between lines of CP_{nontrans} T₁ plants (Fig. 1). The highest level of transcript accumulated in line BG5, which contained 10-fold more than line BG4, which expressed the least. The variation in amounts of transgene RNA transcript between lines was, in general, similar with both CP_{trans} and CP_{nontrans} lines. ELISA of leaf tissue from plants of CP_{nontrans} lines gave no A₄₀₅ values above background and confirmed that the mutated transgene was not translatable. In preliminary tests we found that seedlings of two CP_{nontrans} lines had no resistance to inoculation with PMTV and all plants developed characteristic symptoms of PMTV infection at approximately 14 DPI. In more extensive tests, about 48 plants of each of five independent CP_{nontrans} lines (BG1, BG2, BG4, BG5, and BG6) were inoculated with PMTV; 97.9% of the control nontransgenic plants and 97.1% of the transgenic plants became infected. PMTV was readily detected by both ELISA and infectivity assay of inoculated transgenic plants. In these tests symptoms appeared on all infected plants but they developed 2 to 5 days later on plants of some transgenic lines, compared with the control nontransgenic plants. Furthermore, symptoms on transgenic plants were usually milder than those on control plants. It seems that transformation with the CP_{nontrans} trans-

gene did not confer resistance to PMTV but may ameliorate the symptoms.

DISCUSSION

In work reported here and by Reavy et al. (1995), we have tested 10 CP_{trans} lines in which CP could be detected in very different amounts, but all of which can be regarded as immune or strongly resistant to PMTV. A small proportion of transgenic plants become infected and develop symptoms and infectious virus in the same way as control plants. However, we have observed a smaller number of plants in which virus replication was detected by infectivity assay but which failed to develop symptoms of infection. Furthermore, in tests when such plants were subjected to two or three infectivity assays, it was often found that only one assay gave a positive result. Thus, it seems that some viral replication may occur in a small proportion of transgenic plants but its extent may be limited, resulting in lack of symptom expression in some plants and limited replication in all tissues, and leading to failure to detect virus. Data from resistance tests suggest that all lines (T₁ and T₂) of *N. benthamiana* containing the CP_{trans} transgene have a very high level of resistance to manual inoculation. Resistance was apparently stronger in some T₁ lines than in others; indeed, in two lines resistance was expressed in nearly all plants. However, it is possible that the effectiveness of the transgene is partly determined by environmental conditions because we have found more transgenic plants to be resistant in tests conducted in warmer weather than in those made in the winter. Although more comprehensive tests would be required to be certain of this point, it is noteworthy that with potato cv. Saturna transformed with the same transgene, CP expression in some lines is dependent on environment (H. Barker, B. Reavy and K. D. McGeachy, unpublished results).

Beet necrotic yellow vein virus (BNYVV) is fungus transmitted and is a member of the genus *Benyvirus*, which is related to the genus *Pomovirus* (Torrance and Mayo 1997). Sugar beet (*Beta vulgaris* L.) plants have been transformed with the BNYVV CP gene and challenged by bait tests with viruliferous soil; transgenic plants exhibited decreased virus accumulation, compared with nontransgenic plants (Mannerlöf et al. 1996). The PMTV CP_{trans} transgene also confers

Fig. 3. Steady-state levels of coat protein in individual T₂ seedlings of transgenic lines of *Nicotiana benthamiana* detected by enzyme-linked immunosorbent assay. Each bar represents the A₄₀₅ value (vertical axis) given by a leaf extract from an individual transgenic plant. Each panel is labeled with the number of the T₂ transgenic line tested.

Table 3. Resistance in inoculated and systemic leaves of line W5 at three times post inoculation

Experiment/line	Resistance in plants ^a (no. infected/no. tested)		
	7 DPI	14 DPI	21 DPI
Experiment 1			
W5 inoculated leaf	1/25	1/25	NT ^b
W5 systemic leaf	0/25	1/25	1/25
WT control ^c inoculated leaf	4/10	9/9	NT
WT control systemic leaf	1/10	8/10	8/9
Experiment 2			
W5 systemic leaf	1/33	0/33	0/33
WT control systemic leaf	26/27	26/27	27/27

^a All plants were transgenic and identified by polymerase chain reaction screening before use. Leaves were tested at 7, 14, and 21 days post inoculation (DPI).

^b Not tested.

^c Wild-type control, nontransformed plants.

resistance to infection in soil bait tests (Reavy et al. 1995). However, it is difficult to make precise comparisons with PMTV, as only one transgenic line for each of two BNYPV gene constructs was examined.

There are now several recognized resistance responses associated with transformation with CP genes. With translatable CP transgenes, resistance is frequently expressed as a reduction in the number of lesions on inoculated leaves, a reduced rate of systemic disease development, and low levels of virus accumulation (Powell-Abel et al. 1986; Loesch-Fries et al. 1987; Tumer et al. 1987; van Dun et al. 1987; Hemenway et al. 1988). In some cases, resistance is positively correlated with levels of CP in transgenic plants, e.g., tobacco mosaic virus (Nejidat and Beachy 1989; Powell et al. 1990), alfalfa mosaic virus (Loesch-Fries et al. 1987; van Dun et al. 1988; Halk et al. 1989; Hill et al. 1991), and potato virus X (Hemenway et al. 1988; Hoekema et al. 1989). In their experiments with cucumber mosaic virus, Quemada et al. (1991) could detect no correlation between amount of CP produced and resistance, with some high-expressing lines becoming infected. Resistance to PMTV is not correlated with levels of CP. In some cases, resistance conferred by expression of CP can be overcome by inoculation with RNA inocula (Loesch-Fries et al. 1987; Nelson et al. 1987; van Dun et al. 1987; Angenent et al. 1990; Wisniewski et al. 1990). However, we found that, with total leaf RNA extracts from PMTV-infected leaves as inocula, resistance in CP_{trans} line W5 was expressed as effectively as it was against particle inocula (leaf extracts). In other examples, CP-mediated transgenic resistance is positively correlated with steady-state levels of transcript RNA (Farinelli and Malnoë 1993; Barker et al. 1993). Resistance to PMTV is not correlated with steady-state levels of transcript RNA. With nontranslatable CP transgenes or those that are theoretically translatable but from which no CP expression can be detected, quantitative resistance may be expressed against virus accumulation but also can be stronger and can be manifested as immunity to infection (exemption from infection) even with high concentration inocula (Lindbo et al. 1993; Smith et al. 1994, 1995). However, a frequently observed phenomenon with both translatable and nontranslatable CP transgenes is that the most resistant lines are those in which there is only a low steady-state level of transgene RNA transcript or protein (Stark and Beachy 1989; Lawson et al. 1990; Regner et al. 1992; van der Vlugt et al. 1992; Dougherty et al. 1994; Smith et al. 1994, 1995; Cassidy and Nelson 1995; Mueller et al. 1995; Yepes et al. 1996; Wang et al. 1997). To account for these observations, it has been suggested that induction of a sequence-specific, RNA-degrading mechanism is responsible for the resistance observed (Lindbo et al. 1993; Dougherty et al. 1994; Smith et al. 1994). Resistance to PMTV is equally effective in lines expressing high or low levels of RNA transcript and CP. The resistance to PMTV appears to be independent of the number of transgene loci, whereas homology-dependent, RNA-mediated resistance is often dependent on gene dosage (Lindbo and Dougherty 1992; van der Vlugt et al. 1992; Smith et al. 1994; Goodwin et al. 1996).

The resistance that is expressed is strong (many plants are immune to inoculation) and is independent of steady-state levels of transgene RNA transcript or CP accumulation. However, we show that strong resistance is expressed only in lines containing a translatable PMTV CP gene, but only weak re-

sistance that ameliorates the symptoms is conferred by the same CP gene sequence when rendered nontranslatable by the addition of early stop codons. Thus, the strong resistance to PMTV is not RNA mediated. Another feature of RNA-mediated resistance is the existence of the "recovery" phenotype in which inoculated plants initially develop symptoms but subsequent growth is virus free when assayed by ELISA or infectivity assay, and plants are immune to re-infection (Lindbo et al. 1993; Dougherty et al. 1994). It appears that both the immune and recovery phenotypes operate by a single process, as immune lines exhibit a recovery phenotype when inoculated at early developmental stages (Tanzer et al. 1997). The presence of a translation initiation codon has been shown to be important in inducing resistance or a recovery phenotype in the case of peanut stripe potyvirus (Cassidy and Nelson 1995). Both PMTV CP_{trans} and CP_{nontrans} constructs contain initiation codons but resistance was only detected with the fully translatable version of the gene. We have found that all leaves of resistant plants of one CP_{trans} line (including the inoculated leaves) are free of symptoms and infectious virus. This suggests that a recovery phenotype plays no part in resistance conferred by CP_{trans}. CP-MR to PMTV therefore does not appear to fit with any of the categories above and may be unique.

MATERIALS AND METHODS

Vector construction.

The CP_{trans} transgene vector was described by Reavy et al. (1995). A nontranslatable version of the PMTV CP gene was made by PCR with the same primer (A838) complementary to the 3' end of the CP gene as for the translatable version (Reavy et al. 1995) and a new upstream primer (A1649: 5'CGGATCCGAAATGGCTTAATGATAGGTGAGC-3') representing the start of the CP gene (the initiation codon is underlined). Codons 3, 4, and 5 were mutated to termination codons. The PCR product was cloned into pT7Blue-T (Novagen, Madison, WI) and the mutated sequence verified by dye-terminator sequencing with an Applied Biosystems (Foster City, CA) 373 automated sequencer. The CP gene was excised as a *Bam*HI fragment with recognition sites incorporated into the PCR primers, and cloned into ROK2 between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) gene termination sequence.

Plant transformation and determination of number of transgene loci.

Transgenic lines of *N. benthamiana* expressing the CP_{trans} transgene were obtained in the study described by Reavy et al. (1995), although lines W5, W8, W9, and W20 have not been previously described. *N. benthamiana* plants expressing CP_{nontrans} (BG lines) were produced by transformation as described by Benvenuto et al. (1991).

Most experiments were made with plants grown from seed (T₁ generation) arising from self-fertilization of the original transformants (Reavy et al. 1995); a few were made on plants grown from seed (T₂ generation) arising from self-fertilization of some selected T₁ generation plants. Seedlings of transgenic test plants were grown on compost without selection in an aphid-proof glasshouse. In order to determine the characteristic proportion of transgenic seedlings in each CP_{trans} line,

DNA extracts from 40 or 60 seedlings (8- to 9-leaf stage) were tested by PCR with an oligonucleotide primer (5'-AATCATCGCAAGACCGGCAACAGG-3') complementary to a part of the *nos* gene terminator sequence (Bevan et al. 1983) and the PMTV A839 primer essentially as described by Reavy et al. (1997). PCR of transgenic plants gave a DNA product of 620 bp. Analysis of the segregation ratios (transgenic to nontransgenic) by a χ^2 test enabled the number of transgene loci to be estimated, with the premise that *N. benthamiana* is a diploid species.

Virus isolates and propagation.

One Scottish and four Scandinavian PMTV isolates were maintained in *N. benthamiana* as described by Reavy et al. (1997). Virus was transmitted by manual inoculation in which extracts of freshly macerated leaves from infected *N. benthamiana* plants (1 g of leaf per 5 ml of water) were rubbed onto corundum-dusted leaves of test plants.

Virus resistance tests.

Unselected seedlings were used in resistance tests in preference to seedlings screened for kanamycin sensitivity on agar media because (i) such selection proved to give poor segregation into sensitive and nonsensitive seedlings and (ii) the technique was too laborious when selecting large numbers of test plants. For each experiment, young test plants of the T₁ generation of the transgenic lines and of a control nontransgenic line were manually inoculated at the 8- to 9-leaf stage.

For tests on CP_{nontrans} lines, resistance was assessed by symptomatology and ELISA; it was possible to use ELISA at approximately 18 DPI for these lines because there was no endogenous CP expression, which could interfere with the results.

For CP_{trans} lines, extracts of the inoculated plants were tested approximately 18 DPI (unless stated otherwise) by a very sensitive infectivity assay. In this assay, indicator plants of *N. benthamiana* were manually inoculated with macerated leaf tissue obtained from test plants, as described by Reavy et al. (1995). At 18 DPI, the systemic leaves assayed for infectivity were above the inoculated leaves and separated from them on the stem by three untested leaves. Infection of indicator plants was assessed by symptomatology and ELISA approximately 21 DPI.

Quantification of RNA transcript.

A PCR product produced by oligonucleotides A838 and A839, corresponding to the CP gene (Reavy et al. 1995), was labeled with digoxigenin (Boehringer Mannheim, Lewes, UK) and used as a probe to estimate steady-state levels of RNA transcript in total leaf RNA extracts as described by Webster and Barker (1998). Total leaf RNA was extracted from samples pooled from 24 transgenic plants (selected by PCR) of each transgenic line at the 10- to 12-leaf stage, and analyzed by Northern blotting on nylon membranes as described by Webster and Barker (1998). Bands of polyadenylated RNA transcript of the estimated size (650 to 700 nucleotides) were identified in the Northern blots and quantified by densitometry of scanned images on exposed film, with the Intelligent Quantifier PC software (BioImage, Ann Arbor, MI). RNA transcript was also quantified by nucleic acid spot hybridization and densitometry as above. Two inde-

pendent sets of RNA extracts were prepared and when analyzed by both methods gave very similar estimates of the relative amounts of transcript.

ELISA.

Accumulation of endogenous PMTV CP in transgenic plants and PMTV particle antigen in inoculated infectivity assay plants was assessed by triple antibody sandwich ELISA. This used a polyclonal antibody (1 µg/ml) to coat microtiter plates; monoclonal antibody SCR69 (1 µg/ml) to detect trapped antigen; alkaline phosphatase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) to detect bound monoclonal antibody; and 4-nitrophenyl phosphate (Boehringer Mannheim) at 1 mg/ml as substrate, essentially as described by Torrance et al. (1993). Substrate absorbance signals were measured at 405 nm (A₄₀₅) in a Dynatech (Billingshurst, UK) MR7000 microplate reader.

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