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Immunity to Potato Mop-Top Virus in *Nicotiana benthamiana* Plants Expressing the Coat Protein Gene Is Effective Against Fungal Inoculation of the Virus

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***Nicotiana benthamiana* stem tissue was transformed with *Agrobacterium tumefaciens* harboring a binary vector containing the potato mop-top virus (PMTV) coat protein (CP) gene. PMTV CP was expressed in large amounts in some of the primary transformants. The five transgenic lines which produced the most CP were selected for resistance testing. Flowers on transformed plants were allowed to self-fertilize. Transgenic seedlings selected from the T_1 seed were mechanically inoculated with two strains of PMTV. Virus multiplication, assayed by infectivity, was detected in only one transgenic plant of 98 inoculated. T_1 plants were also highly resistant to graft inoculation; PMTV multiplied in only one plant of 45 inoculated. Transgenic T_1 seedlings were challenged in a bait test in which they were grown in soil containing viruliferous spores of the vector fungus *Spongospora subterranea*. In these tests only two plants out of 99 became infected. Of the five transgenic lines tested, plants of three lines were immune to infection following manual, graft, or fungal inoculation.**

Additional keywords: fungus transmission, furovirus, transgenic resistance.

Resistance to virus infection as the result of transgenic expression of a virus coat protein (CP) gene in a plant (coat protein–mediated resistance, CP-MR) has been shown to be effective with a large number of virus systems (for some reviews see Beachy *et al.* 1990; Wilson 1993). Most work has involved assay of resistance following mechanical transmission of virus, and there have been few studies in which the relative efficacy of CP-MR to manual and vector transmission of viruses has been assessed. CP-MR has been reported to be effective against manual and aphid inoculation of potato virus Y (PVY) (Lindbo and Dougherty 1992; van der Vlugt and Goldbach 1993) and cucumber mosaic virus (CMV) (Quemada *et al.* 1991). However, there are examples in which CP-MR was effective against manually inoculated virus but not against vector-borne virus (Lawson *et al.* 1990; Ploeg *et al.* 1993).

The furovirus group of fungus-transmitted, rod-shaped viruses is responsible for economic losses in a variety of crops, such as potato (potato mop-top virus, PMTV), wheat (soil-borne wheat mosaic virus), and sugar beet (beet necrotic yellow vein virus, BNYVV) (Brunt and Richards 1989). There have been few attempts to obtain transgenic resistance to these viruses, although it has been shown that beet protoplasts expressing BNYVV CP are resistant to the virus (Kallerhoff *et al.* 1990). PMTV is transmitted by motile zoospores of the plasmodiophoromycete fungus *Spongospora subterranea* (Jones and Harrison 1969). Infection with the virus causes some yield loss and, more importantly, qualitative damage in the form of brown arcs and circles, known as spraing, in the flesh of tubers of susceptible cultivars (Harrison and Jones 1971). Some potato cultivars are particularly sensitive to PMTV infection and produce tubers with severe spraing symptoms. In other cultivars the foliage may become infected but the tubers remain free of spraing symptoms. Although this suggests a genetic basis for the reaction of *Solanum tuberosum* to PMTV infection, no sources of resistance or tolerance to PMTV have been deliberately used in breeding programs (R. M. Solomon-Blackburn, personal communication). The genome of PMTV consists of three RNA species (Scott *et al.* 1994). Nucleotide sequence analysis has shown that the CP gene is contained in RNA 3 along with a possible read-through protein, which would be expressed by leaky termination of the CP gene stop codon (Kashiwazaki *et al.*, in press). We describe the use of the PMTV CP gene to obtain resistance by stable genetic transformation of the host plant *Nicotiana benthamiana*. This novel form of resistance to PMTV will have important implications for control of PMTV in potato crops.

RESULTS

Virus isolates.

Two isolates of PMTV were used in these studies. Isolate T came from Scotland (Todd 1965); its properties were described by Harrison and Jones (1970). Isolate S was isolated recently from a farm near Auchterader, Perthshire, Scotland (Arif *et al.* 1994). The CP genes of these isolates are 98% identical, and there is no difference in their reactivities with

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the monoclonal antibodies used in these studies (L. Torrance, personal communication).

Plant transformation.

N. benthamiana stem tissue was transformed with *Agrobacterium tumefaciens* carrying a binary vector containing the PMTV CP gene of isolate T. Nineteen primary transformed plants (T_0 generation) were obtained, from which self-fertilized seed (T_1) was collected. Total leaf RNA extracted from primary transformants was assayed for the PMTV CP gene transcript by reverse transcriptase polymerase chain reaction (PCR) using oligonucleotide primers A838 and A839. Fourteen gave a PCR product of the expected size (566 bp) for the CP gene sequence. The five transgenic lines (W1, W2, W7, W16, and W25) which produced the most CP, as assessed by enzyme-linked immunosorbent assay (ELISA), were selected for resistance testing. In batches of transgenic T_1 plants representing different lines, assayed by ELISA for CP accumulation in leaves, the mean level of CP varied over an eightfold range up to 3.75 μg/g of leaf (fresh weight); the lines ranked in the order W16, W2, W1, W7, and W25 (from lowest to highest CP expressor). The amount of CP produced by individual T_1 plants of a particular line also varied over a five- to 10-fold range. CP was readily detected in roots, but the concentration was not determined.

T_1 seed from the five lines which germinated on kanamycin-containing media segregated to give a very close fit to a ratio of 3:1 between resistant and sensitive seedlings. This indicated that there was a single independent locus of the T-DNA containing the NPTII gene in each line. In tests in which seed was not selected on kanamycin but was screened by ELISA for CP expression, all lines except W1 segregated to give a very close fit to a ratio of 3:1 between CP-expressing and non-CP-expressing seedlings, confirming the hypothesis that these lines contain a single independent locus of integrated T-DNA, each with an active copy of the CP gene. W1 gave a ratio of approximately 7:1, suggesting that W1 may contain two T-DNA loci integrated on the same chromosome, which led to linkage, but that the NPTII gene at one locus is inactive.

Table 1. Infectivity assay and enzyme-linked immunosorbent assay (ELISA) for detection of virus replication in coat protein transgenic lines of *Nicotiana benthamiana* after manual inoculation with potato mop-top virus (PMTV) isolates S and T

	PMTV-T[a]		PMTV-S[a]	
Line	Infectivity assay[b]	ELISA[c]	Infectivity assay[b]	ELISA[c]
W1	0/8	0/8	0/6	0/6
W2	0/12	2/12	0/12	3/12
W7	0/12	1/12	0/12	1/12
W16	0/6	0/6	0/6[d]	1/12
W25	0/6	1/6	0/6	2/6
Wild type (control)[e]	48/48	48/48	48/48	48/48

[a] Number of plants with positive assay results per number of plants tested.
[b] The infectivity assay was performed 4 wk after inoculation.
[c] ELISA was performed three times after inoculation at approximately 7- to 10-day intervals. Positive samples are those with A_{405} values three times those given by noninoculated plants of the same line.
[d] Six test plants died before the infectivity assay was completed.
[e] Lines W2 and W7 were tested independently of lines W1, W16, and W25; 24 nontransgenic wild-type plants were used as controls on each occasion.

Manual inoculation.

Up to 12 plants from the T_1 generation of each of the lines selected for resistance tests were challenged by manual inoculation with sap from plants infected with PMTV-T or PMTV-S. Approximately 1 wk after inoculation, all the nontransgenic control plants displayed visible symptoms of PMTV infection, whereas none of the inoculated transgenic plants displayed symptoms during the 4 wk of the experiment. Growth of the inoculated plants was terminated after 4 wk, and virus replication was assessed by a sensitive biological assay (recovery of infectious virus by inoculation of indicator test plants with leaf sap). The dilution end-point of this assay is approximately 10^{-3} (Harrison and Jones 1970),

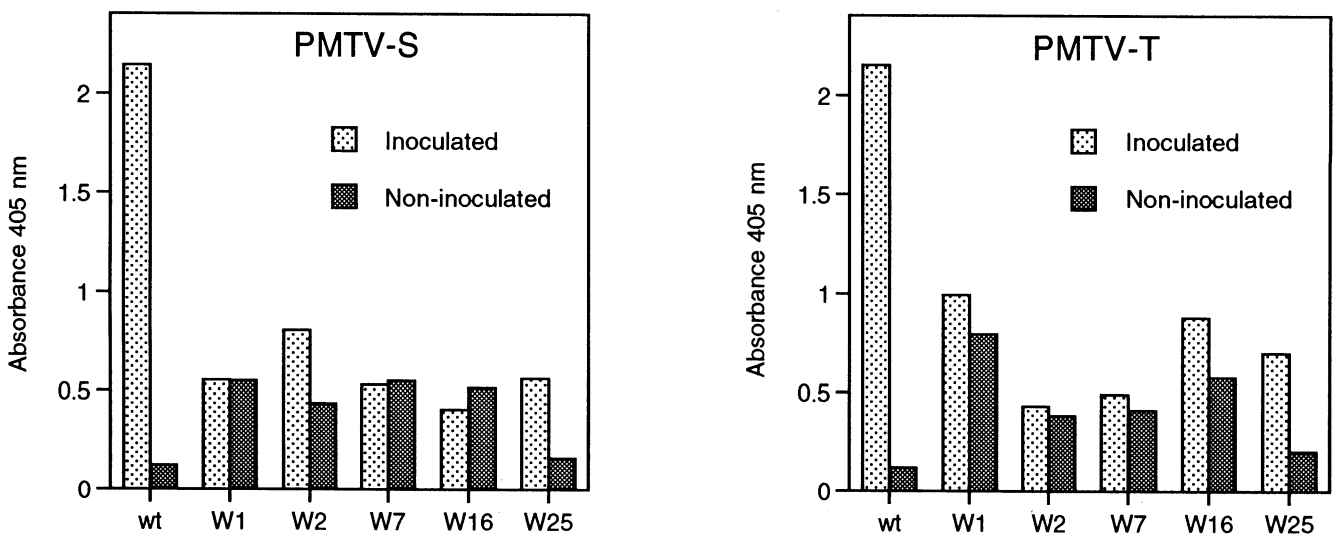

Fig. 1. Enzyme-linked immunosorbent assay of *Nicotiana benthamiana* transgenic plants and wild-type (wt) control plants either left uninoculated or 4 wk after inoculation with potato mop-top virus (PMTV) isolate S or T. Measurements of absorbance (A_{405}) were made after the substrate had reacted at room temperature for 2 hr and then at 4° C for 14 hr. The data are means of extracts from six to 12 transgenic plants and 48 wild-type plants.

allowing detection of virus concentrations equivalent to 0.1–1% of that found in infected susceptible plants. This infectivity assay showed that none of the inoculated transgenic plants contained infectious PMTV, although all of the inoculated control plants were infected (Table 1). The data suggest that the transgenic plants were immune to mechanical inoculation.

Inoculated plants were also assayed by ELISA. Extracts of systemically infected leaves of nontransgenic control plants gave high A_{405} values in ELISA (Fig. 1). Most inoculated transgenic plants did not show any increase in ELISA values in comparison with values given by noninoculated transgenic plants. On occasions, higher ELISA values were obtained from a few plants of all lines. In most cases this was within the range of variability in CP content found in noninoculated plants, and generally these higher ELISA values were not sustained in individual plants throughout the course of the experiment. Thus, the mean values (shown in Fig. 1) obtained from ELISA of the inoculated transgenic plants were occasionally greater than those given by noninoculated plants. However, in line W25 the ELISA values from several inoculated plants were up to 40-fold greater than in noninoculated transgenic plants of this line. Indeed, in one inoculated plant of line W25, in three consecutive tests as much CP was detected as in infected nontransgenic control plants.

The results of the infectivity assay indicate that no infectious virus could be detected in any inoculated transgenic plants. However, the possibility was considered that the elevated level of CP detected by ELISA in inoculated plants of line W25 was due to abnormal virus replication which did not result in the production of infectious virus. To examine this possibility, leaf extracts of four plants from line W25 that gave very high ELISA values (including the plant that consistently accumulated almost as much CP as infected control plants) were examined by immunosorbent electron microscopy. No PMTV-like particles were detected in the transgenic plants, but they were readily detected in infected control plants. In further tests, total RNA extracts of these four transgenic W25 plants did not give a signal by reverse transcriptase PCR using primers specific for the CP read-through domain. This method readily detected viral RNA in infected control plants. We therefore concluded that virus replication leading to the production of infectious virus was not the cause of these high ELISA values.

Tests were made to confirm that resistance to PMTV infection in T_1 seedlings was due to the expression of the CP gene rather than somaclonal variation in the transformed lines. This was done by inoculating populations of transgenic and nontransgenic seedlings which segregated from the T_1 seed of four of the five selected lines. These populations were obtained by growing seed on a kanamycin-free medium and screening by ELISA to identify seedlings which expressed CP. A selection of CP-expressing (transgenic) and non-CP-expressing (nontransgenic) seedlings was manually inoculated with PMTV-S. All of the nontransgenic T_1 seedlings were susceptible to infection, whereas the transgenic seedlings were generally resistant; only one W16 transgenic T_1 plant became infected (Table 2).

Table 2. Infectivity assay of transgenic and nontransgenic seedlings in T_1 populations of transformed *Nicotiana benthamiana* lines after manual inoculation with potato mop-top virus isolate S

Line	Transgenic seedlings[a]	Nontransgenic seedlings[a]
W1	0/3	7/7
W7	0/3	8/8
W16	1/3	6/6
W25	0/3	3/3
Wild type (control)	...	7/7

[a] Number of plants with positive assay results per number of plants tested. Infectivity assay was performed 3 wk after inoculation. Transgenic and nontransgenic plants were selected by triple-antibody sandwich enzyme-linked immunosorbent assay to detect coat protein production in a population of T_1 seedlings which were grown on kanamycin-free medium.

Table 3. Infectivity assay of coat protein transgenic lines of *Nicotiana benthamiana* after graft inoculation with potato mop-top virus (PMTV) isolates S and T

Line	PMTV-S[a]	PMTV-T[a]
W1	0/6	[b]NT[b]
W2	1/12	0/6
W7	0/6	0/6
W16	0/3	NT
W25	0/6	NT
Wild type (control)	12/12	6/6

[a] Number of plants with positive assay results per number of plants tested. Infectivity assay was performed 4 wk after inoculation.
[b] NT = not tested.

Graft inoculation.

Transgenic plants of the five lines were challenged by graft inoculation with scions from plants infected with PMTV-S or PMTV-T to assess the resilience of the resistance identified by manual inoculation. Notwithstanding the high inoculum pressure thus exerted, infectivity assay of leaf tissue from graft-inoculated plants of lines W1, W7, W16, and W25 showed that none had become infected 4 wk after inoculation, although one plant of line W2 did produce infectious virus (Table 3). All control plants were infected as a result of graft inoculation.

Fungal inoculation.

Transgenic plants of the five lines were exposed to viruliferous *S. subterranea* in a bait test to assess their resistance to PMTV transmitted by its fungal vector. Infectivity assay was used to determine if viral replication had occurred in leaf tissue at 3–4 wk postinoculation and in both leaf and root tissue

Table 4. Infectivity assay of coat protein transgenic lines of *Nicotiana benthamiana* after inoculation with potato mop-top virus isolate S by the fungal vector *Spongospora subterranea*

Line	Leaf tissue[a]	Leaf and root tissue[a]
W1	0/23	0/23
W2	0/23	0/23
W7	0/22	0/22
W16	1/18	2/18
W25	0/13	0/13
Wild type (control)	60/60	60/60

[a]Number of plants with positive assay results per number of plants tested. Infectivity assays were performed on leaf tissue 3–4 wk after the start of fungal inoculation and on leaf and root tissue 6–8 wk after the start of fungal inoculation.

6–8 wk postinoculation. Virus was detected in all inoculated nontransformed control plants 3–4 wk after exposure to the fungal vector. By contrast, viral replication was not detected in leaves of any plants of four of the transgenic lines, even 6–8 wk postinoculation, and it was detected in only two plants of line W16 (Table 4). Infectivity assay of roots was used to determine if limited virus replication had occurred only in root tissues of the transgenic plants after fungal inoculation. Virus replication was detected in roots of all inoculated control plants and in roots of the two plants in line W16 in which virus was also detected in leaf tissue, but in no other transgenic plants (Table 4).

Tests were made to determine whether transgenic plants had not become infected with PMTV in these bait tests because of a nonspecific resistance to the fungus vector. At the time of the second infectivity assay (6–8 wk postinoculation) roots of three randomly selected plants from each transgenic line tested in the bait test were examined by light microscopy and staining with phenol–cotton blue to detect the presence of the plasmodial stage of *S. subterranea*. Root hairs of all 15 transgenic plants had moderate to heavy infection by the fungus, as did nine nontransgenic control plants. Thus transgenic expression of the PMTV CP gene in *N. benthamiana* did not appear to prevent infection and infestation by the fungus vector, and we can be certain that resistance is expressed to some stage of PMTV infection and/or replication.

DISCUSSION

Expression of virus CP genes in plants has provided extremely strong resistance on a number of occasions. For example, no symptoms of infection could be detected after manual inoculation of transgenic tobacco plants expressing the alfalfa mosaic virus CP gene, and virus was not detected by infectivity assay (van Dun *et al.* 1987; Tumer *et al.* 1987) Similarly, transgenic expression of potato virus S CP provided extremely strong resistance in *Nicotiana debneyi,* in which no virus symptoms or antigen accumulation occurred after manual inoculation (MacKenzie and Tremaine 1990), and in potato plants after graft inoculation (MacKenzie *et al.* 1991). Tobacco plants expressing untranslatable transcripts of the tobacco etch virus CP gene are immune to mechanical inoculation; no disease symptoms are observed, and no virus could be detected by infectivity assays (Lindbo and Dougherty 1992). Immunity to CMV transmitted by aphid inoculation but not by manual inoculation has been reported in plants expressing the CMV CP gene (Quemeda *et al.* 1991). The only reported case of immunity to both manual and vector transmission of virus is that described by Lawson *et al.* (1990), in which one line of potato plants expressing the CP genes of PVY and potato virus X was resistant to PVY transmitted by both forms of inoculation. Our results demonstrate that similar immunity to a fungus-transmitted virus can also be produced. The other major group of soil-inhabiting plant virus vectors are nematodes, and in the case of tobacco rattle virus (TRV), inoculation with viruliferous nematodes overcame resistance mediated by the TRV CP gene, which was effective against manual inoculation (Ploeg *et al.* 1993). This breakdown of resistance to nematode-transmitted virus may be due to some aspect of the vector-virus interaction rather than vector–root cell interaction, because nematodes were able to transmit TRV into both roots and leaves of CP-transgenic plants. Our results indicate that root cells do not represent a "weak spot" for transgenic resistance, and suggest that transmission by fungi and transmission by nematodes may be fundamentally different with respect to this type of protection. We have no reason to believe that there is any difference in the mechanism of fungal transmission of PMTV in the roots of *N. benthamiana* and *S. tuberosum* and therefore anticipate that this type of resistance could be introduced into sensitive potato cultivars. It seems likely that effective resistance could also be engineered to other economically important fungus-transmitted viruses, such as BNYVV, in which resistance mediated by the BNYVV CP gene has been shown to be effective in beet protoplasts (Kallerhoff *et al.* 1990).

A peculiarity of a few of the plants we examined was an apparent large increase in the amount of antigen following inoculation, even though this was not associated with virus production. A transgenic tobacco plant expressing the CMV CP gene apparently accumulated large amounts of antigen while remaining asymptomatic, and this was thought to represent suppression of symptoms rather than inhibition of virus multiplication (Quemada *et al.* 1991). Although in our tests a few inoculated plants accumulated large amounts of antigen, infectious PMTV was not detected by infectivity assay. It therefore appears that, in some cases, inoculation of plants expressing a CP gene can result in increased accumulation of antigen, but without the production of viable virus. We are not able at this stage to determine whether the increased antigen accumulation represents a stimulation of CP expression from the transgene, whether it is a product of defective virus replication, or whether it is due to some other cause.

MATERIALS AND METHODS

Vector construction.

PCR was used to amplify the PMTV-T CP gene from a plasmid clone containing cDNA encoding most of RNA 3 of isolate T of PMTV (Kashiwazaki *et al.*, in press). The CP gene starts at nucleotide 289 and ends at nucleotide 817. The primers used were

A838 (5′-CGGGATCCTTATGCACCAGCCCAGCGT-3′)

which is complementary to the 3′ end of the CP gene (nucleotides 801–818), and

A839 (5′-TCGGATCCTCTCGGATACCACCCTT-3′)

which is the same as the 5′ untranslated region immediately upstream of the CP gene (nucleotides 268–284). Both primers incorporated additional nucleotides (underlined) to create *Bam*HI restriction sites. In addition, A838 changes the natural CP gene termination codon from TAG to TAA to minimize possible read-through. The PCR product was cloned into the *Bam*HI site of pBluescript II KS(+) (Stratagene) and subsequently excised from that vector as a *Bam*HI fragment and cloned into the *Bam*HI site of the binary vector pROK2. Clones with the CP gene in the sense orientation with respect to the cauliflower mosaic virus 35S promoter were identified by restriction enzyme mapping to give clone PMTV-T

CP/ROK2. This vector was mobilized from *Escherichia coli* into *A. tumefaciens* strain LBA4404 by triparental mating; pRK2013 was used as a helper. Recombinant clones of agrobacteria were isolated by selection on kanamycin-rifampicin plates and the presence of PMTV-T CP/ROK2 confirmed by restriction enzyme mapping of plasmid minipreps. The sequence of the CP gene was not determined at this stage or in transgenic plants, and the possibility that the gene contains mutations not detectable by restriction enzyme mapping cannot be excluded.

Plant transformation.

Pieces of *N. benthamiana* stem tissue were transformed as described by Benvenuto *et al.* (1991). Rooted plantlets of the primary transformants (T_0) were transferred to an aphid-proof glasshouse kept at 20° C; their flowers were allowed to self-fertilize, and seed was collected. In order to select transgenic seedlings (T_1) for manual inoculation experiments, seeds were germinated on medium containing kanamycin sulfate (400 μg/ml) as described by Barker *et al.* (1993). Kanamycin-sensitive and kanamycin-resistant seedlings were counted before the transgenic seedlings were selected for propagation in the glasshouse and resistance testing. For the fungal transmission and graft inoculation experiments, seeds were germinated in compost without selection. At the four- to five-leaf stage, a leaf from each seedling was tested by ELISA, and transgenic seedlings were identified by the production of CP.

ELISA.

CP production in noninoculated transgenic plants and accumulation of PMTV antigen in inoculated plants were assessed by triple antibody sandwich ELISA using monoclonal antibody SCR69 (Torrance *et al.* 1993).

Virus inoculation.

Cultures of PMTV isolates T and S (Harrison and Jones 1970; Arif *et al.* 1994) were maintained in *N. benthamiana.* Virus was transmitted by manual inoculation in which freshly extracted sap from infected *N. benthamiana* plants (1 g of leaf per 5 ml of water) was rubbed onto Carborundum-dusted leaves of test plants. For graft inoculation, infected scions (shoot apices) from PMTV-infected *N. benthamiana* plants were cleft-grafted onto test plants from which the shoot apex had been removed. PMTV was transmitted by fungal inoculation by the use of soil from a site (a farm at Braco, near Auchterader, Perthshire, Scotland) known to be infested with PMTV-containing resting spores of *S. subterranea.* This was the same site from which PMTV isolate S was obtained (Arif *et al.* 1994). The soil was air-dried for 2–3 wk before use, then ground to a fine powder, and passed successively through 250- and 50-μm filters. A hole 20–30 mm in diameter and 40–60 mm deep was made in sterile compost in a 100-mm^2 pot; the hole was then filled with the infested soil powder. One test plant was put into each filled hole and maintained on a regime which involved five cycles, each of 3 days of standing in water followed by 4 days of free draining to stimulate the production of zoospores of *S. subterranea.* Following this period of zoospore stimulation, the plants were maintained on a normal watering regime. The infective nature of the soil was confirmed with *N. debneyi* plants before it was used in experiments with transgenic plants. In this trial experiment, 100% of the plants became infected after growing in the soil as described above.

Infectivity assay of inoculated plants.

Assessment of viral replication in inoculated test plants was performed by inoculating indicator plants of *N. debneyi* with sap extracted from test plants. Infection of indicator plants was assessed by symptomatology and ELISA 3–4 wk after inoculation.

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