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Engineering High Level of Coat Protein-Mediated Resistance To Fungal Transmission of Potato Mop-top Furovirus in *Nicotiana benthamiana*

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Abstract
Coat protein-mediated resistance (CP-MR) to potato mop-top furovirus (PMTV) was incorporated by transforming *Nicotiana benthamiana*, a susceptible host of PMTV, with sequences encoding the coat protein (CP) gene of the virus. The transgenic plants of *N. benthamiana*, expressing the PMTV gene were challenged with isolate T and isolate S of PMTV by mechanical, graft, and fungal inoculation. CP-MR against PMTV was extremely effective in coat protein gene transformed *N. benthamiana* plants. Transgenic T_1 seedlings were inoculated in a bait test in which they were grown in soil containing viruliferous spores of the vector fungus *Spongospora subterranea f. sp. subterranea*. Virus transmission by its natural vector could only infect one or two plants of one transgenic line (W16) whereas four lines (W1, W2, W7, W25) were immune to fungally-mediated PMTV-infection. This type of resistance in potato crop will be very valuable because there is no other source of resistance or tolerance to PMTV.

Introduction
Potato mop-top furovirus (PMTV) has fragile tubular rod-shaped particles of two predominant lengths (100-150 nm and 250-300 nm), a tripartite, single-stranded plus RNA genome (Harrison and Jones, 1970; Scott *et al.*, 1994). The coat protein (CP) gene of PMTV is encoded on the smallest genomic RNA species (RNA 3) (Kashiwazaki *et al.*, 1995). PMTV occurs in Northern and Central Europe, the Andean region of South America, Israel, Japan (Jones, 1988) and Canada (J. MacDonald, personal communication) and possibly in all other countries where the fungus vector is found. In Pakistan, both virus and vector are found in Northern potato growing areas (M. Arif, unpublished) but the etiology of the disease is not yet confirmed. The virus is transmitted in soil by the plasmodiophorid 'fungus' *Spongospora subterranea* f. sp. *subterranea* (Jones and Harrison, 1969; Arif *et al.*, 1995) and is retained for several years in the resting spores of the fungus (Jones and Harrison, 1969).

PMTV causes severe qualitative damage in the form of brown arcs and circles (spraing) in the flesh of susceptible potato cultivars. The control of the virus is extremely difficult. No source of resistance to PMTV has been identified for use in breeding programmes. Transformation of plants with copies of virus coat protein gene confer protection known as coat protein-mediated resistance (CP-MR) (Beachy *et al.*, 1990; Wilson, 1993; Lomonossoff, 1995). However, nothing was known before initiation of these studies about the effectiveness of CP-MR to powdery scab fungus-mediated infection of PMTV. We have cloned the coat protein gene of PMTV under the control of the cauliflower mosaic virus 35S promoter, and introduced it into test species *Nicotiana benthamiana* by *Agrobacterium*-mediated transformation. The preliminary results of these studies have already been published (Barker *et al.*, 1994; Reavy *et al.*, 1995). We report here, the engineering of CP-MR and its effectiveness to mechanical, graft and particularly natural transmission of the virus by its fungal vector, *S. subterranea*. This novel form of resistance to PMTV could effectively be used for the control of fungally transmitted and economically important viruses of other crops.

Materials and Methods
Virus isolates: Isolate T was obtained from Scotland and its properties were described by Harrison and Jones (1970). Isolate S was isolated from a farmer's field at Braco approximately 10km west of Auchterarder, Perthshire Scotland (Arif *et al.*, 1994). The viruliferous soil used in bait tests, was also collected from the same site from which previously PMTV-S was obtained (Arif *et al.*, 1994).

Vector construction and plant transformation: Polymerase chain reaction (PCR) was used to amplify the PMTV CP gene from a plasmid clone containing cDNA encoding most of RNA 3 of PMTV-T (Kashiwazaki *et al.*, 1995). The coat protein gene starts at nucleotide 289 and ends at nucleotide 817 (Figure 1). The primers used were A837 (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 incorporate additional nucleotides (underlined) to create BamHI 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 BamHI site of pBluescript II KS(+) (Stratagene) and subsequently excised from that vector as a BamHI fragment and cloned into the BamHI site of the binary vector pRO1 (Fig. 1). Clones with the CP gene in the sense orientation with respect to the cauliflower mosaic virus (CaMV) 35

promoter were identified by restriction enzyme mapping to give clone PMTV-T CP/ROK2. This vector was mobilized from *Escherichia coli* into *Agrobacterium tumefaciens* LBA4404 by triparental mating using pRK 2103 as a helper. Recombinant clones of agrobacteria were isolated by selection on kanamycin/ rifampicin plates and the presence of PMTV-T CP/ROK2 was confirmed by restriction enzyme mapping of plasmid mini-preparations. The sequence of the CP gene was not determined at this stage or in transgenic plants and the possibility of the gene mutations not detectable by restriction enzyme mapping cannot be excluded.

Pieces of *N. benthamiana* stem tissues were transformed (Benvenuto *et al.*, 1991) by incubating with a 48 hour culture of agrobacteria containing PMTV-T CP/ROK2 then transferred on medium containing cefotaxime for 2 days to remove agrobacteria contamination. Transgenic callus and shoots were regenerated on medium containing kanamycin essentially as described by Barker *et al.* (1993). Rooted plantlets of the primary transformants (T_0) were transferred to an aphid-proof glass house at 20°C; the flowers were allowed to self-fertilize and seed was collected. To select transgenic seedlings (T_1) for manual inoculation experiments, seed were germinated on medium containing 400 μg/ml kanamycin-sulfate as described by Barker *et al.* (1993). The proportions of kanamycin-sensitive and kanamycin-resistant seedlings were counted before selecting the transgenic seedlings for propagation and screening resistance. For the fungal-transmission and graft inoculation experiments, seeds were germinated in compost without selection. At the 4/5 leaf stage, a leaf from each seedling was tested by ELISA and transgenic seedlings were identified by the production of coat protein.

Assessment of CP production and detection of virus replication: CP production in non-inoculated transgenic plants by triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) using monoclonal antibody SCR 69 (Torrance *et al.*, 1993) and method as described by Torrance (1992).

Virus replication was assessed in manually inoculated CP-transformed and non-transformed (wild-type) *N. benthamiana* plants by TAS-ELISA and reverse transcription and polymerase chain reaction (RT-PCR) (Arif *et al.*, 1994). The method used for TAS-ELISA was described by Arif *et al.* (1994). For RT-PCR, total RNA was isolated from pooled samples of both CP-transformed and wild-type *N. benthamiana* leaves by the method of Verwoerd *et al.* (1989) and resuspended in 30 μl sterile distilled water. Five micrograms of total RNA was used as a template for first cDNA synthesis using a 27-nt primer (TRT352) from the read-through domain (2296-nt - 2315-nt) of PMTV RNA 3 which does not contain the coat protein gene. First strand cDNA was synthesized by the methods of Arif *et al.* (1994). The total cDNA product was amplified by PCR. The PCR reaction contained 1 μg of each of the amplification primers (TRT 352, 5'-GCGGTACCTCGTCCCTTTCGAGACTGG-3'; TRT66, 5'-TACGCTGGGCTGGTGCATAG-3'), 200 μM each of four deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.3, in a 50 μl volume and 2.5 units of Taq DNA polymerase (AmpliTaq^R, Perkin Elmer). PCR amplification was done for 30 cycles, each of 94° for 30 sec (denaturing), 55° for 30 sec (annealing) and 72° for 1 min (synthesis) in a thermal cycler (Perkin Elmer 3600). Five microlitres of each PCR reaction were loaded onto a 1% agarose gel and separated by electrophoresis in tris-borate ethylenediamine tetraacetic acid buffer containing 0.5 μg/ ml ethidium bromide, molecular size markers were DRIgest III™ (Pharmacia).

Assessment of viral replication in inoculated *N. benthamiana* test plants was also done by back inoculation of indicator plants of *N. debneyi*. Infection of indicator plants was assessed by characteristic symptom development and by TAS-ELISA 3-4 weeks after inoculation.

Virus inoculation: Cultures of PMTV isolates T and S, used in this study (Harrison and Jones, 1970; Arif *et al.*, 1994), were maintained in plants of *N. benthamiana*. Virus was transmitted by manual inoculation using freshly-extracted sap from infected *N. benthamiana* plants (1 g / 5 ml of water) rubbed onto carborundum-dusted leaves of test plants. Cleft graft inoculation was performed using infected scions (shoot apices) of PMTV-infected *N. benthamiana* plants from which the shoot apex had been removed.

PMTV was transmitted through PMTV infested resting spores of *S. subterranea* using soil from a farmer's field at Braco, Perthshire, Scotland. This was the same site from where PMTV isolate S was obtained previously (Arif *et al.*, 1994). The soil was air-dried for 2-3 weeks before use, then ground to a fine powder and passed successively through 250 μm, 100 μm and 50 μm filters. A hole of 20-30 mm in diameter and 40-60 mm deep was made in sterile compost contained in a 100 mm square pot, and 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 cycle consisting of 3 days standing in water followed by 4 days free-draining to stimulate *S. subterranea* zoospore production. Following this period of zoospore stimulation, plants were maintained on a normal watering regime. The infective nature of the soil was confirmed by *N. debneyi* before planting with transgenic plants. In this trial experiment, 100 per cent of the plants became infected after growing in the soil as described above.

Results and Discussion

Five transgenic lines of *N. benthamiana* were selected on the basis of high coat protein expression. Transgenic and non-transgenic plants were inoculated manually with two PMTV isolates. Approximately 1 week after inoculation, all of the non-transgenic control plants displayed visible

symptoms of PMTV infection, whereas none of the inoculated transgenic plants displayed symptoms during 4 week of the experiment (Fig 2). Growth of the inoculated plants was terminated after 4 week, and virus replication was assessed by sensitive infectivity assay. 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). No virus-like particles were detected by electron microscopic examination and northern analysis of inoculated transgenic plants but they were readily detected in infected control plants. In further tests, RT-PCR using primers specific for CP read-through domain, was unable to detect virus particles in inoculated transgenic plants. This method readily detected viral RNA in infected control plants (Fig 3). Transgenic plants of the five lines were challenged by graft inoculation with scions from PMTV-infected plants to assess the resilience of the resistance identified by manual inoculation. In spite of the high inoculum pressure, the graft-inoculated plants of lines W1,W7,W16 and W25 showed that none had become infected 4 weeks after inoculation, only one plant of line W2 was found infected. All control plants were infected as a result of graft inoculation (Table 1).

To test the effectiveness of this CP-MR to fungus transmission, transgenic plants of five lines were exposed to viruliferous *S. subterranea* in a bait-test. Infectivity assay was used to determine if viral replication had occurred in leaf tissue at 3-4 week post-inoculation and in both leaf and root tissue 6-8 weeks post-inoculation. Virus was detected in all inoculated non-transformed control plants, 3-4 weeks after exposure to the viruliferous fungus vector. By contrast, viral replication was not detected in leaves of any plants of four of the transgenic lines, even 6-8 weeks post-inoculation and virus replication was found in only two plants of line W16 (Table 2). Infectivity assay of roots was used to determine if limited virus replication had occurred only in root tissues of the transgenic plants and in roots of two plants in line W16 in which virus was also detected in leaf tissue, but not in plants of other transgenic lines (Table 2).

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

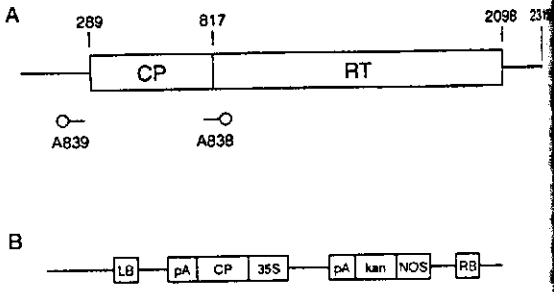

Fig. 1: Schematic representation of (A) the plasmid clon containing cDNA encoding most of PMTV RNA 3 and (B) the pROK2 plant expression vecto containing the cloned coat protein gene. In (A) th position of coat protein (CP) and possible read through (RT) genes are shown with the nucleotide positions; also shown are the relativ positions of the oligonucleotide primers A839 an A838 used to clone the CP gene by polymeras chain reaction. In (B) LB = left border; RB = righ border; pA = polyadenylation signal; CP = PMT coat protein gene; 35S = cauliflower mosaic viru 35S promoter; kan = Tn5 neomyc phosphotransferase gene (NPTII); NOS = nopalin synthase gene promoter.

Expression of CP gene in plants has provided extreme strong resistance on number of occasions. For example, n symptoms were detected after manual inoculation transgenic tobacco plants expressing the alfalfa mosa alfamovirus CP gene, and the virus was not detected infectivity assay (van Dun *et al.*, 1987; Tumer *et al.*, 198 Similarly, transgenic expression of potato virus S carlavir CP provided extremely strong resistance in *N. debneyi*, which no symptoms or antigen accumulation occurred aft manual inoculation (MacKenzie and Tremaine, 1990) a

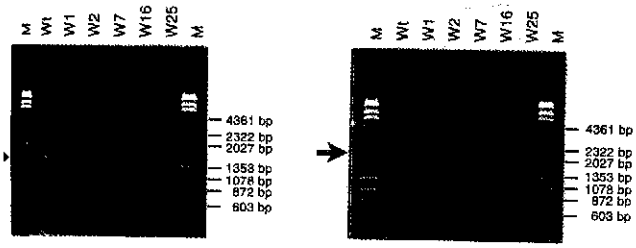

Fig. 2: Comparison of symptoms developed in coat protein (CP) transformed (A, right) and non-transformed (WT) control (A, left) *Nicotiana benthamiana* plants following inoculation with potato mop-top virus (PMTV). Plants were mechanically inoculated with 1:10 (w/v) homogenate prepared from upper leave of a PMTV-infected plants. By 3 wk after inoculation, non-transformed control plants were severely stunted (A, left: B, left) and showed the classic symptoms of systemic PMTV infection (mosaic & distortion), whereas CP-transformed plants (A, right: B; right) were free from obvious symptoms.

Fig. 3: Reverse transcription-polymerase chain reaction analysis of coat protein transformed and non-transformed *Nicotiana benthamiana* plants which were manually inoculated with PMTV-T (A) and PMTV-S (B). RNA extracts were made from leaves of pool samples of five randomly selected transformed and non-transformed *N. benthamiana* plants. PCR products were electrophoresed in 1% agarose gel and stained with 0.5 μg/ml ethidium bromide. Lanes contains: M: DRIgest III *molecular* marker (Boehringer-Mannheim); wt: wild type (non-transformed), W1, W2, W7, W16, W25 CP-transformed *N. benthamiana* lines. Arrows indicate the positions of the PMTV specific bands. Conditions and primers used for PCR amplification are described in the text.

Table 1: Detection of virus replication using infectivity assay and enzyme linked immunosorbent assay (ELISA) of coat protein transgenic lines of *Nicotiana benthamiana* after mechanical and graft inoculation with potato mop-top virus (PMTV) isolates T and S.

	Detection of Virus Replication					
	PMTV-T[a]			PMTV-S[a]		
	Mechanical		Graft	Mechanical		Graft
Transgenic lines	Infectivity[b] assay	ELISA[c]	Infectivity[b] assay	Infectivity[b] assay	ELISA[c]	Infectivity[b] assay
W1	0/8	0/8	Nt[d]	0/6	0/6	0/6
W2	0/12	2/12	0/6	0/12	3/12	1/12
W7	0/12	1/12	0/6	0/12	1/12	0/6
W16	0/6	0/6	NT	0/6[e]	1/12	0/3
W25	0/6	1/6	NT	0/6	2/6	0/6
Wild-type control)[f]	48/48	48/48	6/6	48/48	48/48	12/12

[a] Number of plants with positive assay results per number of plants tested. [b] Infectivity assay was performed 4 weeks after inoculation. [c] ELISA was performed three times after inoculation at approximately 7 to 10 days intervals. Positive samples were those with A_{405} values three times those given by non-inoculated plants of the same line. [d] NT = not tested. [e] Six test plants died before the infectivity assay was completed. [f] Lines W2 and W7 were tested independently of lines W1, W16, and W25; 24 non-transgenic wild-type plants were used as controls on each occasion.

potato plants after graft inoculation (MacKenzie *et al.*, 1991). Extreme resistance or immunity to cucumber mosaic cucumovirus, transmitted by aphids inoculation, was reported but no manual inoculation has been reported in plants expressing CMV CP gene (Quemeda *et al.*, 1991). Lawson *et al.* (1990) reported the only case of immunity to both manual and vector transmission of the virus, in which one line of potato plants expressing the CP gene of potato virus Y potyvirus (PVY) was resistant to PVY transmitted by both forms of inoculations. Our results demonstrate that similar immunity to a fungally-transmitted virus can be produced. Tests were made to confirm that resistance to PMTV-infection in transgenic plants was due to expression of coat protein gene rather than somaclonal variation in transformed lines. This was done by inoculating population of transgenic and non-transgenic 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. All of non-transgenic T_1 seedlings were susceptible to virus, whereas transgenic seedlings were generally resistant, only one plant of line W16 became infected (results not shown). These results further confirmed that the resistance was developed due to expression of CP gene. It was generally observed that higher the protein expression, greater was the protection in transformed plants.

Table 2: Assessment of virus replication using 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*.

	Infectivity assay			
	Leaf tissue[a]		Leaf and root tissue[a]	
	F[b]	%age infection	F[b]	%age infection
W 1	0/23	0	0/23	0
W 2	0/23	0	0/23	0
W 7	0/22	0	0/22	0
W 16	1/18	5.5	2/18	11.11
W 25	0/13	0	0/13	0
wild-type (control)	60/60	100	60/60	100

[a]Infectivity assays were performed on leaf tissue 3-4 weeks after the start of fungal inoculation and on leaf and root tissue 6-8 weeks after the start of fungal inoculation.

[b] Frequency of virus transmission: number of plants with positive assay results per number of plants tested.

CP-MR against PMTV is extremely effective in *N. benthamiana*; indeed three transgenic lines were immune to infection following mechanical, graft or fungus inoculation. Inoculation of virus by a fungus vector into the roots of the transgenic plants cannot overcome CP-MR against PMTV. We have, therefore, developed an extremely effective and novel form of resistance to PMTV which is being incorporated into potato. It seems likely that effective resistance could also be engineered to other economically important fungally-transmitted viruses, such as beet necrotic yellow vein, beet soil-borne and peanut clump viruses.

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References

Arif, M., L. Torrance and B. Reavy, 1994. Improved efficiency of detection of potato mop-top furovirus in potato tubers and in the roots and leaves of soil-bait plants. Potato Res., 37: 373-381.

Arif, M., L. Torrance and B. Reavy, 1995. Acquisition and transmission of potato mop-top furovirus by a culture of *Spongospora subterranea* f. sp. *subterranea* derived from single cystosorus. Annals of Applied Biology, 126: 439-503.

Barker, H., B. Reavy, K. D. Webster, C. A. Jolly, A. Kumar and M. A. Mayo, 1993. Relationship between transcript production and virus resistance in transgenic tobacco expressing the potato leaf roll virus coat protein gene. Plant Cell Reports, 13: 54-58.

Barker, H., B. Reavy, M. Arif, K. D. Webster, and M. A. Mayo, 1994. Towards immunity to potato leafroll virus and potato mop-top virus using transgenic and host gene-mediated forms of resistance. Aspects of Applied Biology 39: 189-194.

Beachy, R. N., S. Loesch-Fries and N. E. Tumer. 1990. Coat protein-mediated resistance against virus infection. Annual Review of Phytopathology 28: 451-474.

Benvenuto, E., R. J. Ordas, R. Tavazza, G. Ancora, S. Biocca, A. Cattaneo and P. Galeffi, 1991. 'Phytoantibodies': a general vector for expression of immunoglobulin domains in transgenic plants. Plant Molecular Biology, 17: 865-874.

Harrison, B. D. and R. A. C. Jones, 1970. Host range and properties of potato mop-top virus. Annals of Applied Biology, 65: 393-402.

Jones, R. A. C., 1988. Epidemiology and control of potato mop-top virus. In Developments in Applied Biology 1: Viruses with Fungal Vectors, pp: 255-270. Eds. J. Cooper and M. J. C. Asher. Wellesbourne, UK: Association of Applied Biologists.

Jones, R. A. C. and B. D. Harrison, 1969. The behaviour of potato mop-top virus in soil, and evidence for transmission by *Spongospora subterranea* (Wall.) Lagerh. Annals of Applied Biology, 63: 1-17

Kashiwazaki, S., K. P. Scott, B. Reavy and B. D. Harrison, 1995. Sequence analysis and gene content of potato mop-top virus RNA 3: further evidence of heterogeneity in the genome organization of furoviruses. Virology 20

701-706.

Lawson, C., W. Kaniewski, L. Haley, R. Rozman, C. Newell, P. Sanders and N. E. Tumer, 1990. Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/Technology, 8: 127-134.

Lomonossoff, G. P., 1995. Pathogen-derived resistance to plant viruses. Annual Review of Phytopathology 33: 323-343.

MacKenzie, D. J. and J. H. Tremaine, 1990. Transgenic *Nicotiana debneyi* expressing viral coat protein are resistant to potato virus S infection. Journal of General Virology 71: 2167-2170.

MacKenzie, D. J., J. H. Tremaine and J. McPherson, 1991. Genetically engineered resistance to potato virus S in potato cultivar Russet Burbank. Molecular Plant-Microbe Interactions , 4: 95-102.

Quemada, H. D., D. Gonsalves and J.L. Slightom, 1991. Expression of coat protein gene from cucumber mosaic virus strain C in tobacco: protection against infection by CMV strains transmitted mechanically or by aphids. Phytopathology, 81: 794-802.

Reavy, B., M. Arif, S. Kashiwazaki, K.D. Webster and H. Barker, 1995. Immunity to potato mop-top virus in *Nicotiana benthamiana* plants expressing the coat protein gene is effective against fungal inoculation of the virus. Molecular Plant-Microbe Interactions, 8: 286-291.

Scott, K. P., S. Kashiwazaki, B. Reavy and B. D. Harrison, 1994. The nucleotide sequence of potato mop-top virus RNA 2: a novel type of genome organization for a furovirus. J. Gen. Virology, 75: 3561-3568.

Torrance, L., 1992. Serological methods to detect plant viruses: production and use of monoclonal antibodies. In Techniques for the Rapid Detection of Plant Pathogens, pp: 7-33. Eds. J. M. Duncan & L. Torrance. Blackwell Scientific Publications, Oxford.

Torrance, L., G. H. Cowan, and L. G. Pereira, 1993. Monoclonal antibodies specific for potato mop-top virus and some properties of the coat protein. Annals of Applied Biology, 122, 311-322.

Tumer, N. E., K. M. O'Connell, R. S. Nelson, P. R. Sanders, R. N. Beachy, R. T. Fraley and D. M. Shah, 1987. Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco expressing AlMV coat protein. The EMBO Journal 6: 1181-1188.

van Dun, C. M. P., J. F. Bol, and L. van Vloten-Doting, 1987. Expression of alfalfa mosaic virus and tobacco rattle virus coat protein gene in transgenic tobacco plants. Virology, 159: 299-305.

Verwoerd, T. C., B. M. M. Dekker and A. Hoekema, 1989. A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acid Research, 17: 2362.

Wilson, T. M. A., 1993. Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. Proceedings of the National Academy of Sciences, USA. **90**, 3134-3141.