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EXPRESSION OF HELENIUM VIRUS S COAT PROTEIN IN *ESCHERICHIA COLI, IN VITRO* IN RABBIT RETICULOCYTE LYSATE AND TRANSGENIC TOBACCO

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Summary. – The coat protein open reading frame (ORF) sequence of Helenium virus S (HelVS) was cloned and expressed in *E. coli*, rabbit reticulocyte and transgenic tobacco. In *E. coli* the size of the protein was identical to that obtained for the coat protein from purified virus particles and less than that predicted for the fusion protein. This may be due to ribosome binding at a potential ribosome binding site present on the viral sequence, approximately 45 nucleotides upstream from the initiating methionine of the coat protein ORF. This region of HelVS, equivalent to the 1.5 kb subgenomic RNA, also produced high levels of protein when transcribed and translated *in vitro*. When introduced into *Nicotiana tabacum* by leaf disk transformation via *Agrobacterium tumefaciens,* high levels of stable coat protein were detected which were identical in molecular weight to that of HelVS coat protein and constituted approximately 0.1–0.5 % of the total extracted protein.

Key words: HelVS; carlavirus; coat protein; expression; E. coli; transgenic tobacco

Introduction

Helenium virus S is a member of the carlavirus group of plant viruses (Kuschki *et al.*, 1978; Wetter and Milne, 1981) and is composed of a single-stranded genomic RNA molecule of 7.4 kb and two subgenomic RNAs of 3.3 kb and 1.5 kb which are encapsidated in a 32 K coat protein into slightly flexuous filamentous particles 650 nm, 320 nm and 180 nm in length (Foster and Mills, 1990*a*; Foster *et al.*, 1990). *In vitro* translation of the 1.5 kb subgenomic RNA molecule resulted in the synthesis of the viral coat protein of approximate size 33 K (Foster and Mills, 1990*a*) and the nucleotide sequence data for this region of

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HelVS revealed two major ORFs encoding proteins of M_r 33 K and 12.6 K (Foster *et al.*, 1990). The 33 K protein shared marked homology with the coat proteins of potexviruses and other carlaviruses such as potato virus S and potato virus M (MacKenzie *et al.*, 1989; Rupasov *et al.*, 1989). Whilst the coat protein has been identified and a subgenomic RNA isolated which encodes it, little more is known on the method of expression from this low level RNA to produce high levels of coat protein when translated *in vitro* (Foster and Mills, 1990*a*).

In this paper we describe the expression of the HelVS coat protein *in vitro* in rabbit reticulocyte, *Escherichia coli* and transgenic tobacco.

Materials and Methods

Virus purification and RNA extraction. HelVS was propagated and purified from *Chenopodium quinoa* as previously described by Foster and Mills (1990*b*). RNA was extracted from purified virus assentially as reported by Shields and Wilson (1987).

Construction of cDNA clones. Double-stranded cDNA was synthesized using reverse transcriptase and oligo$(dT)_{12\text{-}18}$ as the primer for the first strand synthesis, followed by DNA polymerase I from *E. coli* and ribonuclease H treatment for the second strand synthesis (Gubler and Hoffman, 1983) using a commercial kit (Amersham). Double stranded cDNA, blunt-ended by treatment with T4 DNA polymerase, was ligated into *Sma*I-cut, bacterial alkaline phosphatase-treated vector pUC13 (Pharmacia) using T4 DNA ligase and transformed into competent *E. coli* JM109 cells. Plasmids expressing the HelVS coat protein fused to the alpha peptide of the *lac*Z gene of pUC13 (Fig. 1) were detected by colony hybridization using HelVS polyclonal antiserum and the inserts subcloned into the transcription vector pBS SK- (Stratagene). These inserts were subsequently subcloned into the binary plant expression vector pROK2 between the CaMV35S promoter and the NOS terminator. This construct was then introduced into *A. tumefaciens* strain LBA4404 by a triparental mating technique as described by Draper *et al.* (1990) and the resultant transconjugants isolated by selecting for both kanamycin and rifampicin resistance.

In vitro transcription and translation. In vitro transcription was carried out as previously described by Foster and Mills (1990*c*) with RNA translated in rabbit reticulocyte lysate (Promega) as outlined by Foster and Mills (1991). Antiserum to HelVS, kindly provided by Dr. D. J. Barbara, was used for immunoprecipitation essentially as described by Mayo and Reddy (1985).

Screening for plasmids expressing HelVS coat protein and Western blot analysis of expressed fusion proteins. Bacterial colonies were grown overnight on nitrocellulose filters pre-soaked in 10 mmol/l IPTG. The colonies were lysed and incubated in a blocking buffer (5 % dried milk powder in TBS, 0.05 mol/l Tris.HCl pH 7.5, 0.2 mol/l NaCl) overnight at room temperature. Filters were washed 5 times in TBS containing 0.05 % Tween-20 and incubated with HelVS coat protein antiserum (diluted 1:500 in blocking buffer) for 90 min. After several washes with TBS-Tween, bound antibody was detected by incubation with alkaline phosphatase conjugated goat anti-rabbit Ig (Sigma) for 90 min followed by several washes and colour development with Fast Red. Overnight cultures of antibody positive colonies grown in the presence of 5 mmol/l IPTG were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Cells (0.5 ml) were harvested by centrifugation and the pellet resuspended in 50 μl of SDS-PAGE loading buffer (2 % SDS (w/v), 5 % mercaptoethanol (v/v), 10 % glycerol (v/v) in 0.125 mol/l Tris.HCl pH 6.8). Samples were boiled, separated on 12.5 % polyacrylamide gels and electroblotted onto nitrocellulose using a Bio-Rad Transblot cell. The blots were placed in blocking buffer and developed as described above.

Plant transformation and analysis. Leaf disks of *N. tabacum* were transformed using *A. tumefaciens* carrying the HelVS coat protein insert from pHel19 essentially as described by Draper *et al.* (1990). Transformed shoots were subsequently rooted and transferred to soil as described by Draper *et al.* (1990).

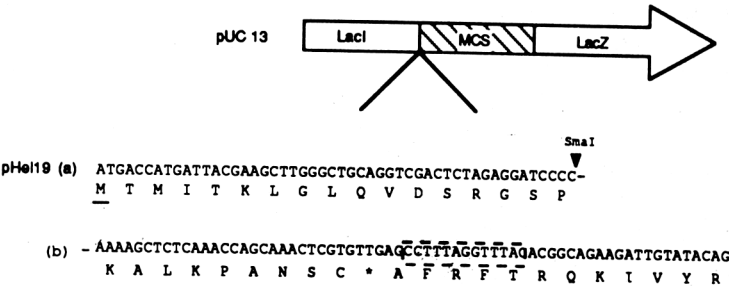

Fig. 1

Diagram of a part of cloning vector pUC13 and partial sequences of recombinant clone pHel19 MCS, multiple cloning site of pUC13. The sequence including the start methionine of the *lac*Z protein and the coat protein within MCS are underlined. pHel19 (a) includes the vector sequences, with pHel19(b) showing the viral sequences cloned within the *Sma*I site. The ORF which is in frame with the *lac*Z start methionine is shown below the DNA sequence. Termination codons are indicated (*). The position of the potential RBS is contained within the hatched box.

The ability of transgenic *N. tabacum* to produce HelVS coat protein was assessed by Western blot analysis specific polyclonal antiserum to the coat protein. Samples of leat tissue were flash frozen in liquid nitrogen, homogenized in SDS-PAGE loading buffer and boiled for 2 min. Samples were then separated on 12,5 % polyacrylamide gels, blotted and developed as described above.

Protein concentration was determined by comparing dilutions of known standards with dilutions of HelVS coat protein and total plant protein extracts. Samples were dotted into Whatman 3MM filter paper and stained in 0.1 % Coomassie blue, 25 % methanol and 10 % acetic acid for 30 min at 37 °C. Filters were then destained in 25 % methanol and 10 % acetic acid to allow visual scoring.

Results

Expression of HelVS coat protein E. coli

Double stranded cDNA to HelVS RNA was ligated into *Sma*I digested pUC13 vector and trasformed into competent *E. coli* cells. Recombinants were analyzed by colony hybridization, using HelVS polyclonal antisera (Fig. 2).

Two clones, pHel19 and pHel23, expressing coat protein via an in-frame fusion protein with the alpha peptide of *lac*Z were detected by colony hybridization using polyclonal antisera. Western blots of bacterial cell lysates from these clones revealed protein bands similar in size to that found for HelVS coat protein from purified virus particles (Fig. 3). Restriction enzyme analysis of the clones revealed inserts of 1.4 kb and 1.6 kb for pHel19 and pHel23, respectively. Sequence analysis revealed that both clones contained a stretch of poly(A) at one terminus which suggested that their difference in nucleotide lengths were at the 5' equivalent of their RNA within the clones. This raised the question as to why the sizes of the *lac*Z fusion proteins were the same in both clones even though the fusion protein from pHel23 should have been up to 7 K larger than from pHel19.

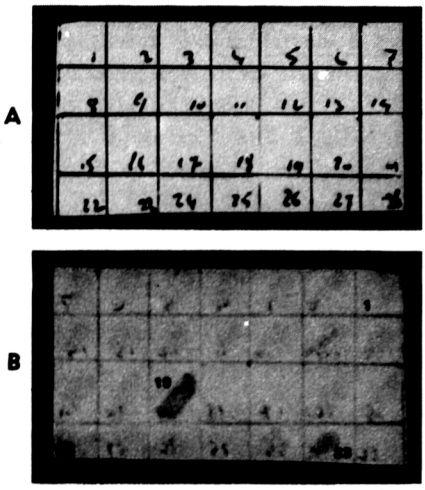

Fig. 2
Colony hybridization using HelVS polyclonal antisera
Colonies were streaked onto nitrocellulose (A) and grown overnight prior to screening. Note, colour development is evident on reverse to side on which colonies were streaked (B). Colony 28 represents pUC13 non-recombinant control.

When the nucleotide sequence of the region covering pHel19 was determined (Foster *et al.*, 1990) it revealed two stop codons which were in frame between the initiating methionine of the *lacZ* protein and the initiating methionine of coat protein ORF (Fig. 1). Indeed, stop codons were present in all three reading frames between the two methionines thus raising the question as to how the

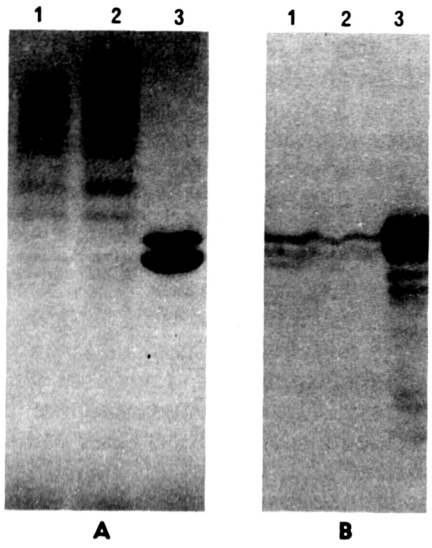

Fig. 3
Western blot analysis of clones expressing HelVS coat protein
A: Coomassie blue stained gel of bacterial lysates and HelVS coat protein; B: Western blot analysis with HelVS polyclonal antisera. Lane 1, lysate of pHel19; lane 2, lysate of pHel23; lane 3, coat protein extracted from purified HelVS. Positions of the coat protein related products are indicated with arrows. No signals were obtained in Western blot experiments from untransformed bacterial cell lysates.

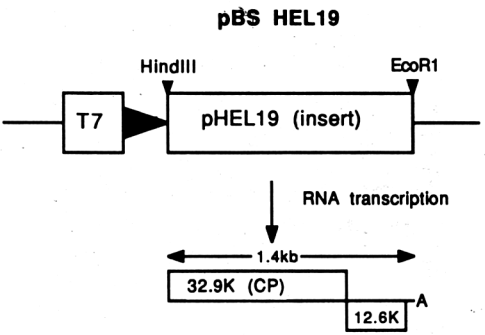

Fig. 4
Partial diagram of pBSHel19
Orientation of the cDNA to HelVS coat protein in relation to the T7 promoter producing the positive-sense RNA. Position and size of the ORFs as determined by sequence analysis are indicated.

coat protein ORF was being efficently expressed in *E. coli* as it was clearly not using the Shine-Dalgarno sequence (Shine and Dalgarno, 1975) and initiating methionine of the *lacZ* protein. When the nucleotide sequence upstream from the coat protein was examined more closely a potential ribosome binding site (RBS) was identified (Foster and Mills, 1991*b*) based around the sequence CCTTTAGGTTTA. This RBS present in pHel19 and pHel23 may be binding to the 3'-terminal region of the 16 S rRNA of *E. coli* allowing subsequent inititation

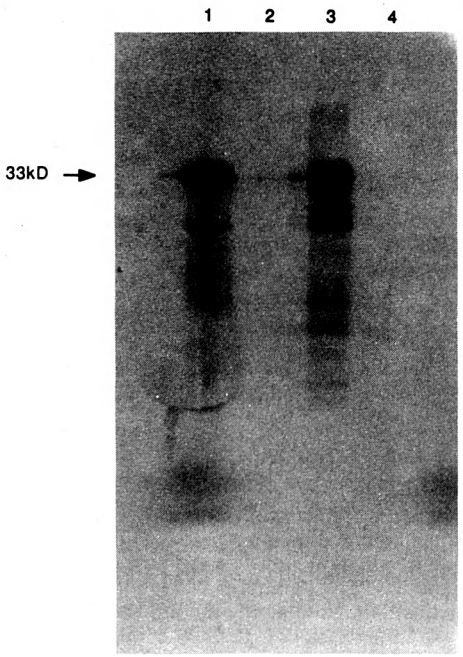

Fig. 5
In vitro translation of pBSHel19
Translation and immunoprecipitation of T7 transcripts of pBSHel19 in rabbit reticulocyte lysate. Lane 1, translation of pBSHel19 RNA (< 0.2 μg); lane 2, blank; lane 3, immunoprecipitation of translation products of pBSHel19 with HelVS antiserum; lane 4, control immunoprecipitation of translation products of pBSHel19 with preimmune antiserum. Position of the 33 K product is indicated with closed arrows.

and elongation from the same methionine of the coat protein ORF, thus producing identical molecular weight products in Western blot analysis.

In vitro translation of pBSHel19

Clone pHel19 was digested with *Hind*III and *Eco*RI and the purified fragment, containing the coat protein gene, 12.6 K gene and all of the 3'-noncoding region, was inserted into the *Hind*III and *Eco*RI sites of Bluescript(pSK-) vector. This clone, designated pBSHel19, allowed a viral sense transcript to be produced from the T7 RNA polymerase promoter (Fig. 4). The polypeptide translated from pBSHel19 comigrated in polyacrylamide gels with the 33 K coat protein from purified virus particles and was specifically immunoprecipitated by antiserum raised against HelVS virus particles (Fig. 5).

High levels of coat protein were synthesized from this uncapped RNA transcript even when levels of transcript were compensated to the low levels of coat protein subgenomic RNA within viral RNA (Foster and Mills, 1990*a*) thus suggesting that this subgenomic RNA is a highly efficent message when translated *in vitro* in a rabbit reticulocyte lysate system. This is similar to the results previously reported by Foster and Mills (1990*c*) for an ordinary strain of potato virus S.

Expression of HelVS coat protein in transgenic tobacco

To further investigate the clone containing the coat protein ORF of HelVS, which was translated efficiently in *E. coli* and *in vitro* in rabbit reticulocyte even though several termination codons were present between the 5' terminus and the initiating methionine, the construct was engineered into tobacco.

The insert from pHel19 was subcloned into the plant transformation vector pROK2 behind the CaMV35S promoter, giving the construct pROK-Hel19 (Fig. 6). This was then transformed into tobacco by *Agrobacterium* mediated transfor

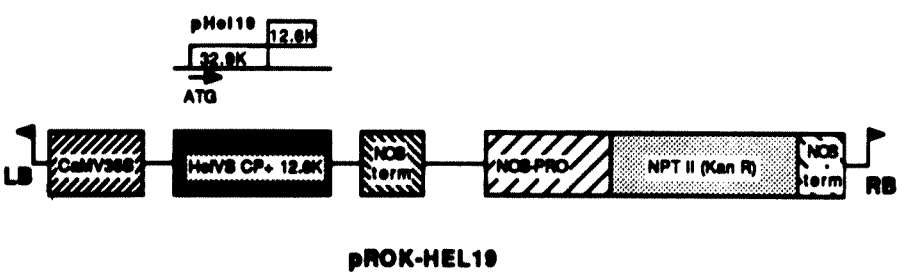

Fig. 6

The construct pROK-HEl19 showing the position of pHel19 within ROK2, and the ORFs of HelVS as determined by sequence analysis

The position of the ATG of the coat protein is indicated. NOS term, nopaline synthase polyadenylation site NOS-PRO, nopaline synthase gene promoter; NPTII, neomycin phosphotransferase-II coding sequence from Tn5; LB and RB with flags indicate the positions of the left and right hand borders of the T-DNA repeats.

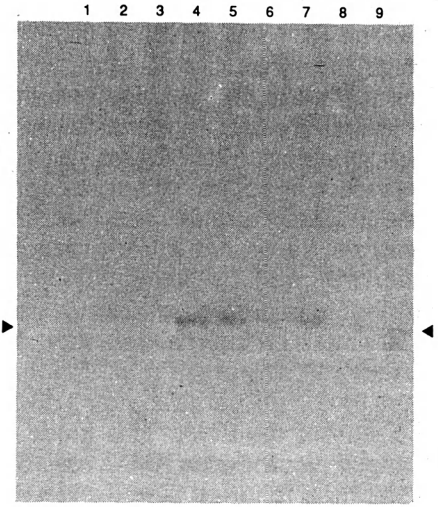

Fig. 7
Western blot analsis of transgenic plants Results for each plant, designated ROK-HEL1, 2, etc., are indicated as, negative(-), weak(+) or strong(++). Lane 1, negative control, tobacco transformed with ROK2 with no HelVS insert (-); lane 2, ROK-HEL6 (+); lane 3, ROK-HEL5 (-); lane 4, ROK-HEL4 (++); lane 5, ROK-HEL3 (++); lane 6, ROK-HEL2 (+); lane 7, ROK-HEL1 (++); lane 8, samples from plants ROK-HEL1 to 4 combined (+); lane 9, positive control, coat protein from purified virus particles of HelVS. Position of the 33 K coat protein is indicated by closed arrows.

mation and whole plants regenerated.

Approximately 80 % of the transgenic plants obtained expressed HelVS coat protein as judged by Western blot analysis. The levels of expression, as shown for 6 individual transformants in Fig. 7, varied from 0.1 % to 0.5 % of total SDS-soluble protein. All transformants produced a coat protein which had an identical electrophoretic mobility to that of the upper band produced by the extraction of coat protein from purified HelVS virus particles. A slightly smaller molecular weight product was also present in coat proteins from purified virus particles which was assumed to be a proteolytic degradation product. Detailed work with potato virus X (PVX) coat protein indicated that the appearance of a second minor band was due to plant proteases (Koening *et al.*, 1978).

Discussion

In this report we described the coat protein ORF sequence of Helenium virus S and its expression *in vivo* in *E. coli* and transgenic tobacco and *in vitro* in rabbit reticulocyte lysate. A clone, pHel19, of 1390 nucleotides was cloned in the plasmid pUC13 which was the equivalent of the polyadenylated subgenomic RNA of 1.5 kb found in encapsidated particles of HelVS of the size of 180 nm. This subgenomic RNA directed the synthesis of the HelVS coat protein when translated *in vitro* in rabbit reticulocyte lysate (Foster and Mills, 1990*a*).

It was surprising to find that pHel19 expressed HelVS coat protein in *E. coli* when induced with IPTG as two stop codons were present between the initiating

methionines of the *lacZ* fusion protein and the coat protein ORF. However, when the intervening sequence was more closely examined a potential Shine-Dalgarno RBS was evident which was highly homologous among a number of carlaviruses (Foster and Mills, 1991*b*). This RBS may interact with the 3' terminus of the prokaryotic ribosome allowing initiation and elongation. When this clone, containing the coat protein ORF was expressed in the eukaryotic rabbit reticulocyte translation system, high levels of translation were evident in the absence of a 5' cap structure and with stop codons in all three reading frames between the 5' terminus and the initiating methionine of the coat protein ORF. Therefore, the Shine-Dalgarno sequence upstream from the coat protein methionine may well be acting in this eukaryotic system to place the initiating methionine in perfect context for initiation and subsequent elongation. A similar situation of internal initiation has already been reported for the animal picornaviruses (Jackson *et al.*, 1990). Translation initiation on picornavirus RNA occurs by the ribosomes binding directly to an internal site rather scanning from the 5'-end of the RNA. This mode of translation is cap-independent, thus the uncapped picornavirus RNAs are able to translate efficiently which is similar to the situation reported here for HelVS and previously for potato virus S (Foster and Mills, 1990*a*).

Results obtained in planta suggests that this unit is a highly efficient message for the production of Helenium virus S coat protein. When the cDNA was expressed via the constitutive CaMV35S promoter in transformed tobacco plants, high levels (up to 0.5 % of total SDS-soluble protein) of coat protein were detected by Western blot analysis.

These results demonstrate that it is possible to clone and express high levels of the coat protein genes of carlaviruses in transgenic plants. It may be possible to use this information to create plants resistant to carlaviruses via the phenomenon of coat protein mediated protection. This was first described by Powell-Abel *et al.* (1986) in which tobacco transformed with and expressing the coat protein gene of tobacco mosaic virus (TMV) displayed reduced and delayed symptom production when challenged with TMV virus. This form of cross protection has now been extended to a wide range of viruses including alfalfa mosaic virus, tobacco streak virus, cucumber mosaic virus and potato virus X (review, Gandani *et al.*, 1990).

References

Draper, D., Scott, R., Armitage, P., and Walden, R. (1990): *Plant genetic transformation: A laboratory manual.* Black-well Scientific Publications, Oxford.

Foster, G. D., and Mills, P. R. (1990*a*): Evidence for the role of subgenomic RNA species in the production of Helenium virus S coat protein. *Virus Res.* **17**, 61–70.

Foster, G. D., and Mills, P. R. (1990*b*): Detection of strains of potato virus S by nucleic acid spot hybridisation (NASH). *Potato Res.* **33**, 487–495.

Foster, G. D., and Mills, P. R. (1990*c*): Investigation of the 5' terminal structures of genomic and subgenomic RNAs of potato virus S. *Virus Genes* **4**, 359–366.

Foster, G. D., Millar, A. W., Meehan, B. M., and Mills, P. R. (1990): Nucleotide sequence of the 3'-terminal region of Helenium virus S RNA. *J. gen. Virol.* **71**, 1877–1880.

Foster, G. D., and Mills, P. R. (1991*a*): Cell-free translation of American hop latent virus. *Virus Genes* **5**, 327–334.

Foster, G. D., and Mills, P. R. (1991*b*): Occurence of chloroplast ribosome recognition sites within conserved elements of the RNA genomes of carlaviruses. *FEBS Letts.* **280**, 341–343.

Gandani, F., Mansky, L. M., Medici, R., Miller, W. A., and Hill, J. H. (1990): Genetic engineering of plants for virus resistance. *Arch. Virol.* **15**, 1–21.

Gubler, U., and Hoffman, B. J. (1983): A simple and very efficent method for generating cDNA libraries. *Gene* **25**, 263–269.

Jackson, R. J., Howell, M. T., and Kaminski, A. (1990): The novel mechanism of initiation of picornavirus RNA translation. *Trends Biochem. Sci.* **15**, 477–483.

Koenig, R., Tremaine, J. H., and Shepard, J. F. (1978): *In situ* degradation of the protein chain of potato virus X at the N- and C- termini. *J. gen. Virol.* **38**, 329–337.

Kuschki, G. H., Koenig, R., Duvel, D., and Kuhne, H. (1978): Helenium virus S and Y: two new viruses from commercially grown helenium hybrids. *Phytopathology* **68**, 1407–1411.

MacKenzie, D. J., Tremaine, J. H., and Stace-Smith, R. (1989): Organisation and interviral homologies of the 3'-terminal portion of potato virus S RNA. *J. gen. Virol.* **70**, 1053–1063.

Mayo, M. A., and Reddy, D. V. R. (1985): Translation products of RNA from Indian peanut clump virus. *J. gen. Virol.* **66**, 1347–1351.

Powell-Abel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. (1986): Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein. *Science* **232**, 738–743.

Rupasov, V. V., Morozov, S. Yu., Kanyuka, K. V., and Zavriev, S. K. (1989): Partial nucleotide sequence of potato virus M RNA shows similarities to potexviruses in gene arrangement and the encoded amino acid sequences. *J. gen. Virol.* **70**, 1861–1869.

Shields, S. A., and Wilson, T. M. A. (1987): Cell free translation of turnip mosaic virus RNA. *J. gen. Virol.* **68**, 169–180.

Shine, J., and Dalgarno, L. (1975): Determination of cistron specificity in bacterial ribosomes. *Nature* **254**, 34–38.

Wetter, C., and Milne, R. G. (1981): In E. Kurstak (Ed.): *Handbook of Plant Virus Infections Comparative Diagnosis.* Elsevier/North Holland Biomedical Press, Amsterdam pp. 695–730.