Virology 267, 29–35 (2000)
doi:10.1006/viro.1999.0098, available online at http://www.idealibrary.com on IDEAL®

metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Efficient Expression of Foreign Proteins in Roots from Tobravirus Vectors

Stuart A. MacFarlane[1] and Alexandra H. Popovich

Virology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

Received October 1, 1999; returned to author for revision November 8, 1999; accepted November 17, 1999

Viral vectors were constructed from infectious cDNA clones of each of the three tobraviruses, tobacco rattle virus (TRV), pea early-browning virus (PEBV), and pepper ringspot virus (PepRSV). RNA2 of each of the three viruses was modified to carry an additional coat protein subgenomic promoter and was used to express green fluorescent protein (GFP) when inoculated to plants. The tobravirus expression vectors have a wide host range and were able to express GFP in, for example, *Nicotiana* species, tomato, pea, arabidopsis, and sugar beet. The TRV vector was able to invade and express GFP very efficiently in roots, whereas the widely used PVX vector was not. © 2000 Academic Press

Key Words: plant virus vectors; root delivery; plant biotechnology; tobraviruses.

INTRODUCTION

Several recent studies have demonstrated the use of plant viruses as vehicles to introduce and express non-viral genes in plants (Donson *et al.,* 1991; Chapman *et al.,* 1992; Dolja *et al.,* 1998). Many plant viruses multiply to high levels in plants, leading to concomitantly high levels of nonviral gene expression. Virus delivery does not lead to permanent incorporation of the transgene into plants, nevertheless, depending on which virus is used, virus multiplication (and gene expression) can continue for long periods (weeks or months). Plant virus expression vectors have several other potential advantages over the more commonly used transgenic plant technology. Firstly, plant RNA viruses often are small in size (between 3000 and 10,000 nucleotides) making them easy to manipulate *in vitro*. Secondly, infection of plants with engineered virus leads to the immediate expression of the heterologous gene with no requirement for the lengthy and complex tissue culture procedures that are necessary when producing stably transformed plants. Different viruses have been described that can infect plants from most of the taxonomic groups. Some viruses in particular have very wide host ranges themselves. Thus it is likely that plant virus gene vectors can be developed that would allow foreign gene expression in whichever plant species was required. Several strategies have been employed in the development of plant virus expression vectors (Lacomme *et al.,* 1998). Often the foreign gene either is carried as a replacement for a nonessential virus gene or is inserted as an additional gene linked to a duplicated viral promoter. Other plant viruses have been adapted to express foreign sequences fused to one end of, or within, the virus coat protein gene, or as part of a polyprotein that is cleaved during maturation. These approaches have been used to express a variety of different nonviral proteins, e.g., marker proteins—GUS (Dolja *et al.,* 1992) and GFP (Baulcombe *et al.,* 1995), α-trichosanthin (ribosome inactivating protein; Kumagai *et al.,* 1993), phytoene synthase (metabolic pathway enzyme; Kumagai *et al.,* 1995), myb-like transcription factor (Sablowski *et al.,* 1995), avr9 (fungal elicitor of disease resistance; Hammond-Kosak *et al.,* 1995) and several antigenic peptides for use in vaccine development (Porta *et al.,* 1994; Joelson *et al.,* 1997; Brennan *et al.,* 1999).

Plant virus expression vector technology can be improved along several lines. New vectors are needed to expand the range of plants in which these systems can be used. Different viruses might be more tolerant of sequence manipulations and may be less prone to insert instability. New vectors might be developed with increased carrying capacity or with the ability to express more than one nonviral protein. In addition, vectors based on alternative viruses might have different tissue tropisms allowing selective foreign gene expression in specific parts of the plant.

The tobraviruses tobacco rattle virus (TRV), pea early-browning virus (PEBV), and pepper ringspot virus (PepRSV) have a number of properties that suggested that they might be developed as gene expression vectors. These viruses have two positive-sense, single-stranded genomic RNAs that are separately encapsidated in rod-shaped particles (Harrison and Robinson, 1986). RNA1 encodes the viral proteins that are responsible for replication and movement of the virus in plants. Indeed, RNA1 can cause infection (a so-called NM-infection) in plants in the complete absence of the second

[1] To whom reprint requests should be addressed. Fax: +44 1382 562426. E-mail: s.macfarlane@scri.sari.ac.uk.

0042-6822/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

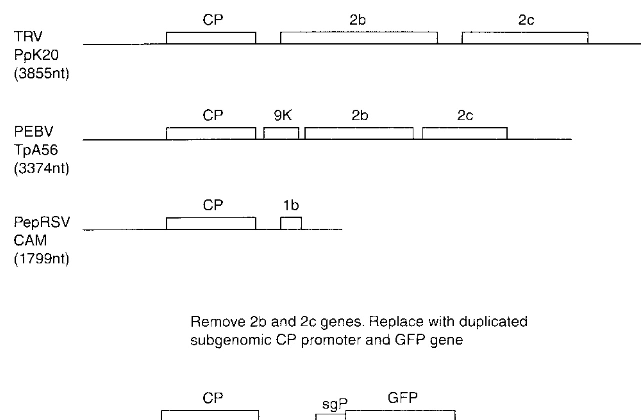

FIG. 1. Strategy for tobravirus vector construction. Gene organisation of RNA2 of isolates of TRV, PEBV, and PepRSV used in this study. CP, coat protein. The 2b and 2c genes encode nonstructural proteins involved in nematode transmission and are deleted from the vector constructs. A duplicated tobravirus CP gene subgenomic RNA promoter (sgP) is inserted upstream of the GFP gene. PepRSV RNA2 includes a partial copy of the RNA1-encoded 1b gene.

RNA (RNA2). RNA2 varies in size, according to virus isolate, and encodes the virus coat protein and sometimes one or more other, nonstructural proteins.

As RNA2 is inessential for infection of plants, we anticipated that it could be engineered to express nonviral genes. Previously, experiments using infectious cDNA clones of PEBV and TRV RNA2 showed that removal of the nonstructural 2b and 2c genes (Fig. 1) did not prevent movement to and replication of the viruses in systemic tissue (MacFarlane *et al.,* 1996; Hernandez *et al.,* 1997). Prior to the work described in this paper, modification of PepRSV had not been undertaken. The nonstructural genes on RNA2 have been shown to be necessary for transmission of tobraviruses by vector nematodes; thus constructs in which these genes are deleted should be effectively contained in the plants under test. In this paper we demonstrate the utility of TRV, PepRSV, and PEBV as gene expression vectors

RESULTS AND DISCUSSION

Analysis of tobravirus vectors expressing GFP

Clones of RNA2 of TRV isolate PpK20 and PEBV isolate TpA56 were modified to remove the nonstructural 2b and 2c genes (Fig. 1). RNA2 of PepRSV isolate CAM is a naturally occurring recombinant molecule lacking these two genes but carrying at its 3′ end a partial copy of the RNA1-derived 16K gene (Bergh *et al.,* 1985). To avoid homology-driven instability, a second coat protein (CP) gene subgenomic RNA (sgRNA) promoter, derived from a different tobravirus isolate was inserted downstream of each virus CP gene. Previous studies showed that TRV and PEBV can activate expression of each others CP sgRNA promoter (Mueller *et al.,* 1997); however, similar studies have not been carried out with PepRSV. The TRV and PepRSV expression vectors were engineered to contain a second, PEBV CP sgRNA promoter, whereas the PEBV vector carried a second, TRV promoter. In each virus vector, the duplicated promoter was used to drive expression of the green fluorescent protein (GFP) gene (Fig. 1).

When combined with RNA1 of the appropriate, homologous virus, transcripts of the three vector constructs caused a productive infection in inoculated plants. TRV-GFP infection produced fluorescent foci visible by eye in inoculated leaves of *Nicotiana benthamiana* by 3 days postinoculation (dpi) (Fig. 2A). GFP fluorescence often was apparent in upper, noninoculated leaves at 4 dpi. PEBV-GFP and PepRSV-GFP produced fluorescence in the inoculated leaves slightly more slowly (3–4 dpi) and also moved into noninoculated leaves slightly later (5–7 dpi) (Figs. 2A and 2B). A similar pattern of infection was seen when these viruses were inoculated to *N. clevelandii,* although systemic infection by PEBV-GFP was infrequent in this species. The intensity of GFP-fluorescence also differed between the viruses. TRV-GFP produced the brightest fluorescence, with PEBV-GFP being less bright and PepRSV-GFP producing the least fluorescence. The three engineered viruses were also examined for their ability to express GFP in plants other than *Nicotiana* species. TRV-GFP was able to infect the greatest number of plant species, moving locally in sugar beet and systemically in tomato, petunia, and arabidopsis. PepRSV-GFP and PEBV-GFP had reduced host ranges, although PepRSV-GFP infected tomato efficiently and PEBV-GFP expressed GFP in systemically infected pea leaves (Figs. 2C and 2D).

CP and GFP expression in inoculated and systemic infected leaves of *N. benthamiana* plants infected with the three viruses was examined by Western blotting (Fig. 3). TRV-GFP and PepRSV-GFP produced more CP in systemic rather than inoculated tissue, probably reflecting a more uniform infection of systemic tissue. TRV produced more GFP in systemic rather than inoculated tissue, whereas with PepRSV there was very little difference in the GFP content of these two tissues. For PEBV, systemic and inoculated tissues contained similar amounts of CP, whereas systemic tissue contained much more GFP than did inoculated tissue. The sensitivities of the virus CP-specific antibodies are not equivalent, thus they cannot be used to compare the amount of each virus in the samples; however, TRV produced the most GFP, both in inoculated and in systemic tissue.

Expression of GFP in roots

Tobraviruses are transmitted in the field by root-feeding nematodes from the genera *Trichodorus* and *Paratrichodorus* (Taylor and Brown, 1997). Consequently these viruses possess the ability to move efficiently within the

A B C D 2

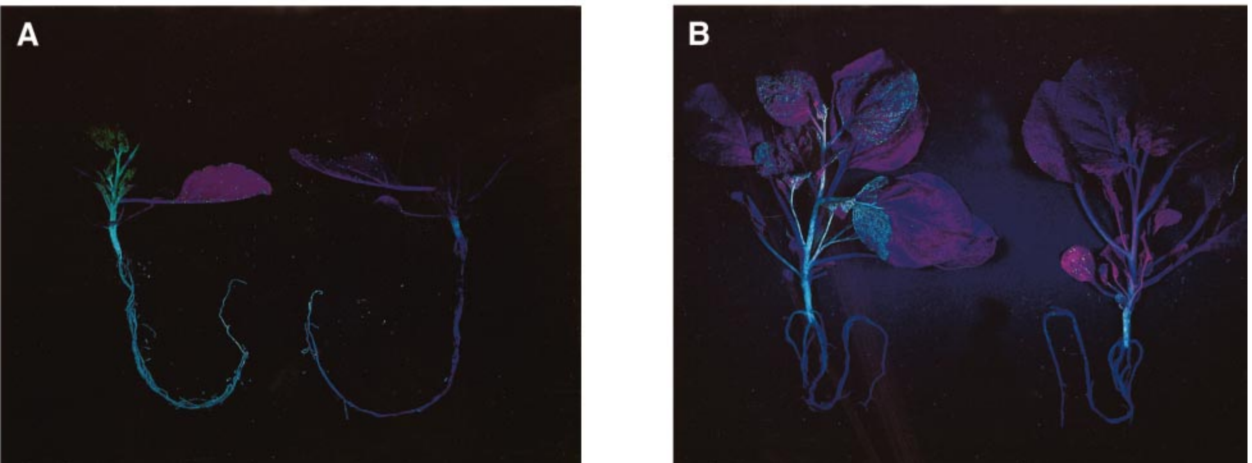

FIG. 2. Visualisation of GFP expression by UV illumination of tobravirus vector-infected plants. (A) Systemically infected leaves. Top row, *N. clevelandii*; bottom row, *N. benthamiana*. Left: TRV-GFP; right: PepRSV-GFP. (B) *N. benthamiana* systemically infected with PEBV-GFP. (C) Pea (*Pisum sativum* cv. Rondo) inoculated leaf infected with PEBV-GFP. (D) Systemic PEBV-GFP infection of pea.

FIG. 4. Comparison by UV illumination of TRV-GFP and PVX-GFP infection of *N. benthamiana*. (A) TRV-GFP at 6 dpi (left), mock-inoculated (right). (B) PVX-GFP at 9 dpi (left), mock-inoculated (right).

roots of infected plants, particularly to the region at the growing tip. We reasoned that significant benefits could be gained if tobraviruses could be used to express biologically active, nonviral proteins in plant roots. Such a system could have numerous applications including, for example, the testing of proteins involved in resistance to soil pests and pathogens, the bioremediation of contaminated soil and perhaps also the testing of candidate genes to manipulate the protein content/quality in root and tuber crops.

Examination of the roots of plants infected with each of the GFP-expressing tobraviruses revealed intense fluo-

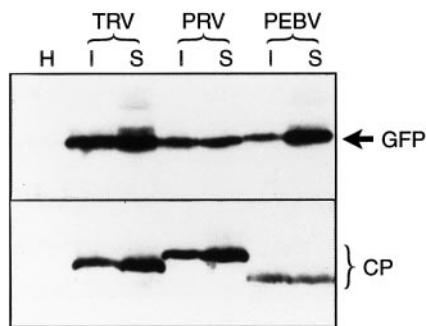

FIG. 3. Western blot of *N. benthamiana* infected with tobravirus vectors. Lanes were loaded with equal amounts of tissue extract. H, uninfected plant sample; I, inoculated leaf (sampled at 5 days postinoculation). S, systemically infected leaf, sampled at 7 dpi (TRV and PepRSV) and 8 dpi (PEBV). Top panel probed with GFP-specific antibodies. Bottom panel probed with a mixture of antibodies raised against TRV isolate PLB, PepRSV CAM, and PEBV SP5.

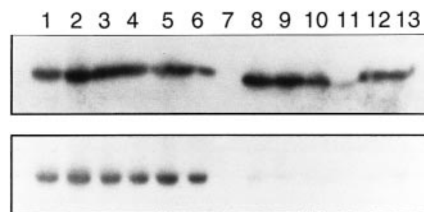

FIG. 5. Western blot of *N. benthamiana* infected with TRV-GFP and PVX-GFP. Lanes were loaded with equal amounts of tissue extract. Lanes 1–6, TRV-GFP infected plants at 6 dpi; lane 7, uninfected plant; lanes 8–13, PVX-GFP infected plants at 9 dpi. Top panel is extracts of leaves showing highest GFP-fluorescence, bottom panel is extracts of the complete root system excluding the hypocotyl. Blot probed with GFP-specific antibodies.

rescence particularly at root tips showing that the alterations made to RNA2 did not affect the pattern of virus movement (data not shown). The tobravirus vectors are not unique in their ability to express nonviral proteins in roots. For example tobacco etch potyvirus expressed high levels of GUS in tobacco lateral roots (Dolja *et al.,* 1992). Also, a TMV vector (30B-GFP; Shivprasad *et al.,* 1999) was found to produce large amounts of GFP in roots, although unlike TRV, the distribution of TMV in the roots was patchy (Popovich and MacFarlane, unpublished observations) and the virus did not invade the meristematic region (Oparka *et al.,* 1998).

Further experiments were carried out to compare the performance of the TRV expression vector with that of potato virus X (PVX), an alternative expression vector that is in the most widespread use (Chapman *et al.,* 1992; Baulcombe *et al.,* 1995).

Transcripts of TRV-GFP or PVX-GFP were inoculated to *N. benthamiana* plants, and extracts of systemically infected leaves exhibiting GFP-fluorescence were collected. These extracts were use to inoculate other *N. benthamiana* plants. Examination of whole plants by UV illumination showed that both viruses exhibited extensive GFP-fluorescence in infected leaves. In contrast, TRV produced significant GFP-fluorescence throughout the root system (Fig. 4A) whereas with PVX, root fluorescence was only apparent in the hypocotyl region, corresponding to an area of autofluorescence also found in the mock-inoculated plants (Figs. 4A and 4B). Root and leaf samples were taken from these plants for Western blotting when systemic GFP-fluorescence was at its strongest (TRV, 6 dpi; PVX, 9 dpi). In these experiments, TRV and PVX expressed GFP to similar levels in systemically infected leaves (Fig. 5). By contrast, however, TRV expressed 10- to 25-fold more GFP (as calculated from scanning densitometry of the blot) in the roots than did PVX.

Stability of a nonviral insert cloned into TRV

A possible limitation of plant viruses as gene expression vectors is the tendency for nonviral sequences to be deleted during virus multiplication in the plant. Tobraviruses are known to be subject to recombination *in vivo* (Hernández *et al.,* 1996; MacFarlane, 1997). However, because the tobravirus RNA2 is dispensable for systemic infection of plants, it might be more amenable to the insertion of nonviral sequences than are some of the other, usually monopartite, plant viral vectors. In keeping with our proposal that TRV could be used to test anti-pest proteins, we decided to examine the stability in roots of a 12.5-kDa lectin (GNA) derived from snowdrop (*Galanthus nivalis* L.). This particular lectin has been shown to be inhibitory to insects (Powell *et al.,* 1995; Rao *et al.,* 1998) and might also, like some other lectins, be effective against nematodes (Marban-Mendoza *et al.,* 1987; Gupta and Sharma, 1993). Thus the TRV-GFP construct was modified by replacement of the GFP gene by the GNA gene. Plants were infected with TRV expressing GNA by inoculation to the leaves. Subsequently, at intervals, the roots of individual infected plants were collected and examined by Western blotting. GNA was expressed at uniform levels in different plants inoculated at the same time (data not shown). In these plants, GNA accumulated to ~10 μg per gram wet weight of root tissue. GNA was first detected in roots usually between 4 and 6 dpi and remained detectable for ≤24 dpi (the last time point to be

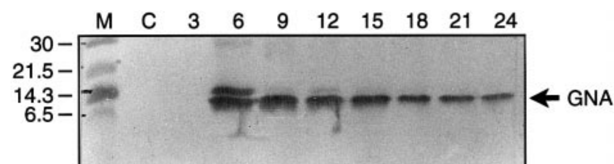

FIG. 6. Western blot of time course of GNA expressed from the TRV vector in roots of *N. benthamiana*. M denotes protein size markers (kDa). Equal amounts of root extract were loaded in each lane. C, uninfected plant sample. Other lanes are infected plants sampled at 3–24 days postinoculation. The blot was probed with GNA-specific antibodies.

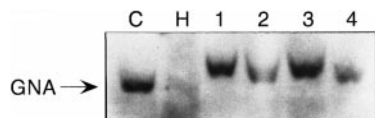

FIG. 7. Western blot showing stability of GNA expression by TRV vector. Lanes 1–4 are equal amounts of root extracts of *N. benthamiana* infected with TRV-GNA. Lane 1 is first passage (7 dpi), lane 2 is second passage (14 dpi), lane 3 is third passage (11dpi), lane 4 is fourth passage (9 dpi). C, 50-ng purified GNA protein. H, root extract from a plant infected with TRV containing a frameshifted GNA gene. The blot was probed with GNA-specific antibodies.

analyzed) (Fig. 6). The longer term stability of the TRV-GNA virus construct was assessed by passaging extracts of infected roots to the leaves of other plants at intervals of between 7 and 14 days, with root samples being taken at each passage. In this experiment, root expression of GNA was maintained for four passages, at which time the experiment was terminated (Fig. 7). In addition, tissue printing of plants inoculated with TRV-GNA showed that GNA expression was widespread throughout the root system (Fig. 8).

Key features of tobravirus vectors

The tobraviruses have a number of features that make them attractive as gene expression vectors. The smaller viral RNA, RNA2, is nonessential for systemic infection of plants by the virus, which means that it can be extensively modified without affecting virus viability. The CP gene sgRNA promoters of these viruses are, to an extent, interchangeable, which allows the construction of relatively stable constructs containing additional promoters. This raises the possibility that constructs might be built that can express more than one nonviral protein. Tobraviruses, particularly TRV, have a wide host range, suggesting that they could be used as gene vectors in many plant species. Lastly, in contrast to many other plant viruses, tobraviruses are adapted for efficient movement into the root system. This property makes them particularly useful as delivery vectors for testing a wide variety of proteins that may be active in plant-soil/pathogen interactions.

MATERIALS AND METHODS

Clone construction

The TRV-GFP construct was derived from a full-length clone of RNA2 of TRV isolate PpK20 (pTR9598), which carries an additional *Apa*I restriction site downstream of the 2c gene at nucleotide 3470 (Mueller *et al.,* 1997). The 3′ part of the 2b gene and all of the 2c gene were removed by digestion with *Bst*EII (nucleotide 1636) and *Apa*I (nucleotide 3467), and replaced with a *Apa*LI–*Sma*I fragment containing the PEBV CP promoter [nucleotides 273–509, pFLA56 (MacFarlane and Brown, 1995)] linked to the GFP gene derived from plasmid pBAD-GFPcycle3 (Crameri *et al.,* 1996). The initiation codon of the GFP gene precisely replaced that of the viral CP gene. The cycle3 GFP gene contains a *Nhe*I site immediately after the initiation codon (ATGGCTAGC) and *Eco*RI and *Kpn*I sites downstream of the termination codon. Thus the GFP gene can be removed from TRV-GFP by digestion with *Nhe*I and *Eco*RI or *Kpn*I and replaced with other genes of choice. An additional vector (TRV-GFPc) includes a *Nco*I site at the GFP initiation codon (ccATGG-CTAGC), allowing a more precise replacement of the GFP gene.

The PEBV-GFP construct was derived from pT72A56, a full-length cDNA clone of RNA2 of PEBV isolate TpA56 (MacFarlane *et al.,* 1996). The 2b and 2c genes were removed by digestion with *Bst*BI (nucleotide 1377) and *Eco*RI (nucleotide 2871), and replaced with a *Cla*I–*Eco*RI PCR fragment containing the TRV PpK20 CP promoter (nucleotides 346–556, pT72K20; Mueller *et al.,* 1997) linked to the GFP gene. As above, the GFP gene can be removed by digestion with *Nhe*I and *Eco*RI or *Kpn*I.

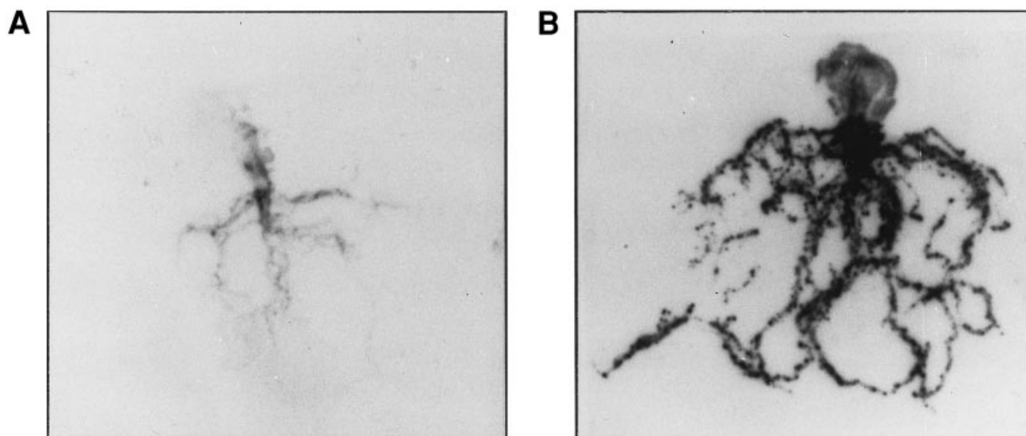

FIG. 8. Tissue print of *N. benthamiana* roots. (A) Mock-inoculated plant. (B) Plant infected with TRV expressing GNA, 21 days postinoculation. The prints were probed with antibodies raised against GNA.

The PepRSV-GFP construct was derived from a full-length clone (pT7Blue-PepRSV12) of RNA2 of PepRSV isolate CAM, which was obtained by long template-PCR (MacFarlane, 1996). A synthetic linker was used to introduce *Bsu*36I and *Bsi*WI sites at nucleotide 1251, downstream of the CP gene. A *Cel*II–*Kpn*I PEBV CP promoter-GFP fragment, derived from TRV-GFP, was introduced between the *Bsu*36I and *Bsi*WI sites. RNA2 of PepRSV isolate CAM carries at its 3′ end a partial copy of the RNA1-derived 16K gene. Thus in the PepRSV-GFP construct, this sequence is located 100 nucleotides downstream of the GFP gene.

The GFP gene was removed from TRV-GFP by digestion with *Nhe*I and *Kpn*I and replaced with the snowdrop lectin GNA gene (pGNA2; Gatehouse *et al.,* 1997) that had been PCR amplified to include a *Avr*II site at the second codon of the N-terminal signal sequence and a *Kpn*I site downstream of the termination codon.

Inoculation and analysis of plants

The TRV-GFP, TRV-GNA, and PEBV-GFP constructs were linearised at the 3′ terminus of the viral sequence by digestion with *Sma*I. PepRSV-GFP was linearised with *Spe*I, which cleaves the plasmid vector 10 bases downstream of the virus sequence. Transcript RNA was synthesised using T7 RNA polymerase as described before (MacFarlane *et al.,* 1996). Transcript RNA2 was mixed with RNA isolated from a plant infected only with RNA1 of each of the three viruses, a so-called NM-infection (Mueller *et al.,* 1997), and inoculated to *N. benthamiana* plants. Alternatively, infectious RNA1 transcripts can be synthesised from full-length cDNA clones of PEBV isolate SP5 (MacFarlane *et al.,* 1991) and TRV isolate PpK20 (S.MacFarlane, unpublished). The plants were examined at regular intervals for GFP fluorescence by illumination with long-wave UV light. Plants were photographed using Kodachrome 200 film with a HOYA G filter (for leaves) or without a filter (for whole plants). Extracts of transcript-inoculated plants were used to infect further plants. Leaf and root samples from infected plants were homogenised in 1 ml/g 1× PBS for analysis by Western blotting (Schmitt *et al.,* 1998). Tissue printing of roots was carried out as described before (Mansky *et al.,* 1990).

ACKNOWLEDGMENTS

We thank Walter Robertson, Vivian Blok, and Mark Phillips (Nematology Department, SCRI) for help and advice with the nematode bioassays. We also thank John and Angharad Gatehouse (Durham University) for providing us with the GNA plasmid clone, GNA2 protein, and GNA-specific antiserum. The GFP-specific antiserum and PVX-GFP were kindly provided by Simon Santa Cruz (Virology Department, SCRI). SCRI is grant-aided by the Scottish Office Environment Agriculture and Fisheries Department.

REFERENCES

Baulcombe, D.C., Chapman, S., and Santa Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for plant virus infections. *Plant J.* **7**, 1045–1053.

Bergh, S. T., Koziel, M. G., Huang, S. C., Thomas, R. A., Gilley, D. P., and Siegel, A. (1985). The nucleotide sequence of tobacco rattle virus RNA2 (CAM strain). *Nucleic Acids Res.* **13,** 8507–8518.

Brennan, F. R., Bellaby, T., Helliwell, S. M., Jones, T. D., Kamstrup, S., Dalsgaard, K., Flock, J.-I., and Hamilton, W. D. O. (1999). Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *J. Virol.* **73,** 930–938.

Chapman, S., Kavanagh, T., and Baulcombe, D. (1992). Potato virus X as a vector for gene expression in plants. *Plant J.* **2,** 549–557.

Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. C. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* **14,** 315–319.

Dolja, V. V., McBride, H. J., and Carrington, J. C. (1992). Tagging of plant potyvirus replication and movement by insertion of β-glucuronidase into the viral polyprotein. *Proc. Natl. Acad. Sci. USA* **89,** 10208–10212)

Dolja, V. V., Peremyslov, V. V., Keller, K. E., Martin, R. R., and Hong, J. (1998). Isolation and stability of histidine-tagged proteins produced in plants via potyvirus gene vectors. *Virology* **252,** 269–274.

Donson, J., Kearney, C. M., Hilf, M. E., and Dawson, W. O. (1991). Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. USA* **88,** 7204–7208.

Gatehouse, A. M. R., Davison, G. M., Newell, C. A., Merryweather, A., Hamilton, W. D. O., Burgess, E. P. J., Gilbert, R. J. C., and Gatehouse, J. A. (1997). Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: Growth room trials. *Mol. Breed.* **3,** 49–63.

Gupta, R., and Sharma, N. K. (1993). A study of the nematicidal activity of allicin—an active principle in garlic, *Allium sativum* L., against root-knot nematode, *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949. *Int. J. Pest Manag.* **39,** 390–392.

Hammond-Kosack, K. E., Staskawicz, B. J., Jones, J. D. G., and Baulcombe, D. C. (1995). Functional expression of a fungal avirulence gene from a modified potato virus X genome. *Mol. Plant-Microbe Interact.* **8,** 181–185.

Harrison, B. D., and Robinson, D. J. (1986). Tobraviruses. *In* "The Plant Viruses" (M. H. V. van Regenmortel and H. Fraenkel-Conrat, Eds.), Vol. 2, pp. 339–369. Plenum Press, New York.

Hernández, C., Carette, J. E., Brown, D. J. F., and Bol, J. F. (1996). Serial passage of tobacco rattle virus under different selection conditions results in deletion of structural and non-structural genes in RNA2. *J. Virol.*, **70,** 4933–4940.

Hernández, C., Visser, P. B., Brown, D. J. F., and Bol, J. F. (1997). Transmission of tobacco rattle virus isolate PpK20 by its nematode vector requires one of the two non-structural genes in the viral RNA2. *J. Gen. Virol.* **78,** 465–467.

Joelson, T., Akerblom, L., Oxelfelt, P., Strandberg, B., Tomenius, K., and Morris, T. J. (1997). Presentation of a foreign peptide on the surface of tomato bushy stunt virus. *J. Gen. Virol.* **78,** 1213–1217.

Kumagai, M. H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. K. (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. USA* **92,** 1679–1683.

Kumagai, M. H., Turpen, T. H., Weinzettl, N., Della-Cioppa, G., Turpen, A. M., Donson, J., Hilf, M. E., Grantham, G. L., Dawson, W. O., Chow, T. P., Piatak, M., and Grill, L. K. (1993). Rapid, high-level expression of biologically active α-trichosanthin in transfected plants by an RNA viral vector. *Proc. Natl. Acad. Sci. USA* **90,** 427–430.

Lacomme, C., Smolenska, L., and Wilson, T. M. A. (1998). Genetic engineering and the expression of foreign peptides or proteins with plant virus-based vectors. *In* "Genetic Engineering" (J. K. Setlow, Ed.), Vol. 20, pp. 225–237. Plenum Press, New York.

MacCullock, L. A. (1991). "Chemotactic and electrotactic localisation of

plant roots by parasitic nematodes." Ph.D. thesis, University of Aberdeen, Aberdeen, Scotland.

MacFarlane, S. A. (1996). Rapid cloning of uncharacterised tobacco rattle virus isolates using long template (LT) PCR. *J. Virol. Methods* **56,** 91–98.

MacFarlane, S. A. (1997). Natural recombination among plant virus genomes: Evidence from tobraviruses. *Semin Virol.* **8,** 25–31.

MacFarlane, S. A., and Brown, D. J. F. (1995). Sequence comparison of RNA2 of nematode-transmissible and nematode non-transmissible isolates of pea early-browning virus suggests that the gene encoding the 29 kDa protein may be involved in nematode transmission. *J. Gen. Virol.* **76,** 1299–1304.

MacFarlane, S. A., Wallis, C. V., and Brown, D. J. F. (1996). Multiple genes involved in the nematode transmission of pea early browning virus. *Virology* **219,** 417–422.

MacFarlane, S. A., Wallis, C. V., Taylor, S. C., Goulden, M. G., Wood, K. R., and Davies, J. W. (1991). Construction and analysis of infectious transcripts synthesized from full-length cDNA clones of both genomic RNAs of pea early-browning virus. *Virology* **182,** 124–129.

Mansky, L. M., Andrews, R. E., Durand, D. P., Jr., and Hill, J. H. (1990). Plant virus location in leaf tissue by press blotting. *Plant Mol. Biol. Rep.* **8,** 13–17.

Marban-Mendoza, N., Jeyaprakash, A., Jansson, H. B., Damon Jr., R. A., and Zuckerman, B. M. (1987). Control of root-knot nematodes on tomato by lectin. *J. Nematol.* **19,** 331–335.

Mueller, A-M., Mooney, A. L., and MacFarlane, S. A. (1997). Replication of *in vitro* tobravirus recombinants shows that the specificity of template recognition is determined by 5′ non-coding but not 3′ non-coding sequences. *J. Gen. Virol.* **78,** 2085–2088.

Oparka, K. J., Boevink, P., Roberts, A. G., Blackman, L., Palukaitis, P., MacFarlane, S. A., and Santa Cruz, S. (1998). The ins and outs of systemic virus transport. "Workshop on Plasmodesmata and Transport of Plant Viruses and Plant Macromolecules." Instituto Juan March, Centre for International Meetings on Biology, Madrid, Spain.

Porta, C., Spall, V. E., Loveland, J., Johnson, J. E., Barker, P. J., and Lomonossoff, G. P. (1994). Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides. *Virology* **202,** 949–955.

Powell, K. S., Gatehouse, A. M. R., Hilder, V. A., and Gatehouse, J. A. (1995). Antifeedent effects of plant lectins and an enzyme on the adult stage of the rice brown planthopper *Nilaparvata lugens. Entomol. Exp. Appl.* **75,** 51–59.

Rao, K. V., Rathore, K., Hodges, T. K., Fu, X., Stoger, E., Sudhakar, D., Williams, S., Christou, P., Bharathi, M., Bown, D. P., Powell, K. S., Spence, J., Gatehouse, A. M. R., and Gatehouse, J. A. (1998). Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown plant hopper. *Plant J.* **15,** 469–477.

Sablowski, R. W. M., Baulcombe, D. C., and Bevan, M. (1995). Expression of a flower-specific Myb protein in leaf cells using a viral vector causes ectopic activation of a target promoter. *Proc. Natl. Acad. Sci. USA* **92,** 6901–6905.

Schmitt, C., Mueller, A.-M., Mooney, A., Brown, D. J. F., and MacFarlane, S. A. (1998). Immunological detection and mutational analysis of the RNA2-encoded nematode transmission proteins of pea early browning virus. *J. Gen. Virol.* **79,** 1281–1288.

Shivprasad, S., Pogue, G. P., Lewandowski, D. J., Hidalgo, J., Donson, J., Grill, L. K., and Dawson, W. O. (1999). Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. *Virology* **255,** 312–323.

Taylor, C. E., and Brown, D. J. F. (1997). "Nematode Vectors of Plant Viruses." CAB International, New York.