11
Viruses as Vectors for the Expression of Foreign Sequences in Plants

CLAUDINE PORTA[1]* AND GEORGE P. LOMONOSSOFF[2]

[1]*Horticulture Research International-Wellesbourne, Warwick CV35 9EF, UK and*
[2]*John Innes Centre, Colney, Norwich NR4 7UH, UK*

Introduction

Since the development, in the 1970s, of methods for manipulating genes (recombinant DNA technology), there has been great interest in the design of systems for expressing proteins in contexts or in amounts in which they do not occur naturally. The first such expression systems were based on micro-organisms (bacteria or yeast) and these

*To whom correspondence may be addressed (Claudine.Porta@hri.ac.uk)

General abbreviations: Ac, transposon activator unit; bp, base pair; BMS, Black Mexican sweetcorn; CAT, chloramphenicol acetyl transferase; Cd, cadmium; ChsA, chalcone synthase A; CP, coat protein; CR, common region; DHFR, dihydrofolate reductase; DI RNA, defective interfering RNA; dRNA, defective RNA; ds, double-stranded; Ds, dissociation element; βgal, β-galactosidase; GFP, green fluorescent protein; kbp, kilobase pair; GR, glutathione reductase; GS, glutamine synthase; GUS, β-glucuronidase; HC-Pro, helper component-proteinase; HPT, hygromycin phosphotransferase; IFN-αD, interferon αD; IFN-γ, interferon γ; LIR, large intergenic reagion; luc, luciferase; MP, movement protein; MTII, metallothionein II; nos, nopaline synthase; NPT-II, neomycin phosphotransferase type II; OAS, origin of assembly; ocs, octopine synthase; ORF, open reading frame; ori, origin of replication; PAT, phosphinothricin acetyl transferase; PDS, phytoene desaturase; PSY, phytoene synthase; PTGS, post-transcriptional gene silencing; Rz, ribozyme; ss, single-stranded; *N. tabacum, Nicotiana tabacum; N. benthamiana, Nicotiana benthamiana;* RBS, ribulose biphosphate carboxylase; ScFv, single-chain variable fragment; sg, subgenomic; SIR, small intergenic region; TGB, triple gene block; VIGS, virus-induced gene silencing; VLPs, virus-like particles. Note: In the text names of genes are given in lower case italics, names of the corresponding gene products in upper case.
Abbreviations for virus names: ACMV, African cassava mosaic virus; AlMV, alfalfa mosaic virus; BaMV, bamboo mosaic virus; BMV, brome mosaic virus; BSMV, barley stripe mosaic virus; BYV, beet yellows virus; CaMV, cauliflower mosaic virus; CCMV, cowpea chlorotic mottle virus; ClYVV, clover yellow vein virus; CMV, cucumber mosaic virus; CPMV, cowpea mosaic virus; CyRSV, cymbidium ringspot virus; FMDV, foot-and-mouth disease virus; HIV-1, human immunodeficiency virus 1; MiSV, miscanthus streak virus; MSV, maize streak virus; ORSV, odontoglossum ringspot virus; PEBV, pea early browning virus; PepRSV, pepper ringspot virus; PMMoV, pepper mild mottle virus; PPV, plum pox virus; PVV, potato virus V; PVX, potato virus X; RABV, rabies virus; RHDV, rabbit haemorrhagic disease virus; SHMV, sunn hemp mosaic virus; sTRSV, satellite of tobacco ringspot virus; TAV, tomato aspermy virus; TBSV, tomato bushy stunt virus; TEV, tobacco etch virus; ToMV, tomato mosaic virus; TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus; TYDV, tobacco yellow dwarf virus; TGMV, tomato golden mosaic virus; TRV, tobacco rattle virus; TSWV, tomato spotted wilt virus; TCV, turnip crinkle virus; WDV, wheat dwarf virus; WSMV, wheat streak mosaic virus.

Biotechnology and Genetic Engineering Reviews – Vol. 19, July 2002
0264–8725/02/19/245–291 $20.00 + $0.00 © Intercept Ltd, PO Box 716, Andover, Hampshire SP10 1YG, UK

systems are now in routine use. Systems based on cultures of cells from higher organisms, such as insects or mammals, were subsequently developed and have also proved to be extremely useful. More recently, expression systems based on complete higher organisms, either animals or plants, have been developed. Plants represent an attractive expression medium for a number of reasons: they can produce enormous biomass, they require only simple inputs to grow (light, carbon dioxide and a source of nitrogen which, in certain cases, can be provided from the atmosphere) and their propagation does not require the use of complex and expensive fermentation apparatus.

There are currently two systems for expressing foreign sequences in plants: stable genetic transformation and the use of plant virus-based vectors. The transgenic approach involves integration of heterologous genes into the chromosomes of a host plant. Expression of the integrated gene is under the control of a promoter positioned immediately upstream of the coding region. This method of foreign gene expression in plants is widely used and has the advantage that the integrated sequence is heritable. There are, however, a number of disadvantages with this approach: the process of regenerating transformed plants can be difficult and time consuming, the levels of expression reached are often relatively low, and undesirable phenomena, such as transgene silencing, often occur. The alternative approach to foreign gene expression in plants is the use of virus-based vectors. One of its attractions is that plant viruses multiply within infected cells. Thus, any foreign gene incorporated within the viral genome should be concomitantly amplified, leading to high levels of expression.

The purpose of this article is to review the progress that has been made in the development of virus-based vectors for the expression of foreign genes in plants. However, to keep this review to a manageable length, we will discuss only those systems which have been used to express sequences of substantial length, e.g. full-length proteins. Thus, we will not discuss strategies by which the coat proteins of plant viruses have been modified to display short peptides (epitope-presentation systems) and the reader is referred to recent reviews to gain information about this related technology (Porta and Lomonossoff, 1998; Lomonossoff and Hamilton, 1999). As the reader will quickly realize, attempts have been made to develop a wide variety of different viruses into vectors. We have, therefore, decided to discuss the vectors under headings dealing with different virus genera, and have also chosen to discuss these in approximately the order in which the vectors were first developed.

DNA viruses

The first viruses to be proposed as potential gene vectors were those whose genomes consist of DNA (Szeto *et al.*, 1977; Hull, 1978). Though these make up only a minority of plant viruses, at the time they were the only viruses for which genetic manipulation was possible. Thus, viruses from two families, the *Caulimoviridae* (circular double-stranded (ds) DNA genomes) and the *Geminiviridae* (circular single-stranded (ss) DNA genomes), were extensively investigated as potential vectors in the 1980s in the hope that they could effectively be developed as plant 'plasmids'.

CAULIMOVIRIDAE

This family of plant viruses contains 6 genera. The *Caulimoviridae* are pararetroviruses,

replicating their double-stranded DNA genomes through an RNA intermediate. Members of the genus *Caulimovirus* are usually transmitted by aphids and have a rather narrow host range. The type member *cauliflower mosaic virus* (CaMV), which infects mostly crucifers, was the first plant virus to be considered as a potential vector for gene expression in plants. An important step in the development of CaMV-based vectors was the demonstration that cloned viral DNA, propagated in *E. coli*, was still infectious to plants providing it was released from its plasmid vector by restriction enzyme digestion prior to inoculation (Howell *et al.*, 1980). This established that the discontinuities found in the DNA isolated from virions (see *Figure 11.1*), and which are sealed in the infected cell, are not essential for infectivity, and opened the way for the manipulation of the CaMV genome in bacteria.

CaMV has a genome consisting of 8 kbp of dsDNA encapsidated in isometric particles of 50 nm. Following entry into the cell's nucleus, host DNA-dependent RNA polymerases transcribe the coding strand into a more-than-genome-length transcript. This 35S RNA serves as the template both for reverse transcription (Pfeiffer and Hohn, 1983) and for expression of six out of the seven virus-encoded genes, with open reading frame (ORF) VI translated from its own 19S transcript (*Figure 11.1*).

Initially, bacterial DNA sequences were used to probe the CaMV genome for potential sites for the insertion of heterologous genes. A 65 bp fragment was inserted into a naturally occurring *Xho*I site located within gene II (see *Figure 11.1*), a region covered by a deletion in a variant strain of CaMV and therefore not essential for viral infection (Howarth *et al.*, 1981). Inoculated turnip plants (*Brassica campestris* L.) developed a systemic infection and the insert was maintained for three to five passages from plant to plant. Maximum insertion capacity in this position was found to be around 250 bp and was assumed to reflect packaging limitations by CaMV particles (Gronenborn *et al.*, 1981).

The first functional gene to be cloned into CaMV was the dihydrofolate reductase gene (*dhfr*) from an *E. coli* plasmid, which encodes a methotrexate-insensitive enzyme. *Dhfr*, which is 234 bp long, was used to replace gene II (470 bp) and resulted in recombinant CaMV, which could be propagated in turnip plants for three cycles of infection with viral DNA (Brisson *et al.*, 1984). Expression of the *dhfr* gene was monitored by Western blot analysis of leaf extracts with a DHFR-specific antibody and yields were estimated to be about 8 μg/g of fresh tissue by comparison with the signal obtained with a known amount of DHFR purified from *E. coli*. DHFR activity was assessed by insensitivity of systemically infected leaves to methotrexate, which normally impairs RNA and DNA synthesis.

Next, gene II was replaced by a 200 bp mammalian gene encoding metallothionein II (MTII) from Chinese hamster, a protein which selectively binds heavy metals and can confer resistance to cadmium (Cd) when overexpressed. As observed following *dhfr* insertion, infectivity of the recombinant virus bearing the gene for MTII was similar to that of the parental virus, with local lesions appearing within two weeks and a systemic infection within four weeks of inoculation of turnip plants with plasmid-released DNA (Lefebvre *et al.*, 1987). The *mtII* gene insert was also maintained during three consecutive passages using crude leaf extracts of infected plants to rub-inoculate healthy plants. Production of MTII was detected by immunoblot and the MTII content of infected leaves estimated to represent 0.5% of soluble leaf proteins. Function of MTII in plants was determined by the ability of excised infected leaves to

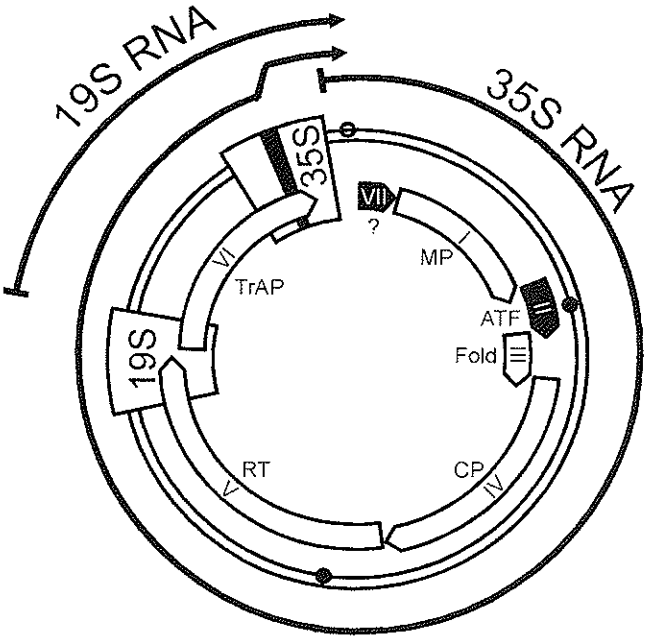

Figure 11.1. Genome organization of *cauliflower mosaic virus* (CaMV). The circular dsDNA (8 kbp) is represented by two concentric circles and contains three discontinuities adjacent to replication priming sequences. One (open circle) is on the (+) strand, while the other two (full circles) are on the (–) strand. Outer arrows represent transcripts from the (+) strand by host DNA-dependent RNA polymerases: the 35S RNA (a more-than-genome-length mRNA), which serves as a template for both reverse transcription and translation of genes I to V, and the 19S RNA from which gene VI is expressed. Inner arrows represent ORFs which encode the movement protein (MP), the aphid transmission factor (ATF), a protein which has a putative role in viral DNA folding during encapsidation (Fold), the coat protein (CP), the reverse transcriptase (RT), and the translational transactivator protein (TrAP). Sequences which are dispensable for virus infection and which can be deleted and/or replaced with foreign genes are shown in black.

survive exposure to a 10 mM $CdCl_2$ solution, linked to increased sequestration of Cd by the expressed MTII.

Subsequently, CaMV was used to express *in planta* the 501 bp human gene encoding interferon αD (IFN-αD). This was again by replacement of ORF II, but into a vector from which ORF VII (see *Figure 11.1*), also dispensable for virus viability (Dixon and Hohn, 1984), had been deleted, therefore potentially increasing the insert size that CaMV can carry successfully. Interferon content of leaves with primary and systemic symptoms was quantified by an *in vitro* antiviral activity assay and found to reach a maximum level of 2 μg of IFN-αD/g of fresh tissue (DeZoeten *et al.*, 1989). However, no protection against superinfection with *turnip yellow mosaic virus* (TYMV), a ss positive-sense RNA virus, was obtained. Interferons are produced by animal cells upon virus infection and induce the synthesis of dsRNA-activated enzymes. This activation trigger can be provided by the replication intermediate of ssRNA viruses and, in animal tissues, results in endoRNase activity and selective inhibition of viral mRNA translation mediated by protein kinases termed PKR. However, no down-regulation of TYMV synthesis was observed when exposed to interferon expressed via CaMV in doubly-infected plant cells.

From insertional analysis into the CaMV genome it has been inferred that, in order to get packaged into particles, recombinant viral DNA could not exceed 8300 bp in size (Daubert *et al.*, 1983). Following deletion of all non-essential sequences: ORF II, ORF VII and a small part of the large intergenic region (see *Figure 11.1*; Penswick *et al.*, 1988), the maximal payload would therefore be 1000 bp. In order to overcome this size limitation, attempts were made at genetic complementation between deletion mutants. Co-infection of plants with species of CaMV DNA bearing non-overlapping deletions invariably resulted in the recovery of wild-type virus (Walden and Howell, 1982; Choe *et al.*, 1985) as a result of either homologous recombination or template switching, which occurs during replication by reverse transcription. A spontaneous CaMV mutant missing ORF I and part of ORF II was combined with a mutant from which an area covering ORF II and ORF III had been deleted. The deletions borne by these mutants therefore overlap over part of ORF II. When inoculated separately, none of these mutants was infectious. When inoculated onto plants as a pair, symptoms developed and restriction analysis of DNA isolated from infected leaves revealed a digestion pattern compatible with the presence of a mixture of both mutants (Hirochika and Hayashi, 1991). However, application of these overlapping deletion mutants for the cloning of foreign genes was not reported at a later date.

Size restrictions were not a constraint for the delivery of a 107 bp *cis* hairpin ribozyme (Rz) fragment which was inserted into the *Xho*I site of gene II. Transcription of a *cis* Rz within the viral mRNA should result in its cleavage and therefore reduce the amount of template available for reverse transcription. Following inoculation of plants, a one-week delay in the appearance of systemic symptoms was observed by comparison to a construct harbouring an inactive Rz sequence. Lysate RNase protection assays demonstrated that the Rz was active in extracts from whole infected plants (Borneman *et al.*, 1995). However, failure to analyse the sequence of PCR products covering the Rz insert left open the possibility that a sequence alteration in the Rz was responsible for the increase in severity of systemic symptoms which was observed during the later stages of the experiment.

Over the years, a number of *cis*-acting elements had been identified within gene II: a (+) strand replication primer (Pietrzak and Hohn, 1985), a splice acceptor site (Kiss-Laszlo *et al.*, 1995), and sequences linking its translation with adjacent genes. As an alternative to insertions into gene II, Noad *et al.* (1997) explored the insertion of functional sequences into domains of the 35S promoter. This approach was based on the observation that within the 35S enhancer (sequences between –207 and –56 from the cap site), domains E3 (–207 to –150) and E2 (–95 to –56) are not simultaneously required for viral infectivity (Turner *et al.*, 1996). E3 was replaced by an artificial (+) strand priming sequence, based on the polypurine tract located in gene II. Functionality in this ectopic site was shown by the creation of one more discontinuity on the (+) strand of progeny virion DNA. Into a clone from which 421 bp from the middle of gene II had been deleted, a 300 bp sequence from the 5' untranslated leader of the *Arabidopsis* phytoene desaturase (*pds*) gene was inserted at the left border of a complete 35S enhancer. Analysis of viral DNA isolated from infected turnip plants demonstrated that the insert had lost 198 pb, consistent with correct splicing of the resident intron. CaMV replicons can therefore be used to deliver to specific cellular compartments – the cytoplasm for recognition of the

artificial replication primer, the nucleus for intron splicing – elements which target either viral and host functions.

Based on the observation that a CaMV ORF I mutant defective for movement can be complemented by wild-type virus for systemic spread (Thomas *et al.*, 1993), a complementation strategy was used to express β-glucuronidase (GUS) from CaMV. A deletion reaching from the *Xho*I site within gene II to within the 5' end of gene V was made in the CaMV genome in order to accommodate the 2 kb *gus* gene without increase in size over that of the wild-type (Viaplana *et al.*, 2001). Upon co-inoculation with wild-type CaMV, expression of GUS was observed but was limited to local lesions, whilst co-inoculation with helper virus disabled for expression of ORF I rapidly produces wild-type virus by recombination.

Recently, an insertion strategy into the CaMV genome has been designed that allows the stable expression of the 720 bp green fluorescent protein (*gfp*) gene (Simon Covey, personal communication).

GEMINIVIRIDAE

These viruses derive their name from the geminate nature of their virions, which consist of two joined incomplete icosahedra, with dimensions of 18 × 30 nm. Their genome is comprised of either one or two molecules of circular ssDNA, 2.5–3.0 kb in size. The viral-sense ssDNA genome replicates in the nucleus of infected cells via a dsDNA intermediate that is used as the template for rolling-circle replication (Stenger *et al.*, 1991). Transcription starts from an intergenic region and proceeds in both directions, i.e. from both the virion-sense and complementary-sense strands. Given the small size of their genomes, which facilitates cloning in *E. coli* plasmids, their unique – amongst plant viruses – place and mode of replication and the fact that, collectively, they infect a wide range of hosts, *Geminiviridae* have attracted interest as gene vectors.

Classification into genera is based on the number of molecules of DNA (1 or 2), the insect vector (leaf/treehoppers or aphids) and the host (monocots or dicots).

Begomoviruses

Viruses belonging to this genus are whitefly transmitted, infect dicotyledonous hosts and the majority have a genome made up of two molecules of ssDNA of similar size, termed DNA A and B. The intergenic region, which is shared by the two molecules of DNA and therefore termed common region (CR), contains the site for initiation and the signals for termination of DNA replication, as well as the promoters for bi-directional transcription. Whilst the two proteins encoded by DNA B are required for virus movement, they are dispensable for viral replication. The coat protein (CP) of bipartite begomoviruses, encoded by DNA A, is dispensable both for viral DNA replication and systemic spread (Stanley and Townsend, 1986; Gardiner *et al.*, 1988). This triggered widespread interest into its replacement by foreign genes (*Figure 11.2*). Because DNA A encodes all the viral proteins involved in replication, it can self-replicate either transiently in protoplasts (Townsend *et al.*, 1986), or when expressed from an integrated sequence in transgenic plants (Rogers *et al.*, 1986; Meyer *et al.*, 1989).

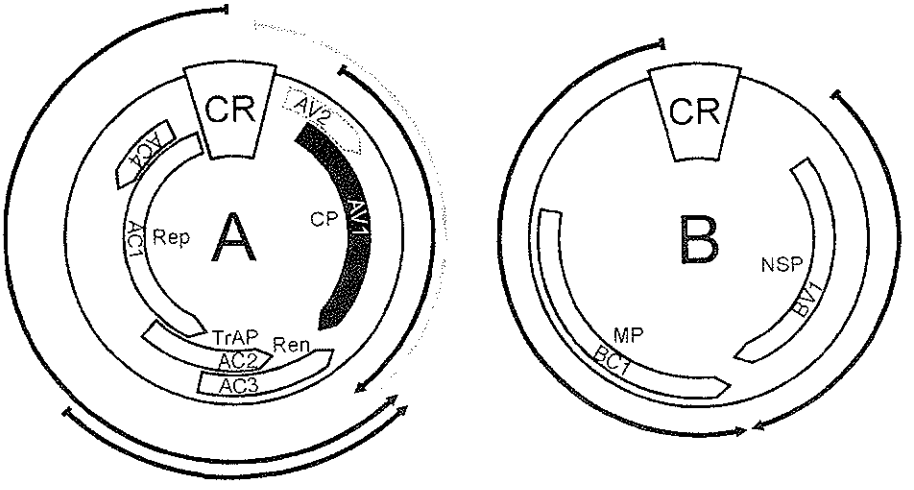

Figure 11.2. Generic genome organization of bipartite begomoviruses. The two molecules of circular ssDNA (2.5–2.8 kb in size each) are represented by individual circles and share approximately 200 b of sequence in the area covered by an open box (termed common region; CR), in which replication starts. Transcripts are synthesized bi-directionally, as shown by the outer arrows. Inner arrows correspond to the ORFs, which are denoted by the genome component – A or B – and the DNA strand – virion (V) or complementary (C) sense – which encodes them. Where known, their functions are indicated: coat protein (CP), replication-associated protein (Rep), transcriptional activator protein (TrAP), replication enhancer (Ren), nuclear shuttle protein (NSP), and movement protein (MP). ORF AV2 and the corresponding virion-sense transcript, both shown in grey, are found in ACMV but not in TGMV. ORF AV1, which has been replaced by foreign sequences, is filled in black.

1. *African cassava mosaic virus (ACMV)*

ACMV DNA A encodes two proteins in the virion-sense: the CP, AV1, and a smaller upstream protein, AV2, of unknown function (*Figure 11.2*). Full-length genomic clones of DNA A and B are infectious by mechanical inoculation following excision of unit-length dsDNA from the cloning vector (Stanley, 1983).

Ward *et al.* (1988) deleted the coding sequence for AV1, leaving just 7 amino acids at the N-terminus. At the C-terminal end, 25 bp of the AV1 coding sequence were preserved in order to avoid disrupting the expression of complementary-sense genes. Deletion of the sequence for the CP resulted in loss of infectivity for *Nicotiana benthamiana* plants, whilst its restoration, even in the opposite orientation, re-established infectivity. When the CP gene was replaced by that of the bacterial chloramphenicol acetyl transferase (CAT), which resulted in a net increase of only 58 bp over the size of wild-type DNA A, symptoms typical of ACMV infection appeared both on the inoculated leaves and on newly expanding leaves. This confirmed that for infectivity it is necessary to preserve the size of the naturally occurring DNA component, despite abolition of the constraints imposed by packaging, through deletion of the CP gene. CAT expression (detected by Western blot analysis and enzyme activity assay) was maintained over a period of four weeks, as well as after mechanical transmission of viral DNA to healthy plants. Given the role of the CP in aphid transmission (Briddon *et al.* 1990), CP replacement constructs would be easy to

contain. However, the usefulness of the system was further limited by poor sap transmissibility of CAT-encoding ACMV, probably as a result of degradation of the unencapsidated viral DNAs.

There are two ways to circumvent the limitations imposed by establishing a begomovirus infection in plants: they involve the introduction of DNA A-based vectors into either single cells, or by expression of DNA A from an integrated copy in transgenic plants.

a. Protoplasts

Longer than unit-length derivatives of ACMV DNA A containing a duplication of the CR can be used for direct electroporation of protoplasts with supercoiled plasmid. The presence of this sequence duplication allows the release of monomers of DNA A either by intramolecular recombination or, more efficiently, by replicational release. The latter is considered to be evidence that geminiviruses replicate by a rolling-circle mechanism (Stenger *et al.*, 1991).

A CP replacement construct expressing CAT from DNA A and a CAT-specific hammerhead Rz similarly inserted into a second DNA A-based construct were delivered simultaneously into *Nicotiana tabacum* cells. *In vivo* CAT mRNA cleavage by the Rz increased when the latter was embedded into the anticodon loop of a $tRNA^{Lys}$: this was due to a 150-fold increase in the steady state level of Rz by transcription from the endogenous tRNA promoter over that from the ACMV CP promoter (tRNAs are abundantly transcribed by RNA polymerase III, while the promoter for the CP of ACMV is recognized by RNA polymerase II). ACMV therefore only served to replicate the Rz and its target *in vivo* (Perriman *et al.*, 1995).

The satellite RNA of *tobacco ringspot virus* (sTRSV) sequence, which has no messenger activity, was substituted for most of the CP in DNA A of ACMV. The inserts varied in size between 460 and 692 bp and allowed the study *in vivo* of the sequences involved in the formation of circular forms of sTRSV, following self-cleavage from subdimeric sTRSV transcripts. For the shortest of these sTRSV inserts, addition of a pol III promoter to their 5' side was again required in order to accumulate detectable amounts of the corresponding transcripts and their derivatives. One possible explanation for this requirement is that smaller inserts could not be efficiently expressed from the ACMV vector (Chay *et al.*, 1997).

b. Transgenic plants

Meyer *et al.* (1992) made use of the fact that, in plants carrying integrated dimers of DNA A, autonomous replication of this component is obtained. A 969 bp fragment spanning the sequence for the CP was deleted from the 2.7 kb DNA A and later substituted by a 660 bp fragment from the maize transposon *En/Spm* or a 751 bp fragment containing the *attB* insertion site of *E. coli*. Extrachromosomal copies of both the deleted and recombinant monomers could be detected in transgenic *N. tabacum* plants. This expression strategy produces symptomless plants, precludes virus transmission by aphids (given the absence of CP) but does not depend on viral spread in the plant. However, activity of the foreign sequences was not assessed, nor were attempts made to generate recombinant molecules larger than 2.7 kb.

The transcription of virion-sense genes of ACMV DNA A has been shown to be transactivated by complementary-sense-encoded protein AC2 (see *Figure 11.2*;

Haley *et al.*, 1992). This led to the development of a transactivatable expression system in transgenic plants. The reporter gene *gus* cloned downstream of the virion-sense promoter, AV1Pro, in frame with the CP initiation codon, was used to transform *N. benthamiana* plants. Levels of constitutive expression from AV1Pro were significantly above those of plants transgenic for *gus* but lacking the viral promoter. Upon infection with ACMV, wild-type symptoms developed and levels of GUS expression increased between 2- and 30-fold, although great fluctuations were observed both between plant lines and between plants from the same line. When dianthin, a ribosome-inactivating protein which exhibits antiviral activity against a broad range of viruses, was substituted for GUS, the resulting transgenic plants (AV1Pro-DIA) displayed normal growth. Following challenge with ACMV, necrotic lesions were observed on the inoculated leaves and systemic symptoms were absent or mild. This virus resistance was confined to ACMV isolates, demonstrating that transactivation of AV1Pro by AC2 homologues from other geminiviruses is not efficient (Hong *et al.*, 1996). Confirmation of the specific role of AC2 in the transactivation of dianthin expression in AV1Pro-DIA plants was sought by mutagenesis. Given the inability of ACMV with mutated AC2 sequences to infect plants systemically (Etessami *et al.*, 1991), the *potato virus X* (PVX) vector described in a subsequent section was used to deliver AC2 sequences *in planta*. Only wild-type AC2 produced a 15-fold increase in dianthin activity over the basic level obtained in plants inoculated with unmodified PVX (Hong *et al.*, 1997).

2. *Tomato golden mosaic virus (TGMV)*

The genome organization of TGMV is similar to that of ACMV with the difference that, in the virion-sense, DNA A encodes only AV1, the CP (*Figure 11.2*). Although dsDNA clones of the viral genome, excised from recombinant plasmids, are infectious when mechanically inoculated (Hamilton *et al.*, 1983), the most efficient route of inoculation is via *Agrobacterium* transformed with Ti plasmids carrying partial or complete dimers of each of the two DNAs (Hayes *et al.*, 1988a).

a. Leaf discs

Hanley-Bowdoin *et al.* (1988) inoculated leaf discs from petunia with *Agrobacterium* carrying a binary vector with 1.5 copies of DNA A from TGMV. The *cat* or *gus* genes were used in turns to replace the CP gene. Transcription of the *gus* gene was found to be only 21% lower than that of the CP gene, despite a concomitant increase in the size of DNA A of 1.1 kb. This demonstrated that replication of TGMV DNA A in individual cells is not regulated by tight size constraints. The fact that *cat* transcripts accumulated 57% less than CP transcripts (from which they differ in size by only 25 bp) was attributed to their instability in plant cells. Because the level of CP transcription depends on DNA A copy number, a transcript for AC1 (for position see *Figure 11.2*) was used as an internal standard. Both bacterial gene replacement constructs produced 60% less AC1 transcript than wild-type DNA A. This was interpreted as reflecting the availability of lower amounts of recombinant DNA A template.

Agrobacterium-mediated transfection of tobacco leaf discs with DNA A was used to study the splicing of pre-mRNAs (McCullough *et al.*, 1991). Full-length exon/intron/exon cassettes were inserted between the CP promoter and terminator sequences.

The corresponding transcripts could be produced in large amounts as a result of autonomous replication of the TGMV-based vector, coupled with efficient transcription from the CP promoter. By RT-PCR analysis and DNA sequencing, accurate excision of introns from the ribulose biphosphate carboxylase (*rbs*) gene was demonstrated. The efficiency of intron splicing from a pea and wheat *rbs* gene, determined by quantitative RNAse protection assays, was respectively 85% and 73%, which is a reflection of the different selectivities of the RNA processing systems in monocot and dicot plants. Expression of the proteins encoded by the spliced RNAs was not analysed.

In order to develop a system for translational suppression in plants, non-replicating plasmids encoding a reporter gene were co-transformed into *N. benthamiana* cell cultures with plasmids expressing tRNAs. Nonsense (amber) and missense mutations of the codon for a Lys essential for luciferase (luc) activity could be suppressed by $tRNAs^{Lys}$ with cognately mutated anticodons (Chen *et al.*, 1998). Suppression increased with the quantity of tRNA-encoding DNA and was therefore found to be higher when tRNA genes replaced the CP gene of TGMV DNA A than when they were delivered by a phagemid, which cannot replicate in plants.

Kanevski *et al.* (1992) cloned DNA A as a partial tandem repeat in a pUC vector. The gene for the CP was replaced by that for neomycin phosphotransferase type II (NPT-II), termed *kan* as it confers resistance to kanamycin. Expression of NPT-II was controlled either by the CP signals or by the 5' region of the nopaline synthase (*nos*) gene and the 3' region of the octopine synthase (*ocs*) gene. Replication of these vectors was compared to that of wild-type DNA A following transfection of protoplasts; it was found to be two-fold higher for the *kan* only bearing construct, and two-fold lower for the *nos/kan/ocs* cassette insertion construct. Biolistic transformation of tobacco suspension culture cells followed by selection for kanamycin resistance yielded 10% of clones in which replicating viral genomes could be detected after four months of culture. The maximum number of unit-length vectors/cell was in the range 500–1000 and corresponded to an approximate 20-fold increase in the amount of NPT-II enzyme over that of a cell line transformed with a non-replicating *kan*-bearing plasmid (less than 10 integrated *kan* gene copies). This study demonstrated that DNA A-based vectors delivered biolistically into single cells can be used to establish clonal cell lines in which replicating viral DNA can mediate the enhanced expression of foreign genes.

b. Whole plants

The bacterial *neo* gene (identical to the *kan* gene described above) was substituted for the CP in a plasmid bearing a partial dimer of DNA A (pA1.6*neo*). Plant infections were obtained by agroinoculation either of transgenic *N. tabacum* carrying a head-to-tail dimer of DNA B (B2 plants) with pA1.6*neo* or of non-transformed plants with a derivative of pA1.6*neo* simultaneously bearing a dimer of DNA B (pA1.6*neo*B2). The first method was more effective than the second one and pA1.6*neo*-infected B2 plants were shown to present an average of 490 copies of the *neo* gene/genome, versus 160 copies in pA1.6*neo*B2-infected non-transgenic plants. NPT expression paralleled gene copy number and was found to be three times higher in the first than in the second set of plants (Hayes *et al.*, 1988b).

Infection of intact plants can also be achieved by particle gun bombardment with a

combination of plasmids carrying tandem copies of DNA A and DNA B. This method was used by Kjemtrup *et al.* (1998) who set out to use TGMV as a source of high copy DNA episomes for the delivery of sequences homologous to chromosomal genes in order to study gene silencing. The CP gene was replaced by the 5' sense fragment of the *su* gene, which encodes the nucleotide binding subunit of magnesium chelatase, an enzyme required in chlorophyll biosynthesis. Symptoms distinct from those of a wild-type infection were induced on *N. benthamiana* plants: yellow spots appeared on the inoculated leaves and yellow-white or light green variegation developed on new leaves and stems. This phenotype was maintained with constructs encoding 5' antisense and 3' sense sequences of the *su* gene. Absence from these fragments of a functional sequence for the transit peptide that is required for import into the chloroplast eliminated interference of mutant proteins with the formation of the multisubunit enzymatic complex as the explanation for the lack of chlorophyll synthesis. Variegation could be maintained through tissue culture but was lost after meiosis, indicating that recombinant TGMV sequences had not significantly integrated into chromosomal DNA. Accumulation of the endogenous *su* gene transcript was prevented by both the 5' sense *su* construct and a frameshift derivative. Similarly, levels of transcription from a transgene were found to be greatly reduced when *N. benthamiana* plants stably transformed for the *luc* gene were infected with TGMV in which the CP gene had been replaced by *luc* 5' sense and antisense fragments. Down-regulation of both an endogenous gene (*su*) and a transgene (*luc*) therefore occurred at the RNA or DNA level and had been mediated by a nuclear localized DNA virus (the use of cytoplasmic RNA viruses in gene silencing is described below).

c. Transgenic plants

pA1.6*neo* was also used by Hayes *et al.* (1988b) to transform *N. tabacum*, leading to extrachromosomal amplification of the *neo* gene and an increase in the level of NPT (levels were half those observed in non-transformed plants infected with pA1.6*neo*B2). In this case, DNA B was not required for spread from cell to cell as DNA A was integrated in the genome of each cell. Part of the CP promoter encompassing the TATA box could be deleted from pA1.6*neo* and its replacement with the CaMV 35S promoter more than doubled NPT expression without affecting vector replication (Hayes *et al.*, 1989). The CP promoter of TGMV is therefore amenable to modification allowing higher expression levels. As in the case of ACMV, expression of the CP of TGMV is transactivated by AC2 (Sunter and Bisaro, 1991), but the regulatory elements involved in promoter activity have to this date not been mapped.

The size of the *neo* gene (0.79 kbp) was close to that of the CP gene (0.74 kbp). Hayes *et al.* (1989) next replaced it by the *gus* gene in pA1.6*gus*, and transgenic *N. tabacum* plants were produced. As previously observed by agroinoculation of leaf discs (Hanley-Bowdoin *et al.*, 1988), the increase in the size of DNA A due to the *gus* gene was well tolerated (no deletions were observed), *gus* gene copy numbers varied between 85 and 140, and were paralleled by the level of GUS activity. However, agroinoculation of the pA1.6*gus* construct onto transgenic B2 plants (see above) led to a range of deletions in the DNA A vector, down to the size of the native molecule. This selection for deletion mutants no smaller than DNA A could be governed by a mechanism of competitive replication with molecules of DNA B (Lazarowitz, 1987) of which pA1.6*gus* transgenic plants are devoid.

Mastreviruses

Viruses belonging to this genus are transmitted via leafhoppers, and most of them infect only monocotyledonous plants. Their genomes are monopartite and comprise both a large intergenic region (LIR), which is the equivalent of the CR of begomoviruses, and a small intergenic region (SIR; see *Figure 11.3*). A short complementary primer for second-strand synthesis binds within the SIR, which also comprises polyadenylation signals for transcript termination. The gene arrangement of mastreviruses is similar to that of DNA A of begomoviruses. The complementary-sense strand encodes proteins C1 and C1:C2, which is generated by processing of an intron between ORFs C1 and C2. C1:C2 has been shown to be the only viral protein necessary for replication (Dekker *et al.*, 1991; Morris *et al.*, 1992; Wright *et al.*, 1997). The virion sense strand encodes not only the CP, V2, which protects the viral genome and is required for insect transmission, but also the cell–to–cell movement protein (MP), V1 (*Figure 11.3*). Both V1 and V2 are needed for systemic spread (Lazarowitz *et al.*, 1989; Woolston *et al.*, 1989). Their replacement by foreign sequences will therefore limit the use of the resulting vectors to the infection of cells in culture. Unlike begomoviruses, mastreviruses are not mechanically transmissible and DNA clones need to be inoculated via *Agrobacterium* (agroinoculated) onto plants in order to produce an infection (Grimsley *et al.*, 1987; Woolston *et al.*, 1988).

1. *Wheat dwarf virus (WDV)*

a. Protoplasts

For the transfection of protoplasts, Matzeit *et al.* (1991) demonstrated that a tandem dimer of the WDV genome can be used in closed circular DNA, while a monomeric construct needs to be released from the bacterial plasmid as a linear fragment which will undergo circularization by cellular ligases.

ORF 2 on the virion-sense strand (V2), which encodes the CP (see *Figure 11.3*), was alternatively replaced by three reporter genes encoding NPT-II (845 bp), CAT (798 bp) and β-galactosidase (βgal; 3007 bp). In wheat protoplasts, all three replacement constructs were able to replicate and to express the encoded bacterial enzymes demonstrating that, as for DNA A of begomoviruses, no narrow limit is imposed on the size of the viral DNA for replication in suspension culture cells (Matzeit *et al.*, 1991). Expression levels of NPT-II were 20 times higher from a replication-competent vector than from a non-linearized monomeric construct, or from a frameshift mutant in C1 (both unable to replicate). NPT-II expression from replicating WDV was also obtained in non-host suspension culture cells of maize and rice (at a level half that observed in wheat protoplasts), demonstrating that replication is not the limiting factor in host specificity.

WDV expressing the *neo* gene as a replacement for the CP gene was used by Töpfer *et al.* (1989) to verify whether imbibition of dry embryos of cereals with plasmid DNA constitutes a valid method for gene transfer. NPT-II expression was 15 to 20 times higher when the *neo* gene was delivered by WDV than when expressed from the CaMV 35S promoter. This increase in NPT-II activity was dependent on the capacity of the WDV vector to replicate, and occurred only when it was cloned as a dimer but not as a monomer, therefore confirming true cellular uptake of the DNA.

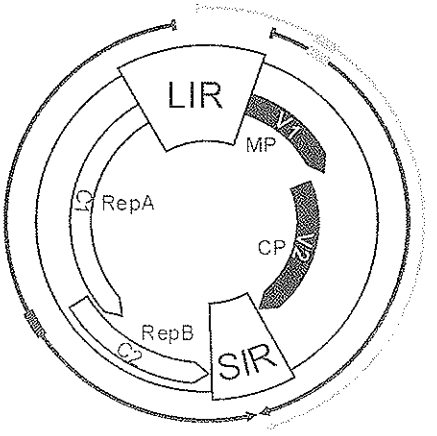

Figure 11.3. Generic genome organization of *Mastreviruses*. The single molecule of circular ssDNA (2.6–2.8 kb in size) is represented by a circle with open boxes for the large (LIR) and small intergenic region (SIR). Bi-directional transcripts (outer arrows) initiate within the LIR, terminate within the SIR and, in some cases, contain introns (shown as closed rectangles). ORFs (inner arrows) are named according to the DNA strand – viral (V) or complementary (C) sense – by which they are encoded and their function is given: movement protein (MP), coat protein (CP); RepA is a regulatory protein whilst the RepA:RepB fusion protein, translated from the spliced complementary-sense transcript, is the replication-associated protein. The transcript and introns shown in grey have been identified for MSV but not for WDV and TYDV. ORFs filled in black have been substituted by foreign sequences.

The *gus* gene was substituted for the CP gene in a series of constructs containing a partial tandem duplication of WDV sequences encompassing the LIR and the replication-associated gene. Following bombardment of plasmid-coated particles into cultured wheat cells, the level of GUS expression was found to be correlated to the relative amount of newly replicated viral DNA. Replicon size was, however, identified as the major determinant of the efficiency of accumulation: plasmids smaller than the genome of WDV were engineered by removing the GUS sequence and found to replicate to the highest levels (Suarez-Lopez and Gutierrez, 1997).

Laufs *et al.* (1990) studied the expression of transposons in several types of monocot protoplasts using WDV. The maize transposon Activator unit (Ac), which is 4579 bp long, and deletion mutants thereof down to 627 bp in size, were substituted for most of the virion-sense transcription unit, i.e. the genes for both V1 and V2 (see *Figure 11.3*). Insertion of the autonomous Ac element rapidly led to the appearance of empty WDV replicons: sequence analysis verified that this was not due to recombination but to Ac-specific excision. By contrast, non-autonomous Ac derivatives, Dissociation (Ds) elements, lacking sequences from within the 5' leader, and reaching across the ORF of the transposase down to the 3' untranslated region, could be stably replicated via WDV. Excision of these Ds elements from the genome of WDV could be rescued by an active transposase, expressed in *trans* from the CaMV 35S promoter of a co-transfecting plasmid. By demonstrating that Ds elements could only be excised from replicative-competent WDV vectors, Wirtz *et al.* (1997) confirmed that transposition is linked to replication.

Another mastrevirus, *miscanthus streak virus* (MiSV), was similarly used as a gene vector for the transposition of maize Ds elements into rice protoplasts (Sugimoto *et al.*, 1994). V1 and V2 were replaced by an artificial 1.4 kb Ds element, which was

stably maintained in the MiSV vector during replication and which could be selectively and efficiently excised by co-electroporation with a plasmid expressing the Ac-encoded transposase under the control of an enhanced CaMV 35S promoter. When the gene for hygromycin phosphotransferase (HPT) was inserted into the Ds element, integration of the resulting Ds::HPT sequence into the rice genome could be monitored by regeneration of transfected protoplasts in the presence of hygromycin. In 1/3 of the resistant calli, direct transposition of Ds::HPT excised from MiSV had occurred, as demonstrated by the presence of flanking 8 bp direct repeats, which result from target site duplication upon Ac/Ds integration. This low efficiency of transposition was interpreted in terms of limiting amounts of transposase.

b. *E. coli*–plant cell shuttle vectors

Bifunctional plasmids combining *E. coli* and WDV sequences have been developed. They simplified transfection experiments, as release of viral monomers from plasmid sequences was no longer required, and could serve as shuttle vectors between bacterial and plant cells.

Genes V1 and V2 can be deleted without affecting the ability of WDV to replicate in wheat protoplasts. Using such a deletion mutant, entire bacterial plasmids derived from pBR322 (ColE1 replicons) were inserted into the WDV genome (Kammann *et al.*, 1991). The resulting vectors were able to replicate in wheat protoplasts. Plant DNA was isolated seven days after transfection. Digestion with a restriction enzyme which only cleaves its recognition site when it is methylated in *dam+* bacteria ensured that residual input bacterial DNA was eliminated, whilst *de novo* plant-synthesized DNA was left intact. Following transformation of *E. coli* with this DNA, the bacterial colonies obtained harboured plasmid DNA, which could be used to successfully transfect fresh protoplasts. No differences in the restriction pattern of the original vector appeared at any point during these passages between bacterial and plant cells, making it a suitable tool for the study of DNA rearrangements in monocotyledonous plants.

In WDV, the genes for V1 and V2 are translated from a unique virion-sense transcript by frame-shifting (Dekker *et al.*, 1991; see *Figure 11.3*). The gene for the CP (V2) was replaced by that for NPT-II fused to the CaMV 35S terminator and followed by the sequence for the p15 origin of replication (*ori*) from an *E. coli* plasmid (Ugaki *et al.*, 1991). The resulting construct pWI-11 was able to replicate extrachromosomally in *E. coli* via *ori*, and in maize protoplasts via the replication-associated proteins encoded by the complementary-sense strand of WDV. Presence of the *npt-II* gene allowed selection for kanamycin resistance in *E. coli*, but not in maize endosperm cells because of their slow rate of growth during the culture period used for the study (9 days). The gene for GUS under the control of a CaMV 35S promoter and terminator was inserted into pWI-11 between the *npt-II* gene fusion and *ori* sequence to produce pWI-GUS. Expression levels of GUS obtained in plant cells could be correlated with active replication of the vector.

By *in vivo* labelling, vector pWI-11 was shown able to replicate in non-dividing protoplasts of maize, whilst co-transfection of pWI-11 with replication-deficient derivatives of pWI-GUS demonstrated that the replicase of WDV can act in *trans*. Up to 30 000 copies of the shuttle vector accumulated per cell. Insertion of the 3 kb GUS expression cassette brought the copy number down by a factor of twelve to 2500, and

only resulted in a ten-fold increase in GUS expression over a non-replicating control plasmid: this suggests that high level replication of the vector titrates out factors needed for gene expression (Timmermans *et al.*, 1992).

Using the same cloning strategy as for insertion of the *gus* gene, expression cassettes for the maize Lc gene, which transcriptionally activates anthocyanin biosynthetic enzymes, were cloned into vector pWI-11 (Neuhaus-Url *et al.*, 1994). Maize callus-derived protoplasts were PEG transformed with different Lc constructs, and transactivation of anthocyanin expression was assessed by counting the number of protoplasts which turned red. A correlation between replication of the vector and transactivation was found when the Lc gene was under the control of the constitutive CaMV 35S promoter, but not when using the maize phosphoenolpyruvate carboxylase (PEPC) promoter, which is mesophyll-specific and poorly transactivated in callus cells. Applications of these transactivating, self-replicating vectors for short-term cell lineage studies in maize were, however, not reported.

2. *Maize streak virus (MSV)*

It was with the purpose of delineating essential viral functions in MSV that the genes for V2, or for both V1 and V2, were first deleted and then replaced by the reporter genes *cat* and *hpt* (Lazarowitz *et al.*, 1989). Following agroinoculation of maize plants, the deletion mutants were unable to replicate. By contrast, mutation of the start codon of V1 or replacement of either the V2 (CP) gene, or of the V1 + V2 genes with foreign sequences, produced replicating MSV DNA, which remained confined to inoculated leaves, did not generate classical disease symptoms, and was not leafhopper transmissible. A polar effect of the mutation at the start of the V1 coding sequence on expression of the V2 gene was excluded because it was thought that MSV transcribes genes V1 and V2 from separate 3' co-terminal transcripts, with the V2 transcript starting in the middle of the V1 sequence (Morris-Krsinich *et al.*, 1985). Later on, it was discovered that some of the MSV V2 transcripts actually result from splicing of an intron within the V1 gene (Wright *et al.*, 1997; *Figure 11.3*). The following conclusions were drawn by Lazarowitz *et al.* (1989): i) all the information required for viral replication is comprised in the complementary-sense genes and intergenic regions; ii) both the CP and V1 are required for systemic spread, symptom development and insect transmission; and iii) there are no inherent size limitations in the absence of CP, as MSV gene substitution mutants 200 b smaller (V1 + V2 deletion mutant with CAT insert) and up to 500 b larger (V2 deletion mutant with HPT insert) than wild-type MSVcould be stably replicated. Failure of the deletion mutants to replicate was likely due to the two intergenic regions being brought into close proximity. Only CAT activity was assayed for, and could be detected at low levels in leaves inoculated with the V2 replacement mutant. V1 was later confirmed by Boulton *et al.* (1993) as the viral MP.

a. Whole plants

Given that the V1 and V2 genes are required for virus spread, their replacement could not produce systemically moving expression vectors. An alternative strategy was used by Shen and Hohn (1994). It was based on the observation that the SIR of MSV could tolerate insertion of the 405 b maize transposable element Ds1, which was

replicated along with the viral genome. However, symptoms of virus infection only appeared after Ds excision, when Ac functions were provided either endogenously by Ac+ maize lines or by co-inoculation with *Agrobacterium* containing a genomic Ac clone (Shen and Hohn, 1992). The gene for GUS (flanked by CaMV 35S promoter and *nos* terminator sequences) was substituted for the Ds1 sequence. Following agroinoculation of maize plants, symptoms were only observed in the single plant in which the *gus* gene had been deleted from the vector. In non-symptomatic plants, the number of blue spots which lined up on the veins of GUS-positive shoots was four times higher on plants agroinoculated with the MSV-GUS construct than on those which had been inoculated with a binary vector containing the *gus* gene alone. GUS expression was correlated to replication of MSV-GUS, but remained limited to leaves whose primordia were present in the coleoptilar node at the time of inoculation. The pattern of distribution for GUS spots remained unchanged when genes V1 and V2 were deleted, therefore confirming the absence of systemic movement.

Next Shen and Hohn (1995) replaced the *gus* gene with the bialophos resistance gene *bar* (under the transcriptional control of the CaMV 35S promoter and terminator) in both the intact MSV genome and in the V1 + V2 deletion mutant. As with the GUS constructs, no viral symptoms developed on maize seedlings agroinfected with the *bar* constructs. After repetitive spraying with the herbicide Basta, to which expression of the *bar* gene confers resistance, 25% of the plants displayed green sectored leaves 18–21 days after inoculation. However, all the plants which survived (5–10%) became sensitive to Basta 40 days after inoculation, confirming that reporter gene expressing-MSV had not spread systemically. Given the non-destructive nature of the Basta assay in resistant tissues, the copy number of *bar*-containing MSV vectors could be estimated: it ranged between 50 and 500 molecules per cell and was correlated with genome size, being highest for the smallest vector, i.e. that based on the V1 + V2 deletion mutant.

b. Cell cultures

Palmer *et al.* (1999) also expressed the *bar* gene from an MSV replicon, but as a replacement for genes V1 + V2 with only transcription termination signals provided by the MSV SIR. Transcription initiation of the *bar* gene was from either a 790 bp CaMV 35S 'long' promoter, or from a 430 bp CaMV 35S 'short' promoter fused to the TMV Ω leader (68 bp) and maize *Adh*I intron (550 bp); in the latter format, the size of the insert was further increased by fusion of the *E. coli gor* gene for glutathione reductase (GR) in frame with the *bar* gene (= *bargor*). Dimers of these constructs were delivered biolistically into suspension cultures of non-regenerable black Mexican sweetcorn (BMS) cells. Several months after transformation, total DNA was isolated from transformed callus lines growing on bialaphos; the presence of replicons was assessed by Southern blotting and their copy number per maize haploid genome determined as varying between 500–750 to over 1000 copies. This was linked to the detection of high levels of *bar*-specific transcripts by Northern blot, and to increased expression of *bar* gene-encoded phosphinothricin acetyl transferase (PAT) over control cell lines transformed with non-replicating plasmids. There was, however, no significant difference between the level of PAT activity obtained from replicons harbouring the 'long' 35S promoter and those presenting a 'short' 35S promoter + expression-enhancing TMV Ω and maize intron sequences. In addition, PAT activity

was reduced by fusion to the GR protein. The fact that differently sized recombinant episomes reached similar copy numbers and were structurally stable (absence of significant levels of deletion mutants) validates the use of these constructs for long-term gene amplification in transformed cell cultures.

3. *Tobacco yellow dwarf virus (TYDV)*

The need for systemic spread can be obviated by a geminivirus-based vector designed to be stably integrated into the plant genome. Transformation of monocot species being more challenging than that of dicot plants, this approach was used with TYDV, a mastrevirus which infects *N. tabacum* (Needham *et al.*, 1998). The complementary sense ORFs for C1 and C2 and the SIR were flanked by LIR sequences to allow replicational release from the T-DNA of a binary vector. The viral-sense ORFs V1 and V2, which are not essential for replication, were left out from the construct. Following *Agrobacterium*-mediated transformation, none of the tobacco plants transgenic for this construct displayed symptoms associated with TYDV infection; in about half of these plants, episomal vector molecules could be detected by Southern blot analysis of undigested genomic DNA. After incubation with *Eco*RI, which cleaves the TYDV vector once between the LIR and SIR sequences, a 1.5 kb linear fragment of the size expected for a replicating episome (following release from the T-DNA) was detected. This is in contrast to findings made with MSV, which was unable to replicate in agroinoculated maize plants when genes V1 and V2 had been deleted from a dimeric construct (see above: Lazarowitz *et al.*, 1989).

Expression of GUS from this TYDV vector was characterized following insertion of a *uidA-nos3'* cassette under the transcriptional control of the V1 gene promoter (Needham *et al.*, 1998). Despite a size of 3.9 kb, i.e. an increase of 1.3 kb over that of the wild-type TYDV genome, plant lines transgenic for this construct displayed heritable GUS expression (from the *uidA* gene). This was as a speckled pattern with the number of GUS foci proportional to the level of replication of the recombinant episome. For the latter to be released from the T-DNA and subsequently replicated, Rep (C1:C2) is required. In transgenic plants, expression of Rep is limited by the temporal and spatial specificity of its promoter, and might therefore not be able to take place in every cell, resulting in non-uniform GUS expression throughout a leaf.

A variant of the TYDV vector described above, with the gene for V2 deleted and that for V1 inactivated by frameshifting, was used to transform petunia plants (Atkinson *et al.*, 1998). Again, no viral symptoms were displayed by transformants in which episomal vector of 1.8 kb could be detected. The gene for petunia chalcone synthase A (*Chs*A), under the control of a CaMV 35S promoter and an *ocs* transcriptional terminator, was cloned into this vector. Following transformation of *Petunia hybrida* with TYDV-*Chs*A, 5.7 kb episomes could be detected in transformants, of which some produced white-spotted flowers instead of purple ones. ChsA being a key enzyme in anthocyanin production, this loss of pigmentation was due to gene silencing by a homology-dependent process termed co-suppression by Napoli *et al.* (1990). Again, a correlation between severity of the phenotype and episome copy number was found. The *Csh*A transcription level in white-spotted transgenic flower tissue, determined by nuclear run-on analysis, was unaffected when compared to that of the purple flowers of wild-type plants; however, RNA blot analysis revealed that

steady-state levels of *Chs*A mRNAs were down-regulated. This demonstrated that white spots resulted from post-transcriptional gene silencing of both the *Chs*A endogene and transgene, and that this phenomenon could be induced by multicopy nuclear episomes of a DNA virus.

RNA viruses

The majority of plant viruses have genomes that consist of one or more strands of positive-sense RNA. These viruses can grow in a wide range of hosts and, in a number of instances, reach extremely high titres. For these reasons, it is very attractive to base gene vectors on such viruses. However, the development of such vectors was initially hampered by the difficulties in manipulating an RNA, as opposed to a DNA, genome. The advent of efficient *in vitro* transcription systems dramatically changed the situation, and the construction of full-length cDNA clones of *brome mosaic virus* (BMV) from which infectious transcripts could be obtained, was reported in 1984 (Ahlquist and Janda, 1984; Ahlquist *et al.*, 1984). Subsequently, full-length cDNA clones of many other RNA plant viruses were produced. After transcription *in vitro* with the appropriate RNA polymerase, many of these produced RNAs which were infectious on plants, thus opening the way to the development of several RNA viruses as vectors. More recently, it has been shown that, by cloning the virus-specific sequence downstream of a CaMV promoter, it is possible to produce cDNA clones which are directly infectious, either when applied as purified DNA or when introduced by 'agroinoculation'.

In addition to the technical difficulties in developing RNA virus-based vectors, there was some concern expressed initially as to whether such vectors would be very useful, due to the high rate of mutation of RNA viruses (Van Vloten-Doting *et al.*, 1985). This view was disputed at the time (Siegel, 1985), and the genetic stability of RNA viruses has actually proved to be quite adequate. As a result, RNA viruses from numerous families have been developed as vectors for a variety of uses. Many of the initially engineered viruses underwent gene replacement. In this approach, a sequence encoding a function not essential for RNA replication was replaced by the sequence of interest. This produced vector systems, which could multiply in protoplasts, but were generally debilitated for the infection of whole plants. Though not very suitable for the high-level expression of foreign proteins, such systems have been useful in the elucidation of the functions of various viral genes. More recently, autonomously-replicating vector systems have been based either on gene addition or on expression of the foreign gene from a satellite or defective RNA whose replication is dependent on a wild-type helper virus. Lastly, there have been several attempts to develop complementation systems in which essential viral functions are provided by transgenes. These four types of systems will be discussed in different subsections.

GENE REPLACEMENT SYSTEMS

Bromoviruses

Members of the family *Bromoviridae* have a tripartite genome, which is encapsidated in isometric particles. RNA 1 and RNA 2 are both monocistronic and encode proteins

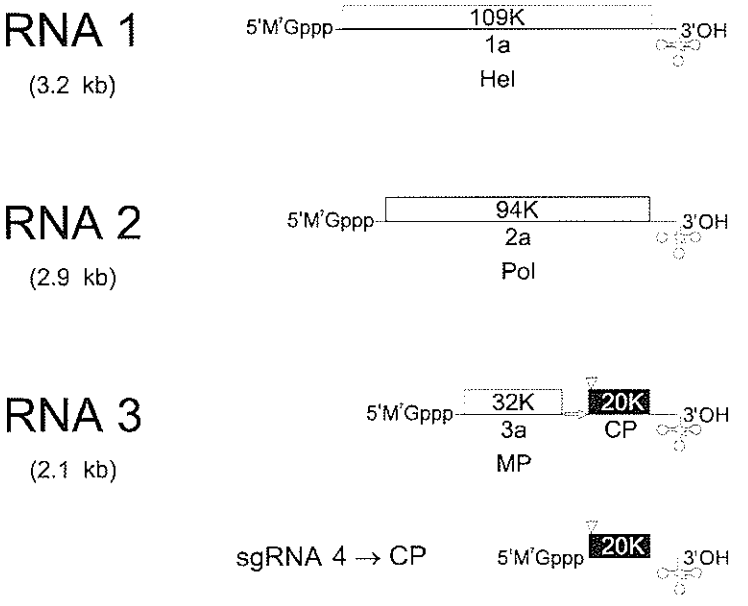

Figure 11.4. Genome organization of *brome mosaic virus* (BMV). RNAs 1, 2 and 3 make up the viral genome, whilst sgRNA 4 is the messenger for the coat protein. The 3' termini of the RNAs are aminoacylatable by Tyr. ORFs are represented by open bars. Below each bar, the common name of the gene product and/or its function is given: helicase (Hel), polymerase (Pol), movement protein (MP), coat protein (CP). The ORF for the CP which has been replaced by foreign sequences is shown as a filled bar. The open arrow represents the sg promoter for the CP. The open triangle indicates the position of the start codon for the truncated CP produced by strain ATCC6 (see text).

involved in the replication of the viral RNAs. RNA 3 is bicistronic and encodes the MP at the 5' proximal end, followed by the CP, the latter being expressed from a subgenomic (sg) mRNA termed RNA 4 (*Figure 11.4*). The type member of the genus *Bromovirus*, BMV was the first RNA plant virus to be developed as a gene vector (French *et al.*, 1986). When the region of RNA 3 encoding the viral CP was replaced with a sequence encoding CAT, the modified RNA 3 was still able to replicate in barley protoplasts, albeit to a lower level than wild-type RNA 3. CAT activity could be detected in extracts from infected cells, providing the first evidence that a heterologous gene could be expressed from a modified RNA virus. No attempt was made to infect whole plants with the construct, since deletion of the CP abolishes the ability of the virus to move in an infected plant.

A similar approach to using BMV as a vector was subsequently adopted by Mori *et al.* (1993a) to express interferon γ (IFN-γ). In this case, strain ATCC6 of BMV, which produces a long and a short form of the CP, was used. The two forms of CP derive from the use of alternative codons for initiation of translation (*Figure 11.4*). By replacing the coding region of the short form of the CP with the gene for IFN-γ, a modified RNA 3 was created, which replicated well in tobacco protoplasts in the presence of RNA 1 and RNA 2. The amount of IFN-γ produced was estimated to be about 0.5% of total protein extracted from protoplasts. Several forms of the protein were detected, and these probably result from a number of post-translational modifications, including glycosylation. The specific activity of the plant cell-expressed

IFN-γ was estimated to be approximately 1% of that extracted from animal cells. Once again, no attempt to infect whole plants was reported.

To date, vectors based on bromoviruses have been used exclusively to investigate the functions of genes from other members of the genus, or more distantly related plant viruses. For example, a BMV RNA 3 construct in which the native CP was replaced with the CP and origin of assembly (OAS) of the tobamovirus, *sunn hemp mosaic virus* (SHMV), was able to replicate in protoplasts in the presence of RNAs 1 and 2 (Sacher *et al.*, 1988). Furthermore, the hybrid RNA 3 was encapsidated into rod-shaped particles by the SHMV CP. When the SHMV OAS was added to RNA 1 and RNA 2, as well as RNA 3, all three modified BMV RNAs were encapsidated by the SHMV CP. Another example was the replacement of the MP on RNA 3 of the bromovirus, *cowpea chlorotic mottle virus* (CCMV), by the SHMV MP (De Jong and Ahlquist, 1992). The resulting hybrid virus was competent for systemic infection of cowpea, demonstrating that the SHMV MP can fully substitute for the native CCMV MP, despite the fact that the proteins are derived from different virus families.

Tobamoviruses

The type member of the tobamovirus group, *tobacco mosaic virus* (TMV), has a number of properties which potentially made it ideal for development as a gene vector. It is one of the best-studied plant viruses, it grows to extremely high titres in susceptible plants and, since it has rod-shaped particles, there are no constraints on the size of RNA which can be packaged. The genome of TMV consists of a single molecule of RNA of 6.4 kb, which contains separate ORFs for the polymerase functions, the viral MP and CP. The latter two proteins are synthesized from sgRNAs (see top section of *Figure 11.8*). The first attempt to use TMV as a vector involved substitution of the CP ORF by a sequence encoding CAT (Takamatsu *et al.*, 1987). The construct was capable of causing local lesions when inoculated onto tobacco, but the infection was unable to spread systemically. CAT expression could be detected in extracts from the inoculated leaves. In view of the problems of spread in the absence of CP, much of the subsequent work on the development of TMV-based vectors concentrated on the gene addition approach (see below). However, Spitsin *et al.* (1999) made use of the fact that TMV constructs without CP are deficient in long-distance movement to demonstrate that expression of the CP from *alfalfa mosaic virus* (AlMV) in such constructs could not only complement long-distance movement, but also extend the host-range of TMV.

Hordeiviruses

Barley stripe mosaic virus (BSMV) has a genome consisting of 3 RNAs (α, β and γ) encapsidated in rod-shaped virions. RNAs α and γ encode proteins essential for replication, while RNA β contains four open reading frames, termed βa, βb, βc and βd, from the 5' end of the RNA. ORF βa encodes the viral CP, while ORFs βb, βc and βd are overlapping and together encode the 'triple gene block' (TGB) proteins involved in viral movement within an infected plant (*Figure 11.5*). When ORF βb was replaced with the sequence encoding LUC and the modified RNA β transfected into either maize or tobacco protoplasts in the presence of RNAs α and γ, expression of LUC

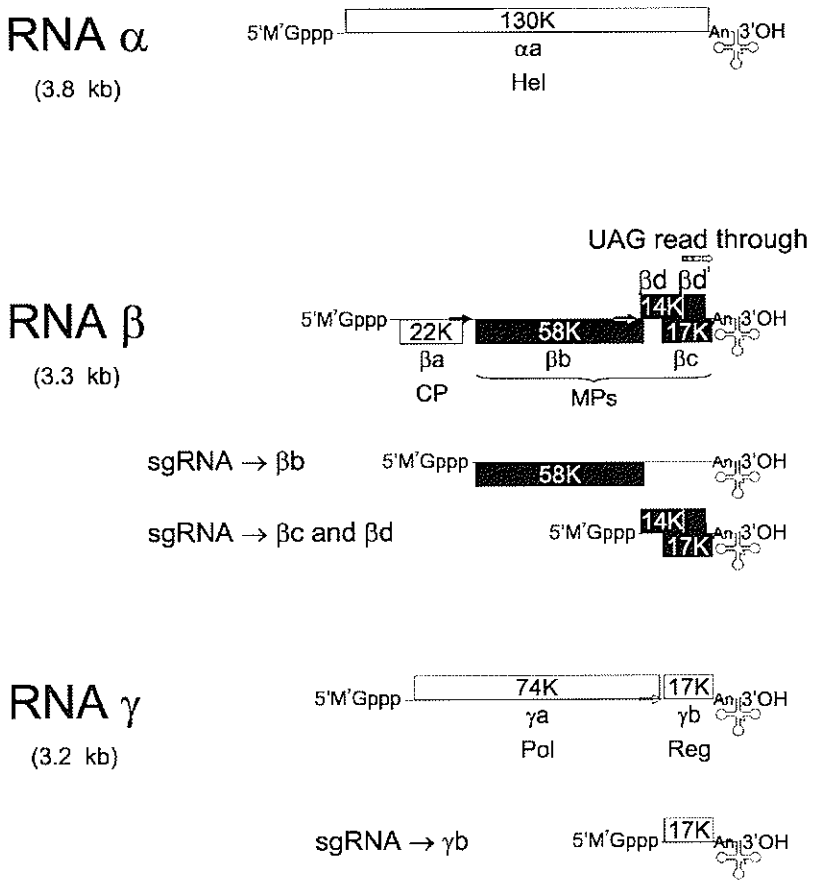

Figure 11.5. Genome organization of *barley stripe mosaic virus* (BSMV). RNAs α, β and γ are the genomic RNAs. Proteins βb and γb are expressed from individual sgRNAs, while βc is expressed from the same sgRNA as βd via a leaky scanning mechanism; read-through of βd results in βd' of unknown function. The 3' termini of all RNAs present a short poly(A) tract, followed by a $tRNA^{Tyr}$-like structure. ORFs are represented by open bars. The TGB proteins (βb, βc and βd), which have been successfully replaced by foreign sequences, are shown as filled bars. Below each bar, the common name of the gene product and its function is given: helicase (Hel), coat protein (CP), movement proteins (MPs), polymerase (Pol), expression regulator for genes encoded by RNA β (Reg). The positions of promoters for the production of sgRNAs are indicated by arrows.

could be detected (Joshi *et al.*, 1990). The levels found were between 20- and 123-fold higher than those detected when cells were transfected with the modified RNA β alone. These data are consistent with the fact that the RNA β-LUC was capable of being replicated by the proteins encoded by RNAs α and γ.

Building on the demonstration that BSMV RNA β can be modified to express heterologous sequences, a vector based on this RNA has been used to examine the function of the MPs of several plant viruses. In this approach, putative MPs were inserted in the BSMV RNA β in place of some or all of the native TGB proteins, and the ability of the heterologous MP to complement movement in tobacco was assessed. Complementation was found with the MPs of tobamo- and dianthoviruses (Solovyev

et al., 1996, 1997) and, to a lesser extent, the HSP70-like protein from a closterovirus (Agranovsky *et al.*, 1998).

Tombusviruses

The genomes of this group consist of a single molecule of RNA of around 4 kb, which is encapsidated in isometric particles. The CP is not essential for infection of certain *Nicotiana* species, though its presence does enhance systemic movement. This fact was utilized to produce constructs derived from the type member of the genus *Tombusvirus*, *tomato bushy stunt virus* (TBSV), in which most of the region encoding the coat protein was replaced with that encoding either GUS or CAT (Scholthof *et al.*, 1993; *Figure 11.6*). Both constructs produced high levels of the appropriate protein in inoculated leaves, though the level of RNA accumulation was substantially reduced in the case of the GUS-containing virus. A refined version of the TBSV vector was subsequently produced in which the CP was replaced with a polylinker (Scholthof, 1999). This, coupled with improvements to the infection process, permitted the facile expression of heterologous sequences in the inoculated leaves of plants.

TBSV has recently been used to express the nucleocapsid protein p24 from *human immunodeficiency virus 1* (HIV-1) as a fusion with the 5' terminal portion of the CP gene (Zhang *et al.*, 2000). The modified TBSV RNA was shown to be capable of replicating in both protoplasts and in the inoculated leaves of whole plants. Accumulation of the CP-p24 fusion protein could be detected in inoculated leaves.

Similar experiments with *turnip crinkle virus* (TCV), a member of the genus *Carmovirus*, showed that when the CP was replaced with GFP, the modified virus could still spread from cell to cell in *Arabidopsis*, but systemic movement was abolished (Cohen *et al.*, 2000).

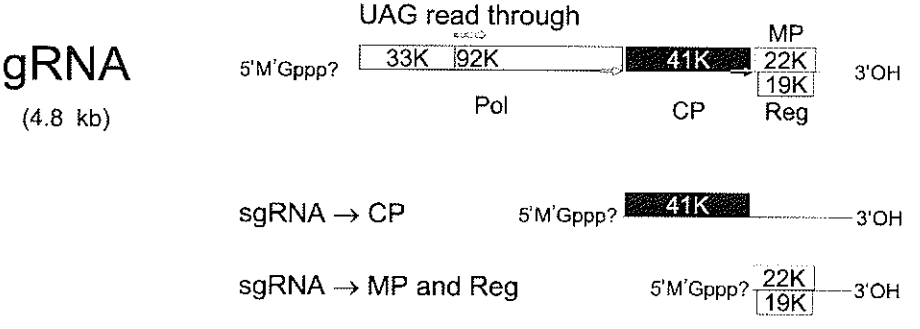

Figure 11.6. Genome organization of *tomato bushy stunt virus* (TBSV). Capping of the 5' end of the genome has been hypothesized while the 3' end has been shown to be devoid both of a poly(A) tract and of a tRNA-like structure. The products of the 5' terminal ORF and its read-through are polymerases (Pol). The coat protein (CP) is expressed from its own sgRNA. The movement protein (MP), and the protein which regulates the fidelity of viral replication (Reg), are translated from the same sgRNA by ribosome scanning. The ORF for the CP, represented by a filled bar, has been replaced by foreign genes.

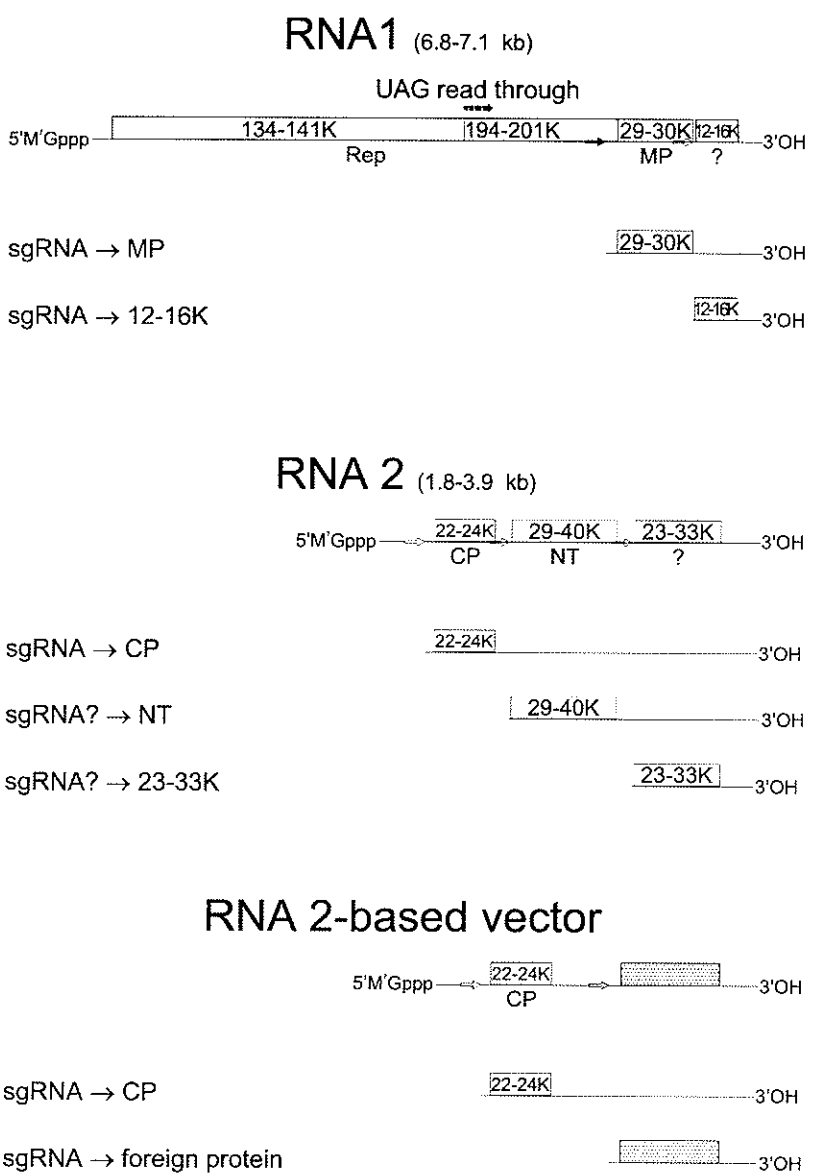

Figure 11.7. Generic genome organization of *Tobraviruses* and derived gene substitution vectors. Genomic RNAs 1 and 2 are capped at their 5' terminus but do not have a poly(A) tract at their 3' terminus. Translation of the first ORF of RNA 1 occurs directly from the genomic RNA and by a process of read-through of an opal stop codon produces two proteins involved in replication (Rep). Expression of the second and third ORFs of RNA 1 is via sgRNAs, for which the promoters are represented by a black arrow for the movement protein (MP) and an open arrow for the 12–14K protein. Only in the case of PEBV has a role in seed transmission been identified for the latter. Translation of the first ORF of RNA 2, which encodes the coat protein (CP), requires synthesis of a sgRNA, for which the promoter is indicated by a white arrow. The number of additional downstream ORFs on RNA 2 varies between 3 for PEBV strain TpA56, 2 for TRV strain PpK20, and none for PepRSV; translation of these ORFs via sg promoters and involvement of their products in nematode transmission (NT) have only been partially demonstrated. In RNA 2-based tobravirus vectors, existing ORFs downstream of the CP were deleted and replaced by a foreign gene (stipled bar) placed under the control of the CP sg promoter of another tobravirus isolate (striped arrow).

Tobraviruses

Tobraviruses are bipartite rod-shaped viruses. The larger RNA 1 encodes proteins involved in replication and movement of the virus while RNA 2 encodes the CP and a variable number of additional non-essential ORFs, some of which are involved in nematode transmission (*Figure 11.7*). It has been shown in the case of each of the three members of the genus *Tobravirus, tobacco rattle virus* (TRV), *pea early browning virus* (PEBV) and *pepper ringspot virus* (PepRSV), that it is possible to replace these non-essential ORFs with GFP (under the control of a duplicated CP sg promoter) without affecting the ability of the viruses to multiply and spread in plants (MacFarlane and Popovich, 2000; bottom section of *Figure 11.7*). All the vectors were able to express GFP efficiently in both leaves and roots, the latter being a property particularly associated with tobraviruses. The ability of the TRV vector to express a protein which might be useful in protecting plants against nematodes was tested by inserting the sequence encoding a lectin (GNA) from snowdrop. Significant levels of the lectin could be detected in the roots of inoculated *N. benthamiana* plants.

GENE ADDITION

In these systems, no viral sequences are removed during vector construction and a heterologous sequence is introduced as an addition to the viral genome. The viability of such systems relies critically on there being a reasonable amount of flexibility in the size of the viral RNAs which can be replicated and packaged. In regard to the latter consideration, viruses with rod-shaped particles should theoretically be more amenable than those with isometric capsids to development as gene addition vectors.

In theory, any viral RNA into which a heterologous sequence has been inserted could be considered a gene addition 'vector'. However, this definition would include the many cases where a marker gene, commonly GFP or GUS, has been inserted, either as a separate ORF or as a fusion to a particular viral protein, in order to facilitate examination of virus movement or protein localization within an infected plant. This method of protein localization is so broadly used that to list all the instances where it has been utilized to elucidate the properties and functions of RNA plant viruses is inappropriate to this review. The readers are referred to reviews on a particular virus genus if they wish to obtain information on this type of work. The only instances where work on the addition of marker genes will be described are those where it was carried out for the purposes of vector development.

Tobamoviruses

To overcome the problems of limited spread with TMV vectors in which the CP is replaced by a foreign gene, Dawson *et al.* (1989) inserted a copy of the CAT gene, complete with its own TMV CP sg promoter, between the MP and CP genes. The construct replicated well, CAT activity could be detected and virions of an appropriately increased length were produced (350 as opposed to 300 nm). However, during systemic infection, the insert was precisely deleted and wild-type RNA accumulated. This was undoubtedly a consequence of homologous recombination between the two copies of CP sg promoter. To address this problem, a vector was developed in which a cartridge consisting of the CP sg promoter and CP from the related *Tobamovirus,*

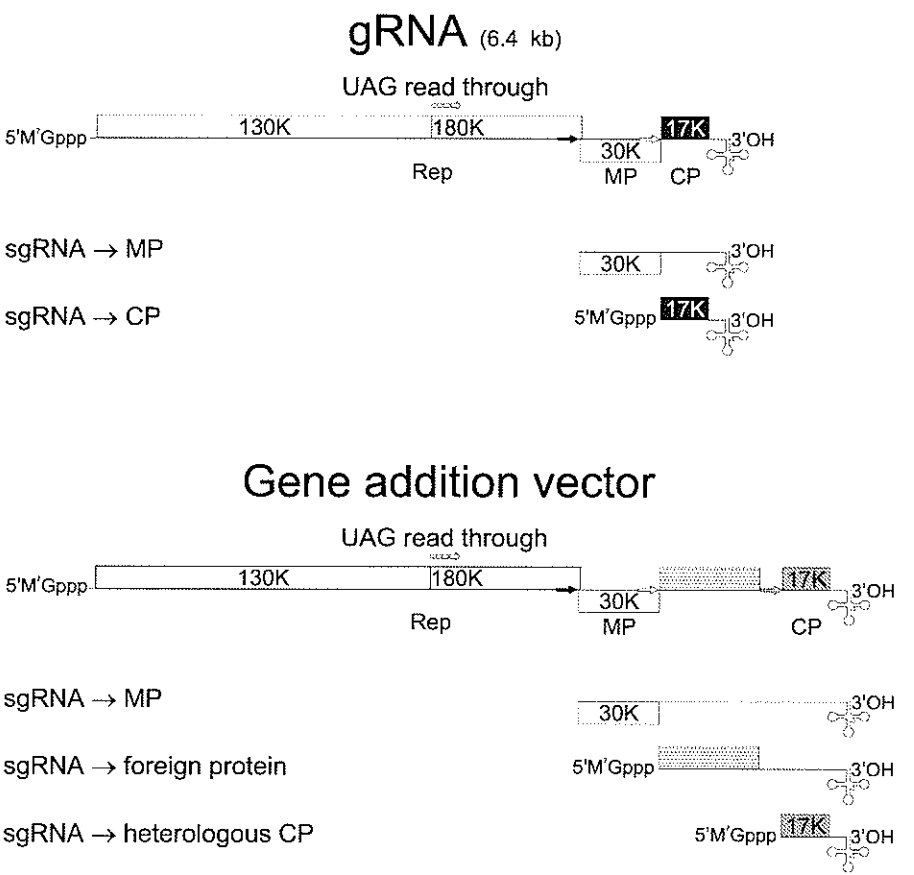

Figure 11.8. Genome organization of *tobacco mosaic virus* (TMV) and derived gene addition vector. The 3' untranslated region folds into a $tRNA^{His}$-like structure. Both the 130K protein and the 180K read-through product of the 5' proximal ORF are involved in replication (Rep). The movement protein (MP) and the coat protein (CP) are translated from separate sgRNAs synthesized from promoters symbolized respectively by a black and a white arrow. In theTMV-based gene replacement vector, a foreign gene is substituted for the ORF of the CP, shown as a filled bar in the upper section of the diagram. In the TMV-based gene addition vector, the foreign sequence (stipled bar) is translated from a sgRNA synthesized from the promoter (white arrow) for the homologous CP, the coding sequence for which has been deleted. A heterologous strain of TMV provides sequence for a CP (striped bar) and its associated sg promoter (striped arrow).

odontoglossum ringspot virus (ORSV; Donson *et al.*, 1991), was inserted into the TMV genome in place of the TMV CP coding region. Expression of a foreign sequence was driven by the native TMV CP sg promoter (see lower section of *Figure 11.8*). Though the ORSV CP sg promoter has only 45% nucleotide sequence identity with that of TMV, it is still active in a TMV background. Likewise, the ORSV CP was able to encapsidate the TMV RNA. The relatively low sequence identity between the CP sg promoters of TMV and ORSV effectively abolished homologous recombination and allowed the stable systemic expression of two bacterial genes (DHFR and NPT-II) in *N. benthamiana*. Detailed analyses of the progeny showed that there was a surprisingly low level of genetic drift within the inserted sequences on serial passaging (Kearney *et al.*, 1993). The detailed requirements for optimal gene expres-

sion with tobamoviral vectors have recently been investigated, allowing a variety of constructs with differing properties to be produced (Shivprasad *et al.*, 1999).

The success in developing genetically stable TMV vectors based on the strategy of using duplicated non-homologous sg promoters has led to such vectors being used to express valuable proteins in plants. The first example of this was the high-level expression (2% of soluble proteins) of a eukaryotic ribosome-inactivating protein (RIP), α-trichosanthin, in *N. benthamiana* (Kumagai *et al.*, 1993).

Subsequently, it has proved possible to use the vectors to produce functional antibodies in plants. These can be either single-chain variable fragments (ScFvs; McCormick *et al.*, 1999) or full-length monoclonal antibodies (Verch *et al.*, 1998). In the latter case, the heavy and light chains of the antibody were separately inserted into a TMV vector and the two constructs used to co-inoculate *N. benthamiana*. Assembly of the separately expressed chains in plants could be detected.

Expression of a rice α-amylase gene from a TMV vector has demonstrated that it is possible to synthesize glycosylated proteins in plants using viral vectors (Kumagai *et al.*, 2000). Analysis of the purified protein revealed that the plant-expressed α-amylase is less heavily glycosylated than when it is expressed in yeast.

In a related approach, Yusibov *et al.* (1997) used a TMV vector with a duplicated CP sg promoter to express modified versions of the CP from AlMV in tobacco. Alterations to the AlMV CP involved the insertion of antigenic sequences from *rabies virus* (RABV) and HIV-1. In infected leaf tissue, the modified AlMV CP subunits assembled into ellipsoid particles, which expressed mutiple copies of the antigenic insert. When purified and injected into mice, these particles elicited the production of neutralizing antibodies. It was subsequently shown that mice immunized with the RABV construct were protected against a normally lethal challenge with the virus (Modelska *et al.*, 1998).

TMV has also been used to produce allergens for diagnostic purposes and possible therapy. Krebitz *et al.* (2000) expressed a major birch pollen antigen (Bet v 1) in *N. benthamiana* and demonstrated that the B cell epitopes from natural Bet v 1 were preserved in the plant-expressed protein. Mice immunized with crude leaf extracts from *N. benthamiana* expressing Bet v 1 generated immunological responses comparable to those induced by the protein expressed in *E. coli* or extracted from birch pollen.

In addition to expressing potentially valuable foreign proteins, TMV-based vectors have been used to express sequences which can perturb plant physiology. One of the first examples of this application was the expression of sequences from the genes for phytoene synthase (PSY) or phytoene desaturase (PDS) in tomato (Kumagai *et al.*, 1995). Both enzymes are part of the carotenoid biosynthetic pathway. A modified TMV vector, in which a cartridge containing the sequence for the CP and its sg promoter from *tomato mosaic virus* (ToMV) replaced that from ORSV, was used to drive expression. Leaves of plants expressing a full-length copy of PSY developed a bright orange colour due to the overexpression of the enzyme. By contrast, leaves of plants infected with the PDS construct became white. This was found to be due to photo-bleaching, caused by suppression of carotenoid synthesis. Thus, it appeared that the presence of high levels of virus-derived PDS sequences in a cell suppresses the synthesis of endogenous PDS. This phenomenon has now been found to be a common one, which has been termed 'virus-induced gene silencing' or VIGS (Ruiz

et al., 1998). Another example of the use of TMV-based gene addition systems to alter plant metabolism was the expression in plants of *Bax*, a cell death-promoting member of the Bcl-2 family of animal proteins (Lacomme and Santa Cruz, 1999). The experiments enabled the identification of those domains of Bax responsible for cell death and provided information about its probable mode of action.

A modified version of the 'duplicated sg promoter' approach has been used to develop a functional screening method to isolate genes involved in the hypersensitive response (HR) pathway (Karrer *et al.*, 1998). A TMV-based vector was created in which sequences could be inserted downstream of the native CP sg promoter, CP synthesis being under the control of the CP sg promoter from the tobamovirus, *tobacco mild green mosaic virus* (TMGMV). A cDNA library from tobacco leaves undergoing HR was cloned into this vector, infectious transcripts were generated *in vitro* and used to inoculate a variety of tobacco which does not display HR. About 0.1% of the resulting infection sites resembled lesions similar to those seen on plants undergoing HR. Sequence analysis of the inserts contained in the recombinants producing these lesions allowed the identification of proteins possibly involved in HR.

Potexviruses

The type member of the genus *Potexvirus*, PVX has been developed into a number of highly successful gene addition vectors. The genome of the virus consists of a single molecule of RNA of 6.4 kb encapsidated in flexuous rod-shaped particles. The viral RNA encodes, in order 5' to 3', the viral replicase, TGB proteins involved in viral movement, and the CP (top section of *Figure 11.9*). When a copy of the *gus* gene coupled to a duplicated copy of the CP sg promoter was inserted into PVX RNA, the resulting construct was able to infect *N. benthamiana* (Chapman *et al.*, 1992; middle section of *Figure 11.9*). The modified virus was able to move systemically and high levels of GUS could be detected. Similar results were subsequently obtained with PVX expressing GFP (Baulcombe *et al.*, 1995). Though some loss of the inserted sequence was found, presumably as a result of homologous recombination across the duplicated CP sg promoter, the vector system has proved stable enough for many applications which require only transient expression. Examples of some of these applications are given below. The fact that the list is not exhaustive testifies to the success of vectors based on PVX with an sg promoter duplication.

1. *Expression of valuable proteins in plants*

Because of their genetic instability, PVX vectors with a duplicated sg promoter have not been widely used as a method for the production of large amounts of valuable proteins in plants. However, they have proved useful in transient studies on gene constructs prior to stable genetic transformation. Examples of this include the expression of ScFv antibodies against proteins from either *potato virus V* (PVV; Hendy *et al.*, 1999), *tomato spotted wilt virus* (TSWV; Franconi *et al.*, 1999) or against granule-bound starch synthase I (Ziegler *et al.*, 2000). These experiments also showed that it is possible to direct PVX-expressed proteins to the secretory pathway. Expression of an unfused version of the major capsid protein, VP6, from a murine *rotavirus* led to

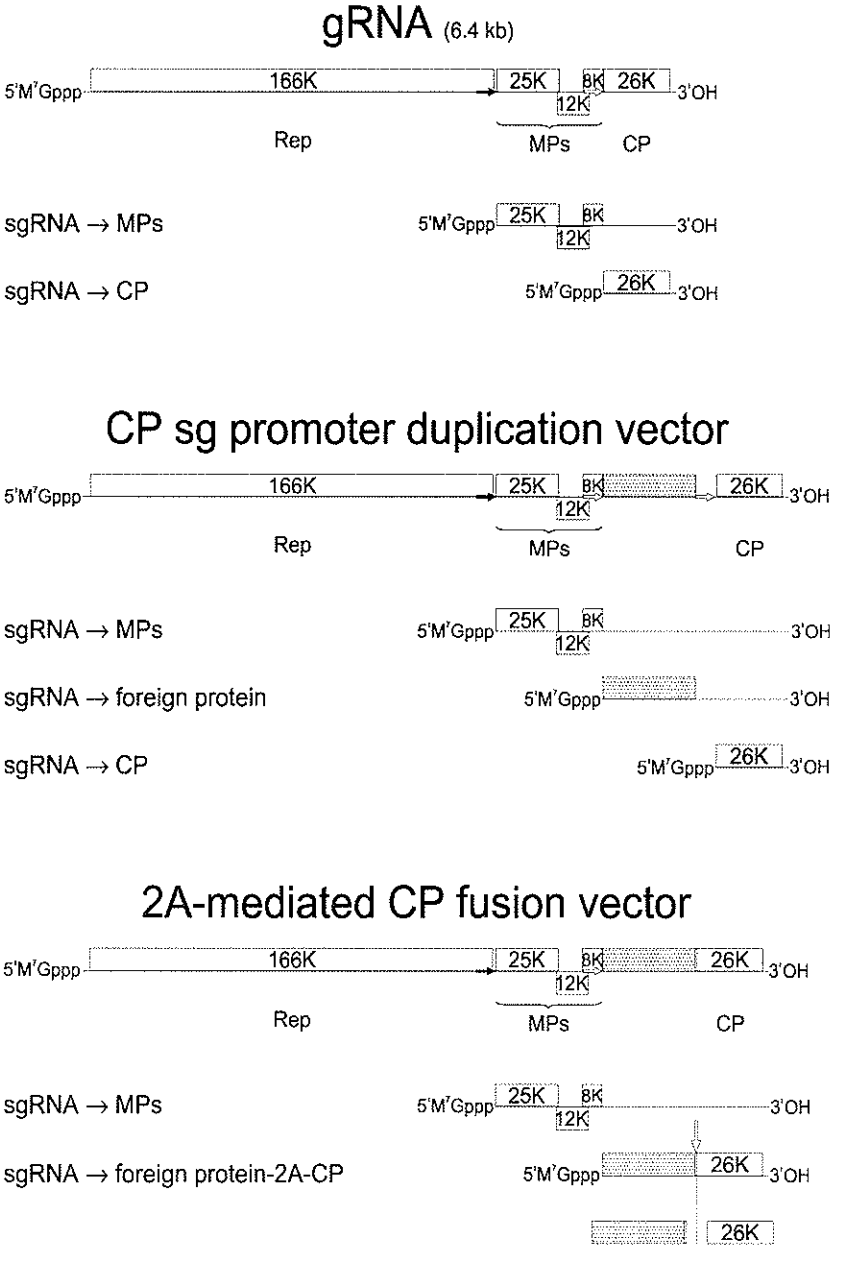

Figure 11.9. Genome organization of *potato virus X* (PVX) and derived gene addition vectors. The RNA polymerase (Rep) is translated directly from the genomic RNA, while the triple gene block movement proteins (MPs) and the coat protein (CP) are expressed from appropriate sgRNAs, the promoters for which are represented by a black and white arrow respectively. In one PVX-based vector, the sg promoter for the CP is duplicated and directs the synthesis of an additional sgRNA from which a foreign gene (stipled bar) can be expressed. In a second type of PVX-based vector, the foreign sequence (stipled bar) is fused to the N-terminus of the CP via a FMDV 2A-derived peptide, which mediates a co-translational cleavage between the foreign protein and the CP (thick vertical white arrow); efficiency of this cleavage is variable and leads to synthesis of foreign protein, some in a free state and some fused to the CP.

the production of VP6 which retained its ability to form trimers (O'Brien *et al.*, 2000). However, the VP6 protein tended to assemble into paracrystalline sheets and tubes rather than virus-like particles (VLPs). This behaviour could, however, be alleviated by expressing VP6 as a fusion to the PVX CP (see below).

As well as being used to produce proteins of immunological interest, PVX with a duplicated CP promoter has recently been used to produce an antimicrobial defensin in *N. benthamiana* (Saitoh *et al.*, 2001). The sequence of the 51 amino acid polypeptide, WT-1, from *Wisabia japonica* had its natural 29 amino acid leader peptide fused to its N-terminus and 6 histidines linked to its C-terminus to aid purification. Though plants expressing WT-1 were not protected from fungal attack, purified WT-1, obtained at a rate of 40 μg per 100 g leaf tissue, did show considerable anti-fungal activity.

2. *Studies on the functions of genes from other plant viruses*

There are a number of examples in which all or part of an ORF from another plant virus has been expressed from a PVX vector in order to obtain information as to its function. Scholthof *et al.* (1995) used PVX to express two nested ORFs (p19 and p22) from TBSV in a variety of hosts in order to gain insight into the functions of these proteins. The results indicated that both p19 and p22 are important symptom determinants. A similar approach was used to determine the role of the CP of *pepper mild mottle virus* (PMMoV) in eliciting a hypersensitive response to infection (Gilardi *et al.*, 1998). The function of the *cucumber mosaic virus* (CMV) 3a protein in virus movement was investigated by expressing the 3a ORF fused to GFP from PVX. The fluorescently labelled CMV 3a protein was then localized within infected cells (Blackman *et al.*, 1998). There are several examples in which the functions of the helper component-proteinase (HC-Pro) from potyviruses have been investigated (Sasaya *et al.*, 2000; Sonoda *et al.*, 2000).

3. *Analysis of sequences required for cross-protection*

Cross-protection is a phenomenon in which infection of a plant with a mild strain of a virus can provide protection against subsequent challenge with a severe strain. The viral CP, or its coding sequence, had previously been implicated in the phenomenon. To further investigate the basis of cross-protection, Culver (1996) expressed the CP from TMV using a PVX vector and then challenged the plants with TMV. The results showed that both the CP and its mRNA are involved but that the effect of the protein is dominant. The same approach was subsequently used to further analyse the CP interactions involved in cross-protection (Lu *et al.*, 1998). Insertion of portions of the replicase gene from TMV into PVX has also been used to investigate the relative contributions of RNA- and protein-mediated mechanisms in the phenomenon of replicase-mediated resistance (Goregacker *et al.*, 2000).

4. *Alterations to plant physiology*

A number of genes which alter the properties of a plant have been expressed using PVX. The first example of this was the expression of a functional fungal avirulence

gene, *avr9*, from a PVX vector (Hammond-Kosack *et al.*, 1995). Expression of the 28 amino acid Avr9 peptide during an infection of tomato plants with *Cladosporium fulvum* results in a necrotic response if the plants possess the *Cf-9* resistance gene. When near-isogenic lines of tomato with and without *Cf-9* were inoculated with PVX expressing Avr9, only those containing *Cf-9* gave the typical necrotic response. This approach was subsequently used extensively to examine the interactions between avirulence proteins and their cognate resistance genes (De Wit *et al.*, 1997; Joosten *et al.*, 1997; Koomann-Gersmann *et al.*, 1997, 1998; Lauge *et al.*, 2000).

PVX has been used to express a gene that confers plants susceptibility to the insecticide fenthion (Rommens *et al.*, 1995). Expression of a mutant form of fenthion identified a domain essential for conferring susceptibility. A PVX construct expressing the virulence factor ECP2 from *Cladosporium fulvum* was instrumental in identifying lines of tomato which give a hypersensitive reaction in response to ECP2 (Lauge *et al.*, 1998).

A PVX-based construct has also been utilized to influence host gene transcription. In this case, a transcription activator, *Myb305*, was expressed in tobacco and shown to be able to activate a promoter for the PAL2 gene which encodes phenylalanine ammonia lyase (Sablowski *et al.*, 1995).

5. *Gene silencing*

PVX-based vectors have probably achieved their most widespread use in the study of post-transcriptional gene silencing (PTGS) due to their ability to stimulate VIGS. The capability of PVX constructs to induce VIGS was first demonstrated by English *et al.* (1996) and Ruiz *et al.* (1998), who showed that, in order for a virus-derived sequence to silence a nuclear gene, homology between the sequences was required. Since that time, PVX has been the vector of choice for probing the mechanism for VIGS and the related phenomenon of RNA-mediated resistance to virus infection (see, for example, Sijen *et al.*, 1996; English and Baulcombe, 1997; Sonoda and Nishiguchi, 2000; Van den Boogaart *et al.*, 2001). PVX has also been instrumental in identifying those viral sequences (suppressors) which can overcome PTGS (Brigneti *et al.*, 1998).

Because of its ability to induce VIGS, expression of gene sequences from PVX has been used to probe the functions of plant genes by effectively creating gene 'knock-outs'. An example of this is cloning of a cDNA fragment of a putative cellulose synthase gene into PVX (Burton *et al.*, 2000). When inoculated on to *N. benthamiana*, the construct silenced the homologous nuclear gene, resulting in the plants developing a dwarf phenotype. The cellulose content of the infected leaves decreased by 25%, leading the authors to conclude that the sequence inserted into the PVX vector was, indeed, derived from a cellulose synthase gene. The potential of this technology is tremendous and has recently been reviewed by Baulcombe (1999).

As described above, the one major weakness of PVX vectors containing duplicated CP sg promoters is their propensity to lose the inserted sequence through homologous recombination. Unfortunately, unlike the tobamovirus situation, there are no species of potexviruses whose CP sg promoters are active in a PVX background. Therefore, an alternative approach was developed to overcome the problem of recombination. In this, the foreign gene, initially GFP, was fused to the N-terminus of the CP gene via

a sequence encoding the 2A catalytic peptide from *foot-and-mouth disease virus* (FMDV; Santa Cruz *et al.*, 1996; bottom section of *Figure 11.9*). The 2A sequence promotes cleavage between the foreign gene insert and the CP. However, the cleavage is not 100% efficient, resulting in some CP subunits still bearing GFP. These were still capable of being integrated into virus capsids, causing the resulting virions to fluoresce green under ultraviolet light. Using this approach, it is possible, with the same construct, to produce a protein of interest in both a free (unfused) state where cleavage by 2A has occurred and as a CP fusion where it is incorporated in PVX particles. The functionality of a foreign protein when incorporated into virions is illustrated by the fact that GFP still fluoresced and that a ScFv expressed as a CP fusion could still bind to its antigen, the herbicide diuron (Smolenska *et al.*, 1998). When the *rotavirus* VP6 sequence was fused to PVX CP via a 2A sequence, the uncleaved VP6-2A-CP assembled into PVX virions, while the VP6-2A cleavage product formed typical VP6 VLPs (O'Brien *et al.*, 2000).

Potyviruses

This, the largest family of RNA plant viruses, has both mono- and bipartite members. Members of the genus *Potyvirus,* which consists of several hundreds of species, have monopartite genomes, which are encapsidated in flexuous rod-shaped particles. The genomic RNA contains a single ORF which encodes a polyprotein. This is self-processed by proteinase domains within it (P1, HC-Pro and NIa) to produce the mature viral proteins (top section of *Figure 11.10*). The first example of a successfully engineered potyvirus was the insertion of the GUS sequence between P1 and HC-Pro of *tobacco etch virus* (TEV; Dolja *et al.*, 1992). The action of the two flanking proteinases ensured that a GUS–HC-Pro fusion protein was released from the polyprotein. The virus was able to spread systemically in plants and accumulated to wild-type levels. Though the insert was retained during rapid serial passaging, deletion of the GUS sequence occurred if the construct was allowed to infect plants for a prolonged period (up to one month). Analysis of the deletions suggested that these occurred through a mechanism involving non-homologous recombination (Dolja *et al.*, 1993). Despite this instability, TEV tagged with GUS has proved useful in monitoring virus movement within a plant (e.g. Valkonen and Somersalo, 1996; Mahajan *et al.*, 1998). A TEV-based vector has also been used to express proteins from the closterovirus, *beet yellows virus* (BYV; Dolja *et al.*, 1997, 1998). As previously, the sequences were expressed between P1 and HC-Pro. The constructs were used to infect tobacco, and histidine-tagged closterovirus proteins could be purified from infected tissue by chromatography on Ni^{2+}-NTA agarose.

In order to develop a potyvirus vector which could potentially infect perennial plant species, Guo *et al.* (1998) inserted GUS between P1 and HC-Pro of *plum pox virus* (PPV). These authors engineered an artificial NIa cleavage site to permit GUS to be cleaved from HC-Pro (middle section of *Figure 11.10*). The resulting construct could multiply in *N. benthamiana* but gave a symptomless infection. After a single passage, deletions in the GUS gene could be detected, a situation similar to that found with the TEV–GUS construct described above. Subsequently, a PPV vector in which the foreign sequence is inserted between the NIb and CP genes was developed and used to express the VP60 structural protein of *rabbit haemorrhagic disease virus* (RHDV)

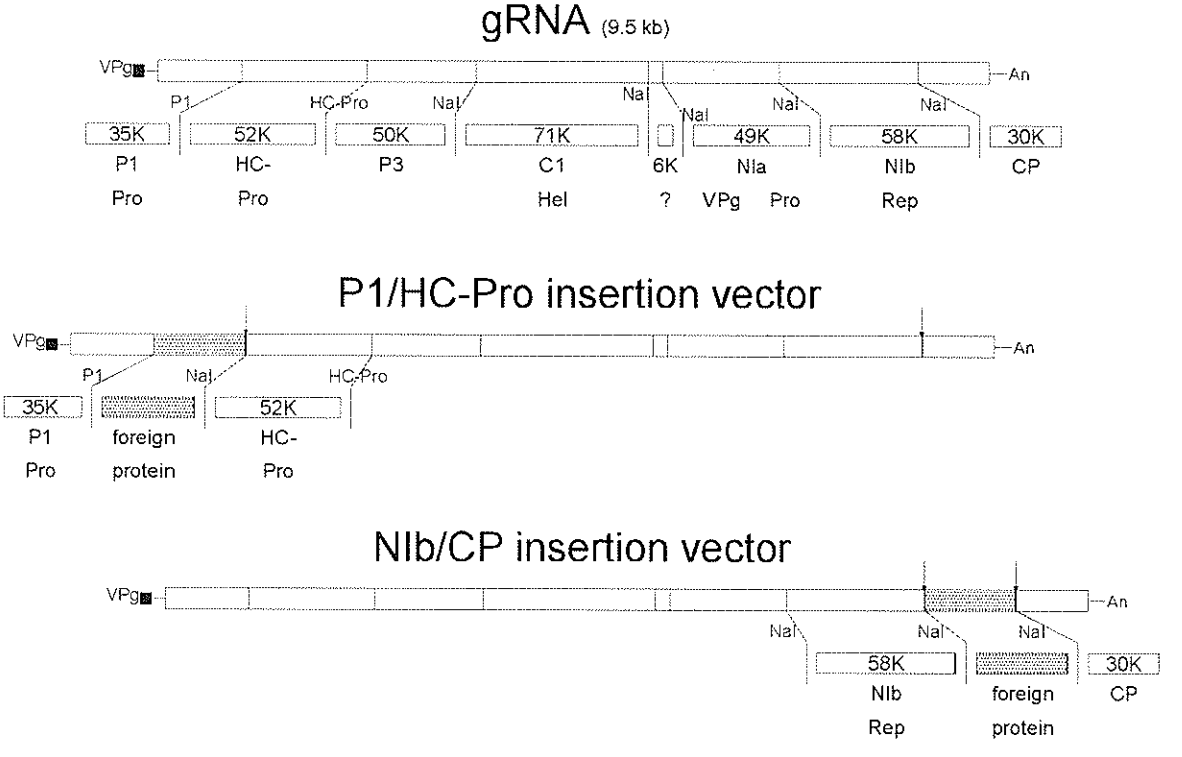

Figure 11.10. Genome organization of potyviruses and derived gene insertion vectors. A viral-encoded protein (VPg) is covalently linked to the 5' terminal nucleotide. The single ORF is translated into a polyprotein which undergoes co- and post-translational processing by proteinases (Pro): P1, HC-Pro and NIa. Below each mature protein, the name of the gene product and, when known, its function is given: helper component for aphid transmission with auto-proteolytic activity at its C-terminus (HC-Pro), helicase (Hel), replicase (Rep), coat protein (CP). Foreign genes (stipled) have been inserted either between the genes for P1 and HC-Pro or between those for NIb and CP. Release from the adjacent proteins can be secured by duplication of the cleavage site for NIa located between NIb and the CP (vertical arrows).

in *N. clevelandii* (Fernandez-Fernandez *et al.*, 2001). Immunization with a crude preparation from infected leaf tissue protected rabbits against subsequent challenge with a lethal dose of RHDV.

Similar experiments have also been carried out to develop *clover yellow vein virus* (ClYVV) as a vector for the production of proteins in legumes (Masuta *et al.*, 2000). Sequences encoding GFP and soybean glutamine synthase (GS) were inserted between P1 and HC-Pro of ClYVV, either separately, in tandem, or to produce a GFP–GS fusion protein. Release of the inserted proteins was achieved by appropriately flanking them with proteinase cleavage sites. The resulting constructs could infect broad bean, kidney bean and soybean, and expression of the foreign proteins could be detected. Plants expressing both GFP and GS were tolerant to the herbicide glufosinate, an indication that the GS gene was functional.

Expression of foreign proteins in cereals is clearly desirable since such crops are widely grown. To this end, Choi *et al.* (2000) developed a vector based on the *Tritimovirus, wheat streak mosaic virus* (WSMV), in which reporter genes (NPT-II and GUS) were inserted between the sequences encoding NIb and CP. The inserts were flanked by NIa cleavage sites to enable processing of the reporter sequence from the polyprotein (lower section of *Figure 11.10*). The constructs were capable of systemically infecting wheat, barley, oat and maize, and expression of the reporter genes could be demonstrated. However, as in the case of the potyvirus vectors described above, deletions rapidly accumulated in the GUS sequence.

Comoviruses

Members of the *Comoviridae* have bipartite genomes, which are separately encapsidated in isometric particles. RNA 1 encodes the proteins involved in replication, while RNA 2 encodes the MP and both the large (L) and small (S) CPs (top half of *Figure 11.11*). Members of the *Comoviridae,* like those of the *Potyviridae*, use a polyprotein processing strategy to express their genome. The type member of the genus *Comovirus*, *cowpea mosaic virus* (CPMV), infects legumes and is very high yielding. For these reasons, CPMV had previously been developed as an epitope-presentation system (Lomonossoff and Hamilton, 1999) and was an attractive candidate for development into a gene-expression vector. Verver *et al.* (1998) demonstrated that it was possible to insert a foreign gene, GFP, between the MP and L. Release of GFP was achieved by flanking it by extensive duplicated MP-L proteinase cleavage sites. Though the modified RNA 2 was as infectious as wild-type RNA 2 and expressed GFP, the construct tended to lose the insert by homologous recombination across the sequences encoding the duplicated cleavage site. To address this problem, the length of repeated sequence consistent with efficient cleavage was minimized, resulting in constructs with increased genetic stability (Gopinath *et al.*, 2000).

In an alternative approach to achieving genetic stability, Gopinath *et al.* (2000) also demonstrated that it was possible to replace either one of the two duplicated proteinase sites with the FMDV 2A sequence. When 2A was inserted between GFP and L, cleavage was only partial, resulting in some GFP–2A–L fusion protein being produced. This fusion protein could be incorporated into virus particles which, as a result, displayed green fluorescence under ultraviolet illumination (see RNA 2-based L vector in *Figure 11.11*). In an alternative approach to using CPMV as a gene

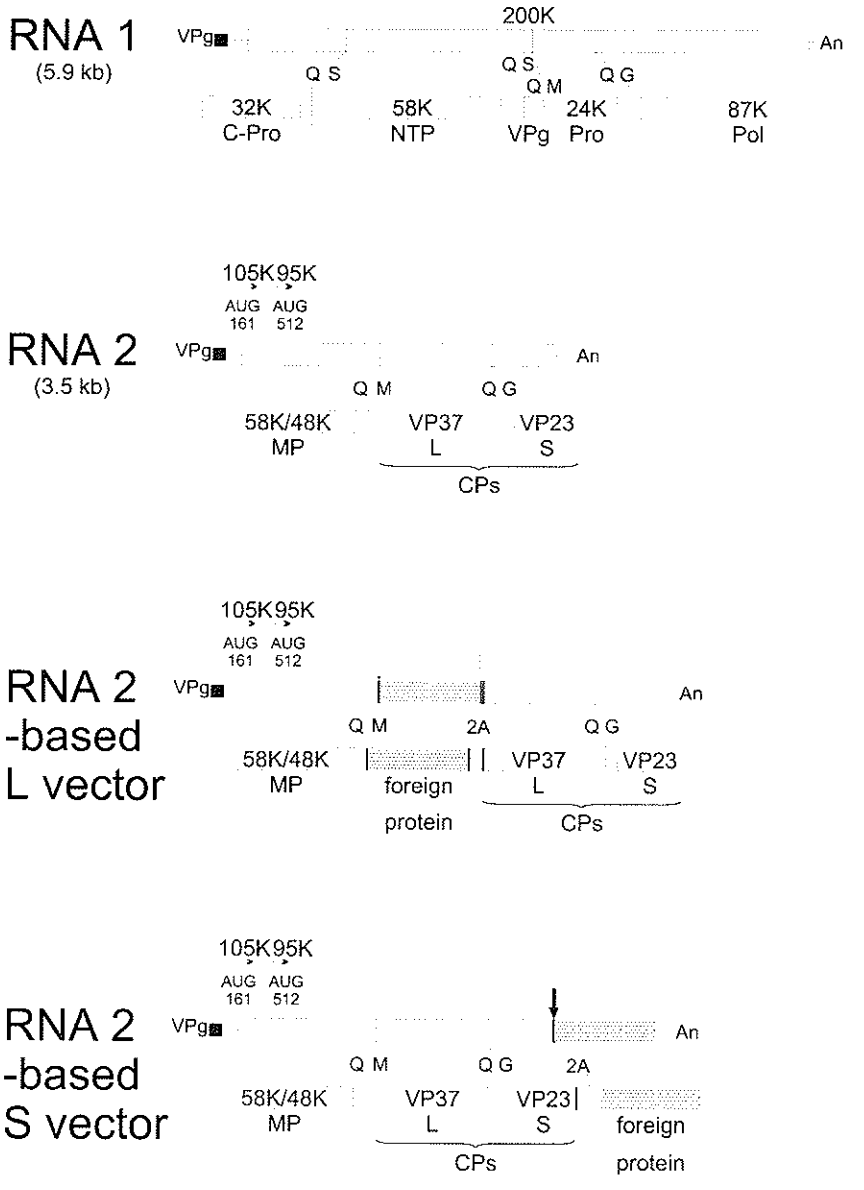

Figure 11.11. Genome organization of *cowpea mosaic virus* (CPMV) and derived gene insertion vectors. Both genomic RNAs are linked to a VPg at their 5' end and are translated into a polyprotein, which undergoes a cascade of cleavages into final products. RNA 1 encodes all the proteins required for RNA replication – nucleotide-binding protein (NTP), RNA polymerase (Pol) – and proteolytic processing – processing regulator (C-Pro), proteinase (Pro). RNA 2 encodes the movement protein (MP) and the large (L) and small (S) coat proteins (CPs). Foreign genes have been inserted into two distinct positions within RNA 2. Between MP and L, release from the polyprotein was achieved by duplicating proteolytic cleavage sequences either side of the insert or, as in the RNA 2 L-based vector shown, by using a duplicated cleavage site (thin vertical arrow) at one end and a FMDV 2A peptide at the other (thick vertical white arrow): the 2A-mediated cleavage was only partial, resulting in both free and L-fused foreign protein. In the RNA 2-based S vector, release of the foreign protein from the carboxy-terminus of S was also mediated by a FMDV 2A sequence (thick vertical black arrow) and was almost complete, therefore producing essentially free protein.

addition vector, Gopinath *et al.* (2000) also fused GFP to the C-terminus of S via a 2A sequence. This construct multiplied well in plants, was genetically stable, and expressed levels of GFP up to 1% of total soluble protein (see RNA 2-based S vector in *Figure 11.11*).

SATELLITE AND DEFECTIVE RNA SYSTEMS

Satellites are small RNA molecules associated with many genera of plant viruses. They are completely dependent on helper viruses, with which they have no sequence homology, for their replication, encapsidation, and movement. Some larger satellites contain ORFs, but others do not appear to act as mRNAs. Though they have no role in the replication of their helper virus, they can influence symptomatology. Because they provide no functions essential to virus replication and spread, satellites are possible candidates for development into useful vectors.

Defective RNAs (dRNAs) are, like satellites, small RNAs, which are completely dependent on a helper virus. However, unlike satellites, they have sequence similarity with their helpers and, indeed, are derived from them through a process of sequence deletion; dRNAs are quite common in nature and their presence during an infection often ameliorates its severity. In certain cases, the presence of a dRNA can interfere with the replication of the helper virus and is then referred to as defective interfering (DI) RNA.

Potexviruses

Bamboo mosaic virus (BaMV) is the only potexvirus with a satellite (satBaMV). This is a linear molecule of 836 nucleotides which contains an ORF capable of encoding a 20 kDa protein and, although this protein can be detected in infected plants, its function is obscure. Lin *et al.* (1996) replaced the 20 kDa ORF of satBaMV with the *cat* gene sequence and showed that the modified satellite could multiply in both barley protoplasts and *C. quinoa* in the presence of helper BaMV. CAT expression reached approximately 2 μg per g of leaf tissue.

Tombusviruses

Burgyan *et al.* (1994) tested a DI RNA from the tombusvirus, *cymbidium ringspot virus* (CyRSV), for its ability to express foreign sequences. They inserted the CP from the unrelated cucumovirus, *tomato aspermy virus* (TAV), into different positions of DI-3 RNA of CyRSV. The modified DI RNA was able to replicate in the presence of helper virus and, when it was inserted at some positions but not others, TAV CP could be detected.

Tobamoviruses

As well as the use of natural dRNAs, there have been attempts to develop artificial dRNAs as a means of expressing foreign genes. The genus *Tobamovirus* was the first to be investigated to this end. Raffo *et al.* (1991) deleted most of the segment of TMV RNA which encodes the replicase proteins and showed that the resulting dRNAs could be replicated by helper TMV and spread systemically in plants. A similar

approach was used to prepare dRNAs of ToMV, though in this case replication in the presence of helper ToMV was only analysed in protoplasts (Ogawa *et al.*, 1992). Detailed analysis subsequently defined those sequences which had to be deleted to allow maximum accumulation of replication-competent artificial TMV dRNAs (Lewandowski *et al.*, 1998). Such dRNAs could form the basis of gene expression systems, though there is currently no report of their use in this regard.

Comoviruses

To develop an alternative CPMV-based gene vector, Verver *et al.* (1998) created two mutants of CPMV RNA 2 bearing complementary deletions in either the MP or CPs. The two deleted forms of RNA 2 could be regarded as artificial dRNAs. In the case of the CP deletion mutant, the CP-encoding sequence was not simply deleted but was replaced with that encoding GFP. Whole plants could be infected with a mixture of RNA 1 and the two deletion mutants, effectively rendering the virus tri- rather than bipartite. GFP expression could be detected in both the inoculated and systemic leaves. Using this tripartite system, expression of foreign genes might be achievable.

TRANSGENE COMPLEMENTATION

Since the demonstration that tobacco, transformed with a full-length cDNA copy of TMV under the control of a CaMV 35S promoter, was persistently infected with the virus (Yamaya *et al.*, 1988), there have been several attempts to devise systems which combine the advantages of stable transformation with those of autonomously replicating vectors. In some systems, a complete viral genome is inserted as a transgene, while in others only a portion is introduced. In the latter systems, additional viral functions are expressed from exogenously applied RNAs, which can only replicate in plants expressing the transgene. These are therefore complementation systems which provide a measure of biological containment.

Alfamoviruses

The first example of a complementation system was based on the alfamovirus, AlMV, which has a similar genome organization to BMV (*Figure 11.4*). Taschner *et al.* (1991) demonstrated that plants co-transformed with, and expressing, the two AlMV proteins, P1 and P2 (which are equivalent to the BMV 1a and 2a proteins), involved in virus replication could support the replication of exogenously applied RNA 3. These transgenic plants (termed P12 plants) have proved extremely valuable in analysing those sequences required for replication of AlMV RNA 3 (see, for example, Van der Vossen *et al.*, 1996). However, as yet, there have been no reports of P12 plants being used to express foreign sequences inserted into RNA 3.

Bromoviruses

The first example of a combined virus–transgenic approach being used to produce a foreign protein involved the bromovirus, BMV. It had been shown that protoplasts derived from tobacco plants transgenic for the 1a and 2a replicase proteins from BMV

(*Figure 11.4*) could support the replication of transfected RNA 3 (Mori *et al.*, 1992). Mori *et al.* (1993b) re-transformed their tobacco plants expressing 1a and 2a with a version of RNA 3 in which the CP sequence had been replaced by that encoding IFN-γ. This RNA 3 construct had previously been shown to be capable of replicating and expressing IFN-γ in tobacco protoplasts (Mori *et al.*, 1993a; see above). The transgene-derived modified RNA 3 was shown to be replicated by the transgene-expressed 1a and 2a proteins, and IFN-γ synthesis could be detected. The level of IFN-γ mRNA was estimated to be 5-fold higher than when the construct was simply expressed from a CaMV 35S promoter.

Potyviruses

The above examples of combined virus–transgenic systems have involved multipartite viruses in which ORFs from individual RNAs have been expressed from transgenes. However, Li and Carrington (1995) demonstrated that it is possible to express part of the single ORF from TEV from a transgene and that the transgene-expressed sequence could complement the replication of defective viral RNAs. In this case, the sequence for the viral replicase (NIb) was integrated into the genome of tobacco. The resulting plants, or protoplasts derived from them, were then tested for their ability to support the replication of TEV derivatives with various mutations in the replicase gene. To aid the assessment of replication, the mutant viral genomes were tagged with GUS (Dolja *et al.*, 1992; see above). The results obtained showed that while some mutants could be complemented more readily than others, the transgene-derived replicase was active *in trans*, could replicate defective TEV genomes, and could promote expression of a heterologous gene.

Potexviruses

In experiments analogous to those undertaken by Yamaya *et al.* (1988) with TMV, Angell and Baulcombe (1997) transformed tobacco with full-length cDNA constructs of either wild-type PVX or PVX carrying GUS. The constructs were intended to be able to replicate in transformed cells and were termed 'amplicons'. However, the resulting plants, far from showing the anticipated symptoms of virus infection and concomitantly high levels of GUS expression, showed phenotypes associated with PTGS (absence of symptoms, extremely low levels of viral RNA, low GUS expression, and resistance to infection with PVX). This silencing phenotype was consistently observed in all the tobacco lines examined. Thus, rather than providing a method for high-level expression, the PVX 'amplicons' have provided an efficient method for gene silencing, and have proved useful in analysing its mechanism (Dalmay *et al.*, 2000).

Conclusion

Since the original proposal in 1978 that plant viruses could be used as a means of expressing foreign sequences in plants, there has been a huge amount of research in this field. It is, perhaps, ironic that the viruses first proposed as vectors, i.e. those that possess DNA genomes, have proved quite difficult to develop into practical vectors.

This is a result of their modes of replication (via a RNA intermediate or through a rolling circle mechanism) being far more complex than originally envisaged. However, although vectors based on caulimo- and geminiviruses have not been used extensively for the production of large amounts of foreign proteins in plants, they have been extremely useful in providing information about such processes as transcription, transactivation of expression, and RNA splicing.

Despite their later development, vectors based on RNA viruses have achieved widespread use within the past 5 years. Some of their applications were quite unforeseen at the time the vectors were developed. For example, the use of TMV- and PVX-based systems to study gene silencing was not anticipated when these viruses were originally engineered for the expression of foreign sequences. However, it still remains to be seen whether vectors based on RNA viruses will be able to deliver on the promise of the commercially viable, large-scale production of valuable proteins in plants.

References

AGRANOVSKY, A.A., FOLIMONOV, A.S., FOLIMONOVA, S.Y., MOROZOV, S.Y., SCHIEMANN, J., LESEMANN, D. AND ATABEKOV, J.G. (1998). Beet yellows closterovirus HSP70-like protein mediates the cell–to–cell movement of a potexvirus transport-deficient mutant and a hordeivirus-based chimeric virus. *Journal of General Virology* **79**, 889–895.

AHLQUIST, P. AND JANDA, M. (1984). cDNA cloning and *in vitro* transcription of the complete brome mosaic virus genome. *Molecular and Cellular Biology* **4**, 2876–2882.

AHLQUIST, P., FRENCH, R., JANDA, M. AND LOESCH-FRIES, S. (1984). Multicomponent RNA plant virus infection derived from cloned viral cDNA. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 7066–7070.

ANGELL, S.M. AND BAULCOMBE, D.C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO Journal* **16**, 3675–3684.

ATKINSON, R.G., BIELESKI, L.R.F., GLEAVE, A.P., JANSSEN, B.J. AND MORRIS, B.A.M. (1998). Post-transcriptional silencing of chalcone synthase in petunia using a geminivirus-based episomal vector. *Plant Journal* **15**, 593–604.

BAULCOMBE, D.C. (1999). Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology* **2**, 109–113.

BAULCOMBE, D.C., CHAPMAN, S. AND SANTA CRUZ, S. (1995). Jellyfish green fluorescent protein as a reporter for virus infections. *Plant Journal* **7**, 1045–1053.

BLACKMAN, L.M., BOEVINK, P., SANTA CRUZ, S., PALUKAITIS, P. AND OPARKA, K.J. (1998). The movement protein of cucumber mosaic virus traffics into sieve elements in minor veins of *Nicotiana clevelandii*. *Plant Cell* **10**, 525–537.

BORNEMAN, J., TRITZ, R., HAMPEL, A. AND ALTSCHULER, M. (1995). Detection of cleavage products from an *in vivo* transcribed *cis* hairpin ribozyme in turnips using the CaMV plant virus. *Gene* **159**, 137–142.

BOULTON, M.I., PALLAGHY, C.K., CHATANI, M., MACFARLANE, S. AND DAVIES J.W. (1993). Replication of maize streak virus mutants in maize protoplasts – evidence for a movement protein. *Virology* **192**, 85–93.

BRIDDON, R.W., PINNER, M.S., STANLEY, J. AND MARKHAM, P.G. (1990). Geminivirus coat protein gene replacement alters insect specificity. *Virology* **177**, 85–94.

BRIGNETI, G., VOINNET, O., LI, W.X., JI, L.H., DING, S.W. AND BAULCOMBE, D.C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO Journal* **17**, 6739–6746.

BRISSON, N., PASZKOWSKI, J., PENSWICK, J.R., GRONENBORN, B., POTRYKUS, I. AND HOHN, T. (1984). Expression of a bacterial gene in plants by using a viral vector. *Nature* **310**, 511–514.

BURGYAN, J., SALANKI, K., DALMAY, T. AND RUSSO, M. (1994). Expression of homologous and

heterologous viral coat protein-encoding genes using recombinant DI RNA from cymbidium ringspot tombusvirus. *Gene* **138**, 159–163.

BURTON, R.A., GIBEAUT, D.M., BACIC, A., FINDLAY, K., ROBERTS, K., HAMILTON, A., BAULCOMBE, D.C. AND FINCHER, G.B. (2000). Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* **12**, 691–705.

CHAPMAN, S., KAVANAGH, T. AND BAULCOMBE, D. (1992). Potato virus X as a vector for gene expression in plants. *Plant Journal* **2**, 549–557.

CHAY, C.A., GUAN, X. AND BRUENING, G. (1997). Formation of circular satellite tobacco ringspot virus RNA in protoplasts transiently expressing the linear RNA. *Virology* **239**, 413–425.

CHEN, Z.H., ULMASOV, B. AND FOLK, W.R. (1998). Nonsense and missense translational suppression in plant cells mediated by tRNA(Lys). *Plant Molecular Biology* **36**, 163–170.

CHOE, K.S., MELCHER, U., RICHARDS, K., LEBEURIER, G. AND ESSENBERG, R.C. (1985). Recombination between mutant cauliflower mosaic virus DNAs. *Plant Molecular Biology* **5**, 281–289.

CHOI, I.R., STENGER, D.C., MORRIS, T.J. AND FRENCH, R. (2000). A plant virus vector for systemic expression of foreign genes in cereals. *Plant Journal* **23**, 547–555.

COHEN, Y., GISEL, A. AND ZAMBRYSKI, P.C. (2000). Cell-to-cell and systemic movement of recombinant green fluorescent protein-tagged turnip crinkle viruses. *Virology* **273**, 258–266.

CULVER, J.N. (1996). Tobamovirus cross protection using a potexvirus vector. *Virology* **226**, 228–235.

DALMAY, T., HAMILTON, A., MUELLER, E. AND BAULCOMBE, D.C. (2000). Potato virus X amplicons in *Arabidopsis* mediate genetic and epigenetic gene silencing. *Plant Cell* **12**, 369–379.

DAUBERT, S., SHEPHERD, R.J. AND GARDNER, R.C. (1983). Insertional mutagenesis of the cauliflower mosaic virus genome. *Gene* **25**, 201–208.

DAWSON, W.O., LEWANDOWSKI, D.J., HILF, M.E., BUBRICK, P., RAFFO, A.J., SHAW, J.J., GRANTHAM, G.L. AND DESJARDINS, P.R. (1989). A tobacco mosaic virus-hybrid expresses and loses an added gene. *Virology* **172**, 285–292.

DE JONG, W. AND AHLQUIST, P. (1992). A hybrid plant RNA virus made by transferring the noncapsid movement protein from a rod-shaped to an icosahedral virus is competent for systemic infection. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6808–6812.

DEKKER, E.L., WOOLSTON, C.J., XUE, Y.B., COX, B. AND MULLINEAUX, P.M. (1991). Transcript mapping reveals different expression strategies for the bicistronic RNAs of the geminivirus wheat dwarf virus. *Nucleic Acids Research* **19**, 4075–4081.

DE WIT, P.J.G.M., LAUGE, R., HONEE, G., JOOSTEN, M.H.A.J., VOSSEN, P., KOOMAN-GERSMANN, M., VOGELSANG, R. AND VERVOORT, J.J.M. (1997). Molecular and biochemical basis of the interaction between tomato and its fungal pathogen *Cladosporium fulvum*. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* **71**, 137–141.

DEZOETEN, G.A., PENSWICK, J.R., HORISBERGER, M.A., AHL, P., SCHULTZE, M. AND HOHN, T. (1989). The expression, localization, and effect of a human interferon in plants. *Virology* **172**, 213–222.

DIXON, L.K. AND HOHN, T. (1984). Initiation of translation of the cauliflower mosaic virus genome from a polycistronic messenger RNA: evidence from deletion mutagenesis. *EMBO Journal* **3**, 2731–2736.

DOLJA, V.V., MCBRIDE, H.J. AND CARRINGTON, J.C. (1992). Tagging of plant potyvirus replication and movement by insertion of beta-glucuronidase into the viral polyprotein. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 10208–10212.

DOLJA, V.V., HERNDON, K.L., PIRONE, T.P. AND CARRINGTON, J.C. (1993). Spontaneous mutagenesis of a plant potyvirus genome after insertion of a foreign gene. *Journal of Virology* **67**, 5968–5975.

DOLJA, V.V., HONG, J., KELLER, K.E., MARTIN, R.R. AND PEREMYSLOV, V.V. (1997).

Suppression of potyvirus infection by coexpressed closterovirus protein. *Virology* **234**, 243–252.

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. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 7204–7208.

ENGLISH, J.J. AND BAULCOMBE, D.C. (1997). The influence of small changes in transgene transcription on homology-dependent virus resistance and gene silencing. *Plant Journal* **12**, 1311–1318.

ENGLISH, J.J., MUELLER, E. AND BAULCOMBE, D.C. (1996). Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**, 179–188.

ETESSAMI, P., SAUNDERS, K., WATTS, J. AND STANLEY, J. (1991). Mutational analysis of complementary-sense genes of African cassava mosaic virus DNA A. *Journal of General Virology* **72**, 1005–1012.

FERNANDEZ-FERNANDEZ, M.R., MOURINO, M., RIVERA, J., RODRIGUEZ, F., PLANA-DURAN, J. AND GARCIA, J.A. (2001). Protection of rabbits against rabbit hemorrhagic disease virus by immunization with the VP60 protein expressed in plants with a potyvirus-based vector. *Virology* **280**, 283–291.

FRANCONI, A., ROGGERO, P., PIRAZZI, P., ARIAS, F.J., DESIDERIO, A., BITTI, O., PASHKOULOV, D., MATTEI, B., BRACCI, L., MASENGA, V., MILNE, R.G. AND BENVENUTO, E. (1999). Functional expression in bacteria and plants of an scFv antibody fragment against tospoviruses. *Immunotechnology* **4**, 189–201.

FRENCH, R., JANDA, M. AND AHLQUIST, P. (1986). Bacterial gene inserted in an engineered RNA virus – efficient expression in monocotyledonous plant cells. *Science* **231**, 1294–1297.

GARDINER, W.E., SUNTER, G., BRAND, L., ELMER, J.S., ROGERS, S.G. AND BISARO, D.M. (1988). Genetic analysis of tomato golden mosaic virus: the coat protein is not required for systemic spread or symptom development. *EMBO Journal* **7**, 899–904.

GILARDI, P., GARCIA-LUQUE, I. AND SERRA, M.T. (1998). Pepper mild mottle virus coat protein alone can elicit the *Capsicum* spp. L-3 gene-mediated resistance. *Molecular Plant-Microbe Interactions* **11**, 1253–1257.

GOPINATH, K., WELLINK, J., PORTA, C., TAYLOR, K.M., LOMONOSSOFF, G.P. AND VAN KAMMEN, A. (2000). Engineering cowpea mosaic virus RNA-2 into a vector to express heterologous proteins in plants. *Virology* **267**, 159–173.

GOREGACKER, S.P., ECKHARDT, L.G. AND CULVER, J.N. (2000). Tobacco mosaic virus replicase-mediated cross-protection: contributions of RNA and protein-derived mechanisms. *Virology* **273**, 267–275.

GRIMSLEY, N., HOHN, T., DAVIES, J.W. AND HOHN, B. (1987). *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. *Nature* **325**, 177–179.

GRONENBORN, B., GARDNER, R.C., SCHAEFER, S. AND SHEPHERD, R.J. (1981). Propagation of foreign DNA in plants using cauliflower mosaic virus as vector. *Nature* **294**, 773–776.

GUO, H.S., LOPEZ-MOYA, J.J. AND GARCIA, J.A. (1998). Susceptibility to recombination rearrangements of a chimeric plum pox potyvirus genome after insertion of a foreign gene. *Virus Research* **57**, 183–195.

HALEY, A., ZHAN, X., RICHARDSON, K., HEAD, K. AND MORRIS, B. (1992). Regulation of the activities of African cassava mosaic virus promoters by the AC1, AC2 and AC3 gene products. *Virology* **188**, 905–909.

HAMILTON, W.D.O., BISARO, D.M., COUTTS, R.H.A. AND BUCK, K.W. (1983). Demonstration of the bipartite nature of the genome of a single-stranded DNA plant virus by infection with the cloned DNA components. *Nucleic Acids Research* **11**, 7387–7396.

HAMMOND-KOSACK, K.E., STASKOWICZ, 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. *Molecular Plant-Microbe Interactions* **8**, 181–185.

HANLEY-BOWDOIN, L., ELMER, J.S. AND ROGERS, S.G. (1988). Transient expression of

heterologous RNAs using tomato golden mosaic virus. *Nucleic Acids Research* **16**, 10511–10528.

HAYES, R.J., COUTTS, R.H.A. AND BUCK, K.W. (1988a). Agroinfection of *Nicotiona* spp with cloned DNA of tomato golden mosaic virus. *Journal of General Virology* **69**, 1487–1496.

HAYES, R.J., PETTY, I.T.D., COUTTS, R.H.A. AND BUCK, K.W. (1988b). Gene amplification and expression in plants by a replicating geminivirus vector. *Nature* **334**, 179–182.

HAYES, R.J., COUTTS, R.H.A. AND BUCK, K.W. (1989). Stability and expression of bacterial genes in replicating geminivirus vectors in plants. *Nucleic Acids Research* **17**, 2391–2403.

HENDY, S., CHEN, Z.C., BARKER, H., SANTA CRUZ, S., CHAPMAN, S., TORRANCE, L., COCKBURN, W. AND WHITELAM, G.C. (1999). Rapid production of single-chain Fv fragments in plants using a potato virus X episomal vector. *Journal of Immunological Methods* **231**, 137–146.

HIROCHIKA, H. AND HAYASHI, K. (1991) A new strategy to improve a cauliflower mosaic virus vector. *Gene* **105**, 239–241.

HONG, Y.G., SAUNDERS, K., HARTLEY, M.R. AND STANLEY, J. (1996). Resistance to geminivirus infection by virus-induced expression of dianthin in transgenic plants. *Virology* **220**, 119–127.

HONG, Y.G., SAUNDERS, K. AND STANLEY, J. (1997). Transactivation of dianthin transgene expression by African cassava mosaic virus AC2. *Virology* **228**, 383–387.

HOWARTH, A.J., GARDNER, R.C., MESSING, J. AND SHEPHERD, R.J. (1981). Nucleotide sequence of naturally occurring deletion mutants of cauliflower mosaic virus. *Virology* **112**, 678–685.

HOWELL, S.H., WALKER, L.L. AND DUDLEY, R.K. (1980). Cloned cauliflower mosaic virus DNA infects turnips (*Brassica rapa*). *Science* **208**, 1265–1267.

HULL, R. (1978). The possible use of plant viral DNAs in genetic manipulation in plants. *Trends in Biochemical Sciences* **3**, 254–256.

JOOSTEN, M.H.A.J., VOGELSANG, R., COZIJNSEN, T.J., VERBERNE, M.C. AND DE WIT, P.J.G.M. (1997). The biotrophic fungus *Cladosporium fulvum* circumvents Cf-4-mediated resistance by producing unstable AVR4 elicitors. *Plant Cell* **9**, 367–379.

JOSHI, R.L., JOSHI, V. AND OW, D.W. (1990). BSMV genome mediated expression of a foreign gene in dicot and monocot plant cells. *EMBO Journal* **9**, 2663–2669.

KAMMANN, M., MATZEIT, V., SCHMIDT, B., SCHELL, J., WALDEN, R. AND GRONENBORN, B. (1991). Geminivirus-based shuttle vectors capable of replication in *Escherichia coli* and monocotyledonous plant cells. *Gene* **104**, 247–252.

KANEVSKI, I.F., THAKUR, S., COSOWSKY, L., SUNTER, G., BROUGH, C., BISARO, D. AND MALIGA, P. (1992). Tobacco lines with high copy number of replicating recombinant geminivirus vectors after biolistic DNA delivery. *Plant Journal* **2**, 457–463.

KARRER, E.E., BEACHY, R.N. AND HOLT, C.A. (1998). Cloning of tobacco genes that elicit the hypersensitive response. *Plant Molecular Biology* **36**, 681–690.

KEARNEY, C.M., DONSON, J., JONES, G.E. AND DAWSON, W.O. (1993). Low level of genetic drift in foreign sequences replicating in an RNA virus in plants. *Virology* **192**, 11–17.

KISS-LASZLO, Z., BLANC, S. AND HOHN, T. (1995). Splicing of cauliflower mosaic virus 35S RNA is essential for viral infectivity. *EMBO Journal* **14**, 3552–3562.

KJEMTRUP, S., SAMPSON, K.S., PEELE, C.G., NGUYEN, L.V., CONKLING, M.A., THOMPSON, W.F. AND ROBERTSON, D. (1998). Gene silencing from plant DNA carried by a Geminivirus. *Plant Journal* **14**, 91–100.

KOOMAN-GERSMANN, M., VOGELSANG, R., HOOGENDIJK, E.C.M. AND DE WIT, P.J.G.M. (1997). Assignment of amino acid residues of the AVR9 peptide of *Cladosporium fulvum* that determine elicitor activity. *Molecular Plant-Microbe Interactions* **10**, 821–829.

KOOMAN-GERSMANN, M., VOGELSANG, R., VOSSEN, P., VAN DEN HOOVEN, H.W., MAHE, E., HONEE, G. AND DE WIT, P.J.G.M. (1998). Correlation between binding affinity and necrosis-inducing activity of mutant AVR9 peptide elicitors. *Plant Physiology* **117**, 609–618.

KREBITZ, M., WIEDERMANN, U., ESSL, D., STEINKELLNER, H., WAGNER, B., TURPEN, T.H., EBNER, C., SCHEINER, O. AND BREITENEDER, H. (2000). Rapid production of the major

birch pollen allergen Bet v 1 in *Nicotiana benthamiana* plants and its immunological *in vitro* and *in vivo* characterization. *FASEB Journal* **14**, 1279–1288.

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 alpha-trichosanthin in transfected plants by an RNA viral vector. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 427–430.

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. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 1679–1683.

KUMAGAI, M.H., DONSON, J., DELLA-CIOPPA, G. AND GRILL, L.K. (2000). Rapid, high-level expression of glycosylated rice alpha-amylase in transfected plants by an RNA viral vector. *Gene* **245**, 169–174.

LACOMME, C. AND SANTA CRUZ, S. (1999). Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 7956–7961.

LAUFS, J., WIRTZ, U., KAMMANN, M., MATZEIT, V., SCHAEFER, S., SCHELL, J., CZERNILOFSKY, A.P., BAKER, B. AND GRONENBORN, B. (1990). Wheat dwarf virus AC/DS vectors – expression and excision of transposable elements introduced into various cereals by a viral replicon. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 7752–7756.

LAUGE, R., JOOSTEN, M.H.A.J., HAANSTRA, J.P.W., GOODWIN, P.H., LINDHOUT, P. AND DE WIT, P.J.G.M. (1998). Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9014–9018.

LAUGE, R., GOODWIN, P.H., DE WIT, P.J.G.M. AND JOOSTEN, M.H.A.J. (2000). Specific HR-associated recognition of secreted proteins from *Cladosporium fulvum* occurs in both host and non-host plants. *Plant Journal* **23**, 735–745.

LAZAROWITZ, S.G. (1987). The molecular characterisation of geminiviruses. *Plant Molecular Biology Reporter* **4**, 177–192.

LAZAROWITZ, S.G., PINDER, A.J., DAMSTEEGT, V.D. AND ROGERS, S.G. (1989). Maize streak virus genes essential for systemic spread and symptom development. *EMBO Journal* **8**, 1023–1032.

LEFEBVRE, D.D., MIKI, B.L. AND LALIBERTE, J.F. (1987). Mammalian metallothionein functions in plants. *Bio-technology* **5**, 1053–1056.

LEWANDOWSKI, D.L. AND DAWSON, W.O. (1998). Deletion of internal sequences results in tobacco mosaic virus defective RNAs that accumulate to high levels without interfering with replication of the helper virus. *Virology* **251**, 427–437.

LI, X.H. AND CARRINGTON, J.C. (1995). Complementation of tobacco etch potyvirus mutants by active RNA-polymerase expressed in transgenic cells. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 457–461.

LIN, N.S., LEE, Y.S., LIN, B.Y., LEE, C.W. AND HSU, Y.H. (1996). The open reading frame of bamboo mosaic potexvirus satellite RNA is not essential for its replication and can be replaced with a bacterial gene. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 3138–3142.

LOMONOSSOFF, G.P. AND HAMILTON, W.D.O. (1999). Cowpea mosaic virus-based vaccines. In: *Current topics in microbiology and immunology* **240**. Eds. J. Hammond, P. McGarvey and V. Yusibov, pp 177–189. Springer.

LU, B., STUBBS, G. AND CULVER, J.N. (1998). Coat protein interactions involved in tobacco mosaic tobamovirus cross-protection. *Virology* **248**, 188–198.

MACFARLANE, S.A. AND POPOVICH, A.H. (2000). Efficient expression of foreign proteins in roots from tobravirus vectors. *Virology* **267**, 29–35.

MAHAJAN, S.K., CHISHOLM, S.T., WHITHAM, S.A. AND CARRINGTON, J.C. (1998). Identification and characterization of a locus (RTM1) that restricts long-distance movement of tobacco etch virus in *Arabidopsis thaliana*. *Plant Journal* **14**, 177–186.

MASUTA, C., YAMANA, T., TACAHASHI, Y., UYEDA, I., SATO, M., UEDA, S. AND MATSUMURA, T. (2000). Development of clover yellow vein virus as an efficient, stable gene-expression system for legume species. *Plant Journal* **23**, 539–546.

MATZEIT, V., SCHAEFER, S., KAMMANN, M., SCHALK, H.J., SCHELL, J. AND GRONENBORN, B. (1991). Wheat dwarf virus vectors replicate and express foreign genes in cells of monocotyledonous plants. *Plant Cell* **3**, 247–258.

MCCORMICK, A.A., KUMAGAI, M.H., HANLEY, K., TURPEN, T.H., HAKIM, I., GRILL, L.K., TUSE, D., LEVY, S. AND LEVY, R. (1999). Rapid production of specific vaccines for lymphoma by expression of the tumor-derived single-chain Fv epitopes in tobacco plants. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 703–708.

MCCULLOUGH, A.J., LOU, H. AND SCHULER, M.A. (1991). *In vivo* analysis of plant pre-messenger RNA splicing using an autonomously replicating vector. *Nucleic Acids Research* **19**, 3001–3009.

MEYER, P., NIEDENHOF, I., HEIDMANN, I. AND SAEDLER, H. (1989). Extrachromosomal forms of CLV DNA1 in transgenic plants are inherited by symptom-free progeny. *Plant Science* **65**, 207–216.

MEYER, P., HEIDMANN, I. AND NIEDENHOF, I. (1992). The use of African cassava mosaic virus as a vector system for plants. *Gene* **110**, 213–217.

MODELSKA, A., DIETZSCHOLD, B., SLEYSH, N., FU, Z.F., STEPLEWSKI, K., HOOPER, D.C., KOPROWSKI, H. AND YUSIBOV, V. (1998). Immunization against rabies with plant-derived antigen. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2481–2485.

MORI, M., MISE, K., OKUNO, T. AND FURUSAWA, I. (1992). Expression of brome mosaic virus-encoded replicase genes in transgenic tobacco plants. *Journal of General Virology* **73**, 169–172.

MORI, M., ZHANG, G.H., KAIDO, M., OKUNO, T. AND FURUSAWA, I. (1993a). Efficient production of human gamma interferon in tobacco protoplasts by genetically engineered brome mosaic virus RNAs. *Journal of General Virology* **74**, 1255–1260.

MORI, M., KAIDO, M., OKUNO, T. AND FURUSAWA, I. (1993b). Messenger-RNA amplification system by viral replicase in transgenic plants. *FEBS Letters* **336**, 171–174.

MORRIS, B.A.M., RICHARDSON, K.A., HALEY, A., ZHAN, X.C. AND THOMAS, J.E. (1992). The nucleotide sequence of the infectious cloned DNA component of tobacco yellow dwarf virus reveals features of geminiviruses infecting monocotyledonous plants. *Virology* **187**, 633–642.

MORRIS-KRSINICH, B.A.M., MULLINEAUX, P.M., DONSON, J., BOULTON, M.I., MARKHAM, P.G., SHORT, M.N. AND DAVIES, J.W. (1985). Bidirectional transcription of maize streak virus DNA and identification of the coat protein gene. *Nucleic Acids Research* **13**, 7237–7256.

NAPOLI, C., LEMIEUX, C. AND JORGENSEN, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell* **2**, 279–289.

NEEDHAM, P.D., ATKINSON, R.G., MORRIS, B.A.M., GARDNER, R.C. AND GLEAVE, A.P. (1998). GUS expression patterns from a tobacco yellow dwarf virus-based episomal vector. *Plant Cell Reports* **17**, 631–639.

NEUHAUS-URL, G., LUSARDI, M.C., IMOBERDORF, R. AND NEUHAUS, G. (1994). Integrative and self-replicating Lc vectors and their transactivation capacity in maize callus protoplasts. *Plant Cell Reports* **13**, 564–569.

NOAD, R.J., TURNER, D.S. AND COVEY, S.N. (1997). Expression of functional elements inserted into the 35S promoter region of infectious cauliflower mosaic virus replicons. *Nucleic Acids Research* **25**, 1123–1129.

O'BRIEN, G.J., BRYANT, C.J., VOOGD, C., GREENBERG, H.B., GARDNER, R.C. AND BELLAMY, A.R. (2000). Rotavirus VP6 expressed by PVX vectors in *Nicotiana benthamiana* coats PVX rods and also assembles into virus-like particles. *Virology* **270**, 444–453.

OGAWA, T., WATANABE, Y. AND OKADA, Y. (1992). *Cis*-acting elements for *in trans* complementation of replication-defective tobacco mosaic virus. *Virology* **191**, 454–458.

PALMER, K.E., THOMSON, J.A. AND RYBICKI, E.P. (1999). Generation of maize cell lines containing autonomously replicating maize streak virus-based gene vectors. *Archives of Virology* **144**, 1345–1360.

PENSWICK, J., HÜBLER, R. AND HOHN, T. (1988). A viable mutation in cauliflower mosaic virus, a retrovirus-like plant virus, separates its capsid protein and polymerase genes. *Journal of Virology* **62**, 1460–1463.

PERRIMAN, R., BRUENING, G., DENNIS, E.S. AND PEACOCK, W.J. (1995). Effective ribozyme delivery in plant cells. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6175–6179.

PIETRZAK, M. AND HOHN, T. (1985). Replication of the cauliflower mosaic virus – role and stability of the cloned delta-3 discontinuity sequence. *Gene* **33**, 169–179.

PORTA, C. AND LOMONOSSOFF, G.P. (1998). Scope for using plant viruses to present epitopes from animal pathogens. *Reviews in Medical Virology* **8**, 25–41.

PFEIFFER, P. AND HOHN, T. (1983). Involvement of reverse transcription in the replication of cauliflower mosaic-virus – a detailed model and test of some aspects. *Cell* **33**, 781–789.

RAFFO, A.J. AND DAWSON, W.O. (1991). Construction of tobacco mosaic virus subgenomic replicons that are replicated and spread systemically in tobacco plants. *Virology* **184**, 277–289.

ROGERS, S.G., BISARO, D.B., HORSCH, R.B., FRALEY, R.T., HOFFMAN, N.L., BRAND, L., ELMER, J.S. AND LLOYD, A.M. (1986). Tomato golden mosaic virus A component DNA replicates autonomously in transgenic plants. *Cell* **45**, 593–600.

ROMMENS, C.M.T., SALMERON, J.M., BAULCOMBE, D.C. AND STASKAWICZ, B.J. (1995). Use of a gene expression system based on potato virus X to rapidly identify and characterize a tomato Pto homolog that controls fenthion sensitivity. *Plant Cell* **7**, 249–257.

RUIZ, M.T., VOINNET, O. AND BAULCOMBE, D.C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **10**, 937–946.

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. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6901–6905.

SACHER, R., FRENCH, R. AND AHLQUIST, P. (1988). Hybrid brome mosaic virus RNAs express and are packaged in tobacco mosaic virus coat protein *in vivo*. *Virology* **167**, 15–24.

SAITOH, H., KIBA, A., NISHIHARA, M., YAMAMURA, S., SUZUKI, K. AND TERAUCHI, R. (2001). Production of antimicrobiol defensin in *Nicotiana benthamiana* with a potato virus X vector. *Molecular Plant-Microbe Interactions* **14**, 111–115.

SANTA CRUZ, S., CHAPMAN, S., ROBERTS, A.G., ROBERTS, I.M., PRIOR, D.A.M. AND OPARKA, K.J. (1996). Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 6286–6290.

SASAYA, T., TORRANCE, L., COWAN, G. AND ZIEGLER, A. (2000). Aphid transmission studies using helper component proteins of Potato virus Y expressed from a vector derived from Potato virus X. *Journal of General Virology* **81**, 1115–1119.

SCHOLTHOF, H.B. (1999). Rapid delivery of foreign genes into plants by direct rub-inoculation with intact plasmid DNA of a tomato bushy stunt virus gene vector. *Journal of Virology* **73**, 7823–7829.

SCHOLTHOF, H.B., MORRIS, T.J. AND JACKSON, A.O. (1993). The capsid protein gene of tomato bushy stunt virus is dispensable for systemic movement and can be replaced for localized expression of foreign genes. *Molecular Plant-Microbe Interactions* **6**, 309–322.

SCHOLTHOF, H.B., SCHOLTHOF, K.B.G. AND JACKSON, A.O. (1995). Identification of tomato bushy stunt virus host-specific symptom determinants by expression of individual genes from a potato virus X vector. *Plant Cell* **7**, 1157–1172.

SHEN, W.H. AND HOHN, B. (1992). Excision of a transposable element from a viral vector introduced into maize plants by agroinfection. *The Plant Journal* **2**, 35–42.

SHEN, W.H. AND HOHN, B. (1994). Amplification and expression of the beta-glucuronidase gene in maize plants by vectors based on maize streak virus. *Plant Journal* **5**, 227–236.

SHEN, W.H. AND HOHN, B. (1995). Vectors based on maize streak virus can replicate to high copy numbers in maize plants. *Journal of General Virology* **76**, 965–969.

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.

SIEGEL, A. (1985). Plant-virus-based vectors for gene transfer may be of considerable use despite a presumed high error frequency during RNA synthesis. *Plant Molecular Biology* **4**, 327–329.

SIJEN, T., WELLINK, J., HIRIART, J.B. AND VAN KAMMEN, A. (1996). RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. *Plant Cell* **8**, 2277–2294.

SMOLENSKA, L., ROBERTS, I.M., LEARMONTH, D., PORTER, A.J., HARRIS, W.J., WILSON, T.M.A. AND SANTA CRUZ, S. (1998). Production of a functional single chain antibody attached to the surface of a plant virus. *FEBS Letters* **441**, 379–382.

SOLOVYEV, A.G., ZELENINA, D.A., SAVENKOV, E.I., GRDZELISHVILI, V.Z., MOROZOV, S.Y., LESEMANN, D.-E., MAISS, E., CASPER, R. AND ATABEKOV, J.G. (1996). Movement of a barley stripe mosaic virus chimera with a tobacco mosaic virus movement protein. *Virology* **217**, 435–441.

SOLOVYEV, A.G., ZELENINA, D.A., SAVENKOV, E.I., GRDZELISHVILI, V.Z., MOROZOV, S.Y., MAISS, E., CASPER, R. AND ATABEKOV, J.G. (1997). Host-controlled cell–to–cell movement of a hybrid barley stripe mosaic virus expressing a dianthovirus movement protein. *Intervirology* **40**, 1–6.

SONODA, S. AND NISHIGUCHI, M. (2000). Delayed activation of post-transcriptional gene silencing and *de novo* transgene methylation in plants with the coat protein gene of sweet potato feathery mottle potyvirus. *Plant Science* **156**, 137–144.

SONODA, S., KOIWA, H., KANDA, K., KATO, H., SHIMONO, M. AND NISHIGUCHI, M. (2000). The helper component-proteinase of Sweet potato feathery mottle virus facilitates systemic spread of Potato virus X in *Ipomoea nil*. *Phytopathology* **90**, 944–950.

SPITSIN, S., STEPLEWSKI, K., FLEYSH, N., BELANGER, H., MIKHEEVA, T., SHIVPRASAD, S., DAWSON, W., KOPROWSKI, H. AND YUSIBOV, V. (1999). Expression of alfalfa mosaic virus coat protein in tobacco mosaic virus (TMV) deficient in the production of its native coat protein supports long-distance movement of a chimeric TMV. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 2549–2553.

STANLEY, J. (1983). Infectivity of the cloned geminivirus genome requires sequences from both DNAs. *Nature* **305**, 643–645.

STANLEY, J. AND TOWNSEND, R. (1986). Infectious mutants of cassava latent virus generated *in vivo* from intact recombinant DNA clones containing single copies of the genome. *Nucleic Acids Research* **14**, 5981–5998.

STENGER, D.C., REVINGTON, G.N., STEVENSON, M.C. AND BISARO, D.M. (1991). Replicational release of geminivirus genomes from tandemly repeated copies – evidence for rolling-circle replication of a plant viral-DNA. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 8029–8033.

SUAREZ-LOPEZ, P. AND GUTIERREZ, C. (1997). DNA replication of wheat dwarf geminivirus vectors: effects of origin structure and size. *Virology* **227**, 389–399.

SUGIMOTO, K., OTSUKI, Y., SAJI, S. AND HIROCHIKA, H. (1994). Transposition of the maize Ds element from a viral vector to the rice genome. *Plant Journal* **5**, 863–871.

SUNTER, G. AND BISARO, D.M. (1991). Transactivation in a geminivirus: AL2 gene product is needed for coat protein expression. *Virology* **180**, 416–419.

SZETO, W.W., HAMER, D.H., CARLSON, P.S. AND THOMAS, C.A. (1977). Cloning of cauliflower mosaic virus (CaMV) DNA in *Escherichia coli*. *Science* **196**, 210–212.

TAKAMATSU, N., ISHIKAWA, M., MESHI, T. AND OKADA, Y. (1987). Expression of bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. *EMBO Journal* **6**, 307–311.

TASCHNER, P.E.M., VAN DER KUYL, A.C., NEELEMAN, L. AND BOL, J.F. (1991). Replication of an incomplete alfalfa mosaic virus genome in plants transformed with viral replicase genes. *Virology* **181**, 445–450.

THOMAS, C.L., PERBAL, C. AND MAULE, A.J. (1993). A mutation of cauliflower mosaic virus gene I interferes with virus movement but not virus replication. *Virology* **192**, 415–421.

TIMMERMANS, M.C.P., DAS, O.P. AND MESSING, J. (1992). *Trans* replication and high copy numbers of wheat dwarf virus vectors in maize cells. *Nucleic Acids Research* **20**, 4047–4054.

TÖPFER, R., GRONENBORN, B., SCHELL, J. AND STEINBISS, H.H. (1989). Uptake and transient expression of chimeric genes in seed-derived embryos. *The Plant Cell* **1**, 133–139.

TOWNSEND, R., WATTS, J. AND STANLEY, J. (1986). Synthesis of viral DNA forms in *Nicotiana plumbaginifolia* protoplasts inoculated with cassava latent virus (CLV): evidence for the independent replication of one component of the CLV genome. *Nucleic Acids Research* **14**, 1253–1265.

TURNER, D.S., MCCALLUM, D.G. AND COVEY, S.N. (1996). Roles of the 35S promoter and multiple overlapping domains in the pathogenicity of the pararetrovirus cauliflower mosaic virus. *Journal of Virology* **70**, 5414–5421.

UGAKI, M., UEDA, T., TIMMERMANS, M.C.P., VIEIRA, J., ELLISTON, K.O. AND MESSING, J. (1991). Replication of a geminivirus derived shuttle vector in maize endosperm cells. *Nucleic Acids Research* **9**, 371–377.

VALKONEN, J.P.T. AND SOMERSALO, S. (1996). Patterns and barriers of cell–to–cell movement and lack of systemic spread of tobacco etch potyvirus (TEV-GUS) in *Solanum brevidens*. *Plant Science* **113**, 221–228.

VAN DEN BOOGAART, T., WEN, F., DAVIES, J.W. AND LOMONOSSOFF, G.P. (2001). Replicase-derived resistance against *Pea early browning virus* in *Nicotiana benthamiana* is an unstable resistance based upon post-transcriptional gene silencing. *Molecular Plant-Microbe Interactions* **14**, 196–203.

VAN DER VOSSEN, E.A.G., NEELEMAN, L. AND BOL, J.F. (1996). The 5' terminal sequence of alfalfa mosaic virus RNA 3 is dispensable for replication and contains a determinant for symptom formation. *Virology* **221**, 271–280.

VAN VLOTEN-DOTING, L., BOL, J.F. AND CORNELISSEN, B. (1985). Plant virus-based vectors for gene transfer will be of limited use because of the high error frequency during viral RNA synthesis. *Plant Molecular Biology* **4**, 323–326.

VERCH, T., YUSIBOV, V. AND KOPROWSKI, H. (1998). Expression and assembly of a full-length monoclonal antibody in plants using a plant virus vector. *Journal of Immunological Methods* **220**, 69–75.

VERVER, J., WELLINK, J., VAN LENT, J., GOPINATH, K. AND VAN KAMMEN, A. (1998). Studies on the movement of cowpea mosaic virus using the jellyfish green fluorescent protein. *Virology* **242**, 22–27.

VIAPLANA, R., TURNER, D.S. AND COVEY, S.N. (2001). Transient expression of a GUS reporter gene from cauliflower mosaic virus replacement vectors in the presence and absence of helper virus. *Journal of General Virology* **82**, 59–65.

WALDEN, R.M. AND HOWELL, S.H. (1982). Intergenomic recombination events among pairs of defective cauliflower mosaic virus genomes in plants. *Molecular and Applied Genetics* **1**, 447–456.

WARD, A., ETESSAMI, P. AND STANLEY, J. (1988). Expression of a bacterial gene in plants mediated by infectious geminivirus DNA. *EMBO Journal* **7**, 1583–1587.

WIRTZ, U., OSBORNE, B. AND BAKER, B. (1997). Ds excision from extrachromosomal geminivirus vector DNA is coupled to vector DNA replication in maize. *Plant Journal* **11**, 125–135.

WOOLSTON, C.J., BARKER, R., GUNN, H., BOULTON, M.I. AND MULLINEAUX, P.M. (1988). Agroinfection and nucleotide sequence of cloned wheat dwarf virus DNA. *Plant Molecular Biology* **11**, 35–43.

WOOLSTON, C.J., REYNOLDS, H.V., STACEY, N.J. AND MULLINEAUX, P.M. (1989). Replication of wheat dwarf virus DNA in protoplasts and analysis of coat protein mutants in protoplasts and plants. *Nucleic Acids Research* **17**, 6029–6041.

WRIGHT, E.A., HECKEL, T., GROENENDIJK, J., DAVIES, J.W. AND BOULTON, M.I. (1997). Splicing features in maize streak virus virion- and complementary-sense gene expression. *Plant Jounal* **12**, 1285–1297.

YAMAYA, J., YOSHIOKA, M., MESHI, T., OKADA, Y. AND OHNO, T. (1988). Expression of tobacco mosaic virus RNA in transgenic plants. *Molecular and General Genetics* **211**, 520–525.

YUSIBOV, V., MODELSKA, A., STEPLEWSKI, K., AGADJANYAN, M., WEINER, D., HOOPER, D.C. AND KOPROWSKI, H. (1997). Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 5784–5788.

ZHANG, G.C., LEUNG, C., MURDIN, L., ROVINSKI, B. AND WHITE, K.A. (2000). *In planta* expression of HIV-1 p24 protein using an RNA plant virus-based expression vector. *Molecular Biotechnology* **14**, 99–107.

ZIEGLER, A., COWAN, G.H., TORRANCE, L., ROSS, H.A. AND DAVIES, H.V. (2000). Facile assessment of cDNA constructs for expression of functional antibodies in plants using the potato virus X vector. *Molecular Breeding* **6**, 327–335.