Use of Viral Replicons for the Expression of Genes in Plants

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Abstract

Autonomously replicating virus-based vectors have been investigated as a means of introducing heterologous genes into plants. This approach has a number of potential advantages over stable genetic transformation, particularly in terms of speed and levels of expression that can be obtained. Several groups of plant viruses, with genomes consisting of both DNA and RNA, have been investigated as possible gene vectors. In the case of DNA viruses, it has generally been possible to identify nonessential regions of the genome that can be replaced by foreign sequences. However, there appear to be limitations on the size of insert which can be tolerated. In the case of RNA viruses, replacement of viral sequences usually has a drastic effect on the viability. However, in several cases it has proved possible to substantially increase the size of the viral genome by the direct insertion of additional sequences while still retaining the ability of the viruses to multiply and spread in plants. These RNA virus-based systems appear to have the greatest potential as gene vectors.

Index Entries: Plant virus; vector; gene transfer; chimeric particles; protein engineering.

1. Introduction

In recent years there has been an increasing level of interest in the possibility of using plants as "bioreactors" for the expression of heterologous genes. The most commonly used approach to achieve such expression involves integrating foreign DNA sequences into the plant genome and regenerating fertile plants expressing the sequence of interest *(1)*. Gene transfer to higher plants can be accomplished using *Agrobacterium*-based vectors or by direct gene transfer techniques that involve physical methods for the delivery of DNA into plant cells *(2)*. Once the foreign sequence is integrated into the plant genome, it is heritable. There are, however, a number of difficulties that can be encountered in the production of transgenic plants: the process is time-consuming, requiring several months, some plant species are refractory to regeneration, the levels of expression of the foreign gene are often low, and undesirable phenomena such as transgene-silencing and cosuppression are often encountered *(3,4)*.

An alternative to genetic transformation involves the use of autonomously replicating virus-based vectors to express the sequence of interest. There are several potential advantages to this approach: the absence of "position effects" in the plant chromosomes, which can result in variable levels of expression of the foreign gene in transgenic plants, the fact that infection with a virus-based vector is far simpler and quicker than the production of stable transformants and, most importantly, the fact that amplification of the foreign gene during multiplication of the viral vector should lead to a concomitant high level of expression of the encoded protein. The development of virus-based vectors for the expression of heterologous sequences in plants is the subject of this review.

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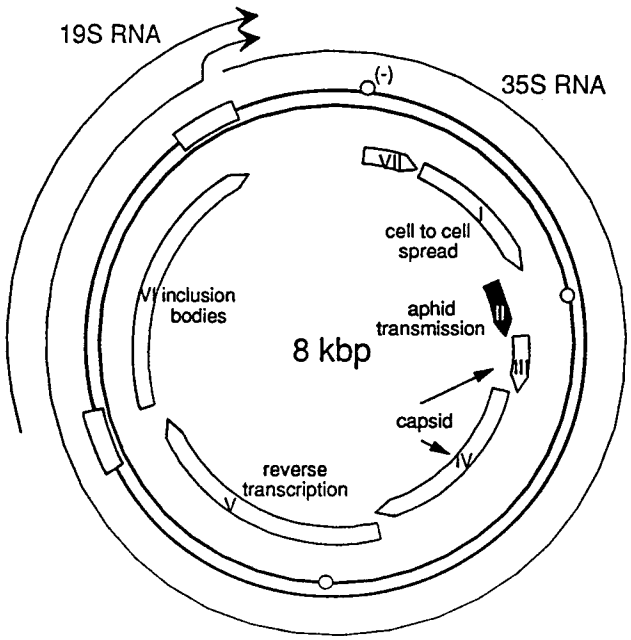

Fig. 1. Organization and features of the genome of CaMV. Inner arrows represent ORFs encoding polypeptides whose functions are indicated. The bold circles correspond to the two DNA strands that contain discontinuities shown by small open circles. On the minus strand, the positions of transcription promoters are indicated by open boxes and the resulting RNA transcripts are shown as outer arrows. Gene II (shown in black) is dispensable for infectivity and has been replaced by a number of foreign genes.

2. Double-Stranded DNA Plant Viruses

There are only two groups of plant viruses that have a genome consisting of double-stranded DNA: the caulimo- and the badnaviruses. So far, only the caulimoviruses have been investigated as potential gene vectors. The caulimoviruses have circular ds DNA genomes of about 8 kbp that contain discontinuities at specific sites and that are packaged in spherical particles. They have narrow host-ranges and infect only dicotyledons. The DNA is transcribed asymmetrically into a genome-length polycistronic 35S RNA with a terminal redundancy of 180 nucleotides which serves as a template for viral replication by means of a virus-encoded reverse transcriptase. The genome organization of the type member of the caulimoviruses, cauliflower mosaic virus (CaMV) is shown in Fig. 1.

CaMV was the first plant virus to be investigated as a vector because it had a number of potentially attractive features: its genomic DNA can be readily manipulated by recombinant DNA techniques, cloned viral DNA is infectious by mechanical inoculation *(5,6)*, and the virus replicates efficiently in host cells with more than 10^6 viral genomes accumulating per cell *(7)*. However, investigations of the replication cycle of CaMV indicated that the virus had a number of properties which might limit its use as a vector. First, a very high recombination rate has been observed *(6)* probably because the viral replication mode involves template switches by the reverse transcriptase. Second, translation of CaMV ORFs from the polycistronic 35S RNA appears to be a very complex process and great care must be taken when making insertions into the genome to avoid disrupting this process *(8,9)*.

Gronenborn et al. *(10)* showed that it was possible to insert foreign DNA sequences into gene II of CaMV, the gene that encodes a protein responsible for aphid transmission of the virus, without abolishing the ability of the virus to infect plants. The maximum length of sequence that could be stably inserted was found to be about 250 bp. The reason for this limit is not clear but may reflect packaging constraints. Systematic deletion and insertional mutagenesis was subsequently used to determine which regions of the CaMV genome it is possible to modify without destroying virus viability *(11,12)*. Although several regions could tolerate small insertions, only gene II and the intergenic region (which contains ORF VII) could accept significant lengths of heterologous DNA. Both gene II and ORF VII were shown to be nonessential for virus infectivity *(9,13)*.

To date, all successful attempts to use CaMV as a vector have involved replacing gene II. Brisson et al. *(14)* cloned the 240-bp dihydrofolate reductase *(dhfr)* gene from plasmid R67 of *Escherichia coli* just downstream of the ORF I termination codon. After inoculation of turnip plants, a full systemic infection was obtained and DHFR enzyme activity could be found in plant extracts. In addition, the infected plants showed an increased tolerance to methotrexate, a toxin

which does not inactivate the R-plasmid-encoded DHFR *(15)*. Lefebvre et al. *(16)* inserted a 200-bp sequence encoding Chinese hamster metallotionein (MTII) into the gene II region. In systemically-infected leaves of turnip plants expression of MTII was estimated to represent 0.5% of the soluble leaf protein. De Zoeten et al. *(17)* introduced a 501-bp sequence encoding human interferon-αD (IFN-αD) into a CaMV vector from which both genes II and VII had been deleted. Expression levels of IFN-αD in inoculated turnip plants reached 2 μg/g of tissue fresh weight and the *in planta* produced interferon was shown to be biologically active.

It has been estimated that if all the nonessential regions of the viral genome were deleted, it should be possible to increase the maximum length of sequence which can be inserted into CaMV to approx 1000 bp *(18)*. If this proves possible it would greatly increase the potential usefulness of the virus as a vector. An alternative approach to increasing the maximum size of insert by using a pair of vectors containing overlapping deletions has recently been suggested *(19)*, but as yet, there are no reports of the successful application of such a system.

3. Single-Stranded DNA Viruses

Geminiviruses are a group of plant viruses with twinned quasiicosahedral particles. Their genomes consist of either one or two small (2.6–3 kb) circles of ssDNA. The bipartite geminiviruses, such as African cassava mosaic virus (ACMV) and tomato golden mosaic virus (TGMV) are generally whitefly-transmitted and infect dicotyledonous hosts while the monopartite geminiviruses, such as maize streak virus (MSV) and wheat dwarf virus (WDV), are transmitted by leafhoppers and generally infect monocots. Transcription of the genome is bidirectional and ORFs have been identified on both the virion (encapsidated) and the complementary strands *(20–23)*; these will be given the prefixes V and C, respectively.

The fact that geminiviruses reach high copy numbers in infected cells and replicate in the nucleus made them attractive candidates as gene vectors, especially as some members of the group infect agriculturally important monocots. However, although in some cases (e.g., ACMV) viral DNA is infectious when mechanically inoculated onto plants, the DNA of many geminiviruses, especially those infecting monocots is not infectious by this route and it is only since the development of the technique of agroinoculation *(24)* that their evaluation as potential gene vectors has been possible. In this technique, the viral genome is arranged as a tandem repeat or partial dimer within the T-DNA borders of an *Agrobacterium* plasmid. This is necessary to ensure that the cloned genome is able to form a complete replicative unit in cells by way of homologous recombination or a replicative mechanism.

3.1. Bipartite Geminiviruses

The genome organization of a bipartite geminivirus is presented in Fig. 2, the two genomic components being designated DNA A and DNA B. The viral coat protein, which is by far the most abundant viral protein in infected cells, is encoded by ORF AV1. Coat protein gene deletion mutants of ACMV *(25)* and TGMV *(26)* can spread systemically, causing symptoms in the host. Since the coat protein is not required for virus infection and movement, the ability of bipartite geminiviruses to act as gene vectors has been investigated by substituting a number of foreign sequences for AV1.

When the chloramphenicol acetyltransferase *(cat)* gene was substituted for AV1 in ACMV, the virus maintained its infectivity *(27)*. CAT activity could be detected in both the inoculated and the systemically infected leaves and was maintained over a period of 4 wk. Activity could also be detected after mechanical transmission of the viral DNA from infected leaves to healthy plants. In similar experiments the coat protein coding region of TGMV DNA A was replaced by the bacterial neomycin phosphotransferase *(nptII)* gene to give TA*neo* *(28)*. *Nicotiana tabacum* plants agroinoculated with TA*neo* and DNA B did not show symptoms but systemic spread of TA*neo* could be demonstrated by hybridization of leaf DNA with labeled TGMV probes. Enzyme activity could be correlated with the average copy number of the *neo* gene.

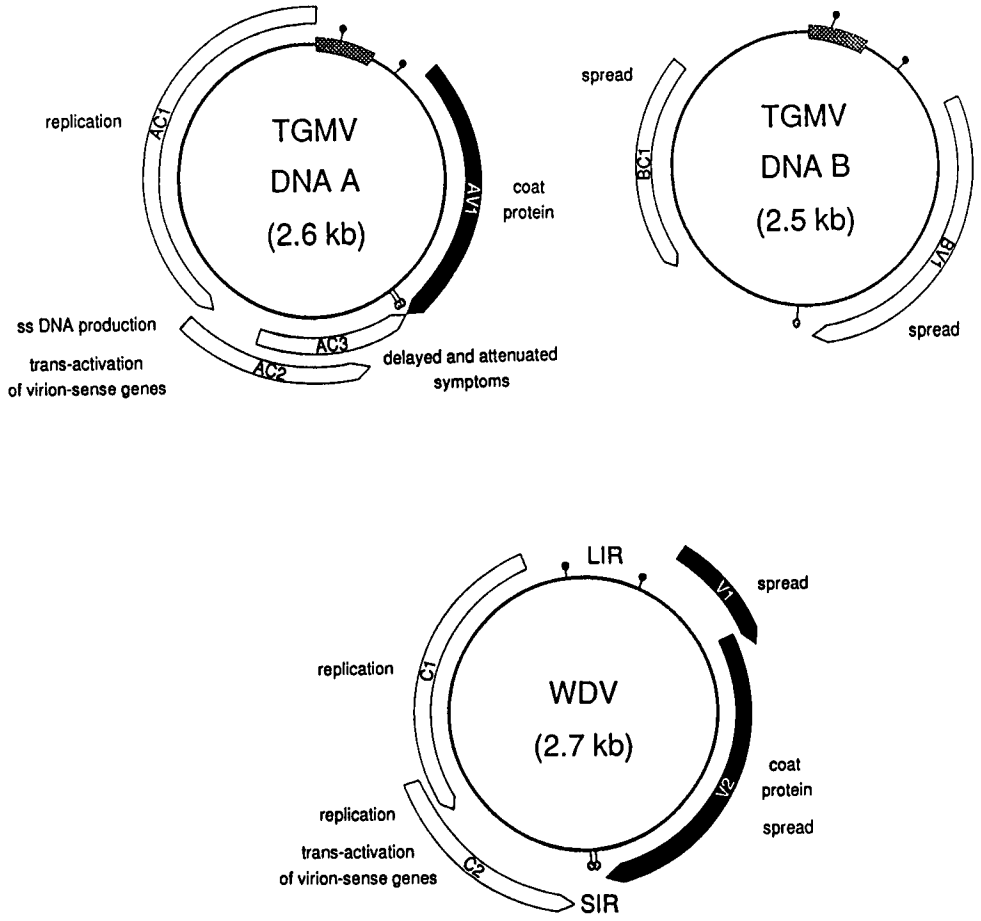

Fig. 2. Genome organization of a bipartite (TGMV) and a monopartite (WDV) geminivirus. The functions of viral ORFs are indicated next to the corresponding arrows. The single-stranded genomic DNA is represented by the bold circle on which the location of transcription initiation signals are marked by small solid filled circles. Polyadenylation signal sequences are shown by small shaded circles. The region common to both the A and B component of TGMV is indicated by a shaded box. In WDV, SIR = small intergenic region, LIR = large intergenic region. Genes dispensable for viral replication and which have been substituted by foreign sequences have been filled in black.

In the case of both the ACMV *cat* and the TGMV *neo* constructs, the heterologous gene was very similar in size to the coat protein gene which it replaced. To determine if genes significantly larger could be stably substituted for AV1, *N. tabacum* plants transgenic for a head-to-tail dimer of DNA B were agroinoculated with DNA A in which the 1.8-kbp bacterial β-glucuronidase *(gus)* gene replaced the 0.74-kbp coat protein gene *(29)*. Southern blotting of DNA from noninoculated leaves using a TGMV DNA A probe showed that in a high proportion of the infected plants the hybrid DNA A (TA*gus*) had undergone deletions giving rise to molecules down to the size of wild-type DNA A. Absence of GUS activity in leaf extracts of these plants suggested that the deletions had occurred in the reporter gene or its promoter. Using a similar TGMV*gus* vector, Elmer and Rogers *(30)* showed that although the *gus*-substituted DNA A could replicate in leaf disks, it could not move systemically; the only TGMV A derivatives found in systemically infected leaves were the size of wild-type DNA A and sequence analysis showed that they had deletions in the *gus* gene.

The aforementioned results indicate that DNA A molecules harboring heterologous sequences are stable only when the total size of the DNA is similar to that found in the wild-type virus. Since the viral coat protein is dispensible for virus infection, this limitation cannot be due to packaging constraints and it has been suggested that the discrimination against larger DNAs operates at the level of systemic movement in the plant *(30)*. Whatever the reason, the instability found in vectors carrying sequences which increase the total size of the DNA clearly imposes a severe limit on the use of bipartite geminiviruses as vectors.

3.2. Monopartite Geminiviruses

The genome map of a monopartite geminivirus (WDV) is shown in Fig. 2. The coat protein of monopartite geminiviruses (encoded by ORF V2), and the product of ORF V1, are both nonessential for viral replication, but are required for the systemic spread of the virus through the plant *(31–33)*. Thus, though deletions and insertions within the virion-sense transcription unit are tolerated from the point of view of replication *(32,34)*, the resulting viruses cannot spread throughout a plant. The same applies to insertions made in the small intergenic region (SIR) *(35,36)*. This lack of systemic movement has restricted the use of monopartite geminivirus-based vectors to the transient expression of foreign sequences in cell cultures or locally in agroinoculated plants.

In the case of MSV, substitution of the *cat* gene for the V2 gene followed by agroinoculation of the modified virus on to the leaves of maize plants resulted in detectable CAT expression *(31)*. However due to the inability of the viral vector to spread from the site of inoculation, CAT expression from contaminating *Agrobacterium*, instead of episomal MSV, could not be excluded. A construct containing the *gus* gene inserted into the SIR of MSV was able to replicate in maize cells of agroinfected plants, but did not cause symptoms and could not spread systemically *(35)*. It was concluded that the increased size of the modified viral genome may have resulted in failure of encapsidation. A similar MSV construct, containing the *bar* gene instead of *gus* has recently been shown capable of conferring resistance to the herbicide Basta in agroinoculated maize cells *(36)*.

Substitution of the V2 gene of WDV by the *npt*II gene resulted in NPTII expression in seed-derived wheat embryos *(37)*. Wheat protoplasts transfected with a WDV-based vector supported viral replication, when the V2 gene (845 bp) was replaced by the similarly sized *npt*II gene *(38)*. NPTII activity could be detected from 24 h after transfection until d 13 and was about 20 times higher when NPTII was expressed in a replicating vector compared to a nonreplicating C1 mutant. This construct was also able to replicate and express NPTII in protoplasts of maize and rice which are nonhost plants for WDV. V2 replacement constructs containing either the *cat* gene (shorter than V2) or the *lac* Z gene of *E. coli*, (which doubles the size of the WDV genome) were also replication competent, as was a construct in which NPTII was fused to the N-terminus of V2. This indicates that there are not strict limits on the size of the genome which can be replicated.

A 3.7 kbp *E. coli*-plant cell shuttle vector based on WDV has been developed *(39)*. The region encoding V2 and the 3' end of V1 was replaced by the selectable marker gene *npt*II, functional in both plant and *E. coli* cells, and by the p15A replication origin of an *E. coli* plasmid. After electroporation into maize endosperm protoplasts this vector replicated to high copy number and could be rescued into *E. coli* without significant rearrangements. Insertion of the 3 kbp *gus* reporter gene (under the control of the CaMV 35S gene promoter and terminator) between *npt*II and p15 *ori*, resulted in expression of GUS, which correlated with the active amplification of the viral DNA. This shuttle vector proved useful for the study of viral replication in maize cells *(40)* but applications to whole plants remain to be demonstrated.

4. Single-Stranded RNA Viruses

The majority of plant viruses have genomes that consist of one or more molecules of single-stranded RNA. A number of these viruses grow to

extraordinarily high titers in infected plants and could potentially make ideal vectors. However, the development of RNA virus-based vectors was initially precluded by the difficulties involved in genetically manipulating RNA. Demonstration that in vitro transcripts of cloned cDNA copies of brome mosaic virus (BMV) were infectious when inoculated onto plants *(41,42)* radically changed the situation and the genetic manipulation of RNA plant viruses is now routine. More recently it has been demonstrated that plasmids containing full-length cDNA copies of viral RNAs cloned downstream of the CaMV 35S promoter are directly infectious on plants *(43)*, thus facilitating further the development of RNA plant viruses as gene vectors.

4.1. Expression of Unfused Proteins

BMV was the first RNA virus to be engineered for the expression of a foreign coding sequence. This virus, which infects primarily monocots, has icosahedral particles and a tripartite genome. Although all 3 RNAs are required for an infection of whole plants, RNA1 and RNA2 can replicate on their own in protoplasts, allowing alterations to be made to RNA 3 (Fig. 3). When the coat protein was replaced by the *cat* gene *(44)* replication of RNA3 in barley protoplasts was reduced, compared to that of wt RNA3. Nevertheless a higher level of CAT activity was found in the infected cells than was obtained in plants transgenic for the *cat* gene *(45)*. No attempt was made to infect whole plants with the construct.

Barley stripe mosaic virus (BSMV), which has three RNAs encapsidated in rod-shaped virions (Fig. 3) and infects both cereals and dicots was also engineered to serve as an expression vector in protoplasts *(46)*. Replacement of ORFb (translated from RNA β by synthesis of a subgenomic RNA) by the firefly luciferase *(luc)* reporter gene and transfection into tobacco (dicot) and maize (monocot) protoplasts in the presence of both RNAs α and γ, resulted in luciferase activity 20- to 123-fold higher than that obtained after transfection with a *luc* gene mRNA. Replacement of the 5' proximal ORFa by the *luc* gene revealed that the deleted sequences had a *cis*-acting function in the replication of RNA β.

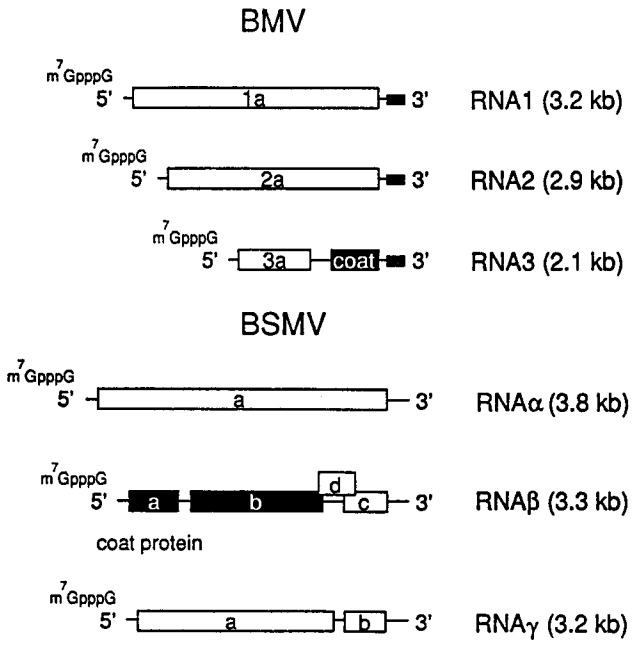

Fig. 3. Schematic maps of the genomes of BMV and BSMV. In the case of BMV, the shaded boxes represent the conserved 3' region between the three RNAs. RNAs 1 and 2 encode proteins involved in viral replication. RNA 3 encodes protein 3a which has a role in viral spread and the coat protein subunit. In the case of BSMV, RNA α and ψ code for polypeptides, which are part of the viral replicase, while RNA β codes for the coat protein (ORFa) and for three nonstructural proteins (ORFs b, c, and d) required for systemic infection. For both viruses, translation of all the genes not 5' proximal on the polycistronic RNAs occurs by synthesis of subgenomic RNAs. The ORFs replaced by foreign sequences are solid filled.

Tobacco mosaic virus (TMV) has rod-shaped, 300-nm long particles containing a single molecule of positive-sense RNA. The genome organization and transcription strategy is shown in Fig. 4. Its ability to reach enormous titers in infected plants, its wide host range and the potential lack of packaging constraints make TMV a particularly attractive candidate as an RNA virus-based vector. When the coat protein (CP) gene is subjected to deletions *(47)* or replaced by the *cat* gene *(48)*, the virus can still produce local symptoms on inoculated leaves but can no longer spread systemically, indicating that the coat protein is essential for long distance virus movement.

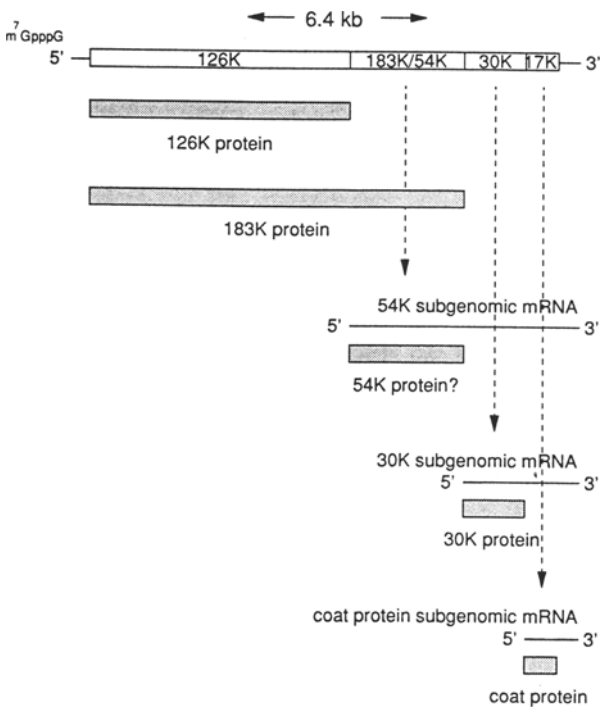

Fig. 4. Diagram of the TMV genome and its mode of translation. The TMV RNA has ORFs for five gene products. The 183K protein results from readthrough of the termination codon of the 126K protein and these N-co-terminal proteins are both involved in viral replication. The other proteins (putative 54K protein, 30K movement protein, and 17K coat protein) whose cistrons do not begin at the 5' end of the viral genome are translated from separate 3' co-terminal subgenomic mRNAs.

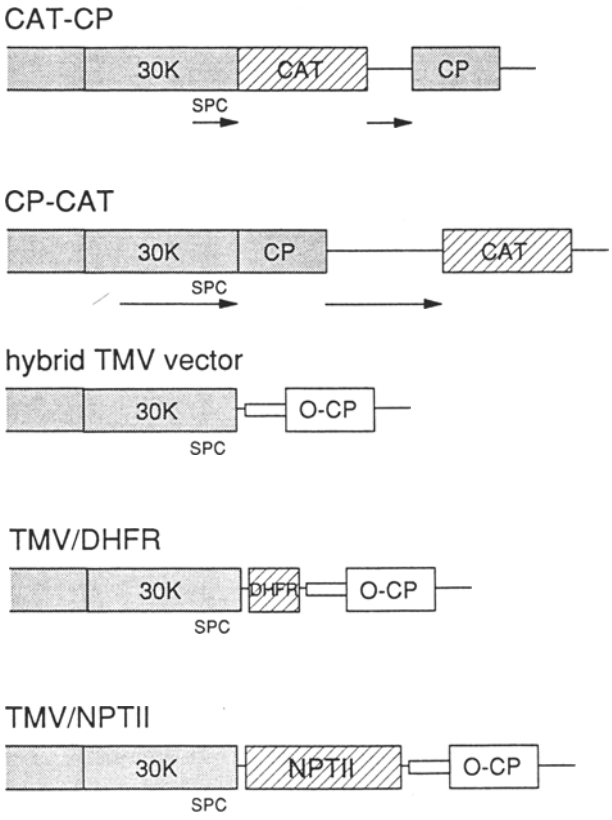

Fig. 5. Schematic diagrams of TMV expression vectors. **(A)** TMV mutants in which the gene coding for CAT is added into the complete TMV-U1 genome. The *cat* gene is fused behind the subgenomic promoter for the coat protein in order to produce an additional subgenomic RNA. The resulting sequence duplications are indicated by arrows underneath each construct. **(B)** Hybrid TMV expression vector in which all ORFs are TMV-U1 sequences, except the coat protein sequence which originates from ORSV and is transcribed into mRNA from its own, ORSV-derived, subgenomic RNA promoter (shown as a small open box). Foreign genes coding for DHFR and NPTII are expressed via subgenomic mRNAs transcribed from the heterologous TMV-U1 promoter located in the 30K ORF. SPC, subgenomic promoter for the TMV-U1 coat protein.

The ability of the TMV genome to tolerate the addition of foreign sequences was explored by introduction of a copy of the TMV CP subgenomic promoter sequence fused to the *cat* gene *(49)*. When this *cat* gene cartridge was placed between the CP gene and the 3' nontranslated region (Fig. 5A; CP-CAT), the hybrid virus replicated poorly and did not spread systemically. By contrast, when the insertion was made between the 30K and the CP genes (Fig. 5A; CAT-CP), replication was efficient, CAT activity was detectable and virus particles of increased length (350 nm) accumulated. However, during systemic infection, progeny virions the size of wild-type TMV (300 nm) also appeared. This was shown to be the result of an exact deletion of the inserted sequences, probably owing to homologous recombination between the two copies of the CP subgenomic promoter, flanking the insert *(50)*. Similar results have also been obtained when the suitability of potato virus X (PVX) to act as a gene vector was investigated *(51)*. As in the case of

TMV, replacement of the coat protein gene by a foreign sequence (in this case the *gus* gene) disabled virus movement. Addition of the *gus* gene coupled to a copy of the promoter for the coat protein subgenomic mRNA resulted in systemic infection and synthesis of GUS in inoculated and systemically infected tissue. This construct, like the homologous TMV-based vector, was unstable owing to duplication of the coat protein promoter. Nonetheless, the PVX-based system has successfully been used to express the fungal avirulence gene, *Avr9*, in plants *(52)*.

To avoid the problem of instability discussed above, a hybrid expression vector containing sequences from two tobamoviruses, TMV-U1 and *Odontoglossum* ringspot virus (ORSV) has been designed *(53)*. Promoters of TMV-U1 and ORSV, which share a low sequence similarity (45%), were used to direct synthesis of the subgenomic RNAs for the foreign gene and the ORSV CP gene respectively (Fig. 5B). Two bacterial sequences were inserted independently between the 30K protein of TMV-U1 and the ORSV CP cartridge: the 238 bp DHFR ORF and the larger 832 bp NPTII ORF. Both chimeras moved systemically in *Nicotiana benthamiana*, but on serial passage, the *npt*II gene was partially deleted, a phenomenon considered insert-specific and not an intrinsic feature of the expression vector. This vector system was subsequently used to express α-trichosanthin, a eukaryotic ribosome-inactivating protein from the roots of a Chinese medicinal plant called *Trichosanthes kirilowii (54)*. α-Trichosanthin purified from systemically infected leaf tissue of *N. benthamiana* was shown to be biologically active in vitro and to represent at least 2% of total soluble protein. This compares very favorably with the expression level obtained in *E. coli*, which reached only 0.01% of total cellular protein *(55)*. A derivative of this vector expressing the CP of tomato mosaic virus (ToMV) instead of that of ORSV has recently been used to manipulate a biosynthetic pathway *in planta (56)*. A cDNA encoding phytoene synthase (PSY) or part of phytoene desaturase (PDS), both isolated from ripening tomato fruit, was placed under the transcriptional control of the TMV-U1 CP subgenomic promoter and the constructs used to alter the carotenoid biosynthetic pathway in *N. benthamiana*. Leaves from plants expressing PSY developed a bright orange phenotype owing to overproduction of the enzyme. Plants transfected with PDS constructs developed a white phenotype. When the partial PDS gene was cloned into the TMV vector such that an antisense RNA transcript *(57)* was generated, the synthesis of carotenoids was inhibited, as expression of the endogenous PDS gene was turned off. Downregulation of this gene was also observed when the PDS cDNA was cloned into the TMV vector in the sense orientation. This was attributed to a phenomenon of "cosuppression" analogous to the inhibition of endogenous genes observed in transgenic plants containing sense RNA *(58,59)*.

4.2. Production of Foreign Peptides Fused to Viral Coat Proteins

One approach to producing novel vaccines is to identify portions of proteins from pathogens that can elicit an immune response in animals and then to chemically synthesize short peptides corresponding to these portions. Such synthetic oligopeptides, although capable of stimulating the production of antibodies against the protein from which they are derived, have generally been found to be insufficiently immunogenic to serve as vaccines. To overcome this problem, epitope-presentation systems have been developed in which the peptide sequence is biochemically fused to a carrier molecule, which is often a protein able to assemble into a macromolecular structure, typically a virus capsid. The high titers to which many RNA plant viruses grow make them attractive candidates for such a use.

Cowpea mosaic virus (CPMV) is an icosahedral virus, 28 nm in diameter, with a bipartite positive-sense RNA genome. The RNAs are initially translated into precursor polyproteins which are subsequently cleaved by a virus-encoded protease (Fig. 6). The virus capsid is made of 60 copies each of a large (L) and a small (S) coat protein, both of which are encoded by the smaller genomic RNA (RNA 2). Knowledge of the 3-dimensional structure of the particles *(60)* allows the site of

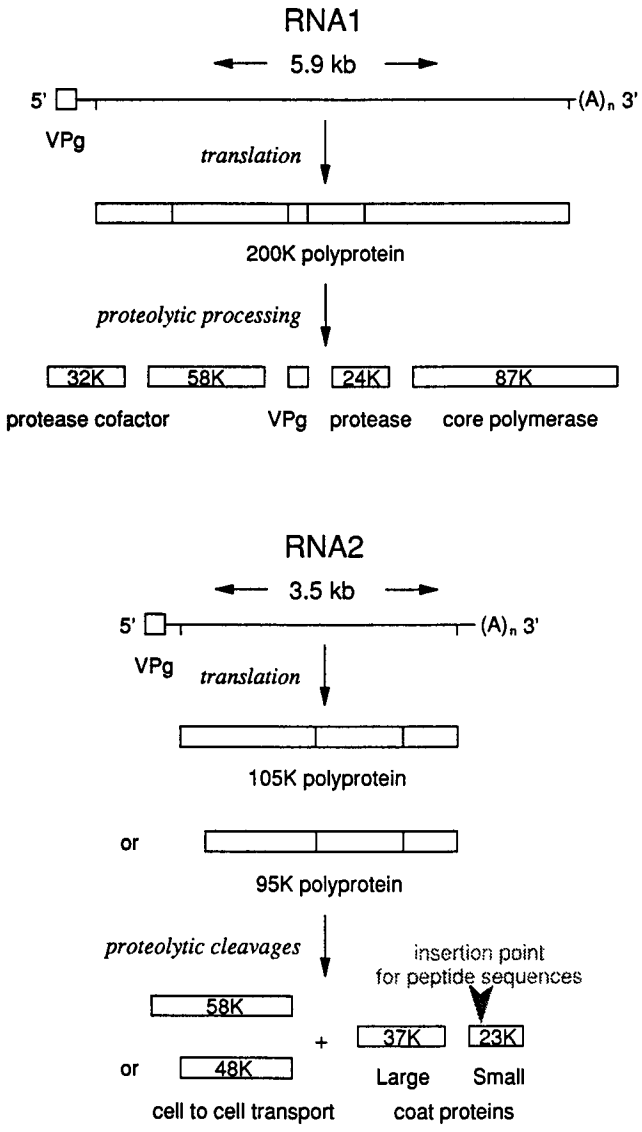

Fig. 6. Diagram of the CPMV genome and its expression products. Each of the two genomic RNAs contains a single long ORF and is translated into a large polyprotein. Maturation of this precursor protein occurs by cleavage with virus-encoded proteases. In the case of RNA2 both a 105K and a 95K protein are produced as a result of secondary initiation downstream of the first in-phase AUG codon. RNA 1 encodes the replication-associated functions and RNA 2 codes for the cell-to-cell movement protein and the two coat proteins. Insertion of foreign peptide sequences have been made in the small coat protein as indicated by the grey arrow.

insertion to be selected in such a way that the heterologous sequence should not interfere with virus assembly. Ease of propagation and purification of the virus makes CPMV an attractive candidate for an epitope-presentation system. Initially an antigenic site derived from foot-and-mouth disease virus (FMDV) was inserted into a portion of RNA 2 encoding a loop on the surface of the S protein *(61)*. When the modified RNA 2 was inoculated onto cowpea plants in the presence of RNA 1, particles were produced that had the antigenic properties of the FMDV insert. Reversion of the initial construct to wild-type on serial passaging, once again probably the result of homologous recombination, was prevented by optimization of the site of insertion and the elimination of repeated sequences *(62)*. This has allowed the production of genetically stable constructs containing epitopes from human immunodeficiency virus type 1 (HIV-1) and human rhinovirus 14 (HRV-14) as well as from FMDV *(62)*. The particles containing the HIV-1 and HRV-14 epitopes could be readily purified, with a yield in excess of 1 mg of virus per gram of leaf tissue, similar to that obtained with wild-type virus. The purified chimeric particles possessed the antigenic properties of the insert as shown by reactivity with sera specifically directed against the foreign epitopes. Upon injection into experimental animals insert-specific antibodies were raised *(62)* and in the case of the HIV-1 chimera, these antibodies were capable of neutralizing the HIV-1 infection of T-cells in vitro *(63)*. CPMV thus appears to have a significant potential as an epitope presentation system leading to the production of vaccines in plants.

The C-terminal portion of the coat protein of TMV projects outwards from the virus particle and a number of foreign peptides have been fused at this position. Leu-enkephalin, a pentapeptide with opiate-like activity, was inserted just before the stop codon of the CP (64). The fusion protein accumulated to 3/10 the yield of the wild-type CP in protoplasts, but only local lesions were obtained in systemic host plants and no virus particles were formed. This indicates that modification of the C-terminus of all

the 2300 subunits which comprise a virus particle can prevent virus assembly. To circumvent this problem, a six-base 3' context sequence *(65)* which permits readthrough of the stop codons was inserted between the stop codon of the TMV CP and the foreign sequence allowing both unfused and fused forms of coat protein to be produced. This approach was used to design a TMV-based construct containing angiotensin-I-converting enzyme inhibitor (ACEI), a 12 amino-acid peptide with antihypertensive effects, fused to the coat protein *(66)*. When the construct was used to infect tobacco and tomato plants, systemic spread occurred and virus particles containing wild-type CP and CP-ACEI fusion protein in a ratio of 20:1 were formed.

A similar approach to that used in the case of the ACEI chimeric TMV, allowed expression of an antigenic site from the *Plasmodium yoelii* circumsporozoite (CS) protein at the C-terminus of the TMV coat protein *(67)*. Another malarial B-cell epitope from *Plasmodium vivax* CS protein was also inserted into the surface loop region of the CP of TMV. RNA transcripts of both mutants induced mosaic symptoms in the upper leaves of Xanthi tobacco from which virus could be recovered in yields ranging from 0.4 to 1.2 mg/g fresh wt. Progeny virus was genetically stable and expression of the respective epitopes was confirmed by Western blot using monoclonal antibodies directed against the sequences selected for surface engineering of TMV.

5. Conclusions

Although it was over 15 yr ago that the use of plant viruses as gene vectors was first proposed *(68,69)*, it is only in the past 2–3 yr that their use in this regard has become a practical reality. The RNA viruses, which were the last to be investigated, rather than the DNA viruses, are proving the most useful. Initial fears that the high rate of mutation of RNA viruses would result in rapid loss of inserted sequences, which are not under selective pressure *(70)*, have dwindled as data about the variability and mutation rate of both wild-type *(71,72)* and recombinant *(73)* tobamoviruses have become available. These data show that plant RNA virus populations are, in fact, rather stable. Thus RNA plant virus-based vectors may well be a viable method for the production of novel proteins in plants.

Acknowledgments

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References

1. Christou, P. (1994) Genetic engineering of crop legumes and cereals: current status and recent advances. *AGRO food INDUSTRY hi-tech* **5,** 17–27.
2. Draper, J. and Scott, R. (1991) Gene transfer to plants, in *Plant Genetic Engineering* (Grierson, D., ed.), Blackie, Glasgow, London, pp. 38–81.
3. Flavell, R. B. (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Natl. Acad. Sci. USA* **91,** 3490–3496.
4. Finegan, J. and McElroy, D. (1994) Transgene inactivation: plants fight back! *Biotechnology* **12,** 883–888.
5. Howell, S. H., Walker, L. L., and Dudley, R. K. (1980) Cloned cauliflower mosaic virus DNA infects turnips *(Brassica rapa). Science* **208,** 1265–1267.
6. Lebeurier, G., Hirth, L., Hohn, B., and Hohn, T. (1982) *In vivo* recombination of cauliflower mosaic virus DNA. *Proc. Natl. Acad. Sci. USA* **79,** 2932–2936.
7. Maule, A. J. (1985) Replication of caulimoviruses in plants and protoplasts, in *Molecular Plant Virology,* vol. 2 (Davies, J. W., ed.), CRC, Boca Raton, FL, pp. 161–190.
8. Sieg, K. and Gronenborn, B. (1982) Introduction and propagation of foreign DNA in plants using cauliflower mosaic virus as a vector, in *NATO Adv. Study Course: Structure and Function of Plant Genomes,* p. 154.
9. Dixon, L. K. and Hohn, T. (1984) Initiation of translation of the cauliflower mosaic virus genome from a polycistronic mRNA: evidence from deletion mutagenesis. *EMBO J.* **3,** 2731–2736.
10. 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.
11. Dixon, L. K., Koenig, I., and Hohn, T. (1983) Mutagenesis of cauliflower mosaic virus. *Gene* **25,** 189–199.
12. Daubert, S., Shepherd, R. J., and Gardner, R. C. (1983) Insertional mutagenesis of the cauliflower mosaic virus genome. *Gene* **25,** 201–208.
13. Howarth, A. J., Gardner, A. C., Messing, J., and Shepherd, R. J. (1981) Nucleotide sequence of naturally occurring deletion mutants of cauliflower mosaic virus. *Virology* **112,** 678–685.
14. Brisson, N., Paszkowski, J., Penswick, J. R., Gronenborn, B., Potrykus, I., and Hohn, T. (1984)

Expression of a bacterial gene in plants. *Nature* **310,** 511–514.
15. Pattishall, K. H., Acar, J., Burchall, J. J., Goldstein, F. W., and Harvey, R. J. (1977) Two distinct types of trimethoprim-resistant dihydrofolate reductase specified by R-plasmids of different compatibility groups. *J. Biol. Chem.* **252,** 2319–2323.
16. Lefebvre, D. D., Miki, B. L., and Laliberte, J. F. (1987) Mammalian metallothionein functions in plants. *Biotechnology* **5,** 1053–1056.
17. De Zoeten, 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.
18. Futterer, J., Bonneville, J. M., and Hohn, T. (1990) Cauliflower mosaic virus as a gene expression vector for plants. *Physiologia Plantarum* **79,** 154–157.
19. Hirochika, H. and Hayashi, K. (1991) A new strategy to improve a cauliflower mosaic virus vector. *Gene* **105,** 239–241.
20. Townsend, R., Stanley, J., Curson, S. J., and Short, M. N. (1985) Major polyadenylated transcripts of cassava latent virus and location of the gene encoding coat protein. *EMBO J.* **4,** 33–37.
21. 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 Res.* **20,** 7237–7256.
22. Sunter, G., Gardiner, W. E., and Bisaro, D. M. (1989) Identification of tomato golden mosaic virus-specific RNAs in infected plants. *Virology* **170,** 243–250.
23. Dekker, E. L., Woolston, C. J., Xue, Y., 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 Res.* **19,** 4075–4081.
24. 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.
25. 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 Res.* **14,** 5981–5998.
26. Gardiner, W., 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 J.* **7,** 899–904.
27. Ward, A., Etessami, P., and Stanley, J. (1988) Expression of a bacterial gene in plants mediated by infectious geminivirus DNA. *EMBO J.* **7,** 1583–1587.
28. Hayes, R. J., Petty, I. T. D., Coutts, R. H. A., and Buck, K. W. (1988) Gene amplification and expression in plants by a replicating geminivirus. *Nature* **334,** 179–182.
29. 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 Res.* **17,** 2391–2403.
30. Elmer, S. and Rogers, S. G. (1990) Selection for wild type size derivatives of tomato golden mosaic virus during systemic infection. *Nucleic Acids Res.* **18,** 2001–2006.
31. 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 J.* **8,** 1023–1032.
32. Woolston, C. J., Reynolds, H., 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 Res.* **17,** 6029–6041.
33. Mullineaux, P. M., Boulton, M. I., Bowyer, P., Van der Vlugt, R., Marks, M., Donson, J., and Davies, J. W. (1988) Detection of a non-structural protein of M_R 11,000 encoded by the virion DNA of maize streak virus. *Plant Mol. Biol.* **11,** 57–66.
34. Boulton, M. I., Steinkellner, H., Donson, J., Markham, P. G., King, D. I., and Davies, J. W. (1989) Mutational analysis of the virion-sense genes of maize streak virus. *J. Gen. Virol.* **70,** 2309–2323.
35. Shen, W.-H. and Hohn, B. (1994). Amplification and expression of the β-glucuronidase gene in maize plants by vectors based on maize streak virus. *Plant J.* **5,** 227–236.
36. Shen, W.-H. and Hohn, B. (1995). Vectors based on maize streak virus can replicate to high copy numbers in maize plants. *J. Gen. Virol.* **76,** 965–969.
37. Töpfer, R., Gronenborn, B., Schell, J., and Steinbiss, H. H. (1989) Uptake and transient expression of chimeric genes in seed-derived embryos. *Plant Cell* **1,** 133–139.
38. Matzeit, W., Schaefer, S., Kammann, M., Schalk, H. S., 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.
39. 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 Res.* **19,** 371–377.
40. 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 Res.* **20,** 4047–4054.
41. Ahlquist, P. and Janda, M. (1984) cDNA cloning and in vitro transcription of the complete brome mosaic virus genome. *Mol. Cell. Biol.* **4,** 2876–2882.
42. Ahlquist, P., French, R., Janda, M., and Loesch-Fries, S. (1984) Multicomponent RNA plant virus infection

derived from cloned viral cDNA. *Proc. Natl. Acad. Sci. USA* **81,** 7066–7070.

43. Mori, M., Mise, K., Kobayashi, K., Okuno, T., and Furusawa, I. (1991). Infectivity of plasmids containing brome mosaic virus cDNA linked to the cauliflower mosaic virus 35S promoter. *J. Gen. Virol.* **72,** 243–246.
44. 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.
45. Herrera-Estrella, L., Van den Broeck, G., Maenhaut, R., Van Montagu, M., and Schell, J. (1984) Light-inducible and chloroplast-associated expression of a chimaeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. *Nature* **310,** 115–120.
46. 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 J.* **9,** 2663–2669.
47. Dawson, W. O., Bubrick, P., and Grantham, G. L. (1988) Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement and symptomatology. *Phytopathology* **78,** 783–789.
48. Takamatsu, N., Ishikawa, M., Meshi, T., and Okada, Y. (1987) Expression of bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. *EMBO J.* **6,** 307–311.
49. 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.
50. King, A. M. Q. (1988) Genetic recombination in positive strand RNA viruses, in *RNA Genetics*, vol 2 (Domingo, E., Holland, J., and Ahlquist, P. eds.), CRC, Boca Raton, FL, pp. 149–165.
51. Chapman, S., Kavanagh, T., and Baulcombe, D. (1992) Potato virus X as a vector for gene expression in plants. *Plant J.* **2,** 549–557.
52. Hammond-Kosack, K. E., Staskawicz, B. J., Jones, J. D. G., and Baulcombe, D. C. (1995) Functional expression of a fungal avirulence gene from a modified potato virus X genome. *MPMI* **8,** 181–185.
53. Donson, J., Kearney, C. M., Hilf, M. E., and Dawson, W. O. (1991) Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. USA* **88,** 7204–7208.
54. 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., Jr., and Grill, L. K. (1993) Rapid, high-level expression of biologically active α-trichosanthin in transfected plants by an RNA viral vector. *Proc. Natl. Acad. Sci. USA* **90,** 427–430.
55. Shaw, P. C., Yung, M. H., Zhu, R. H., Ho, W. K. K., Ng, T. B., and Yeung, H. W. (1991) Cloning of trichosanthin cDNA and its expression in *Escherichia coli*. *Gene* **97,** 267–272.
56. Kumagai, M. H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. K. (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. USA* **92,** 1679–1683.
57. Gray, J., Picton, S., Shabbeer, J., Schuch, W., and Grierson, D. (1992) Molecular biology of fruit ripening and its manipulation with antisense genes. *Plant Mol. Biol.* **19,** 69–87.
58. van der Krol, A. R., Mur, L. A., and Beld, M. (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2,** 291–299.
59. Fray, R. G. and Grierson, D. (1993) Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. *Plant Mol. Biol.* **22,** 589–602.
60. Lomonossoff, G. P. and Johnson, J. E. (1991) The synthesis and structure of comovirus capsids. *Prog. Biophys. Molec. Biol.* **55,** 107–137.
61. Usha, R., Rohll, J. B., Spall, V. E., Shanks, M., Maule, A. J., Johnson, J. E., and Lomonossoff, G. P. (1993) Expression of an animal virus antigenic site on the surface of a plant virus particle. *Virology* **197,** 366–374.
62. Porta, C., Spall, V. E., Loveland, J., Johnson, J. E., Barker, P. J., and Lomonossoff, G. P. (1994) Development of cowpea mosaic virus as a high-yielding system for the presentation of foreign peptides. *Virology* **202,** 949–955.
63. McLain, L., Porta, C., Lomonossoff, G. P., Durrani, Z., and Dimmock, N. J. (1995) Human immunodeficiency virus type 1 neutralizing antibodies raised to a gp41 peptide expressed on the surface of a plant virus. *Aids and Human Retroviruses* **11,** 327–334.
64. Takamatsu, N., Watanabe, Y., Yanagi, H., Meshi, T., Shiba, T., and Okada, Y. (1990) Production of enkephalin in tobacco protoplasts using tobacco mosaic virus RNA vector. *FEBS* **269,** 73–76.
65. Skuzeski, J. M., Nichols, L. M., Gesteland, R. F., and Atkins, F. (1991) The signal for a leaky UAG codon in several plant viruses includes the two downstream codons. *J. Mol. Biol.* **218,** 365–373.
66. Hamamoto, H., Sugiyama, Y., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y., and Okada, Y. (1993) A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. *Biotechnology* **11,** 930–932.
67. Turpen, T. H., Reinl, S. J., Charoenvit, Y., Hoffman, S. L., Fallarme, V., and Grill, L. K. (1995) Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Biotechnology* **13,** 53–57.

68. 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.
69. Hull, R. (1978) The possible use of plant viral DNAs in genetic manipulation in plants. *Trends Biochem. Sci.* **3,** 254–256.
70. 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 Mol. Biol.* **4,** 323–326.
71. Rodríguez-Cerezo, E., Moya, A., and García-Arenal, F. (1989) Variability and evolution of the plant RNA virus pepper mild mottle virus. *J. Virol.* **63,** 2198–2203.
72. Rodríguez-Cerezo, E. and García-Arenal, F. (1989) Genetic heterogeneity of the RNA genome population of the plant virus U5-TMV. *Virology* **170,** 418–423.
73. 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.