
Preface

Altogether, the biochemical, technical and economic limitations on existing prokaryotic and eukaryotic expression systems and the growing clinical demand for complex therapeutic proteins have created substantial interest in developing new expression systems for the production of therapeutic proteins. To that end, plants have emerged in the past decade as a suitable alternative to the current production systems, and today their potential for production of high quality, much safer and biologically active complex recombinant pharmaceutical proteins is largely documented.

The chapters in this volume, contributed by leaders in the field, sum up the state-of-the-art methods for using a variety of different plants as expression hosts for pharmaceutical proteins. Several production platforms are presented, ranging from seed- and leaf-based production in stable transgenic plant lines, to plant cell bioreactors, to viral or *Agrobacterium*-mediated transient expression systems. Currently, antibodies and their derived fragments represent the largest and most important group of biotechnological products in clinical trials. This explains why the potential of most production platforms is illustrated here principally for antibodies or antibody fragments with acknowledged potential for immunotherapy in humans. In addition, a comparison of different plant expression systems is presented using aprotinin, a commercial pharmaceutical protein, as a test system.

Although multiple books and monographs have been recently published on molecular pharming, there is a noticeable dearth of bench step-by-step protocols that can be used quickly and easily by beginners entering this new field. This volume aims to fill the void by presenting detailed protocols for using the main plant expression systems, for rapidly detecting and quantifying recombinant proteins in a crude plant protein extract. Several chapters feature methods to improve the yield and stability of recombinant proteins using targeting to different subcellular organelles, expression of protease inhibitors or fusion to carrier sequences.

Most biopharmaceutical products have the potential to be immunogenic in at least a small population of human subjects. In this respect, the immunogenicity of plant N-glycans in mammals is a major concern. Protocols, extra notes and problem-solving tips are presented to define whether, how and where a pharmaceutical protein expressed in plants is glycosylated. However, until a number of plant-derived therapeutic glycoproteins have completed their clinical development and registration process, the risk related to parenteral administration of these products in humans remains purely theoretical. Principles and methods of biosafety and risk assessment of plant-derived therapeutic proteins for humans and for the environment are detailed in the last two chapters, which are dedicated to the safe development of plant-made pharmaceuticals.

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L. Faye, V. Gomord

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Chapter 2

Cowpea Mosaic Virus-Based Systems for the Expression of Antigens and Antibodies in Plants

Frank Sainsbury, Li Liu, and George P. Lomonosoff

Summary

This chapter describes the use of Cowpea mosaic virus-based vectors for the production of foreign proteins such as antigens and antibodies in plants. The systems include vectors based on both full-length and deleted versions of RNA-2. In both cases, the modified RNA-2 is replicated by coinoculation with RNA-1. The constructs based on full-length RNA-2 retain the ability to spread systemically throughout an inoculated plant and the infection can be passaged. The vector based on a deleted version of RNA-2 can stably incorporate larger inserts but lacks the ability to move systemically. However, it has the added advantage of biocontainment. In both cases, vector constructs modified to contain a foreign gene of interest can be delivered by agroinfiltration to obtain transient expression of the foreign protein. If required, the same constructs can also be used for stable nuclear transformation. Both types of vector have proved effective for the production in plants of a diverse range of proteins including antigens and antibodies.

Key words: CPMV, Full-length viral vector, Deleted viral vector, Agroinfiltration, Suppressor of silencing, Virus passaging

2.1. Introduction

A number of RNA viruses have been developed over the last 20 years as vectors for the expression of foreign peptides and polypeptides (1, 2), including antigens and antibodies (3–5), in plants. The advantages of using viruses for expression include the fact that (i) viral genomes are small and infectious cDNA copies are, therefore, easy to manipulate, (ii) infection of plants with modified viruses is much simpler and quicker than the regeneration of stably transformed lines of plants, and (iii) a sequence inserted into a virus vector will be highly amplified during viral

replication. Unavoidably, there are also some potential disadvantages: there are likely to be size constraints on the sequences which can be inserted while retaining virus viability, the inserted sequence might be susceptible to “genetic drift” during virus replication, and there are biocontainment concerns over the use of vectors based on fully competent viruses. As a result, attention has recently turned toward the development of plant virus-based expression systems that can alleviate some of these disadvantages while retaining the advantageous features of viral vectors. These are often based on defective versions of viral RNAs which, though able to replicate, cannot spread in the environment (6, 7).

Cowpea mosaic virus (CPMV) is the type member of the Genus *Comovirus* in the family *Comoviridae*. It infects a number of legume species and grows to particularly high titers in its natural host, cowpea (*Vigna unguiculata*). It also infects the experimental host, *Nicotiana benthamiana*. The genome of CPMV consists of two separately encapsidated positive-strand RNA molecules of 5889 (RNA-1) and 3481 (RNA-2) nucleotides. The RNAs each contain a single open reading frame (ORF) and are expressed through the synthesis and subsequent processing of precursor polyproteins (Fig. 2.1a). RNA-1 encodes proteins involved in the replication of viral RNAs and polyprotein processing. RNA-2 encodes the 48K movement protein and the two coat proteins, which are essential for cell to cell movement and systemic spread. The enzyme responsible for processing both the RNA-1- and the RNA-2-encoded polyproteins is the RNA-1-encoded 24K proteinase. Importantly, infectious cDNA clones of both viral RNAs are available, allowing manipulation of the viral genome and the generation of viral infection by agroinfiltration (8). The fact that CPMV infects edible plants and grows to very high titers (yields of particles reaching ~1 g/Kg infected cowpea tissue) makes CPMV a very attractive system for the production of pharmaceutical proteins. It has already enjoyed considerable success as an “epitope presentation” system in which short antigenic sequences are expressed on the surface of assembled virus particles (9, 10). More recent work has concentrated on the development of CPMV as a method of expression whole polypeptides in plants and this application is the subject of this chapter.

The development of CPMV-based systems for the expression of whole proteins has so far focused entirely on modifying the sequence of RNA-2. Replication functions are provided by coinoculating the RNA-2 constructs with unmodified RNA-1. Two types of system have been developed based on either full-length or deleted versions of RNA-2. In the first instance, the foreign protein to be expressed is added to the normal complement of RNA-2 proteins, resulting in an increase in size of RNA-2. Since all the normal functions are retained, the resulting modified

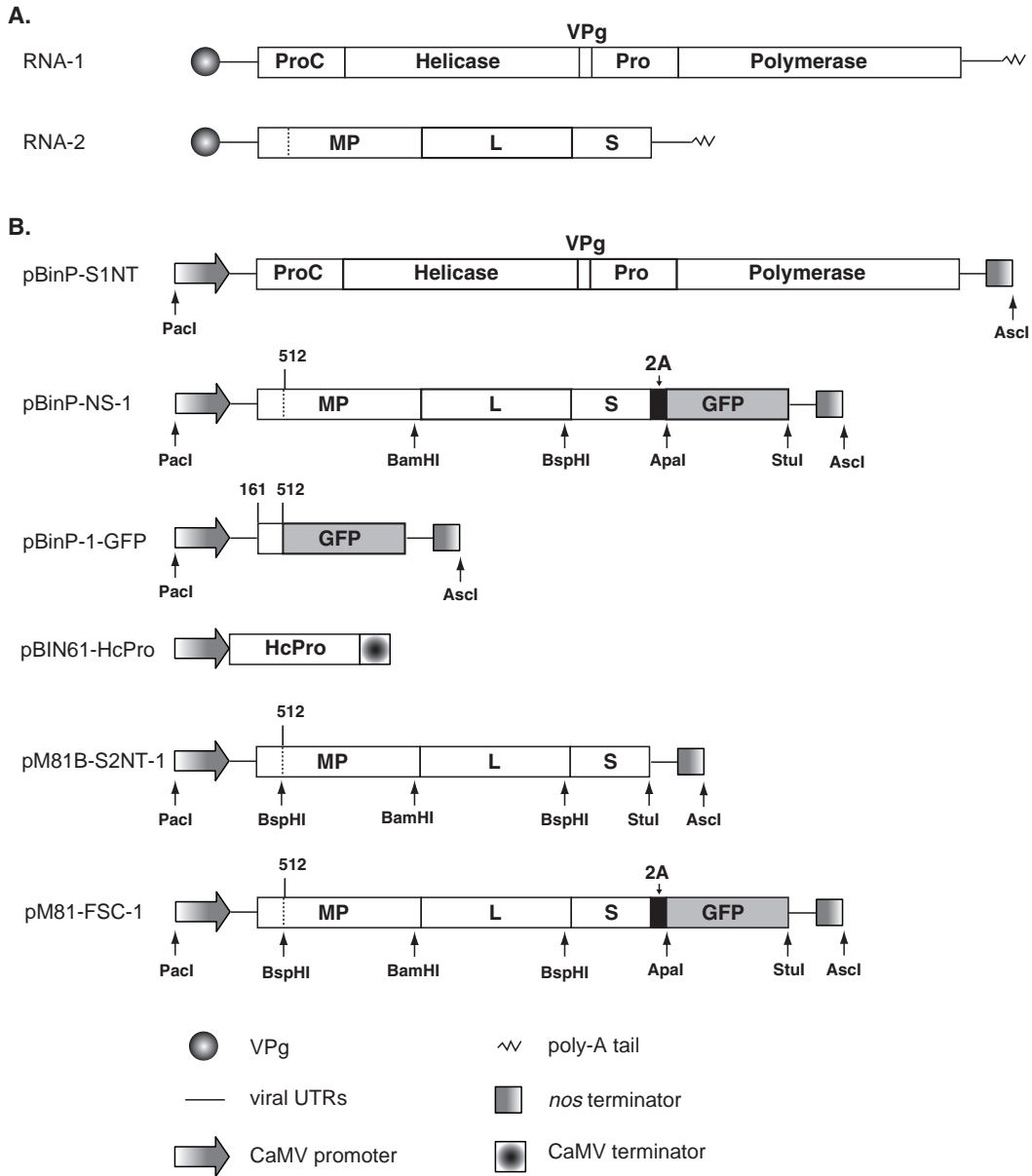


Figure 2.1. (a) Organization of CPMV genomic RNAs. The functions of the regions of the polyproteins are indicated. Abbreviations used are as follows: *ProC* proteinase co-factor, *Pro* proteinase, *MP* movement protein, *L* large coat protein, *S* small coat protein. (b) Structure of constructs used for the expression of foreign proteins. Relevant restriction sites are indicated.

virus can spread systemically within the inoculated plant and can be transmitted to other plants. In the second case, the foreign gene replaces most of the RNA-2 ORF, permitting larger inserts to be incorporated but abolishing the ability of the virus to spread both within and between plants.

2.1.1. Vectors Based on Full-Length CPMV RNA-2

In these systems, the sequence encoding the polypeptide is fused in-frame with the RNA-2-encoded polyprotein. This means that an appropriate site for the insertion of the foreign sequence had to be identified and a method developed for the release of the inserted protein from the viral polyprotein. While a number of approaches have been investigated, the most successful has involved the insertion of the foreign sequence at the C-terminus of the RNA-2 polyprotein (i.e. immediately after the S protein). Release of the foreign sequence is achieved through the action of 2A catalytic peptide sequence from foot-and-mouth-disease virus inserted between the S protein and the foreign sequence (11). The utility of this approach was first demonstrated with GFP when the pUC9-based construct, pCP2/S-2A-GFP, was shown to be able to systemically infect cowpea plants in the presence of RNA-1 and to express GFP at a level which represented ~1–2% of total soluble protein (11). Plasmid pCP2/S-2A-GFP was designed so that the sequence of GFP could be removed by digestion with *ApaI* and *StuI* and replaced by a sequence with *ApaI*- and *StuI* (blunt)-compatible ends. The use of *ApaI* is particularly significant as its recognition sequence, GGGCCC, encodes the dipeptide G-P which is cleaved by the action of the 2A catalytic peptide. Thus, the inserted sequence (originally GFP) possesses only a single additional proline residue at its N-terminus.

To enable the pCP2/S-2A-GFP system to be used in conjunction with agroinfiltration, the modified RNA-2 sequence was inserted into a modified version of the binary vector pBINPLUS (12) in which the *ApaI* at position 9005 had been removed (13). This gave rise to the vector pBinP-NS-1 in which the sequence of GFP can be replaced with any foreign sequence via the unique *ApaI* and *StuI* restriction sites in a one-step procedure (13). The pBinP-NS-1 derivative can then be used to agroinfiltrate leaves of *N. benthamiana* or cowpea in the presence of the RNA-1 construct pBinP-SINT (8). Initially, inoculated cowpea plants rarely, if ever, develop symptoms of infection, and it is generally necessary to passage the infections on to further plants to obtain sufficient material for characterization.

One of the advantages of using the foot-and-mouth-disease virus 2A sequence to achieve release of the foreign protein is that the processing is co- rather than posttranslational. This means that it is possible to achieve targeting of the expressed protein to the secretory pathway by the addition of an appropriate signal peptide. The expressed protein can also be retained in the endoplasmic reticulum if a retention signal is added to the C-terminus of the protein (Nicholson, Sainsbury, Porta, and Lomonosoff, unpublished data).

The full-length RNA-2-based vector pBinP-NS-1 has been successfully used to express assembled Hepatitis B virus core antigen (HBcAg) particles (14) and small immune proteins (SIPs);

15, 16). In the latter case, the plant-expressed protein was shown to be capable of passively immunizing pigs against challenge.

2.1.2. Deleted Versions of RNA-2

Two of the major limitations on the use of plant viral vectors based on full-length RNA molecules are concerns over biocontainment and size constraints on the inserted sequence. While the ability of infections derived from pBinP-NS-1 derivatives to spread both within a host plant and to other healthy plants is advantageous in terms of scaling-up the manufacture of the expressed protein, it simultaneously raises the possibility of unintentional environmental spread. The problem of the size constraints relates to the fact that longer RNA-2 molecules replicate more slowly than the corresponding wild-type molecule. This is reflected both in terms of low yield and the genetic stability of the inserted sequence. These problems have indeed been observed with full-length RNA-2 molecules modified to express both HBcAg and SIP molecules.

To address these concerns, a system based on a defective form of CPMV RNA-2 has been developed. The concept is based on the observation that the sequences necessary for replication of RNA-2 by the RNA-1-encoded replicase lie exclusively at the 5' and 3' ends of the RNA (17). This allows most of the RNA-2 ORF to be deleted without affecting the ability of RNA-2 to be replicated. However, while the essential 3' terminal sequence lies exclusively within the 3' UTR, the 5' region extends beyond the first in-frame AUG (position 161), which means that initiation of translation of the foreign gene should be driven by the second in-frame AUG at position 512.

The ability of a deleted version of RNA-2 carrying a foreign gene to be replicated by RNA-1 was tested by creating construct pBinP-1-GFP (Fig. 2.1b) in which expression of GFP was driven by the AUG at 512 (7). It was found that RNA-1 alone was unable to replicate RNA derived from pBinP-1-GFP as the deleted version of RNA-2 lacked the region encoding the CPMV suppressor of silencing which resides at the C-terminus of the S protein (18, 19). This problem could be relieved by simultaneously supplying the suppressor of silencing, HcPro, from *Potato virus Y* (pBIN61-HcPro in Fig. 2.1b; 20). The replication of pBinP-1-GFP-derived RNA in the presence of both RNA-1 and a suppressor allowed high levels of GFP to be expressed. Replication of pBinP-1-GFP by RNA-1 in the presence of a suppressor could be achieved in several different formats: by supplying all three components simultaneously to nontransgenic leaves by agroinfiltration, by transforming plants with some of the components and inducing expression by agroinfiltration with the others, or by transforming plants with all three components (7).

To create a useful cloning vector for the expression of foreign proteins from a pBinP-1-GFP-based plasmid, the complete sequence of RNA-2 flanked by the Cauliflower mosaic virus

(CaMV) 35S promoter and nopaline synthase (*nos*) terminator from pBinP-S2NT (8) was inserted into mutagenesis plasmid pM81W (21) as an *AscI*/*PacI* fragment. The resulting plasmid, pM81W-S2NT, was subjected to a single round of mutagenesis which simultaneously introduced four changes (*see* method in *ref.* 21) to give pM81B-S2NT-1. The mutagenesis removed two *Bsp*HI sites from the vector backbone and introduced a *Bsp*HI site (TCATGA) around AUG 512 and a *Stu*I site (AGGCCT) after UAA 3299, the termination codon for the RNA-2-encoded polyprotein. Subsequently, the *Bam*HI/*AscI* fragment was excised from pBinP-NS-1 and ligated into similarly digested pM81B-S2NT-1, yielding pM81-FSC-1 (**Fig. 2.1b**). This vector allows the whole of the RNA-2 ORF downstream of AUG 512 to be excised by digestion with *Bsp*HI and *Stu*I and replaced with any sequence with *Bsp*HI and *Stu*I (blunt)-compatible ends. The use of the *Bsp*HI site is important as it preserves the AUG at 512 and this initiator is used to drive translation of the inserted gene.

To express the foreign gene in plants, the pM81-FSC-1-derived plasmid is digested with *AscI* and *PacI* and the fragment containing the foreign sequences transferred to similarly digested pBINPLUS and the resulting plasmids are finally transformed into *Agrobacterium tumefaciens*. In its simplest format, an *Agrobacterium* suspension containing the deleted version of RNA-2 harboring the inserted gene can be coinfiltrated into leaves in the presence of suspensions of bacteria containing RNA-1 and HC-Pro. This approach has been used successfully to express HBcAg (Mechtcheriakova, personal communication) and to express two proteins simultaneously by coinfiltration with two separate RNA-2-based constructs. In this case, fully formed IgG molecules have been assembled in leaves by coinfiltrating with separate constructs containing the heavy and light chains systems (22).

The use of deleted versions of RNA-2, lacking the regions encoding both the movement protein and the viral coat proteins, provides the high levels of gene amplification associated with viral vectors without the possibility of the modified virus spreading in the environment. Recent results show that even in the presence of wild-type RNA-2, the construct 1-GFP is not transencapsidated indicating that even accidental coinfection with wild-type CPMV would not lead to the spread of the foreign sequence (Sainsbury and Lomonosoff, unpublished data). Furthermore, this system should support replication of much larger inserts as the RNA-2-based replicon is intrinsically smaller than wild-type RNA-2.

The lack of ability of constructs based on pM81-FSC-1 to spread either within or between plants also has certain disadvantages. Infections are not able to spread systemically throughout a plant, with the site of expression of the foreign protein being limited to the infiltrated region. Similarly, it is not possible to bulk up material simply by passing an infection to healthy plants.

However, these disadvantages can potentially be overcome by incorporating a construct based on the deleted version of RNA-2 as transgene and inducing expression by crossing with plants transgenic for RNA-1 and a suppressor (7). Clearly, such an approach is more time-consuming than a purely transient system based on agroinfiltration but has the potential to generate lines of plants expressing high levels of a foreign protein throughout the plant.

2.2. Materials

2.2.1. Plants, Plasmids, and Bacterial Strains

1. *N. benthamiana* and cowpea plants (*Vigna unguiculata*) are grown in greenhouses with supplemental light to 16 h at a constant temperature of 24°C for *N. benthamiana* and 25°C for cowpea.
2. Plasmids described for cloning, subcloning, and *Agrobacterium* transformation are prepared using the QIAprep mini-prep Kit (Qiagen, West Sussex, UK) for high purity.
3. *Escherichia coli* strain used is DH5 α .
4. *Agrobacterium tumefaciens* strain LBA4404 (23) is used for all transformations.

2.2.2. Enzymes, Materials, and Commercial Kits

1. *Apa*I (New England Biolabs, Hertfordshire, UK)
2. *Bsp*HI (New England Biolabs)
3. *Stu*I (New England Biolabs)
4. *Pac*I (New England Biolabs)
5. *Asc*I (New England Biolabs)
6. Alkaline Phosphatase, Calf Intestinal (New England Biolabs)
7. T4 DNA ligase (Roche Diagnostics, East Sussex, UK)
8. QIAprep Miniprep Kit (Qiagen)
9. QIAquick Gel Extraction Kit (Qiagen)
10. RNeasy Plant Mini Kit (Qiagen)
11. Protoscript First Strand cDNA synthesis Kit (New England Biolabs)
12. Butter muslin
13. Carborundum (Sigma-Aldrich, Cambridgeshire, UK)

2.2.3. Buffers and Solutions

1. Luria-Bertani (LB) media: 10 g/L Bacto-tryptone, 10 g/L NaCl, and 5 g/L Yeast extract, pH 7.0.
2. MMA: 10 mM MES (2-[N-morpholino]ethanesulfonic acid; Sigma-Aldrich) pH 5.6, 10 mM MgCl₂, 100 μ M Acetosyringone (Sigma-Aldrich).

3. Sodium Phosphate buffer, 0.1 M, pH 7.0.
4. Protein extraction buffer: 50 mM Tris-HCl pH 7.25, 150 mM NaCl, 2 mM EDTA, 0.1% (v/v) Triton X-100 (Sigma-Aldrich).

2.3. Methods

2.3.1. Restriction Site Cloning

Cloning and subcloning into both vector systems is straightforward with PCR-generated inserts amplified with appropriate restriction sites at both ends (*see Note 1*). As mentioned in the Introduction, cloning into the full-length RNA-2 vector can be done in a single step with pBinP-NS-1 (*see Note 2*). For the deleted RNA-2 vector, the entire expression cassette including the CaMV promoter and *nos* terminator is released from pM81-FSC-1 following the insertion of the gene of interest and inserted into pBINPLUS (*12*).

2.3.1.1. Cloning into Full-Length RNA-2

1. Digest both the insert and plasmid pBinP-NS-1 with *ApaI* and *StuI* by mixing components including the appropriate buffer according to the enzyme manufacturer's recommendation in a 1.5 mL microcentrifuge tube (*see Note 3*).
2. Dephosphorylate vector with alkaline phosphatase following the manufacturer's instruction (*see Note 4*).
3. Resolve digests on a 1% agarose gel and purify vector and inserts using QIAquick gel extraction kit (*see Note 5*).
4. Combine in ligase buffer with T4 DNA ligase and incubate according to manufacturer's recommendations.
5. Transform competent *E. coli* and plate onto an LB agar plate with kanamycin (50 µg/mL) selection.
6. Colonies may be screened by PCR or restriction analysis. Positive clones are grown overnight and plasmids are extracted for sequencing, confirmation of insertion, and *Agrobacterium* transformation.

2.3.1.2. Cloning into the Deleted Vector

1. Digest both the insert and plasmid pM81-FSC-1 with *BspHI* and *StuI* by mixing components including the appropriate buffer according to the enzyme manufacturer's recommendation in a 1.5 mL microcentrifuge tube and incubate at 37°C for 1 h.
2. Dephosphorylate vector with alkaline phosphatase following the manufacturer's instruction (*see Note 4*).
3. Resolve digests on a 1% agarose gel and purify vector and inserts using QIAquick gel extraction kit (*see Note 6*).

4. Combine in ligase buffer with T4 DNA ligase and incubate according to manufacturer's recommendations.
5. Transform competent *E. coli* and plate onto an LB agar plate with carbenicillin (100 µg/mL) selection.
6. Colonies may be screened by PCR or restriction analysis. Positive clones are grown overnight, and plasmids are extracted for sequencing, confirmation, and subcloning.
7. Upon confirmation of clones, digest plasmid with *PacI* and *AscI* by mixing components including the appropriate buffer according to the enzyme manufacturer's recommendation in a 1.5 mL microcentrifuge tube and incubate at 37°C for 1 h. Simultaneously digest pBINPLUS or pBINPLUS-derived plasmid, for example, pBinP-NS-1, in the same way.
8. Dephosphorylate vector with alkaline phosphatase following the manufacturer's instruction (*see Note 4*).
9. Resolve digests on a 1% agarose gel and purify vector and inserts using QIAquick gel extraction kit (*see Note 7*).
10. Combine in ligase buffer with T4 DNA ligase and incubate according to manufacturer's recommendations.
11. Transform competent *E. coli* and plate onto an LB agar plate with kanamycin (50 µg/mL) selection.
12. Colonies may be screened by PCR or restriction analysis. Positive clones are grown overnight and plasmids are extracted for sequencing, confirmation of insertion, and *Agrobacterium* transformation.

2.3.2. *Agrobacterium* Infiltration and Monitoring Expression

For CPMV-based expression of foreign proteins, agroinfiltration is an integral step. It is used to initiate viral infection of both cowpea and *N. benthamiana* plants in the case of full-length RNA-2 and for transient expression using the deleted RNA-2 system. In both case, RNA-1 must be provided and a suppressor of silencing is also required in the case of the deleted version of RNA-2.

2.3.2.1. *Agrobacterium* Infiltration (Agroinoculation)

1. *Agrobacterium* cultures are grown to stationary phase from glycerol stocks, fresh liquid, or solid media cultures.
2. Gently spin cells (~2,000 g) at 4–10°C for 20 min to pellet, and discard media.
3. Resuspend in MMA to an OD₆₀₀ of 1.0 (*see Note 8*).
4. Incubate for at least 2 h at room temperature or overnight at 4°C if necessary.
5. Mix equivalent volumes of equal-density cultures, with or without a suppressor of silencing (*see Note 9*).
6. Prick underside of leaf (*see Note 10*) with a razor blade or small pipette tip and gently pressure infiltrate the wound

against a counter pressure (provided by a finger) with the *Agrobacteria* mixture using a 1 or 2 mL syringe (*see* **Note 11**).

2.3.2.2. Monitoring Expression

Protein production in infiltrated leaf tissue is generally at a very high level and in the case of GFP, is visible *in vivo* under UV illumination. Therefore, it is sensible to include inoculation with a GFP-expressing construct in each experiment. Using both the full-length and deleted RNA-2-based vectors, expression of GFP in *N. benthamiana* becomes visible after 2 days, reaches a peak at 5–6 days, and remains at or near this level for at least 12 days. For constructs based on full-length RNA-2, symptoms of systemic spread appear after 8–10 days and continue to spread throughout the plant for the life of the plant. A pattern of high-GFP expression levels detectable under UV illumination follows that of systemic symptoms. Because of the thick cuticle of cowpea leaves, considerable damage is sustained during agroinoculation rendering the fluorescence of GFP indistinguishable from that of autofluorescence caused by the damage. Therefore, expression levels of GFP can be monitored under UV illumination only when the viral particles produced are passaged. Fluorescent infection foci appear on leaves inoculated with virus particles after ~10 days and systemic symptoms develop after 2–3 weeks.

2.3.3. RT-PCR Confirmation of Genetic Stability

When using full-length RNA-2-based vectors, RT-PCR of RNA extracted from infected leaves provides a useful check on the genetic stability of the foreign sequence within the viral genome. This step will also check for any possible WT CPMV contamination.

1. Harvest up to 100 mg of leaf tissue showing systemic symptoms and grind to a powder under liquid nitrogen.
2. Extract total RNA using the RNeasy Plant Mini Kit.
3. cDNA is synthesized from 1 µg of RNA using the Proto-script First Strand cDNA synthesis Kit.
4. PCR is carried out in a 20 µL reaction using standard reaction conditions (24) with 4 µL of the cDNA synthesis reaction as a template.

2.3.4. Virus Preparations and Passaging (Cowpea)

Passaging of viral infections is an important means for scaling-up the production of foreign proteins in cowpea plants. Obviously, this is only relevant for constructs based on full-length CPMV RNA-2. For passaging, highly purified virus preparations are not required. The following protocol describes a procedure for extracting virions sufficient for passaging. A detailed protocol for obtaining preparations of CPMV of higher purity can be found in *ref.* 25.

1. Harvest inoculated or systemically infected leaves from cowpea or *N. benthamiana* and grind to a powder under liquid nitrogen.
2. Add 2× volume of sodium phosphate buffer and vortex.

3. Filter through two layers of butter muslin and centrifuge at 3,500*g* for 25 min at 4°C.
4. Take supernatant and add ¼ volume of 1 M NaCl with 20% PEG 6000 (w/v, Sigma-Aldrich).
5. Incubate for 1 h at room temperature.
6. Centrifuge at 15,000*g* for 15 min at 4°C and discard supernatant.
7. Re-suspend pellet in 1/10 (10 mM) sodium phosphate buffer (0.1–1 mL/g of starting material, *see Note 12*)
8. Dust the first true leaves of young cowpea plants with carborundum (*see Note 13*).
9. Apply 100 µL of virus preparation and gently spread over the leaf with forefinger.
10. Symptoms should appear on inoculated leaves after 10–14 days and on upper trifoliate leaves 2–3 weeks of passaging.

2.3.5. Protein Extractions

Following extraction, recombinant proteins produced using the vectors described in this chapter are generally detectable by coomassie blue staining of SDS-PAGE separated samples. Other commonly used techniques for detection for antigens and antibodies expressed from CPMV include western blotting, enzyme-linked immunosorbant assays, or as in the case of HBcAg, electron microscopy (14). Further detailed characterization of the recombinant proteins produced by CPMV-based vectors should be carried out using techniques specific to the protein. Below is a general protocol for the extraction of soluble proteins from tissue inoculated with CPMV vectors that is compatible with most protein analysis methods.

1. Harvest inoculated or systemically infected leaf tissue.
2. Grind tissue to a powder under liquid nitrogen.
3. Suspend in 1–2× volume (w/w) of EB.
4. Centrifuge at ~15,000*g* for 10 min at 4°C.
5. Collect supernatant and store at 4°C for up to 12 h or frozen for longer periods (*see Note 14*).

2.4. Notes

1. The *Bsp*HI site is also compatible with *Nco*I and *Pci*I. The choice of restriction enzymes permits different nucleotides to be incorporated immediately after the ATG start codon. The recognition sites for the enzymes are T/CATGA for *Bsp*HI, C/CATGG for *Nco*I, and A/CATGT for *Pci*I, permitting the incorporation of an A, G, or T after the ATG. The enzyme

FatI (/CATG) allows any base to follow the start codon, but it is of limited use as this enzyme has a four base recognition site. To incorporate a C after the ATG, the insert may be digested with *SphI* (GCATG/C) followed by removal of the 3' overhang by "polishing." The blunt end fragment can then be ligated into *BspHI*/*StuI*-cut pM81-FSC-1 after the 5' overhang of the *BspHI* has been filled in.

2. Low copy number and the large size of pBINPLUS-based plasmids may increase the difficulty of directly cloning into them. Therefore, it is also possible to use pM81-FSC-1 for cloning into the full-length vector with subsequent subcloning into pBINPLUS as for the deleted version of RNA-2. To do this, follow 3.1.2 using *ApaI* and *StuI* restriction sites.
3. Despite the manufacturer's recommendation to carry out *ApaI* digests at 25°C, we find that the *ApaI*/*StuI* double digest is effective at 37°C.
4. Calf intestinal alkaline phosphatase from New England Biolabs may be used with any of the buffers supplied with the enzymes described in this chapter. Therefore, following heat inactivation of the restriction enzymes, phosphatase may be added to the digest reaction mixture and incubated for a further 60 min.
5. *ApaI*/*StuI*-cut pBinP-NS-1 will yield a fragment ~15 kb long, corresponding to the vector, and a fragment of 0.7 kb corresponding to the GFP sequence.
6. *BspHI*/*StuI*-cut pM81-FSC-1 will result in three fragments: the 4.5 kb vector and two fragments of 2.0 and 1.6 kb corresponding to the RNA-2 coding region (including the 2A and GFP sequences), which contains an internal *BspHI* site.
7. *PacI*/*AseI*-cut pM81-FSC-1 clones will result in a vector fragment of 2.9 kb. If the insert into the deleted RNA-2 is between 1.2 and 2.0 kb, it will be necessary to further digest the vector backbone. Provided there is no recognition sequence in the insert for *BglI*, this enzyme will reduce the backbone to three fragments of 1.4, 1.2, and 0.3 kb. *PacI*/*AseI*-cut pBINPLUS plasmids will result in a vector fragment of 11 kb. If pBin-NS-1 is used, another fragment of ~5.2 kb, corresponding to full-length RNA-2 plus GFP, will also be present.
8. Resuspension to OD600 of 1.0 usually requires 1–1.5 times volume of MMA to that of the starting culture. Although each culture tends to grow at a different rate, allowing cultures to grow to stationary phase generally ensures that all cultures have an equal density.
9. Including the suppressor of silencing will increase efficiency of viral infection initiated by the full-length vector and increase protein yield in infiltrated tissue. For the deleted

vector, the presence of a suppressor of silencing is essential. A number have been cloned into expression cassettes within pBIN61 (D. Baulcombe, pers. comm.). We have seen varying efficacy with different suppressors in transient assays using pBinP-1-GFP.

10. For cowpea plants, the first true leaves should be agroinoculated immediately before the first set of trifoliolate leaves appear. This stage is normally reached about 10 days after sowing. For *N. benthamiana*, viral infection initiated by agroinoculation of autonomously replicating vectors is most efficient in young plants that have 4–6 true leaves and are ~8 cm across at their widest point. These plants are generally about 14 days old. Cotransformation with a suppressor of silencing broadens the window for successful initiation of systemic viral infection and allows transient transformation of infiltrated tissues in much older plants (potentially up to the floral transition) by both CPMV-based vector systems.
11. Videos of the agroinoculation technique can be seen at <http://www.plantsci.cam.ac.uk/baulcombe/protocols.html>.
12. Viral particles prepared by this method should be used within no more than 48 h and should be kept at 4°C if not used immediately.
13. Passaging of CPMV in cowpea is most efficient when the first trifoliolate are just appearing and no more than 10 mm in size.
14. Optimal storage conditions of protein extracts will vary according to the recombinant protein of interest and the requirements of its future uses. For useful discussions on protein extraction and storage conditions and analysis *see* ref. 26.

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