

## **Cowpea mosaic virus: the plant virus-based biotechnology workhorse**

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## INTRODUCTION

The first plant viruses to be developed as expression vectors in the early 1980s were those with DNA genomes (for reviews, see 42, 56). This was due to the fact that, at the time, only the DNA genomes could be manipulated and the technology for creating infectious cDNA copies of viruses with RNA genomes did not exist. However, the vast majority of plant viruses have genomes that consist of one or more strands of positive-sense RNA. These viruses infect a wide range of hosts and some can reach extremely high titres. Following the construction of the first full-length cDNA clones shown to be infectious (1), the past 25 years has seen a large number of RNA viruses developed as vectors for the expression of foreign sequences and other uses, such as gene silencing (10, 31, 42, 55, 56, 70).

Many proteins have been successfully expressed with virus vectors and significant progress in vector design has been driven by the demands of this application. Generally developed with the expression of fluorescent marker proteins, RNA virus-based vectors have become a highly effective means of producing recombinant heterologous protein in plant tissue within a short time frame (28, 35, 44, 67). In addition to their use as vectors for the production of heterologous polypeptides and as gene silencing vectors, plant RNA viruses have also provided a source of particles for various applications. Virus capsids provide nano-scale particles with consistent size and shape, which can be exploited for a number of chemical and biological applications (72). For example, a number of systems make use of the repetitive geometry of plant virus capsids to present multiple copies of antigenic sequences, to increase their potential as a source of novel vaccines (10, 39, 58). Also, both wild type and genetically modified capsids of plant viruses are also being used as biotemplates for novel materials in nanotechnology (88).

Cowpea mosaic virus (CPMV) has borne witness to most of the aforementioned biotechnological uses of RNA viruses. The year 2009 marks the 50<sup>th</sup> anniversary of the first description of CPMV as a pathogen of cowpeas (*Vigna unguiculata*) in West Africa (13). As a result of its ease of propagation, high yield and the stability of the viral particles, CPMV rapidly became an object of intense scientific research. Early studies revealed the bipartite nature of the viral genome (7, 78), the structural similarities between CPMV and the animal picornaviruses (87) and the mechanism of gene expression (polyprotein processing; 53). Subsequent work resulted in the determination of the nucleotide sequences of both genomic segments (81, 43), a realisation of the genetic similarities between CPMV and picornaviruses (21), an atomic resolution structure of the virus particles (32, 33), and the creation of infectious cDNA clones (16, 25, 84). A crucial step for the development of practical CPMV-based expression systems was the creation of vectors that could be inoculated by agroinfiltration (38), and this approach is now the method of choice for introducing CPMV-based constructs into plants.

This review covers the development of CPMV vectors since their first use as an epitope display platform in the early 1990s. The first plant virus to be successfully developed for this purpose (59, 77), CPMV virions continue to find many uses in bio-nanotechnology (20, 73). Through various incarnations as replicating viral vectors for polypeptide expression (9, 24, 66, 82) and the successful production of a number of pharmaceutically relevant proteins (46, 47, 49, 51, 65), investigation of limitations imposed by CPMV biology have yielded technological advances. Very recent developments have seen an extremely high-level expression system based on non-replicating CPMV sequences (67) being used to generate CPMV capsids devoid of RNA, thereby allowing CPMV capsids, which is where the biotechnology of CPMV began, to be exploited in novel ways.

## COWPEA MOSAIC VIRUS – THE BASICS

*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 titres 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, both of which are required for infection. Both RNAs are polyadenylated and possess a small protein (VPg) covalently linked to their 5' termini. The RNAs each contain a single open reading frame and are expressed through the synthesis and subsequent processing of precursor polyproteins (Fig. 1a). RNA-1 encodes proteins involved in the replication of viral RNAs and polyprotein processing, including the RNA-dependent RNA polymerase, a helicase, the 24K proteinase, and a proteinase cofactor. RNA-2 encodes proteins essential for cell to cell movement and systemic spread, namely, the 48K movement protein and the two coat proteins, large (L) and small (S). Initiation at an upstream in-frame AUG results in a protein, the 58K protein which shares most of its sequence with 48K protein apart from an N-terminal extension. Further details of the molecular genetics of CPMV can be found in Goldbach and Wellink (23). The virus particles are composed of sixty copies each of the L and S coat proteins arranged with icosahedral symmetry (Fig 1b and c), and the particle structure is known to atomic resolution (32, 33). Particle formation is essential for the virus to spread both locally and systemically within plants (85) and for its transmission by its beetle vectors.

## CPMV AS A PEPTIDE-PRESENTATION SYSTEM

The initial motivation for using CPMV as platform to express peptides was the observation that the presentation of multiple copies of short antigenic peptides (epitopes) on the surface of a macromolecular carrier protein often enhances the immunogenicity of such peptides (41). Because of this, a number of systems, including CPMV, have been developed in which epitopes were genetically fused to self-assembling macromolecules, such as viruses, with the objective of creating novel vaccines. The ability of CPMV to grow to high titres in plants, the robustness of its particles and its lack of infectivity in mammalian cells made CPMV an attractive candidate for use as an epitope presentation system. Knowledge of the detailed three-dimensional structure of the particle allowed potential sites for the insertion of foreign peptides to be identified and the availability of infectious cDNA clones enabled the necessary genetic changes to be made. Thus CPMV became the first plant virus to be successfully developed as a peptide presentation platform (26, 40, 59, 77). Since the original reports describing the construction of chimaeric viruses, a large variety of epitopes have been expressed on the surface of CPMV particles. In most cases, the foreign sequence has been inserted into the most exposed loop of the virus surface, the  $\beta$ B- $\beta$ C loop of the S protein, resulting in the display of 60 copies of inserted peptide on the surface the particle (Fig. 1a-c). Other sites, such as the  $\beta$ C'- $\beta$ C'' loop of the S protein and the  $\beta$ E- $\alpha$ B loop of the L protein, have also been used successfully (5, 14, 75, 57). Provided the inserted peptide is less than 40 amino acids and has a pI below 9.0 (57) the yields of modified particles are generally similar to those obtained with wild-type CPMV (up to 1mg of particles per gram of infected leaf tissue). When purified virions were analysed by SDS-polyacrylamide gel electrophoresis, coat proteins of the appropriately increased size were observed. However, additional, smaller proteins, not seen with wild-type virus, were also routinely observed. These proteins arise through a proteolytic cleavage event near the carboxy-termini of the inserted sequence, which appears to be position-, rather than sequence-, dependent (75, 76). This cleavage does not

immediately result in the loss of the epitope from the surface of the virion but does lead to it being anchored at only its N-terminus (34); however, proteolytic trimming of the peptide can subsequently occur, leading to the loss of most, if not, all of the insert (N. Montague and G.P. Lomonosoff, unpublished data).

A number of CPMV-based chimaeras have been subjected to immunological analyses. Where appropriate antisera were available, detection of the inserted epitope on the modified coat protein subunits was straightforward (59, 77), demonstrating the antigenicity of the chimaeric particles. To demonstrate that the particles are capable of raising an immune response against the presented peptide, purified virus particles have been administered to experimental animals, usually by intramuscular injection, followed by assessment of the properties of the resulting antisera. The first CPMV chimaera, and indeed, the first modified plant virus particle, to be assessed for the immunogenicity of a foreign peptide contained a 14 amino acid epitope (the NIm1A site) from VP1 of *Human rhinovirus 14* (HRV-14). The modified particles proved capable of raising specific antibodies against the insert when supplied parenterally to rabbits (59). Subsequently, a number of chimaeras expressing epitopes from a variety of pathogens have been analysed for their ability to raise specific antibodies, culminating in the demonstration that a chimaera displaying a 17-amino acid epitope from the major capsid protein of mink enteritis virus (MEV) could protect mink or dogs against challenge with the pathogen (15, 30). This work provided first example of plant-produced vaccine, albeit an experimental one. Since then, protective immunity has been generated in several animal species against a number of pathogens, both viral and bacterial. A summary of the results obtained from these types of experiments are shown in Table 1.

An additional attractive feature of using CPMV to present epitopes was the fact that the conditions for crystallisation of the virus were well established, potentially allowing crystallographic analyses of the structure of presented peptides to be undertaken. That this was a practical proposition was demonstrated by the crystallisation and structural analysis of the first CPMV chimaera expressing the NIm-1A site of HRV-14 (34; Fig. 1c). The NIm-1A sequence was found to adopt an extended conformation on the virus surface and displayed high mobility, which was attributable to the freedom of movement resulting from the cleavage at the C-terminus of the peptide. This was in marked contrast to the convoluted structure that the NIm-1A epitope adopts on the surface of HRV-14 itself and probably explained why antisera raised against this chimaera could bind to denatured HRV-14 VP1 but not intact HRV-14 particles. In an attempt to correlate the structure and mobility of the NIm-1A epitope with the type of antibodies elicited, a series of chimaeras was produced with the sequence inserted in different positions on the CPMV surface (75). Only when the mobility of the NIm-1A peptide was restricted by preventing cleavage at the C-terminus of the epitope were antisera obtained that could bind to intact HRV-14 particles.

Though the initial motivation for using CPMV particles to present peptides was to create novel vaccines, the same technology has recently been used for applications in nanotechnology. The observation that certain peptide sequences can stimulate the deposition of specific inorganic compounds led to an investigation as to whether CPMV-based chimaeric virus technology could be adapted for the templated production of nanoparticles. By incorporating specific peptides on the virus surface, it was demonstrated that either silica or an iron-platinum alloy could be deposited on the virus surface (71, 74). These experiments represent but a small fraction of the many investigations into the use of virus particles,

including CPMV, in nanotechnology. However, research in this field has been reviewed extensively recently (73) and will therefore not be discussed further in this review.

## CPMV AS A GENE EXPRESSION SYSTEM

### *Fully functional replicating vectors*

CPMV-based systems for the expression of whole genes have so far focussed exclusively on modifying the sequence of RNA-2 and co-inoculating the vector constructs with unmodified RNA-1 to provide polyprotein processing and viral replication functions. The primary reason for this is that increasing the size of RNA-2 above its normal length of 3.5kb, at least as far as the size of RNA-1 (6kb), should not affect its encapsidation and should therefore allow systemic spread of the modified virus. In addition, it has proved extremely difficult to make extensive modifications to RNA-1 without destroying virus viability. Two main approaches have been taken for the creation of autonomously replicating RNA-2-based expression systems, both of which have been assessed by the expression of the green fluorescent protein (GFP) from *Aequorea victoria*.

The first published attempt to express a foreign protein from a CPMV-based vector used a gene replacement strategy that saw the exchange of most of the coat protein-coding region of RNA-2 with the sequence of GFP (82). Release of GFP was achieved by processing at the natural 48K/L junction, leaving 29 amino acids from the L protein fused to its amino terminus (Figure 2a). Although this construct was replicated by RNA-1 and GFP could be detected in individual cells, no infection was generated since no capsid proteins were produced. Systemic infection and GFP expression was achieved by co-inoculation with a complementary RNA-2 construct lacking the 48K movement protein, which supplied a

source of coat proteins (Figure 2a). This approach effectively created a tripartite virus and, therefore, was the first deconstructed plant virus vector. However, after several passages, expression of GFP was lost and RNA analysis showed that recombination had occurred between sequences which were present in both of the defective RNA-2 molecules.

The second strategy used for protein expression was that of gene addition. In one format, a series of constructs was created in which the sequence encoding GFP was inserted between gene products of RNA-2 such that the full complement of viral genes was maintained (24, 82). To achieve release of GFP from the polyprotein, it was flanked by duplicated Q/M cleavage sites that would be recognised by the 24K proteinase from RNA-1 (Figure 2b). Although these vectors could spread systemically and expression levels of the released GFP reached 1-2% of total soluble protein (24), the duplicated cleavage sites resulted in the released GFP having N- and C-terminal extensions. The presence of these additional amino acids is of particular concern in relation to the production of recombinant pharmaceuticals where close to, if not completely, authentic protein is required. Attempts to reduce the size of these sequences by minimising the size of the cleavage sites, resulted in inefficient processing and limited movement of the virus. The use of longer duplications, while giving effective processing, resulted in a higher frequency of homologous recombination and, therefore, loss of the insert and reversion to wild-type virus. The problems of the presence of additional sequences on the expressed protein and the genetic instability of the vectors have limited the use of the constructs to intracellular localisation studies (48).

As an alternative to the use of duplicated 24K proteinase sites to achieve post-translational release of an inserted sequence, a number of RNA-2-based constructs have been produced which enable co-translational release via the action of the 2A catalytic peptide from foot-and-

mouth-disease virus (FMDV). Incorporation of a 2A sequence, which can be as short as 16 amino acids, between two proteins leads to an efficient co-translational separation of nascent polypeptides (18, 64); the use of such a sequence to achieve processing obviates the need to duplicate CPMV sequences and hence has advantages in terms of the genetic stability of constructs. Cleavage at a 2A site occurs between the last two amino acids of the sequence, resulting in the N-terminal product having all but one of the 2A amino acids attached to its C-terminus, while the C-terminal product has only an additional N-terminal proline.

Initially, the 2A sequence was substituted for the one of duplicated proteinase site at either the N- or C-terminal side of the GFP sequence inserted between the 48K movement protein and L coat protein sequence in RNA-2 (Figure 2c). Only when the 2A sequence was inserted at the C-terminal side of the GFP insert were the constructs capable of efficient systemic spread in plants. This was probably a result of the 2A-mediated cleavage leaving a 16 amino acid extension on the C-terminus of the 48K movement protein when it is inserted to the N-terminal side of the GFP sequence, with negative effects on the correct functioning of the movement protein. However, when the 2A sequence was positioned to the C-terminal side of the GFP sequence, most of the 2A peptide remained fused to the C-terminus of GFP. Though this does not interfere with the movement of the virus, it does have consequences for the authenticity of the expressed protein. In addition, because of the incompleteness of the cleavage mediated by the 2A sequence used, approximately 1 in 6 of the L subunits carried GFP fused to their N-termini and these fusion proteins can be incorporated into virus particles (24).

Subsequently, GFP was fused to the C-terminus of the S protein via the 2A sequence (Figure 2d). The resulting recombinant virus was able to systemically infect cowpea plants and

expressed GFP at approximately 1% of total soluble protein with less than 10% of GFP remaining fused to the S coat protein (24). A particular advantage of this approach is that the inserted sequence (in this case GFP) possesses only the single additional proline residue at its N-terminus and an authentic C-terminus. The co-translational nature of the 2A-mediated cleavage means that the C-terminal product can be directed to the plant secretory pathway by the inclusion of appropriate targeting sequences. Since the leader peptide is cleaved off during such targeting, the N-terminal proline left by the 2A-mediated cleavage is lost. Thus, this full-length RNA-2-based construct was further developed into a practical vector for protein expression (CPMV NS-1) by modifications which permitted the one-step replacement of the sequence of GFP by any gene of interest and the use of agroinfiltration to initiate an infection (36)

#### *Pharmaceutical protein expression with CPMV NS-1*

Both Small Immune Proteins (SIPs) and full size antibodies have been expressed in plants from the autonomously replicating CPMV NS-1 vector. The SIPs consisted of single-chain antibody specific for transmissible gastroenteritis virus (TGEV) linked to either the  $\epsilon$ -CH4 domain from human IgE ( $\epsilon$ -SIP; 49) or the  $\alpha$ -CH3 domain from human IgA ( $\alpha$ -SIP; 2) to promote dimerisation. The expressed molecules could neutralise TGEV in tissue culture and extracts from cowpea leaves inoculated with the CPMV-based vectors fed to piglets protected the animals against challenge with TGEV (49). Thus the crude leaf extracts could potentially be used to passively immunise newborn animals against TGEV. Subsequently, the production of a full-length IgG was achieved by co-inoculating separate CPMV NS-1 constructs containing the heavy and light chains of the blood group typing IgG, C5-1, in the presence of RNA-1 (65). In the above studies, all the antibody constructs were directed to the secretory pathway by positioning a native mammalian signal peptide between the 2A sequence and

immunoglobulin chain. Furthermore the antibodies could be retained within the endoplasmic reticulum with the use of a C-terminal HDEL or KDEL sequence, resulting in increased levels of accumulation.

The ability of vectors based on CPMV to allow the simultaneous synthesis of proteins from multiple RNA-2 molecules in co-inoculated tissue, as is required for the production of a full-sized antibody, is probably a consequence of the bipartite nature of the virus. This stands in contrast to the situation when vectors based on monopartite viruses are used. For example, the use of multiple constructs based on TMV to express different proteins results in segregation of the expression of each protein in agro-infiltrated tissue (22). This exclusion phenomenon can also affect foreign protein expression from some combinations of different monopartite viruses (17).

In addition to the production of antibodies, CPMV-NS-1 has been used to express the hepatitis B virus core antigen (HBcAg), which was shown to self-assemble into core-like particles in cowpeas (47). HBcAg has a demonstrated potential to significantly enhance the immunogenicity of epitopes displayed on its surface and is, in itself, a potential source of novel vaccines (12, 60). Thus, autonomously replicating vectors based on CPMV have displayed the ability to produce pharmaceutically valuable polypeptides including those requiring glycosylation, and therefore, targeting to the secretory pathway. However, the inclusion of targeting sequences at the N-terminus of the inserted protein can have unpredictable consequences on the replication of the resulting RNA-2 constructs (L. Nicholson and G.P. Lomonosoff, unpublished data).

**Insert Side Bar:** *Non-coding sequences as encapsidated mimics*

### *Replicating deleted versions of RNA-2*

Though clearly able to direct the expression of biologically active molecules, the use of CPMV vectors based on full-length RNA-2 molecules has a number of disadvantages. Such vectors produce infectious virus particles which are capable of spreading not only within a plant but between plants. Though this ability could be considered to be advantageous, especially where large amounts of plant material expressing the desired protein are required, it raises concerns about undesirable environmental spread. Moreover, it has transpired that the ability to spread systemically is less useful than originally thought, as an inserted sequence tends to be lost when such spread occurs (49) and co-inoculated constructs tend to segregate in systemically infected tissue (65). Thus, in reality, only inoculated leaves have been used as the source of the expressed protein. Therefore, elimination of the ability to spread systemically by creating vectors based on defective versions of RNA-2 has the advantage of bio-containment with little penalty in terms of the amount of material that can be produced, provided that a highly efficient method of inoculation, such as agro-infiltration is used. Furthermore, reducing the size of the RNA should have advantages in terms of the maximum size of insert that can be stably expressed.

To develop a system based on a defective version of CPMV RNA-2, most of the RNA-2 coding sequence was removed, leaving only the 5' and 3' sequences previously identified as being required for RNA-2 to be replicated by RNA-1 (63). To optimise the design of the vector, a series of deleted versions of RNA-2 (delRNA-2) was created in which the sequence of GFP was flanked by three different lengths from the 5' region of RNA-2 and the complete 3' untranslated region (UTR). The three constructs were designed to initiate translation of GFP at AUG161 (the initiation codon for the 58K protein), AUG512 (the initiation codon for

the 48K protein) or AUG524 (the next in-frame AUG) of the RNA-2 sequence giving rise to constructs 5'-3'-GFP; 1-GFP and 2-GFP, respectively (Figure 2e; 9). Replication of 1-GFP and 2-GFP, but not 5'-3'-GFP, was observed when the constructs were co-infiltrated into *N. benthamiana* leaves in the presence of RNA-1 and a suppressor of gene silencing. Furthermore enhanced levels of GFP fluorescence were observed in the case of the 1-GFP and 2-GFP, but not the 5'-3'-GFP construct, when RNA-1 and a suppressor were supplied. These results are consistent with the polymerase encoded by RNA-1 being able to replicate only versions of RNA-2 when the sequence between AUG161 and AUG512, as well as the 5'UTR, is present (25, 63, 79). For all subsequent research, delRNA-2 constructs based on the design of 1-GFP (initiation at 512) have been used.

#### *Pharmaceutical protein expression with replicating delRNA-2*

The usefulness of the delRNA-2 system has been demonstrated through the successful expression of pharmaceutically active proteins. In the first instance, delRNA-2 constructs containing the sequences of either the H or L chain IgG C5-1 were co-infiltrated into *N. benthamiana* leaves in the presence of RNA-1 and the heterologous potyviral silencing suppressor HC-Pro (6). Fully formed IgG molecules were obtained at levels (74mg/Kg wet weight leaf tissue) which substantially exceeded those obtained when the same antibody was expressed using full-length RNA-2 vectors (19mg/Kg wet weight leaf tissue; 65). As well as the expression of antibodies, the delRNA-2 approach has also been used successfully to express the core antigen of hepatitis B virus (HBcAg; 46) and the enzyme human glutamic acid decarboxylase (hGAD-65; M.C. Canizares, L. Avesani and G.P. Lomonossoff, unpublished data), which has potential applications in preventing Type I diabetes .

In addition to giving higher levels of protein expression than full-length RNA-2 vectors in inoculated tissue, the delRNA-2 system removes the possibility of modified viral particles spreading in the environment. Recent results have shown that even in the presence of wild-type RNA-2, the RNA from construct 1-GFP is not trans-encapsidated, indicating that even accidental coinfection with wild-type CPMV would not lead to the spread of the foreign sequence (F. Sainsbury and G.P. Lomonosoff, unpublished data).

#### *Combined transgene/viral vector system*

Despite the high levels of expression obtained using delRNA-2 vectors, the use of defective viruses suffers from the limitation that expression is restricted to inoculated tissue. This disadvantage can be overcome by simply increasing the amount of tissue that is infiltrated using a technique such as the vacuum infiltration of whole plants. However, an alternative would be a system in which the replicating construct is produced in all cells of a plant from a transgene, and such a system has been developed for CPMV (9).

The first stage in the development of a CPMV transgene/virus vector expression system was to check if an integrated full-length copy of RNA-2 containing a foreign gene could be replicated by supplying the RNA-1. To this end, *N. benthamiana* was transformed with a full-length version of RNA-2 containing GFP (CPMV NS-1). The resulting plants were identical in appearance to non-transgenic *N. benthamiana* and showed no detectable green fluorescence. When these plants were agroinfiltrated with RNA-1, they developed localized green fluorescence around the area of inoculation and this fluorescence subsequently spread beyond the inoculated leaves to give patches on the stems and on the upper, uninoculated leaves. In addition, when the plants transformed with CPMV NS-1 were crossed with an RNA-1-containing transgenic line, the resulting plants showed a high level of GFP

production throughout (9). These results showed that RNA-1 supplied either exogenously or expressed from a transgene is capable of replicating a transgene-derived RNA-2 molecule containing a foreign gene. To prevent the production infectious virus, the system was improved by adapting it for use with delRNA-2 constructs. When RNA-1 alone was supplied by agroinfiltration to plants transgenic for the three delRNA-2 constructs described above, no replication of any of the transgene-derived delRNA-2 molecules could be detected. However, when a suppressor of silencing was simultaneously supplied, either in the form of the natural CPMV suppressor encoded by full-length RNA-2 (11, 37) or HC-pro, an increase in the levels of fluorescence was seen in the case of the 1-GFP and 2-GFP, but not the 5'3'-GFP, transgenic plants. These results are consistent with the conclusion that enhanced GFP expression requires replication of the transgene-derived mRNA, which can only occur when both RNA-1 and a suppressor of silencing are present simultaneously.

To produce a system in which delRNA-2 molecules can be expressed and replicated throughout an entire plant, *N. benthamiana* transgenic for both CPMV RNA-1 and HC-Pro were produced. These were shown to be capable of supporting the replication of agroinfiltrated 1-GFP and of giving high levels of GFP expression. Furthermore, when the RNA-1 and HC-Pro double transgenic plants were crossed with those transgenic for 1-GFP, the progeny containing all three transgenes were brightly fluorescent throughout the plant, whereas those transgenic for just HC-Pro and 1-GFP were not (9). The level of GFP expression enhancement over plants transgenic for 1-GFP alone was estimated to be at least 10-fold and the presence of negative-strand delRNA-2 in the plant tissue showed this was associated with replication of the transgene-derived 1-GFP RNA by RNA-1.

The combined transgene/virus system based on delRNA-2 has several advantages in addition to that of biocontainment. Among these is the fact that every cell in a transgenic plant will contain a “master copy” of the foreign sequence embedded in the delRNA-2 construct. This should lead to an enhancement in the genetic stability of heterologous sequences since propagation of the foreign sequence throughout the plant will not rely on successive rounds of RNA viral replication. This, in turn, may permit the stable expression of larger proteins than is possible when delRNA-2 sequences are used in a transient format. In addition, it should be possible to produce larger amounts of plant tissue compared with when the transient system based on agroinfiltration is used. On the downside, the production of lines of transformed plants is a considerably longer process than transient expression via agroinfiltration.

#### *Hyper-translatable RNA-2 5' UTR*

The 5' region of CPMV RNA-2 has a somewhat complicated structure. Though most protein synthesis, which ultimately results in the production of the 48K movement protein, initiates at AUG512, some initiation occurs at the upstream, in-frame AUG at position 161, giving rise to the 58K protein. In addition there is a further upstream, out of phase AUG at position 115, which can potentially direct the synthesis of a 20 amino acid peptide (Figure 3a). The method by which CPMV RNA-2 escapes the first-AUG rule (29) and initiates at both AUG161 and AUG512 is not clear but may involve a combination of leaky scanning and reinitiation. The AUG at 115 is known to initiate translation *in vitro* (86) although the resulting peptide has no known function. Preserving frame continuity between the AUGs at 161 and 512 is required for replication of RNA-2, though the precise reason for this is unclear (25, 63, 79).

The need to preserve frame continuity between AUG 161 and 512 in order to retain the replication ability of RNA-2-based vectors complicates their construction. However, while the replication of delRNA-2 constructs is essential to achieve high levels of expression when the RNA-2-based mRNA is derived from a transgene (9), it is less important for transient expression since mRNA can accumulate to very high levels in agroinfiltrated tissue, particularly in the presence of a suppressor of silencing. With the goal of improving the ease of cloning, the necessity of maintaining frame continuity between AUG 161 and 512 for high level expression of foreign proteins from delRNA-2 in transient assays was investigated using variants of the construct 1-GFP (67). The continuity of reading frame between the AUG for GFP translation initiation and AUG161 was altered by adding nucleotides immediately upstream of AUG512. The effect of these changes was determined by agroinfiltrating the constructs into *N. benthamiana* leaves in the presence of the suppressor of silencing P19 (83) and assessing the level of GFP expression. Adding one or two nucleotides, so translation occurs from positions 513 and 514, reduced GFP expression relative to that which occurs when the AUG is at 512, while adding 3 nucleotides so translation occurred at position 515 restored the expression to the original level (Figure 3b; F. Sainsbury and G.P. Lomonosoff, unpublished data). These observations suggest a dependency on frame continuity between AUGs 161 and 512 for translation to occur efficiently from AUG512. To investigate whether this dependency could be obviated, a series of mutants was constructed by systematically removing one or both of AUGs at 115 and 161 from each of three reading frame variants (Figure 3a). While removal of AUG115 ( $\Delta$ 115) led to a reduction of GFP expression from all reading frame variants, deletion of AUG161 ( $\Delta$ 161) led to a massive increase in GFP expression, which was further enhanced by the simultaneous elimination of AUG115 (Figure 3b; 67). This great enhancement of expression levels, which was seen in all three reading frame variants, did not result from elevated mRNA levels but was due to the mRNA

molecules being “hyper-translated” relative to the wt leader. For this reason, the RNA-2 leaders lacking either AUG161, or both AUG115 and AUG161, are referred to as the hyper-translatable or *HT* leaders.

*Extremely high level protein expression easily and quickly*

The levels of foreign protein produced using the *HT* leaders far exceeded those achieved from replicating full-length or delRNA-2 vectors in transient expression studies. To exploit this phenomenon, a number of pharmaceutically relevant proteins have been expressed using the CPMV-*HT* system. They include approximately 1g per kg of agroinfiltrated tissue of assembled HBcAg particles (67), and up to 0.4g per kg of the human anti-human immunodeficiency virus IgG, 2G12 (68). The fact that these levels are attained without the need for viral RNA polymerase to amplify transcripts reduces the chance of mutations being introduced during replication. It could also extend the range of hosts which can be used beyond those species that support CPMV replication.

The most recent development of the CPMV-*HT* expression system has seen the components of the system (the CPMV-*HT* expression cassette and the P19 sequence) placed onto a single vector for *Agrobacterium*-mediated delivery to plant cells. This was achieved by constructing a new binary vector backbone derived from pBINPLUS (80), which is based on the highly successful pBIN19 (3). The new pEAQ vectors are less than half the size of the parent plasmid while retaining all the essential components for efficient transient expression (68). Furthermore, the removal of reading frame dependency, necessary for replication and evidently adversely affecting translation (Figure 3b), has permitted the use of a multiple cloning site for direct insertion into the most effective CPMV-*HT* vector, pEAQ-*HT*. In

addition, it has been possible to construct pEAQ-*HT* variants that are compatible with the GATEWAY system of recombination-based cloning (68).

The extremely high level expression of therapeutic proteins by CPMV-*HT*, achievable within a few days, has great potential for use in the high-throughput screening of vaccine candidates. Combined with the simplicity afforded by the pEAQ series of binary vectors, which permit one-step and optional high-throughput cloning, milligram quantities of foreign protein can be produced within 2 to 3 weeks of receiving a DNA sequence. This means that a significant number of target protein variants can be screened within a very short time-frame.

#### GENERATION OF EMPTY CAPSIDS

As previously mentioned, while the CPMV capsid-based technologies have enjoyed considerable successes, the range of applications has been restricted by the need to propagate the virus by the infection of plants. This means that viral particles must be fully competent for movement from cell-to-cell, placing limitations on sequences that can be successfully expressed on the particle surface (57). Furthermore, the particles must retain their ability to package the genomic RNA, as this is essential for the virus to move between cells. As a result, the majority of particles isolated from plants contain either RNA-1 or RNA-2 meaning that preparations remain infectious, raising biosafety issues. A further disadvantage is that, being full of RNA, the particles cannot be loaded with heterologous material. The CPMV-*HT* expression system has provided a means of producing CPMV capsids in plants without the need for infection, thereby overcoming these concerns and limitations.

To achieve capsid formation in the absence of infection, the sequence of the precursor of both the L and S coat proteins, VP60, was expressed in plants using pEAQ-*HT*. Processing to the mature L and S coat proteins was achieved by co-infiltration with either full-length RNA-1 (to provide the 24K proteinase function) or just the 24K proteinase, also expressed from pEAQ-*HT* (Figure 4a; 69). In both cases efficient processing of VP60 to the L and S proteins was observed, and virus-like particles (VLPs), similar in structure to wild-type CPMV, could be purified from the inoculated tissue. Analyses of these VLPs showed that using RNA-1 to process VP60 resulted in the encapsidation of RNA-1 in a proportion of the VLPs (Figure 4b,c). In contrast, using just the 24K proteinase to process VP60 results in the production of exclusively empty or eVLPs (Figure 4b,c). The fact that it is possible to produce preparations completely devoid of RNA demonstrates that, unlike some other plant virus-based VLPs (8), CPMV VLPs do not encapsidate host-derived RNA (69).

The development of a method for the generation of eVLPs should prove very valuable to researchers developing technologies based on CPMV capsids. Previously, irradiation with ultraviolet light (30, 61) or chemical treatment (54) has been used to eliminate the infectivity viral RNAs. However, although addressing biosafety concerns, these processes risk altering the structural properties of the particles and do not actually remove the RNA from within the particles. An alternative approach has been to treat a natural population of CPMV at high pH, which results in the loss of RNA from the particles (52). However, as all these methods make use of particles originally produced through the infection of plants, they do not address the limitations imposed by the need to maintain the ability of the capsids to assemble with RNA and move throughout the plant. In addition to alleviating biosafety concerns, the production of eVLPs via the CPMV-*HT* system no longer requires that the particles be able to package RNA or to spread between plant cells; they need only retain their ability to assemble. This

should greatly increase the range of mutations that it is possible to introduce on to the outer surface of coat proteins and will make it possible to modify the inner capsid surface. As a result, these easily generated eVLPs should find use as nano-sized reaction vessels for chemical and bionanotechnological applications (20). This opens the possibility of combining internal modification/loading with exterior functionalisation, introduced genetically or chemically.

#### CLOSING REMARKS

With the use of the non-replicating CPMV-*HT* expression system to produce viral capsids, the use of CPMV in biotechnology appears to have come full circle. Through the development of a CPMV-based expression system, the promises of CPMV capsid-based technologies have been renewed by harnessing modified elements of the virus to generate a more useful version of itself. Thus, the “Ouroboros” that represents the development of CPMV-based expression strategies has breathed new life into some of the earliest technologies for which this versatile virus has been used.

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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## ARTICLE COMPONENTS

**Keywords:** viral vector, molecular farming, epitope display, vaccine, bionanotechnology, virus-like particles

### **Abstract:**

In the 50 years since it was first described, Cowpea mosaic virus (CPMV) has become one of the most intensely studied plant viruses. Research in the past 15-20 years has shifted from studying the underlying genetics and structure of the virus, into focussing on ways in which it can be exploited in biotechnology. This work led first to the use of the virus particles to present peptides, through the creation of a variety of replicating virus vectors, and thence, to the development of a highly efficient protein expression system that does not require viral replication. Finally, the circle has been completed by the use of the latter system to create empty particles for peptide presentation and other novel uses. Thus, the history of CPMV in biotechnology can be likened to an “Ouroborus”, an ancient symbol depicting a snake or dragon swallowing its own tail, thus forming a circle.

### **Terms/Definitions:**

- Epitope display – The presentation of antigenic peptides on the surface of a carrier molecule.
- Bipartite – Having a genome consisting of two molecules of nucleic acid.
- Agroinfiltration – A method of delivering DNA to plant nuclei by pressure-infiltrating *Agrobacterium* suspensions
- Ouroborus – a symbol, in the form of a snake or dragon swallowing its own tail that represents something constantly re-creating itself.

**Acronyms list:**

- GFP – Green Fluorescent Protein from the jellyfish, *Aequorea victoria*
- 2A – co-translationally cleaved peptide from foot and mouth disease virus
- HBcAg – Hepatitis B core Antigen
- Ig – Immunoglobulin
- *HT* – Hyper-Translated
- UTR – Untranslated Region
- VLP – Virus-Like Particle
- eVLP – Empty VLP

**Summary Points:**

1. In the 50 years since the first identification of CPMV, this simple and robust bipartite RNA virus has proved to be useful in many and varied areas of biotechnology.
2. CPMV was the first plant virus to be developed as a peptide display system and a number of chimaeric particles displaying epitopes from pathogens have been shown to provide protective immunity.
3. As a nanoparticle for which the structure is very well known, CPMV has attracted interest for medical and chemical applications.
4. Replicating vectors based on CPMV have been used to produce a variety of pharmaceutically relevant proteins.
5. The non-replicating CPMV-*HT* system provides a means of producing milligrams of recombinant proteins in plant tissue within days on a bench scale.

6. The efficient production of eVLPs through the use the CPMV-*HT* system will expand the range of genetically generated surface modifications which it is possible to introduce.
7. The absence of RNA in eVLPs will enable the interior of the capsid to be modified and heterologous material to be loaded. Thus eVLPs have potential as reaction vessels or drug delivery vehicles.

**Future Issues:**

1. As with all plant-made pharmaceuticals, there will be significant regulatory hurdles to be overcome before any of the products made using the CPMV-based systems described in this review will be licensed for human or veterinary use.
2. A particular issue that will need to be addressed is the batch-to-batch variation which may occur when transient expression systems, such as those described in this review, are used for pharmaceutical production.
3. Even if all the regulatory hurdles are overcome, it will be necessary to demonstrate that the production of proteins using CPMV-based systems has significant advantages (eg. In terms of cost or quality of product) over other production systems.
4. The non-competing nature of CPMV-*HT* expression vectors raises the possibility of producing protein complexes or mimicking metabolic pathways within plants. Exploration of the full-range of possibilities has only just begun.
5. The availability, for the first time, of a source of empty CPMV capsids (eVLPs) which are not produced by infections, opens a whole new area of research into the potential uses of the virus.

**Side Bar:***Non-coding sequences as encapsidated mimics*

Though the majority of work on full-length RNA-2 vectors has concentrated on the expression of proteins, it is also possible to insert non-coding sequences between the C-terminus of the polyprotein and the 3'UTR. Such sequences are packaged into RNA-2-containing CPMV virions and are therefore, like the genomic RNAs, protected from nuclease degradation. As a result, particles harbouring the modified RNA-2 molecules, known as CPMV RNA mimics, can be used as in-tube internal controls in diagnostic real-time reverse-transcription PCR (rRT-PCR) assays for the detection of RNA viruses (27). False negative results are clearly undesirable when screening for diseases and CPMV RNA mimics can be used to verify negative assay results by confirming the absence of rRT-PCR inhibitors from clinical samples. Moreover, primers pairs for diagnosis of multiple viruses may be incorporated into a single CPMV RNA mimic.

## FIGURE LEGENDS

**Figure 1.** CPMV as a peptide-presentation system. The  $\beta$ B- $\beta$ C loop of the S coat protein (indicated by red arrows in a and b) is the most commonly used site for the insertion of foreign peptides. (a) Genome organisation of CPMV RNAs. ProC – proteinase co-factor; VPg – genome-linked protein; Pro – 24K proteinase; 48/58K - movement protein; L – large coat protein; S – small coat protein. (b) Ribbon diagram of the icosahedral asymmetric unit, consisting of the two domains of the large coat protein (cyan and green) and the small coat protein (dark blue). (c) Space-filling drawing of the CPMV capsid displaying an epitope from HRV-14 inserted into the  $\beta$ B- $\beta$ C loop of the S protein shown in red; kindly provided by Dr. T. Lin and Prof. J.E. Johnson, The Scripps Research Institute, La Jolla, USA.

**Figure 2.** CPMV as a polypeptide expression system. RNA-2-based vector improvement has been advanced by the development of systems compatible with agroinfiltration. (a) A gene replacement approach whereby the foreign sequence (GFP) replaces the coat proteins and systemic movement is restored by providing the appropriate sequences in a third construct. (b) A gene addition approach in which GFP is placed between duplicated 48K/L glutamine-methionine (QM) cleavage sites. (c) A gene addition approach where duplication QM cleavage sites and the FMDV 2A sequence are used. (d) Full-length RNA-2 construct in which GFP is placed at the end of the open reading frame following the 2A catalytic peptide. (e) Series of deleted RNA-2 constructs where initiation of translation starts at AUG 161, 512, or 524. Grey arrow indicates CaMV 35S promoter, zig-zag indicates poly-A signal, and grey box indicates the *nos* terminator.

**Figure 3.** Expression from modified RNA-2 5'UTRs. The unusual translation strategy employed by CPMV RNA-2 was investigated using a series of 5'UTR mutants. (a) Schematic representation of the RNA-2 5'UTR variations each with three initiation site options for the translation of GFP from different reading frames. (b) Visualisation of GFP expression from 5'UTR variants in leaves 5 days after agroinfiltration using UV light.

**Figure 4.** Production of CPMV eVLPs. CPMV-*HT* was used to produce CPMV capsids devoid of RNA by providing large amounts of the coat protein precursor, VP60, and the 24K proteinase. (a) Schematic representation of the constructs used to create VLPs. Grey arrow indicates CaMV 35S promoter, and grey box indicates the *nos* terminator. (b) Illustration of the different types of particles generated by the coexpression of various components as indicated, where RNA-2 + RNA-1 represents a natural infection. (c) Analysis of nucleic acid content of CPMV VLPs produced by different approaches using an ethidium bromide-stained agarose gel. Section (c) reprinted from reference 69, Copyright (2009), with permission from Elsevier.

**Table 1. Immunological properties of CPMV chimaeras created by genetic modification of capsid genes**

<b>Site of insertion</b>	<b>Sequence inserted</b>	<b>Immunological properties</b>
$\beta$ B- $\beta$ C loop S protein	HRV-14 VP1 epitope	Raises non-neutralising antibodies against HRV-14 (59, 75)
$\beta$ C'- $\beta$ C'' loop S protein	HRV-14 VP1 epitope	Raises non-neutralising antibodies against HRV-14 (75)
$\beta$ B- $\beta$ C loop S protein	HIV-1 gp41 Epitope	(59,45) Raises neutralising antibodies against HIV-1 (19) stimulates mucosal immune response in mice
$\beta$ B- $\beta$ C loop S protein	MEV VP2 Epitope	15) Protects mink against MEV (30) protects dogs against CPV (50) stimulates mucosal immune response in mice
$\beta$ E- $\alpha$ B loop L protein	<i>P. aeruginosa</i> F protein epitope	(5) Protects mice against infection with <i>P. aeruginosa</i> (4) stimulates mucosal immune response in mice
$\beta$ B- $\beta$ C loop S protein	<i>S. aureus</i> D2 domain of FnBN	Protects rats against secondary infection by <i>S. aureus</i> (62)

Figure 1.

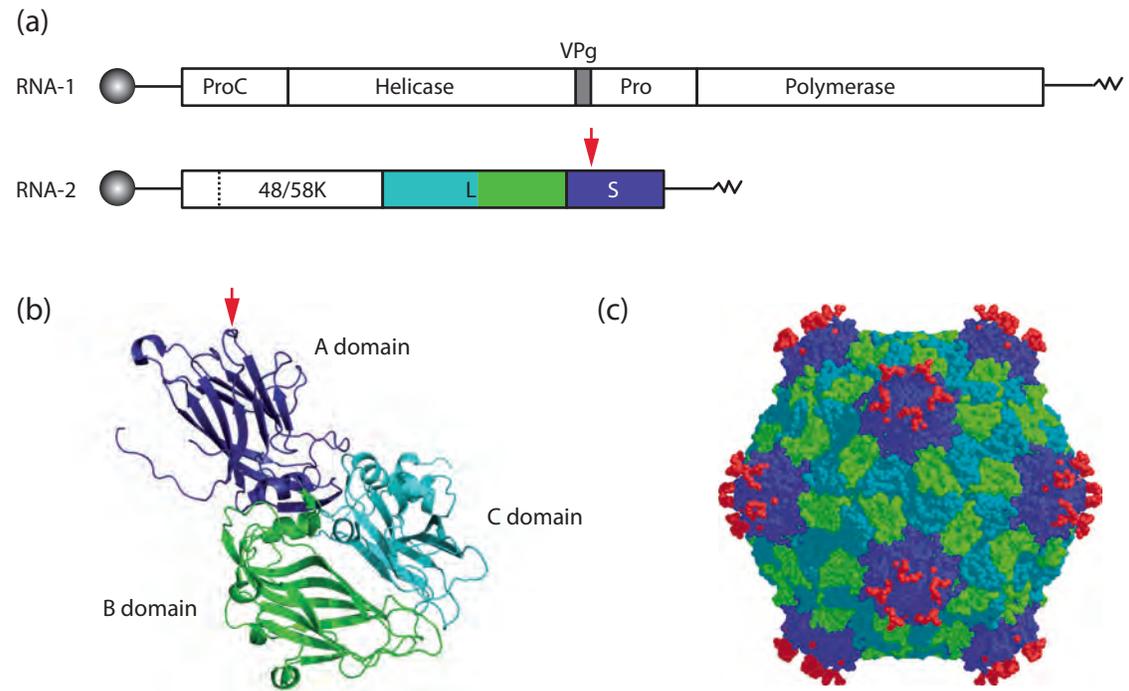


Figure 2.

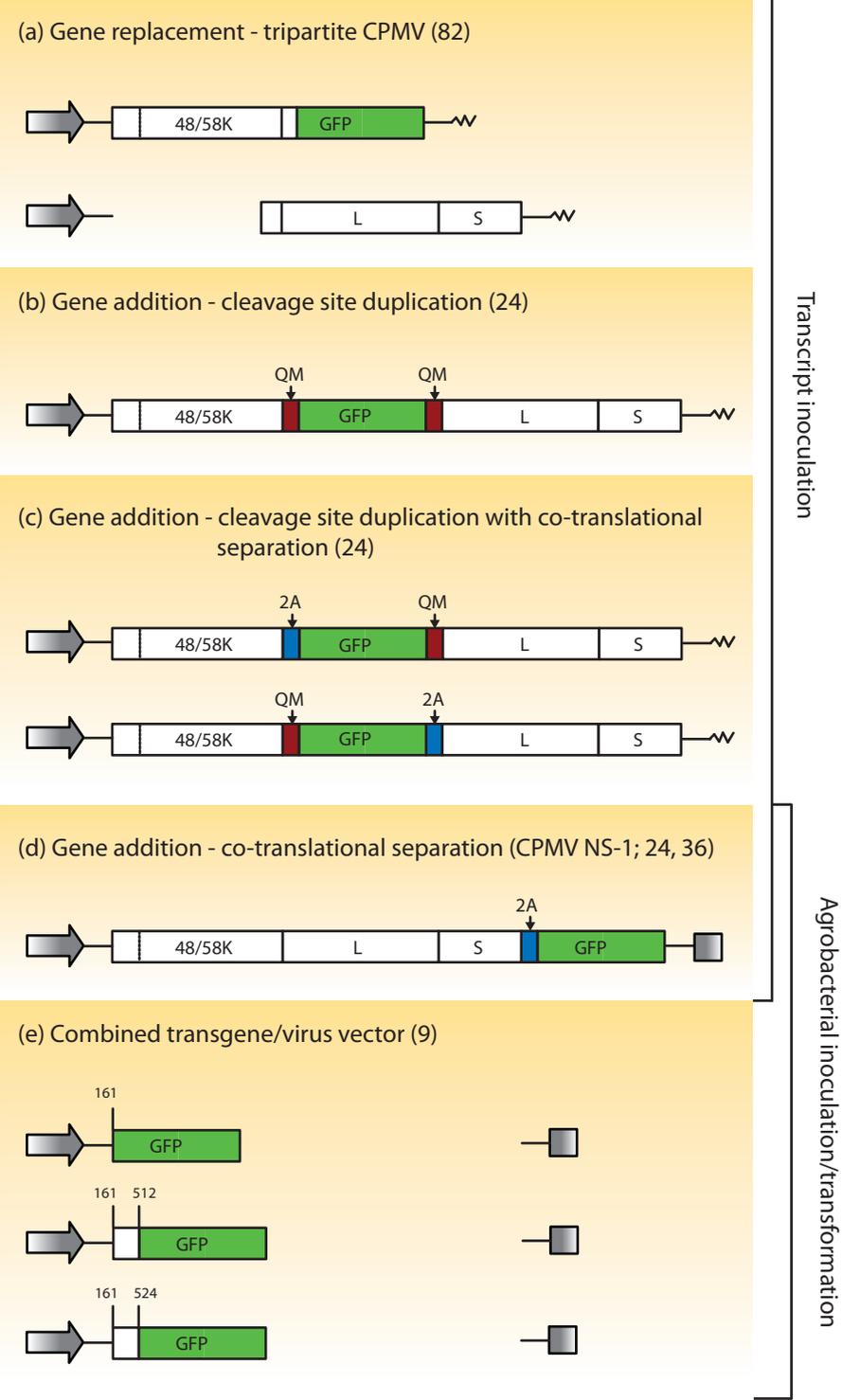


Figure 3.

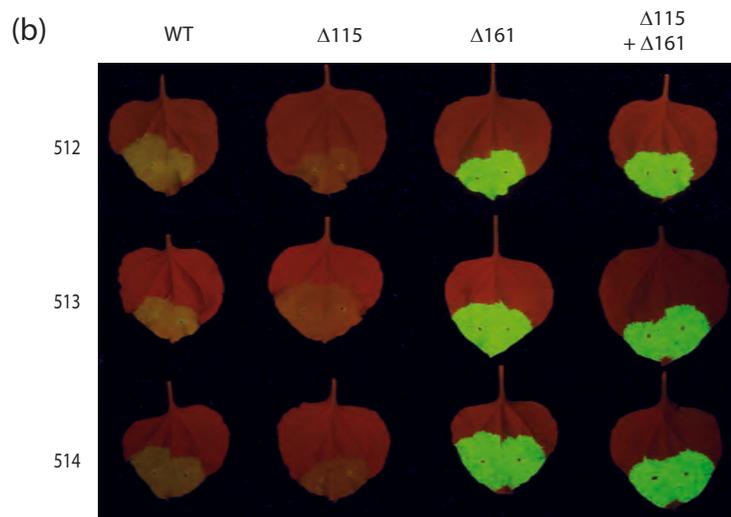
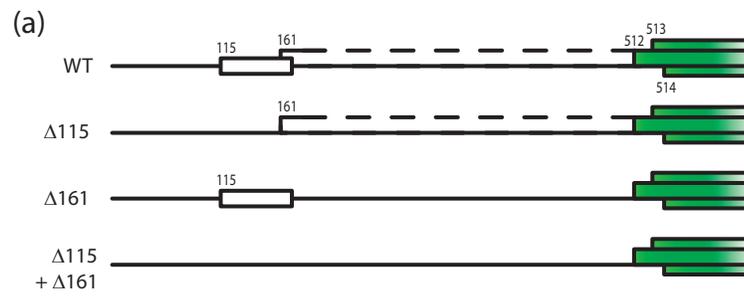


Figure 4.

