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(54) Titre : PROCEDES DE PRODUCTION DE PROTEINES EMPLOYANT DES SEQUENCES AMPLIFICATRICES D'EXPRESSION A PARTIR DU SEGMENT RNA Z D'UN VIRUS A ARN BIPARTITE COMOVIRIDAE

(54) Title: METHODS FOR PROTEIN PRODUCTION EMPLOYING EXPRESSION ENHANCER SEQUENCES DERIVED FROM THE RNA-Z SEGMENT OF A COMOVIRIDAE BIPARTITE RNA VIRUS

(57) Abrégé/Abstract:

The inventions is based on an expression enhancer sequence derived from the RNA-2 genome segment of a bipartite RNA virus, in which a target initiation site in the RNA-2 genome segment has been mutated. Deletion of appropriate start codons upstream of the main RNA2 translation initiation can greatly increase in foreign protein accumulation without the need for viral replication. Also provided are methods, vectors and systems, including the 'hyper-translatable' Cowpea Mosaic Virus ('CPMV-HT) based protein expression system.

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(54) Title: PROTEIN EXPRESSION SYSTEMS

(57) Abstract: The inventions is based on an expression enhancer sequence derived from the RNA-2 genome segment of a bipartite RNA virus, in which a target initiation site in the RNA-2 genome segment has been mutated. Deletion of appropriate start codons upstream of the main RNA2 translation initiation can greatly increase in foreign protein accumulation without the need for viral replication. Also provided are methods, vectors and systems, including the 'hyper-translatable' Cowpea Mosaic Virus ('CPMV-HT') based protein expression system.

METHODS FOR PROTEIN PRODUCTION EMPLOYING EXPRESSION ENHANCER SEQUENCES DERIVED FROM THE RNA-2 SEGMENT OF A *COMOVIRIDAE* BIPARTITE RNA VIRUS

FIELD OF THE INVENTION

The present invention relates generally to methods and materials, and particularly viral derived sequences, for boosting gene expression in plants and other eukaryotic cells, for example of heterologous genes encoding proteins of interest.

BACKGROUND OF THE INVENTION

Comoviruses (CPMV)

Comoviruses are RNA viruses with a bipartite genome. The segments of the comoviral RNA genome are referred to as RNA-1 and RNA-2. RNA-1 encodes the VPg, replicase and protease proteins (Lomonossoff & Shanks, 1983). The replicase is required by the virus for replication of the viral genome. The RNA-2 of the comovirus *cowpea mosaic virus* (CPMV) encodes a 58K and a 48K protein, as well as two viral coat proteins L and S.

Initiation of translation of the RNA-2 of all comoviruses occurs at two different initiation sites located in the same triplet reading frame, resulting in the synthesis of two carboxy coterminal proteins. This double initiation phenomenon occurs as a result of ‘leaky scanning’ by the ribosomes during translation.

The 5’ terminal start codons (AUGs) in RNA-2 of CPMV occur at positions 115, 161, 512 and 524. The start codons at positions 161 and 512 are in the same triplet reading frame. Initiation at the start codon at position 161 results in the synthesis of a 105K polyprotein while initiation at the start codon at position 512 directs the synthesis of a 95K polyprotein. As the synthesis of both polyproteins is terminated at the same stop codon at position 3299, the 105K and the 95K proteins are carboxy coterminal. The AUG codon at position 524 can serve as an initiator if the AUG at 512 is deleted. However, in the presence of the AUG 512 it does not serve this function and simply codes for the amino acid methionine (Holness *et al.*, 1989; Wellink *et al.*, 1993). The start codon at position 115 is not essential for virus replication (Wellink *et al.*, 1993).

The 105K and 95K proteins encoded by CPMV RNA-2 genome segment are primary translation products which are subsequently cleaved by the RNA1-encoded proteolytic activity to yield either the 58K or the 48K protein, depending on whether it is the 105K or 95K polyprotein that is being processed, and the two viral coat proteins, L and S. Initiation of translation at the start codon at position 512 in CPMV is more efficient than initiation at position 161, resulting in the production of more 95K polyprotein than 105K polyprotein.

The start codon at position 115 in CPMV RNA-2 lies upstream of the initiation sites at positions 161 and 512 and is in a different reading frame. As this start codon is in-phase with a stop codon at position 175, initiation at this site could result in the production of a 20 amino acid peptide. However, production of such a peptide has not been detected to date.

Necessity of maintaining the frame between AUGs

Mutagenesis experiments have shown that maintenance of the frame between the initiation sites at positions 161 and 512 in CPMV RNA-2 is essential for efficient replication of RNA-2 by the RNA-1-encoded replicase (Holness *et al.*, 1989; van Bokhoven *et al.*, 1993; Rohll *et al.*, 1993; Wellink *et al.*, 1993). This requirement restricts the length of sequences which can be inserted upstream of the 512 start codon in expression vectors based on CPMV RNA-2 (see below), making the cloning of foreign genes into such vectors more difficult than would be ideal. For example it precludes the use of polylinkers as their use will often alter the open reading frame (ORF) between these initiation sites.

CPMV vectors

CPMV has served as the basis for the development of vector systems suitable for the production of heterologous polypeptides in plants (Liu *et al.*, 2005; Sainsbury *et al.*, 2007). These systems are based on the modification of RNA-2 but differ in whether full-length or deleted versions are used. In both cases, however, replication of the modified RNA-2 is achieved by co-inoculation with RNA-1. Expression systems based on a full-length version of RNA-2 involve the fusion of the foreign protein to the C-terminus of the RNA-2-derived polyproteins. Release of the N-terminal polypeptide is mediated by the action of the 2A catalytic peptide sequence from foot-and-mouthdisease virus (Gopinath *et al.*, 2000). The resulting RNA-2 molecules are capable of spreading both within and between plants. This strategy has been used to express a number of recombinant proteins, such as the Hepatitis B core antigen (HBcAg) and Small Immune Proteins (SIPs), in cowpea plants (Mechtcheriakova *et al.*, 2006; Monger *et al.*, 2006; Alamillo *et al.*, 2006). Though successful, the use of a full-length viral vector has disadvantages in terms of size constraints of inserted sequences and concerns about biocontainment.

To address these, a system based on a deleted version of CPMV RNA-2 has recently been developed (Cañizares *et al.*, 2006). In this system the region of RNA-2 encoding the movement protein and both coat proteins has been removed. However, the deleted molecules still possess the *cis*-acting sequences necessary for replication by the RNA-1-encoded replicase and thus high levels of gene amplification are maintained without the concomitant possibility of the modified virus contaminating the environment. With the inclusion of a suppressor of gene silencing, such as HcPro from PVY, (Brigneti *et al.*, 1998) in the inoculum in addition to RNA-1, the deleted CPMV vector can be used as a transient expression system (WO/2007/135480) Bipartite System, Method And Composition For The Constitutive And Inducible Expression Of High Levels Of Foreign Proteins In Plants; also Sainsbury *et al.*, 2009). However, in contrast to the situation with a vector based on full-length RNA-2, replication is restricted to inoculated leaves. These CPMV vectors have been used to express multi-chain complexes consisting of a single type of polypeptide.

Multiple copies of vectors based on either full-length or deleted versions of CPMV RNA-2 have also been shown to be suitable for the production of heteromeric proteins in plants (Sainsbury *et al.*, 2008). Co-infiltration of two full-length RNA-2 constructs containing different marker genes into *Nicotiana benthamiana* in the presence of RNA-1 has been used to show that two foreign proteins can be efficiently expressed within the same cell in inoculated tissue. Furthermore, the proteins can be co-localised to the same sub-cellular compartments, which is an essential prerequisite for heteromer formation.

The suitability of different CPMV RNA-2 vectors for the expression of heteromeric proteins in plants has also been investigated. Insertion of the heavy and light chains of an IgG into full-length and deleted versions of RNA-2 showed that both approaches led to the accumulation of full-size IgG molecules in the inoculated tissue but that the levels were significantly higher when deleted RNA-2 vectors were used. The ability of full-length RNA-2 constructs to spread systemically therefore seems to be irrelevant to the production of heteromeric proteins and the use of deleted versions of RNA-2 is clearly advantageous, especially as they also offer the benefit of biocontainment.

Thus, known CPMV based vector systems represent useful tools for the expression of a heterologous gene encoding a protein of interest in plants. However, there is still a need in the art for optimised vector systems which improve, for example, the yield of the heterologous proteins expressed and the ease of use of the vector.

SUMMARY OF INVENTION

The present inventors have surprisingly found that mutation of the start codon at position 161 in a CPMV RNA-2 vector strongly increases the levels of expression of a protein encoded by a gene inserted after the start codon at position 512. The levels of protein expression were increased about 20-30 fold compared with expression of the same protein from a CPMV RNA-2 vector differing only in that the start codon at position 161 was intact (Sainsbury and Lomonossoff, 2008). The present invention allows the production of high levels of foreign proteins without the need for viral replication.

The inventors have also found that mutation of the start codon at position 161 negates the need for maintaining the frame between the position of the mutated start codon at position 161 and the start codon at position 512, thus allowing insertion of sequences of any length after the mutated start codon at position 161. This is particularly advantageous as it allows polylinkers of any length to be inserted into RNA-2 vectors after the mutated start codon, which can then be used to facilitate cloning of a gene of interest into the vector.

In addition, the inventors have found that despite the increase in protein expression, plants transformed with a CPMV RNA-2 vector comprising a mutated start codon at position 161 looked healthier, i.e. showed less necrosis, than plants transformed with known CPMV RNA-2 vectors. Plant health is an important factor in the expression of proteins from plants as healthy plants survive for longer periods of time. In addition, plant health is also important in the purification of proteins from plants as tannins released as a result of necrosis can interfere with protein purification (Sainsbury and Lomonossoff, 2008).

Thus the present invention relates to improved protein production systems and methods, based on modified bipartite virus sequences.

In accordance with one aspect of the present invention, there is provided a gene expression construct comprising:

(a) an expression enhancer sequence derived from the RNA-2 genome segment of a *Comoviridae* bipartite RNA virus, in which an initiation site in the RNA-2 genome segment has been mutated,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame, and wherein the mutated intiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV),

wherein the expression enhancer sequence either:

(i) comprises at least a 100 nucleotide sequence of the comoviral RNA-2 genome segment which includes the mutated initiation site, or

(ii) has at least 70% identity to the full length of nucleotides 1 to 507 of the CPMV RNA-2 genome segment sequence shown in SEQ ID NO: 1, and wherein the initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated; and

(b) a heterologous sequence for facilitating insertion of a gene encoding a protein of interest into the gene expression construct, wherein the heterologous sequence is located downstream of the mutated target initiation site in the enhancer sequence.

In accordance with another aspect of the present invention, there is provided a expression construct comprising:

(a) an expression enhancer sequence derived from the RNA-2 genome segment of a *Comoviridae* bipartite RNA virus, in which an initiation site in the RNA-2 genome segment has been mutated,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame, and wherein the mutated intiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV),

wherein the expression enhancer sequence either:

(i) comprises at least a 100 nucleotide sequence of the comoviral RNA-2 genome segment which includes the mutated initiation site, or

(ii) has at least 70% identity to the full length of nucleotides 1 to 507 of the CPMV RNA-2 genome segment sequence shown in SEQ ID NO: 1, and wherein the initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated; and

(b) a heterologous gene encoding a protein of interest, wherein the gene encoding the protein of interest is located downstream of the enhancer sequence.

In accordance with another aspect of the present invention, there is provided a gene expression construct comprising:

(a) a promoter;

(b) nucleotides 1 to 507 of the cowpea mosaic virus RNA-2 genome segment sequence shown in SEQ ID NO: 1, wherein the AUG at position 161 has been mutated to ACG, located downstream of the promoter;

(c) a gene encoding a protein of interest located downstream of the sequence defined in (b);

(d) nucleotides 3302 to 3481 of the cowpea mosaic virus RNA-2 genome segment sequence shown in SEQ ID NO: 1, located downstream of the gene encoding the protein of interest; and

(e) a nopaline synthase terminator located downstream of the sequence defined in (d).

In accordance with another aspect of the invention, there is provided a gene expression construct comprising:

(a) a first gene construct comprising a sequence derived from an RNA-2 segment of a *Comoviridae* bipartite virus genome carrying at least one foreign gene encoding a heterologous protein of interest operably linked to promoter and terminator sequences, wherein the gene construct comprises a mutated initiation site upstream of the foreign gene,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame

(a) a first gene construct comprising a sequence derived from an RNA-2 segment of a *Comoviridae* bipartite virus genome carrying at least one foreign gene encoding a heterologous protein of interest operably linked to promoter and terminator sequences, wherein the gene construct comprises a mutation in a translation initiation site upstream of the foreign gene such that it no longer functions as a translation initiation site,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame

wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of CPMV; and

(b) a second gene construct comprising a heterologous suppressor of gene silencing operably linked to promoter and terminator sequences.

In accordance with another aspect of the present invention, there is provided a process for increasing expression or translational enhancing activity of a sequence derived from an RNA-2 genome segment of a *Comoviridae* bipartite virus, comprising mutating a translation initiation site therein,wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame,

wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV),

wherein the mutating is performed either by:

(i) a point mutation in the initiation site, or

(ii) a deletion of up to 50 nucleotides in length including the initiation site,

wherein the mutation enhances the expression of a heterologous ORF to which the sequence is attached.

In accordance with another aspect of the present invention, there is provided a method for expressing a heterologous protein of interest in a host organism using the gene expression construct of the invention, wherein the host organism is a plant or insect.

In accordance with another aspect of the present invention, there is provided a method of enhancing the translation of a heterologous protein of interest from a gene or open reading frame

(ORF) encoding the heterologous protein of interest, which gene or ORF is operably linked to an RNA2- genome segment of a *Comoviridae* bipartite virus derived sequence,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame,

comprising mutating the first of these two initiation sites in the RNA2-derived sequence, wherein the mutated translation initiation site corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV),

wherein the mutating is performed either by:

(i) a point mutation in the initiation site, or

(ii) a deletion of up to 50 nucleotides in length including the initiation site.

In accordance with another aspect of the present invention, there is provided a method of expressing a heterologous protein in a plant comprising the steps of:

(a) introducing into a plant cell a gene expression construct comprising a first gene construct comprising a sequence derived from an RNA-2 segment of a *Comoviridae* bipartite virus genome carrying at least one foreign gene encoding a heterologous protein of interest operably linked to promoter and terminator sequences,

wherein the gene construct comprises a mutation in a translation initiation site upstream of the foreign gene such that it no longer functions as a translation initiation site,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame,wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV); and

(b) introducing a second gene construct comprising a heterologous suppressor of gene silencing operably linked to promoter and terminator sequences into the plant cell.

In accordance with another aspect of the present invention, there is provided a plant or insect host cell obtained by a method of the invention, which host cell comprises the gene expression construct, and wherein the gene encoding the protein of interest is expressed in the host cell.

In accordance with another aspect of the present invention, there is provided a plant host cell transiently transfected with, and comprising, the gene expression construct of the invention.

In accordance with another aspect of the present invention, there is provided an insect host cell transiently transfected with, and comprising, the gene expression construct of the invention.

In accordance with another aspect of the present invention, there is provided a transgenic plant or insect host cell stably transformed with, and comprising, the gene expression construct of the invention.

In accordance with another aspect of the present invention, there is provided a method for generating a protein of interest, comprising using the plant or insect host cell of the invention and harvesting a tissue in which the protein of interest has been expressed and isolating the protein of interest from the tissue.

Thus in various aspects of the invention there is provided or utilised an expression enhancer sequence, which sequence is derived from (or shares homology with) the RNA-2 genome segment of a bipartite RNA virus, such as a comovirus, in which a target initiation site has been mutated

The present invention further provides processes for increasing the expression or translational enhancing activity of a sequence derived from an RNA-2 genome segment of a bipartite virus, which processes comprise mutating a target initiation site therein.

Some particular definitions and embodiments of the invention will now be described in more detail.

“Enhancer” sequences (or enhancer elements), as referred to herein, are sequences derived from (or sharing homology with) the RNA-2 genome segment of a bipartite RNA virus, such as a comovirus, in which a target initiation site has been mutated. Such sequences can enhance downstream expression of a heterologous ORF to which they are attached. Without limitation, it is believed that such sequences when present in transcribed RNA, can enhance translation of a heterologous ORF to which they are attached.

A “target initiation site” as referred to herein, is the initiation site (start codon) in a wild-type RNA-2 genome segment of a bipartite virus (e.g. a comovirus) from which the enhancer sequence in question is derived, which serves as the initiation site for the production (translation) of the longer of two carboxy coterminal proteins encoded by the wild-type RNA-2 genome segment.

As described above, production of the longer of the two carboxy coterminal proteins encoded by CPMV RNA-2, the 105K protein, is initiated at the initiation site at position 161 in the wild-type CPMV RNA-2 genome segment. Thus, the target initiation site in enhancer sequences derived from the CPMV RNA-2 genome segment is the initiation site at position 161 in the wild-type CPMV RNA-2.

Mutations around the start codon at position 161 may have the same (or similar) effect as mutating the start codon at position 161 itself, for example, disrupting the context around this start codon may mean that the start codon is by-passed more frequently.

In one aspect of the present invention, a target initiation site may therefore be 'mutated' indirectly by mutating one or more nucleotides upstream and/or downstream of the target initiation site, but retaining the wild-type target initiation site, wherein the effect of mutating these nucleotides is the same, or similar, to the effect observed when the target initiation site itself is mutated.

As target initiation sites serve as the initiation site for the production of the longer of two carboxy coterminal proteins encoded by a wild-type RNA-2 genome segment, it follows that target initiation sites are in-frame (in phase) with a second initiation site on the same wild-type RNA-2 genome segment, which serves as the initiation site for the production of the shorter of two carboxy coterminal proteins encoded by the wild-type RNA-2. Two initiation sites are in-frame if they are in the same triplet reading frame.

The target initiation site in enhancer sequences derived from the wild-type CPMV RNA-2 genome segment, i.e. the initiation site at position 161, is in frame with the initiation site at position 512, which serves as the initiation site for the production of the shorter of the two carboxy coterminal proteins encoded by CPMV RNA-2 (the 95K protein) in the wild-type CPMV RNA-2 genome segment.

Thus, a target initiation site is located upstream (5') of a second initiation site in the wild-type RNA-2 genome segment from which the enhancer sequence is derived, which serves the initiation site for the production of the shorter of two carboxy coterminal polyproteins encoded by the wild-type RNA-2 genome segment. In addition, a target initiation site may also be located downstream (3') of a third initiation site in the wild-type RNA-2 genome from which the enhancer sequence is derived. In CPMV the target initiation site, i.e. the initiation site at position 161, is located upstream of a second initiation site at position 512 which serves as the initiation site for the production of the 95K protein and downstream of a third initiation site at position 115.

A target initiation site in an enhancer sequence derived from the RNA-2 genome segment of a bipartite virus is therefore the first of two initiation sites for the production of two carboxy coterminal proteins encoded by the wild-type RNA-2. 'First' in this context refers to the initiation site located closer to the 5' end of the wild-type RNA-2 genome segment.

More than one initiation site in the sequence may be mutated, if desired. For example the 'third' initiation site at (or corresponding to) position 115 may also be deleted or altered. It has been shown that removal of AUG 115 in addition to the removal of AUG 161, further enhances expression (Sainsbury and Lomonossoff, 2008) .

The enhancer sequences of the present invention are based on modified sequences from the RNA-2 genome segments of bipartite RNA viruses.

A bipartite virus, or virus with a bipartite genome, as referred to herein may be a member of the *Comoviridae* family. All genera of the family *Comoviridae* appear to encode two carboxy-coterminal proteins. The genera of the *Comoviridae* family include Comovirus, Nepovirus, Fabavirus, Cheravirus and Sadwavirus. Comoviruses include *Cowpea mosaic virus* (CPMV), Cowpea severe mosaic virus (CPSMV), Squash mosaic virus (SqMV), Red clover mottle virus (RCMV), Bean pod mottle virus (BPMV). Preferably, the bipartite virus (or comovirus) is CPMV.

The sequences of the RNA-2 genome segments of these comoviruses and several specific strains are available from the NCBI database under the accession numbers listed in brackets: *cowpea mosaic virus* RNA-2 (NC_003550), *cowpea severe mosaic virus* RNA-2 (NC_003544), *squash mosaic virus* RNA-2 (NC_003800), *squash mosaic virus* strain Kimble RNA-2 (AF059533), *squash mosaic virus* strain Arizona RNA-2 (AF059532), *red clover mottle virus* RNA-2 (NC_003738), *bean pod mottle virus* RNA-2 (NC_003495), *bean pod mottle virus* strain K-Hopkins1 RNA-2 (AF394609), *bean pod mottle virus* strain K-Hancock1 RNA-2 (AF394607), *Andean potato mottle virus* (APMoV: L16239) and *Radish mosaic virus* (RaMV; AB295644). There are also partial RNA-2 sequences available from *bean rugose mosaic virus* (BRMV; AF263548) and a tentative member of the genus Comovirus, *turnip ringspot virus* (EF191015). Numerous sequences from the other genera in the family *Comoviridae* are also available.

To date, all comoviruses which have been investigated have been shown to have two alternative start codons for the expression of two carboxy coterminal polyproteins form their RNA-2 genome segments. In particular, the RNA-2 genome segments of CPMV, CPSMV, BPMV, SqMV and RCMV are known to comprise two alternative start codons for the expression of two carboxy coterminal polyproteins.

Target initiation sites in other comoviruses, which are equivalent to the initiation site at position 161 in the wild-type RNA-2 segment of CPMV (i.e. correspond to it) can therefore be identified by methods known in the art. For example, target initiation sites can be identified by a sequence alignment between the wild-type RNA-2 genome segment sequence of CPMV and the RNA-2 genome segment sequence of another comovirus. Such sequence alignments can then be used to identify a target initiation site in the comoviral RNA-2 genome segment sequence by identifying an initiation site which, at least in the alignment, is near, or at the same position as, the target initiation site at position 161 in the wild-type CPMV RNA-2.

Target initiation sites in other comoviruses may also be identified by determining the start codon which serves as the initiation site for the synthesis of the longer of two carboxy coterminal proteins encoded by the wild-type comoviral RNA-2 genome segment. This approach can also be used in combination with an alignment as described above, i.e. this approach can be used to confirm that a comoviral initiation site identified by means of an alignment with CPMV RNA-2 is a target initiation site.

Of course, the above methods can also be used for identifying initiation sites in other comoviral RNA-2 genome segments, which are equivalent to the initiation site at position 512 in the wild-type CPMV RNA-2 genome segment. However, instead of identifying the start codon which serves as the initiation site for the synthesis of the longer of two carboxy coterminal proteins encoded by the wild-type comoviral RNA-2 genome segment, the start codon which serves as the initiation site for the synthesis of the shorter of two carboxy coterminal proteins encoded by the wild-type comoviral RNA-2 genome segment, is identified.

Once two comoviral RNA-2 initiation sites which are likely to be equivalent to the initiation sites at positions 161 and 512 in CPMV RNA-2 have been identified, the identification of the target initiation site can be confirmed by checking that the two initiation sites are in the same frame, i.e. in the same triplet reading frame, as they can only serve as initiation sites for the production of two carboxy coterminal proteins if this is the case.

In one embodiment of the invention, the enhancer sequence comprises nucleotides 1 to 512 of the CPMV RNA-2 genome segment (see Table 1), wherein the target initiation site at position 161 has been mutated. In another embodiment of the invention, the enhancer sequence comprises an equivalent sequence from another comovirus, wherein the target initiation site equivalent to the start codon at position 161 of CPMV has been mutated. The target initiation site may be mutated by substitution, deletion or insertion. Preferably, the target initiation site is mutated by a point mutation.

In alternative embodiments of the invention, the enhancer sequence comprises nucleotides 10 to 512, 20 to 512, 30 to 512, 40 to 512, 50 to 512, 100 to 512, 150 to 512, 1 to 514, 10 to 514, 20 to 514, 30 to 514, 40 to 514, 50 to 514, 100 to 514, 150 to 514, 1 to 511, 10 to 511, 20 to 511, 30 to 511, 40 to 511, 50 to 511, 100 to 511, 150 to 511, 1 to 509, 10 to 509, 20 to 509, 30 to 509, 40 to 509, 50 to 509, 100 to 509, 150 to 509, 1 to 507, 10 to 507, 20 to 507, 30 to 507, 40 to 507, 50 to 507, 100 to 507, or 150 to 507 of a comoviral RNA-2 genome segment sequence with a mutated target initiation site. In other embodiments of the invention, the enhancer sequence comprises nucleotides 10 to 512, 20 to 512, 30 to 512, 40 to 512, 50 to 512, 100 to 512, 150 to 512, 1 to 514, 10 to 514, 20 to 514, 30 to 514, 40 to 514, 50 to 514, 100 to 514, 150 to 514, 1 to 511, 10 to 511, 20 to 511, 30 to 511, 40 to 511, 50 to 511, 100 to 511, 150 to 511, 1 to 509, 10 to 509, 20 to 509, 30 to 509, 40 to 509, 50 to 509, 100 to 509, 150 to 509, 1 to 507, 10 to 507, 20 to 507, 30 to 507, 40 to 507, 50 to 507, 100 to 507, or 150 to 507 of the CPMV RNA-2 genome segment sequence shown in Table 1, wherein the target initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated.

In further embodiments of the invention, the enhancer sequence comprises nucleotides 1 to 500, 1 to 490, 1 to 480, 1 to 470, 1 to 460, 1 to 450, 1 to 400, 1 to 350, 1 to 300, 1 to 250, 1 to 200, or 1 to 100 of a comoviral RNA-2 genome segment sequence with a mutated target initiation site.

In alternative embodiments of the invention, the enhancer sequence comprises nucleotides 1 to 500, 1 to 490, 1 to 480, 1 to 470, 1 to 460, 1 to 450, 1 to 400, 1 to 350, 1 to 300, 1 to 250, 1 to 200, or 1 to 100 of the CPMV RNA-2 genome segment sequence shown in Table 1, wherein the target initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated.

Enhancer sequences comprising at least 100 or 200, at least 300, at least 350, at least 400, at least 450, at least 460, at least 470, at least 480, at least 490 or at least 500 nucleotides of a comoviral RNA-2 genome segment sequence with a mutated target initiation site are also embodiments of the invention.

In addition, enhancer sequences comprising at least 100 or 200, at least 300, at least 350, at least 400, at least 450, at least 460, at least 470, at least 480, at least 490 or at least 500 nucleotides of the CPMV RNA-2 genome segment sequence shown in Table 1, wherein the target initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated, are also embodiments of the invention.

Alternative embodiments of the invention are enhancer sequences having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, or 50% identity to the CPMV RNA-2 genome segment sequence shown in Table 1, wherein the target initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated.

The terms "percent similarity", "percent identity" and "percent homology" when referring to a particular Sequence are used as set forth in the University of Wisconsin GCG software program. Enhancer sequences may thus specifically hybridise with the complementary sequence of the CPMV RNA-2 genome segment sequence shown in Table 1, with the proviso that the target initiation site corresponding to position 161 in the wild-type CPMV RNA-2 genome segment has been mutated.

The phrase "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. "Complementary" refers to the natural association of nucleic acid sequences by base-pairing (A-G-T pairs with the complementary sequence T-C-A). Complementarity between two single-stranded molecules may be partial, if only some of the nucleic acids pair are complementary; or complete, if all bases pair are complementary. The degree of complementarity affects the efficiency and strength of hybridization and amplification reactions.

A target initiation site in an enhancer sequence of the invention may be mutated by deletion, insertion or substitution, such that it no longer functions as a translation initiation site. For example, a point mutation may be made at the position of the target initiation site in the enhancer sequence. Alternatively, the target initiation site in the enhancer sequence may be deleted either partially or in its entirety. For example, a deletion spanning the target initiation site in the enhancer sequence may be made. Deletions spanning the initiation site may be up to 5, up to 10, up to 15, up to 20, up to 25, up to 30, up to 35, up to 40, up to 45, or up to 50 nucleotides in length, when compared with the sequence of the wild-type RNA-2 genome segment from which the enhancer sequence is derived.

Without wishing to be bound by theory, mutation of the start codon at position 161 in CPMV is thought to lead to the inactivation of a translational suppressor, which results in enhanced initiation of translation from start codons located downstream of the inactivated translational suppressor.

Thus, the present invention further provides an enhancer sequence derived from an RNA-2 genome segment of a bipartite virus, wherein the enhancer sequence comprises an inactivated translational suppressor sequence.

The present invention further provides a process for increasing the expression or translational enhancing activity of a sequence derived from an RNA-2 genome segment of a bipartite virus, which process comprises inactivating a translational suppressor sequence therein.

As already mentioned above, mutation of the initiation site at position 161 in the CPMV RNA-2 genome segment is thought to lead to the inactivation of a translation suppressor normally present in the CPMV RNA-2.

A translational suppressor sequence, as referred to herein, is a sequence in the wild-type RNA-2 genome segment of the bipartite virus (e.g. a comovirus) from which the enhancer sequence in question is derived, which comprises, or consists of, the initiation site for the production (translation) of the longer of two carboxy coterminal proteins encoded by the wild-type RNA-2 genome segment.

Translational suppressor sequences in enhancer sequences derived from the CPMV RNA-2 genome segment, are sequences comprising, or consisting of, the target initiation site described above. Thus, translational suppressor sequences comprise, or consist of, a target initiation site as defined above, and may be inactivated by mutagenesis as described above.

The enhancer sequences defined above may be used in various aspects and embodiments of the invention as follows.

Thus in one aspect of the present invention there is provided or utilised an isolated nucleic acid consisting, or consisting essentially of, an expression enhancer sequence as described above.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" Is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated.

For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e., in cells or tissues). An "isolated nucleic acid" (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

The nucleic acid may thus consist or consist essentially of a portion, or fragment, of the RNA-2 genome segment of the bipartite RNA virus from which the enhancer is derived. For example, in one embodiment the nucleic acid does not comprise at least a portion of the coding region of the RNA-2 genome segment from which it is derived. The coding region may be the region of the RNA-2 genome segment encoding the shorter of two carboxy coterminal proteins. The nucleic acid may consist or consist essentially of the portion of an RNA-2 genome segment of a bipartite virus extending from the 5' end of the wild-type RNA-2 genome segment to the initiation site from which production (translation) of the shorter of two carboxy coterminal proteins encoded by the wild-type RNA-2 genome segment is initiated.

The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID NO. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence. For example, when used in reference to a nucleic acid, the phrase includes the sequence per se and minor changes and\or extensions that would not affect the enhancer function of the sequence, or provide further (additional) functionality.

The invention further relates to gene expression systems comprising an enhancer sequence of the invention.

Thus, in another aspect the present invention provides a gene expression system comprising an enhancer sequence as described above.

The gene expression system may also comprise a gene encoding a protein of interest inserted downstream of the enhancer sequence. Inserted sequences encoding a protein of interest may be of any size.

In a further aspect the present invention therefore provides a gene expression system comprising:

(a) an enhancer sequence as described above; and (b) a gene encoding a protein of interest, wherein the gene is located downstream of the enhancer sequence.

The gene and protein of interest may be a heterologous i.e. not encoded by the wild-type bipartite RNA virus from which the enhancer sequence is derived.

Gene expression systems may be used to express a protein of interest in a host organism. In this case, the protein of interest may also be heterologous to the host organism in question i.e. introduced into the cells in question (e.g. of a plant or an ancestor thereof) using genetic engineering, i.e. by human intervention. A heterologous gene in an organism may replace an endogenous equivalent gene, i.e. one which normally performs the same or a similar function, or the inserted sequence may be additional to the endogenous gene or other sequence.

Persons skilled in the art will understand that expression of a gene of interest will require the presence of an initiation site (AUG) located upstream of the gene to be expressed. Such initiation sites may be provided either as part of an enhancer sequence or as part of a gene encoding a protein of interest.

The host organism may be a plant. However, as translational mechanisms are well conserved over eukaryotes, the gene expression systems may also be used to express a protein of interest in eukaryotic host organisms other than plants, for example in insect cells as modified baculovirus vectors, or in yeast or mammalian cells.

Gene expression systems may be operably linked to promoter and terminator sequences.

Thus, gene expression systems may further comprise a termination sequence and the gene encoding a protein of interest may be located between the enhancer sequence and the termination sequence, i.e. downstream (3') of the enhancer sequence and upstream (5') of the termination sequence.

Thus the invention further provides an expression cassette comprising:
(i) a promoter, operably linked to
(ii) an enhancer sequence as described above
(iii) a gene of interest it is desired to express

(iv) a terminator sequence.

Preferably the promoter used to drive the gene of interest will be a strong promoter. Examples of strong promoters for use in plants include:

(1) p35S: Odell *et al.*, 1985

(2) Cassava Vein Mosaic Virus promoter, pCAS, Verdaguer *et al.*, 1996

(3) Promoter of the small subunit of ribulose biphosphate carboxylase, pRbcS: Outchkourov *et al.*, 2003.

Other strong promoters include pUbi (for monocots and dicots) and pActin.

In a preferred embodiment, the promoter is an inducible promoter.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus.

The termination (terminator) sequence may be a termination sequence derived from the RNA-2 genome segment of a bipartite RNA virus, e.g. a comovirus. In one embodiment the termination sequence may be derived from the same bipartite RNA virus from which the enhancer sequence is derived. The termination sequence may comprise a stop codon. Termination sequence may also be followed by polyadenylation signals.

Gene expression cassettes, gene expression constructs and gene expression systems of the invention may also comprise an untranslated region (UTR). The UTR may be located upstream of a terminator sequence present in the gene expression cassette, gene expression construct or gene expression system. Where the gene expression cassettes, gene expression constructs or gene expression systems comprises a gene encoding a protein of interest, the UTR may be located downstream of said gene.

Thus, the UTR may be located between a gene encoding a protein of interest and a terminator sequence. The UTR may be derived from a bipartite RNA virus, e.g. from the RNA-2 genome segment of a bipartite RNA virus. The UTR may be the 3' UTR of the same RNA-2 genome segment from which the enhancer sequence present in the gene expression cassette, gene expression construct or gene expression system is derived. Preferably, the UTR is the 3' UTR of a comoviral RNA-2 genome segment, e.g. the 3' UTR of the CPMV RNA-2 genome segment.

As described above, it was previously shown to be essential for efficient replication of CPMV RNA-2 by the CPMV RNA-1-encoded replicase that the frame between the initiation sites at positions 161 and 512 in the RNA-2 was maintained, i.e. that the two initiation sites remained in the same triple reading frame (Holness *et al.*, 1989; van Bokhoven *et al.*, 1993; Rohll *et al.*, 1993). This requirement limited the length of sequences which could be inserted upstream of the initiation site at position 512 in expression vectors based on CPMV. In particular, it precluded the use of polylinkers as their use often altered the open reading frame (ORF) between the two initiation sites.

The present inventors have shown that maintenance of the reading frame between the initiation sites at positions 161 and 512 in CPMV RNA-2 is also required for efficient initiation of translation at the initiation site at position 512, i.e. it is required for efficient expression of the shorter of the two carboxy coterminal proteins encoded by CPMV (the 95K protein).

However, the present inventors have also demonstrated that mutation of the initiation site at position 161 in CPMV RNA-2 allows insertion of sequences upstream of the initiation site at position 512, which alter the frame between the mutated start codon and the initiation site at position 512, without any negative effect on the level of expression of the 95K protein. Consequently, mutation of the initiation site at position 161 means that there is no longer any restriction on the length of sequences that can be inserted upstream of the initiation site at position 512.

Where maintenance of the reading frame between initiation sites coding for two carboxy-coterminal proteins is also required in other bipartite viruses, this requirement may also be overcome by mutating the AUG which serves as the initiation site for productions of the longer of the two carboxy-coterminal proteins encoded by the viral

RNA-2 genome segment.Thus, in another aspect the present invention provides a gene expression construct comprising:
(a) an enhancer sequence as described above; and
(b) a heterologous sequence for facilitating insertion of a gene encoding a protein of interest into the gene expression system, wherein the heterologous sequence is located downstream of the mutated target initiation site in the enhancer sequence.

The heterologous sequence may be located upstream of the start codon from which production of the shorter of two carboxy coterminal proteins is initiated in the wild-type RNA-2 genome segment from which the enhancer sequence of the gene expression system is derived. Alternatively, the heterologous sequence may be provided around the site of the start codon, or replace the start codon, from which production of the shorter of two carboxy coterminal proteins is initiated in the wild-type RNA-2 genome segment from which the enhancer sequence of the gene expression system is derived. In a gene expression system with an enhancer sequence derived from the RNA-2 of CPMV, the heterologous sequence may be provided upstream of, around the site of, or replace, the start codon which is at position 512 in the wild-type RNA-2 CPMV genome segment.

The heterologous sequence may be a polylinker or multiple cloning site, i.e. a sequence which facilitates cloning of a gene encoding a protein of interest into the expression system.

For example, as described hereinafter, the present inventors have provided constructs including a polylinker between the 5' leader and 3' UTRs of a CPMV-based expression cassette. As described below, any polyinker may optionally encode one or more sets of mutliple x Histidine residues to allow the fusion of N- or C terminal His-tags to facilitate protein purification.

Preferably the expression constructs above are present in a vector, and preferably it comprises border sequences which permit the transfer and integration of the expression cassette into the organism genome.

Preferably the construct is a plant binary vector. Preferably the binary transformation vector is based on pPZP (Hajdukiewicz, *et al.* 1994). Other example constructs include pBin19 (see Frisch, D. A., L. W. Harris-Haller, et al. (1995). "Complete Sequence of the binary vector Bin 19." Plant Molecular Biology 27: 405-409).

As described herein, the invention may be practiced by moving an expression cassette with the requisite components into an existing pBin expression cassette, or in other embodiments a direct-cloning pBin expression vector may be utilised.

For example, as described hereinafter, the present inventors have modular binary vectors designed for (but not restricted to) use with the enhancer sequences described herein. These are based on improvements to the pBINPLUS vector whereby it has been shown that it is possible to drastically reduce the size of the vector without compromising performance in terms of replication and TDNA transfer. Furthermore, elements of the enhancer system (as exemplified by the so-called "CPMV-HT" system) have been incorporated into the resulting vector in a modular fashion such that multiple proteins can be expressed from a single T-DNA. These improvements have led to the creation of a versatile, high-level expression vector that allows efficient direct cloning of foreign genes.

These examples represent preferred binary plant vectors. Preferably they include the ColEI origin of replication, although plasmids containing other replication origins that also yield high copy numbers (such as pRi-based plasmids, Lee and Gelvin, 2008) may also be preferred, especially for transient expression systems.

If desired, selectable genetic markers may be included in the construct, such as those that confer selectable phenotypes such as resistance to antibiotics or herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

Most preferred vectors are the pEAQ vectors described below which permit direct cloning version by use of a polylinker between the 5' leader and 3' UTRs of an expression cassette including a translational enhancer of the invention, positioned on a T-DNA which also contains a suppressor of gene silencing and an NPTII cassettes. The polylinker also encodes one or two sets of 6 x Histidine residues to allow the fusion of N- or C terminal His-tags to facilitate protein purification.

An advantage of pEAQ-derived vectors is that each component of a multi-chain protein such as an IgG can automatically be delivered to each infected cell.

The present invention also provides methods of expressing proteins, e.g. heterologous proteins, in host organisms such as plants, yeast, insect or mamalian cells, using a gene expression system of the invention.

The present invention further provides a method of enhancing the translation of a heterologous protein of interest from a gene or ORF encoding the same which is operably linked to an RNA2-derived sequence as described above, the method comprising mutating a target initiation site in the RNA2-derived sequence.

The enhancer sequences described herein may also be used with bipartite expression systems as described in WO/2007/135480. The invention therefore also relates to gene expression systems based on truncated RNA-2 gene segments, optionally further comprising a second gene construct encoding a suppressor of gene silencing operably linked to promoter and terminator sequences.

In a further aspect the present invention therefore relates to a gene expression system comprising:
(a) a first gene construct comprising a truncated RNA-2 of a bipartite virus genome carrying at least one foreign gene encoding a heterologous protein of interest operably linked to promoter and terminator sequences, wherein the gene construct comprises a mutated target initiation site upstream of the foreign gene; and optionally
(b) a second gene construct comprising RNA-1 of said bipartite virus genome operably linked to promoter and terminator sequences; and optionally
(c) a third gene construct, optionally incorporated within said first gene construct, said second gene construct or both, comprising a suppressor of gene silencing operably linked to promoter and terminator sequences.

The presence of a suppressor of gene silencing in a gene expression system (including any of those described above) of the invention is preferred but not essential. Thus, a gene expression system, as defined above, preferably comprises a third gene construct, optionally incorporated within said first gene construct, said second gene construct or both, comprising a suppressor of gene silencing operably linked to promoter and terminator sequences.

Thus, in another aspect the present invention provides a method of expressing a protein in a plant comprising the steps of:

(a) introducing a gene expression construct of the invention into a plant cell; and optionally

(b) introducing a second gene construct comprising RNA-1 of said bipartite virus genome operably linked to promoter and terminator sequences into the plant cell; and optionally

(c) introducing a third gene construct, optionally incorporated within said first gene construct, said second gene construct or both, comprising a suppressor of gene silencing operably linked to promoter and terminator sequences into the plant cell.

Preferably, a method of expressing a protein in a plant, as defined above, comprises the step of introducing a third gene construct, optionally incorporated within said first gene construct, said second gene construct or both, comprising a suppressor of gene silencing operably linked to promoter and terminator sequences into the plant cell.

The present invention also provides methods comprising introduction of such a construct into a plant cell.

The present inventors have shown very high expression levels by incorporating both a gene of interest and a suppressor of silencing onto the same T-DNA as the translational enhancer. Preferred embodiments may therefore utilise all these components are present on the same T-DNA.

Additionally it should be understood that the RNA-1 is not required for high level expression in the systems described herein, and indeed the "CPMV-HT" system described herein is not by the action of RNA-1.

Thus in a further aspect the present invention therefore relates to a gene expression system comprising:

(a) a first gene construct comprising a truncated RNA-2 of a bipartite virus genome carrying at least one foreign gene encoding a heterologous protein of interest operably linked to promoter and terminator sequences, wherein the gene construct comprises a mutated target initiation site upstream of the foreign gene; and optionally

(b) a second gene construct optionally incorporated within said first gene construct, a suppressor of gene silencing operably linked to promoter and terminator sequences.

Thus, in another aspect the present invention provides a method of expressing a protein in a plant comprising the steps of:

(a) introducing a gene expression construct of the invention into a plant cell; and optionally

(b) introducing a second gene construct optionally incorporated within said first gene construct, comprising a suppressor of gene silencing operably linked to promoter and terminator sequences into the plant cell.

Suppressors of gene silencing useful in these aspects are known in the art and described in WO/2007/135480. They include HcPro from Potato virus Y, He-Pro from TEV, P19 from TBSV, rgsCam, B2 protein from FHV, the small coat protein of CPMV, and coat protein from TCV. Most preferably, the RNA-2 of the system is truncated such that no infectious virus is produced.

A preferred suppressor when producing stable transgenic plants is the P19 suppressor incorporating a R43W mutation.

In a further aspect of the invention, there is disclosed a host cell containing a heterologous construct according to the present invention.

Gene expression vectors of the invention may be transiently or stably incorporated into plant cells.

For small scale production, mechanical agroinfiltration of leaves with constructs of the invention. Scale-up is achieved through, for example, the use of vacuum infiltration.

In other embodiments, an expression vector of the invention may be stably incorporated into the genome of the transgenic plant or plant cell.

In one aspect the invention may further comprise the step of regenerating a plant from a transformed plant cell.

Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148). Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809). If desired, selectable genetic markers may be included in the construct, such as those that confer selectable phenotypes such as resistance to antibiotics or herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

Nucleic acid can be introduced into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984; the floral dip method of Clough and Bent, 1998), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11. Ti-plasmids, particularly binary vectors, are discussed in more detail below.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. However there has also been considerable success in the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (see e.g. Hiei *et al.* (1994) *The Plant Journal* 6, 271-282)). Microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium alone is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice.

It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration. In experiments performed by the inventors, the enhanced expression effect is seen in a variety of integration patterns of the T-DNA.

Thus various aspects of the present invention provide a method of transforming a plant cell involving introduction of a construct of the invention into a plant tissue (e.g. a plant cell) and causing or allowing recombination between the vector and the plant cell genome to introduce a nucleic acid according to the present invention into the genome. This may be done so as to effect transient expression.

Alternatively, following transformation of plant tissue, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewd in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

The generation of fertile transgenic plants has been achieved in the cereals such as rice, maize, wheat, oat, and barley plus many other plant species (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, *et al.* (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

Regenerated plants or parts thereof may be used to provide clones, seed, selfed or hybrid progeny and descendants (e.g. F1 and F2 descendants), cuttings (e.g. edible parts), propagules, etc.

The invention further provides a transgenic organism (for example obtained or obtainable by a method described herein) in which an expression vector or cassette has been introduced, and wherein the heterologous gene in the cassette is expressed at an enhanced level, The invention further comprises a method for generating the protein of interest, which method comprises the steps of performing a method (or using an organism) as described above, and optionally harvesting, at least, a tissue in which the protein of interest has been expressed and isolating the protein of interest from the tissue.

Specifically, the present invention therefore provides a transgenic plant or plant cell transiently transfected with an expression vector of the invention.

In a further aspect, the present invention also provides a transgenic plant or plant cell stably transformed with an expression vector of the invention.

The invention also provides a plant propagule from such plants, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. It also provides any part of these plants which includes the plant cells or heterologous DNA described above.

Thus in various aspects (and without limitation) the invention provides:

- Nucleic acids consisting or consisting essentially of an enhancer sequence of the invention (which enhancer sequence may (for example) consist of nucleotides 1 to 512 of the CPMV RNA-2 genome segment, or be derived from that, or from another RNA-2 genome segment of a bipartite RNA virus, in each case in which the target initiation site corresponding to CPMV RNA-2 position 161 is mutated).

- Gene expression systems comprising such enhancer sequences, for example upstream of an ORF encoding a protein of interest, or a polylinker, and optionally terminator.

- Bipartite expression systems as described in WO/2007/135480 modified according to the present invention to use enhancer sequences described herein.

- Expression cassettes comprising: (i) a promoter, operably linked to (ii) an enhancer sequence as described above (iii) a polylinker or gene of interest it is desired to express (iv) the cognate 3' UTR (i.e. from the 3' UTR of the CPMV RNA-2 genome segment), (v) a terminator sequence.

- Methods of expressing proteins, e.g. heterologous proteins, in host organisms such as plants using gene expression systems or vectors of the invention.

- Host cells and organisms (e.g. plants or yeasts) expressing proteins from the gene expression systems or vectors of the invention and methods of producing the same.

"Gene" unless context demands otherwise refers to any nucleic acid encoding genetic information for translation into a peptide, polypeptide or protein. Thus unless context demands otherwise it used interchangeably with "ORF".

The genes which it may be desired to express may be transgenes or endogenes.

Genes of interest include those encoding agronomic traits, insect resistance, disease resistance, herbicide resistance, sterility , grain characteristics, and the like. The genes may be involved in metabolism of oil, starch, carbohydrates, nutrients, etc. Thus genes or traits of interest include, but are not limited to, environmental- or stress- related traits, disease-related traits, and traits affecting agronomic performance. Target sequences also include genes responsible for the synthesis of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, toxins, carotenoids, hormones, polymers, flavonoids, storage proteins, phenolic acids, alkaloids, lignins, tannins, celluloses, glycoproteins, glycolipids, etc.

Most preferably the targeted genes in monocots and/or dicots may include those encoding enzymes responsible for oil production in plants such as rape, sunflower, soya bean and maize; enzymes involved in starch synthesis in plants such as potato, maize, cereals; enzymes which synthesise, or proteins which are themselves, natural medicaments such as pharmaceuticals or veterinary products.

Heterologous nucleic acids may encode, *inter alia*, genes of bacterial, fungal, plant or animal origin. The polypeptides may be utilised *in planta* (to modify the characteristics of the plant e.g. with respect to pest susceptibility, vigour, tissue differentiation, fertility, nutritional value etc.) or the plant may be an intermediate for producing the polypeptides which can be purified therefrom for use elsewhere. Such proteins include, but are not limited to retinoblastoma protein, p53, angiostatin, and leptin. Likewise, the methods of the invention can be used to produce mammalian regulatory proteins. Other sequences of interest include proteins, hormones, growth factors, cytokines, serum albumin, haemoglobin, collagen, etc.

Thus the target gene or nucleotide sequence preferably encodes a protein of interest which is : an insect resistance protein; a disease resistance protein; a herbicide resistance protein; a mammalian protein.

"Vector" is defined to include, inter alia, any plasmid, cosmid, phage, viral or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication). The constructs used will be wholly or partially synthetic. In particular they are recombinant in that nucleic acid sequences which are not found together in nature (do not run contiguously) have been ligated or otherwise combined artificially. Unless specified otherwise a vector according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

“Binary Vector”: as is well known to those skilled in the art, a binary vector system includes (a) border sequences which permit the transfer of a desired nucleotide sequence into a plant cell genome; (b) desired nucleotide sequence itself, which will generally comprise an expression cassette of (i) a plant active promoter, operably linked to (ii) the target sequence and\or enhancer as appropriate. The desired nucleotide sequence is situated between the border sequences and is capable of being inserted into a plant genome under appropriate conditions. The binary vector system will generally require other sequence (derived from *A. tumefaciens*) to effect the integration. Generally this may be achieved by use of so called "agro-infiltration" which uses Agrobacterium-mediated transient transformation. Briefly, this technique is based on the property of *Agrobacterium tumefaciens* to transfer a portion of its DNA ("T-DNA") into a host cell where it may become integrated into nuclear DNA. The T-DNA is defined by left and right border sequences which are around 21-23 nucleotides in length. The infiltration may be achieved e.g. by syringe (in leaves) or vacuum (whole plants). In the present invention the border sequences will generally be included around the desired nucleotide sequence (the T-DNA) with the one or more vectors being introduced into the plant material by agro-infiltration.

"Expression cassette" refers to a situation in which a nucleic acid is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial or plant cell.

A "promoter" is a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter.

"Plant" species of interest include, but are not limited to, corn (Zea mays),Brassica sp. (e.g., B. napus, B. rapa, B. juncea), particularly those Brassica species useful as sources of seed oil, alfalfa (Medicago sativa), rice (Oryza sativa), rye (Secale cereale), sorghum (Sorghum bicolor, Sorghum vulgare), millet (e.g., pearl millet (Pennisetum glaucum)), proso millet (Panicum miliaceum), foxtail millet (Setaria italica), finger millet, (Eleusine coracana)), sunflower (Helianthus annuus), safflower (Carthamus tinctorius), wheat (Triticum aestivum), soybean (Glycine max), tobacco (Nicotiana tabacum), Nicotiana benthamiana , potato (Solanum tuberosum), peanuts (Arachis hypogaea), cotton (Gossypium barbadense, Gossypium hirsutum), sweet potato (Ipomoea batatus), cassava (Manihot esculenta), coffee (Coffea spp.), coconut (Cocos nucifera), pineapple (Ananas comosus), citrus trees (Citrus spp.), cocoa (Theobroma cacao), tea (Camellia sinensis), banana (Musa spp.), avocado (Persea americana), fig (Ficus casica), guava (Psidium guajava), mango (Mangifera indica), olive (Olea europaea), papaya (Carica papaya), cashew (Anacardium occidentale), macadamia (Macadamia integrifolia), almond (Prunus amygdalus), sugar beets (Beta vulgaris), sugarcane (Saccharum spp.), oats, barley, vegetables, ornamentals, and conifers. The skilled person will appreciate that the tropism of the viral vectors disclosed herein varies. However, determining suceptibility to such viruses is well within the purview of the skilled person. Moreover, it may be possible to alter such specificity by recombinantly expressing receptors which facilitate viral entry into a plant cell.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic diagram of CPMV expression vectors 00, 10, 01 and 11. In 00 expression vectors the initiation sites at positions 115 and 161 are intact. In 10 expression vectors the initiation site at positions 115 has been mutated but the initiation site at position 161 is intact. In 01 expression vectors the initiation site at positions 161 has been mutated but the initiation site at position 115 is intact. In 11 expression vectors the initiation sites at positions 115 and 161 are both mutated. CPMV expression vectors 00, 10, 01 and 11 also comprise an initiation site at either position 512 (FSC2-152), 513 (FSC2-513) or 514 (FSC2-514). Bars are used to indicate the initiation sites from which protein expression is expressed to occur.

Figure 2 shows the level of soluble green fluorescent protein (GFP) expressed in plants transfected with the CPMV expression vectors schematically illustrated in Figure 1. In expression vectors FSC2-512, FSC2-513 and FSC2-514, the gene encoding GFP was inserted after the initiation codon at position 512, 513 and 514, respectively. The lanes of the SDS-PAGE gels are marked 00, 10, 01 and 11, depending on which of the initiation sites in the CPMV vector, at positions 115 and 161, are intact or mutated. The lane marked '500ng' shows the position of a band corresponding to 500ng of GFP protein and thus indicates the expected position of GFP protein expressed from the CPMV expression vectors. The left hand lane of each SDS-PAGE gel shows the position of protein size markers.

Figure 3 shows the level of GFP expression in *Nicotiana benthamiana* leaves transfected with the same CPMV expression vectors used in the experiment illustrated in Figure 2. The pale regions at the tips of the leaves correspond to regions of GFP expression. Mutations made in order to inactivate the initiation sites at positions 115 and/or 161 in expression vectors 10, 01 and 11 are also indicated.

Figure 4 shows a comparison of Del-RNA-2 (expression vector 00 [FSC2-512] in Figure 1) and *HT* (expression vector 01 [FSC2-512] in Figure 1) for transient expression of green fluorescent protein (GFP), *Discosoma* red fluorescent protein (DsRed), and the hepatitis B core antigen (HBcAg). delRNA-2 or *HT* clones for each protein were infiltrated with the silencing suppressor P19. **(A)** Tissue 7 days after infiltration with delRNA-2 constructs becomes necrotic when DsRed or HBcAg is expressed whereas this is not the case for *HT* driven expression. In fact, tissue expressing DsRed by *HT* looks visibly red under day light conditions. **(B)** Coomassie-stained SDS-PAGE analysis of protein expression. The prominent bands corresponding to recombinant proteins as indicated were confirmed by western blotting. 1- marker, 2- uninfiltrated tissue, 3- delRNA-2 construct, 4- *HT* construct, 5- commercial standard where available. Crude extracts from approximately 5 mg of infiltrated tissue were loaded per lane as was 2 μg of GFP standard and 2 μg of HBcAg standard. No standard for DsRed was available at the time.

Figure 5 shows an initial comparison of Del-RNA-2 (expression vector 00 [FSC2-512] in Figure 1) and *HT* (expression vector 01 [FSC2-512] in Figure 1) for transient expression of the human anti-Human Immunodeficiency Virus antibody 2G12. The IgG Heavy chain was either in natural form (HL) or ER-retained (HEL) and infiltrated with the light chain and P19. **(A)** Expression of 2G12-HEL by del-RNA-2 leads to necrosis of infiltrated tissue whereas this does not occur for *HT* expression. **(B)** SDS-PAGE analysis of crude extracts of tissue infiltrated with the antibody heavy chains (delRNA-2 or *HT*) plus P19. For each sample crude extract from 5 mg of infiltrated tissue was loaded as was 1 μg of standard human IgG. A band corresponding to 2G12 is easily visible after coomassie staining. **(C)** Accumulation of antibody 2G12 after 5 days was measured by capture to protein A and surface plasmon resonance spectroscopy and represents the concentration following extraction in 2 volumes of buffer (PBS, 5 mM EDTA). Therefore, we can derive fresh weight concentrations approaching 150 mg/kg (for *HT* HEL) without any optimisation of plant incubation or extraction. Three samples were measured for each treatment.

Figure 6 shows an electron micrograph of assembled HBcAg particles, which were expressed using the *HT* (expression vector 01 [FSC2-512] in Figure 1) expression system described herein. The assembled HBcAg particles appear as hollow spheres, about 30 nm in diameter. The sap containing the HBcAg particles was not concentrated before the electron micrograph was taken, although unwanted salts were removed. Therefore, the electron micrograph represents the concentration of HBcAg particles in the sap.

Figure 7 shows the vector pM81-FSC1.

Figure 8 shows the vector pM81-FSC2.

Figure 9 shows a schematic representation of the construction of pEAQ. (A) Starting pBINPLUS-based plasmid with extraneous sequence shown in grey. (B) PCR products containing essential elements of the binary vector. (C) Intermediate plasmid following 3-part ligation of end-tailored PCR products. (D) Final plasmid following amplification and subsequent ligation of two fragments from the intermediate.

Figure 10 shows a schematic representation of the T-DNAs of the main pEAQ derivatives. The T-DNAs contain either or both of the P19 and NPTII cassettes as indicated leaving possible cloning into restriction sites as indicated.

Figure 11 shows expression levels of GFP generated by pEAQ vectors compared to its parent plasmid pBB-FSC2-512-HT. Tissue was analysed 6 days after infiltration with P19 and the vector indicated except for pEAQexpress, which was infiltrated alone at the standard OD, or at a two-fold dilution. (A) Leaves visualised under UV light, (B) coomassie-stained 12% SDS-PAGE, and (C) spectrofluorometric analysis.

Figure 12 shows the ability of P19(R43W) to enhance GFP expression compared to wild type P19. Tissue was analysed 6 days after infiltration with pEAQselectK at two-fold dilution (selK -P19), pEAQselectK and P19 (selK), pEAQspecialK (spK), pEAQspecialK at two-fold dilution (spK 1:2), pEAQspecialKm (spKm) , pEAQspecialKm at two-fold dilution (spKm 1:2), pEAQexpress (ex), and pEAQexpress at two-fold dilution (ex 1:2). (A) Leaves visualised under UV light and (B) spectrofluorometric analysis.

Figure 13 shows expression of the full size IgG, 2G12, with a single pEAQ plasmid. (A) Schematic representation of the two pEAQexpress-derived plasmids constructed to express 2G12. (B) Infiltration scheme indicating dilutions and their respective ODs for each plasmid combination, and the concentration of protein extracts made after infiltrations at each OD (± SD). (C) Coomassie-stained 12-4% SDS-PAGE analysis and (D) immunological detection of 2G12 heavy (γ) and (E) immunological detection of 2G12 Fab region (Fab) chain 8 days after infiltration. M, marker with sizes indicated; C, control extract; Std, CHO-produced 2G12. For coomassie-staining, protein from the equivalent of 3 mg of infiltrated tissue is loaded in each lane with 1 µg of CHO2G12 and for western blotting the equivalent of 0.75 mg of tissue in each lane with 250 ng of CHO2G12. Estimated assembly/degradation products are indicated.

Figure 14 shows the cloning and expression of GFP from pEAQ-HT in native and his-tagged variants. (A) Schematic representation of the pEAQ-HT T-DNA with polylinker detail. (B) Spectrofluorometric analysis of GFP expression. spK = pEAQspecialK-GFP-HT, GFP, HisGFP, and GFPHis = pEAQ-HT clones. (C) 12% SDS-PAGE and western analysis of GFP expression. C = control extract.

Figure 15 shows a nucleic construct of the present invention which is suitable for use in insect cells as part of a baculovirus vector.

EXAMPLES

Example 1

1.1 METHODS

Creation of expression vector FSC2 and its derivatives

A useful cloning vector for the expression of foreign proteins from a pBinP-1-GFP-based plasmid (*Cañizares et al.*, 2006) was created by excising the complete sequence of RNA-2 flanked by the Cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (*nos*) terminator from pBinP-S2NT (Liu and Lomonossoff, 2002) and inserting it into mutagenesis plasmid pM81W (Liu and Lomonossoff, 2006) as an *Asc*I/*Pac*I fragment. The resulting plasmid, pM81W-S2NT, was subjected to a single round of mutagenesis which simultaneously introduced four changes (*see* method in Liu and Lomonossoff, 2006) to give pM81B-S2NT-1. The mutagenesis removed two *Bsp*HI sites from the vector backbone and introduced a *Bsp*HI site (T/CATGA) around AUG 512 and a *Stu*I site (AGG/CCT) after UAA 3299, the termination codon for the RNA-2-encoded polyprotein. Subsequently, the *Bam*HI/*Asc*I fragment was excised from pBinP-NS-1 (Liu *et al.*, 2005) and ligated into similarly digested pM81B-S2NT-1, yielding pM81-FSC-1. 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 *Asc*I and *Pac*I and the fragment containing the expression cassette including the foreign sequences transferred to similarly digested pBINPLUS and the resulting plasmids are finally transformed into *A. tumefaciens*.

To improve the ease of cloning, expand the choice of applicable restriction enzymes, and to investigate the effect of reading frame on foreign gene expression, the whole RNA-2 ORF was replaced with a short polylinker. A combination of oligonucleotide insertion and site-directed mutagenesis resulted in pM81-FSC-2, which allows cloning with *Nru*I (TCG/CGA) and either *Xho*I (C/TCGAG) or *Stu*I. The terminal adenine of the *Nru*I site lies at position 512 thereby preserving the AUG found here. The modifications altered nucleotides immediately 5' to the AUG at 512, however, a good context was maintained. Cloning GFP into pM81-FSC-2 such that its translation was initiated from an AUG at 512, 513, 514, or 515 gave the pM81-FSC-1 derived constructs pM81-FSC2-512, pM81-FSC2-513, pM81-FSC2-514, and pM81-FSC2-515. These pM81-based plasmids are the cloning vectors containing the expression cassettes which were then transferred into the binary vector to produce the expression vectors FSC2-512, FSC2-513 and FSC2-514 used in the Experiments shown in Figures 2 and 3. Differences between the sequence of the wt RNA-2 genome segment of CPMV and the pM81-FSC1 and pM81-FSC2 vectors are shown in Table 3. Nucleotides altered in the vectors compared with the wt CPMV sequence are shown as capital letters.

Agrobacteria-mediated transient transformation following mobilisation into pBINPLUS (as outlined above for pM81-FSC-1) showed that lower protein levels are obtained when frame continuity between AUG 161 and the downstream AUG is not maintained. There was a significant decrease in the amount of GFP translated from the +1 and +2 positions relative to AUGs 161 and 512, whereas translation from the +3 position (that is, from 515 and back in frame) was as efficient as translation from an AUG at 512. To show that this was not due to weakened contexts of the AUGs at 513 and (to a lesser extent) 514, FSC2-515+ was created to initiate from +3 position but with the same poor context as FSC2-513. Expression from FSC2-515+ was as high as that achieved from FSC2-512 or 515, indicating that inferior context does not explain the reduction in expression from FSC2-513 and 514.

Given that the known mechanisms by which translation can escape the first-AUG rule are not known to require frame continuity, it is intriguing that efficient translation from a deleted RNA-2-based vector depends on frame continuity between AUG 161 and the downstream AUG. In order to understand, and hopefully overcome this phenomenon, a series of mutants were created with modifications to the 5' sequence of RNA-2. Complement pairs of oligonucleotides (see Table 2) were used in the site-directed mutagenesis of pM81-FSC2-512, 513, and 514. The mutations removed either AUG 115 (the start codon for the uORF), AUG 161 (without changing the amino acid sequence of the uORF), or both of these upstream initiation sites. Double mutations were made by mutagenizing the A115G mutants with the U162C oligos (Table 2).

Transient expression from these mutant transcripts was carried out as described for previous pM81-FSC-2 constructs. Analysis of expression of GFP from these mutants using coomassie-stained SDS-PAGE (Figure 2) or UV light to visualise whole leaves (Figure 3) shows a strong increase in expression in the absence of the AUG at 161. Furthermore, the removal of AUG 161 alone or both AUGs 115 and 161 alleviates the dependence on frame continuity between AUG 161 and the downstream AUG. In contrast, removal of just AUG 115 appears to enhance this dependency as well as generally inhibit translation. In conclusion, the uORF appears to function to down-regulate translation from AUG 161, which is both generally inhibitory and confers dependence on frame continuity.

Electron microscopy of sap containing HBcAg particles

The sap used for the electron micrograph of the assembled HBcAg particles shown in Figure 6 was prepared as follows. Leaf tissue was extracted in 2 volumes of Tris/NaCl buffer and exchanged for TE without concentration on a 100 kDa MWCO column. The final concentration of HBcAg was approximately 0.2 mg/ml as judged by comparison to standard on a coomassie stained SDS-PAGE gel.

1.2 RESULTS

(1) Effect of altering relative phases of the initiation sites at position 161 (AUG161)and 512 (AUG512).

To achieve this extra nucleotides were inserted immediately upstream of AUG512 (FSC2-512) to move the AUG to position 513, 514 and 515 (FSC2-513, FSC2-514 and FSC2-515) (Figure 1). Putting AUG512 out-of-phase with AUG161 (FSC2-513 and FSC2-514) gave less GFP expression as judged by fluorescence (Figure 3) and Coomassie-stained gels (Figure 2). Restoring the phase (FSC2-515) brought expression back to levels seen with the natural situation (FSC2-512). The conclusion is that when AUG161 is present, initiation at a downstream AUG is most efficient when it is in-phase with the AUG at position 161.

(2) Removal of the initiation site at position 115 (AUG115) coupled with altering relative phases of the initiation sites at position 161 (AUG161)and 512 (AUG512).

Removal of AUG115 has little or no effect when GFP expression is driven from AUG512 i.e. when this second AUG is in phase with AUG161 (see lanes labelled 10 in Figures 2 and 3). However, deletion of AUG115 when the second AUG is out-of-phase with AUG161 (513, 514) results in virtually no GFP expression (see lanes labelled 10 in Figures 2 and 3). Conclusion: AUG115 is somehow involved in the ability of ribosomes to by-pass AUG161 and reach AUG512. However, this requires the downstream AUG to be in the correct phase.

(3) Effect of removal of the initiation site at position 161 (AUG161)

The effect of this mutation is incredibly dramatic with GFP expression levels reaching 20-30 times the amount found when AUG161 is present (see lanes labelled 01 in Figures 2 and 3). Furthermore, it no longer appears to matter which phase AUG512 is in, though in the absence of AUG161, the idea of phase does not mean much. In addition the presence or absence of AUG115 makes no difference (see lanes labelled 11 in Figures 2 and 3).

When using the delRNA-2 (expression vector 00 [FSC2-512] in Figure 1) constructs for DsRed and HBcAg expression, within 5 days infiltrated patches have lost turgor pressure and become chlorotic (pale). By 7 days the tissue appears grey and is completely dead. When using *HT* (expression vector 01 [FSC2-512] in Figure 1), the tissue remains turgid after 7 days and the only sign of stress is slight chlorosis of HBcAg expressing tissue. When the heavy chain of the 2G12 IgG is expressed by delRNA-2 and retained in the ER, chlorosis is evident after 7 days, whereas for *HT* this is not observed. The level of necrosis seen in plants when using *HT* to express a heterologous protein is thus much lower, despite the higher level of heterologous protein expression achieved, than when using delRNA-2 to express the heterologous protein.

DISCUSSION

Very high levels of foreign gene expression can be expressed from the delRNA-2 constructs by deleting AUG161. At present, using GFP, we estimate the levels as 25-30% of total soluble protein (TSP) or approximately 1 gram expressed protein per Kg leaves. This is a tremendous level and the approach we use is extremely simple. The fact that we no longer need to preserve a reading frame means that user-friendly vectors with polylinkers can be produced.

Example 2

2.1 BACKGROUND

As described in Example 1, to investigate the features necessary for the 5' untranslated region (UTR) of CPMC RNA-2 necessary for efficient expression, the present inventors addressed the role of two AUG codons found within the 5' leader sequence upstream of the main initiation start site. The inventors demonstrated that deletion of an in-frame start codon (161) upstream of the main translation initiation site (512) led to a massive increase in foreign protein accumulation.

Using this system the inventors have shown that by 6 d postinfiltration, a number of unrelated proteins, including a full-size IgG and a self-assembling virus-like particle, were expressed to >10% and 20% of total extractable protein, respectively. Thus, this system provides an ideal vehicle for high-level expression that does not rely on viral replication of transcripts.

This new system (as exemplified by expression vector 01 [FSC-512] in Figure 1) has been called "CPMV-HT" for hyper-translatable Cow Pea Mosaic Virus protein expression system.

The HT-CPMV system shows dramatic increases in protein levels and thus is an excellent method for the rapid, high-level expression of foreign proteins in plants.

A growing array of binary vectors has been developed for plant transformation over the past 25 years (Hellens et al., 2000b; Veluthambi et al., 2003; Lee and Gelvin, 2008). The main aim of these developments has thus far focused on improving stable integration by, for example, expanding the host range for Agrobacteria (Hiei et al., 1994), the creation of a series of vectors that allow a choice of selectable markers, expression cassettes and fusion proteins (exemplified by the pCAMBIA range of open source binary vectors; http://www.cambia.org/daisy/bioforge_legacy/3725.html), or by developing systems for minimising extraneous DNA integration and marker-free transformation (for example pCLEAN; Thole et al., 2007, Plant Physiology 145(4):1211-1219).

Binary vectors have also been engineered to replicate at low copy numbers to reduce the frequency of multiple integration events of the same transgene, as this can lead to gene silencing (Johansen and Carrington, 2001).

However, for transient expression, ensuring efficient integration into the host nucleus and the presence of marker for in planta selection are not strictly required. Furthermore, upon agro-infiltration each cell is flooded with T-DNA molecules, which are thought to be transcriptionally competent in the nucleus even without genome integration (Janssen and Gardner, 1989; Narasimhulu et al., 1996). This suggests that transient expression could benefit from higher copy number binary plasmids.

Another area of improvement of binary vectors has been the reduction in size of the vector backbone. Two prominent examples that continue to demonstrate the benefits of smaller plasmids are pPZP (Hajdukiewicz et al., 1994) and pGREEN (Helens et al., 2000a). In addition to improving the efficiency of cloning procedures and bacterial transformation, these vectors have provided templates for expression systems that rely on multiple cassettes present on a single T-DNA (Tzfira et al., 2005; Thole et al., 2007).

The present example discloses non-obvious refinements of this vector which facilitates its practical use by permitting the cloning to be done in a single step, rather than requiring subcloning of expression cassettes between the cloning vector (e.g. pM81-FSC2) and expression systems (e.g. PBINPLUS). More specifically, the results herein show it was possible to drastically reduce the size of pBINPLUS without compromising performance in terms of replication and TDNA transfer. Furthermore, elements of the CPMV-HT system have been incorporated into the resulting vector in a modular fashion such that multiple proteins can be expressed from a single T-DNA. These improvements have led to the creation of a versatile, high-level expression vector that allows efficient direct cloning of foreign genes.

2.2 MATERIALS AND METHODS

pBD-FSC2-512-U162C (HT), contains the FSC2-512-U162C cassette (see Example 1) inserted into the PacI/AscI sites of pBINPLUS (van Engelen et al., 1995). The essential segments of this plasmid (see below) were amplified with the high fidelity polymerase, PHUSION (New England Biolabs) using oligonucleotides encoding unique restriction enzyme sites for re-ligation (Table 4.1). The T-DNA region was amplified with a sense primer homologous to sequence upstream of a unique AhdI site (pBD-LB-F) and an antisense primer that included an ApaI site (pBD-RB-ApaI-R). A region including the ColEI origin of replication, the NPTIII gene, and the TrfA locus was amplified with a sense primer that included an ApaI site (pBD-ColEI-ApaI-F), and an antisense primer that included a SpeI site (pBD-TrfA-SpeI-R). The RK2 origin of replication (OriV) was amplified with a sense primer that included a SpeI site (pBD-oriVSpeI-F) and an antisense primer that included an AhdI site (pBD-oriV-AhdI-R). Following purification, the products were digested according to the unique restriction sites encoded at their termini and mixed for a three-point ligation. This resulted in the plasmid pEAQbeta, for which the ligation junctions were verified by sequencing. A deletion of approximately 1.2 kb from the T-DNA which had removed a portion of the nos terminator of the CPMV-GFP-HT cassette was detected. Therefore, a portion of the terminator including the right border from pBD-FSC2-GFP-HT was re-amplified with primers pMini>pMicroBIN-F2 and pBD-RB-ApaI-R, as was the pEAQbeta backbone, including the right border, using primers pBD-ColEI-ApaI-F and pMini>pMicroBIN-R (Table 4.1). The purified products were digested with ApaI and FseI and ligated to give pEAQ (Figure 9).

The P19 gene flanked by 35S promoter and 35S terminator was amplified from pBIN61-P19 (Voinnet et al., 2003) using either 35SP19-PacI-F and 35SP19-AscIR, or 35SP19-FseI-F and 35S-P19-FseI-R as primers (Table 4.1). The NPTII gene flanked by the nos promoter and terminator was amplified from pBD-FSC2-GFPHT using primers pBD-NPTII-FseI-F and pBD-NPTII-FseI-R (Table 4.1). Following A-tailing, the amplified cassettes were ligated into pGEM-T easy (Promega). The P19 cassette excised from pGEM-T easy with FseI was ligated into FseI-digested pEAQ-GFP-HT to give pEAQexpress-GFP-HT. The NPTII cassette excised with FseI was ligated into FseI-digested pEAQ-GFP-HT in both directions to give pEAQselectK-GFP-HT and pEAQselectK(rev)-GFP-HT. The NPTII cassette was also excised with PacI/AscI and ligated into the AsiSI/MluI sites of pEAQselectK-GFP-HT to give pEAQspecialK-GFP-HT. The P19 in pGEM-T was subjected to site-directed mutagenesis by the QUICKCHANGE method (Stratagene) to effect the conversion of Arginine43 to a tryptophan residue using primers P19-R43W-F and P19-R43W-R. The mutant P19 cassette was released with PacI/AscI digest and inserted into the AsiSI/MluI sites of pEAQselectK-GFP-HT to give pEAQspecialKm-GFP-HT.

Oligonucleotides encoding the sense and antisense strands of a short polylinker (Table 4.1) were annealed leaving the downstream half of an NruI site at the 5'end and an overhang matching that of XhoI at the 3' end. The annealed oligos were ligated with NruI/XhoI digested pM81-FSC2-A115G-U162C (see above) to give pM81-FSC2-POW. The NruI site was removed from the P19 cassette in pGEM-T by site-directed mutagenesis (QUICKCHANGE; Stratagene) with the primers P19-ΔNruI-F and P19-ΔNruI-R, and was re-inserted into the AsiSI/MluI sites of pEAQselectK-GFP-HT to give pEAQspecialKΔNruI-GFP-HT which showed no reduction in expression compared to pEAQspecialK-GFP-HT (data not shown). The PacI/AscI fragment from pM81-FSC2-POW was then released and inserted into similarly digested pEAQspecialKΔNruI-GFP-HT thereby replacing the GFP HT expression cassette and yielding pEAQ-HT. GFP was amplified from pBD-FSC2-GFP-HT with a set of four primers (Table 4.1) in three combinations for insertion into pEAQ-HT: GFP-AgeI-F and GFP-XhoI-R; GFP-AgeI-F and GFP-XmaI-R; and GFP-XmaI-F and GFP-XhoI-R. Purified PCR products were digested with the enzymes specified in their primers and inserted into appropriately digested pEAQ-HT to give pEAQ-HT-GFP, pEAQ-HT-GFPHis, and pEAQ-HT-HisGFP.

Table 4.1. Oligonucleotides used for amplification and mutagenesis. Restriction enzyme sites, or parts thereof, are shown in lower case, and mutations underlined in bold.

Oligo	Sequence	Function
pBD-LB-F	GCCACTCAGCTTCCTCAGCGGCTTT	Sense primer for amplification of the region 6338-12085 of pBD-FSC2-GFP-HT
pBD-RB-ApaI-R	TATTAgggcccCCGGCGCCAGATCTGGGGAACCCTGTGG	Antisense primer for amplification of the region 6338-12085 of pBD-FSC2-GFP-HT with ApaI site
pBD-ColEI-ApaI-F	GACTTAgggcccGTCCATTTCCGCGCAGACGATGACGTCACT	Sense primer for amplification of the region 1704-5155 of pBD-FSC2-GFP-HT with ApaI site
pBD-TrfA-SpeI-R	GCATTAactagtCGCTGGCTGCTGAACCCCCAGCCGGAACTGACC	Antisense primer for amplification of the region 1704-5155 of pBD-FSC2-GFP-HT with SpeI site
pBD-oriV-SpeI-F	GTAGCactagtGTACATCACCGACGAGCAAGGC	Sense primer for amplification of the region 14373-670 of pBD-FSC2-GFP-HT with SpeI site
pBD-oriV-AhdI-R	CAGTAgacaggctgtcTCGCGGCCGAGGGGCGCAGCCC	Antisense primer for amplification of the region 14373-670 of pBD-FSC2-GFP-HT with AhdI site
pMini>pMicroBIN-F2	ggccggccacgcgtTATCTGCAGAgcgatcgcGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACC	Sense primer for amplification of the region 2969-85 of pEAQbeta with FseI-MluI-AsiSI sites
pMini>pMicroBIN-R	gcgatcgcTCTGCAGATAacgcgtggccggccCTCACTGGTGAAA	Antisense primer for amplification of the region

	AGAAAAACCACCCCAGTACATTAAAAACGTCC	2969-85 of pEAQbeta with AsiSI-MluI-FesIsites
35SP19-PacI-F	ttaattaaGAATTCGAGCTCGGTACCCCCCTACTCC	Sense primer for amplification of the 35S-P19 cassette with PacI site
35SP19-AscI-R	ggcgcgccATCTTTTATCTTTAGAGTTAAGAACTCTTTCG	Antisense primer for amplification of the 35S-P19 cassette with AscI site
35SP19-FseI-F	ggccggccGAATTCGAGCTCGGTACCCCC	Sense primer for amplification of the 35S-P19 cassette with FseI site
35SP19-FseI-R	ggccggccATCTTTTATCTTTAGAGTTAAG	Antisense primer for amplification of the 35S-P19 cassette with FseI site
pBD-NPTII-FseI-F	ggccggccTACAGTATGAGCGGAGAATTAAGGGAGTCACG	Sense primer for amplification of the NPTII cassette from pBD-FSC2-GFP-HT with FseI site
pBD-NPTII-FseI-R	ggccggccTACAGTCCCGATCTAGTAACATAGATGACACCGCGC	Antisense primer for amplification of the NPTII cassette from pBD-FSC2-GFP-HT with FseI site
P19-R43W-F	CGAGTTGGACTGAGTGGTGGCTACATAACGATGAG	Sense primer for mutagenesis of arginine 43 of P19 to a tryptophan residue
P19-R43W-R	CTCATCGTTATGTAGCCACCACTCAGTCCAACTCG	Antisense primer for mutagenesis of arginine 43 of P19 to a tryptophan residue
P19-ΔNruI-F	CCGTTTCTGGAGGGTCTCGAACTCTTCAGCATC	Sense primer for the silent mutagenesis of the NruI restriction site within P19
P19-ΔNruI-R	GATGCTGAAGAGTTCGAGACCCTCCAGAAACGG	Antisense primer for the silent mutagenesis of the

		NruI restriction site within P19
POW-F	cgaccggtATGCATCACCATCACCATCATcccgggCATCACCATCACCATCACTAGc	Sense oligo for polylinker, POW
POW-R	tcgagCTAGTGATGGTGATGGTGATGcccgggATGATGGTGATGGTGATGCATaccggttcg	Sense oligo for polylinker, POW
GFP-AgeI-F	atcggaccggtatgactagcaaaggagaagaac	Sense oligo for amplification of GFP with AgeI site
GFP-XmaI-F	atccgacccgggactagcaaaggagaagaacttttcac	Sense oligo for amplification of GFP with XmaI site and no start codon
GFP-XmaI-R	atccgacccgggtttgtatagttcatccatgcc	Antisense oligo for amplification of GFP with XmaI site and no termination codon
GFP-XhoI-R	cgatcctcgagttatttgtatagttcatccatgcc	Antisense oligo for amplification of GFP with XhoI site

2.3 RESULTS

2.3.1 pBINPLUS contains at least 7.4kb of extraneous sequence

Expression from CPMV-HT enables the production of extremely high levels of recombinant proteins. Nevertheless it was desired to further improve the system and its use for transient transformation.

The first area of improvement relates to the fact that small plasmids are more efficient than larger ones in ligation reactions and bacterial transformation procedures. Comparisons with the structures of smaller binary vectors indicated that pBINPLUS likely contains significant amounts of extraneous sequence. Four elements of pBINPLUS were determined to be essential for proper function as a binary vector: the T-DNA, the RK2 (OriV) broad host range replication origin, the NPTIII gene conferring resistance to kanamycin (Trieu-Cuot and Courvalin, 1983), and TrfA from RK2 that promotes replication (Figure 9). Bioinformatic analysis of the remaining backbone sections show them to be artefacts of the construction of pBIN19, which relied on the presence of appropriate restriction sites within parent plasmids (Bevan, 1984). These observations are confirmed by a report on the complete sequencing of pBIN19 (Frisch et al., 1995). pBINPLUS includes the non-essential ColEI replication origin for higher copy number in E. coli. Approximately 2.6kb of superfluous DNA can be found within the T-DNA. This includes the NPTII selectable marker for plant transformation that is not required for transient expression. Overall, the total amount of extraneous sequence within pBINPLUS appears to be in excess of 7.2kb.

2.3.2 pEAQ series construction

In order to monitor the effects on expression resulting from modifications to vector, we chose to start with the pBINPLUS-derived plasmid, pBD-FSC2-512-U162C(HT). Three regions, consisting of the T-DNA, the RK2 (OriV) replication origin, and a segment containing the ColEI origin, NPTIII, and TrfA, were amplified by PCR from pBD-FSC2-GFP-HT. Ligation of these three fragments resulted in the plasmid pEAQbeta (Figure 9), which is 4584 bp smaller than its parent plasmid. A further round of PCR amplification of pEAQbeta removed 2639 bp of non-essential sequence from the T-DNA region and inserted three unique restriction sites, AsiSI, MluI, and FseI. AsiSI/MluI digestion is compatible with the insertion of PacI/AscI fragments, and is therefore, extremely useful for cloning multiple cassettes from all previous CPMV cloning vectors. FseI provides a unique 8-base recognition site useful for interchanging different selection markers or silencing suppressor cassettes. The resulting pEAQGFP-HT plasmid is less than half the size of pBINPLUS and without the CPMVHT expression cassette would be only 5137 bp, making it one of the smallest known binary vectors (Figure 9). The entire pEAQ plasmid was sequenced and it was discovered that the RK2 origin of replication was in the reverse orientation to that previously reported (Frisch et al., 1995) and is therefore indicated in the correct orientation in pEAQ-GFP-HT.

pEAQ-GFP-HT was used as a starting point for the inclusion of various additional features into the T-DNA (Figure 10). The NPTII cassette from pBINPLUS was re-inserted into the FseI site of pEAQ in both the forward and reverse orientations relative to the GFP-HT cassette to give pEAQselectK-GFP-HT and pEAQselectK(rev)-GFP-HT.

The 35S-P19 cassette was inserted into the FseI site to give pEAQexpress-GFP-HT. Finally, the 35S-P19 cassette was inserted into the Mlu/AsiSI sites of pEAQselectK-GFP-HT to give pEAQspecialK-GFP-HT. Thus, a series of small binary vectors for easy and quick transient expression were constructed.

2.3.3 Reduction in size does not compromise transient expression from pEAQ

Agro-infiltration of the pEAQ series of vectors shows that the large reduction in size does not significantly compromise expression levels in transient assays. Coinfiltration of pEAQ-GFP-HT, and pEAQselectK(rev)-GFP-HT with P19 provided by pBIN61-P19, resulted in levels of expression not significantly different to the co-infiltration of pBD-FSC2-512-HT and P19. This can be seen under UV illumination (Figure 11A), SDS-PAGE (Figure 11B), and spectrofluorescence measurements of GFP in protein extracts (Figure 11C). Interestingly, the orientation of the NPTII cassette within the T-DNA appears to affect expression level. pEAQselectK shows a marked improvement compared to the otherwise identical pEAQselectK(rev), which results in a reduction in GFP accumulation.

Theoretically, the incorporation of a suppressor of silencing cassette into pEAQ should not affect its ability to improve transient expression level from a foreign gene to be expressed from the same T-DNA. Indeed, the infiltration of pEAQexpress-GFP-HT alone also resulted in expression levels similar to, or better than, pBD-FSC2-GFP-HT (Figure 7.3). Furthermore, to test the efficiency of pEAQexpress, the Agrobacterium culture was diluted two-fold, such that the final optical density (OD) was that of each individual culture of the coinfiltrations.

As expected, this resulted in similarly high expression levels and demonstrates that incorporating both the gene of interest and the suppressor of silencing onto the same T-DNA allows the use of half the amount of Agrobacteria (Figure 11). Therefore, CPMV-HT may be used to express high levels of foreign protein when all components are present on the same T-DNA.

2.3.4 Mutant P19 can suppress silencing of a transgene in a transient assay

In order to take advantage of the increase in expression afforded by the forward orientation of the NPTII cassette within the T-DNA, the P19 cassette was inserted between the AsiSI and MluI sites in pEAQselectK-GFP-HT to give pEAQspecialK-GFP-HT (Figure 10). The presence of P19 on the same T-DNA as the sequence of GFP results in similar levels of expression to pEAQselectK-GFPHT co-infiltrated with P19 (Figure 12). This is more than the expression generated by pEAQexpress-GFP-HT, and appears to be due to the presence of the NPTII cassette (Figure 12). On the other hand, the lower expression from pEAQexpress could be due to the different position and orientation of the P19 cassette within the T-DNA. Nevertheless, as with pEAQexpress, pEAQspecialK vectors give high-level expression with Agrobacteria suspensions at half the final OD of that used when two cultures must be co-infiltrated.

Combining the foreign gene expression cassette with a P19 cassette and a selectable marker makes it possible to test the performance of CPMV-HT in transgenic plants. However, the constitutive expression of suppressors of silencing like P19 can result in severe phenotypes due to their interference with endogenous gene silencing associated with developmental processes (Silhavy and Burgyán, 2004). A recently characterised mutation of P19 (R43W) has been proposed to have a reduced activity towards endogenous gene silencing and therefore may be a better candidate for the suppression of transgene silencing in stable transformants (Scholthof, 2007). To investigate the feasibility of stable transformation with the CPMV-HT system, both wt and the mutant P19 were inserted into the T-DNA of pEAQselectK-GFP-HT to assay the variants transiently. As shown by, UV illumination of infiltrated leaves, SDS-PAGE of protein extracts, and spectrofluorometric measurements of GFP levels, the mutant P19 in pEAQspecialKm is approximately half as effective in improving foreign gene expression as the wt P19 in pEAQspecialK (Figure 12). This represents the first study on the effect of the R43W mutation in P19 on the ability to suppress silencing of a transgene.

Example 3

High level IgG expression from a single plasmid

In order to take advantage of the modular nature of the pEAQ series, CPMV-HT expression cassettes containing the ER-retained heavy chain (HE) and light chain (L) of the human anti-HIV IgG, 2G12 were inserted into the PacI/AscI and AsiSI/MluI sites of pEAQexpress. To determine whether the site of insertion influences expression levels, the L and HE chains were inserted into both positions yielding pEAQex-

2G12HEL and pEAQex-2G12LHE (Figure 13A). Infiltration of N. benthamiana with single Agrobacterium cultures containing the above plasmids resulted in the formation of fully assembled 2G12 antibodies identical in size to 2G12 produced by mixing three Agrobacterium cultures which each expressed the individual components, L, HE and P19 (Figure 13C). The protein loaded in each lane represents 1/30 of the extract obtained from 90 mg of infiltrated tissue or 1/333 of the protein potentially obtainable from 1 g of tissue. The maximum amount of assembled IgG produced from the 3-strain mixture corresponds to 1μg of CHO-produced 2G12 on the coomassie-stained non-reduced SDS-PAGE gel. This suggests an expression level of 2G12 in excess of 325 mg/kg of fresh weight tissue, which is in agreement with the SPR-measured concentrations. The use of pEAQex-2G12HEL appears to surpass this already high-level of antibody accumulation.

An advantage of pEAQ-derived vectors is that each component of a multi-chain protein such as an IgG can automatically be delivered to each infected cell. Therefore, high expression levels should be maintained at higher dilutions of Agrobacteria suspensions than if multiple cultures have to be used. To test if this is the case in practice, cultures that were initially resuspended to OD 1.2, and mixed where necessary, were subjected to two serial three-fold dilutions (Figure 13B). This resulted in final ODs of each individual culture in the three-culture mix being 0.4, 0.13, and 0.04. Single cultures harbouring the pEAQexpress constructs were infiltrated at ODs of 1.2, 0.4, and 0.13. When three separate cultures were used, the level of assembled 2G12 decreases markedly on serial dilution. In contrast, 2G12 expression from pEAQex-2G12HEL and pEAQex-2G12LHE, was maintained at a consistently high level, with any reduction on dilution being very modest (Figure 13C - E). The lack of sensitivity to dilution confirms the improved efficiency afforded by placing all three expression cassettes on the same T-DNA. Interestingly, the amount of total protein extracted from the infiltrated tissue was almost halved when the OD of the infiltrate was reduced from 1.2 to 0.4. This suggests that a significant fraction of the protein in extracts from tissue in which the higher OD suspension has been infiltrated can consist of Agrobacteria-derived protein or plant proteins produced in response to the higher concentrations of Agrobacteria.

Inspection of Figure 13C suggests that the relative position of a cassette within the T-DNA can affect the expression levels. The overall expression from pEAQex-2G12LHE was slightly lower than from pEAQex-2G12HEL. This was confirmed by western blotting of the non-reduced samples, which also indicated some differences in the abundance of degradation products and unincorporated immunoglobulin chains (Figure 13C - E). Tissue infiltrated with pEAQex-2G12LHE appears to lack a heavy chain-specific degradation product of approximately 70 to 80 kDa (Figure 13D). Also, there appears to be much less of the HL2 assembly intermediate, as well as more free light chain (Figure 13E). Since, the heavy chain is known to be limiting in 2G12 assembly in plants (Markus Sack, pers. comm., RWTH, Aachen, Germany), which is confirmed by the lack of discernable free heavy chain in all samples, these results indicate that pEAQex-2G12LHE produces less heavy chain than pEAQex-2G12HEL. This could be due to reduced expression from the CPMV-HT cassette closer to the left border of the T-DNA.

In other experiments (data not shown) the CPMV-HT system has also been successfully used in the transient format in *N. benthamiana* to express:

- Bluetongue Virus (serotype 10) VP2, VP3, VP5, VP7 and NS1.
- Rotavirus NSP5.
- Calmodulin from *Medicago truncatula* (which was subsequently purified).
- The difficult-to-express ectodomain of human Fc gamma receptor 1 (CD64) – which has been purified and shown to be functional in antibody binding studies.
- The CPMV Small (S) and Large (L) coat proteins were co-expressed and shown to assemble into virus-like particles (data not shown)

Example 4

Direct cloning into a CPMV-HT expression vector

Although combining elements of the system on to a single plasmid, the vectors described hereinbefore still required a two-step cloning procedure to introduce a sequence to be expressed into the binary plasmid. The present example provides a binary plasmid into which a gene of interest could be directly inserted. The plasmid incorporates a polylinker that not only permits direct insertion into the pEAQ-based plasmid, but also permits the fusion of a C- or N-terminal histidine tag if desired (pEAQ-HT; Figure 14A). The polylinker was first inserted as annealed oligonucleotides into pM81-FSC2-512(A115G)(U162C) giving pM81-FSC-POW. This construct can still be used for the standard two-step cloning procedure for the generation pEAQ-based constructs for the expression of multiple polypeptides. Furthermore, use of the double mutated 5' leader may enable even higher expression levels to be obtained than is possible with the single mutation. The CPMV-HT cassette was then transferred into pEAQspecialK via the PacI/AscI sites to give pEAQ-HT. Insertion of GFP into all three positions within the polylinker of pEAQ-HT resulted in an un-tagged GFP, and 5' (HisGFP) and 3' (GFPHis) His-tag fusions.

As expected, untagged GFP was expressed to a level even higher than that obtained with pEAQspecialK-GFP-HT and in excess of 1.6 g/kg FW tissue (Figure 14B). This is likely due to the fact that the CPMV 5' leader of pEAQ-HT contains the extra mutation which removes AUG 115 which, when removed in addition to AUG 161, further enhances expression.

The presence of the His-tag as detected by western blotting confirmed the correct fusion at both the N- and C-terminus of the amino acid residues encoded by the polylinker. All three GFP variants were detectable with anti-GFP antibodies whereas only HisGFP and GFPHis were detectable with anti-His antibodies (Figure 14C), and the presence of the His-tag reduced the mobility of the GFP band in SDS-PAGE by the expected amount. The tag also reduced the amount of GFP detected by the analysis of fluorescence (Figure 14B). This effect was more pronounced for N-terminal His tag. The intensity of the coomassie-stained bands suggests that this represents a reduction in tagged GFP accumulation (Figure 14C), rather than interference with the fluorogenic properties of GFP. Nevertheless, the levels of the His-tagged proteins were still very high yielding in excess of 0.6 and 1.0 g of GFP per kg FW tissue.

Discussion of Examples 2-4

To improve the ease of use and performance of the CPMV-HT expression system, a modular set of vectors has been created for easy and quick plant expression.

Removing more than half of the plasmid backbone from the binary vector, pBINPLUS, and some of the T-DNA region not essential for transient expression resulted in one of the smallest binary Ti plasmids known with no compromise on expression levels.

A similar proportion of the backbone had previously been removed from pBIN19 without a loss of performance (Xiang et al., 1999). However, pBINPLUS possesses two significant improvements over pBIN19 (van Engelen et al., 1995); an increased copy number in E. coli owing to the addition of the ColEI origin of replication and a reoriented T-DNA ensuring the gene of interest is further from the left border that can suffer extensive deletions in planta (Rossi et al., 1996). While the smaller size of pEAQ plamids had no noticeable effect on their copy number, they give greatly improved yields during cloning procedures using commercial plasmids extraction kits as these are most efficient for plasmids below 10 kb (data not shown).

The modular nature of the pEAQ binary vector adds functionality to CPMV-HT expression by allowing any silencing suppressor and/or marker gene, if required, to be co-expressed with one or two CPMV-HT cassettes. For example, insertion of a second HT cassette containing a heterologous sequence into the AsiSI/MluI sites of pEAQexpress-GFP-HT would allow tracking of expression with GFP fluorescence.

Furthermore, the flexibility of the vectors simplifies the system for transient expression by only requiring the infiltration of a single Agrobacterium construct, and improves efficiency by reducing the amount of infiltrate required in proportion to the number of expression cassettes present within the T-DNA. With P19 occupying the FseI site, the presence of two cloning sites for accepting HT cassettes from cloning vectors (such as pM81-FSC2-U162C) also allows even more efficient expression of multi-subunit proteins such as full-size antibodies.

The effect of P19 on enhancing expression levels of transgenes is well characterised (Voinnet et al., 2003). However, this study presents the first demonstration of its effectiveness when co-delivered to each cell on the same TDNA. A previous study has reported the co-delivery of P19 from a separate TDNA within the same Agrobacterium as the transgene-containing T-DNA (Hellens et al., 2005). However, there was no effect of P19 until 6 days after infiltration, suggesting inefficient transfer of T-DNA. The present study also demonstrates the first use of the R43W mutant P19 to enhance the expression of a transgene. The finding that the mutant was about half as effective in enhancing the expression of GFP as wt P19 agrees with its known reduction in activity, which compromises both the infectivity of TBSV (Chu et al., 2000), and the ability of the protein to bind the smaller class (21 – 22 nts) of short interfering RNAs (Omarov et al., 2006). However, it is possible that this feature potentially makes the R43W mutant more suitable for applications involving stable transformation. The micro RNAs associated with development are also in the smaller size class (Vaucheret, 2006;

Zhang et al., 2006) and, therefore, developmental processes may not be as severely affected by the presence of the mutant P19 as they would by the wt version (Scholthof, 2007). Furthermore, the mutant may provide a way of controlling the transient expression of potentially cytotoxic foreign proteins.

The expression of 2G12 from a single plasmid represents the highest reported yield of an antibody from plant tissue infiltrated with a single Agrobacterium culture. Apart from using 3 Agrobacterium cultures for CPMV-HT expression, the only way of achieving similar levels with another system involved the infiltration of 6 separate cultures and a virus vector approach (Giritch et al., 2006). Furthermore, the use of a single plasmid affords a reduction in the amount of bacteria needed to ensure co-delivery of multiple expression cassettes, which would provide a significant cost saving at industrial production levels. The infiltration process is also physically easier to carry out with more dilute cultures due to less clogging of the intercellular spaces of leaf tissue. In addition, the dilution to a total OD of 0.4 reduced the amount of infiltration-derived protein contaminants. Analysis of nine separate infiltrations at each OD showed a reduction in the protein concentrations of the extracts from 2.7 ± 0.2 to 1.5 ± 0.1 mg/ml when the OD of the cultures was reduced from 1.2 to 0.4. Since the use of pEAQexpress generates as much 2G12 at OD 0.4 as the three-culture system does at an infiltrate OD of 1.2, the recombinant target protein must be purified from only half the amount of contaminating protein using pEAQexpress. This provides a very useful and unexpected advantage for downstream processing. Expression of 2G12 from pEAQexpress also indicates an effect of position of an expression cassette within the T-DNA of pEAQ vectors on the level of expression obtained. The increase in free light chain accumulation from pEAQex-2G12LHE suggests that less heavy chain is expressed with this construct, which appears to result in less assembled antibody. This could be due to the arrangement of expression cassettes on the T-DNA. Alternatively, a proportion of the T-DNAs are susceptible to nucleolytic degradation at the left border (Rossi et al., 1996). The reinsertion of the NPTII cassette within the T-DNA appeared to have a marked effect on expression depending on its orientation. During cloning manipulations it became apparent that pEAQselectK-GFP-HT reached a plasmid copy number in E. coli of approximately 1.5 times that of pEAQselectK(rev)- GFP-HT (determined from yield measurements of three separate plasmid preparations performed with the QIAprep kit, QIAGEN). This loosely correlates to the difference in expression levels observed between the two vectors. It is not known what contributes to the increased copy number, or indeed whether the difference also exists when the plasmids are transferred to Agrobacteria. However, these observations suggest that plasmid copy number may be an important for efficient Agrobacterium mediated transient expression. In this respect, the use of the RK2 origin (oriV in Figure 9) by pBIN19 and its derivatives makes it a good choice for transient expression as RK2 plasmids are known to accumulate to 7 to 10 copies in Agrobacterium (Veluthambi et al., 1987). This is similar to the pVS1 origin utilised by pPZP and about 2-5 times higher than is generated by the pSa origin (Lee and Gelvin, 2008), which is present in the widely used pGREEN binary vector (Helens et al., 2000). Plasmids containing replication origins that yield higher copy numbers such as pRi-based plasmids (Lee and Gelvin, 2008) maybe even better suited to transient expression.

To make high-level expression with pEAQ vectors easily accessible for labs with no previous experience with CPMV-based expression or indeed, plant-based expression in general, a direct cloning version of pEAQ was created. This was achieved by inserting a polylinker between the 5' leader and 3' UTRs of a CPMVHT expression cassette, which was the positioned on a T-DNA which also contained P19 and NPTII cassettes. The NPTII cassette was included because its presence appeared to appreciably enhance expression (see above). The polylinker also encodes two sets of 6 x Histidine residues to allow the fusion of N- or C terminal His-tags to facilitate protein purification. The resulting constructs also benefit from the second mutation in the 5' leader which enhances expression relative to HT.

These enhanced expression cassettes may also be sub-cloned from the cloning vector pM81-FSC-POW into any pEAQ plasmid. The use of pEAQHT led to increased GFP expression compared with pEAQspecialK, which contains just the single mutation (U162C). Furthermore, the polylinker design also allowed the expression of His-tagged variants using a one step cloning procedure. The modular binary vectors presented here are specifically designed for, but not restricted to, use with CPMV-HT expression. Extremely high-level expression has been coupled with improved cloning efficiency and ease of use. The system provides the most effective and straightforward method for transient expression of value-added proteins in plants without the complications of viral amplification. It allows milligram quantities of recombinant protein within two weeks of sequence identification in any molecular biology lab with access to plant growth facilities. Therefore, it is anticipated that it will provide an extremely valuable tool in both academic and industrial settings.

Example 5

Stable integration with pEAQ plasmids and transgenic plants

Although the pEAQ vector series was designed with transient expression in mind, the reinsertion of the NPTII cassette into the T-DNA to provides a selectable marker for genome integration. This potentially allows these smaller and more useful binary vectors to be used for stable plant and plant cell culture transformation. When used to transform N. benthamiana leaf discs, pEAQ vectors containing the NPTII cassette within the T-DNA were able to induce callus formation under selection with the same efficiency as pBINPLUS-based constructs. Furthermore, GFP expression was detectable in these tissues under UV light (data not shown. This demonstrates that multi-cassette T-DNA molecules from pEAQ vectors can stably integrate into the plant genome and drive the expression of foreign genes.

Fluorescent plants have also been regenerated. The leaves of the primary transformants (T_0) were fluorescent under uv light indicating high levels of GFP expression. The seed from the self-fertilised T_0 plants were viable, and the resulting T_1 seedlings harbouring the transgene are also fluoresecent (results not shown).

Example 6

Use of the CPMV-based HT system with baculovirus vectors

Figure 15 shows a construct suitable for utilising the CPMV-based HT system with baculovirus vectors in insect cells. Under control of the p10 promoter, the HyperTrans CPMV RNA-2 UTRs also enhance the expression of GFP in insect cells using the Baculovirus expression system. An approximately 5-fold enhancement of fluorescence levels in baculovirus-infected sf21 cells, as measured by flow cytometry, was obtained in comparison to a construct without the CPMV-HT cassette.

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Table 1

The complete CPMV RNA-2 genome segment (nucleotides 1 to 3481)

1 tattaaaatc ttaataggtt ttgataaaag cgaacgtggg gaaacccgaa ccaaaccttc
61 ttctaaattc tctctcatct ctcttaaagc aaacttctct cttgtctttc ttgc**atg**agc
121 gatcttcaac gttgtcagat cgtgcttcgg caccagtaca **atg**ttttctt tcactgaagc
181 gaaatcaaag atctctttgt ggacacgtag tgcggcgcca ttaaataacg tgtacttgtc
241 ctattcttgt cggtgtggtc ttgggaaaag aaagcttgct ggaggctgct gttcagcccc
301 atacattact tgttacgatt ctgctgactt tcggcgggtg caatatctct acttctgctt
361 gacgaggtat tgttgcctgt acttctttct tcttcttctt gctgattggt tctataagaa
421 atctagtatt ttctttgaaa cagagttttc ccgtggtttt cgaacttgga gaaagattgt
481 taagcttctg tatattctgc ccaaattgga a**atg**gaaagc att**atg**agcc gtggtattcc
541 ttcaggaatt ttggaggaaa aagctattca gttcaaacgt gccaaagaag ggaataaacc
601 cttgaaggat gagattccca agcctgagga tatgtatgtg tctcacactt ctaaatggaa
661 tgtgctcaga aaaatgagcc aaaagactgt ggatctttcc aaagcagctg ctgggatggg
721 attcatcaat aagcatatgc ttacgggcaa catcttggca caaccaacaa cagtcttgga
781 tattcccgtc acaaaggata aaacacttgc gatggccagt gattttattc gtaaggagaa
841 tctcaagact tctgccattc acattggagc aattgagatt attatccaga gctttgcttc
901 ccctgaaagt gatttgatgg gaggcttttt gcttgtggat tctttacaca ctgatacagc
961 taatgctatt cgtagcattt ttgttgctcc aatgcgggga ggaagaccag tcagagtggt
1021 gaccttccca aatacactgg cacctgtatc atgtgatctg aacaatagat tcaagctcat
1081 ttgctcattg ccaaactgtg atattgtcca gggtagccaa gtagcagaag tgagtgtaaa
1141 tgttgcagga tgtgctactt ccatagagaa atctcacacc ccttcccaat tgtatacaga
1201 ggaatttgaa aaggagggtg ctgttgttgt agaatactta ggcagacaga cctattgtgc
1261 tcagcctagc aatttaccca cagaagaaaa acttcggtcc cttaagtttg actttcatgt
1321 tgaacaacca agtgtcctga agttatccaa ttcctgcaat gcgcactttg tcaagggaga
1381 aagtttgaaa tactctattt ctggcaaaga agcagaaaac catgcagttc atgctactgt
1441 ggtctctcga gaaggggctt ctgcggcacc caagcaatat gatcctattt tgggacgggt
1501 gctggatcca cgaaatggga atgtggcttt tccacaaatg gagcaaaact tgtttgccct
1561 ttctttggat gatacaagct cagttcgtgg ttctttgctt gacacaaaat tcgcacaaac
1621 tcgagttttg ttgtccaagg ctatggctgg tggtgatgtg ttattggatg agtatctcta
1681 tgatgtggtc aatggacaag attttagagc tactgtcgct tttttgcgca cccatgttat
1741 aacaggcaaa ataaaggtga cagctaccac caacatttct gacaactcgg gttgttgttt

1801 gatgttggcc ataaatagtg gtgtgagggg taagtatagt actgatgttt atactatctg
1861 ctctcaagac tccatgacgt ggaacccagg gtgcaaaaag aacttctcgt tcacatttaa
1921 tccaaaccct tgtggggatt cttggtctgc tgagatgata agtcgaagca gagttaggat
1981 gacagttatt tgtgtttcgg gatggacctt atctcctacc acagatgtga ttgccaagct
2041 agactggtca attgtcaatg agaaatgtga gcccaccatt taccacttgg ctgattgtca
2101 gaattggtta ccccttaatc gttggatggg aaaattgact tttccccagg gtgtgacaag
2161 tgaggttcga aggatgcctc tttctatagg aggcggtgct ggtgcgactc aagctttctt
2221 ggccaatatg cccaattcat ggatatcaat gtggagatat tttagaggtg aacttcactt
2281 tgaagttact aaaatgagct ctccatatat taaagccact gttacatttc tcatagcttt
2341 tggtaatctt agtgatgcct ttggttttta tgagagtttt cctcatagaa ttgttcaatt
2401 tgctgaggtt gaggaaaaat gtactttggt tttctcccaa caagagtttg tcactgcttg
2461 gtcaacacaa gtaaacccca gaaccacact tgaagcagat ggttgtccct acctatatgc
2521 aattattcat gatagtacaa caggtacaat ctccggagat tttaatcttg gggtcaagct
2581 tgttggcatt aaggattttt gtggtatagg ttctaatccg ggtattgatg gttcccgctt
2641 gcttggagct atagcacaag gacctgtttg tgctgaagcc tcagatgtgt atagcccatg
2701 tatgatagct agcactcctc ctgctccatt ttcagacgtt acagcagtaa cttttgactt
2761 aatcaacggc aaaataactc ctgttggtga tgacaattgg aatacgcaca tttataatcc
2821 tccaattatg aatgtcttgc gtactgctgc ttggaaatct ggaactattc atgttcaact
2881 taatgttagg ggtgctggtg tcaaaagagc agattgggat ggtcaagtct ttgtttacct
2941 gcgccagtcc atgaaccctg aaagttatga tgcgcggaca tttgtgatct cacaacctgg
3001 ttctgccatg ttgaacttct cttttgatat catagggccg aatagcggat ttgaatttgc
3061 cgaaagccca tgggccaatc agaccacctg gtatcttgaa tgtgttgcta ccaatcccag
3121 acaaatacag caatttgagg tcaacatgcg cttcgatcct aatttcaggg ttgccggcaa
3181 tatcctgatg cccccatttc cactgtcaac ggaaactcca ccgttattaa agtttaggtt
3241 tcgggatatt gaacgctcca agcgtagtgt tatggttgga cacactgcta ctgctgctta
3301 actctggttt cattaaattt tctttagttt gaatttactg ttatttggtg tgcatttcta
3361 tgtttggtga gcggttttct gtgctcagag tgtgtttatt ttatgtaatt taatttcttt
3421 gtgagctcct gtttagcagg tcgtcccttc agcaaggaca caaaaagatt ttaattttat
3481 t

The start codons at positions 115, 161, 512 and 524 of the CPMV RNA-2 genome segment are shown in bold and underlined.

Table 2

Oligonucleotides used in the mutagenesis of the 5' region of pM81-FSC-2 clones

Oligonucleotide	Sequence	Mutation
A115G-F	CTTGTCTTTCTTGC**G**TGAGCGATCTTCAACG	Removes AUG (→GUG) at 115 eliminating translation from uORF
A115G-R	CGTTGAAGATCGCTCA**C**GCAAGAAAGACAAG	
U162C-F	GGCACCAGTACAA**C**GTTTTCTTTCACTGAAGCG	Removes AUG (→ACG) at 161 eliminating translation from AUG 161 while maintaining amino acid sequence of uORF
U162C-R	CGCTTCAGTGAAAGAAAAC**G**TTGTACTGGTGCC	

The mutant nucleotide of the oligonucleotides used in the mutagenesis of the 5' region of pM81-FSC-2 clones are shown in bold

Table 3

CPMV wt sequence from Table 1	tatattctgc ccaaatttga aatggaaagc attatgagcc gtggtattcc
Mutated sequence in pM81-FSC-1	tatattctgc ccaaatttgT CatgAaaagc attatgagcc gtggtattcc 509 BspH1
Mutated sequence in pM81-FSC-2	tatattctgc ccaaattCGC GACGATCGTA CTCTCGAGGC CT 507 Nru1 Xho1

Nucleotide differences between the sequence of the pM81-FSC-1 and pM81-FSC-2 vectors and the CPMV wt sequence from Table 1 and are shown as capital letters.

Nucleotide Sequence of pM81-FSC-1

```
LOCUS       pM81-FSC1              7732 bp    DNA      circular
10-OCT-2007

FEATURES             Location/Qualifiers
     5'UTR           342..501
                     /vntifkey="52"
                     /label=CPMV\RNA2\5'UTR
     promoter        27..341
                     /vntifkey="29"
                     /label=CaMV\35S\promoter
     terminator      4669..4921
                     /vntifkey="43"
                     /label=Nos\Terminator
     mat_peptide     3712..4422
                     /vntifkey="84"
                     /label=GFP
     3'UTR           4432..4615
                     /vntifkey="50"
                     /label=CPMV\RNA2\3'UTR
     CDS             complement(5944..6804)
                     /vntifkey="4"
                     /label=AmpR
     misc_feature    complement(7391..7546)
                     /vntifkey="21"
                     /label=lacZ_a
     promoter        complement(6846..6874)
                     /vntifkey="30"
                     /label=AmpR\promoter
     rep_origin      complement(7067..7373)
                     /vntifkey="33"
                     /label=f1_origin
     rep_origin      complement(5170..5789)
                     /vntifkey="33"
                     /label=pBR322_origin
     mat_peptide     502..1878
                     /vntifkey="84"
                     /label=CPMV\Movement\Protein
     mat_peptide     1879..2999
```

```
                     /vntifkey="84"
                     /label=CPMV\Lg.\Coat\Protein
     mat_peptide     3000..3638
                     /vntifkey="84"
                     /label=CPMV\Sm.\Coat\Protein
BASE COUNT     2105 a     1682 c     1770 g     2175 t
ORIGIN

1 ttaattaaga attcgagctc caccgcggaa acctcctcgg attccattgc ccagctatct
61 gtcactttat tgagaagata gtggaaaagg aaggtggctc ctacaaatgc catcattgcg
121 ataaaggaaa ggccatcgtt gaagatgcct ctgccgacag tggtcccaaa gatggacccc
181 cacccacgag gagcatcgtg gaaaaagaag acgttccaac cacgtcttca aagcaagtgg
241 attgatgtga tatctccact gacgtaaggg atgacgcaca atcccactat ccttcgcaag
301 acccttcctc tatataagga agttcatttc atttggagag gtattaaaat cttaataggt
361 tttgataaaa gcgaacgtgg ggaaacccga accaaacctt cttctaaact ctctctcatc
421 tctcttaaag caaacttctc tcttgtcttt cttgcatgag cgatcttcaa cgttgtcaga
481 tcgtgcttcg gcaccagtac aatgttttct ttcactgaag cgaaatcaaa gatctctttg
541 tggacacgta gtgcggcgcc attaaataac gtgtacttgt cctattcttg tcggtgtggt
601 cttgggaaaa gaaagcttgc tggaggctgc tgttcagccc catacattac ttgttacgat
661 tctgctgact ttcggcgggt gcaatatctc tacttctgct tgacgaggta ttgttgcctg
721 tacttctttc ttcttcttct tgctgattgg ttctataaga aatctagtat ttctttgaa
781 acagagtttt cccgtggttt tcgaacttgg agaaagattg ttaagcttct gtatattctg
841 cccaaatttg tcatgaaaag cattatgagc cgtggtattc cttcaggaat tttggaggaa
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961 aagcctgagg atatgtatgt gtctcacact tctaaatgga atgtgctcag aaaaatgagc
1021 caaaagactg tggatctttc caaagcagct gctgggatgg gattcatcaa taagcatatg
1081 cttacgggca acatcttggc acaaccaaca acagtcttgg atattcccgt cacaaaggat
1141 aaaacacttg cgatggccag tgattttatt cgtaaggaga atctcaagac ttctgccatt
1201 cacattggag caattgagat tattatccag agctttgctt cccctgaaag tgatttgatg
1261 ggaggctttt tgcttgtgga ttctttacac actgatacag ctaatgctat tcgtagcatt
1321 tttgttgctc caatgcgggg aggaagacca gtcagagtgg tgaccttccc aaatacactg
1381 gcacctgtat tatgtgatct gaacaataga ttcaagctca tttgctcatt gccaaactgt
1441 gatattgtcc agggtagcca agtagcagaa gtgagtgtaa atgttgcagg atgtgctact
1501 tccatagaga aatctcacac cccttcccaa ttgtatacag aggaatttga aaaggagggt
1561 gctgttgttg tagaatactt aggcagacag acctattgtg ctcagcctag caatttaccc
1621 acagaagaaa aacttcggtc ccttaagttt gactttcatg ttgaacaacc aagtgtcctg
1681 aagttatcca attcctgcaa tgcgcacttt gtcaagggaa aaagtttgaa atactctatt
1741 tctggcaaag aagcagaaaa ccatgcagtt catgctactg tggtctctcg agaaggggct
1801 tctgcggcac ccaagcaata tgatcctatt ttgggacggg tgctggatcc acgaaatggg
1861 aatgtggctt ttccacaaat ggagcaaaac ttgtttgccc tttctttgga tgatacaagc
```

1921 tcagttcgtg gttctttgct tgacacaaaa ttcgcacaaa ctcgagtttt gttgtccaag
1981 gctatggctg gtggtgatgt gttattggat gagtatctct atgatgtggt caatggacaa
2041 gattttagag ctactgtcgc ttttttgcgc acccatgtta taacaggcaa aataaaggtg
2101 acagctacca ccaacatttc tgacaactcg ggttgttgtt tgatgttggc cataaatagt
2161 ggtgtgaggg gtaagtatag tactgatgtt tatactatct gctctcaaga ctccatgacg
2221 tggaacccag ggtgcaaaaa gaacttctcg ttcacattta atccaaaccc ttgtggggat
2281 tcttggtctg ctgagatgat aagtcgaagc agagttagga tgacagttat ttgtgtttcg
2341 ggatggacct tatctcctac cacagatgtg attgccaagc tagactggtc aattgtcaat
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2461 cgttggatgg gaaaattgac ttttccccag ggtgtgacaa gtgaggttcg aaggatgcct
2521 ctttctatag gaggcggtgc tggtgcgact caagctttct tggccaatat gcccaattca
2581 tggatatcaa tgtggagata ttttagaggt gaacttcact ttgaagttac taaaatgagc
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2941 gtggtatagg ttctaatccg ggtattgatg gttcccgctt gcttggagct atagcacaag
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3181 cgtactgctg cttggaaatc tggaactatt catgttcaac ttaatgttag gggtgctggt
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4261 aatactccaa ttggcgatgg ccctgtcctt ttaccagaca accattacct gtccacacaa

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SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 04900-89 seq 10-07-08 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

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<220>
<223> Synthetic sequence: Oligonucleotide POW-F

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<213> Artificial sequence

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cg 62

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<212> DNA
<213> Artificial sequence

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<223> Synthetic sequence: Oligonucleotide GFP-XhoI-R

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CLAIMS

1. A gene expression construct comprising:

(a) an expression enhancer sequence derived from the RNA-2 genome segment of a *Comoviridae* bipartite RNA virus, in which an translation initiation site in the RNA-2 genome segment has been mutated such that it no longer functions as a translation initiation site,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame, and wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV),

wherein the expression enhancer sequence either:

(i) comprises at least a 100 nucleotide sequence of the comoviral RNA-2 genome segment which includes the mutated initiation site, or

(ii) has at least 70% identity to the full length of nucleotides 1 to 507 of the CPMV RNA-2 genome segment sequence shown in SEQ ID NO: 1, and wherein the initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated; and

(b) a heterologous sequence for facilitating insertion of a gene encoding a protein of interest into the gene expression construct, wherein the heterologous sequence is located downstream of the mutated target initiation site in the enhancer sequence.

2. The gene expression construct according to claim 1 further comprising a 3' UTR.

3. A gene expression construct comprising:

(a) an expression enhancer sequence derived from the RNA-2 genome segment of a *Comoviridae* bipartite RNA virus, in which an translation initiation site in the RNA-2 genome segment has been mutated such that it no longer functions as a translation initiation site,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame, and wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV), wherein the expression enhancer sequence either:

(i) comprises at least a 100 nucleotide sequence of the comoviral RNA-2 genome segment which includes the mutated initiation site, or

(ii) has at least 70% identity to the full length of nucleotides 1 to 507 of the CPMV RNA-2 genome segment sequence shown in SEQ ID NO: 1, and wherein the initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated; and

(b) a heterologous gene encoding a protein of interest, wherein the gene encoding the protein of interest is located downstream of the enhancer sequence.

4. The gene expression construct according to claim 3, wherein the gene encoding the protein of interest is operably linked to promoter and terminator sequences.

5. The gene expression construct according to claim 4, wherein the gene encoding the protein of interest is located downstream of the enhancer sequence and upstream of the terminator sequence.

6. The gene expression construct according to any one of claims 2 to 5 comprising or further comprising a 3' UTR.

7. The gene expression construct according to any one of claims 2 to 6, wherein the bipartite RNA virus is a comovirus.

8. The gene expression construct according to claim 7, wherein the comovirus is CPMV.

9. The gene expression construct as claimed in claim 8 wherein the AUG at position 115 is also mutated.

10. The gene expression construct according to any one of claims 7 to 9, wherein the enhancer sequence comprises at least nucleotides 10 to 512, 20 to 512, 30 to 512, 40 to 512, 50 to 512, 100 to 512, 150 to 512, 1 to 514, 10 to 514, 20 to 514, 30 to 514, 40 to 514, 50 to 514, 100 to 514, 150 to 514, 1 to 511, 10 to 511, 20 to 511, 30 to 511, 40 to 511, 50 to 511, 100 to 511, 150 to 511, 1 to 509, 10 to 509, 20 to 509, 30 to 509, 40 to 509, 50 to 509, 100 to 509, 150 to 509, 1 to 507, 10 to

507, 20 to 507, 30 to 507, 40 to 507, 50 to 507, 100 to 507, or 150 to 507 of a comoviral RNA-2 genome segment sequence with said mutated initiation site.

11. The gene expression construct according to claim 10 wherein the enhancer sequence comprises nucleotides 10 to 512, 20 to 512, 30 to 512, 40 to 512, 50 to 512, 100 to 512, 150 to 512, 1 to 514, 10 to 514, 20 to 514, 30 to 514, 40 to 514, 50 to 514, 100 to 514, 150 to 514, 1 to 511, 10 to 511, 20 to 511, 30 to 511, 40 to 511, 50 to 511, 100 to 511, 150 to 511, 1 to 509, 10 to 509, 20 to 509, 30 to 509, 40 to 509, 50 to 509, 100 to 509, 150 to 509, 1 to 507, 10 to 507, 20 to 507, 30 to 507, 40 to 507, 50 to 507, 100 to 507, or 150 to 507 of the CPMV RNA-2 genome segment sequence shown in SEQ ID NO: 1, wherein the initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated.

12. The gene expression construct according to any one of claims 2 to 11, wherein the enhancer sequence has at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, or 75% identity to the full length of nucleotides 1 to 507 of the CPMV RNA-2 genome segment sequence shown in SEQ ID NO: 1, wherein the initiation site at position 161 in the wild-type CPMV RNA-2 genome segment has been mutated.

13. A gene expression construct comprising:
(a) a promoter;
(b) nucleotides 1 to 507 of the cowpea mosaic virus RNA-2 genome segment sequence shown in SEQ ID NO: 1, wherein the AUG at position 161 has been mutated to ACG, located downstream of the promoter;
(c) a gene encoding a protein of interest located downstream of the sequence defined in (b);
(d) nucleotides 3302 to 3481 of the cowpea mosaic virus RNA-2 genome segment sequence shown in SEQ ID NO: 1, located downstream of the gene encoding the protein of interest; and
(e) a nopaline synthase terminator located downstream of the sequence defined in (d).

14. The gene expression construct according to any one of claims 2 to 12, wherein the gene expression construct comprises:
(a) a promoter;
(b) an expression enhancer sequence with at least 70% identity to the full length of nucleotides 1 to 507 of the cowpea mosaic virus RNA-2 genome segment sequence shown in SEQ ID NO: 1, wherein the AUG at position 161 has been mutated, located downstream of the promoter;

(c) a gene encoding a protein of interest located downstream of the sequence defined in (b);
(d) nucleotides 3302 to 3481 of the cowpea mosaic virus RNA-2 genome segment sequence shown in SEQ ID NO: 1, located downstream of the gene encoding the protein of interest; and
(e) a nopaline synthase terminator located downstream of the sequence defined in (d).

15. The gene expression construct as claimed in any one of claims 2 to 14 further encoding a heterologous suppressor of gene silencing operably linked to promoter and terminator sequences.

16. A gene expression construct comprising:

(a) a first gene construct comprising a sequence derived from an RNA-2 segment of a *Comoviridae* bipartite virus genome carrying at least one foreign gene encoding a heterologous protein of interest operably linked to promoter and terminator sequences, wherein the gene construct comprises a mutation in a translation initiation site upstream of the foreign gene such that it no longer functions as a translation initiation site,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame

wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of CPMV; and

(b) a second gene construct comprising a heterologous suppressor of gene silencing operably linked to promoter and terminator sequences.

17. The gene expression construct as claimed in claim 16, wherein the second gene construct is incorporated within said first gene construct.

18. The gene expression construct as claimed in any one claims 2 to 17 which is a DNA binary vector.

19. A process for increasing expression or translational enhancing activity of a sequence derived from an RNA-2 genome segment of a *Comoviridae* bipartite virus, comprising mutating a translation initiation site therein,wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame,

wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV),

wherein said mutating is performed either by:

(i) a point mutation in the initiation site, or

(ii) a deletion of up to 50 nucleotides in length including said initiation site,

wherein said mutation enhances the expression of a heterologous ORF to which the sequence is attached.

20. The process according to claim 19, wherein the bipartite RNA virus is a comovirus.

21. The process according to claim 20, wherein the comovirus is cowpea mosaic virus.

22. A method for expressing a heterologous protein of interest in a host organism using the gene expression construct according to any one of claims 2 to 18, wherein the host organism is a plant or insect.

23. The method according to claim 22, wherein the host organism is a plant.

24. A method of enhancing the translation of a heterologous protein of interest from a gene or open reading frame (ORF) encoding the heterologous protein of interest, which gene or ORF is operably linked to an RNA2- genome segment of a *Comoviridae* bipartite virus derived sequence,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame,

comprising mutating the first of these two initiation sites in the RNA2-derived sequence, wherein the mutated translation initiation site corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV),

wherein said mutating is performed either by:

(i) a point mutation in the initiation site, or

(ii) a deletion of up to 50 nucleotides in length including said initiation site.

25. A method of expressing a heterologous protein in a plant comprising the steps of:

(a) introducing into a plant cell a gene expression construct comprising a first gene construct comprising a sequence derived from an RNA-2 segment of a *Comoviridae* bipartite virus genome carrying at least one foreign gene encoding a heterologous protein of interest operably linked to promoter and terminator sequences,

wherein the gene construct comprises a mutation in a translation initiation site upstream of the foreign gene such that it no longer functions as a translation initiation site,

wherein the wild-type RNA2 genome segment of the *Comoviridae* virus encodes two carboxy coterminal proteins through two different translation initiation sites located in the same triplet reading frame,wherein the mutated initiation site is the first of these two initiation sites and corresponds to the initiation site at position 161 in the wild-type RNA-2 segment of cowpea mosaic virus (CPMV); and

(b) introducing a second gene construct comprising a heterologous suppressor of gene silencing operably linked to promoter and terminator sequences into the plant cell.

26. The method as claimed in claim 25, wherein the second gene construct is incorporated within said first gene construct.

27. The method according to claim 22 or claim 23, comprising the step of introducing the gene expression construct into the host organism.

28. A plant or insect host cell obtained by a method according to any one of claims 25 to 27, which host cell comprises the gene expression construct, and wherein the gene encoding the protein of interest is expressed in said host cell.

29. A plant host cell transiently transfected with, and comprising, the gene expression construct according to any one of claims 2 to 18.

30. An insect host cell transiently transfected with, and comprising, the gene expression construct according to any one of claims 2 to 18.

31. A transgenic plant or insect host cell stably transformed with, and comprising, the gene expression construct according to any one of claims 2 to 18.

32. A method for generating a protein of interest, comprising using the plant or insect host cell according to any one of claims 28 to 31 and harvesting a tissue in which the protein of interest has been expressed and isolating the protein of interest from the tissue.

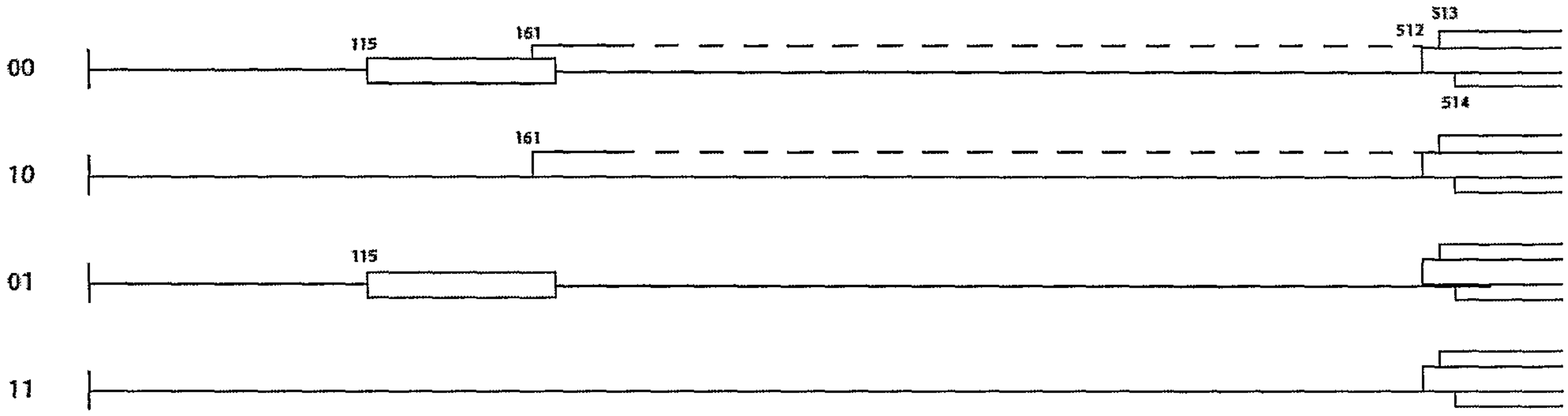


FIGURE 1

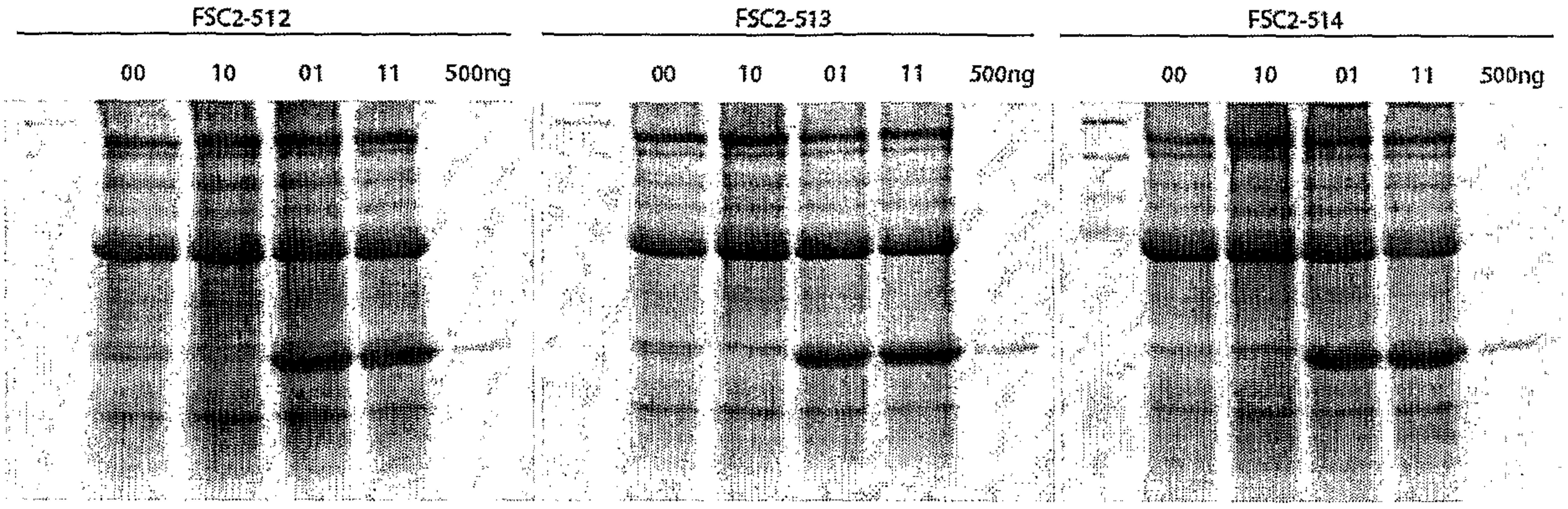

FSC2-512
00
10
01
11
500ng
FSC2-513
00
10
01
11
500ng
FSC2-514
00
10
01
11
500ng

FIGURE 2

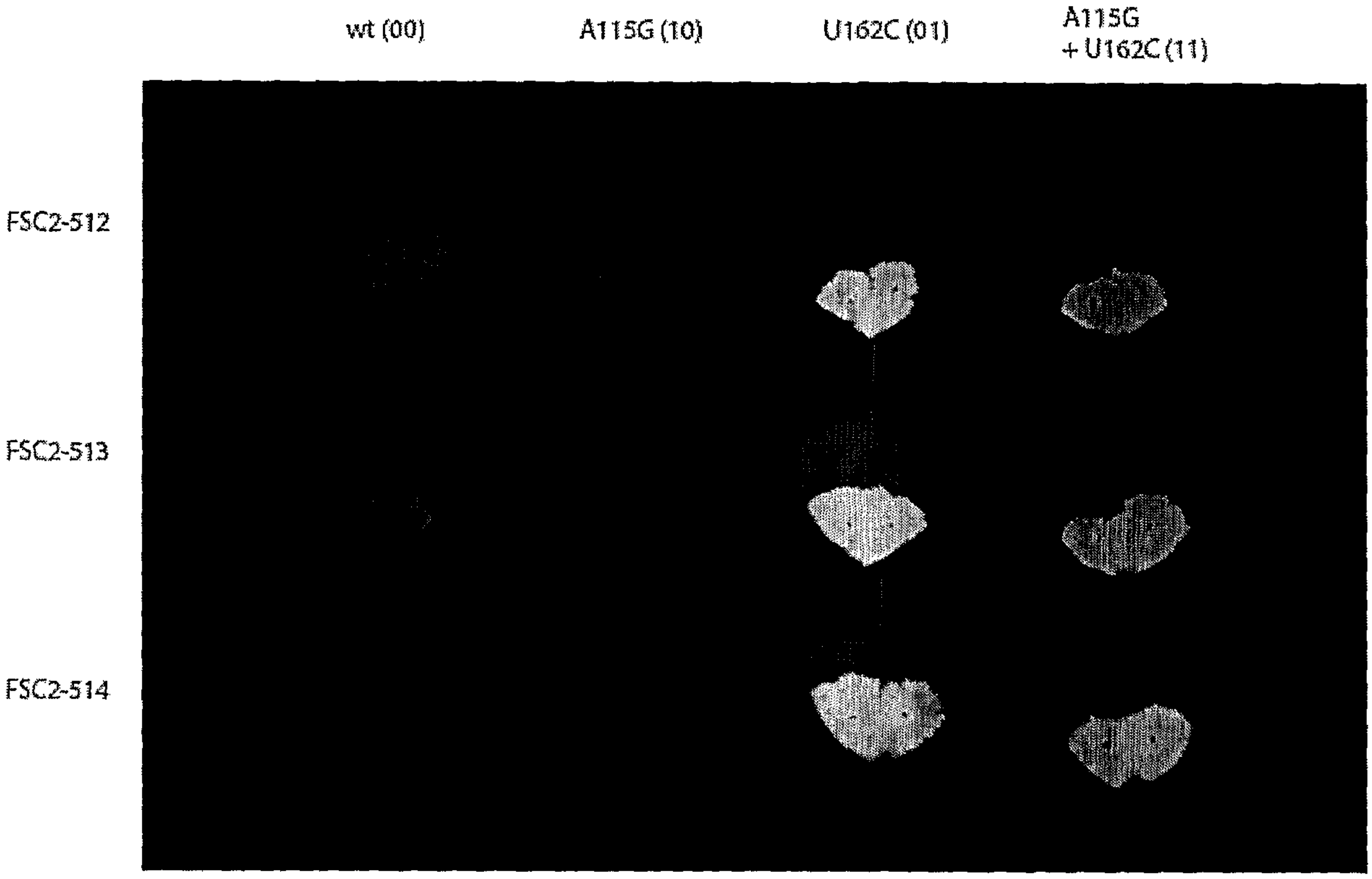

wt (00)
A115G (10)
U162C (01)
A115G
+ U162C (11)
FSC2-512
FSC2-513
FSC2-514

FIGURE 3

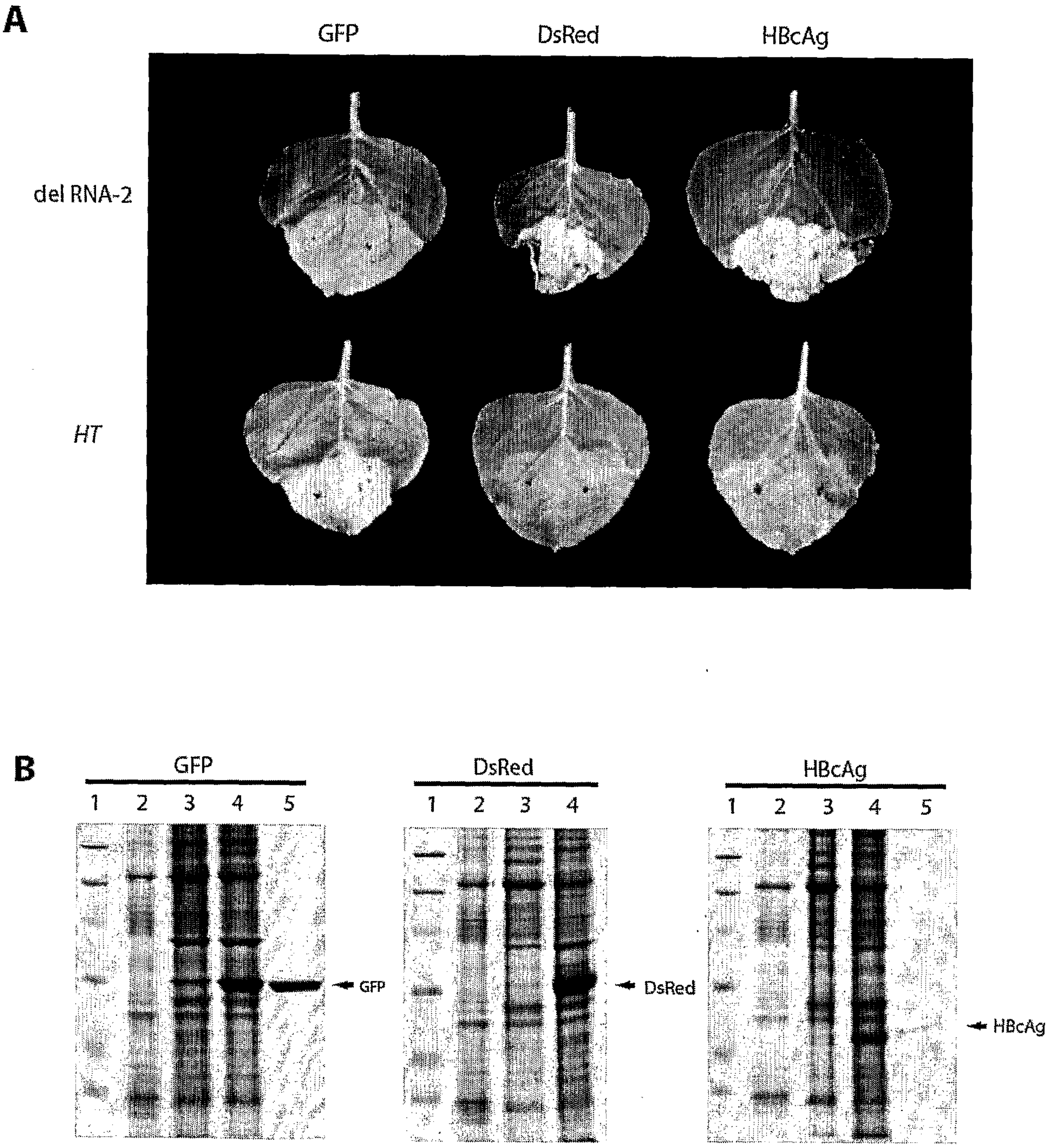

A
GFP
DsRed
HBcAg
del RNA-2
HT
B
GFP
1 2 3 4 5
GFP
DsRed
1 2 3 4
DsRed
HBcAg
1 2 3 4 5
HBcAg

FIGURE 4

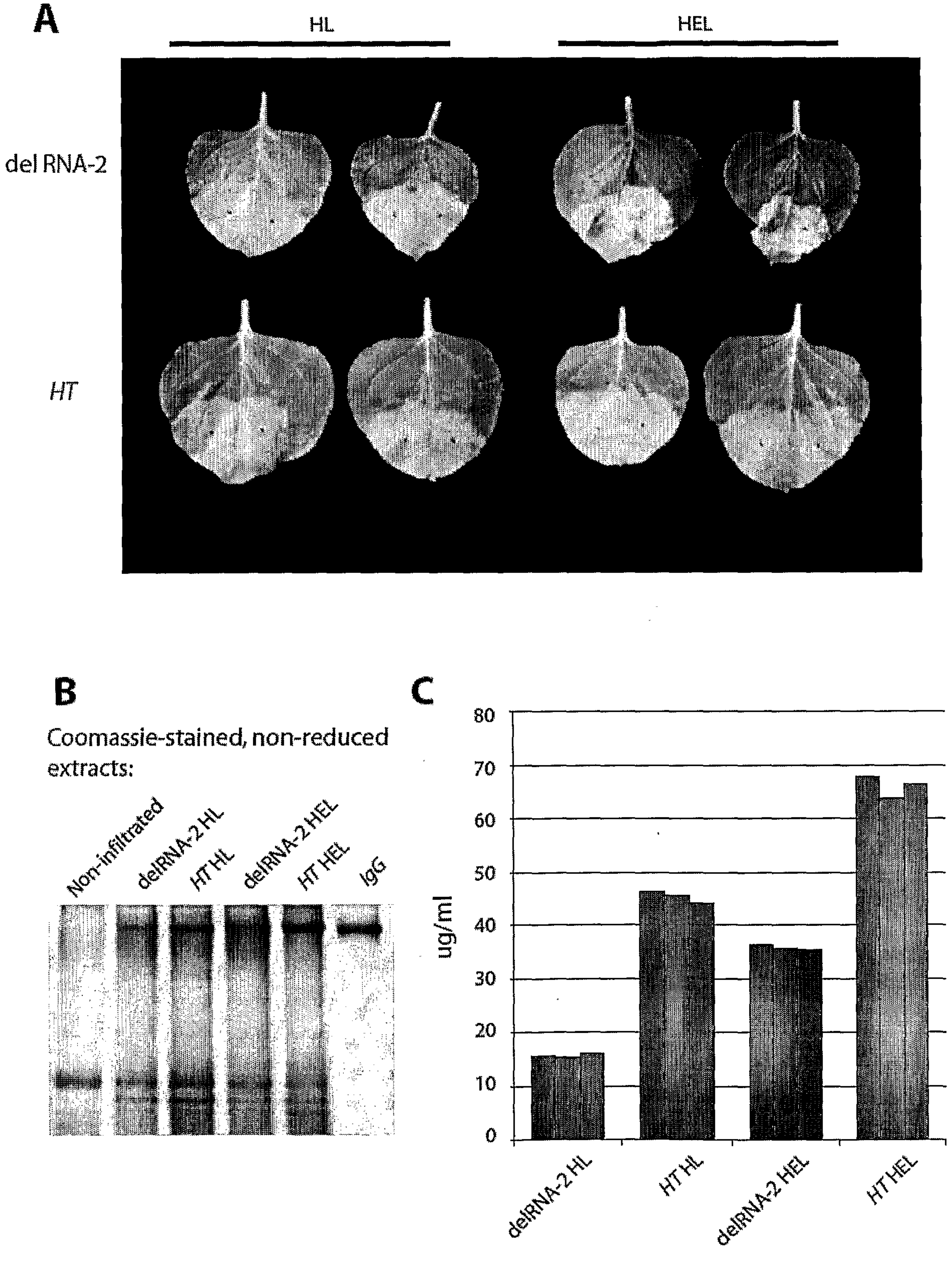

A
HL
HEL
del RNA-2
HT
B
Coomassie-stained, non-reduced extracts:
Non-infiltrated
delRNA-2 HL
HT HL
delRNA-2 HEL
HT HEL
IgG
C
80
70
60
50
40
30
20
10
0
ug/ml
delRNA-2 HL
HT HL
delRNA-2 HEL
HT HEL

FIGURE 5

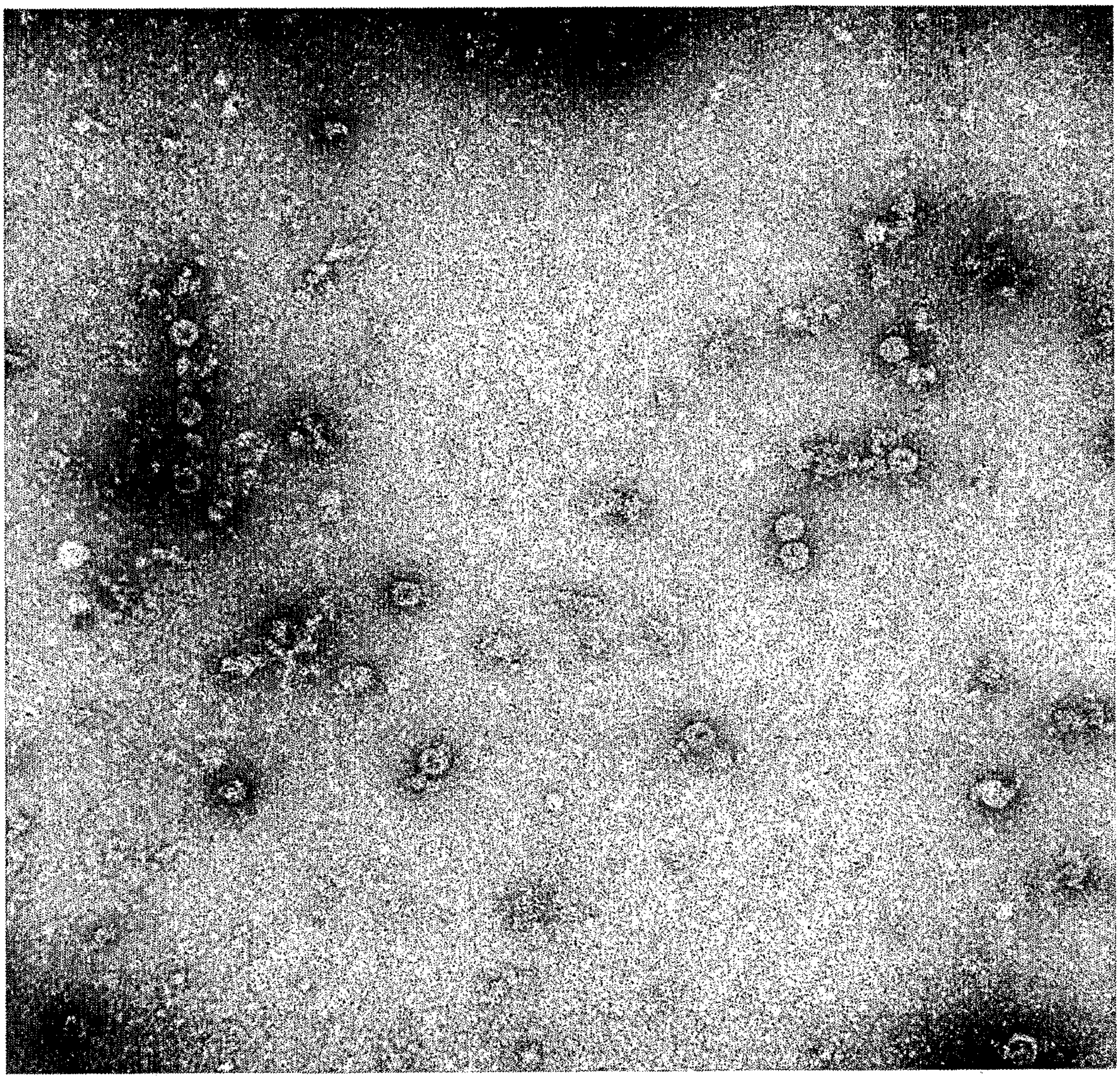


FIGURE 6

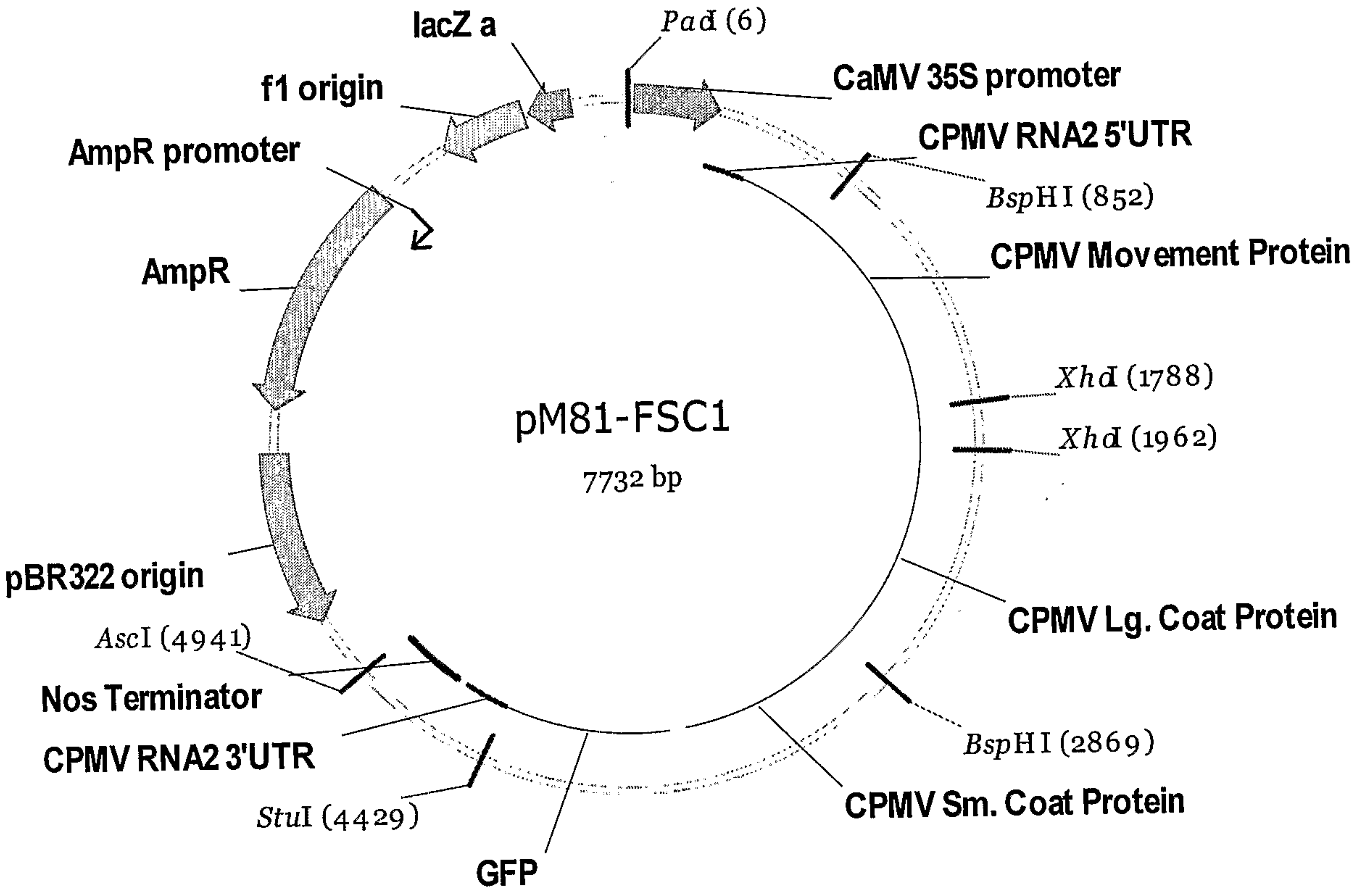

lacZ a
PacI (6)
CaMV 35S promoter
f1 origin
CPMV RNA2 5'UTR
AmpR promoter
BspHI (852)
CPMV Movement Protein
AmpR
XhoI (1788)
pM81-FSC1
7732 bp
XhoI (1962)
pBR322 origin
CPMV Lg. Coat Protein
AscI (4941)
Nos Terminator
BspHI (2869)
CPMV RNA2 3'UTR
StuI (4429)
CPMV Sm. Coat Protein
GFP

FIGURE 7

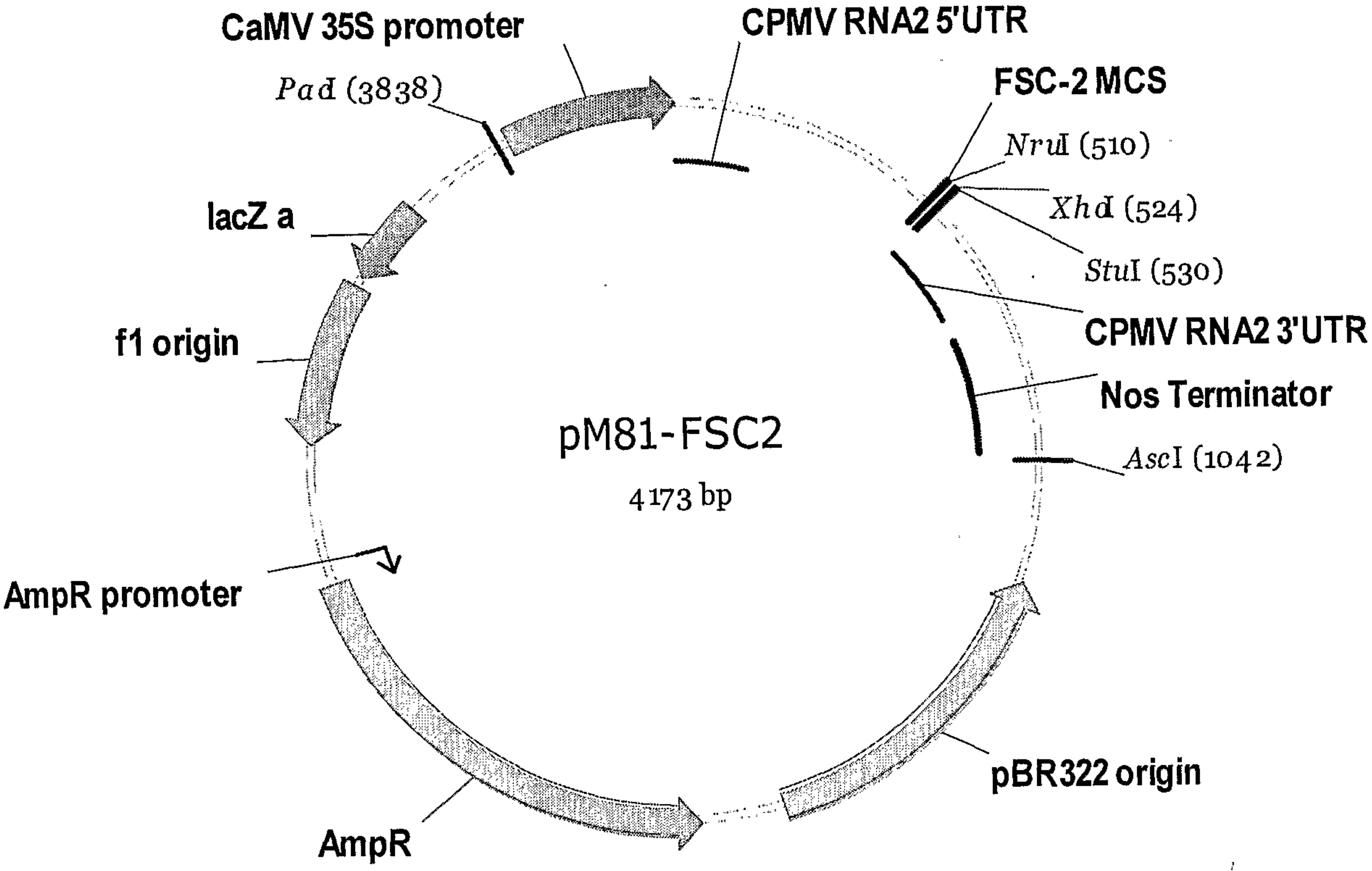

CaMV 35S promoter
PacI (3838)
CPMV RNA2 5'UTR
FSC-2 MCS
NruI (510)
XhoI (524)
StuI (530)
lacZ a
CPMV RNA2 3'UTR
f1 origin
Nos Terminator
pM81-FSC2
4173 bp
AscI (1042)
AmpR promoter
pBR322 origin
AmpR

FIGURE 8

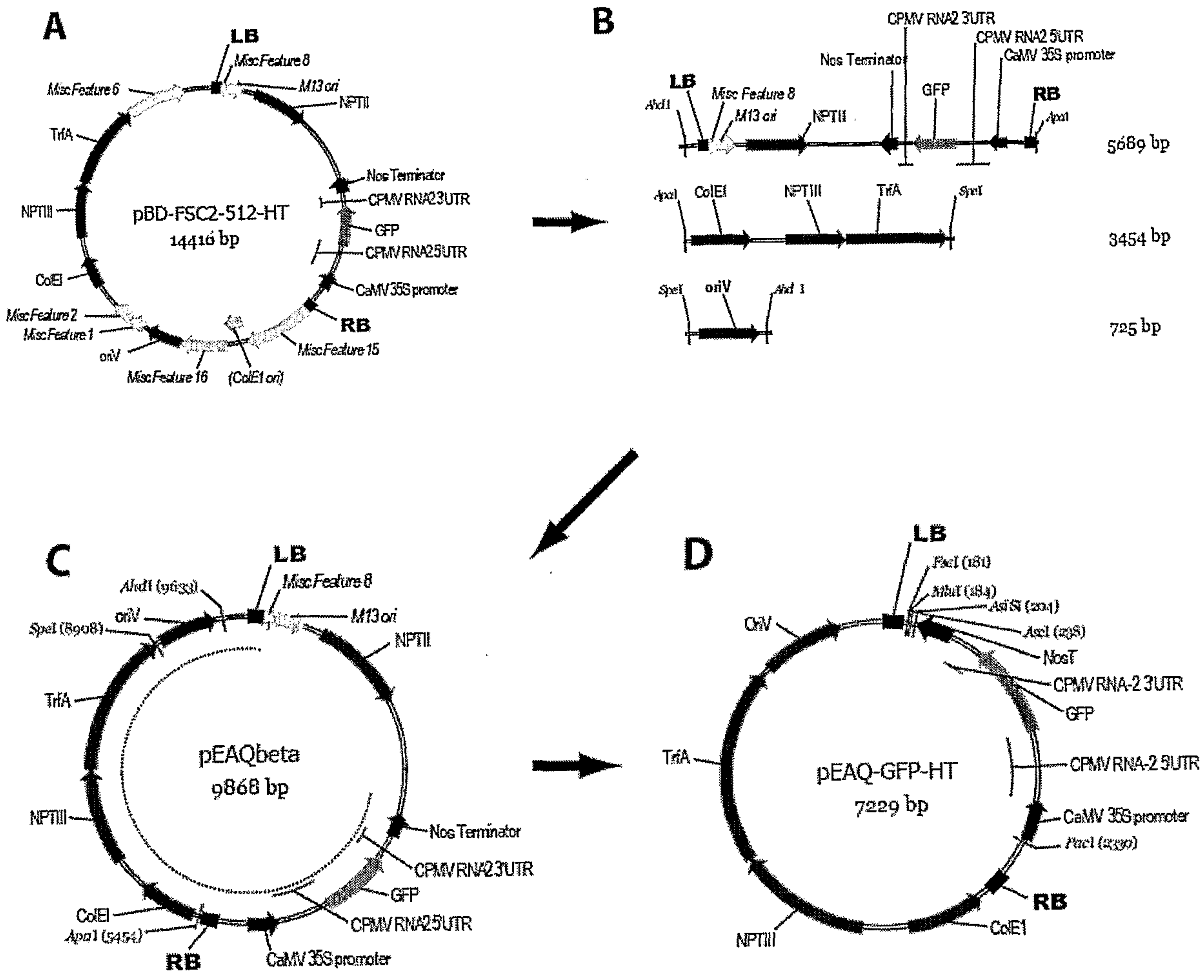

A
LB
Misc Feature 8
M13 ori
NPTII
Misc Feature 6
TrfA
NPTIII
pBD-FSC2-512-HT
14416 bp
Nos Terminator
CPMV RNA2 3UTR
GFP
CPMV RNA2 5UTR
CaMV 35S promoter
RB
ColEI
Misc Feature 2
Misc Feature 1
oriV
Misc Feature 16
(ColE1 ori)
Misc Feature 15
B
CPMV RNA2 3UTR
CPMV RNA2 5UTR
Nos Terminator
CaMV 35S promoter
LB
Misc Feature 8
AhdI
M13 ori
NPTII
GFP
RB
ApaI
5689 bp
ApaI
ColEI
NPTIII
TrfA
SpeI
3454 bp
SpeI
oriV
Ahd I
725 bp
C
LB
AhdI (9633)
Misc Feature 8
oriV
M13 ori
SpeI (8908)
NPTII
TrfA
pEAQbeta
9868 bp
NPTIII
Nos Terminator
CPMV RNA2 3UTR
ColEI
GFP
ApaI (5454)
CPMV RNA2 5UTR
RB
CaMV 35S promoter
D
LB
PacI (181)
MluI (184)
AsiSI (204)
OriV
AscI (238)
NosT
CPMV RNA-2 3UTR
GFP
TrfA
pEAQ-GFP-HT
7229 bp
CPMV RNA-2 5UTR
CaMV 35S promoter
PacI (2390)
RB
NPTIII
ColE1

FIGURE 9

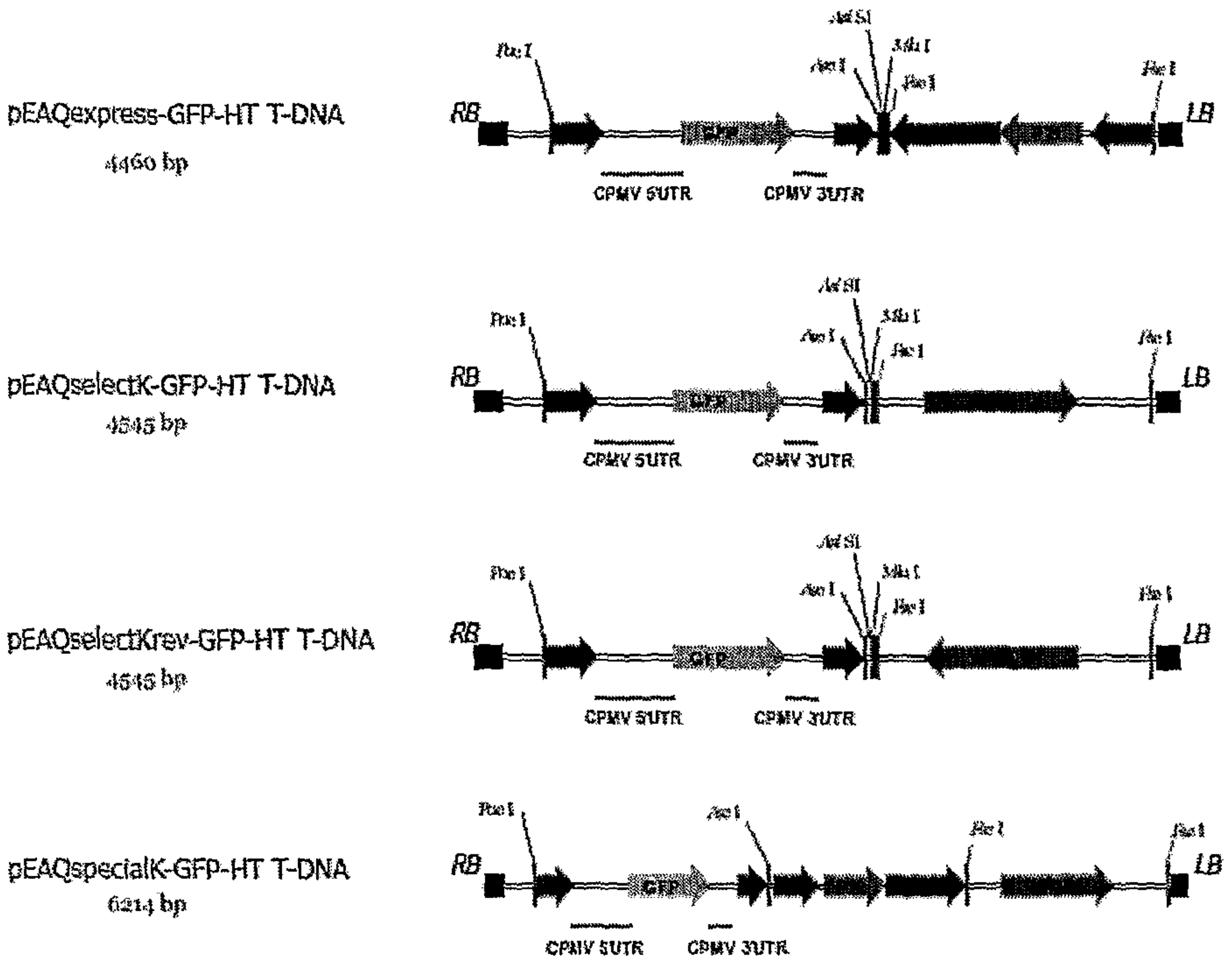


FIGURE 10

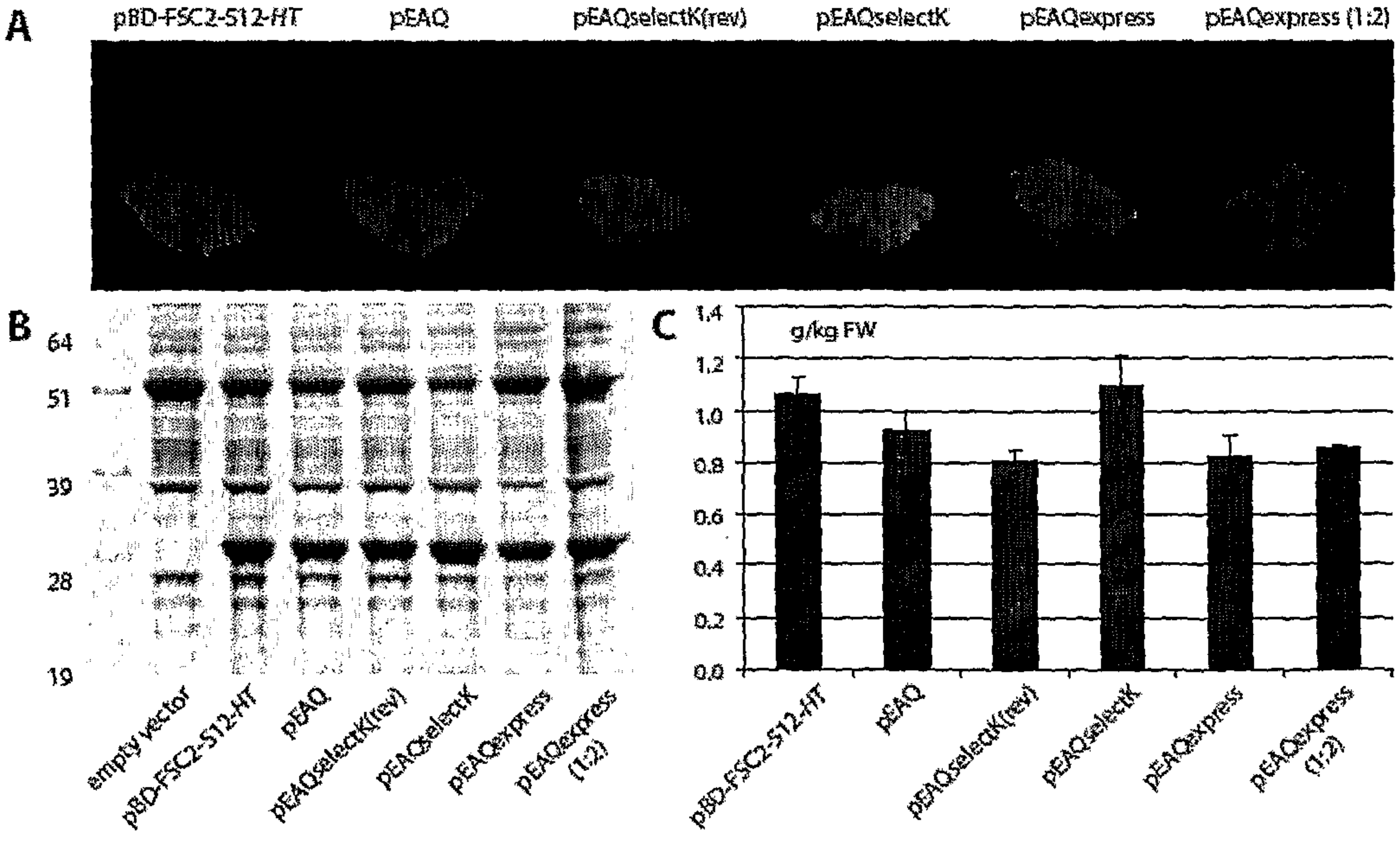


FIGURE 11

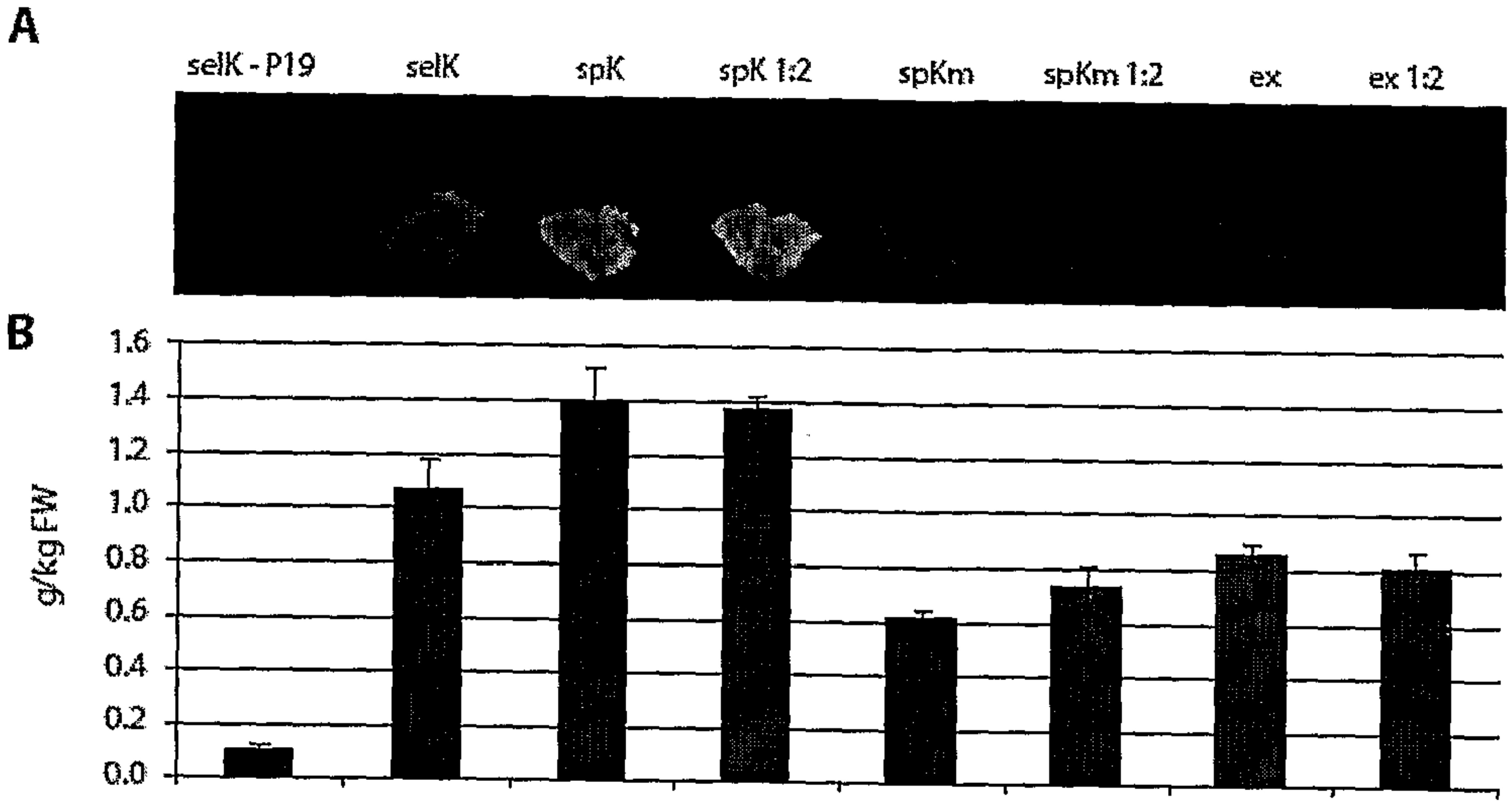
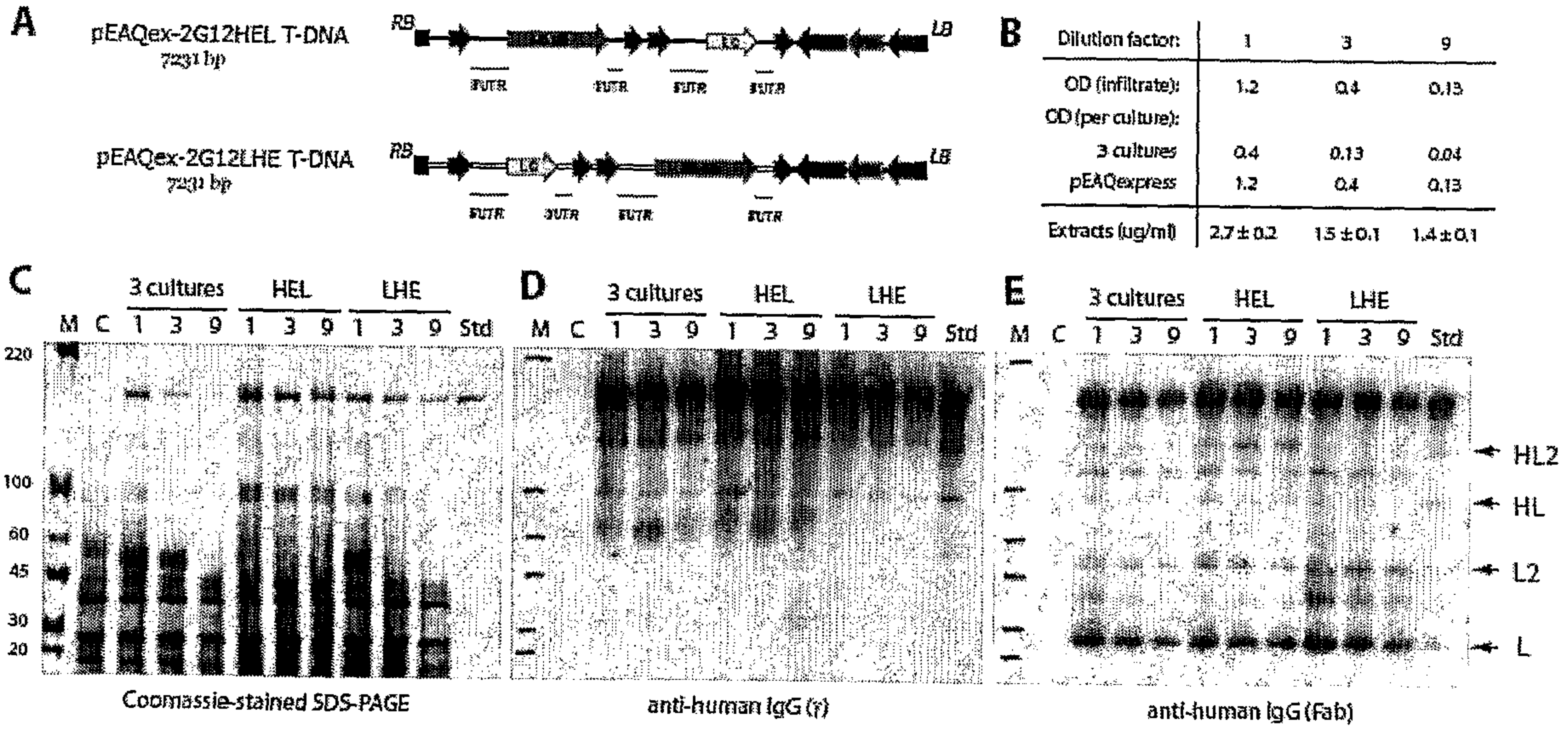


FIGURE 12



Dilution factor:	1	3	9
OD (infiltrate):	1.2	0.4	0.13
OD (per culture):			
3 cultures	0.4	0.13	0.04
pEAQexpress	1.2	0.4	0.13
Extracts (ug/ml)	2.7 ± 0.2	1.5 ± 0.1	1.4 ± 0.1

FIGURE 13

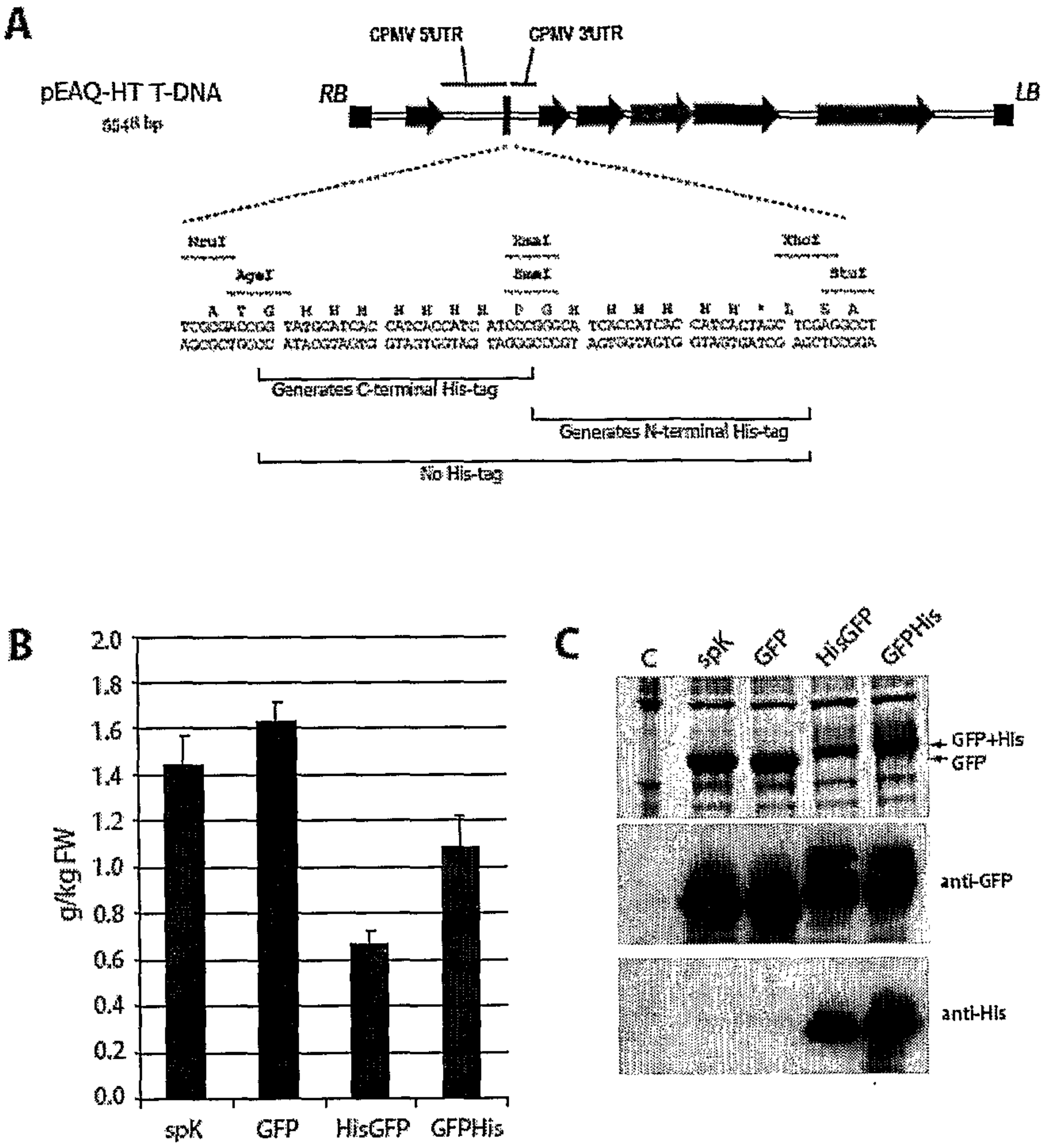


FIGURE 14

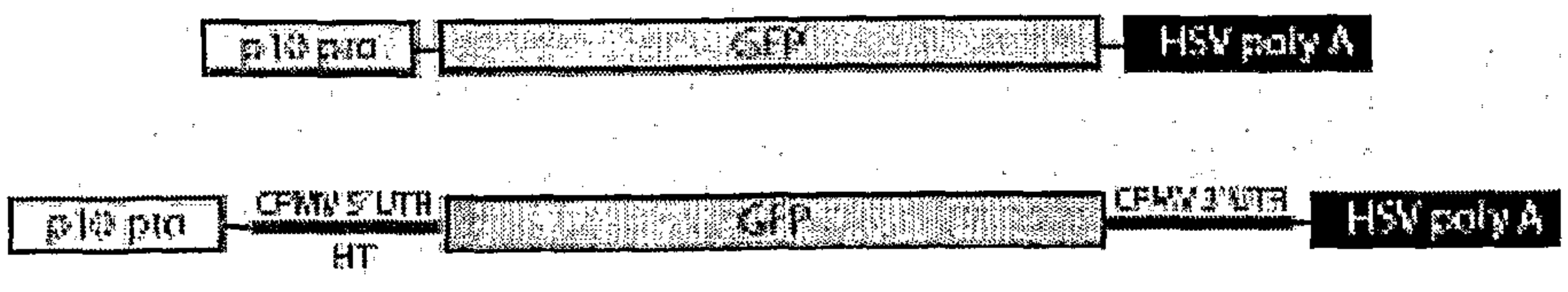


FIGURE 15