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High expression of foreign proteins from a biosafe viral vector derived from *Turnip mosaic virus*

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Abstract

A vector derived from an original infectious clone of *Turnip mosaic potyvirus* (TuMV) has been developed. The vector (p35Tunos-vec01) was made through the creation of a new unique insertion site for foreign genes between the NIb and the CP genes of the viral genome. The jellyfish green fluorescent protein (GFP) and *E. coli* β-glucuronidase (GUS) were expressed in *Arabidopsis thaliana* plants infected with chimeric vectors carrying their corresponding genes. In the case of GUS, expression levels that were 15-50 fold higher than nuclear transgenic *A. thaliana* lines carrying the same gene were attained. Acceptable levels of gene stability were found in the infected plants, compatible with the use of the vector for protein production in plants. A non-aphid transmissible version of the vector was also made and its lack of aphid transmission was extensively shown. The vector was not transmitted through seeds or by contact between plants.

Additional key words: aphid transmission, *Arabidopsis thaliana,* biofactories, environment, potyvirus.

Resumen

Altos niveles de expresión de proteínas foráneas a partir de un vector viral bioseguro derivado del virus del mosaico del nabo

Se desarrolló un vector a partir del clon infectivo original del potyvirus del mosaico del nabo (TuMV). El vector (p35Tunos-vec01) se construyó mediante la introducción de un nuevo y único sitio de restricción para la inserción de genes foráneos entre los genes virales que codifican para las proteínas NIb y CP. La proteína verde fluorescente de la medusa *Aequorea victoria* (GFP) y la β-glucuronidasa (GUS) de *Escherichia coli* se expresaron en plantas de *Arabidopsis thaliana* infectadas con los vectores quiméricos que contenían los genes correspondientes. En el caso de GUS, los niveles de expresión obtenidos fueron 15-50 veces superiores a los medidos en plantas transgénicas de *A. thaliana* que contenían el mismo gen. Los niveles de estabilidad del gen foráneo en las plantas infectadas fueron aceptables, lo cual es compatible con la utilización del vector en la producción de proteínas en plantas. Se desarrolló una versión del vector no transmisible por pulgones. El vector tampoco fue transmisible por semillas o por contacto entre plantas.

Palabras clave adicionales: *Arabidopsis thaliana,* biofactorías, medio ambiente, potyvirus, transmisión por pulgones.

Introduction[1]

The expression of heterologous genes in plants has become a central issue in plant biotechnology. Genes from many biological origins have already been expressed in plants, giving rise to a wide variety of applications and tools for the study of plant biology. The most frequent strategy takes advantage of transgenic expression in plants after genetic transformation (Herrera-Estrella *et al.*, 2005). However, transient expression is increasingly being used for many different purposes. Both agro-inoculation and gene bombardment have proved efficient

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[1] Abbreviations used: cDNA(complementary DNA), CP (coat protein), DAE (aspartic acid, alanine, glutamic acid), DAG (aspartic acid, alanine, glycine), dpi (days post inoculation), GFP (green fluorescent protein), GUS (β-glucuronidase), HC-Pro (helper component-protease), IC-RT-PCR (immunocapture-reverse transcription-polymerase chain reaction), IgG (immunoglobulin G), NAT (non aphid-transmissible), NBT-BCIP (nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate), PAGE (polyacrylamide gel electrophoresis), PDS (phytoene desaturase), SDS (sodium dodecyl sulfate).

in foreign expression, and are invaluable tools in modern plant biotechnology (Fischer *et al.*, 1999).

Viruses are important vehicles for heterologous gene expression in plants. For about 20 years (French *et al.*, 1986), different research groups have developed a range of virus-derived vectors which have been used to express a variety of gene products (Pogue *et al.*, 2002; Grill *et al.*, 2005). Main advantages of using viruses as vectors for heterologous gene expression include speed, low cost, no plant transgenic technology, and very high levels of heterologous protein production, among others. Although some specific points such as foreign gene stability within the context of the viral genome and insert size are still an issue, virus-derived vectors are an important biotechnological tool for foreign gene expression, and their development is making progress. In addition to heterologous gene expression, viral vectors are now an indispensable tool for many studies involving plant virus replication, accumulation and movement, mostly by means of the expression of reporter genes, or pieces of them. Within plant viruses, vectors derived from tobacco viruses are mostly used for commercial purposes (Grill *et al.*, 2005).

Potyviruses are the most extensively represented group within plant viruses, being about 30% of the whole universe of these pathogens (Shukla *et al.*, 1994). Members of the *Potyviridae* family have been found in practically all know taxonomical families of cultivated and non-cultivated plants. Virions of this group of viruses are flexuous rods, made up of ca. 2000 repeats of a single viral protein (the coat protein, CP), covering up a molecule of RNA, about 10 kb in size, the viral RNA genome (Shukla *et al.*, 1994). This RNA encodes a single large polyprotein, proteolytically cleaved by proteinases represented within it, upon the process of viral gene expression in infected cells (Urcuqui-Inchima *et al.*, 2001). Potyviruses are transmitted by aphids in a non-persistent manner. Viral proteins mediating aphid transmission are the CP, involving its DAG triplet, and the helper component (HC-Pro), in which several residues have been revealed as essential for transmission (Maia and Bernardi, 1996; Maia *et al.*, 1996).

Some potyviral traits are advantageous for the exploitation of potyviruses as viral-derived vectors. For instance, their rod shape make them in principle less restrictive to accommodate large inserts that increase the encapsidated chimeric new viral genome; they should express foreign proteins as free molecules cleaved out of the polyprotein by the viral proteinases, but the virion flexibility should facilitate the expression of foreign peptides fused to the viral CP, if desired. On top of the geometrical considerations, other attractive features of potyvirus-derived vectors are the fact that at least some potyviruses are infectious as cloned cDNA, thus facilitating the inoculation process, and viral expressed proteins can reach high percentages of the final protein present in the infected cells. All these traits have prompted several research groups to derive vectors form members of the Potyviruses (Dolja *et al.*, 1992; Guo *et al.*, 1998; Choi *et al.*, 2000; German-Retana *et al.*, 2000; Masuta *et al.*, 2000; Arazi *et al.*, 2001; Beauchemin *et al.*, 2005).

Turnip mosaic virus (TuMV) is one of the most prevalent potyviruses. As a pathogen it has been cited as the second most important viral pathogen in vegetables, only preceded by the ubiquitous *Cucumber mosaic virus* (Tomlinson, 1987). Of the potyviruses described to date, TuMV has the second largest host range. Over 300 plant species have been cited as TuMV hosts (Edwardson and Christie, 1991). TuMV is an attractive candidate as a vector because it infects practically all species in the family *Brassicaceae*, which includes some of the largest (biomass) vegetable plants. Importantly, TuMV infects *Arabidopsis thaliana*, the well known plant model (Martínez-Herrera *et al.*, 1994). Infectious cloned cDNAs of TuMV have been previously described (Sánchez *et al.*, 1998), which are increasingly being used as tools in studies of plant-virus interactions. This paper reports the derivation of the original TuMV infectious clone, p35Tunos, into a vector (p35Tunos-vec01), useful for the expression of heterologous genes in TuMV hosts. While this work was in progress, other researchers have also used p35Tunos as a basis for the development of TuMV-based vectors, whose utility has been shown for the expression of foreign reporter proteins in *Brassica perviridis* (Beauchemin *et al.*, 2005). Their results and ours strongly support that TuMV-derived vectors are a sound basis for the effective expression of foreign proteins in plants.

Material and Methods

Construction of p35Tunos-vec01

An infectious cDNA clone of TuMV, p35Tunos (Sánchez *et al.*, 1998) was modified to introduce a *Ngo*MIV restriction site at the NIb-CP junction. There are no *NgoM*IV restriction sites in p35Tunos. The site

(GCCGGC) was introduced through the substitution of third-base nucleotides in the codons of the first two amino acids (Ala and Gly) of the CP cistron, by PCR mutagenesis. Two fragments were generated by PCR using p35Tunos as a template with the following oligonucleotide pairs: 5'AGACCTCTGGTTTATGTC GCACC3'/5'CAAGCGTTTC*GCCGGC*CTG3' that amplified a ~ 400 bp fragment covering genomic positions 8377-8774 of TuMV UK1 cDNA (Accession: AF169561), and 5'GC*C*GG*C*GAAACGCTTGATGCA GGTTTG3'/5'GCGCGCGGTGTCATCTATCTTACT A3' that amplified a ~1,200 bp fragment covering genomic positions 8759-3' end of the *nos* terminator (*Ngo*MIV site underlined, mutated nucleotides in italics). PCR products were digested with *Ngo*MIV, ligated and digested with *Mlu*I and *Sal*I. The fragment (~1,300 bp) containing the engineered *Ngo*MIV site was used to replace the wt *Mlu*I-*Sal*I fragment in p35Tunos. The resulting plasmid was named p35Tunos-vec01 (vec01 in short).

Cloning reporter genes in p35Tunos-vec01

The reporter gene GUS (*uidA*) was amplified by PCR using plasmid pBI101.3 (Clontech) as a template, and the following primers: upper primer, 5'GCCGGC ATGTTACGTCCTGTAGAAAC3'; lower primer, 5'GCCGGC**TTGATGGTATACGCATGCT**TGTTTG CCTCCCTG3' (a *Ngo*MIV restriction site is underlined and the sequence coding for NIa protease recognition site is represented in bold). The PCR product (5' *Ngo*MIV-GUS gene-NIa proteolytic cleavage site-*Ngo*MIV 3') was cloned into the pCR2.1-TOPO (Invitrogen) and sequenced. The GUS cassette was excised with *Ngo*MIV and inserted into the vec01 to obtain p35Tunos-vec01-GUS (vec01-GUS).

A similar strategy was followed to generate p35Tunos-vec01-GFP (vec01-GFP) using plasmid psmGFP (CD3-326, obtained from the Arabidopsis Biological Resource Center at The Ohio State University) (Davis and Vierstra, 1998) as a template, and the following primers: upper primer, 5'GCCGGCATGAGTAAAGG AGAAGAAC3', lower primer, 5'GCCGGC**TTGATG GTATACGCATGCT**TTGTATAGTTCATCC3' (underlined and bold indicate the *Ngo*MIV and the sequence coding for NIa protease recognition site respectively).

The phytoene desaturase (PDS) gene of *A. thaliana* was kindly provided by Dr. David Baulcombe. An internal fragment of the gene that did not contain stop codons was amplified with the following primers: upper primer, 5'GCCGGCGATGAACTGTCAATGC3', lower primer, 5'GCCGGCTTGATGGTATACGCATGCTAT ATGAACATTAATAACTGG3'. The PCR product was inserted in vec01 to obtain p35Tunos-vec01-PDS (vec01-PDS).

Construction of p35Tunos-vec01-NAT1 (vec01-NAT1)

The non aphid-transmissible (NAT) mutation was introduced by PCR site-directed mutagenesis in the DAG motif (located at residues 6-8 of CP) using the following primers: upper primer, 5'G CCGGCGAAAC GCTTGATGCAG**AG**TTG3' (*Ngo*MIV site underlined; mutated nucleotides in bold) and lower primer 5'GCGCGCGGTGTCATCTATCTTACTA3'. The PCR product was cloned into pCR2.1 TOPO (Invitrogen). The resulting clones were double-digested with *Ngo*MIV and *Sal*I, and the mutated fragment was purified and transferred into vec01, previously also *Ngo*MIV-*Sal*I double-digested. The introduced mutations were sequence-verified.

Plant inoculation. Stability of reporter genes

Purified vec01-GUS and vec01-GFP plasmid DNAs were resuspended in TE (10 mM Tris-HCl, pH 8.0. 1 mM EDTA) at 1-3 mg mL^{-1}. Two leaves per plant of *A. thaliana*, ecotype RLD, were dusted with carborundum and manually inoculated with 6 µL of the diluted samples with the help of glass rods.

The presence and integrity of foreign sequences after plant infection with each chimeric vector was analysed by immunocapture-RT-PCR from extracts of infected plants (Nolasco *et al.*, 1993) using the monoclonal antibody anti-POTY (Agdia) to capture the virus. Primers used were the following: upper primer 5'CAAGCAATCTTTGAGGATTATG3' and lower primer 5'TATTTCCCATAAGCGAGAATAC3'. The amplicon corresponds to the 3'region of NIb gene (nt 8704-8726) and the 3'UTR (nt 9668-9646) in the TuMV genome, respectively. Extracts from plant leaves rendering only the complete amplicon (without any additional smaller band) were used for the successive passage, in a serial passage scheme, using now cotton sticks. Extracts for crude sap inoculation were done with 50 mM phosphate buffer, pH 7.5 (1/10, w/v)

(Martínez-Herrera *et al.*, 1994). This type of crude sap inoculation was also used onto leaves of Indian mustard (*Brassica juncea*) and oilseed rape (*B. napus*, cvs. Drakka and Lizard) (plants were a generous gift from Dr. John Walsh).

Detection of GFP fluorescence

GFP expression *in planta* was analysed with an inverted epifluorescence microscope (Leica Microsystems), using a FITC filter.

GUS quantitative and qualitative detection

Quantitative GUS activity was assayed in a buffer consisting of 50 mM sodium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 0.1% (w/v) sarkosyl, 10 mM EDTA, 0.1% (v/v) Triton X-100, and 1 mM p-nitrophenyl-β-D-glucuronide (Jefferson *et al.*, 1986). Final volume was 200 µL at 37°C, and the reaction was terminated by the addition of 2.5 M 2-amino-2-methylpropanediol (80 µL). The assays were carried out in microtitre plates, and p-nitrophenol absorbance was measured at 415 nm using an ELISA plate reader (Titertek Multislan PLUS MKII).

For GUS histochemistry, plant leaves were immersed in a solution of 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D glucuronide (X-Gluc) in 50 mM sodium phosphate buffer (pH 7.2), 0.1% (v/v) Triton X-100, 10 mM EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide (Stomp, 1992). A visible colorimetric reaction was obtained in a time range of a few minutes to overnight incubation at 37°C in the darkness. For comparisons of activity and amount of protein produced (next heading), the results were compared with equivalent extracts of different transgenic Arabidopsis plants expressing GUS under the 35S promoter (a generous gift from Dr. David Martínez-Herrera, who obtained these by *Agrobacterium* root transformation).

Protein extraction and western blotting

Total soluble protein was extracted from Arabidopsis leaf tissue. Tissue was finely ground in liquid nitrogen and the resulting powder was suspended in a 2-fold volume of extraction buffer (50 mM sodium phosphate (pH 7.2), 0.1% (w/v) sarkosyl, 0.1% (v/v) Triton X-100 and 10 mM 2-mercaptoethanol). The homogenate was spinned for 10 min at 13,000 g, at 4°C. The supernatant was stored in aliquots at –20°C until use. Protein concentrations were determined according to Bradford (1976) with bovine serum albumin as a standard. For western blotting of GUS and GFP producing plants, total proteins were resolved in 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad) using an electroblotting apparatus (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). After membrane blocking with 5% skim milk in phosphate-buffered pH 7.4, blots were probed with a polyclonal antibody to GUS (Molecular Probes), diluted 1:500, followed by treatment with an alkaline-phosphatase-conjugated goat anti-rabbit IgG (Chemicon International), diluted 1:200. Blots were developed using the NBT-BCIP substrate system (Roche) as described by the manufacturer.

Aphid transmission assays

TuMV, like other potyviruses, is transmitted naturally by aphids in a non-persistent manner. No other form of transmission has been reported for TuMV. Aphid transmissibility of potyviruses depends on the presence of functional amino acid motifs in two of its genes (HC-Pro and CP) mediating the transmission (Gal-On *et al.*, 1992; Pirone and Blanc, 1996). Naturally-occurring non-aphid transmissible (NAT) potyvirus isolates that have been subjected to molecular analysis present mutations in these motifs, and artificial NAT potyvirus variants have been obtained away from potyvirus infectious clones through site-directed mutagenesis in them (Atreya *et al.*, 1991, 1995; Gal-On *et al.*, 1992; Wylie *et al.*, 2002). A particularly efficient and stable NAT mutation is DAG to DAE in the N-terminal region of the CP of potyviruses (T. Pirone, personal communication). We introduced this mutation in vec01 to produce vec01-NAT1 (Table 1) and tested infectivity, foreign gene expression, and aphid transmissibility.

Aphid clones of *Myzus persicae* Sulzer, *Brevicoryne brassicae* and *Aphis fabae,* maintained on turnip (*Brassica rapa* var. rapa L.), cabbage (*Brassica oleracea* L.) and bean (*Phaseolus vulgaris* L.) plants respectively, were used as vectors. Approximately 100 aphids, previously starved for 2 h, were placed on leaves of vec01-NAT1 infected *Arabidopsis* source plants and allowed to probe for a 5-min acquisition access period. Groups of five aphids were then placed on each of 24 Arabidopsis

Table 1. Nucleotide and amino acid sequence of part of the 5´-region of the CP gene. The sequence encoding the DAG motif is in italics, and nucleotides differing between p35Tunos-vec01 and p35Tunos-vec01-NAT1 are shown in bold

Sequence	Virus name
5'GCC GGC GAA ACG CTT *GAT GCA G**GT*** TTG ACA 3' A G E T L D A G L T	p35Tunos-vec01(wild type)
5'GCC GGC GAA ACG CTT GAT GCA G**AG** TTG ACA 3' A G E T L D A E L T	p35Tunos-vec01-NAT1

test plants and allowed a 2-h inoculation access period before insecticide application. Vec01 source plants were used as positive control. Test plants were analysed by ELISA and IC-RT-PCR, 15 days later.

Results

A viral vector derived from an infectious TuMV clone

The vector for heterologous protein expression in plants was derived from an infectious TuMV clone under control of the 35S promoter (p35Tunos), previously reported by us (Sánchez *et al.*, 1998). To convert p35Tunos into an expression vector, a *Ngo*MIV site (unique restriction site) was created by oligonucleotide-directed mutagenesis, between the NIb and CP viral cistrons. This was done introducing third-base changes in the codons corresponding to the first two CP amino acids (Fig. 1). The infectivity of this new clone (vec01) was tested by inoculation of plasmid DNA onto *A. thaliana* plants as described for p35Tunos (Sánchez *et al.*, 1998). Vec01-induced systemic symptoms were indistinguishable from those of p35Tunos or TuMV UK 1 (Martín-Martín *et al.*, 1999), first appearing 7-10 days post inoculation (dpi).

Plants were analysed for the presence of TuMV CP by ELISA and IC-RT-PCR. A perfect correlation was found between symptom induction and positive analytical results by both techniques (results not shown).

Cloning and expression of reporter genes

The reporter genes, *uidA* and *gfp* were inserted in vec01, generating recombinant plasmids vec01-GUS and vec01-GFP (Fig. 1). A cloning strategy was designed to generate the following array (a-f) in the NIb-foreign gene-CP region: (a)- an unchanged NIb-protein coding sequence; (b)- *Ngo*MIV restriction site; (c)- foreign gene without stop codon; (d)- a nucleotide sequence encoding the NIa protease recognition sequence (ACVYHQ), with third-base codon changes to minimize recombination risks; (e)- *Ngo*MIV restriction site; and (f)- CP coding sequence. To obtain this array, *Ngo*MIV restriction sites were added to the PCR primers used to amplify the heterologous gene, the stop codon was eliminated, and the nucleotide sequence encoding the NIa protease recognition sequence was added in the 3' terminal sequence. Thus, NIa protease recognition sequences are present on both sides of the reporter protein, which should be consequently processed out of the polyprotein by the viral proteinase.

Recombinant plasmids were directly inoculated onto *Arabidopsis* plants resulting in a high percentage of infected plants (60-80% in the different independent experiments). The overall infection rate and symptom induction were indistinguishable from results obtained in parallel infections with vec01, and ELISA results again correlated perfectly with symptoms in the diseased plants. Molecular analysis by IC-RT-PCR of systemically infected tissue showed that the amplified fragments were of the size expected for the constructs carrying the foreign genes in most cases for *uidA* and in all cases for *gfp*, indicating the stability of the foreign gene within the replicating recombinant virus (Fig. 2).

Activity of the proteins encoded by both reporter genes, *uidA* and *gfp*, was detected in the corresponding infected plants. Autofluorescence of the GFP protein was easily detected by epifluorescence microscopy in leaves infected with the chimeric virus (not shown). For GUS activity, histochemical detection resulted in blue-stained vec01-GUS infected leaves, whereas control leaves (vec01 infected leaves) got whitish (not shown).

The heterologous proteins should be processed out of the viral polyprotein upon translation, because they are flanked by two NIa-Pro recognition sites. The integrity of the foreign protein was analyzed by western blot in the case of GUS (Fig. 3). Plants infected with vec01-GUS gave a band reacting with GUS-specific antibodies of the approximate size expected for the

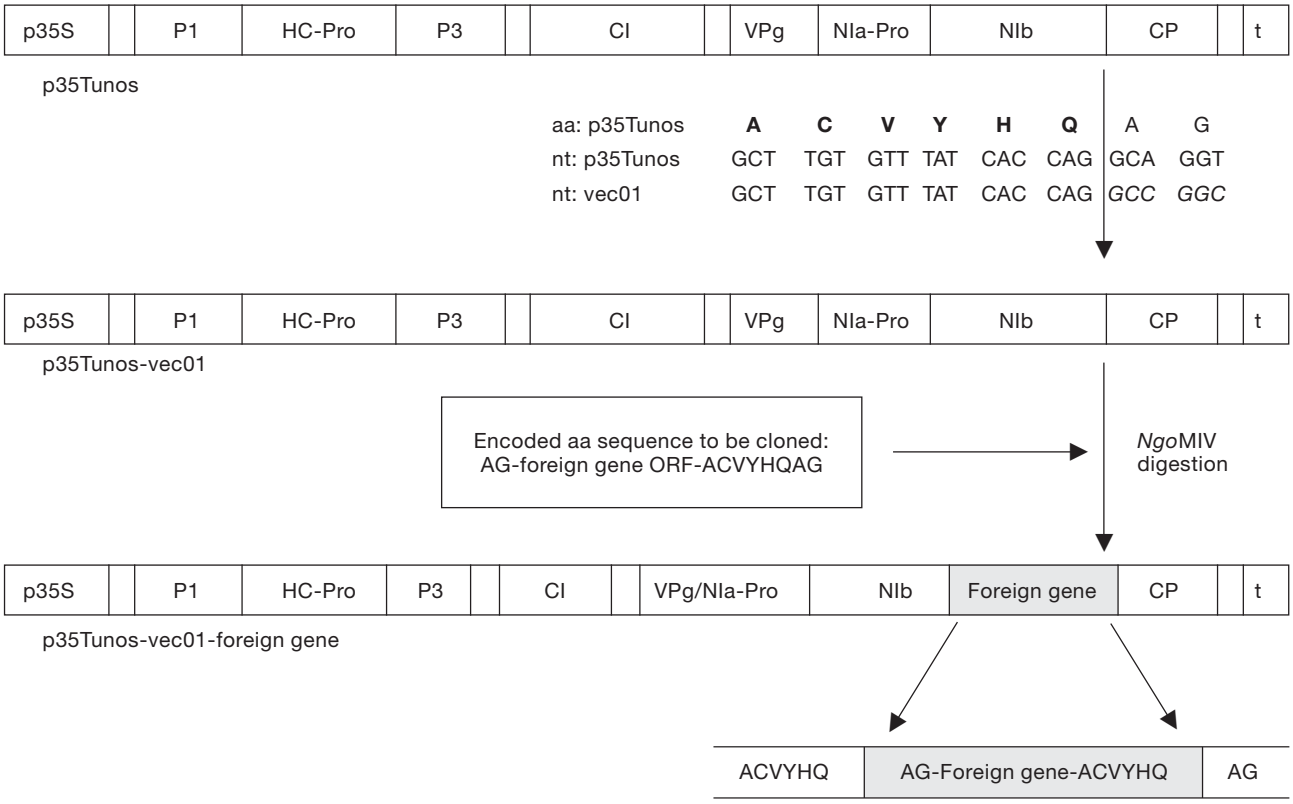

Figure 1. Construction of p35Tunos-vec01 (vec01) and cloning process of a foreign gene. Construction details in the text. The insertion site for foreign genes in the TuMV genomic map (between NIb and CP), and the corresponding sequences (amino acid and nucleotide) at this site are shown. The amino acid sequence recognized by the NIa protease is in bold. The newly created *Ngo*MIV recognition sequence (*GCCGGC*) is in italics.

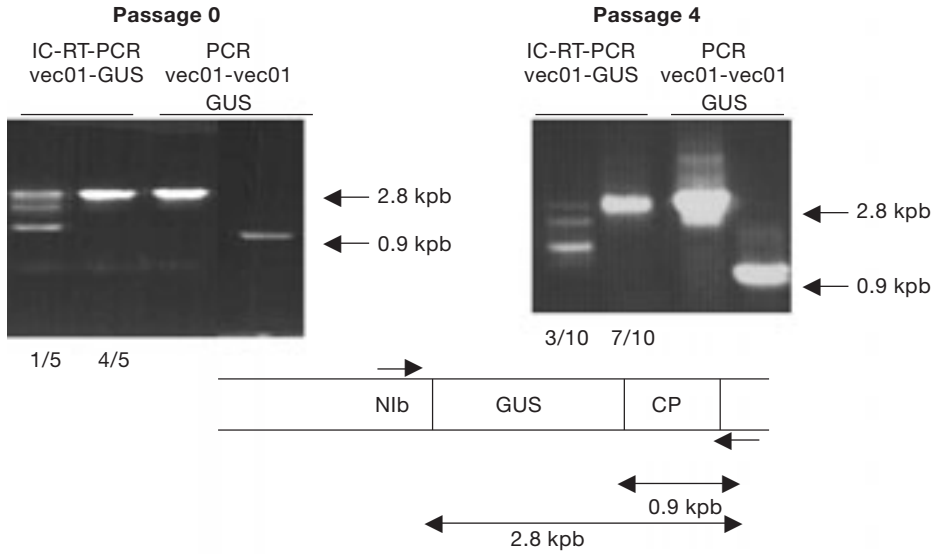

Figure 2. Integrity of the *uidA* gene (GUS) in infected *Arabidopsis thaliana* plants. IC-RT-PCR was performed on extracts of plants inoculated with p35Tunos-vec01-GUS (vec01-GUS) DNA (passage 0), or at passage 4 after a serial passage using crude sap from plants of the previous passage as inoculum. A scheme showing the positions of the PCR primers and the amplicons is shown below the agarose gels. Arrows mark the position in the gels of the expected amplicons. The last two lanes in each gel show the PCR amplification products using plasmid DNA as templates. Fractions below lanes represent number of plants rendering a particular pattern in each passage over the total analysed in the experiment.

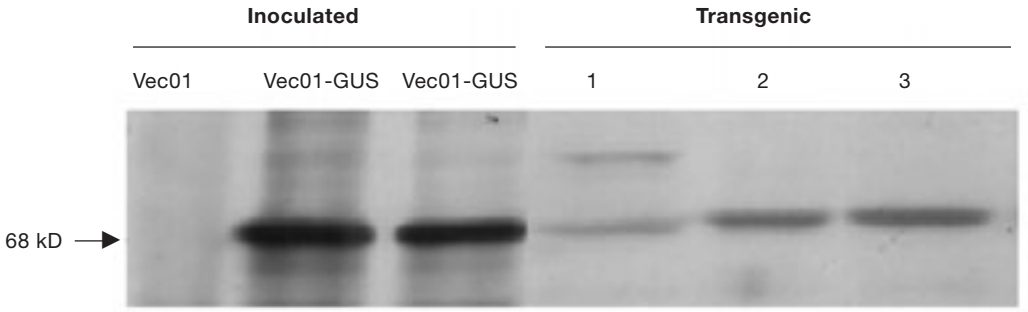

Figure 3. Western blot analysis of GUS in *Arabidopsis thaliana* protein extracts. Total soluble protein was prepared from plants inoculated with p35Tunos-vec01-GUS (vec01-GUS) or from GUS transgenic plants. Numbering of transgenic lines 1-3 as in Table 1. Samples contained the following amounts of total soluble protein: vec01-GUS = 4 µg, vec01 and GUS transgenic plants (1-3) = 40 µg. The arrow marks the position of GUS in the gel obtained by parallel running of commercial GUS, and confirmed with molecular weight markers (not shown). Vec01 infected plants were used as negative control.

free protein, which was absent in vec01-infected plants. No proteins of lower or higher mobility were visible in the membranes, thus discarding either inefficient protein processing or degradation of the GUS protein.

For a relative quantitative assessment of the efficiency of the vec01 expression system in the production of free protein, the intensity of the GUS band was compared in extracts of Arabidopsis plants infected with vec01-GUS, with the bands detected in equivalent extracts of different transgenic Arabidopsis plants expressing GUS under the 35S promoter. Five different transgenic lines were used for comparison to take into account the widely reported phenomenon of different expression levels of the same genetic constructs in similar, but independent, transgenic lines due to positional effects or other reasons (Bhat and Srinivasan, 2002). The amount of GUS in vec01-GUS infected plants was significantly higher than in any of the transgenic lines tested, ranging approximately 15-50 fold higher, as estimated by image analysis of the blots (results not shown). GUS activity was also subjected to relative quantitative assessment in order to verify that the foreign protein activity was active in similar proportions in both expression systems. For this purpose, quantitative GUS activity assays were performed with a colorimetric GUS substrate. Results (Table 2, Fig. 4) were in agreement to band image analysis in the western. Again, large variation was found between five transgenic lines, and GUS activity in the vec01-GUS infected plants was in the same approximate 15-50 fold higher range, indicating that the amount of active GUS produced was similar in both systems.

Plant viral vectors have been widely used to induce silencing of both transgenes and endogenous plant genes, in a process termed virus-induced gene silencing or VIGS (Burch-Smith *et al.*, 2004). Potyvirus-derived

Table 2. β-glucuronidase activity (in nmol product μg^{-1} protein) in extracts of *Arabidopsis thaliana* plants inoculated with p35Tunos-vec01-GUS or GUS-transgenic plants

	Time (min)						
	0	**5**	**10**	**25**	**45**	**1,020**	**1,440**
Buffer	9.2 ± 0.3	8.7 ± 0.5	9.1 ± 0.4	10.3 ± 0.4	8.4 ± 0.6	9.4 ± 1.1	10.1 ± 0.8
p35Tunos-Vec01	9.1 ± 0.4	9.1 ± 0.8	9.3 ± 0.9	10.7 ± 1.3	11.4 ± 0.4	8.8 ± 0.8	9.1 ± 0.4
p35Tunos-Vec01-GUS	31.4 ± 12.1	28.2 ± 5.1	42.1 ± 3.6	44.1 ± 5.7	193.5 ± 15.6	350.0* ± 0.0	350.0* ± 0.0
GUS-Transgenic 1	8.2 ± 0.8	7.9 ± 1.3	8.3 ± 0.7	9.7 ± 0.3	10.5 ± 0.4	15.3 ± 1.1	17.1 ± 2.2
GUS-Transgenic 2	9.1 ± 0.6	11.8 ± 0.5	14.2 ± 0.6	16.5 ± 0.6	17.5 ± 0.8	30.6 ± 1.6	45.3 ± 6.5
GUS-Transgenic 3	8.8 ± 0.8	8.3 ± 0.6	7.8 ± 0.2	11.1 ± 1.3	10.2 ± 0.8	41.4 ± 0.8	55.6 ± 0.5
GUS-Transgenic 4	10.5 ± 0.8	6.9 ± 4.2	7.8 ± 0.5	9.7 ± 0.6	8.7 ± 0.8	19.9 ± 1.8	15.7 ± 3.0
GUS-Transgenic 5	8.7 ± 0.5	10.4 ± 0.6	13.5 ± 0.7	14.4 ± 0.6	19.8 ± 0.6	31.4 ± 3.0	52.8 ± 3.0

This table provides the results obtained in one of three similar assays, differing slightly in the times allowed for reaction development. Values with their standard deviation are the mean of two parallel readings from the extract of one plant. GUS-transgenic (1-5) represent five different transgenic lines. * Values estimated directly from the readings of the spectrophotometer. Actual values are likely larger, since the curve has already reached saturation.

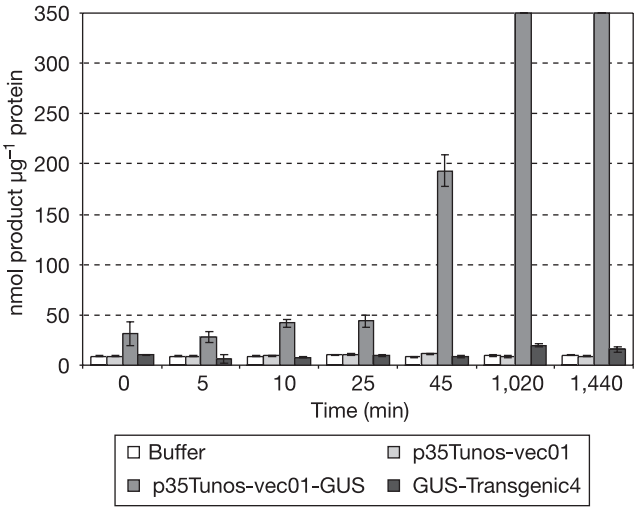

Figure 4. β-glucuronidase activity in extracts of *Arabidopsis thaliana* plants inoculated with p35Tunos-vec0-GUS or GUS-transgenic plants (from a particular transgenic line, taken as an example). Assays were done in duplicate for each sample. The bars indicate standard deviations. Details of the assay in the text.

vectors are not good potential candidates to induce VIGS, since they encode HC-Pro, one of the most potent silencing suppressors (Qu and Morris, 2005). However, even a partial level of silencing could be of biotechnological interest for external control of the modulation of gene expression. Thus, the ability of vec01 to induce VIGS of both transgenes (uid A, GUS) and endogenous genes (phytoene desaturase, PDS) was tested. The GUS-expressing transgenic lines referred to previously, were used to test transgene silencing induced by vec01-GUS. Infection by vec01-GUS was confirmed in transgenic inoculated plants by ELISA and IC-RT-PCR. The putative effect of vec01-GUS on the expression of the transgenic GUS gene was evaluated by histochemical analysis, but no obvious staining differences could be found between inoculated and non-inoculated plants (not shown). Similar negative results were obtained for the inoculations for possible silencing of the endogenous PDS gene. No whitish region was detected in plants infected with the vec01-PDS-derived constructs.

Stability of foreign gene upon successive passages on plants

Genetic stability of the GUS insert within the TuMV-GUS genome, after plant infection, was tested by IC-RT-PCR in experiments of plant to plant serial passages, using virus originally recovered from a plant infected with DNA (passage 0). The inocula used in each passage was derived exclusively from plants that had not shown any deletion in the previous passage. Four serial passages were performed by mechanical inoculation. In all cases, 100% infection was obtained and typical symptoms were observed in all plants throughout the passages (12 plants per passage). For each passage, the presence and stability of the recombinant virus were monitored. The results showed that the presence of recombinant virus was maintained throughout the passages (7/10, passage 4). In the fourth passage, 30% of plants rendered fragments smaller than expected, indicating partial deletions (Fig. 2).

The stability of the GFP insert was tested in a similar way. Fifteen to 20% of plants showed no fluorescence and fragments smaller than expected in IC-RT-PCR tests (not shown).

Biosafety issues

Contact and seed transmission

Assays were performed to check transmissibility of vec01-NAT1-GFP by contact and seed. Fifty *Arabidopsis* plants were grown in a 15 cm diameter pot. Twenty-eight days after sowing, five of the plants (at different places of the pot's surface) were inoculated with vec01-NAT1-GFP. Forty dpi, plants were tested for fluorescence and presence of potyvirus by ELISA. Only inoculated plants were fluorescent and gave positive results in the ELISA test, indicating that no contact transmission had taken place from the infected plants. Seeds collected from four infected plants (one plant did not produce seeds) were surface-sterilized and sown onto Petri plates containing MS (Murashige and Skoog medium). Eighty individual seedlings (20 seedlings from each inoculated plant) were analyzed and gave negative results when assayed for the presence of the potyvirus by ELISA, thus showing that the recombinant virus was not seed-transmitted.

Aphid transmission

A NAT TuMV mutant which had a DAE motif in its CP, instead of the natural DAG was able to infect Arabidopsis plants. Time course of infections and type of symptoms induced were indistinguishable from vec01. The stability of the mutated sequence (Gly to

Glu) in the progeny virus was verified by nucleotide sequencing after 7 passages.

The effect of the DAE mutation on TuMV aphid transmission was tested using different species of aphids and Arabidopsis plants. The results are shown in Table 3. While *Myzus persicae* transmitted vec01 with an efficiency close to 100%, and other aphid species were also able to transmit it (although less efficiently), none of the assayed aphids were able to transmit vec01-NAT1. In a derived experiment using now only *M. persicae* as the most efficient TuMV vector, this derivative remained NAT after seven serial passages by mechanical inoculation. After the seven passages, attempts were made to aphid-transmit the progeny virus with no success, indicating the stability of this mutation. In less rigorously conducted subsequent tests, involving thousands of plants over several years, no escapes from vec01-NAT1 infected plants have been detected, even though several episodes of aphid attacks have taken place in our greenhouses and growth chambers.

Expression from plants other than Arabidopsis

A. thaliana is of course an excellent host for laboratory studies, but its biotechnological potential to be used as a biofactory is limited, due to its small size. A biotechnological tool like vec01-NAT1 will have to be used on other plant species for production purposes. Testing and evaluating its potential in this context is out of the scope of this paper. However, as a preliminary approach to test the possibility of the vector in this regard, we have inoculated vec01-NAT1-GUS on Indian mustard and oilseed rape. Around 20 plants of each of these species and cultivars were inoculated and evaluated. TuMV symptoms developed in all of them after about 15 dpi in the greenhouse, and the infection was confirmed by ELISA. All of them stained blue in the histochemical assay, confirming the potential of this system to express foreign proteins in large plants.

Table 3. Aphid transmission of wild type (p35Tunos-vec01) and mutant TuMV virus (p35Tunos-vec01-NAT1)

	Aphid (species)		
	M. persicae	***B. brassicae***	***A. fabae***
	No. of infected plants/ No. of inoculated plants		
Control (without virus)	0/8	0/8	0/8
p35Tunos-Vec01	24/24	19/24	3/24
p35Tunos-Vec01-NAT1	0/24	0/24	0/24

Discussion

The present paper describes the development of a biosafe viral vector derived from an infectious TuMV clone (p35Tunos), previously reported (Sánchez *et al.*, 1998). Its potential for the systemic expression of high levels of foreign proteins in *A. thaliana* plants, and its lack of transmissibility by seeds, plant contact, or aphids, is shown.

Potyviral-based vectors expressing full-length individual proteins have been developed previously (Dolja *et al.*, 1992; Guo *et al.*, 1998; Choi *et al.*, 2000; German-Retana *et al.*, 2000; Masuta *et al.*, 2000; Arazi *et al.*, 2001; Fernández-Fernández *et al.*, 2001; Beauchemin *et al.*, 2005). However, the utility of most constructs designed for the overproduction of proteins of interest could be limited by their genetic instability due to RNA recombination events that rapidly eliminate foreign sequences (Dolja *et al.*, 1993; Guo *et al.*, 1998; Choi *et al.*, 2000; German-Retana *et al.*, 2000). The stability of chimeras based on wheat streak virus, which uses the NIb-CP junction as the insertion site appears to depend on the gene inserted and the host (Choi *et al.*, 2000). The NIb-CP junction has also been used as the cloning site in order to obtain stable chimeras (Fernández-Fernández *et al.*, 2001). Some authors (Arazi *et al.*, 2001) claim that a foreign gene inserted between the NIb and CP genes is relatively less prone to recombination events than genes inserted between the P1 and HC-Pro.

The vector developed, vec01, exploits the NIb-CP junction as the insertion site and its viability has been analyzed by the development of two different chimeras. Our results show that chimeric virus harboring reporter genes *uidA* and *gfp* are viable. The incorporation of the foreign genes in the virus genome does not affect the ability of TuMV to infect Arabidopsis plants. Both chimeras were infectious in Arabidopsis, with characteristics (infectivity and symptomatology) similar to the wild-type virus. With regard to the stability of the foreign genes, our results support the results described previously for p35Tunos-derived vectors (Beauchemin *et al.*, 2005), *gfp* gene being more stable than the *uidA*. The sequence encoding GFP was shown to be stable when cloned in vec01-GFP.This construct has already shown its usefulness in our own work (Lunello *et al.*,

2007). In the case of the sequence encoding GUS, it was also quite stable, although deleted forms appeared in some plants (Fig. 2). In other potyviral-based vectors it was observed that *uidA* suffered spontaneous deletions after prolonged propagation or subsequent plant passage (Dolja *et al.*, 1993; Guo *et al.*, 1998; Choi *et al.*, 2000; German-Retana *et al.*, 2000). Although the instability of *uidA* may be caused by the length of the foreign sequence, some results indicate that other factors affect insert stability (Choi *et al.*, 2000; Arazi *et al.*, 2001; Beauchemin *et al.*, 2005).

The use of plant virus-based vectors instead of virus-free plant transformation for the production of foreign proteins presents several advantages (Scholthof *et al.*, 1996; Arntzen, 1997), although both systems are of great value under different circumstances. Plant virus-based vectors usually allow the quicker production of higher and more reproducible amounts of the protein of interest (Pogue *et al*., 2002). Our results show a relative quantitative assessment of the efficiency of the vec01 expression system in the production of free protein. The higher amount of GUS in vec01-GUS infected plants in relation to GUS transgenic plants revealed the potency of our vector, demonstrating that, at least in the case of GUS protein, the production is significantly higher than in any of the transgenic lines tested (15-50 fold higher range). The quantitative GUS activity assays showed similar results. In both experiments, large variation was found among transgenic lines, probably due to positional effects or other reasons (Bhat and Srinivasan, 2002). Moreover, silencing, which is a potential problem in the nuclear transgenic approach, was never detected.

Environmental safety and risk assessment of recombinant virus-vectors are crucial for their future use. When using viral vectors to express foreign proteins in plants, an important environmental issue is the contention of the expressed genes only in the inoculated plants. Since no plant genome modification is involved in this approach, the only concern with respect to gene spread is virus transmission. So, the three possible major ways of potyvirus transmission were tested for the recombinant viruses. The biosafety level of the vector was directly addressed experimentally. Since TuMV is an aphid-transmitted virus a mutant was created in the CP aphid transmissibility motif, which rendered it non-aphid transmissible. This trait has shown its stability over the years. The possibility of seed or contact transmission was also tested, even though no TuMV transmission has been reported by these means. The results confirmed the vector biosafety also from these standpoints. We think that this high level of environmental biosafety is an important added value of vec01-NAT1.

Plants have several important advantages as large-scale bioreactors for proteins. Our preliminary results in large *Brassica* plants, showing GUS activity upon inoculation of the GUS-expressing vector are quite promising for the further enforcement of TuMV-derived vectors for commercial applications.

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