

PROTOCOL EXCHANGE | COMMUNITY CONTRIBUTED Expression and accumulation of heterologous molecules in the protein storage vacuoles of soybean seeds

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Abstract

The production of recombinant molecules in seeds plant has presented interesting results related to the synthesis, secretion, compartmentalisation, post-translational modifications and purification, scalability, stable expression, the absence of contaminants and human pathogens, and knowledge of the growth, harvest, storage and processing practices. In this context, the soybean plant has emerged as an option to express recombinant proteins. We obtained several transgenic soybean plant lines, expressing different types of recombinant molecules, under the control of the tissue-specific soybean seed storage β -conglycinin promoter. This promoter is the most abundant protein in soybean seeds, directing the expression of the molecules that accumulate in the seed storage tissue. Cotyledonary immunocytochemical analysis of the seeds demonstrated that the targeted proteins effectively drove polypeptide accumulation into the protein storage vacuoles (PSVs). Here, we provide a detailed protocol to reproduce the results described above and properly direct the expression of recombinant protein into the PSVs.

Subject terms: [Cell biology](#) [Genetic modification](#) [Protein analysis](#)

[Immunological techniques](#) [Plant biology](#)

Keywords: [transgenic soybean](#) [recombinant protein](#) [seed storage promoter](#)

Introduction

Plants have emerged as a viable alternative to microbial fermentation and mammalian cell culture for the industrial production of biopharmaceuticals. They offer scalability and low production costs and are free of human pathogens and toxins (Fiedler et al., 1997; Evangelista et al., 1998; Hood et al., 2002; Ma et al., 2003; Stoger et al., 2005; Verma et al., 2008; Orzáez et al., 2009; Woodard et al., 2009; Dreesen et al., 2010). In addition, plant enzymatic machinery can provide a method of biosynthesis, folding and assembly of complex proteins and promote proper post-translational

modifications, resulting in high quality recombinant products (Ma et al., 2003).

The genetic transformation of a broad range of vegetable species and the efficient production of more than 100 different recombinant proteins using plant systems has confirmed the potential to utilise transgenic plants as efficient bioreactors of several relevant biomolecules, such as antigens, vaccines, antibodies and growth factors (Perrin et al., 2000; Ma et al., 2003; Stoger et al., 2005).

As a potential, low-cost platform for recombinant protein biosynthesis, soybeans [*Glycine max* L. (Merrill)] might constitute one of the least expensive systems for the large-scale production of biopharmaceuticals. Their high biomass capacity, short life cycle, low allogamy frequency and photoperiod sensitivity are intrinsic and unique characteristics (Abud et al., 2003; 2007; Cunha et al., 2010b). Under greenhouse conditions, utilising a photoperiod of 19 h of light can increase soybean seed production 10-fold when compared with seed production under field conditions as a consequence of an extended vegetative growth and flowering delay (Cunha et al., 2010b).

Soybean seeds are a rich source of protein, which can reach up to 40% of the dry weight of these organs (Cantoral et al., 1995; Moravec et al., 2007; Boothe et al., 2010), and, unlike leaves, they can be easily transported and stored for extended periods without significant protein degradation and do not require special storage conditions (Leite et al., 2000; Stoger et al., 2005; Moravec et al., 2007; Lau & Sun, 2009; Cunha et al., 2010a).

Because seeds generally express a wide range of genes related to abundant endogenous storage proteins, it is advisable to use a seed-specific promoter with high specificity and strong transcriptional activity to maximise transgene expression and reach economic scalability for the production of recombinant proteins (Stoger et al., 2000, 2005). Monocot promoters, such as the commonly used rice glutelin GluA-2 (Gt-1), rice globulin, barley D hordein, and maize zein promoters, are usually employed to drive the expression of genes related to storage proteins that are localised in the endosperm, the main storage tissue of monocot seeds. For this reason, expression cassettes using monocot promoters are specifically optimised for gene expression in monocot seeds, resulting in expression levels reaching up to 15% of the total seed protein in transgenic seeds (Takagi et al., 2005, Yang et al., 2006 and Yang et al., 2007).

Among the dicot promoters, legume promoters, such as the seed-specific promoters from the *Phaseolus vulgaris* phaseolin and arcelin-5 genes, soybean lectin, glycinin, the β -conglycinin α' subunit, pea legumin (legA) and the bean unknown seed protein (USP), have emerged as a suitable choice for recombinant protein production in dicot seeds (Boothe et al., 2010). In a study that examined the expression of a functional, active single chain antibody in *Arabidopsis* seeds, impressive results were achieved when both *Phaseolus* promoters were used, accumulating up to 36 times more immunoglobulin than was observed in transgenic *Arabidopsis* seeds that were transformed with the CaMV 35S promoter (De Jaeger et al., 2002).

Following mRNA translation, the accumulation of newly synthesised proteins in seeds is particularly sensitive to subcellular targeting and, for certain proteins, it can have a major effect on

the final yields of the recombinant product by avoiding unintended proteolysis and achieving accumulation levels suitable for economic production (Ma et al., 2003; Stoger et al., 2005; Streatfield, 2007; Semenyuk et al., 2010; Boothe et al., 2010). For example, protein targeting to the secretory pathway components can lead to a considerable increase in the final protein yield, which can be as much as 10-fold higher than the accumulation in the cytosol alone (Conrad & Fiedler, 1998; Avesani et al., 2003).

The most utilised targeting approaches are to retrieve newly synthesised proteins in the endoplasmic reticulum (ER) by adding N- or C-terminal signal peptides to the nascent proteins or to direct them to other subcellular organelles, such as the mitochondria, the chloroplasts, and the many components of the secretory pathway, especially the main sites for protein accumulation in seeds, namely, the protein storage vacuoles (PSVs) (Stoger et al., 2000; Jiang & Sun, 2002; Stoger et al., 2005; Vitale & Pedrazzini, 2005; Boothe et al., 2010).

Plant vacuoles are the intracellular endpoints of the plant secretory pathway, the final destination of proteins bypassing the ER and travelling through the Golgi complex (Jolliffe et al., 2005). They can be divided into two main categories: (i) LVs (lytic vacuoles), which are acidic compartments that are rich in hydrolases and can be regarded as the equivalent of mammalian lysosomes, and (ii) PSVs, the ER-derived cisternae, which are found in the storage organs, mainly seeds, and are the main specialised storage sites of large amounts of protein that will later be used as a source of aminated compounds during seed germination (Yoo & Chrispeels, 1980; Vitale & Hinz, 2005; Jolliffe et al., 2005). Cotyledonary PSVs constitute an excellent subcellular target for the long-term storage of recombinant proteins as their lumen are characterised by a non-acidic environment with low concentrations of amino peptidases, thus minimising protein degradation (Zheng et al., 1992; Muntz, 1998; Jolliffe et al., 2005; Takaiwa et al., 2007; Cunha et al., 2010c).

PSVs also contain large amounts of the major seed storage globulins 7S and 11S and toxic proteins, such as lectins, protease inhibitors and ribosome inactivating proteins, which probably evolved to protect the seeds from predators (Herman & Larkins 1999; Vitale & Hinz 2005).

The mechanism related to the transport of recombinant proteins to the PSVs has not been well studied compared to that of the LVs (Nishawa et al., 2003). However, the addition of N-terminal signal peptides without any additional signal leads the protein of interest to be targeted to the ER, followed by further delivery along the secretory pathway and to the PSVs and apoplast (Vitale & Pedrazzini, 2005).

Another model for protein targeting to plant vacuoles is based on recent advances in understanding the role of a restricted group of cargo proteins, named vacuolar sorting signals (VSS), that can mediate protein targeting to the PSVs (Jolliffe et al., 2005). VSSs of barley lectin, common bean phaseolin, and the soybean β -conglycinin α' subunit have already been identified as potential mediators for recombinant protein targeting to the PSV (Bednarek & Raikhel, 1991; Frigerio et al., 1998; Nishizawa et al., 2003; Robinson et al., 2005). Although they are sophisticated targeting tools, few studies have reported the interference of the high flow of seed

storage proteins to the PSVs in the correct targeting mediated by VSSs, which resulted in the unintended accumulation of proteins in non-targeted organelles (Lau & Sun, 2009).

The beta-conglycinins (7S) and the glycinins (11S) are the major storage proteins of the soybean, which can account for up to 70% of the total seed protein (Chen et al., 1986; 1988; Nishizawa et al., 2003). Beta-conglycinin is a multimeric protein and consists of three subunits: alpha prime (α'), alpha and beta (Derbyshire et al., 1976; Chen et al., 1986; Doyle et al., 1986). The alpha and α' subunits are synthesised as pre-proteins and the beta subunit as a pre-protein (Nishizawa et al., 2003). The expression of these subunit genes is spatially and temporally regulated, coinciding with the development of the seed and resulting in high accumulation levels of the related proteins during seed maturation.

In this work, we describe results showing a highly efficient, reproducible system for recombinant protein expression in seeds that targets the PSVs, where several molecules with different molecular weights and structures, such as the human Growth Hormone (hGH) (Cunha et al., 2010b), the human coagulation factor IX (hFIX) (Cunha et al., 2010c), two single chain fragment variable (scFVDIR83D4 and anti-CD18), and a potent microbicide against HIV, Cyanovirin-N (CVN), were stably accumulated in the PSVs from transgenic soybean seeds using the α' subunit regulatory sequences of the soybean beta-conglycinin. We successfully utilised two seed-specific expression vectors of soybean construction, containing the following:

- i) the promoter and signal peptide from the α' subunit of beta-conglycinin (with step-by-step directions for how to construct it) (Fig. 1A);
- ii) the promoter from the soybean α' subunit of beta-conglycinin, along with the monocot signal peptide α -Coixin from *Coix lacrima-jobi* L. (responsible for directing the polypeptides to the PSVs of *Coix lacrima-jobi* L. seeds) (Ottoboni et al., 1993) (Fig. 1B).

Reagents

- DNeasy Plant Mini Kit (QIAGEN, cat. no. 69106).
- dNTPs (Invitrogen, cat. no. 10297018).
- Restriction endonucleases.
- A set of primers for sequence amplification (Table 2).
- PCR cloning vector (Promega pGEM-T Easy Vector Systems I, cat. no. A1360).
- T4 DNA ligase (Invitrogen, cat. no. 15224017).
- Platinum Taq DNA Polymerase High Fidelity (Invitrogen, cat. no. 11304-011)
- QIAquick Gel Extraction Kit (QIAGEN, cat. no. 28704)
- Absolute ethanol (Sigma-Aldrich Chemical Co. Ltd., cat. no. 459844)
- Isopropanol (2-propanol; Sigma-Aldrich Chemical Co. Ltd., cat. no. 67630)
- Plasmid DNA isolation Kit (QIAGEN Plasmids Kits, cat. no. 12162)
- Plant material: young, green, healthy soybean leaves from mature seeds from BR-16 cultivar

were used in the protocol (supplied by Embrapa Soybean, Rodovia Carlos João Strass, Distrito de Warta, C.P. 231, CEP 86001-970, Londrina, PR, Brazil).

- Sterile distilled deionised water.
- Tris, ultra pure (Invitrogen, cat. no. 15504-020).
- Paraformaldehyde ($C_7H_{14}O$; J.T. Baker, cat. no. 30525894).
- Glutaraldehyde (25% vol/vol) in Milli-Q water ($C_5H_8O_2$; Electron Microscopy Sciences, cat. no. 16530).
- Sodium Cacodylate Trihydrate ($C_2H_6AsNaO_2 \cdot 3H_2O$; Electron Microscopy Sciences, cat. no. 124652).
- LR White embedding resin and Benzoyl peroxide accelerator (Electron Microscopy Sciences, cat. no. 14381).
- Gold conjugate protein A 5 nm (SPI Supplies, cat. no. 04283-AB).
- Sodium phosphate, dibasic, anhydrous (Na_2HPO_4 , Sigma-Aldrich Chemical Co. Ltd., cat. no. 7558794).
- Potassium phosphate monobasic (KH_2PO_4 , Sigma-Aldrich Chemical Co. Ltd., cat. no. 7778770).
- Sodium chloride (NaCl, Sigma-Aldrich Chemical Co. Ltd., cat. no. 7647145).
- Potassium chloride (KCl, Sigma-Aldrich Chemical Co. Ltd., cat. no. 7447407).
- Bovine serum albumin (BSA, Sigma-Aldrich Chemical Co. Ltd., cat. no. 9048-46-8).

Equipment

- PTC-100 Peltier thermal cycler (Bio-Rad, cat. no. PTC-1196).
- Thin wall 0.2 ml PCR tubes (Midwest Scientific, cat. no. AVTW2).
- Refrigerated microcentrifuge (Eppendorf 5415R, Fisher, cat. no. 05-401-05).
- 1.5-ml Polypropylene microcentrifuge tubes (Fisher Scientific Ltd., cat. no. 05-669-32).
- Wide-mouth bottles, 250 ml (Fisher Scientific Ltd., cat. no. 02911916).
- Filters, 0.22 μm (Fisher Scientific Ltd., cat. no. MPGL02GH2).
- NanoDrop 3300 (Thermo Scientific).
- Ultramicrotome (model EM UCT, Leica Microsystems, Vienna, Austria).
- Transmission electron microscope (model DSM 962, Carl Zeiss, Oberkochen, Germany).

Procedure

A. Plant material

Genomic DNA of the variety BR-16 (Embrapa) was isolated from young leaves with the DNeasy Plant Kit (QIAGEN), following the manufacturer's instructions, and quantified with a NanoDrop 3300 (Thermo Scientific). The concentration was confirmed by analysis with a 1% agarose gel stained with ethidium bromide and adjusted to 10 ng/ μl with distilled water. The DNA was kept at $-20^\circ C$ until it was analysed using polymerase chain reaction (PCR).

■ PAUSE POINT Genomic DNA and PCR products can be stored at $-20^\circ C$ for several days

without a loss of quality.

B. Cloning of the regulatory sequences

For amplification of the signal peptide and the promoter region of the alpha' subunit from the beta-conglycinin soybean protein, the primers CongF and CongR were constructed. These primers were chosen based on the alpha' subunit promoter and signal peptide sequence available in GenBank (accession number: M13759). The primer sequences can be viewed in Table 1 and include sites for the restriction enzymes *Xho* I and *Hin_d* III. *The primers anneal to the start of the promoter region and to the end of the signal peptide, respectively, resulting in the amplification of a 1,148-bp fragment when using Platinum _Taq DNA Polymerase High Fidelity*, according to the manufacturer's (Invitrogen) instructions, and a PTC-100 Peltier thermal cycler (Tables 2 and 3). The PCR fragment was purified using a QIAquick Gel Extraction Kit (QIAGEN) and quantified using a NanoDrop. The final concentration was adjusted with nuclease free water to 5 ng/ul and utilised for cloning into the pGEM-T Easy cloning vector (Promega) (Table 4).

The vector pGEM-T Easy, containing the amplified fragment, was digested with the restriction enzymes *Xho* I and *_Hin_d* III, and the resulting fragment was cloned into the pBluescript KS⁺ (Stratagene), utilising the same sites (Table 4).

After generation of the intermediate vector, two other primers (named 35STF and 35STR) were designed to amplify a 228-bp fragment containing the CaMV 35S terminator (Table 1), utilising the pCAMBIA 2300 vector (<http://www.cambia.org/daisy/cambia/585.html>) as a template and creating the sites *Eco* RI and *Bam* HI. The PCR fragment was generated, purified from 1% agarose gel as described above, cloned into a pGEM-T Easy vector and digested by *Bam* HI and *Eco* RI. After purification, the insert was cloned into the previously obtained vector to generate the vector pbetaCong1.

The vector pbetaCong1 was utilised as a model to clone ten different genes that encoded various molecules (Table 5). We always utilised *Hin_d* III and *_Eco* RI sites to clone these genes (Fig. 2). Each construct was utilised at the concentration of 1 ug.ul⁻¹ in the soybean transformation experiments. All of the vectors obtained were completely sequenced by MacroGen Corp. (USA). We also tested the vector pbetaCong3 (Fig. 1B, kindly provided by Roger N. Beachy) to express four different genes in the PSVs of soybean seeds. The pbetaCong3 vector contains the promoter from the soybean alpha' subunit of beta-conglycinin and the CaMV35S poly A sequence. Below, we describe each construct in greater detail:

a) pbetaCong3hGH – The sequence that contains the human growth hormone and the coix signal peptide was digested from pRT-PGK-PShGH (Leite et al., 2000) with the restriction enzymes *Nco* I and *Bam* HI and cloned into the pbetaCong3 utilising the same sites.

b) pbetaCong3scFv – The sequence that contains the scFv and the coix signal peptide was digested from pRT-PGK-PSscFv (kindly provided by Adilson Leite, Unicamp), with the restriction enzymes *Nco* I and *Bam* HI and cloned into the pbetaCong3 vector utilising the same sites.

The fragment containing the alpha-coixin signal peptide and the 805-bp fragment *scfv* (Oppezzo et al., 2000) was cloned into the vector pbetaCong3, utilising the restriction enzymes *Nco* I and *Bam* HI, to generate the 4,826-bp vector pbetaCong3scFv (Fig. 1). The recombinant protein included a carboxy terminal 6xHis-tag. The plasmids pbetaCong3scFv and pAC 321 were utilised for soybean genetic co-transformation experiments in a 1:1 ratio.

c) pbetaCong3CD18 – We constructed primers to add the monocot signal peptide α -coixin from *Coix lacrima-jobi* L., which permitted the addition of *Nco* I and *Bam* HI sites before and at the terminus of each molecule of CD-18 (Table 6).

d) pbetaCong3FIX – We constructed primers to add the monocot signal peptide α -coixin from *Coix lacrima-jobi* L., which permitted the addition of *Nco* I and *Bam* HI sites before and at the terminus of FIX (Table 6).

The construction scheme is shown in Fig. 3.

C. Soybean transformation

The vector that was constructed was utilised to obtain transgenic soybean plants that expressed several genes of interest to molecular farming (Table 5) by bombardment of the somatic embryonic axes from mature soybean seeds cv. BR-16, utilising a particle bombardment procedure. The genetic transformation process, greenhouse plant cultivation and molecular analysis were conducted according to an article published in *Nature Protocols* (<http://www.nature.com/nprot/journal/v3/n3/full/nprot.2008.9.html>).

D. Immunocytochemical analysis

For immunolocalisation of the cited heterologous molecules mature transgenic and nontransgenic seeds were sliced (2 mm thick) and fixed for 4 h at 4 °C in a 0.05 M sodium cacodylate buffer solution containing 2% paraformaldehyde and 0.5% glutaraldehyde at pH 7.2. After fixation, the seed slices were washed three times in fixation buffer and dehydrated for 5 h in a series of ethanol solutions (30, 50, 70, 95 and 100%, 1 h each, under -20 °C and partial vacuum). Following ethanol desaturation, the samples were infiltrated with increased concentrations (30 to 100%) of ethanol used to dilute LR White resin (SPI Supplies, USA) in 3 h increments, followed by an 8 h incubation period in pure LR White resin. Inclusion was done by transferring the samples to 1.98% benzoyl peroxide in LR White resin and incubating at 4 °C under UV light for 72 h. Ultra-thin sections (50 nm thick) were collected in 400 mesh nickel nets. The nets were then incubated for 1 h at room temperature with 1X PBS-T (10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, 0.5% Tween 20 and 2% bovine serum albumin (BSA)) and for 2 h with rabbit polyclonal anti-specific molecule (1:500, 24 ng/ul) diluted in PBS-T. The samples were washed for 1 h in 1X PBS and incubated in gold conjugate protein A (SPI Supplies) in PBS solution (20 nM) for 2 h at room temperature. After being washed in PBS and dried for 24 h, the samples that were contrasted with 1% uranyl acetate in a 0.1 M PBS solution were observed under a Zeiss DSM 962 transmission

electron microscope (Carl Zeiss, Germany).

Anticipated Results

Seeds have the intrinsic capability for stable protein accumulation that makes them a suitable bioreactor platform for the manufacture of different recombinant molecules. Exploiting the capacity of soybean seeds regulatory sequences it is possible to manipulate and induce the synthesis of functional proteins. The results presented in this protocol allowed the introduction of genes for expressing different biomolecules of pharmaceutical or industrial interest. The construction of a seed-specific transformation vector should result in a fully functional plasmid to express different recombinant proteins in the soybean seeds protein storage vacuoles. Transgenic shoots harboring the seed-specific expression vector should regenerate under the selectable conditions described by Rech et al., 2008.

Analytical assays demonstrated that the recombinant proteins presented in total soluble protein extracts from transgenic soybean seeds are expected to present the correct molecular weights in western blot and be detected only in the seeds, being absent in roots, stem, flowers and leaves (Cunha et al., 2010b and 2010c). The subcellular compartmentalization obtained by the utilization of the promoter and the signal peptide from the α' portion of the β -conglycinin or the α -coixin cotyledonary vacuolar signal peptide from *Coix lacryma-jobi* (Poaceae), resulted in the stable accumulation of the recombinant proteins in the transgenic seeds, even when these seeds remained stored for six years under room temperature. Also, the sequencing of the protein fragments by trypsin digestion followed by nanoLC-MS assay can confirm the expected monoisotopic masses and positions of the portions covered by the spectrum assay, without the PSV targeting signal peptide detection (Cunha et al., 2010b and c).

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Figures

Figure 1: Figure 1

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Figure 1

A) Sequence of the vector pbetaCong1 that contains the alpha' subunit promoter and signal peptide from soybean beta-conglycinin and CaMV35S poly A. B) Sequence of the vector pbetaCong3 that contains the alpha' subunit promoter and the signal peptide α -coixin from *Coix lacrima-jobi* L and alpha' subunit beta-conglycinin poly A. The promoters are in black, the signal peptides are in red, and the terminators are in blue. Underlining denotes the sites included in the sequences to clone all the regulatory and coding sequences. CTCGAG, *Xho* I; AAGCTT, *Hin*_d III; GAATTC, *Eco* RI ;

GGATCC, *Bam* HI; CCATGG, *Nco* I.

Figure 2: Figure 2

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Figure 2

Schematic representations of the vectors used for soybean seed heterologous protein expression. These maps show the alpha' subunit promoter and signal peptide from soybean beta-conglycinin and CaMV35S poly A. The sites *Hin_d III* and *_Eco* RI indicate the site for cloning the gene of interest. A) PβCong1; B) pβCong3.

Figure 3: Figure 3

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Figure 3

Scheme for construction of the vector pβCong1 used for heterologous expression of biomolecules in PSVs from soybean seeds, based on the use of the promoter and signal peptide portion of the α' subunit from β-conglycinin from soybean.

Table 1: Table 1

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Table 1

Primer sequences used for cloning the regions of interest in the soybean beta-conglycinin and CaMV 35S poly A genes, as well as their accession numbers in GenBank (<http://www.ncbi.nlm.nih.gov>). Underlining denotes the restriction sites utilised for cloning.

Table 2: Table 2

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Table 2

Platinum Taq DNA polymerase high fidelity PCR reaction conditions to amplify the sequences utilised in this work.

Table 3: Table 3

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Table 3

PCR Platinum high-fidelity reaction conditions utilised to amplify the fragments.

Table 4: Table 4

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Table 4

Ligation conditions to clone fragments into the pGEM-T Easy vector (Promega).

Table 5: Table 5

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Table 5

Different heterologous molecules expressed into the PSVs of soybean seeds and their respective constructs.

Table 6: Table 6

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Table 6

Primer sequences utilised for cloning four different molecules and the signal peptide from alpha-coix. Underlining denotes the restriction sites utilised for cloning.

Figure 4: Figure 4

Download Figure 4

Figure 4

Evaluation by ultrastructural immunocytochemistry of the accumulation of different recombinant proteins in protein storage vacuoles (PSVs) in ultra-thin sections of soybean cotyledon of transgenic seeds. (A) Microbicide cyanovirin-N; (B) human Growth Hormone; (C) single chain fragment variable of the anti-Tn monoclonal antibody; (D) mutated cyanovirin-N; (E) human blood coagulation factor IX; (F) single chain fragment variable of an Anti-CD18 antibody, and (G) negative control.

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Competing financial interests

The authors declare no competing financial interest.

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