Transgenic Res (2011) 20:841–855
DOI 10.1007/s11248-010-9461-y

ORIGINAL PAPER

Accumulation of functional recombinant human coagulation factor IX in transgenic soybean seeds

Nicolau B. Cunha · André M. Murad · Gustavo L. Ramos · Andréia Q. Maranhão · Marcelo M. Brígido · Ana Cláudia G. Araújo · Cristiano Lacorte · Francisco J. L. Aragão · Dimas T. Covas · Aparecida M. Fontes · Gustavo H. M. F. Souza · Giovanni R. Vianna · Elíbio L. Rech

Received: 14 May 2010 / Accepted: 24 October 2010 / Published online: 11 November 2010

Abstract The seed-based production of recombinant proteins is an efficient strategy to achieve the accumulation, correct folding, and increased stability of these recombinant proteins. Among potential plant molecular farming systems, soybean [*Glycine max* (L.) Merrill] is a viable option for the production of recombinant proteins due to its high protein content, known regulatory sequences, efficient gene transfer protocols, and a scalable production system under greenhouse conditions. We report here the expression and stable accumulation of human coagulation factor IX (hFIX) in transgenic soybean seeds. A biolistic process was utilised to co-introduce a plasmid carrying the *hFIX* gene under the transcriptional control of the α' subunit of a β-conglycinin seed-specific promoter and an α-Coixin signal peptide in soybean embryonic axes from mature seeds. The 56-kDa hFIX protein was expressed in the transgenic seeds at levels of up to 0.23% (0.8 g kg^{-1} seed) of the total soluble seed protein as determined by an enzyme-linked immunosorbent assay (ELISA) and western blot. Ultrastructural immunocytochemistry assays indicated that the recombinant hFIX in seed cotyledonary cells was efficiently directed to protein storage vacuoles. Mass spectrometry characterisation confirmed the presence of the hFIX recombinant protein sequence. Protein extracts from transgenic seeds showed a blood-clotting activity of up to 1.4% of normal plasma. Our results demonstrate the correct processing and stable accumulation of functional hFIX in soybean seeds stored for 6 years under room temperature conditions (22 ± 2°C).

Keywords Biolistic-mediated transformation · Clotting assay · Human coagulation factor IX · Transgenic soybean seeds

N. B. Cunha · A. M. Murad · G. L. Ramos · A. C. G. Araújo · C. Lacorte · F. J. L. Aragão · G. R. Vianna · E. L. Rech (✉)
Embrapa Genetic Resources and Biotechnology, Parque Estação Biológica (PqEB), Av. W5 Norte, Brasília, DF 70770-917, Brazil
e-mail: rech@cenargen.embrapa.br

N. B. Cunha · G. L. Ramos · A. Q. Maranhão · M. M. Brígido
Department of Cellular Biology, University of Brasília, Brasília, DF 70910-900, Brazil

D. T. Covas · A. M. Fontes
Blood Center of Ribeirão Preto, Center of Cellular Therapy—FMRP/USP, Rua Tenente Catão Roxo 2501, Monte Alegre, Ribeirão Preto, SP 14051-140, Brazil

G. H. M. F. Souza
MS Applications Research and Development Laboratory, Waters Corporation, Alameda Tocantins, 125, 27th floor, West Side, Alphaville, São Paulo, SP 06455-020, Brazil

Introduction

Human coagulation factor IX (hFIX) is a vitamin K-dependent serine-protease glycoprotein that is

synthesised and stored in liver cells as a zymogen (Ragni et al. 2002). When secreted into the blood, hFIX plays a key role in the intrinsic blood coagulation pathway (Bristol et al. 1993). In addition to hFIX, two other serine-proteases, factors X (FX) and XI (FXI), are also directly involved in clot formation. Through a series of peptide bond cleavages and enzyme activation, FX and FXI cause the structural modification of pro-thrombin and fibrinogen, the two essential proteins involved in blood clotting (Osterud and Rapaport 1977; Furie and Furie 1988). Genetic mutations that lead to hFIX dysfunction or impaired synthesis are X-linked, recessive traits. These mutations cause Type B Christmas disease, the second most frequent haemophilia variant, affecting 1 in 30,000 men (Roth et al. 2001).

Current treatment of X-linked bleeding disorders is based on the intravenous infusion of recombinant or plasma-derived coagulation factors. Despite the efficacy of this approach, coagulation therapy based on purified hFIX from pooled plasma is closely associated with high costs and prion and virus contamination risks (Lisauskas et al. 2008). In contrast, the adjusted continuous infusion of licensed recombinant hFIX (BeneFix) has proved to be inherently free of human blood-borne pathogens and efficacious in the treatment of Type B Christmas disease over the last 10 years (Lambert et al. 2007).

Biologically active hFIX has been successfully expressed in bacteria (Kurachi and Davie 1982), milk of transgenic mice (*Mus musculus* L.) (Jallat et al. 1990), transgenic fibroblasts (Liu et al. 1993); cultured Chinese hamster (*Cricetulus griseus* G. Fischer) ovary cells (Kaufman et al. 1986), haematopoietic stem cells (Chen et al. 2006), transgenic hamster kidney cells (Wajih et al. 2005) and murine, dog (*Canis lupus familiaris* L.) and macaque (*Macaca mulatta* Zimmermann) muscle cells (Miao et al. 2001; Nathwani et al. 2002; Arruda et al. 2004). We recently demonstrated the functional secretion of hFIX in the milk of transgenic mice, reaching levels of up to 3% of the total soluble protein (TSP; Lisauskas et al. 2008). Despite the correct post-translational processing of the recombinant hFIX synthesised in bacteria and animal systems, the production of biopharmaceuticals in these platforms may incur significantly high overall costs that are due—at least partially—to a long production timescale, low scale-up capacity and expensive storage conditions (Kusnadi et al. 1997; Ma et al. 2003).

The utilisation of plants as bioreactors constitutes one of the most cost-effective systems for the large-scale production of recombinant proteins (Ma et al. 2003; Twyman et al. 2003). In addition, plant enzymatic machinery can provide complex protein folding and assembly and promote proper post-translational modifications, with minor but avoidable glycosylation changes, resulting in high-quality recombinant products (Ma et al. 2003; Howard 2005).

The functional activity of hFIX has already been demonstrated in transgenic tomato (*Solanum lycopersicum* L.) plants using an *Agrobacterium tumefaciens*-based system (Zhang et al. 2007). In this case, protein extracts from the tomato fruits were able to promote effective blood coagulation. However, the maximum level of the recombinant protein was only 0.016% of the total fruit protein content. Recently, a recombinant bioencapsulated human FIX fused to the cholera toxin β-subunit (producing up to 3.8% TSP in transplastomic tobacco plants) that was orally delivered to mice showed a high efficiency in preventing inhibitory antibodies associated with the pathogenic immune response and elimination of anaphylactic reactions (Verma et al. 2010).

Seed-based platforms are particularly useful for the production of recombinant proteins due to their high protein content and potential for long-term storage of functionally stable recombinant proteins (Hood et al. 2002; Ma et al. 2003; Stoger et al. 2005). The retrieval of proteins to the endoplasmic reticulum (ER) lumen or targeting them to a variety of subcellular organelles of the seeds, such as the vacuoles, are key factors in achieving satisfactory yields and the stable accumulation of recombinant biopharmaceuticals produced in transgenic plants (Drakakaki et al. 2006; Moravec et al. 2007; Streatfield 2007).

Soybean [*Glycine max* (L.) Merrill] seeds provide a rich source of protein, which can reach approximately 40% of the dry weight of these organs due to abundant seed-specific protein accumulation organelles, such as protein storage vacuoles (PSVs) (Takaiwa et al. 2007). The α' subunit of the β-conglycinin tissue-specific promoter, utilised in this work to accumulate hFIX in soybean seeds, controls the expression of the most abundant protein stored in embryonic axes and cotyledons of developing soybean seeds (Yamada et al. 2008). The activity of this promoter is modulated by complex binding interactions between enhancer

sequences rich in G + C, located approximately 270 nucleotides upstream of the transcription initiation site, with specific *trans*-acting factors previously activated during seed development (Chen et al. 1986; Doyle et al. 1986). The efficacy of this regulatory sequence has been demonstrated by the accumulation of a methionine-rich delta-zein in soybean PSV seeds (Kim and Krishnan 2004) and the seed-specific expression of novokinin, an efficient anti-hypertensive hybrid molecule (Yamada et al. 2008).

Here, we report and characterise the stable expression of the biologically active recombinant hFIX and its subcellular targeting to seed protein storage organelles, mediated by an expression cassette containing the regulatory sequences of the α′ subunit of β-conglycinin seed-specific promoter and an α-Coixin signal peptide.

Materials and methods

DNA amplification and cloning

The hFIX coding sequence (GenBank accession number: M11309) was amplified by PCR from a human liver cDNA library (λ TriplEx; Clontech, Mountain View, CA). *Nco*I and *Bam*HI restriction sites were included as 5′ extensions in the forward and reverse primers, respectively. To facilitate ER targeting, the forward primer also contained an extension corresponding to the α-Coixin signal peptide from *Coix lacryma jobi* (Ottoboni et al. 1993). The fragment was amplified by PCR using Precision Plus Taq polymerase (Invitrogen, Carlsbad, CA) and cloned into the *Nco*I and *Bam*HI restriction sites of the pβcong3 vector to obtain the pβcong3FIX vector (Fig. 1a). This pBlue Script-based vector (Promega, Madison, WI) contains the α′ subunit of the β-conglycinin promoter and the terminator from *G. max* (Chen et al. 1986; Doyle et al. 1986), resulting in an expression cassette of 2,737 bp. The acid-acetic-hydroxy-synthase gene (*ahas*), which confers tolerance to the herbicide Imazapyr, was cloned into the pAC321 plasmid under the control of the *ahas* promoter and a 3′ polyadenylation signal, generating an expression cassette of 4,435 bp, as described previously (Rech et al. 2008) (Fig. 1a). The pβcong3FIX and pAC321 plasmids were utilised for subsequent soybean genetic transformation experiments in a 1:1 ratio, as described below.

Soybean genetic transformation

The pβcong3FIX and pAC321 vectors were co-bombarded (ratio 1:1) into somatic embryonic axes from mature soybean seeds cv. BR16 utilising a particle bombardment procedure, as previously described (Rech et al. 2008).

DNA extraction and PCR analysis

Genomic DNA of regenerated (R_0) plants and their progeny was isolated from leaf disks utilising the cetyl trimethylammonium bromide (CTAB) procedure (Stewart and Via 1993). Each amplification reaction contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 160 μM of each dNTP, 200 nM of each hFIX specific primer (GATGGAGATCAGT GTGAGTC and TAACGATAGAGCCTCCACAG), 2U Taq polymerase (Invitrogen) and 20 ng of genomic DNA. PCR mixes were covered with mineral oil, and the DNA was pre-denatured at 95°C for 5 min and amplified in a DNA thermal cycler (MJ Peltier; MJ Research, Watertown, MA) for 35 cycles at 95°C for 1 min, 55°C for 1 min, and 73°C for 1 min, with a final cycle at 72°C for 7 min. The primers ahasp127 (ACTA GAGATTCCAGCGTCAC) and ahas 534c (GTGGCTA TACAGATACCTGG) were used under the same reaction conditions as above for detection of the *ahas* gene. Amplification reactions were resolved by electrophoresis on 1% agarose gels containing ethidium bromide and visualised under UV light.

Southern blot analysis

Genomic DNA of transgenic R_1 plants was isolated according to the Dellaporta et al. procedure (Dellaporta et al. 1983). A 15-μg aliquot of genomic DNA was digested with *Hin*dIII (150 U), separated on a 1% agarose gel and transferred to a nylon membrane (Hybond N^+; Amersham Biosciences, Arlington Heights, IL). The probe was a 495-bp internal fragment of the *hFIX* gene, obtained by PCR amplification utilising the same primers as those used for transgene detection, labelled with α-[^{32}P]dCTP (3,000 Ci mol^{-1}) using the Ready-To-Go DNA Labelling kit (GE Healthcare, Waukesha, WI). The

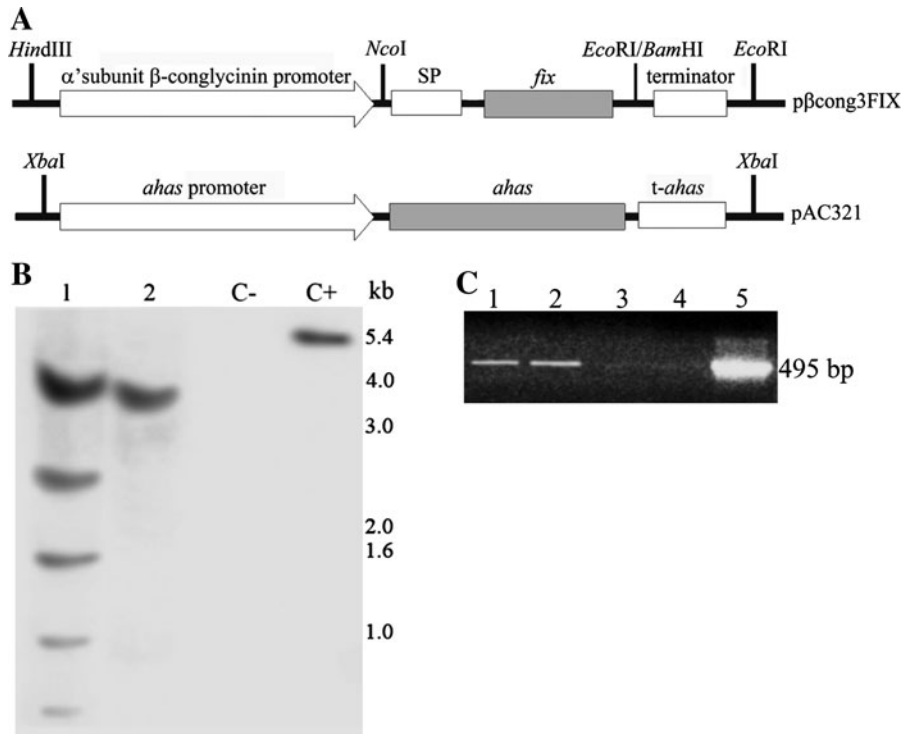

Fig. 1 **a** Schematic representation of the expression cassettes of the pβcong3FIX and pAC321 plasmids used for particle bombardment transformation of soybean embryos. The human coagulation factor (*hfix*) gene fused to the α-Coixin signal peptide (*SP*) is under the control of the α′ subunit of the β-conglycinin promoter and 3′ region (*terminator*). In the pAC321 plasmid, the acid-acetic-hydroxy-synthase (*ahas*) gene is controlled by the *ahas* promoter and the 3′ region (*t-ahas*). **b** Southern blot analysis of transgenic R_1 soybean plants from lines 1 and 2 (*lanes 1* and *2*, respectively), a non-transgenic plant (*C-*) and pβcong3FIX plasmid (100 pg) (*C+*). **c** Reverse transcription (RT)-PCR analysis of R_2 immature seeds of transgenic lines 1 and 7 (*lanes 1* and *2*, respectively), non-transformed seed (*lane 3*), negative control with no template (*lane 4*) and pβcong3FIX plasmid (*lane 5*). The molecular weights were estimated using a 1-kb DNA ladder (Promega)

analysis was conducted as described by Sambrook and Russell (2001).

Reverse transcription-PCR analysis

Sixty days after pollination, immature transgenic R_1 seeds were analysed for the presence of hFIX primary transcripts. Total RNA from a pool of immature seeds (250 mg) was isolated utilising a total RNA Purification System (Invitrogen) according to the manufacturer's protocol. Genomic DNA was eliminated by sample digestion with 2 U of DNase 1 (Ambion, Foster City, CA) for 10 min at 37°C. First strand cDNA synthesis was carried out from 2 µg of the total treated RNA using a SuperScript II RNase H-Reverse Transcriptase kit (Invitrogen) as per the manufacturer's instructions. After cDNA construction, PCR was performed using 1.0 U of Taq polymerase (Invitrogen) with 0.4 mmol l^{-1} of both hFIX specific primers, 125 µmol l^{-1} of dNTP in 1× PCR buffer (Invitrogen) and water to a total volume of 25 µl. The amplification was performed in a DNA thermal cycler (MJ Peltier; MJ Research) under the same conditions as those used for the standard PCR.

TSP extraction from soybean seeds

Total soluble protein extracts from soybean leaves, stems, flowers, roots and seeds (each seed weighed approximately 200 mg) were obtained by homogenising 1.5 g of seeds in 10 ml PBS buffer (10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.2). Samples were homogenised on ice with three 30-s pulses on the lowest setting of a Polytron PT homogeniser (Kinematica, Bohemia, NY) and immediately frozen at −80°C until use. Prior to analysis,

samples were thawed on ice and centrifuged at 12,000 *g* for 10 min at 4°C. The aqueous supernatant was collected and filtered through a Millipore (Billerica, MA) 0.22-μm polyethersulfone disc filter under sterile conditions. Protein concentrations were determined using a Pierce BCA Protein Assay Reagent (Quantum Scientific, Murarrie, Australia) as per the manufacturer's instructions. Pools of three seeds were used for the enzyme-linked immunosorbent assay (ELISA). Approximately 50 seeds were used for the blood-clotting assay. TSP extracts were concentrated approximately sixfold using a Falcon 100-μm nylon sieve column (Amicon series; Millipore) and quantified using a protein assay kit (Bio-Rad, Hercules, CA).

Western blot analysis

About 100 μg TSP was incubated at 95°C for 10 min with loading buffer [20% sodium dodecyl sulfate (SDS), 0.5 M Tris-HCl, 10% β-mercaptoethanol, 20% glycerol, 0.04% bromophenol blue] and separated by SDS-polyacrylamide gel electrophoresis (PAGE; 5% stacking gel and 15% resolving gel). Proteins were transferred to nitrocellulose membranes (C-Extra; Hybond, Amersham Biosciences) using a transblot SD-electrotransfer unit (Bio-Rad). Transferred membranes were blocked with 5% non-fat milk in 0.02 M Tris base and 0.137 M NaCl, pH 7.6, for 16 h at 4°C and incubated for 4 h at room temperature with 4 ng μl^{-1} rabbit polyclonal anti-hFIX (Santa Cruz Biotechnology, Santa Cruz, CA). After washing twice with phosphate buffered saline (PBS) buffer as described above, the blotted membranes were incubated with 0.2 ng μl^{-1} alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG). Proteins were visualised using the chemiluminescent substrate CSPD (Applied Biosystems, Foster City, CA) as recommended by the manufacturer.

Quantification of hFIX in soybean transgenic seeds

An ELISA was performed to determine the hFIX concentration in the TSP extracts. For each sample, 20 μg of TSP was diluted 1:10 in PBS and quantified using an Asserachrom IX:AG ELISA kit (Diagnostica Stago, Parsippany, NJ) as per the manufacturer's protocol. Concentrations of hFIX proteins were determined based on a standard curve of hFIX and ranged from 0.17 to 5.0 μg ml^{-1}. Plate washing, incubation with rabbit anti-hFIX peroxidase conjugate (Diagnostica Stago) and detection using ortho-phenylene-diamine with hydrogen peroxide (OPD/H_2O_2; Diagnostica Stago) were performed according to the manufacturer's protocols. Reactions were stopped with 3 M H_2SO_4 and the absorbance was read at 492 nm in a microplate Reader model 3550 (Bio-Rad).

Immunocytochemical analysis

For the immunolocalisation analysis of hFIX in cotyledons of transgenic plant lines, mature R_1 transgenic seeds were sliced (2 mm thick), fixed (2% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2) for 4 h at 4°C, washed three times in fixation buffer and dehydrated for 5 h in an ethanol series (30, 50, 70, 95 and 100% for 1 h each at 20°C and with a partial vacuum). Samples were infiltrated with increasing concentrations (30–100%) of ethanol-diluted LR White resin (SPI Supplies, West Chester, PA) for 3 h, followed by an 8-h incubation in pure LR White resin. Inclusion was performed by transferring the samples to 1.98% benzoylperoxide in LR White resin and incubating them 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 incubated for 1 h at room temperature with 1× PBS-T (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, 0.5% Tween 20, 2% bovine serum albumin) and for 2 h with 24 ng μl^{-1} rabbit polyclonal anti-hFIX diluted in PBS-T. Samples were washed for 1 h in PBS and incubated in Gold Conjugate Protein A (SPI Supplies) for 2 h at room temperature. After washing in PBS and drying for 24 h, samples were contrasted with 1% uranile acetate in 0.1 M PBS and observed under a Zeiss EM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

Sample preparation for mass spectrometry analysis

Soybean seed proteins were extracted following the Sussulini et al. (2007) protocol. A 100-mg sample of soybean seeds was ground with liquid N_2 and washed for 15 min with 1 ml petroleum ether. The solvent was removed and the procedure repeated. The

proteins were extracted using 1 ml of 50 mM Tris-HCl (pH 8.8), 1.5 mM potassium chloride, 10 mM DTT, 1.0 mM PMSF and SDS 0.1% (m/v) in an ice bath shaker for 10 min and then centrifuged for 5 min at 5,000 rpm and 4°C. The supernatant was then precipitated by adding cold acetone in a 1:4 ratio on ice for 1 h and then centrifuged at 13,000 rpm for 10 min. The pellet was solubilised by adding 500 µl of 50 mM ammonium bicarbonate, quantified by Qubit (Invitrogen) and diluted to a 1 µg μl^{-1} concentration. A 50-µl sample was transferred to a new centrifuge microtube, and 10 µl of 50 mM ammonium bicarbonate was added followed by 25 µl of a 0.2% solution of RapiGest SF (Waters Corp, Milford, MA). The solution was then vortexed. The sample was then incubated at 80°C in a dry ice bath for 15 min, and then 2.5 µl of a 100 mM DTT solution was added and incubated for 30 min at 60°C. The sample was then cooled at room temperature, 2.5 µl of 300 mM iodoacetamide was added and the sample was incubated in a dark room for 30 min. Ten microlitres of trypsin (Promega) in 50 mM ammonium bicarbonate (50 ng μl^{-1}) was used for digestion at 37°C overnight. Following the digestion and to hydrolyze the RapiGest, 10 µl of 5% trifluoroacetic acid was added to the sample, vortexed and incubated at 37°C for 90 min. The sample was centrifuged at 14,000 rpm at 6°C for 30 min and the supernatant was transferred to a Waters Total Recovery vial (Waters Corp). A 5-µl sample of 1 pmol μl^{-1} MassPREP Digestion Standard [yeast alcohol dehydrogenase (ADH); Waters Corp] was added, followed by 85 µl of 3% acetonitrile (ACN) with 0.1% formic acid (FA). The final concentration of the protein and the ADH was 250 ng μl^{-1} and 25 fmol μl^{-1}, respectively.

NanoLC–MS^E acquisition

Nanoscale liquid chromatography (LC) separation of tryptic peptides was performed using a nanoAcquity system (Waters Corp) equipped with a Symmetry C18 5 µm, 5-mm × 300-µm pre-column and a nanoEase BEH130 C18 1.7 µm, 100 µm × 100 mm analytical reversed phase column (Waters Corp). The samples were initially transferred with an aqueous 0.1% FA solution to the pre-column with a flow rate of 15 µl min^{-1} for 1 min. The mobile phase A was water with 0.1% FA, and the mobile phase B was 0.1% FA in ACN. The peptides were separated with a gradient of 3–40% mobile phase B over 90 min with a flow rate of 600 nl min^{-1} followed by a 10-min rinse with 90% of mobile phase B. The column was re-equilibrated to the initial conditions for 20 min. The column temperature was maintained at 35°C. The lock mass was delivered from the auxiliary pump of the nanoAcquity pump with a constant flow rate of 150 nl min^{-1} at a concentration of 100 fmol of human [Glu^1]-Fibrinopeptide (GFP) B (Sigma–Aldrich, St. Louis, MO) to the reference sprayer of the NanoLockSpray source of the mass spectrometer (MS). All samples were analysed in triplicate. Analysis of tryptic peptides was performed using a Synapt HDMS mass spectrometer (Waters Corp). This instrument has a hybrid quadrupole/ion mobility/orthogonal acceleration time-of-flight (oa-TOF) geometry. For all measurements, the mass spectrometer was operated in the V-mode of analysis with a typical resolving power of at least 10,000 full-width half-maximum (FWHM). All analyses were performed using positive nanoelectrospray ion mode (nanoESI+). The TOF analyser of the mass spectrometer was externally calibrated with GFP b^+ and y^+ ions from *m/z* 50–1,990 with the data post acquisition lock mass corrected using the GFP monoisotopic precursor ion $[M + 2H]^{2+} = 784.8426$. The reference sprayer was sampled with a frequency of 30 s. Exact-mass retention-time (EMRT) (Silva et al. 2005) nanoLC-MS^E data were collected in an alternating low-energy and elevated-energy mode of acquisition. The continuum spectra acquisition time in each mode was 1.5 s, with a 0.1 s interscan delay. In the low-energy MS mode, data were collected at constant collision energy of 3 eV. In the elevated-energy MS mode, the collision energy was ramped from 12 to 45 eV during each 1.5-s spectrum. The radio frequency applied to the quadrupole mass analyser was adjusted such that ions from *m/z* 50–2,000 were efficiently transmitted ensuring that any ions less than *m/z* 50 observed in the LC-MS data only arose from dissociations in the TRAP T-wave collision cell.

Data processing and protein identification

Mass spectrometry data obtained from LC-MS^E were processed and searched using ProteinLynx Global Server (PLGS) version 2.4v (Waters Corp). Protein identifications were obtained with the embedded ion

accounting algorithm of the software and searching a *G. max* database with MassPREP digestion standards (MPDS) UniProtKB/Swiss-Prot sequences [Phosphorylase: P00489 (PHS2_RABIT); bovine hemoglobin: P02070 (HBB_BOVIN); ADH: P00330 (ADH1_ YEAST), bovine serum albumin: P02769 (ALBU_ BOVIN)] and human coagulation factor IXa [UniProtKB/Swiss-Prot P00740 (FA9_HUMAN)] appended to the database. Identifications and quantitative data packaging were generated by the use of dedicated algorithms (Silva et al. 2006) and searching against a species-specific database. The ion detection, clustering and log-scale parametric normalisation procedures were performed in PLGS with an Expression^{E} license installed. Intensity measurements are typically adjusted on those components; that is, deisotoped and charge state-reduced EMRTs that replicate throughout the complete experiment for analysis at the EMRT's cluster level. Components are typically clustered with a 10-ppm mass precision and a 0.25-min time tolerance. Alignment of elevated-energy ions with low-energy precursor peptide ions is conducted with an approximate precision of 0.05 min. For analysis of the protein identification and quantification level, the observed intensity measurements are normalised to the intensity measurement of the identified peptides of the digested internal standard.

hFIX blood clotting assay

The functional activity of the recombinant hFIX protein produced in soybean seeds was evaluated by the activated partial thromboplastin (aPTT) time assay (Arruda et al. 2004). For each sample, 600 μg TSP was incubated with 200 μl vitamin K_1 (Sigma, St. Louis, MO) solution (100 μg ml^{-1} in PBS) for 24 h at 37°C. Samples were then incubated with 150 μl hFIX-deficient substrate plasma (Helena Laboratories, Beaumont, TX) and 150 μl Veronal Buffer (Dade–Behring, Marburg, Germany) for 3 min at 37°C to activate the hFIX. A volume of 150 μl of 25 mM TriniCLOT aPTT $CaCl_2$ (Trinity Biotech, Carlsbad, CA) was added, and clotting time formation was measured in an ACL 200 System Coagulometer (Instrumental Laboratory, USA). A reference standard curve (dilution series of 1:80, 1:160, 1:320, 1:640, 1:1,280, 1:2,560 and 1:5,120) was generated utilising Verify (BioMérieux, Marcy L'Etoile, France), a lyophilised human plasma reagent with characteristics similar to those of fresh normal plasma, in order to determine the relative clotting activity of the recombinant hFIX expressed in transgenic seeds.

Results

Generation and molecular analysis of transgenic soybean plants

A co-bombardment transformation procedure was used to generate transgenic soybean plants. The pβcong3FIX plasmid contained the cassette with hFIX in a translational fusion to the signal peptide from the α-*Coixin* gene for ER targeting. This fusion was under control of the seed-specific α' subunit of the β-conglycinin promoter. After microparticle bombardment, the embryonic axes were cultured in vitro for 6 weeks before the regenerated plantlet lines could be transferred to soil. The 495-bp *hFIX* gene fragment was detected by PCR in 11 independent soybean R_0 lines (data not shown). The time from bombardment of the embryonic axes until the plants set seeds and were ready for harvesting was about 7–10 months. Segregation patterns of the R_1 seed lines were also evaluated by PCR. Lines 1 and 7 showed a Mendelian segregation pattern and were chosen for further molecular and biochemical characterisation. All transgenic lines, R_0, and R_1, also showed the presence of the *ahas* gene (data not shown). Southern blot analysis of the R_1 plants from these two transgenic lines revealed an integration profile consistent with a multiple insertion event in transgenic line 1 plants and a single-copy insertion event in transgenic line 7 plants (Fig. 1b). The use of a radio-labelled probe, specific to an internal region of the *hFIX* gene, demonstrated that transgenic line 1 had at least four to five copies of the integrated gene in its genome (Fig. 1b). Reverse transcription (RT)-PCR analysis showed the expression of hFIX at the transcriptional level. The expected 495-bp fragment was observed in lines 1 and 7 (Fig. 1c).

Expression and localisation of recombinant hFIX in transgenic soybean seeds

Expression of hFIX in R_1 seed lines was confirmed by a western blot using total protein extracts from PCR-positive seeds (Fig. 2a). Protein extracts from R_1 transgenic seed lines 1 and 7 had the expected 56-

kDa hFIX protein fragment. The 56-kDa hFIX protein was observed in the seeds, but as expected, not in transgenic soybean roots, leaves, stems or flowers (Fig. 2b).

The kinetics of hFIX protein accumulation during R_1 seed development was evaluated at 2, 4, 6 and 8 weeks after pollination. hFIX protein accumulation increased during seed development. Western blots indicated that the accumulation was higher in the immature seeds collected 6 weeks after pollination and in the dried mature seeds obtained 8 weeks after pollination (Fig. 2c).

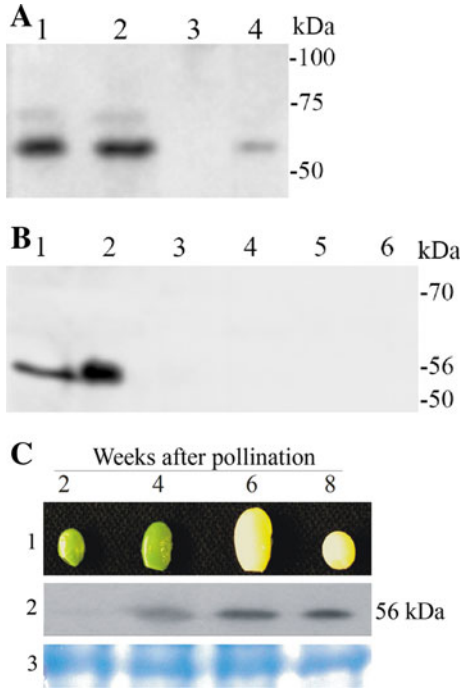

Fig. 2 **a** Western blot analysis of transgenic soybean seeds expressing recombinant hFIX protein. *Lanes*: *1*, *2* Total soluble protein (TSP) extracts (100 µg) from transgenic R_1 lines 1 and 7, respectively, *3* a non-transgenic seed extract, *4* 40 ng of standard hFIX (Sigma). **b** Western blot demonstrating the organ-specific expression of hFIX in transgenic soybean seeds from line 1. *Lanes*: *1* 40 ng of standard hFIX, *2–6* 100 µg TSP from seed, flower, leaf, stem and root, respectively. **c** Western blot analysis showing the accumulation of hFIX in soybean seeds at different phenological stages. *Lanes*: *1* Seeds at 2, 4, 6, and 8 weeks after pollination, *2* accumulation of the hFIX protein in each seed developmental stage, *3* sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading controls of each protein extract sample (approx. 80 µg). Molecular weights were estimated with the marker Precision Plus Protein Standards All Blue (Bio-Rad)

The subcellular localisation of hFIX protein in mature soybean seeds was analysed by ultrastructural immunocytochemistry. Electron microscopy images of ultra-thin seed sections treated with hFIX-specific antibodies indicated that hFIX accumulation in transgenic soybean seeds from line 1 was directed to the PSVs, as evidenced by the 20-nm gold particles (Fig. 3a, b). No significant gold particle accumulation was found in the apoplast and the starch grains.

Quantification of hFIX in soybean transgenic seeds

An ELISA was performed to determine the concentration of hFIX protein in the TSP extracts from transgenic seed lines 1 and 7. The concentrations of hFIX were 0.138 mg ml^{-1} (0.2% TSP) and 0.158 mg ml^{-1} (0.23% TSP), respectively (data not shown).

Mass spectrometry

Recombinant hFIX protein present in soybean TSP extracts was detected and identified utilising a nanoLC-MS assay. The results demonstrated the expected—correct—hFIX peptide sequences in transgenic soybean TSP extracts and their respective monoisotopic expected masses and positions, with no detection of post-translational modifications in the fragment covered by the spectrum assay (Fig. 4). Carbamidomethyl+C modification to two hFIX peptide sequences was caused by the protein digestion protocol described in Materials and methods.

Functional activity of hFIX in transgenic soybean seed extracts

The clotting activity of the recombinant hFIX protein present in the extracts of plants of soybean seed lines 1 and 7 was evaluated by the aPTT method, which permits evaluation of the activity of components required for plasma coagulation in terms of the time taken to form a stable fibrin clot. To interpret the clotting activity of the recombinant hFIX expressed in soybean seeds, we performed a dilution series with lyophilised human plasma containing properly activated FIX. This approach allowed for a comparison with the pooled normal plasma standard, whose reference concentration of FIX was 1 IU ml^{-1} (international unit ml^{-1}) or 5 µg ml^{-1} of blood. This

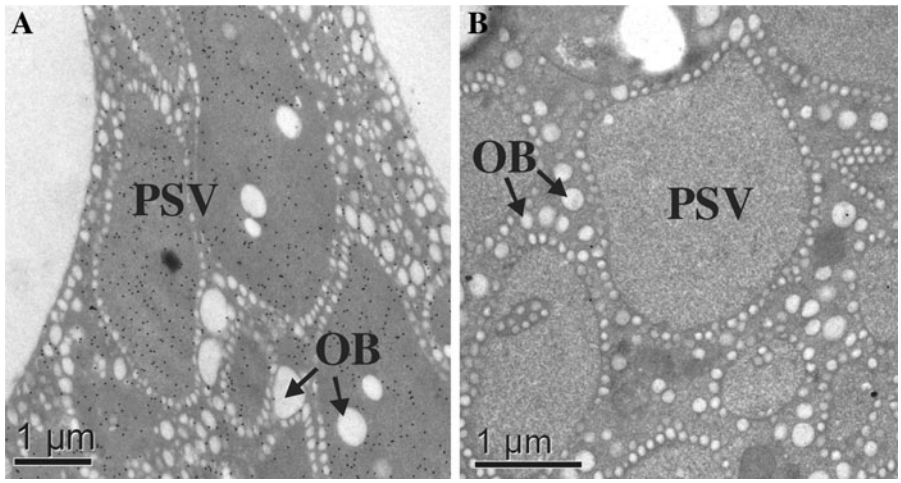

Fig. 3 Ultrastructural immunocytochemistry evaluation of recombinant hFIX in ultra-thin sections of soybean cotyledon. **a** Subcellular accumulation of recombinant hFIX in protein storage vacuoles (*PSV*) of transgenic seeds from line 1. **b** Analysis of non-transgenic seeds. *OB* oil bodies

Fig. 4 Full hFIX recombinant protein sequence expressed in soybean. *Vertical black lines* Digestion sites of trypsin, *shaded boxes* peptide sequences confirmed by tandem mass spectrometry spectra, with the darker shaded box indicating hFIX peptide sequences with expected masses and the *lighter shaded box* indicating sequences with carbamidomethyl+C modification caused by protein digestion protocol. *Numbers above shaded boxes* indicate the monoisotopic mass of the corresponding peptide

concentration can stimulate a clotting activity of 100% in a healthy adult. Recovered recombinant hFIX showed a relative clotting activity of 1 (line 1) and 1.4% (line 7), and a clotting time of 119.5 and 126.0 s, respectively. TSP extracts from non-transgenic soybean seeds did not show clotting activity (Table 1).

Discussion

As a bioreactor, transgenic soybeans can express biologically active recombinant hFIX protein. Mature seeds were subjected to co-transformation utilising a biolistic process. PCR analysis showed the expected 495-bp *hFIX* fragment in 11 independent, putative, transgenic soybean lines, all of which generated seeds (data not shown). The transformation frequency was consistent with those reported previously (Rech et al. 2008). Among the 11 transgenic lines obtained, two lines had progenies with Mendelian segregation patterns, in addition to multiple transgene copies integrated into the genomes of R_1 soybean plants. This hypothesis was corroborated by Southern blot analysis, which detected four to five copies of the *fix* gene integrated in R_1 plants from line 1 and a single copy in R_1 plants from line 7 (Fig. 1b).

Our results indicate that the *fix* copies present in transgenic soybean line 1 segregated in a linked way as a result of multiple-copy integration in one or a few transgenic loci, resulting in structurally complex transgene blocks inherited in a Mendelian fashion. This is not the most common inheritance pattern observed in plant genetic transformations mediated by particle bombardment, but examples in transgenic cereals and tobacco attest to the occurrence of such exceptions (Campbell et al. 2000; Popelka et al. 2003; Yin et al. 2004; Travella et al. 2005).

Table 1 Clotting activity of recombinant human coagulation factor protein expressed in transgenic soybean seeds

Sample	Clotting time (s)	Relative hFIX activity of normal human blood (%)
Standard dilution series of hFIX in normal human plasma[a]		
1 (1:80)	40.5	75.5
2 (1:160)	45.0	28.7
3 (1:320)	50.1	10.7
4 (1:640)	54.8	4.7
5 (1:1280)	60.3	1.9
6 (1:2560)	63.1	1.3
7 (1:5120)	64.9	1.0
Soybean seeds		
Transgenic line 1	119.5	1.0
Transgenic line 7	117.5	1.4
Non-transgenic	254	0

hFIX, Human coagulation factor

[a] Samples were homogenised with hFIX-deficient substrate plasma, submitted to in vitro inactivation, and the relative time for clot formation was measured by a coagulometer. A dilution series with lyophilised human plasma containing the reference concentration of FIX (1 IU ml^{-1} or 5 μg ml^{-1}) was performed in order to interpret the clotting activity. The clotting time was associated with the relative hFIX activity to provide blood coagulation (Hoffer et al. 1999; Arruda et al. 2004; Zhang et al. 2007)

Transgenic soybean lines showed a major hybridisation band of 4.0 kb, which corresponds to the internal *Hin*dIII fragment containing the complete expression cassette in addition to adjoining genomic DNA integration sites (Fig. 1b). Several other bands with a low level of hybridisation signal intensity could also be detected, probably explained by the integration of degenerated or partially integrated fragments with a low capacity to hybridise with the specific probe under stringent conditions (Montchamp-Moreau et al. 1993; Luthra and Medeiros 2004). These results are in accordance with previous findings showing that transgene integration, mediated by either particle bombardment or *A. tumefaciens*, is a random process correlated with the position of naturally occurring chromosome breaks (Aragão and Rech 1997; Aragão et al. 2000; Altpeter et al. 2005).

Because superfluous transgene copies integrated in tandem in the host genome can be reflected in low production levels of recombinant protein, primarily by gene silencing on the transcriptional level (Hobbs et al. 1993; Kumar and Fladung 2001; Kohli et al. 2003; Popelka et al. 2003), the transgenic soybean lines were further analysed by RT-PCR to determine hFIX expression on the transcriptional level. It was possible to detect the *hfix* gene primary transcripts in immature soybean R_1 seeds (Fig. 1c), indicating that transgene integration in the host genome was not followed by gene silencing.

Expression and accumulation of recombinant hFIX in transgenic soybean seeds stored for 6 years at room temperature (22 ± 2°C) was demonstrated in R_1 progeny lines 1 and 7 (Fig. 2a). This result corroborated the finding that the α′ subunit of the β-conglycinin tissue-specific promoter and α-Coixin signal peptide were sufficient to drive the expression of recombinant hFIX to the PSVs of transgenic soybean seeds. Similar approaches have been utilised to improve the nutritional quality of Lupin (*Lupinus angustifolius* L.) by inducing the expression of a methionine-rich protein (Molvig et al. 1997). The identification of the expected hFIX 56-kDa band indicated that the cleavage of the α-Coixin signal peptide in the ER was correctly performed during protein post-translational processing and that correct subcellular targeting was achieved. Low-intensity bands above the expected molecular mass of the hFIX could also be detected in the western blot assay for both progenies, probably due to an increase in the molecular mass of a fraction of the expressed recombinant hFIX caused by undesirable glycosylation (Jacobs and Callewaert 2009) and/or other post-translational modifications, or even by an inefficient removal of the signal peptide during protein targeting (Hjernø 2007). The α-Coixin signal peptide isolated from a monocot plant has also been observed to direct recombinant hGH to the ER of tobacco (*Nicotiana tabacum* L.) seeds. This result was expected since signal peptides are highly conserved among eukaryotic organisms (Leite et al. 2000). A nanoLC-MS^E tryptic digestion analysis of TSP soybean extracts expressing recombinant hFIX demonstrated the correct and expected peptide sequences of hFIX.

The results from the ultrastructural immunocytochemistry analysis indicated that the α′ subunit of the β-conglycinin promoter and the α-Coixin signal peptide were effective in directing hFIX protein accumulation in mature soybean seeds. The accumulation profile showed detectable hFIX protein restricted to the PSVs, with residual detection in the

apoplast and oil bodies and an absence of detectable hFIX protein in the cell wall and starch grains (Fig. 3a). In contrast, immunolocalisation studies on the expression of delta-zein under the control of the β-conglycinin promoter in transgenic soybean seeds have indicated the formation of two distinct dense protein bodies primarily localised in the cytoplasm, but also in PSVs (Kim and Krishnan 2004). Overall, our results are in agreement with previous findings wherein PSV-specific proteins are formed in the ER and, in many cases, subsequently transported along the secretory pathway through the Golgi complex and dense vesicles before reaching PSVs (Hohl et al. 1996; Müntz 1998; Vitale and Raikhel 1999).

hFIX protein constituted up to 0.23% of TSP extracts from transgenic soybean seeds. This level is consistent with the yields of recombinant biopharmaceuticals produced in transgenic plants (Kusnadi et al. 1997; Daniell et al. 2001) and is significantly higher than the expression level of 0.016% previously reported for tomato fruits (Zhang et al. 2007). Our results on the accumulation of biopharmaceuticals in soybean seeds, driven by two different combinations of regulatory sequences, including promoters and signal peptides, revealed different levels of recombinant protein expression, reaching up to 6% TSP when a glycinin promoter and a PSV signal peptide were utilised to modulate the expression and subcellular targeting of the microbicide cyanovirin. A similar approach was used to obtain the B subunit of the heat-labile toxin of enterotoxigenic *E. coli*, reaching 2.4% of total seed protein content and >7% of the green fluorescent protein (GFP) retained in the ER of soybean seeds (Moravec et al. 2007; Schmidt and Herman 2008).

As a potential low-cost platform for recombinant protein production, transgenic soybean may constitute one of the least expensive systems for the large-scale production of biopharmaceuticals. Under greenhouse conditions, soybean has a relatively high biomass capacity; it is also photoperiod sensitive, which means that increasing the length of the light period will induce flowering delay and, consequently, high vegetative growth (Cavazzoni et al. 1999; Kantolic and Slafer 2007). Utilisation of a 24-h light photoperiod has been found to increase seed production by up to 100-fold (average of 10,000 seeds/plant) compared to seed production under field conditions (average of 100 seeds/plant). This photoperiod sensitivity together with the intrinsic high protein content (40%) in the seeds makes soybean an attractive system for the production of recombinant proteins.

The general estimate of cost effectiveness of plant-derived pharmaceutical proteins expressing about 1% of the dry weight of seeds (assuming at least 50% recovery during protein purification) is 2–10% of that observed in microbial systems (Hood et al. 2002; Demain and Vaishnav 2009; Sharma and Sharma 2009) and 0.1% of that found in mammalian cell culture (Chen et al. 2005). Early studies have shown that soybean may have the lowest cost to production of recombinant proteins ratio in comparison with other crops, such as corn, canola, peanuts, sunflower, alfalfa leaves and potato tubers (Kusnadi et al. 1997).

Our blood-clotting assays confirmed the functionality of the hFIX protein expressed in soybean seeds. A standard curve of the hFIX protein present in normal human plasma was utilised to interpret the clotting activity. When compared to normal human clotting activity (that of adults with a FIX concentration of 5 $\mu g\ ml^{-1}$ of blood), which normally reach 100% when measured with the aPTT method (Hoffer et al. 1999; Acar et al. 2006), the coagulation rates of 1.0–1.4% observed in the soybean TSP extracts were low. However, in most cases, haemophilia B patients usually require FIX supplementations with 10% of clotting activity to provide proper coagulation rates, and a significant haemostatic effect can be achieved with the elevation of the FIX levels in the blood to only 0.8 IU dl^{-1} (Kay et al. 2000; Goldenberg et al. 2008). In addition, experiments have been carried out to obtain purified hFIX from soybean seeds. This, in turn, may contribute to increasing the coagulation rates.

The low clotting activity of the hFIX derived from TSP extracts can be partially attributed to the presence of endogenous proteins in the extracts, which can inhibit proper coagulation (Limentani et al. 1995; Buchacher et al. 1998) and the necessity to improve the in vitro activation of the recombinant FIX protein. This activation is mediated by other coagulation factors and vitamin K and promotes more efficient post-translational modifications required to obtain a fully active fraction of the recombinant protein (Jallat et al. 1990).

One approach to removing the protein of interest from endogenous seed contaminants would be the

addition of N or C-terminal tags to the hFIX, such as histidine tags. This may improve protein recovery by means of partial purification with His-affinity chromatography columns. This additional strategy could result in higher yields of the recombinant protein being obtained, reflecting an increase in the biological evaluation of the hFIX efficiency to promote proper blood clotting (Rohila et al. 2004; Ribeiro et al. 2008). Experiments are currently being run that focus on improving the TSP extraction and purification protocols, such as the separation of the hFIX from abundant seed endogenous proteins, by exploring different protein solubilities in a variety of extraction buffers, among other factors. This assumption is supported by the results of our recent studies evaluating downstream processing steps involved in the purification of β-glucuronidase from transgenic soybean seeds (Robić et al. 2006, 2010). In addition, the results reported here on homozygous soybean lines expressing the hFIX protein and those from our studies on downstream processing of transgenic soybean seeds form a foundation for the further evaluation of transgenic soybean seeds expressing hFIX and their potential economic production.

The molecular strategy presented in this report has a great potential when the primary focus is to minimise recombinant protein degradation and provide options for easy handling and long-term storage. It is supported not only by our results showing that the seed storage period is far longer than those reported in the literature (Fiedler and Conrad 1995; Stoger et al. 2000; Larrick and Thomas 2001; Ramírez et al. 2001), but also by the fact that the storage conditions during this time, such as room temperature (22 ± 2°C) and the absence of air humidity control, were not sufficiently harsh so as to reduce recombinant hFIX yields.

The PSVs of soybean seeds are an attractive option for the production of recombinant proteins because simple plasmid construction and protein engineering allow for the targeting and accumulation of recombinant proteins in seed storage compartments.

Acknowledgements The authors are grateful to Ana C. M. M. Gomes for scanning electron microscopy assistance and Maria A.P. Ottoboni and Maria B.G.S. Braz for clotting assay assistance. The authors thank Prof. Roger N. Beachy (Danforth Center, USA) for providing the α' subunit of β-conglycinin regulatory sequences. This study was supported partially by the Brazilian Agricultural Research Corporation (EMBRAPA) and National Council for Scientific and Technological Development (CNPq).

References

Acar K, Yağci M, Sucak GT, Haznedar R (2006) Isolated prolonged activated partial thromboplastin time in an asymptomatic patient: Fletcher factor deficiency. Thromb Res 118:765–766

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell D, Datta K, Datta S, Dix P, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen T, Nugent G, Raemakers K, Romano A, Somers D, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15:305–327

Aragão FJL, Rech EL (1997) Morphological factors influencing recovery of transgenic bean plants (*Phaseolus vulgaris* L.) of a carioca cultivar. Int J Plant Sci 158:157–163

Aragão FJL, Sarokin L, Vianna GR, Rech EL (2000) Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean [*Glycine max* (L.) Merril] plants at a high frequency. Theor Appl Genet 101:1–6

Arruda VR, Schuettrumpf J, Herzog RW, Nichols TC, Robinson N, Lotfi Y, Mingozzi F, Xiao W, Couto LB, High KA (2004) Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. Blood 103:85–92

Bristol JA, Furie BC, Furie B (1993) Propeptide processing during factor IX biosynthesis. Effect of point mutations adjacent to the propeptide cleavage site. J Biol Chem 268:7577–7584

Buchacher A, Schulz P, Choromanski J, Schwinn H, Josic D (1998) High-performance capillary electrophoresis for in-process control in the production of antithrombin III and human clotting factor IX. J Chromatogr 802:355–366

Campbell BT, Baenziger PS, Mitra A, Sato S, Clemente T (2000) Inheritance of multiple transgenes in wheat. Crop Sci 40:1133–1141

Cavazzoni J, Volk T, Bugbee B, Dougher T (1999) Phasic temperature and photoperiod control for soybean using a modified CROPGRO model. Life Support Biosph Sci 6:273–278

Chen ZL, Schuler MA, Beachy RN (1986) Functional analysis of regulatory elements in a plant embryo-specific gene. Proc Natl Acad Sci USA 83:8560–8564

Chen M, Liu X, Wang Z, Song J, Qi Q, Wang PG (2005) Modification of plant N-glycans processing: the future of producing therapeutic protein by transgenic plants. Med Res Rev 25:343–360

Chen H, Yao H, Huang L, Shen Q, Jia W, Xue J (2006) Expression of human factor IX gene in murine plasma through lentiviral vector-infected haematopoietic stem cells. Clin Exp Pharmac Physiol 33:1196–1201

Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219–226

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA mini-preparation: version II. Plant Mol Biol Rep 1:19–21

Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 27:297–306

Doyle JJ, Schuler MA, Godette WD, Zenger V, Beachy RN, Slightom JL (1986) The glycosylated seed storage proteins of *Glycine max* and *Phaseolus vulgaris*. Structural homologies of genes and proteins. J Biol Chem 261:9228–9238

Drakakaki G, Marcel S, Arcalis E, Altmann F, Gonzalez-Melendi P, Fischer R, Christou P, Stoger E (2006) The intracellular fate of a recombinant protein is tissue dependent. Plant Physiol 141:578–586

Fiedler U, Conrad U (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. Biotechnology 13:1090–1093

Furie B, Furie BC (1988) The molecular basis of blood coagulation. Cell 53:505–518

Goldenberg NA, Bombardier C, Hathaway WE, McFarland K, Jacobson L, Manco-Johnson MJ (2008) Influence of factor IX on overall plasma coagulability and fibrinolytic potential as measured by global assay: monitoring in haemophilia B. Haemophilia 14:68–77

Hjernø K (2007) Protein identification by peptide mass fingerprinting. Methods Mol Biol 367:61–75

Hobbs SL, Warkentin TD, DeLong CM (1993) Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol Biol 21:17–26

Hoffer L, Schwinn H, Josić D (1999) Production of highly purified clotting factor IX by a combination of different chromatographic methods. J Chromatogr A 844:119–128

Hohl I, Robinson DG, Chrispeels MJ, Hinz G (1996) Transport of storage proteins to the vacuole is mediated by vesicles without a clathrin coat. J Cell Sci 109:2539–2550

Hood EE, Woodard SL, Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants-myths and realities. Curr Opin Biotechnol 13:630–635

Howard JA (2005) Commercialization of biopharmaceutical and bioindustrial proteins from plants. Crop Sci 45: 468–472

Jacobs PP, Callewaert N (2009) N-glycosylation engineering of biopharmaceutical expression systems. Curr Mol Med 9:774–800

Jallat S, Perraud F, Dalemans W, Balland A, Dieterle A, Faure T, Meulien P, Pavirani A (1990) Characterization of recombinant human factor IX expressed in transgenic mice and in derived trans-immortalized hepatic cell lines. EMBO J 9:3295–3301

Kantolic AG, Slafer GA (2007) Development and seed number in indeterminate soybean as affected by timing and duration of exposure to long photoperiods after flowering. Ann Bot 99:925–933

Kaufman RJ, Wasley LC, Furie BC, Furie B, Shoemaker CB (1986) Expression, purification, and characterization of recombinant gamma-carboxylated factor IX synthesized in Chinese hamster ovary cells. J Biol Chem 261:9622–9628

Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, Glader B, Chew AJ, Tai SJ, Herzog RW, Arruda V, Johnson F, Scallan C, Skarsgard E, Flake AW, High KA (2000) Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 24:257–261

Kim WS, Krishnan HB (2004) Expression of an 11 kDa methionine-rich delta-zein in transgenic soybean results in the formation of two types of novel protein bodies in transitional cells situated between the vascular tissue and storage parenchyma cells. Plant Biotechnol J 2:199–210

Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol 52:247–258

Kumar S, Fladung M (2001) Controlling transgene integration in plants. Trends Plant Sci 6:155–159

Kurachi K, Davie EW (1982) Isolation and characterization of a cDNA coding for human factor IX. Proc Natl Acad Sci USA 79:6461–6464

Kusnadi AR, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: practical considerations. Biotechnol Bioeng 56:473–484

Lambert T, Recht M, Valentino LA, Powell JS, Udata C, Sullivan ST, Roth DA (2007) Reformulated BeneFix: efficacy and safety in previously treated patients with moderately severe to severe haemophilia B. Haemophilia 13:233–243

Larrick JW, Thomas DW (2001) Producing proteins in transgenic plants and animals. Curr Opin Biotechnol 12: 411–418

Leite A, Kemper EL, da Silva MJ, Luchessi AD, Siloto RMP, Bonaccorsi ED, El-Dorry HF, Arruda P (2000) Expression of correctly processed human growth hormone in seeds of transgenic tobacco plants. Mol Breed 6:47–53

Limentani SA, Gowell KP, Deitcher SR (1995) In vitro characterization of high purity factor IX concentrates for the treatment of hemophilia B. Thromb Haemost 73:584–591

Lisauskas SF, Cunha NB, Vianna GR, Mendes EA, Ramos GL, Maranhao AQ, Brigido MM, Almeida JO, Baptista HA, Motta FL, Pesquero JB, Aragao FJ, Rech EL (2008) Expression of functional recombinant human factor IX in milk of mice. Biotechnol Lett 30:2063–2069

Liu HW, Ofosu FA, Chang PL (1993) Expression of human factor IX by microencapsulated recombinant fibroblasts. Hum Gene Ther 4:291–301

Luthra R, Medeiros LJ (2004) Isothermal multiple displacement amplification: a highly reliable approach for generating unlimited high molecular weight genomic DNA from clinical specimens. J Mol Diagn 6:236–242

Ma JK, Drake PM, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Miao CH, Thompson AR, Loeb K, Ye X (2001) Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. Mol Ther 3:947–957

Molvig L, Tabe LM, Eggum BO, Moore AE, Craig S, Spencer D, Higgins TJ (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. Proc Natl Acad Sci USA 94:8393–8398

Montchamp-Moreau C, Ronsseray S, Jacques M, Lehmann M, Anxolabéhère D (1993) Distribution and conservation of sequences homologous to the 1731 retrotransposon in *Drosophila*. Mol Biol Evol 10:791–803

Moravec T, Schmidt MA, Herman EM, Woodford-Thomas T (2007) Production of *Escherichia coli* heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. Vaccine 25:1647–1657

Müntz K (1998) Deposition of storage proteins. Plant Mol Biol 38:77–99

Nathwani AC, Davidoff AM, Hanawa H, Hu Y, Hoffer FA, Nikanorov A, Slaughter C, Ng CYC, Zhou J, Lozier JN, Mandrell TD, Vanin EF, Nienhuis AW (2002) Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. Blood 100:1662–1669

Osterud B, Rapaport SI (1977) Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc Natl Acad Sci USA 74:5260–5264

Ottoboni LM, Leite A, Yunes JA, Targon ML, Souza Filho GA, Arruda P (1993) Sequence analysis of 22 kDa-like alpha-coixin genes and their comparison with homologous zein and kafirin genes reveals highly conserved protein structure and regulatory elements. Plant Mol Biol 21: 765–778

Popelka JC, Xu J, Altpeter F (2003) Generation of rye (*Secale cereale* L.) plants with low transgene copy number after biolistic gene transfer and production of instantly marker-free transgenic rye. Transgenic Res 12:587–596

Ragni MV, Pasi KJ, White GC, Giangrande PL, Courter SG, Tubridy KL (2002) Use of recombinant factor IX in subjects with haemophilia B undergoing surgery. Haemophilia 8:91–97

Ramírez N, Oramas P, Ayala M, Rodríguez M, Pérez M, Gavilondo J (2001) Expression and long-term stability of a recombinant single-chain Fv antibody fragment in transgenic *Nicotiana tabacum* seeds. Biotechnol Lett 23:47–49

Rech EL, Vianna GR, Aragão FJL (2008) High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. Nat Protoc 3:410–418

Ribeiro CW, Soares-Costa A, Falco MC, Chabregas SM, Ulian EC, Cotrin SS, Carmona AK, Santana LA, Oliva ML, Henrique-Silva F (2008) Production of a His-tagged canecystatin in transgenic sugarcane and subsequent purification. Biotechnol Prog 24:1060–1066

Robić G, Farinas CS, Rech EL, Bueno SMA, Miranda EA (2006) Downstream process engineering evaluation of transgenic soybean seeds as host for recombinant protein production. Biochem Eng J 32:7–12

Robić G, Farinas CS, Rech EL, Miranda EA (2010) Transgenic soybean seed as protein expression system: aqueous extraction of recombinant β-glucuronidase. Appl Biochem Biotechnol 160:1157–1167

Rohila JS, Chen M, Cerny R, Fromm ME (2004) Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. Plant J 38: 172–181

Roth DA, Kessler CM, Pasi KJ, Rup B, Courter SG, Tubridy KL (2001) Human recombinant factor IX: safety and efficacy studies in hemophilia B patients previously treated with plasma-derived factor IX concentrates. Blood 98:3600–3606

Sambrook J, Russell DW (2001) Commonly used techniques in molecular cloning. In: Sambrook J, Russell DW (ed) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 8–A8.20

Schmidt MA, Herman EM (2008) Proteome rebalancing in soybean seeds can be exploited to enhance foreign protein accumulation. Plant Biotechnol J 6:832–842

Sharma AK, Sharma MK (2009) Plants as bioreactors: recent developments and emerging opportunities. Biotechnol Adv 27:811–932

Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, McKenna T, Nold MJ, Richardson K, Young P, Geromanos S (2005) Quantitative proteomic analysis by accurate mass retention time pairs. Anal Chem 77: 2187–2200

Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ (2006) Absolute quantification of proteins by LC-MS^{E}: a virtue of parallel MS acquisition. Mol Cell Proteomics 5:144–156

Stewart CN Jr, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques 14:748–750

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol 42:583–590

Stoger E, Ma JK, Fischer R, Christou P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 16:167–173

Streatfield SJ (2007) Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 5:2–15

Sussulini A, Garcia JS, Mesko MF, Moraes DP, Flores EMM, Pérez CA, Arruda MAZ (2007) Evaluation of soybean seed protein extraction focusing metalloprotein analysis. Mikrochim Acta 158:173–180

Takaiwa F, Takagi H, Hirose S, Wakasa Y (2007) Endosperm tissue is good production platform for artificial recombinant proteins in transgenic rice. Plant Biotechnol J 5: 84–92

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. Plant Cell Rep 23:780–789

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578

Verma D, Moghimi B, LoDuca PA, Singh HD, Hoffman BE, Herzog RW, Daniell H (2010) Oral delivery of bioencapsulated coagulation factor IX prevents inhibitor formation and fatal anaphylaxis in hemophilia B mice. Proc Natl Acad Sci USA 107:7101–7106

Vitale A, Raikhel NV (1999) What do proteins need to reach different vacuoles? Trends Plant Sci 4:149–155

Wajih N, Hutson SM, Owen J, Wallin R (2005) Increased production of functional recombinant human clotting factor IX by baby hamster kidney cells engineered to overexpress VKORC1, the vitamin K 2, 3-epoxide-

reducing enzyme of the vitamin K cycle. J Biol Chem 280:31603–31607

Yamada Y, Nishizawa K, Yokoo M, Zhao H, Onishi K, Teraishi M, Utsumi S, Ishimoto M, Yoshikawa M (2008) Anti-hypertensive activity of genetically modified soybean seeds accumulating novokinin. Peptides 29:331–337

Yin Z, Plader W, Malepszy S (2004) Transgene inheritance in plants. J Appl Genet 45:127–144

Zhang H, Zhao L, Chen Y, Cui L, Ren W, Tang K (2007) Expression of human coagulation factor IX in transgenic tomato (*Lycopersicon esculentum*). Biotechnol Appl Biochem 48:101–107