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ORIGINAL PAPER

Expression of functional recombinant human growth hormone in transgenic soybean seeds

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Abstract We produced human growth hormone (hGH), a protein that stimulates growth and cell reproduction, in genetically engineered soybean [*Glycine max* (L.) Merrill] seeds. Utilising the alpha prime (α') subunit of β-conglycinin tissue-specific promoter from soybean and the α-Coixin signal peptide from *Coix lacryma-jobi*, we obtained transgenic soybean lines that expressed the mature form of hGH in their seeds. Expression levels of bioactive hGH up to 2.9% of the total soluble seed protein content (corresponding to approximately 9 g kg^{-1}) were measured in mature dry soybean seeds. The results of ultrastructural immunocytochemistry assays indicated that the recombinant hGH in seed cotyledonary cells was efficiently directed to protein storage vacuoles. Specific bioassays demonstrated that the hGH expressed in the soybean seeds was fully active. The recombinant hGH protein sequence was confirmed by mass spectrometry characterisation. These results demonstrate that the utilisation of tissue-specific regulatory sequences is an attractive and viable option for achieving high-yield production of recombinant proteins in stable transgenic soybean seeds.

Keywords Protein storage vacuoles · Soybean seeds · Transgenic · Molecular farming · Human growth hormone

In memoriam of Adilson Leite.

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Introduction

Human growth hormone (hGH), known as somatotropin, is a 22-kDa single-chain polypeptide of 191 amino acids that is stabilised by two disulfide bonds. hGH plays a crucial role in the cell cycle and in somatic cell growth through cyclin activation and its effects on protein, carbohydrate and lipid metabolisms (Filikov et al. 2002). Growth hormone biosynthesis in

normal humans suffers a natural decay after 20 years of age, and its absence or low production in children and teenagers (caused by genetic mutations) is the main cause of hypo-pituitary paediatric dwarfism (Bidlingmaier and Strasburger 2010). Although this growth order occurs only infrequently, the high cost of its treatment represents a considerable barrier to treatment. hGH directly purified from human and monkey pituitary glands (Allen 2006) was withdrawn from the market in the 1980s due to its association with a risk of transmission of Creutzfeld–Jacob disease (Houdebine 2009). Recombinant hGH protein is currently commercially produced in transgenic bacterial cell-based systems (Apte-Deshpande et al. 2009) which are associated with high production levels of 6–10% of total cellular secreted protein (Chang et al. 1987). These production levels can increase to 23% when hGH is expressed as an N-terminal fusion system with human tumour necrosis factor alpha (Shin et al. 1998).

The production of recombinant hGH in transgenic bacterial systems has considerably reduced the overall treatment costs of dwarfism and other related growth deficiencies, but the daily administration of *Escherichia coli*-derived hGH is still a very expensive therapy. The estimated annual cost for a child weighing 30 kg receiving intramuscular or subcutaneous injections of recombinant hGH at a dose of 0.18–0.30 mg kg^{-1} per week is approximately US$15,000–20,000, with the prospect of a twofold increase in costs when higher doses are required for adolescents and adults (Allen 2006; Riddick et al. 2009).

The main cause of the high costs associated with the production of recombinant hGH is the overexpression of the molecule synthesised in aggregated, insoluble inclusion bodies, which later requires costly denaturation and refolding to recover functionality (Demain and Vaishnav 2009). Additional production cost-increasing procedures include the need for careful product quality control to avoid the presence of endotoxins and the necessity to store hGH-expressing bacteria under sterile conditions (Daniell et al. 2001; Ma et al. 2003; Demain and Vaishnav 2009).

Expression of hGH has also been reported in diverse organisms, such as yeast (Apte-Deshpande et al. 2009), *Bacillus subtilis* (Ozdamar et al. 2009), rabbits (Lipiński et al. 2003), baculovirus (Cholin et al. 1996), silkworms (Kadono-Okuda et al. 1995; Sumathy et al. 1996), pigs (Hens et al. 2000), sheep (Sanchez et al. 2004), CHO animal cells (Kunaparaju et al. 2005), cows (Salamone et al. 2006) and mice (von Waldthausen et al. 2008). The possibility of producing recombinant proteins in plant systems, the least expensive biomass, is another option being considered as a means to lower the costs associated with the large-scale production of pharmaceuticals and industrial molecules (Kusnadi et al. 1997; Daniell et al. 2001; Ma et al. 2003; Twyman et al. 2003). Production in plants also reduces concerns about contamination by animal or human pathogens (Cañizares et al. 2005; Stein et al. 2009). In addition to being advantageous for the production of hGH, plant systems have a great potential for industrial application in the high-quality biosynthesis of structurally complex proteins (Schillberg et al. 2002). Plant production is enabled by the similarity between human and plant cellular processes, including protein synthesis, folding, assembly and promotion of post-translational modifications in the endoplasmic reticulum (ER) and Golgi apparatus. There are, however, minor but avoidable differences in the glycosylation patterns of these organisms (Ma et al. 2003).

Typical plant glycans, such as α-1,3-fucose and β-1,2-xylose, may be enzymatically added to the amino or carboxyl ends of the protein as well as to the lateral chains of specific amino acid residues of the recombinant proteins produced by transgenic plants (Sethuraman and Stadheim 2006). However, strategies to prevent undesirable N-glycosylation, such as the inactivation of N-glycosylation sites on Asn or Ser and Thr residues (Gomord et al. 2004) or the inhibition of Golgi glycosyltransferases, are also necessary (Wenderoth and von Schaewen 2000). As N-glycan synthesis in the ER is relatively well conserved in eukaryotes, one efficient strategy to avoid the addition of immunogenic N and O-glycans in the Golgi complex is to retrieve the recombinant proteins to the ER lumen by adding N or C-terminal signal peptides to the nascent proteins (Lerouge et al. 2000; Ma et al. 2003; Pagny et al. 2003; Gomord et al. 2004; Faye et al. 2005; Benchabane et al. 2008).

Expression of recombinant hGH protein in plants has been reported in transgenic tobacco (*Nicotiana tabaccum)* seeds (Leite et al. 2000), chloroplasts (Staub et al. 2000), the apoplast of leaves of *Nicotiana benthamiana* (Gils et al. 2005), rice cell

suspensions (Kim et al. 2008), tobacco cell suspension, corn seeds and soybean [*Glycine max* (L.) Merrill] seeds and leaves (Russell et al. 2005). In a recent study by Rabindran et al. (2009), a plant virus-based expression vector was utilised to transiently express a biologically active hGH in *N. benthamiana* plants. Subcutaneous doses of this tobacco-derived hGH were able to elicit a 17-g weight gain in 10 days in hypophysectomised rats.

To avoid the low expression levels of recombinant proteins that are often obtained from different plant systems (one of the major concerns in transgenic plants), strategies to maximise protein accumulation can be adopted to improve the effective yield of the protein of interest (Stoger et al. 2005). Restriction of the nascent recombinant protein to appropriate plant organs, such as seeds, can improve protein stability and considerably reduce the rate of polypeptide degradation (Leite et al. 2000; Stoger et al. 2000, 2005). Seeds are the major storage organs of plants and can accumulate and store a wide range of proteins (Leite et al. 2000). In soybeans, the most abundant proteins in the seeds are seed storage proteins, which represent more than 60% of the total protein content (Mooney et al. 2004). Moreover, heterologous proteins expressed in plant seeds under control of tissue-specific regulatory sequences and directed to specific organelles by protein targeting may effectively increase yield accumulation. This happens once the biochemical environments inside these cellular cisternae are made unfavourable for proteolytic degradation (Müntz 1998; Park et al. 2004; Fischer et al. 2004).

Soybean protein storage vacuoles (PSVs) are temporal extension organelles of the ER, which specialise in accumulating and compartmentalising seed storage proteins (Kim and Krishnan 2004; Robinson et al. 2005; Mori et al. 2009). This intrinsic characteristic results in an absence or low density of aminopeptidases in the lumen, an internally neutral pH and a low protein degradation rate, all of which are condition that allow for long-term storage of immunogenic and fully active recombinant proteins (Takaiwa et al. 2007).

The α' subunit of the β-conglycinin promoter is an efficient tissue-specific regulatory sequence that controls the endogenous accumulation of β-conglycinin, the most abundant protein stored in the cotyledons of developing soybean seeds (Ladin et al. 1987; Yamada et al. 2008). This promoter is temporally regulated by interactions between TRANS-acting factors, previously activated by phytohormones, and enhancer sequences located upstream of the transcription initiation site (Chen et al. 1986; Imoto et al. 2008). This seed-specific regulatory sequence has been successfully utilised to demonstrate the efficacy of accumulating a methionine-rich delta-zein in soybean PSV seeds under control of the β-conglycinin promoter and its signal peptide (Kim and Krishnan 2004). Yamada et al. (2008), taking advantage of the endogenous accumulation of β-conglycinin, recently achieved the expression of an active anti-hypertensive hybrid molecule in soybean seeds. In contrast, the utilisation of a constitutive promoter resulted in a low expression level of hGH protein in soybean seeds and leaves (Russell et al. 2005).

Utilisation of the δ-kafirin promoter under control of the α-Coixin signal peptide from *Coix lacryma jobi* was effective for the accumulation of hGH in transgenic tobacco seeds, accounting for 0.16% of total soluble seed protein (Leite et al. 2000). This signal peptide acts as an additional regulatory sequence that is able to direct alpha-prolamins of Coix, sorghum and maize to the ER (Ottoboni et al. 1993), which can then be trafficked to the PSVs following the default secretory pathway (Neumann et al. 2003; Jolliffe et al. 2005; Boothe et al. 2010). Thus, the aim of this study was to express functional hGH under control of the α' subunit of the β-conglycinin tissue-specific promoter from soybean and the α-Coixin signal peptide from *C. lacryma-jobi* in the PSVs from mature soybean seeds.

Materials and methods

DNA cloning

The 740-bp PShGH fragment containing the mature hGH coding sequence (GenBank accession number: BC075013) and the α-Coixin signal peptide from *C. lacryma-jobi* was removed from the expression vector pPGK-hGH (Leite et al. 2000) using *Nco*I and *Bam*HI and inserted into a vector containing the soybean α' subunit of the β-conglycinin promoter and terminator (Imoto et al. 2008), producing expression vector pβcong3hGH (Fig. 1a). Expression vector pβcong3hGH was utilised to transform soybean

embryos in a co-transformation procedure with the pAC 321 vector (Fig. 1a) that contains the *ahas* gene, previously isolated from *Arabidopsis thaliana,* under control of the *ahas* promoter and 3′ polyadenylation signal. The *ahas* gene has a mutation at position 653 bp, which results in a serine to asparagine substitution that produces a modified AHAS enzyme. This modified enzyme confers tolerance to the herbicide Imazapyr (Sathasivan et al. 1991; Rech et al. 2008).

Soybean genetic transformation

The vectors p*β*cong3hGH and pAC321 were co-bombarded in a 1:1 ratio into the apical meristem of somatic embryonic axes from mature soybean seeds (cv. BR-16) utilising a particle bombardment procedure as previously described (Aragão et al. 2000; Rech et al. 2008). Transgenic R_0 plants were cultivated under greenhouse conditions to produce seed progenies.

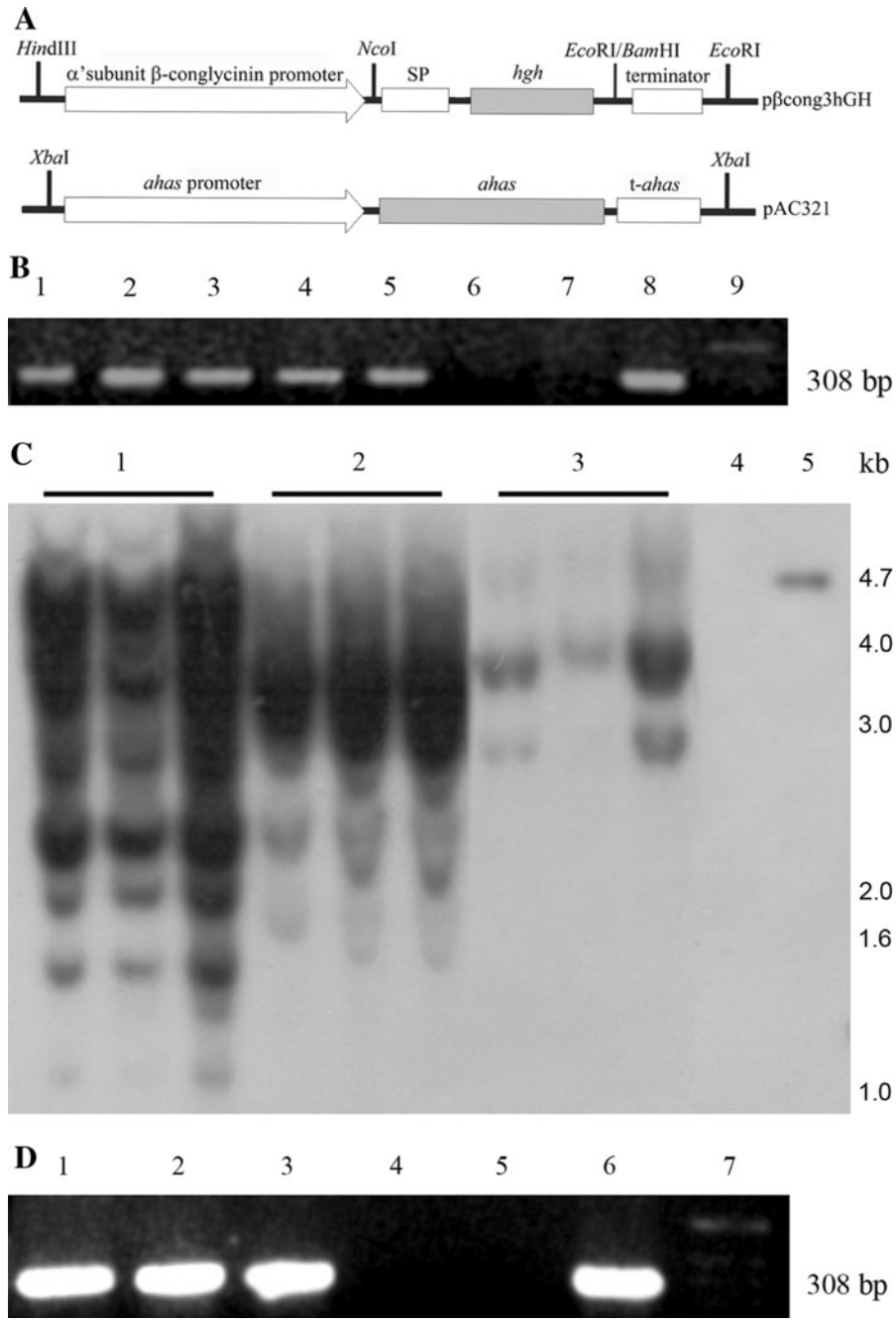
DNA extraction and PCR analysis

DNA of selected plants was isolated from foliar discs according to Bonfim et al. (2007). PCR reactions contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 160 μM of each dNTP, 2 U Taq polymerase (Invitrogen), 20 ng of genomic DNA and 200 nM of each hGH specific primer, namely, HGH273 forward (ATCCAACCTAGAGCTGCTCC) and HGH581 reverse (CCTTGTCCATGTCCTTCC). PCR mixes were covered with mineral oil, and the DNA was pre-denatured at 95°C for 5 min and amplified for 35 cycles in a MJ Peltier DNA thermal cycler (MJ Research, Watertown, MA) under the following conditions: 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. For the detection of the selectable marker gene *ahas*, the primers AHASP124 (ACTAGAGATTCCA GCGTCAC) and AHAS500c (GTGGCTATACAGA-TACCTGG) were utilised under the same reaction conditions as previously mentioned. The expected amplification products using primers HGH273 and HGH581 and AHAS500c and AHASP124 were of 308 and 624 bp, respectively. Amplification reactions were resolved by electrophoresis on 1% agarose gels containing ethidium bromide and visualised under UV light.

Fig. 1 **a** Schematic representation of the expression cassettes of the p*β*cong3hGH and pAC321 plasmids used for particle bombardment transformation of soybean embryos. The human growth hormone (*hgh*) 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 *ahas* gene is controlled by the *ahas* promoter and the 3′ region (*t-ahas*).**b** PCR analysis of the integration of the *hgh* gene into transgenic, regenerated soybean plantlets. *Lanes 1–5* Soybean leaf DNA preparations of 5 transgenic lines showing the amplified fragment (308 bp) corresponding to an internal region of the human *hgh* gene. *Lanes 5*, *6* DNA from a non-transformed seed showing no detectable amplification (*lane 6*) and a negative control with water instead of DNA showing no detectable amplification (*lane 7*). *Lane 8* Band at approximately 308 bp corresponds to an internal amplification of the *hgh* gene presented in the p*β*cong3hGH plasmid (100 ng). *Lane 9* Molecular-weight marker (1-kb DNA ladder; Invitrogen) was used to estimate the electrophoretic migration profile of all samples. **c** Southern blots of transgenic R_1 soybean plants demonstrated multiple-copy insertions in the genome of all transgenic plants analysed. *Lanes 1–3* Plants from lines 3, 9, and 19, respectively, were submitted to a specific hybridisation with an internal *hgh* gene homologous probe. *Lane 4* DNA from a non-transgenic plant showing no hybridisation. *Lane 5* A band corresponding to the 4.7-kb plasmid (100 pg) indicated that the probe utilised in the experiment efficiently recognised an internal transgene segment. Molecular weights were estimated based on the 1-kb DNA ladder (Invitrogen, USA). **d** Reverse transcription (RT)-PCR analysis of R_2 immature seeds from transgenic lines 3, 9 and 19 showed *hgh* gene expression (*lanes 1–3*, respectively). *Lanes 4, 5* Negative controls with DNA from a non-transformed seed (*lane 4*) and water in place of the template (*lane 5*), demonstrating no detectable amplification. *Lane 6* Reaction with the p*β*cong3hGH plasmid showed the amplification of a 308-bp fragment corresponding to an internal sequence of the *hgh* gene. The molecular weight was estimated using a 1-kb DNA ladder (Invitrogen)

Southern blot analysis

Genomic DNA of transgenic R_1 plants from lines 3, 9 and 19 was isolated according to Dellaporta et al.'s procedure (Dellaporta et al. 1983). Approximately 15 μg of genomic DNA was digested with *Hin*d III (150 U), separated by 1% agarose gel electrophoresis and transferred to a nylon membrane (Hybond N^+; Amersham Biosciences, Arlington Heights, IL). The 740 bp fragment of the *hgh* gene and the signal peptide of *Coix* used to obtain the p*β*cong3hGH were used as the probe and labelled with α-[^{32}P]dCTP (3,000 Ci mol^{-1}) using the Ready-To-Go DNA Label kit (GE Healthcare, Waukesha, WI). Analysis was conducted as previously described by Sambrook and Russell (2001).



Reverse transcription-PCR analysis

Sixty days after pollination, immature transgenic R_2 seeds were analysed to detect the presence of hGH primary transcripts. Total RNA from a pool of seeds (250 mg) was isolated utilising the total RNA Purification System (Invitrogen, Carlsbad, CA). Genomic DNA was eliminated by digestion with 2 U of DNAse 1 (Ambion, Foster City, CA). cDNA synthesis was carried out with 2 µg of total RNA using the SuperScript II RNase H-Reverse Transcriptase kit (Invitrogen, USA). After cDNA construction,

PCR was performed using 1.0 U of Taq polymerase (Invitrogen) with 0.4 mmol l^{-1} of both hGH specific primers (HGH273 and HGH581), 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 previously described.

Western blot analysis

For the western blot analysis, total soluble proteins (TSPs) were extracted from soybean leaves, stems, roots and seeds (200 mg of each tissue sample) by grinding samples in liquid N_2. Each gram of sample powder was resuspended in 3 ml of phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.2). The extracts were immediately homogenised by vortexing for 4 h at 4°C, then centrifuged at 10,650 *g* for 40 min; The aqueous supernatant was collected, and the pellet was resuspended in an additional 3 ml of PBS, homogenised and washed four times for maximum TSP recovery. The aqueous supernatants were combined into one TSP extract. Extracts were quantified using the Bradford assay (1976). Approximately 100 µg of 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, Hercules, CA). 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 then incubated for 4 h at room temperature with rabbit polyclonal anti-human hGH in a dilution of 1:2.500 (0.4 ng μl^{-1} in 10 ml of blocking solution). After being washed twice with 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's protocol.

Immunocytochemical analysis

Immunolocalisations of hGH were conducted in soybean seeds. The cotyledons of 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%, 1 h each, at −20°C under partial vacuum). The samples were then 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 then at 4°C under UV light for 72 h. Ultra-thin sections 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 and 2% bovine serum albumin) and for 2 h with 5 ng μl^{-1} rabbit polyclonal anti-hGH diluted in PBS-T. Samples were washed for 1 h in PBS and incubated on Gold Conjugate Protein A (SPI Supplies) for 2 h at room temperature. After being washed in PBS and dried for 24 h, the 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).

Quantification and biological activity of recombinant hGH

For hGH quantification and bioactivity evaluation, TSPs were extracted from soybean seeds by homogenising 1.5 g of seeds in 10 ml of PBS buffer (10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.2). The samples were homogenised on ice with three 30-s pulses on the lowest setting of a Kinematica Polytron PT (Kinematica, Bohemia, NY), and immediately frozen at −80°C until analysis. The samples were thawed on ice and centrifuged at 12,000 *g* for 10 min at 4°C, and the aqueous supernatant was collected. The supernatant was filtered through a Millipore 0.22-µm polyethersulfone disc filter (Millipore, Billerica, MA) under sterile conditions. Protein concentrations were determined by a Pierce BCA Protein Assay Reagent (Quantum

Scientific, Murarrie, Australia) as per the manufacturer's instructions. The biological activity of hGH in soybean transgenic seeds was quantified utilising a GH bioassay (Rowland et al. 2002). Transgenic soybean extracts were serially diluted in assay buffer (RPMI-1640 phenol-red free medium supplemented with 0.5% Serum Supreme newborn calf serum) (Invitrogen) for quantification in a bioassay. A range of recombinant hGH (rhGH) standards were run on each plate, and all protein concentrations were expressed as the final concentration in the assay wells. The results were analysed on Graphpad Prism 5 (Graphpad Software, San Diego, CA), and the trendline was calculated using the log (agonist) versus response curve with a variable slope and a least squares fit.

Sample preparation for mass spectrometry analysis

Soybean seed proteins were extracted following the Sussulini et al. (2007) protocol. A 100-µg aliquot 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 vortexed before being incubated at 80°C in a dry ice bath for 15 min; 2.5 µl of a 100 mM DTT solution was then 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. A 10-µl aliquot of trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate (50 ng μl^{-1}) was used for digestion at 37°C overnight. Following the digestion, 10 µl of 5% trifluoroacetic acid was added to hydrolyze the RapiGest, and the sample then 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). Five microlitres of 1 pmol ul^{-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 with 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% formic acid solution to the pre-column at a flow rate of 15 µl min^{-1} for 1 min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 0.1% formic acid 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 B (GFP) (Sigma-Aldrich, St. Louis, MO) to the reference sprayer of the NanoLockSpray source of the mass spectrometer. All samples were analysed in triplicate. Analysis of tryptic peptides was performed using a Synapt HDMS mass spectrometer (MS; 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 nano-electrospray ion mode (nanoESI+). The TOF analyser of the mass spectrometer was externally calibrated with GFP b^+ and y^+ ions from *m/z* 50 to

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 a 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 to 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) ver. 2.4v (Waters Corp). Protein identifications were obtained with the embedded ion accounting algorithm of the software and searching a soybean 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 (BSA): P02769 (ALBU_BOVIN)] and human somatotropin GH-N [UniProtKB/Swiss-Prot P01241 (SOMA_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 were performed in PLGS with an Expression^E license installed. Intensity measurements are typically adjusted on those components, i.e., 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.

Results

The hGH expression vector

High-level expression of recombinant molecules has been consistently achieved utilising specific regulatory sequences within seed tissues. A co-bombardment transformation strategy to generate transgenic soybean plants allowed us to evaluate the pβcong3hGH plasmid vector (Fig. 1a). The *hgh* gene cloned under control of the α' subunit of the β-conglycinin seed-specific promoter and terminator and the α-Coixin signal peptide was effective in directing the protein to the ER and PSVs. The selection plasmid carried the *ahas* gene under control of the *ahas* constitutive promoter and terminator. The herbicide-resistant *ahas* gene was utilised for selection of the putative transformants on imazapyr, as previously described (Rech et al. 2008). A translational initiation site added at the N-terminus of the α-Coixin signal peptide sequence was efficient in providing the biosynthesis of the mature hGH with the expected molecular weight of 22 kDa.

Generation and analysis of transgenic soybean

Transgenic soybean lines were generated as previously described (Aragão et al. 2000). Following microparticle bombardment, the embryonic axes were cultured in vitro for 6 weeks before the regenerated plantlet lines could be transferred to soil under greenhouse conditions to set seeds. The 308-bp *hgh* gene fragment was detected by PCR in five independent soybean R_0 lines (Fig. 1b). Segregation patterns of the R_1 seed lines were also evaluated by PCR. Lines 3, 9 and 19 showed Mendelian segregation patterns and were chosen for further molecular and biochemical characterisation. All R_0 and R_1 transgenic lines also showed the presence of the *ahas* gene (data not shown). A Southern blot analysis was performed with a probe covering the insertion site of 1,656 bp, corresponding to the signal peptide of *Coix* and the gene of interest after DNA restriction with

*Hin*dIII, as indicated in Fig. 1c. The results showed that the R_1 plants from lines 3, 9 and 19 had integration profiles that were consistent with a multiple insertion event. The utilisation of a radiolabelled probe that was specific to an internal region of the *hgh* gene demonstrated that all lines had at least three to eight copies of the integrated gene in their genomes (Fig. 1c). We have previously reported a similar profile in the case of transgenic soybean expressing the herbicide tolerant *ahas* gene (Aragão et al. 2000). Reverse transcription (TR)-PCR analysis showed the expression of the *hgh* gene on the transcriptional level, with the expected 308-bp fragment observed in lines 3, 9 and 19 (Fig. 1d).

Expression and accumulation of rhGH in transgenic soybean seeds

Western blot analysis was performed to evaluate the expression of the hGH in R_1 seeds from three transgenic lines stored up to 6 years at room temperature (Fig. 2a). Antibodies raised against purified hGH reacted specifically to the 22-kDa hGH molecular-weight components of the protein extracts of seeds from lines 3, 9 and 19. No recombinant hGH protein degradation was detected in the immunoblots, as indicated by the absence of low-molecular-weight hGH fragments below the expected 22 kDa electrophoretic migration position (Fig. 2a). The tissue-specific expression was examined in different organs from an R_1 transgenic soybean plant of line 19. As expected, no signal was observed in protein extracts isolated from transgenic soybean roots, leaves, stems or flowers. The hGH antibody did not recognise any tissue proteins extracted from control soybean plants (Fig. 2b). The kinetics of the hGH protein accumulation during R_1 transgenic seed (line 19) development were evaluated 2, 4, 6, and 8 weeks after pollination (Fig. 2c). The results showed an increased accumulation of hGH protein during seed development. Analysis of the western blots revealed that the accumulation of hGH protein reached its highest level in the immature seeds 6 weeks after pollination. A similar level was maintained in the dried mature seeds that were examined 8 weeks after pollination.

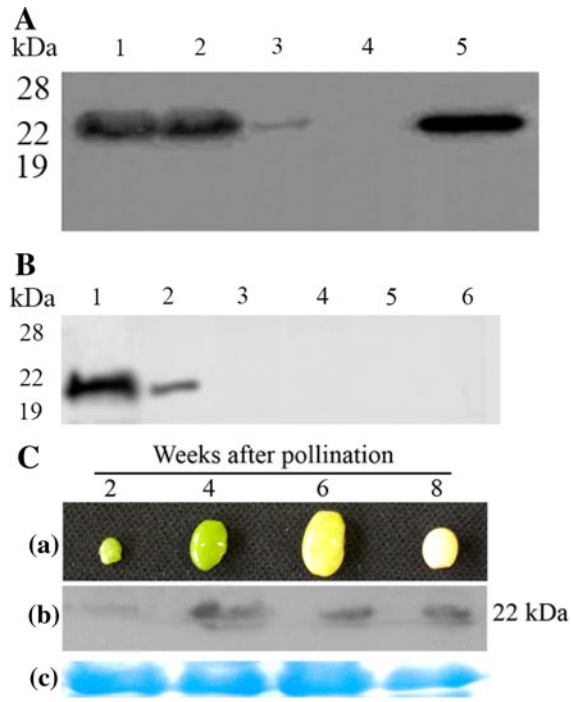


Fig. 2 **a** The accumulation of recombinant human growth hormone (hGH) in transgenic soybean seeds was evaluated by western blot analysis. *Lanes 1–3* Analysis of total soluble protein (TSP) extracts (100 μg) from transgenic R_1 seeds from lines 3, 9 and 19 demonstrated the recognition of the recombinant hGH by specific anti-human hGH primary antibodies. *Lane 4* Extract from a non-transgenic seed, showing no detection of the hGH. *Lane 5* Internal control (100 ng of a recombinant hGH purified from *Escherichia coli*) used to demonstrate the antibody specificity and the electrophoretic migration pattern of the recombinant protein under denaturing conditions. Molecular weight was estimated with the marker Precision Plus Protein Standards All Blue (Bio-Rad). **b** The efficiency of the α' subunit of β-conglycinin promoter to restrict the transgene expression of the transgenic seeds was evaluated by organ-specific western blot analysis. *Lane 1* A total of 100 ng of recombinant hGH purified from *E. coli* was properly detected by primary antibody recognition. *Lanes 2–6* Immunoassays of TSP extracts (100 μg) from seeds (*2*), leaves (*3*), flowers (*4*), stems (*5*) and roots (*6*) of a transgenic plant from line 19 demonstrated the successful detection of the recombinant hGH only in transgenic seeds. All molecular weights were estimated with the marker Precision Plus Protein Standards All Blue (Bio-Rad, USA). **c** (*a*) The hGH kinetics expression in different phenological stages of R_1 soybean seeds from line 19 was demonstrated 2, 4, 6, and 8 weeks after pollination, (*b*) western blot analysis showed the accumulation of hGH protein in each seed developmental stage, (*c*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading controls of each total soluble protein extracts (approx. 100 μg) were utilised to provide a uniform sample electrophoresis

Localisation of rhGH in transgenic soybean seeds

To determine whether the recombinant protein expressed in transgenic soybean accumulated in the PSVs, we observed the subcellular localisation of the hGH protein in mature transgenic soybean seeds from line 19. Electron microscopy images of ultra-thin seed sections treated with hGH-specific antibodies indicated that the recombinant protein in transgenic soybean seeds from lines 9 and 19 mainly accumulated in PSVs, as highlighted by the 20-nm gold particles (Fig. 3a, b). No significant gold particle accumulation was found in the apoplast, starch grains or cytoplasm.

Mass spectrometry

The rhGH protein present in soybean TSP extracts was detected and identified utilising a nanoLC-MS assay. The results demonstrated the correct hGH peptide sequences in transgenic soybean TSP extracts and their respective monoisotopic expected masses and positions (Fig. 4). The carbamidomethyl+C modified peptide sequences were caused by the

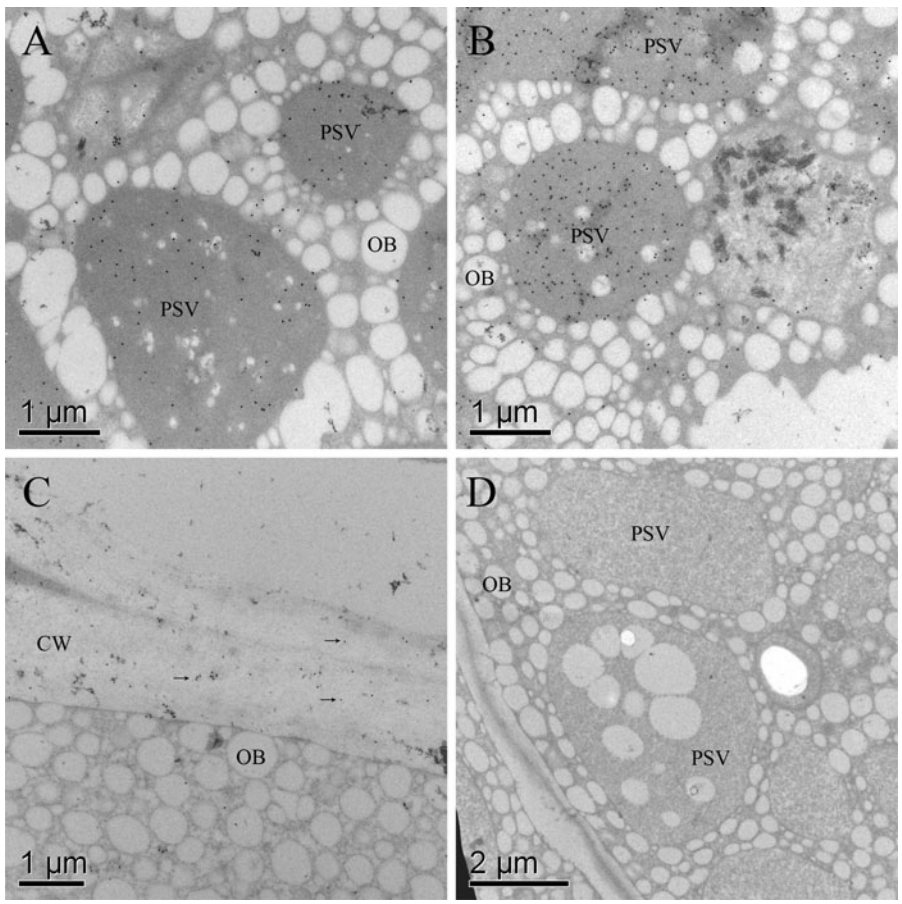


Fig. 3 Protein targeting to protein storage vacuoles (*PSV*) was evaluated by ultrastructural immunocytochemistry of rhGH presence in ultra-thin sections of soybean cotyledons. **a**, **b** Subcellular accumulation of rhGH in the PSV of transgenic R_1 seeds from lines 9 (**a**) and 19 (**b**). **c** Punctate accumulation of rhGH in the cell wall (*CW*) of the transgenic seed from line 9 could also be detected. **d** Recombinant hGH could not be detected in non-transgenic seeds or associated with oil bodies (*OB*)

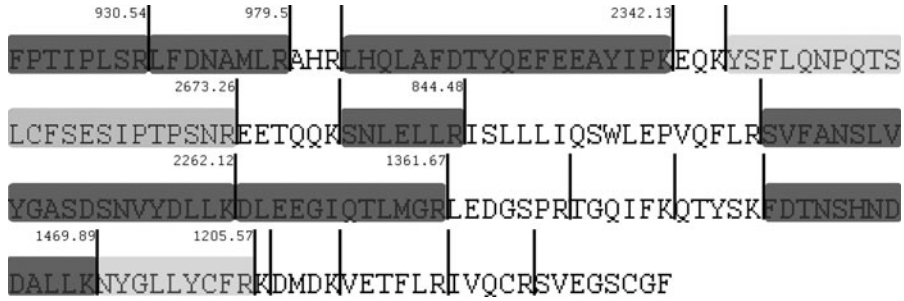


Fig. 4 The full hGH recombinant protein sequence expressed in soybeans. *Black vertical lines* Digestion sites of trypsin, *shaded boxes* sequence peptides confirmed by tandem mass spectrometry (MS/MS) spectra, with *darker shaded boxes* indicating hGH peptide sequences with expected masses and *lighter shaded boxes* indicating sequences with carbamidomethyl+C modification caused by the protein digestion protocol. *Numbers above* the coloured boxes indicate the monoisotopic masses of the corresponding peptide

protein digestion protocol, as described in the Materials and Methods.

Quantification and evaluation of recombinant hGH biological activity

The biological activity of the recombinant hGH present in the extracts of soybean seed lines 3, 9 and 19 was evaluated (Fig. 5). The somatogenic activity bioassay is based on the proliferation of murine BaF/B03-B2B2 clones in response to the presence of active hGH. These stable cells express the wild-type hGH receptor (WT hGHR), and their proliferation is normally dependent on the presence of growth factors, such as hGH. Somatogenic activity was measured by the difference in the cell response in the presence and absence of a high-affinity antagonist able to inhibit hGH activity. Lines 9 and 19 expressed high levels of extracted bioactive hGH, with 2.90 ± 0.069% TSP [mean ± standard error of the mean (SEM) from 3 separate determinations] for line 9 and 2.60 ± 0.01% TSP for line 19 (Fig. 5). The results indicated that seed-derived hGH from both lines acted as efficient spreaders of intracellular signals that led to metabolic activation and the proliferation of B2B2 cells. No significant activity was detected in transgenic seeds from line 3 and non-transgenic seeds.

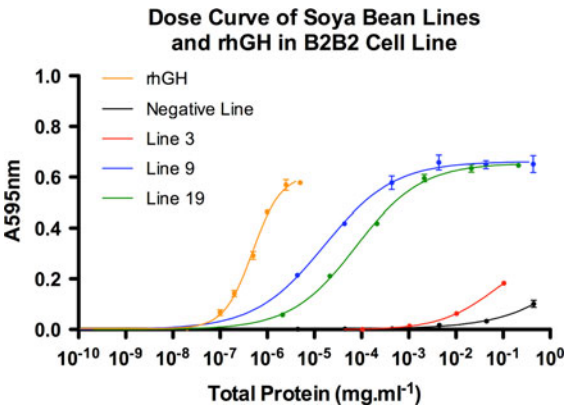


Fig. 5 Proliferation dose-response curve for the rhGH standard and for R_1 seeds of transgenic lines 3, 9 and 19 and non-transgenic seeds (*Negative Line*). Evaluation of the biological activity of rhGH was measured using a somatogenic assay on cells expressing wild-type hGHR

Discussion

We were successful in expressing bioactive hGH in transgenic soybean seeds. A vector carrying a tissue-specific α′ subunit of the β-conglycinin promoter and a α-Coixin signal peptide were evaluated for their abilities to direct hGH to the ER and elicit the accumulation of the recombinant protein in the PSVs. The α′ subunit of the β-conglycinin promoter is tissue-specific and temporally regulated (Chen et al. 1986). This promoter is expressed in both the embryonic axes and cotyledons of developing soybean seeds (Imoto et al. 2008). We obtained five transgenic soybean lines containing both *hgh* and *ahas* genes in a stable integration pattern, as observed in R_1 and R_2 progenies. Stable integration is an essential feature in the development of homozygous lines created for large-scale seed production, protein characterisation and clinical trials. RT-PCR analysis of the immature transgenic seeds demonstrated that expression of the *hgh* gene occurred in all of the

transgenic lines tested, suggesting that the *hgh* gene copies both the position and the integration sites in soybean chromatin, offering minor barriers to RNA polymerase II transcription complex attachment and efficient primary transcript synthesis (Meyer and Saedler 1996). Three transgenic soybean lines displayed Mendelian segregation for the integrated *hgh* and *ahas* genes (data not shown), suggesting that if a high transgene copy number was integrated into the R_0 plants, the alternative DNA copies should be integrated at different loci with a low recombination frequency (Zhao et al. 2007). This result was corroborated in our study by the Southern blot analyses of different R_1 plants from lines 1, 3 and 9, which presented at least eight, four and three *hgh* copies, respectively. One concern related to these high transgene copy numbers that were integrated in the host genome is the possibility of superfluous tandem integration that could result in gene silencing and low recombinant protein production levels (Finnegan and McElroy 1994). Future hybridisation analyses with advanced generation plants will allow for the monitoring of transgene copy segregation in more detail and for associating specific copy patterns with the higher *hgh* gene expression levels. Western blot analysis demonstrated that hGH expression was present in transgenic seeds during all seed developmental stages. As expected, there was no expression of this promoter in soybean roots, stems, leaves or flowers. Regulatory information for the tissue-specific expression mediated by the α' subunit of β-conglycinin promoter and other elements involved in the modulation of the expression of genes associated with seed storage protein has been localised to DNA sequences approximately 250 bp upstream of the transcription start site (Chen et al. 1986; Allen et al. 1989; Fujiwara and Beachy 1994). In our study, the gene encoding the hGH was placed under the control of the α' subunit of β-conglycinin soybean promoter and the α-Coixin signal peptide from *C. lacryma-jobi* (Leite et al. 2000) and introduced into the soybean genome with the aim of stably accumulating and storing hGH in soybean seeds. The accumulation of hGH in transgenic mature seeds from three different lines was detected by western blot, demonstrating that the α' subunit of the β-conglycinin tissue-specific promoter was efficient in directing gene expression to the transgenic seeds. A nanoLC-MS^E tryptic digestion analysis of TSP soybean extracts expressing rhGH demonstrated the correct and expected peptide sequences of hGH. The accumulation levels of the rhGH in mature seeds varied from 2.6 to 2.9% TSP based on the bioassay results. Despite data showing that the average weight of each mature soybean seed (cultivar BR-16) is 200 mg and the average protein content is 40% (Cantoral et al. 1995), our results consistently demonstrated an average TSP content of 30% (data not shown). Consequently, it is possible to estimate that the transgenic seeds could potentially produce up to 9 g hGH kg^{-1} in mature dry seeds. This value markedly exceeds the 0.0008% value reported for soybean expression of hGH driven by the 35S promoter (Russell et al. 2005). In addition, we found no signs of hGH protein degradation within the soybean seeds. These results are in accordance with previous findings that describe the expression of hGH in the chloroplasts of tobacco leaves by an agroinfiltrating-viral vector (Gils et al. 2005). In contrast, significant levels of hGH degradation products were detected in other rhGH plant-derived systems and/or within crops (Russell et al. 2005).

The kinetic accumulation pattern indicates that the temporal modulation of seed hGH availability during the maturation cycle reached its peak by the middle of the phenologic plant cycle (approximately 4 weeks after pollination) and that a steady expression occurred until the dry seed mature stage. This result could also be correlated with the kinetics pattern of the PSV organelles' availability (Yoo and Chrispeels 1980). If this were the case, an increase in hGH accumulation in transgenic soybean seeds during development would be closely related to the increase in the number of PSVs during seed development.

Our studies using ultrastructural immunocytochemistry assays also indicate that the rhGH in seed cotyledonary cells was efficiently directed to the PSVs. rhGH molecules were observed all along the cisterna lumen, but they were absent in the interior of the seed oil bodies. Soybean seeds expressed the expected 22-kDa hGH molecular mass, suggesting that the cleavage and removal of the α-Coixin signal peptide in the ER was correctly performed during protein post-translational processing and that subcellular targeting was correctly promoted. These findings are in accordance with previous ones that evaluated different nuclear transformed plant systems as potential bioreactors (Daniell et al. 2001; Ma et al.

2003; Stoger et al. 2005). Our results corroborate the spatial and temporal transgene expression expected by the molecular strategy adopted our work: the use of the tissue-specific seed α' subunit of the β-conglycinin promoter and α-Coixin signal peptide for recombinant protein subcellular targeting.

In terms of hGH protein storage capability, the utilisation of transgenic soybean seeds to target recombinant proteins may constitute a viable option for a molecular farming system (Giddings 2001; Twyman et al. 2003). Our results show that five transgenic soybean lines generated 6 years ago and stored at room temperature (22 $\pm$ 2°C) still contained bioactive hGH. These results are not only in agreement with previous findings (Fiedler and Conrad 1995; Stoger et al. 2000; Larrick and Thomas 2001; Ramírez et al. 2001) but also expand the previously evaluated storage periods. Based on our findings, we conclude that targeting the PSVs was an effective strategy for achieving stable accumulation of functional hGH protein in seeds for up to 6 years (data not shown). This long-term stability of hGH in soybean seed is an important feature which may allow a temporal downstream processing (Boothe et al. 2010). Due to the potential utilisation of soybean seed-derived hGH for injections in humans, experiments have been carried out to purify hGH and characterise the protein according to the current good manufacturing practice processing (Boothe et al. 2010). In addition, experiments have also been conducted to evaluate the potential utilisation of soybean seeds for producing hGH for animal and fish feed.

The soybean has a number of intrinsic and unique characteristics, including high biomass capacity, short-life cycle and photoperiod sensitivity. Specifically, the photoperiod sensitivity characteristic enables high vegetative growth and flowering delay to be manipulated by simply utilising a photoperiod of 19 h of light. This in turn will cause a tenfold increase in seed production (average of 1,000 seeds/plant) when compared with seed production under field conditions. In addition, the high-protein synthesis capability of soybean make soybean seeds an attractive target system for the production of proteins. Therefore, altering the DNA of soybean seeds to direct recombinant proteins to protein storage vacuoles has a great potential as a practical approach for the commercial production of biopharmaceuticals for humans and animals.

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