

Targeted expression of human serum albumin to potato tubers

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Abstract

Complementary DNA expression of mature human serum albumin was engineered into potato plants under the transcriptional control of patatin B33 promoter and potato proteinase inhibitor II terminator. Protein secretion was achieved by using the signal sequence from potato proteinase inhibitor II. Recombinant albumin accumulated up to 0.2% of total soluble tuber protein in single transformant lines, regardless of the potato cultivar used. Electrophoretic mobility and N-terminal amino acid sequence analysis of partially purified recombinant albumin confirmed proper processing of an immune responsive recombinant albumin, and revealed that the proteinase inhibitor II signal sequence was correctly removed. No further optimisation of these yields was obtained by HSA expression in patatin antisense plants (line Pas58). Subcellular localisation showed that recombinant protein was successfully targeted to the apoplast. Potato tubers may be used, by applying this technology, to produce other heterologous proteins of interest in the biopharmaceutical industry.

Introduction

Human serum albumin (HSA) is the serum protein most widely used in the world for medical purposes. Commercial albumin is obtained basically from human plasma. As albumin demand continuously increases world-wide, finding alternative ways to obtain HSA is highly attractive, both to decrease the dependence on blood banks and to reduce possible health hazards, such as AIDS and hepatitis, that may be transmitted during infusion of contaminated blood-derived products.

There is a wide range of organisms potentially suitable, though not all equally amenable, to produce pharmaceutical proteins. It is essential to develop appropriate expression systems to ensure the production of those proteins that are required in

large amounts. Plant-derived biopharmaceuticals are cheap to produce and store, easy to scale-up for mass production and safer than those derived from animals (Daniell et al., 2001). Moreover, clinical trials are proceeding on the first plant-derived biopharmaceuticals, and one of them is now being commercially produced in Canada (Giddings et al., 2000).

Potato tubers would seem an organ of choice for the production of HSA since the same standard processes used in the starch industry may be adapted with little modifications to separate proteins. Also, the tubers, as most of the storage organs, offer a low hydrolytic profile, which facilitates protein stability. There is one report on recombinant HSA (rHSA) production in both tobacco and potato using the CaMV 35S promoter (Sijmons et al., 1990). The highest level attained was, however, too low for most commercial applications (0.02% of the total soluble protein in

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shoots and young leaves, and five times lower in roots and tubers).

Several genes have been expressed in potato tubers using different techniques with varying degrees of success (Castañón et al., 1999; Artsaenko et al., 1998; Ehsani et al., 1997; Mason et al., 1996). Considerable success has been achieved with the patatin promoter, which confers tuber specificity (Artsaenko et al., 1998; Mason et al., 1996).

Human serum albumin is produced in the liver and rapidly secreted to the extracellular space. Hepatocytes initially synthesise albumin as a prepro-protein of 609 amino acids (Strauss et al., 1977), and subsequent postranslational processes give rise to a mature protein with 585 residues (Dugaiczky et al., 1982). Secretion of HSA appears to be a requirement for proper folding with disulphide bridges. Consequently, a presequence would likely be needed in tubers. It would provide a methionine to initiate the translation and latter would be removed releasing the mature HSA. Targeting the rHSA expression to the secretory pathway would therefore seem appropriate. In the present study, the expression of medium to high levels of rHSA in potato tubers has been undertaken.

Materials and methods

Plasmids, bacteria and plants

The following plasmids, bacterial strains and plants were used throughout this study. Plasmids: pBIN19 (Bevan et al., 1984), pB33Hyg (provided by Dr. L. Willmitzer, Max-Planck Institute for Molecular Plant Physiology, Golm) and pCT58 (Rosahl et al., 1986). Bacterial strains: *E. coli* XL1-Blue (Stratagene) and *Agrobacterium tumefaciens* GV2260 (Deblaere et al., 1985). Plant material: *Solanum tuberosum* sp *tuberosum* L. cv. Désirée, cv. Kennebec and patatin antisense Pas58 transgenic line (Höfgen & Willmitzer, 1992).

Construction of plant rHSA expression vectors

The pTUB3 vector was used to achieve tuber-specific expression of HSA. It was constructed as follows: a 1.5 kb patatin B33 promoter (Rocha-Sosa et al., 1989) was cloned as an *EcoRI/KpnI* fragment in pBIN19 containing at its *SphI* site the 3' end of the potato proteinase inhibitor II (Pin2) gene (Keil et al., 1989) to produce pTUB2. The signal peptide from the Pin2 gene (Sánchez-Serrano et al., 1986) was

subsequently introduced into pTUB2 to provide the signals for protein import into the endoplasmic reticulum. To this end, a 1078 bp-long PCR-fragment of the Pin2 promoter region (from -807 to +271 regarding the transcription start of the Pin2 gene; Keil et al., 1986) was obtained with the oligonucleotides 5'-GCGAATTCAATTTCTACCACCACCCAAA-3' and 5'-GTGCAGGCCTTCGCGTCGACATGC-3' as primers. This latter introduces three mutations to the authentic sequence to provide *SalI* and *StuI* restriction sites at the 3' end of the fragment for subsequent cloning (introduced mutations do not alter the amino acid sequence). Digestion of this fragment with *ScaI* (at position +32) and *StuI* generates a fragment of 232 bp in size that encompasses the putative processing site and the only intron of the Pin2 gene. This fragment was cloned in the *SmaI* site of pTUB2 to generate pTUB3.

The HSA cDNA was amplified by RT-PCR from total RNA derived from human liver cells using the oligonucleotides HSA1 (5'-GCACGCGTCGACGCGGATGCACACAAGAGTGAGGT-3') and HSA2 (5'-GCACGCGTCGACGATGTTATAAGCCTAAGGCA GC-3') as primers, thus introducing for cloning purposes *SalI* sites at both ends of the sequence of the mature HSA. In addition, HSA1 introduces an alanine (A) residue before the first amino acid of the mature HSA to engineer the putative processing site (VDA/DAH) of the Pin2-HSA protein fusion. This PCR fragment, digested with *SalI*, was cloned into the *SalI* sites of pTUB3 to generate pTUB3HSA. Sequencing of the junctions after all cloning steps was performed to assess the fidelity of the constructions.

The 3.7 kb *EcoRI/HindIII* fragment of pTUB3HSA, which contains the HSA expression cassette, was cloned into a pB33Hyg also digested with *EcoRI/HindIII*, to generate pTUB3HSAHyg. The pB33Hyg is a pBIN19-derived plasmid, which contains the hygromycin resistance gene.

Transformation of potato plants and selection of transgenic lines

Vector constructs were transferred into *A. tumefaciens* GV2260 by direct transformation (Höfgen & Willmitzer, 1988). The integrity of the construct was assessed by Southern blot analysis. Potato leaf explants (cvs. Désirée & Kennebec) were transformed with *A. tumefaciens* strain GV2260 harbouring plasmid pTUB3HSA as described (Dietze et al., 1995), except that 1 mg/l of NAA in the callus induction

medium was used for cv. Kennebec. Transgenic line Pas58 was transformed with GV2260 strain harbouring plasmid pTUB3HSAHyg and regenerated explants were selected on hygromycin (Dietze et al., 1995).

The presence of the transgene in transformed plant tissue was determined by PCR on genomic DNA (1 µg) isolated from leaf tissue of transgenic potato plants (Sul & Korban, 1996), using HSA-specific forward and reverse primers (5'-GATGCACACAAGAGTGAGGT-3' and 5'-GATGTTATAAGCCTAAGGCAGC-3', respectively). Positive samples will result in amplification of a 1758 bp fragment of the HSA sequence. Southern blot analysis was performed after digestion of 30 µg of genomic DNA with *Hind*III that cuts only once the T-DNA. A 1.77 kb *Sal*I fragment of pTUB3HSA was used as a ³²P-labelled HSA probe.

All transgenic lines were grown under controlled glasshouse conditions for production of transgenic tubers, which were used in further analysis.

Analysis of HSA gene expression in potato tubers

The transcriptional expression of HSA gene was estimated by northern blotting. Total RNA was extracted from tuber tissue as described (Logemann et al., 1987). Hybridisations were carried out using three different ³²P labelled DNA probes: HSA probe as described above, patatin probe was a 1.5 kb *Pst*I fragment of pcT58 plasmid and constitutive probe, a 3.2 kb *Kpn*I/*Bam*HI DNA fragment derived from the 25S subunit ribosomal RNA. Radioactivity was quantified with a GS-250 Molecular Imager (Bio-Rad).

Gene expression of HSA was also analysed at the protein level. The extraction of total soluble proteins from first generation potato minitubers and subsequent analysis of rHSA by western blot were performed essentially as described (Fütterer et al., 1995). Approximately 40 µg of total protein was separated by electrophoresis on 10% SDS-PAGE and subsequently transferred onto a nitro-cellulose membrane. Primary antibody (rabbit anti-HSA; Nordic Immunology, Tilburg, Netherlands) was used at 1 : 10,000 dilution, with a 1 : 5000 dilution of goat anti-rabbit HRP conjugated secondary antibody. Immunoblot detection was performed by chemiluminescence (ECL western blotting, Amersham). Recombinant HSA protein levels were determined in second generation transgenic potato tubers by a quantitative ELISA assay (Human Albumin ELISA Quantitation Kit; Bethyl, Texas, USA) using high binding micro-

titre plates (Corning Inc.). Different dilutions of duplicate samples were analysed. Extraction of total soluble proteins from potato tubers for rHSA quantification was performed as previously described (Ehsani et al., 1997). Total soluble protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as standard.

Protein purification and N-terminal amino acid sequencing ascertained the identity and proper processing of the expressed protein as a mature rHSA. The procedures described by Goodey et al. (1998) were essentially followed. Two hundred millilitre of 25 mM phosphate buffer (pH 7.4) were added to 100 grams of transgenic fresh tubers, and the mixture was homogenised. After centrifugation (15 min, 10,000 g), the supernatant was loaded onto a column (1.6 × 20 cm) of DEAE Sepharose FF (Pharmacia) and a linear gradient elution followed (NaCl from 0 to 0.6 M). The rHSA containing fractions were located by SDS-PAGE. The partially purified rHSA was desalted with a HiPrep 26/10 desalting column (Pharmacia) and loaded onto an affinity column (2.6 × 18 cm) of Blue Sepharose FF (Pharmacia); rHSA was eluted with 2 M NaCl buffer. The rHSA fractions were pooled, desalted with HiPrep column, concentrated by lyophilisation and transferred to a PVDF membrane. N-terminal amino acid sequence analysis was performed by automated Edman degradation.

Analysis of rHSA secretion to the apoplast

In order to analyse a possible secretion of rHSA to the apoplastic compartment, expression of recombinant protein was induced in transgenic potato leaves by incubation in 8% sucrose solution for 24 h in continuous light (Peña-Cortés et al., 1992). To obtain extracellular fluid, discs of induced leaves were vacuum infiltrated for 15 min in 66 mM phosphate buffer pH 7.0, 100 mM KCl, dried and the extracellular fluid collected by centrifugation (15 min, 600 g). Assays for marker enzyme activity and protein content followed. G6PDH and UGPase were used as cytosolic marker enzymes (Castillo et al., 1984; Sowokinos et al., 1993) and peroxidase as apoplastic marker enzyme (Castillo & Greppin, 1986). Total soluble protein samples were prepared from the same plants, by homogenising in infiltration buffer with insoluble PVP (0.5 g/ml). Equal protein samples from both fractions were analysed by western blotting as described above.

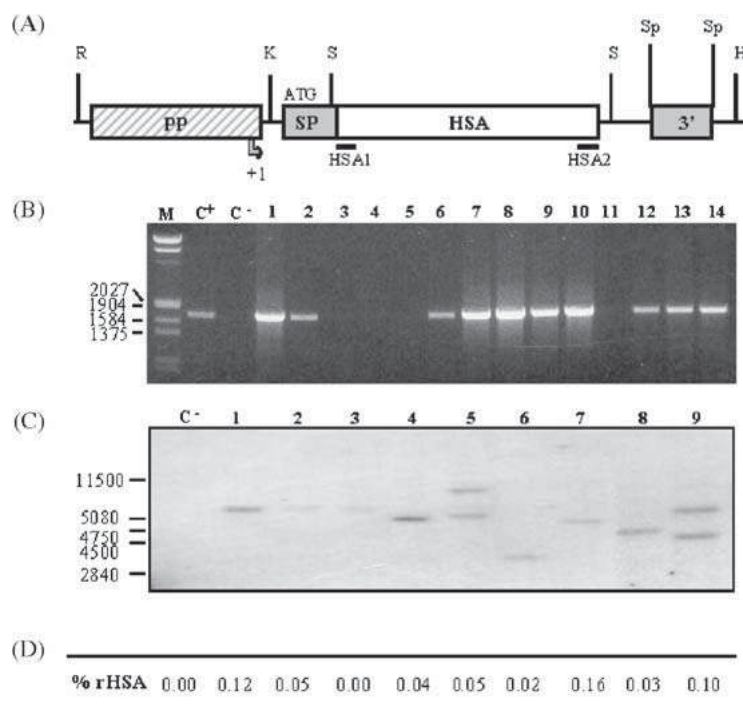


Figure 1. (A) *EcoRI/HindIII* fragment of plasmids pTUB3HSA or pTUB3HSAHyg. PP: patatin B33 promoter. SP: Pin2 signal peptide. HSA: mature human albumin sequence; 3': Pin2 3' end. Main restriction sites are: R-*EcoRI*, K-*KpnI*, S-*SalI*, Sp-*SphI* and H-*HindIII*. +1: transcription start. ATG: translation start. (B) Presence of the HSA gene in transgenic plants was determined by PCR using gene specific primers (HSA1 and HSA2) that amplified a 1758 bp fragment. Genomic DNA was extracted from *in vitro* cultured leaves. Bands were visualised with ethidium bromide in 1% agarose gel. Lanes are M: molecular weight marker λ -*EcoRI/HindIII*; C⁺: PCR product of plasmid pTUB3HSA; C⁻: untransformed plants; 1–14: PCR product of kanamycin regenerated potato plants. (C) Southern blot of genomic DNA, from different PCR+ lines, digested with *HindIII*, and hybridised with a ³²P-labelled HSA probe. (D) rHSA levels (as percentage of rHSA over total soluble protein) in tubers of transgenic plants showed in Figure 1(C).

Statistical analysis

The rHSA level in potato tubers of different transgenic lines was estimated with the statistical program SAS[®] System (SAS Institute). Ln($x+1$) transformation of data was accomplished, and Shapiro–Wilk test (Conover, 1980) was used to test the assumption of normality. Transformed data were analysed by ANOVA and mean separation was performed by Duncan test ($p < 0.05$) (Snedecor & Cochran, 1969).

Results and discussion

Transformation of potato tubers with HSA expression plasmid and stable integration of the transgene

The patatin promoter B33 (Rocha-Sosa et al., 1989) was used to drive expression of a chimeric gene (Figure 1(A)) consisting of a mature HSA sequence fused to a signal peptide derived from the Pin2,

a 12 kDa protein constitutively expressed in potato tubers (Sánchez-Serrano et al., 1986). Since HSA was being expressed under transcriptional control of the patatin promoter, and transgene expression levels may depend on the transformed cultivar (Blundy et al., 1991), two potato hosts (cvs. Désirée & Kennebec) with different patatin levels were chosen for transformation with the expression plasmid pTUB3HSA; this construct contains *nptII* (neomycin phosphotransferase II) as marker gene conferring kanamycin resistance.

There is evidence that the patatin promoter can be repressed in tubers (Bourque et al., 1987; Grierson et al., 1994), possibly through activation of a feedback mechanism when high levels of patatin are reached. Consequently, the use of patatin antisense plants (transgenic line Pas58) as host for transformation with HSA expression plasmid hypothetically could result in higher levels of recombinant protein. To obtain the double transgenics (patatin antisense and HSA transformations), a new HSA expression plasmid

conferring hygromycin resistance, pTUB3HSAHyg, was required because the host line Pas58 already displays kanamycin resistance.

Transgene insertions in genomic DNA were verified by PCR screening. Figure 1(B) shows that transgenic plants (lanes 1–2, 6–10 and 12–14) and false positives (lanes 3–5, 11) are clearly identified. Although, as estimated by Southern blotting, most of the transgenic lines showed a single copy of T-DNA, 20–40% of them contained several copies (Figure 1(C)), depending on the cultivar. As described by other authors (Hobbs et al., 1993) there was no correlation between transgene copy number and protein levels (Figure 1(D)). Each transgenic line showed a distinctive pattern of bands (Figure 1(C)), suggesting different transformation events.

Accumulation of rHSA in transgenic potato tubers

The presence of rHSA in transgenic potato tubers was detected by western blotting using HSA specific antibody. A total of 98 transgenic lines were analysed for cv. Désirée, 30 for cv. Kennebec and 41 for line Pas58. Figure 2 shows the results with a few samples of transgenic plants in which a band with an identical relative mobility to commercial HSA is present. Sometimes additional immuno-positive bands of smaller molecular weight were also observed (Figure 2). These are likely products of proteolytic degradation that may occur during extraction. Accumulation levels were variable among transgenic lines (Figure 2) as expected, due to random integration. Indeed, ELISA analysis showed that rHSA levels among transgenic lines were quite variable within each cultivar (Figure 3), however its distribution was similar within transgenic lines of cultivars Kennebec and Désirée. However, as depicted in Figure 3, the distribution of frequencies for double transgenic lines was comparatively shifted to the left. This would explain the significantly lower rHSA contents found in double transgenic lines (Table 1). Also, the percentage of transformants without rHSA in the tubers of double transgenic lines was almost twice as high as in both single transgenic cultivars (Figure 3). Mean rHSA levels found were 0.051% for cv. Kennebec, 0.056% for cv. Désirée and 0.032% for double transgenics (Table 1). The highest level was 0.2% of the tuber total soluble protein (0.01 g/kg of tuber fresh weight) for line 133 of cv. Désirée (Table 1).

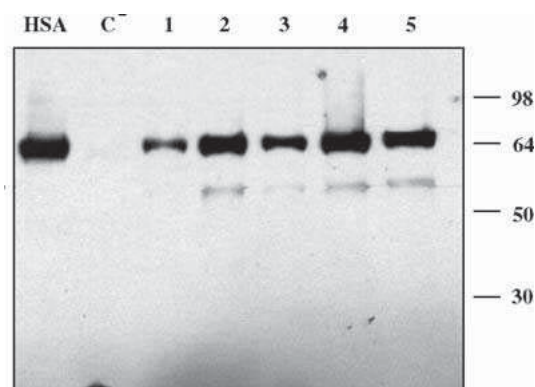


Figure 2. Western blot of first generation transgenic tubers expressing rHSA; 40 µg of tuber protein (Fütterer et al., 1995) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were hybridised with a specific HSA polyclonal antibody (Nordic Immunology). Lanes are HSA: tuber protein from untransformed plants mixed with 40 ng of pure exogenous HSA (Sigma); C⁻: protein extract from untransformed tuber as a negative control; 1–5: tuber proteins from different transgenic lines grown in a glasshouse. To the right, molecular weights in kDa (NovexTM, San Diego, USA).

The similar rHSA levels found in cvs. Désirée and Kennebec, which have different endogenous patatin contents, may be explained by the use of patatin promoter B33, which belongs to subclass IB (Blundy et al., 1991). It is not clear why the rHSA levels in patatin antisense plants are lower than in cvs. Kennebec and Désirée. The low levels may be attributed to the transgenic line Pas58 itself, or to the fact of being a double transgenic. Höfgen and Willmitzer (1992) reported that in line Pas58 there was a decrease in total tuber protein. It could be hypothesised that the total amount of tuber protein might determine the recombinant protein background allowed to accumulate. Alternatively, despite that regulatory sequences (35S promoter and *nos* terminator) of patatin antisense are different from those used for HSA, gene silencing may occur in a double transgenic harbouring other T-DNAs homologous sequences. This may also explain why the percentage of plants containing the transgene but not expressing rHSA in their tubers is almost double than in the other two cultivars (Figure 3).

It is not easy to compare the highest transgenic rHSA levels reported here with those obtained in potato for other proteins for at least two reasons: first, the different expression cassettes used; second, the effect of the protein itself being expressed (Mason et al., 1996; Verwoerd et al., 1995). The highest HSA level reported here (0.2% of the total soluble protein in tubers) is higher than that previously described

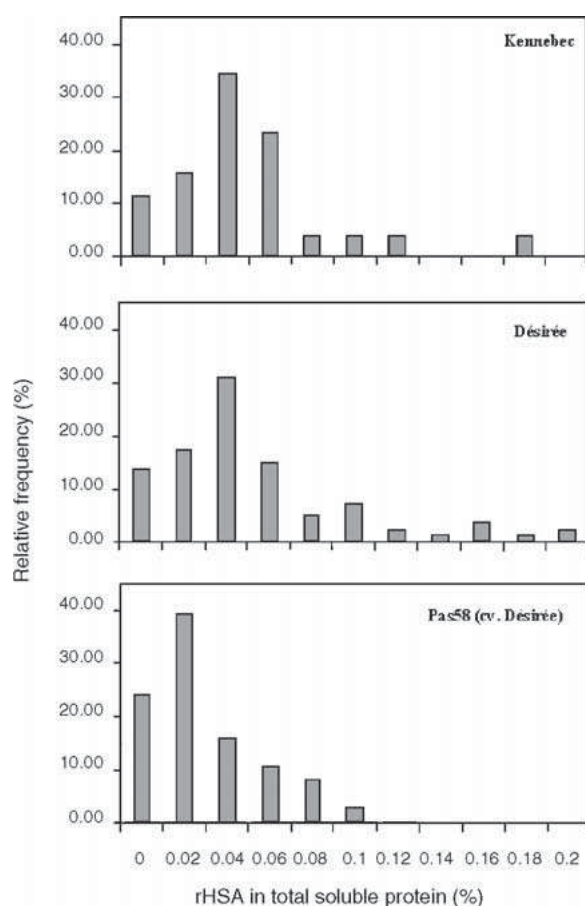


Figure 3. Frequency histogram of percentage of transgenic plants (cv. Kennebec, Désirée and line Pas58) expressing rHSA. Results indicate percentage of transgenic plants (vertical axis) expressing a given percentage of rHSA over total soluble protein from second generation tubers of glasshouse grown plants (horizontal axis). A total of 81 lines of cv. Désirée, 26 of cv. Kennebec and 38 of line Pas58 were analysed.

(Sijmons et al., 1990). Such differences may be attributed to position effects, which are rather common in nuclear transformation, but they do not appear likely after the results of many transgenic lines analysed. The expression cassettes used differ in various ways: (i) B33 patatin promoter versus CaMV 2 × 35S promoter; (ii) Pin2 versus NOS terminator; (iii) and potato tuber Pin2 versus tobacco PR-S signal peptides.

There are data indicating that promoter strength of patatin PS20 and CaMV 2 × 35S are similar in micro-tubers (Castañón et al., 1999; Mason et al., 1996; Wenzler et al., 1989). Other reports comparing different patatin promoters suggest that subclass IB promoters (as B33) improve transgene expression in comparison to other patatin promoters as PS20 (Liu et al., 1991). Thus, patatin promoter B33 may be somehow

responsible for the higher rHSA levels obtained in our work despite the report by Artsaenko et al. (1998), who found lower expression of recombinant antibodies under the control of the B33 compared to the 35S promoter in potato tubers. In addition, the 3' untranslated region of the Pin2 was an efficient terminator for transgene expression (An et al., 1989) due to the presence of 3' region elements acting on the stability and processing of mRNA. Richter et al. (2000) also found that the 3' end of Pin2 gene strongly contributes to enhance expression. Finally, there is substantial evidence that compartmentalisation of heterologous proteins plays a key role in protein accumulation (Schillberg et al., 1999; Conrad and Fiedler, 1998; Schouten et al., 1996). Despite that in both the present work and Sijmons's (Sijmons et al., 1990) rHSA is directed towards the apoplast, the presence of a 117-nucleotide intron, very rich in A+T (83%), within the Pin2 signal peptide sequence (Keil et al., 1986) is noteworthy. Some introns increase transgene expression levels due to greater mRNA stability (Mitsuhara et al., 1996).

HSA transcript levels in transgenic tubers

Transcript levels in tubers varied between transgenic lines, both within each cultivar and between cultivars (Figure 4(A)). Tubers of transgenic lines accumulated HSA mRNA (Figure 4(A), lanes 3–8, 11–15 and 17–23). Messenger RNA was not detected in control untransformed lines (Figure 4(A), lanes 1, 9 and 16), nor in transgenic lines without rHSA in their tubers (Figure 4(A), lanes 2 and 10).

In potato plants transformed with patatin-GUS fusions, there was a good correlation between GUS mRNA levels and enzyme activity in different organs (Blundy et al., 1991; Rocha-Sosa et al., 1989). Eibl et al. (1999) define translation efficiency as the ratio between GUS activity and mRNA levels. Mason et al. (1996) estimated the translation efficiency by the slope of the regression line between protein and mRNA. We found a correlation between transcript levels and the HSA expression in tubers of cv. Kennebec (Figure 4(C)). Also, patatin transcript levels were similar in four different lines of cv. Kennebec (data not shown). This suggests that an active patatin promoter may result in a correlation between transcript and protein levels.

Translation efficiency may be regulated by the preferential use of codons in different organisms (Perlak et al., 1991; Adang et al., 1993). There are little differences in codon usage between humans and

Table 1. Recombinant HSA (rHSA) expression in transgenic potato tubers of cv. Kennebec, Désirée and line Pas58; rHSA levels were quantified by ELISA and expressed as a percentage of total soluble protein from second generation tubers of glasshouse grown plants

Cultivar	PCR+ lines	rHSA expressing lines	Level of expression mean (SE) ¹	Maximum level of expression
Kennebec	26	23	0.051 (0.008) a	0.17
Désirée	81	70	0.056 (0.005) a	0.20
line Pas58	38	29	0.032 (0.005) b	0.10

¹ Values followed by different letter were significantly different by Duncan test ($p < 0.05$).

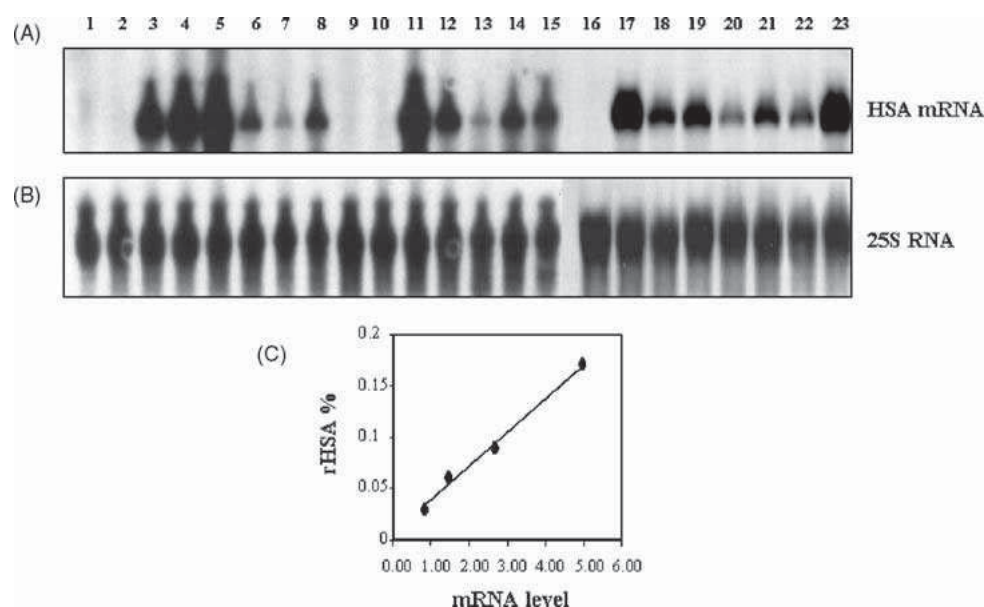


Figure 4. Northern blots of total RNA from transgenic potato tubers and control tubers of cv. Désirée, cv. Kennebec and line Pas58. Blots of 20 µg total RNA from tubers of glasshouse plants were made on a nylon membrane and hybridised with different probes: (A) specific HSA probe; (B) probe for subunit 25S ribosomal RNA. Exposure time for the constitutive probe was 10 times shorter to that for the HSA probe. Lanes: 1, 9 and 16: untransformed Désirée, Kennebec and line Pas58; 2–8: independent transgenic lines of cv. Désirée; 10–15: independent transgenic lines of cv. Kennebec; 17–23: independent transgenic lines of Pas58. (C) relationship between HSA transcript levels (arbitrary units quantified with GS-250 Molecular Imager (Bio-Rad) and corrected by RNA of constitutive band) and percentage of rHSA in transgenic tubers of cv. Kennebec. Recombinant HSA data (%) come from three independent measurements of one tuber. In the graph, each dot represents a transgenic line.

S. tuberosum. The percentage of CGs in albumin (42.89%) is almost identical to *S. tuberosum* (42.95%). The greatest limitation reported in plants derived from the scarcity of CG, is probably due to its involvement in methylation processes in gene regulation (Murray et al., 1989). Codons for amino acids Ala, Pro,

Ser and Thr containing CG for the two last nucleotides are very rare in the albumin sequence. The same occurs for arginine codons starting with CG. Furthermore in cases of codon usage limitations, recoding the transgene 5'-end is sufficient to significantly increase

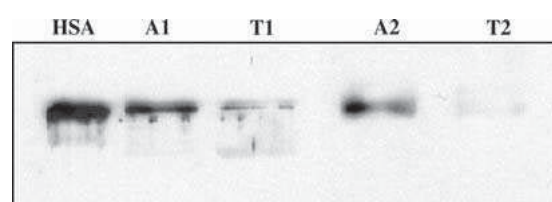


Figure 5. Western blot of apoplastic and total extracts of sucrose-induced leaves for transgenic line Kb18, from two independent extractions (1 and 2). HSA: 40 ng of human albumin (Sigma); A1: 4.0 µg of apoplastic protein extract; T1: 5.2 µg of total protein extract; A2: 2.2 µg of apoplastic protein extract; T2: 2.9 µg of total protein extract.

Table 2. Soluble protein and G6PDH, UGPase and peroxidase activities, in apoplast and total extracts of potato leaf cells

	<i>n</i>	Total extract	Apoplast
G6PDH (nmol NADPH min/g fw)	4	182.5 ± 33.5	0.578 ± 0.042 (0.3)
UGPase (nmol NADH min/g fw)	9	1046.2 ± 76.9	12.2 ± 2.4 (1.2)
Peroxidase (ΔA_{470} min/g fw)	8	43.8 ± 3.6	7.8 ± 0.8 (17.8)
Soluble protein (mg/g fw)	24	7.3 ± 1.0	0.042 ± 0.003 (0.6)

Values are means ± standard error from triplicate samples of *n* independent extractions.

fw: fresh weight; *n*: number of extractions; numbers in brackets are percentage activity in the apoplast in relation to total activity.

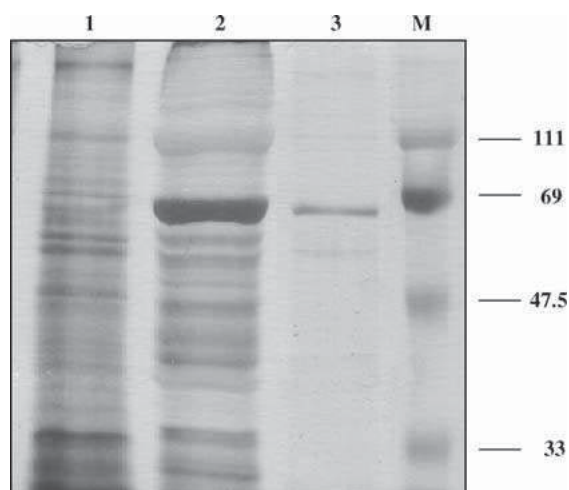


Figure 6. FPLC fractions in silver stained polyacrylamide gel. Lanes: 1: total extract, diluted 1:10; 2: fraction from *DEAE Sepharose*; 3: fraction from *Blue Sepharose*; M: molecular weight marker. Molecular weights, in kDa, appear to the right.

its expression in plants (Batard et al., 2000; Perlak et al., 1991). In this work, the 5'-end of the transgene is correspondent with and has the same codon bias as the signal peptide sequence of Pin2 from potato. All this suggests that codon usage would not be an important limiting factor for albumin translation.

Signal peptide of rHSA fusion protein was properly processed in transgenic tubers

In order to verify both a correct processing of the rHSA signal peptide and an N-terminal end identical to native HSA, rHSA was partially purified, and its N-terminal end sequenced. Purification achieved by the affinity column was sufficient for amino end sequencing (Figure 5, lane 3). Sequencing of the six first amino acids (DAHKSE) of the N-terminal end showed that the signal peptide of the rHSA was

properly processed giving rise to a mature protein identical to native HSA in terms of primary structure.

Some reports using the Pin2 signal peptide fused to the yeast invertase (Heineke et al., 1992; Sonnewald et al., 1991; von Schaewen et al., 1990) did not report the sequence of its N-terminal end. In other work, an unspecific break took place between the potato Pin2 peptide signal and a truncated xylanase gene in tobacco (Herbers et al., 1995). Our results show that the Pin2 signal peptide, fused to the mature HSA sequence, is efficiently and reliably processed in potato.

Subcellular localisation of rHSA

Previous work with yeast invertase fused to Pin2 signal peptide showed apoplastic localisation of the heterologous protein in tobacco and potato transgenic plants (Heineke et al., 1992; von Schaewen et al., 1990). To avoid difficulties inherent to tuber tissue, we assumed that subcellular localisation in tuber and leaves would be similar. We found that the peroxidase activity in the apoplastic fraction of sucrose-induced leaves was 17.8% of the activity in the total extract (Table 2). A low cytosolic contamination, as revealed by G6PDH or UGPase activity (Table 2), was present, as already reported (Castillo et al., 1984; Baroja-Fernández et al., 2000).

Western blots of total soluble protein from total and apoplastic extracts of induced leaves showed a higher amount of rHSA in the apoplast than in the total extract (Figure 6) for two independent extractions, suggesting that rHSA is secreted to the apoplast of sucrose-induced leaves. Given the high degree of conservation of protein compartment signals in cells, we may speculate that rHSA in potato tubers is also secreted to the apoplast.

Secretion to the apoplast offers several advantages. First of all, restriction of rHSA to such a compartment will make subsequent isolation easier since no

membrane breaking detergents would be necessary and, consequently, homogenates would contain lower amounts of other proteins. It will contribute to separate rHSA from cytosolic proteases (Firek et al., 1993) and will provide protein exposure to ER protein disulphide isomerases, which may be needed for proper folding of HSA. Moreover, the signal peptide is naturally processed in the ER, releasing the mature protein without external proteolytic action.

Estimates by industry suggest that the substantial improvement in HSA accumulation obtained in this work, so far, is not cost-effective in pharmaceutical production (a sufficient yield of rHSA for this purpose appears to be ca. 0.1 g of HSA per kg of fresh weight). Expression levels through nuclear transformation could perhaps be increased by the use of Ω sequences (Turner et al., 1999). Though it is not anticipated that the desired levels might be reached, the nuclear transformation technology presented here may be used, without any further improvements, for the production of other pharmaceutical proteins. Another promising alternative could be the use of homologous recombination through plastid transformation (Daniell, 1999).

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