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Alicia Fernández-San Millán
University of Central Florida

Angel Mingo-Castel

Michael Miller

Henry Daniell
University of Central Florida

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Alicia Fernández-San Millán[1], Angel Mingo-Castel[2], Michael Miller[3] and Henry Daniell[1]*

[1]*Department of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Science Building #20, Room 336, Orlando, FL 32816-2360, USA*
[2]*Institute of Agrobiotechnology and Natural Resources, Public University of Navarra-CSIC, Mutilva Baja, 31192 Navarra, Spain*
[3]*Auburn University Research Instrumentation Facility – Advanced Microscopy and Imaging Laboratory, Auburn, AL 36849, USA*

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**Correspondence* (fax +1 407 823 0956;
e-mail daniell@mail.ucf.edu)

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Summary

Human Serum Albumin (HSA) accounts for 60% of the total protein in blood serum and it is the most widely used intravenous protein in a number of human therapies. HSA, however, is currently extracted only from blood because of a lack of commercially feasible recombinant expression systems. HSA is highly susceptible to proteolytic degradation in recombinant systems and is expensive to purify. Expression of HSA in transgenic chloroplasts using Shine-Dalgarno sequence (SD), which usually facilitates hyper-expression of transgenes, resulted only in 0.02% HSA in total protein (tp). Modification of HSA regulatory sequences using chloroplast untranslated regions (UTRs) resulted in hyper-expression of HSA (up to 11.1% tp), compensating for excessive proteolytic degradation. This is the highest expression of a pharmaceutical protein in transgenic plants and 500-fold greater than previous reports on HSA expression in transgenic leaves. Electron micrographs of immunogold labelled transgenic chloroplasts revealed HSA inclusion bodies, which provided a simple method for purification from other cellular proteins. HSA inclusion bodies could be readily solubilized to obtain a monomeric form using appropriate reagents. The regulatory elements used in this study should serve as a model system for enhancing expression of foreign proteins that are highly susceptible to proteolytic degradation and provide advantages in purification, when inclusion bodies are formed.

Introduction

Availability of recombinant human proteins has revolutionized the use of therapeutically valuable proteins in clinical medicine. Plants offer a suitable alternative to microbial or animal expression of biopharmaceutical proteins because of their inexpensive production costs and absence of human pathogens. However, there are some limitations. In particular, expression of human proteins in nuclear transgenic plants has been disappointingly low, e.g. human serum albumin 0.02% of total soluble protein (tsp), human Interferon-β 0.000017% of fresh weight, human epidermal growth factor 0.001% of tsp and erythropoietin 0.0026% of tsp (Daniell *et al*., 2001d). Therefore, it is important to increase levels of expression in order to exploit plant production of pharmacologically important proteins.

As an alternative to nuclear expression, the chloroplast transgenic approach has been developed as an effective tool for the expression of biopharmaceutical proteins in plants (Daniell and Dhingra, 2002; Daniell *et al*., 2001a,b; DeGray *et al*., 2001; Guda *et al*., 2000; Staub *et al*., 2000). After the first demonstration of a protein based polymer expression with varied medical applications (Guda *et al*., 2000), transgenic chloroplasts have been shown to express very small antimicrobial peptides without fusion proteins (DeGray *et al*., 2001), assemble functional oligomers with disulphide bonds of the cholera toxin β-subunit (Daniell *et al*., 2001b), and express a monoclonal antibody with coordinated expression and assembly of heavy and light chain with proper folding and formation of disulphide bridges (Daniell *et al*., 2001a), suggesting that adequate redox environment or required

chaperonins are present within chloroplasts. Expression of functional human somatotropin in transgenic tobacco chloroplasts established that chloroplasts are capable of proper folding of human proteins with disulphide bonds (Staub *et al*., 2000). The ability to express multiple genes in a single transformation event (Daniell and Dhingra, 2002; De Cosa *et al*., 2001), accumulation of exceptionally large quantities of foreign proteins (De Cosa *et al*., 2001), successful engineering of tomato chromoplasts for high level transgene expression in fruits (Ruf *et al*., 2001), coupled to hyper-expression of vaccine antigens (Daniell *et al*., 2001b), and the use of plant derived antibiotic free selectable markers (Daniell *et al*., 2001c), augur well for oral delivery of edible vaccines and biopharmaceuticals that are currently beyond the reach of those who need them most. In addition, chloroplast genetic engineering is an environmentally friendly approach, offering containment of transgenes and a solution to gene silencing and position effect encountered in nuclear transgenic plants (Bogorad, 2000; Daniell and Dhingra, 2002; Daniell *et al*., 2002; Daniell, 2002).

HSA is the most widely used intravenous protein and is prescribed in multigram quantities to replace blood volume in trauma and in various other clinical situations (Peters, 1995). HSA is a monomeric globular prepro-protein whose mature form consists of a single polypeptide chain of 585 amino acids (66.5 kDa with 17 disulphide bonds). The annual world need exceeds 500 tons, representing a market value of more than $1.5 billion. To date, albumin has been produced primarily by the fractionation of blood serum. Lack of glycosylation facilitates production of functional HSA in prokaryotic systems. Although the *HSA* gene and cDNA have been expressed in a wide variety of microbial systems, including *E. coli* (Latta *et al*., 1987), *Bacillus subtilis* (Saunders *et al*., 1987), *Saccharomyces cerevisiae* (Quirk *et al*., 1989), *Kluyveromyces* (Fleer *et al*., 1991) or *Pichia pastoris* (Ohtani *et al*., 1998), no system is yet commercially feasible. Sijmons *et al*. (1990) made the first reported attempt to express HSA in transgenic plants, but very low expression levels were attained (0.02% tsp). HSA could not be detected if expressed in the cytoplasm, suggesting that the protein is not stable in this compartment, due to high susceptibility to proteolytic degradation. A 10-fold increase in HSA accumulation has been reported recently by nuclear transformation of potato plants and targeting the HSA to the tuber apoplast (Farran *et al*., 2002). Estimates by industry, however, suggest that the cost-effective yield for pharmaceutical production is 0.1 mg of HSA per gram of fresh weight (Farran *et al*., 2002).

In addition, good recombinant systems are still not available for many human proteins that are expensive to purify or highly susceptible to proteolytic degradation. It is known that traditional purification of biopharmaceuticals using columns accounts for 30% of the production cost and 70% of the set up cost (Petrides *et al*., 1995). Proteolytic degradation is another serious concern for industrial bioprocessing. The increasing production of proteins in heterologous hosts through the use of recombinant DNA technology has brought this problem into focus; heterologous proteins appear to be more prone to proteolysis (Enfors, 1992). Recombinant proteins are often regarded by a cell as foreign and therefore degraded much faster than most endogenous proteins (Rozkov *et al*., 2000). Proteolytic stability of recombinant proteins is a significant factor influencing the final yield.

This study attempts to develop a more efficient method of recombinant HSA production, which may be used as a model system to enrich or purify biopharmaceutical proteins from transgenic plants, which are highly susceptible to proteolytic degradation.

Results and discussion

Two chloroplast transformation vectors were designed with different 5′ regulatory sequences to direct HSA expression and maximize protein accumulation in transgenic chloroplasts. Basic pLD vector, developed in this laboratory for chloroplast transformation, was used (Daniell *et al*., 1998; Daniell *et al*., 2001b; De Cosa *et al*., 2001; Guda *et al*., 2000; Kota *et al*., 1999). In the plasmid pLDAsdHSA (Figure 1a), the *aadA* gene, which confers spectinomycin resistance, and the *HSA* gene are expressed as a polycistron from the plastid *Prrn* promoter. The Shine-Dalgarno (SD) consensus sequence GGAGG was placed upstream of both genes. High levels of foreign protein expression in chloroplasts (3–21% of tsp) have been shown for different proteins using this 5′ sequence (Daniell *et al*., 2001b; DeGray *et al*., 2001; Kota *et al*., 1999). In the pLDApsbAHSA vector (Figure 1a), the 204 bp tobacco chloroplast DNA fragment containing the promoter and the *psbA* 5′UTR was inserted immediately upstream of the *HSA* coding sequence and downstream of the *aadA* gene. It is well known that foreign genes under the control of the *psbA* promoter and untranslated region are expressed at very high levels (Daniell *et al*., 1990). This enhancement of translation may be due to elements in the 5′UTR (Eibl *et al*., 1999). Vectors were bombarded into tobacco leaves as described previously (Daniell, 1997) and, after 5 weeks, several primary shoots appeared from each bombarded leaf as a result of independent transformation events. Putative transformed shoots were identified by growth on 500 μg/mL of spectinomycin.

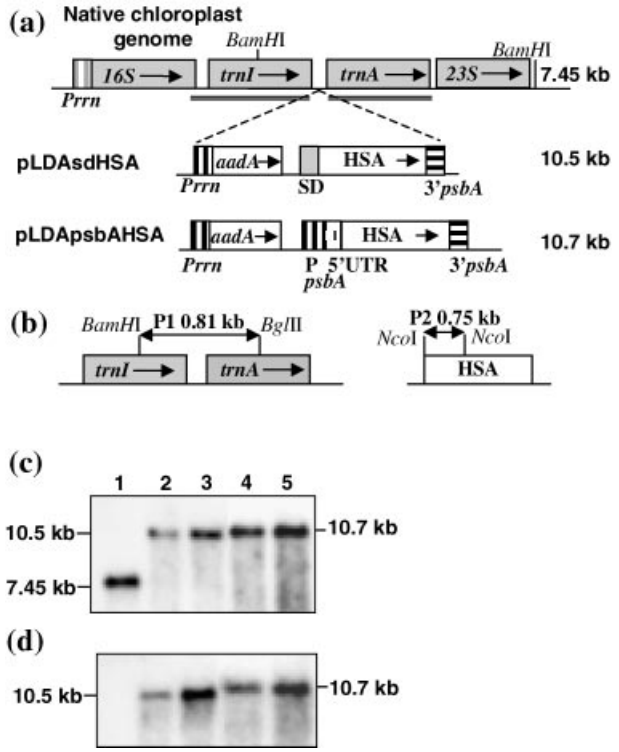

Figure 1 Integration of transgene cassettes into the chloroplast genome and study of homoplasmy. (a) Regions for homologous recombination are underlined in the native chloroplast genome. *HSA* is driven in all cassettes by the *Prrn* promoter upstream of the *aadA* gene for spectinomycin resistance with additional promoters and control elements as described in the text. Arrows within boxes show the direction of transcription. Numbers to the right indicate the predicted hybridizing fragments when total DNA digested with *Bam*HI is probed with probe P1. (b) The 0.81 kb fragment (P1) flanking the cassette and 0.75 kb fragment containing *HSA* coding region (P2) were used as probes for the Southern blot analysis. (c, d) Southern blot analysis. 1: untransformed DNA; DNA from plants transformed with: 2,3: pLDAsdHSA; 4,5: pLDApsbAHSA. Plants for the first (T_0) and second (T_1) generation were analysed. 2,4: T_0 generation. 3,5: T_1 generation. Blots were probed with P1 (c) and P2 (d). *AadA*: aminoglycoside 3′-adenylyl transferase; kb: kilobases; P: promoter; Prrn: 16SrRNA promoter; SD: Shine-Dalgarno.

Integration of the foreign gene cassettes into the chloroplast genome was confirmed by PCR screening of primary shoots. The strategy employed lands one primer on the native chloroplast genome adjacent to the point of integration and the second primer on the *aadA* gene. This PCR product can not be obtained in nuclear transgenic plants or spontaneous mutants, thus both possibilities could be eliminated. It was found that 90% of total shoots obtained were true chloroplast transformants. Confirmed transformants were subjected to a second round of spectinomycin selection to achieve homoplasmy. They were rooted in the presence of spectinomycin and then transferred to pots for further characterization. Southern blot analysis was performed to select homoplasmic T_0 lines and confirm stable maintenance of integrated transgenes in the T_1 generation (Figure 1b–d). The flanking region probe (P1) identified a 7.45 kb fragment in the untransformed control plant, as expected (Figure 1c). In the chloroplast transgenic lines, only transformed genome copies are observed as evidenced by the 10.5 and 10.7 kb hybridizing fragments for pLDAsdHSA and pLDApsbAHSA transgenic lines, respectively. To confirm that the 10.5 and 10.7 kb fragments contained the *HSA* gene, the same blot was reprobed with the *HSA* P2 probe. As expected, hybridization was detected only in the chloroplast transgenic lines (Figure 1d). Absence of other hybridizing fragments eliminates nuclear and chloroplast integration events in the same transgenic line.

HSA quantities in transgenic tobacco chloroplasts were determined by ELISA. More than a 360-fold difference in HSA accumulation was observed between plants transformed with the two different vectors (Figure 3a): 0.02% vs. 7.2% tp in pLDAsdHSA and pLDApsbAHSA transgenic lines, respectively. Chloroplast constructs with the SD sequence have been demonstrated to direct CTB expression very efficiently (up to 4% of tsp; Daniell *et al*., 2001b). Similar constructs, but inserted in other areas of the plastid genome, have also been successful (3–21% tsp; DeGray *et al*., 2001; Kota *et al*., 1999), demonstrating that high protein expression levels can be achieved by using this construct in an operon with a SD sequence. Thus, low levels of HSA expression in the pLDAsdHSA transgenic plants could not be due to the effect of regulatory signals in the construct. Differences in the amounts of HSA could be due to post-transcriptional, translational or post-translational effects. To study differences in HSA expression, transcript abundance was examined by Northern blots, which were performed using the 3′*psbA* region as the probe (Figure 2). The *5′psbA/HSA* monocistron transcript is much more abundant than the *aadA/SD/HSA* dicistron, but such differences do not show a linear correlation with the 360-fold difference in HSA accumulation between both transgenic lines. Such a lack of correlation between transcript abundance and protein accumulation has been reported from several laboratories when the *psbA* 5′UTR is used (Mayfield *et al*., 1995; Staub and Maliga, 1993, 1994), suggesting an important role of the *psbA* 5′UTR in enhancement of translation. Eibl *et al*. (1999) also showed that deletion of the terminal sequences of the *psbA* 5′UTR decreased the ability of the UTR to enhance translation. Thus, efficient translation in the pLDApsbAHSA transgenic line might be an important factor in establishing high levels of HSA accumulation.

There are several studies demonstrating that *psbA* 5′UTR confers light-dependent translation not only to the *psbA*

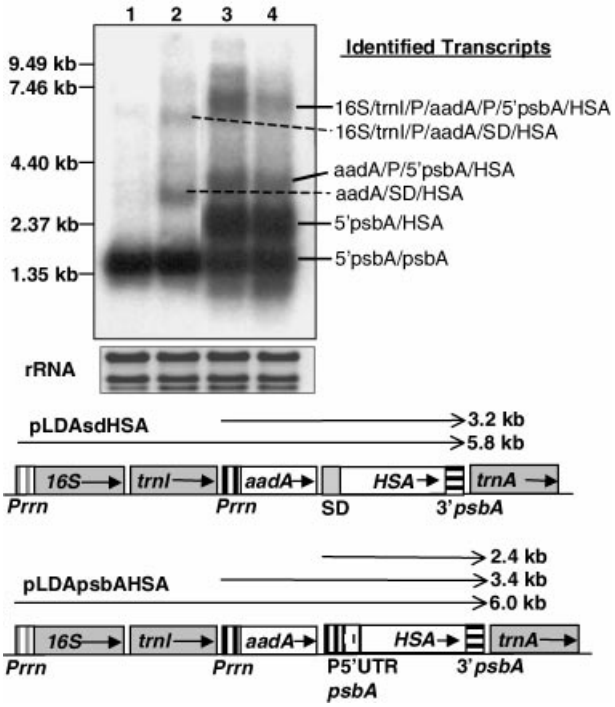

Figure 2 Transcription patterns of transgenic plants. A Northern blot analysis was performed with total RNA extracted from leaves of potted plants. The 3′ of the *psbA* gene was used as probe. 1: untransformed plant; 2: transformed with pLDAsdHSA; 3: transformed with pLDApsbAHSA after illumination or 4: in the dark. Ethidium bromide-stained rRNA was used to assess loading. Identified transcripts are indicated to the right. A scheme of transcription patterns expected for the different cassettes integrated into the chloroplast genome is shown at the bottom of the figure. Horizontal arrows above genes show anticipated transcripts. Arrows within boxes show the orientation of genes within the chloroplast genome. Read through transcripts are not shown in this figure. rRNA: ribosomal RNA.

gene (Zerges, 2000) but also to other heterologous proteins (Eibl *et al*., 1999; Staub and Maliga, 1993, 1994). Expression of *HSA* under the *psbA* 5′UTR control is therefore expected to be light dependent. Changes in HSA accumulation after different periods of illumination were monitored by ELISA (Figure 3b). HSA quantity was observed to be maximum up to 50 h of continuous illumination (11.1% of tp) in mature leaves and a 2–4-fold decrease was observed after the 8 h dark period. Such differences in HSA accumulation were so pronounced that it was detected by staining gels with Coomassie Brilliant Blue (Figure 3c). Staub and Maliga (1993, 1994) and Eibl *et al*. (1999) showed that although translation is arrested in the dark, the *5′psbA/uidA* mRNA turnover was very low. This observation was confirmed for HSA by Northern blot analysis, which showed no major differences between light and dark amount of *5′psbA/HSA* transcripts (Figure 2, lanes 3, 4). Therefore, differences in HSA accumulation between dark and light could not be due to differences in the rate of transcription or transcript stability, but due to the arrest of translation in the dark and the turnover of HSA in the chloroplast.

Proteins from transformed plants were separated to study the pattern of HSA accumulation within transgenic chloroplasts. Western blots confirmed differences in HSA quantities among transgenic lines (Figure 3d). In pLDApsbAHSA transgenic lines, HSA is partially solubilized with the standard buffer used for total protein extraction. This observation suggests formation of HSA aggregates inside transgenic chloroplasts in the pLDApsbAHSA transformants. Electron microscopy and immunogold labelling therefore were performed in transformed and untransformed plants to further investigate this. As expected, electron micrographs of leaf tissues showed formation of large aggregates or inclusion bodies within transgenic chloroplasts of pLDApsbAHSA mature transformed plants (Figure 4b–d). It is interesting to note that chloroplasts containing inclusion bodies increased in size to accommodate large accumulation of HSA (compare Figures 4a,d). However, the phenotype of these plants appeared normal (Figure 5). The amount of HSA in pLDAsdHSA transgenic chloroplasts was so low that it was not possible to detect immunogold labelling above the background. No significant changes in chloroplast size were observed in these plants.

Inclusion bodies have been often observed in the cytosol of prokaryotes and eukaryotes when heterologous proteins are overexpressed. The occurrence of this feature in the chloroplast was first reported by Ketchner *et al*. (1995). It is widely known that protein aggregation into inclusion bodies mostly involves intermolecular associations of partially folded intermediates (Mitraki and King, 1989). High protein concentrations usually lead to conditions that frequently exceed the normal solubility limit. Even the most abundant protein in photosynthetic cells, RuBisCO, forms inclusion bodies in some cases. Many autotrophic bacteria and all cyanobacteria package much of the RuBisCO into inclusion bodies actively involved in the fixation of CO_2, known as carboxysomes (Shively and English, 1991). Our hypothesis, based on the process of formation of inclusion bodies, is that in contrast to pLDAsdHSA transgenic lines, HSA synthesized under the *psbA* 5′UTR forms large aggregates mainly due to the high local concentration of the protein.

Formation of inclusion bodies is one of the strategies for reducing the proteolysis of unstable recombinant proteins (Enfors, 1992). The majority of recombinant proteins studied have been shown to be highly resistant to proteolysis inside inclusion bodies. Although there is protection from proteases within inclusion bodies, some proteolysis can also take place directly on the aggregated protein (Carrio *et al*., 1999). HSA

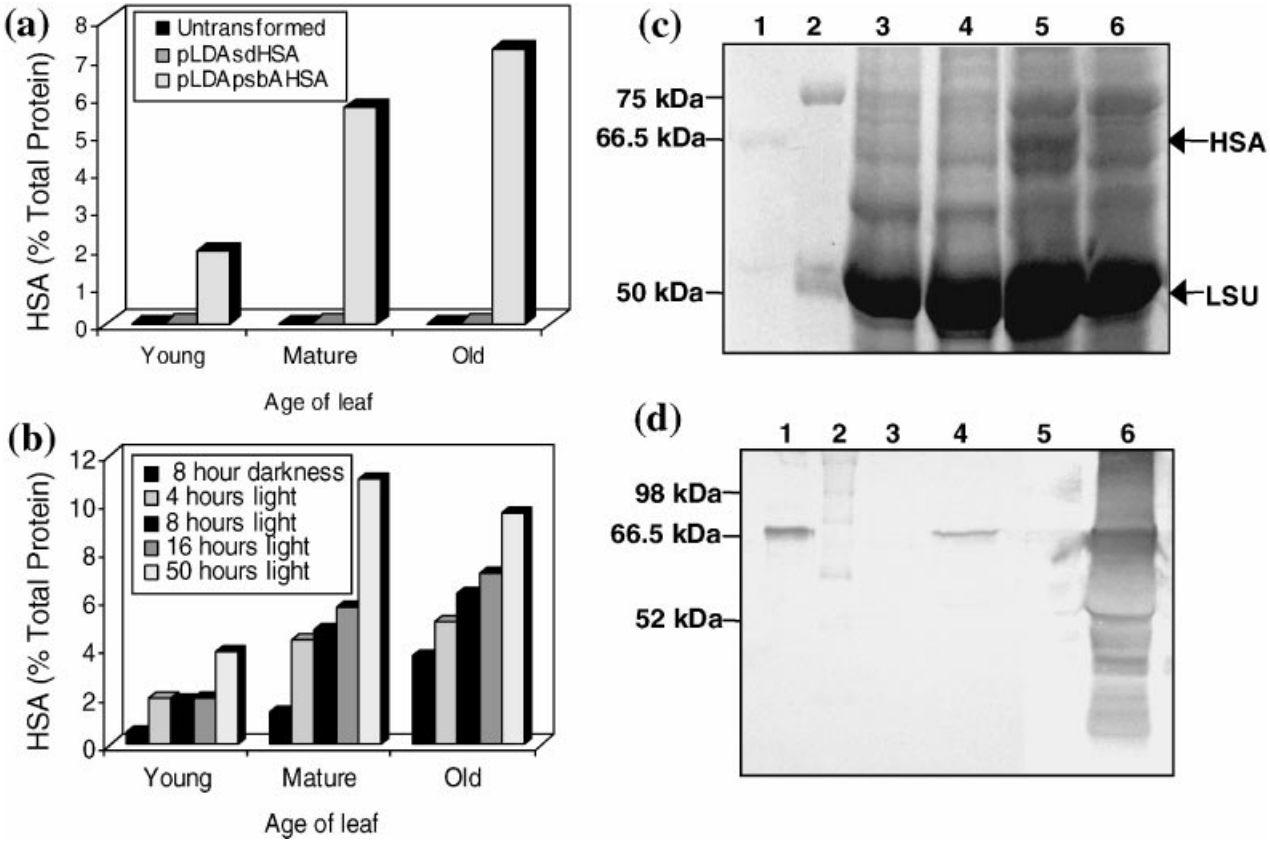

Figure 3 Analysis of HSA accumulation in transgenic chloroplasts. (a) ELISA of HSA accumulation in leaves of potted plants at different stages of development. Samples were collected from untransformed plants or transformed with pLDAsdHSA or pLDApsbAHSA. Expression levels are indicated as a percentage of total protein. (b) Study after different hours of illumination. Samples of leaves were collected from potted plants transformed with pLDApsbAHSA after the 8-h dark period or at indicated hours in the light. (c) Coomassie stained gel to study HSA accumulation in tobacco leaves of potted plants. Total protein extracts were loaded in the gel. 1: 500 ng pure HSA; 2: molecular weight marker; 3: untransformed plant; transformed with 4: pLDAsdHSA; 5: pLDApsbAHSA after 8 h of illumination; 6: pLDApsbAHSA after 8 h of darkness. Between 40 and 50 μg of plant protein were loaded per well. The positions of HSA and RuBisCO large subunit (LSU) are marked. (d) Colorimetric immunoblot detection of tobacco protein extracts from mature leaves in potted plants. Total protein extracts were loaded in the gel. 1: 40 ng pure HSA; 2: molecular weight marker; 3,5: untransformed plant extract; 4: pLDAsdHSA plant extract; 6: pLDApsbAHSA plant extract. Between 40 and 50 μg of plant protein were loaded per well. kDa: kiloDalton; LSU: RuBisCO large subunit.

also appears to be susceptible to some proteolytic degradation within transgenic chloroplasts. However, the net balance between synthesis and degradation is highly favourable, especially after several hours of continuous illumination.

Properly folded HSA can be recovered from inclusion bodies after denaturation for complete solubilization and *in vitro* refolding. Proper refolding of HSA from inclusion bodies is a routine procedure that has been previously demonstrated in several studies with *E. coli* (Latta *et al*. (1987) and *Saccharomyces cerevisiae* (Dodsworth *et al*., 1996; Quirk *et al*., 1989). In these cases, human and recombinant refolded HSA were compared and it was shown that the two proteins were structurally equivalent, demonstrating that HSA may be recovered from inclusion bodies and properly folded. Following the guidelines from these protocols, HSA was extracted from transgenic chloroplasts. Figure 6a shows a silver stained SDS-PAGE gel in which HSA inclusion bodies could be separated from the soluble fraction (lane 3), where most of the cellular proteins are found. After solubilization of inclusion bodies and subsequent refolding, HSA could be completely converted into monomeric forms (Figure 6a, lane 5; Figure 6b, lane 5). Our estimations of HSA yields at the end of the protocol are about 20% of the initial quantities in leaves, although the reported protocol has been performed at the laboratory scale and may be further optimized for industrial production. Expression of HSA in transgenic plants has been estimated to be cost effective with levels of expression as low as 0.1 mg HSA/g fresh weight (Farran *et al*., 2002). The recoveries after solubilizing the inclusion bodies and refolding the HSA are about 0.25 mg HSA/g fresh weight (excluding soluble HSA in transgenic chloroplasts), which exceeds cost effective estimations of pharmaceutical industries.

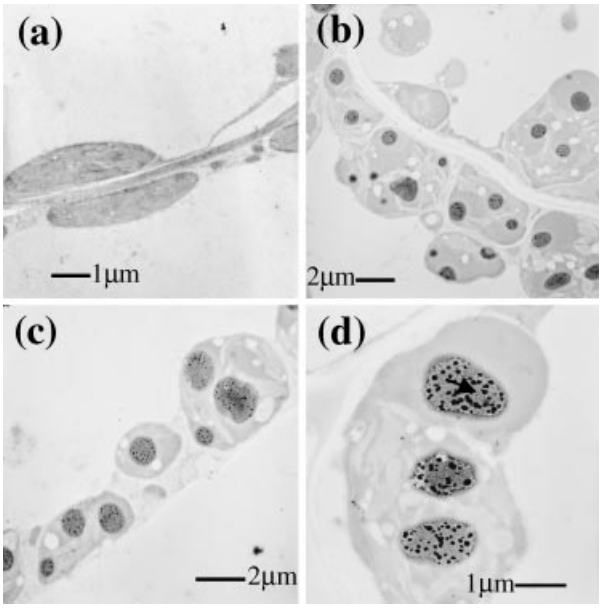

Figure 4 Study of HSA accumulation into inclusion bodies. (a–d) Electron micrographs of immunogold labelled tissues from untransformed (a) and transformed mature leaves with the chloroplast vector pLDApsbAHSA (b–d). Note presence of inclusion bodies (b–d) marked with an arrow in (d). Scale bars indicate μm. Magnifications are a × 10 000; b × 5000; c × 6300; d × 12 500.

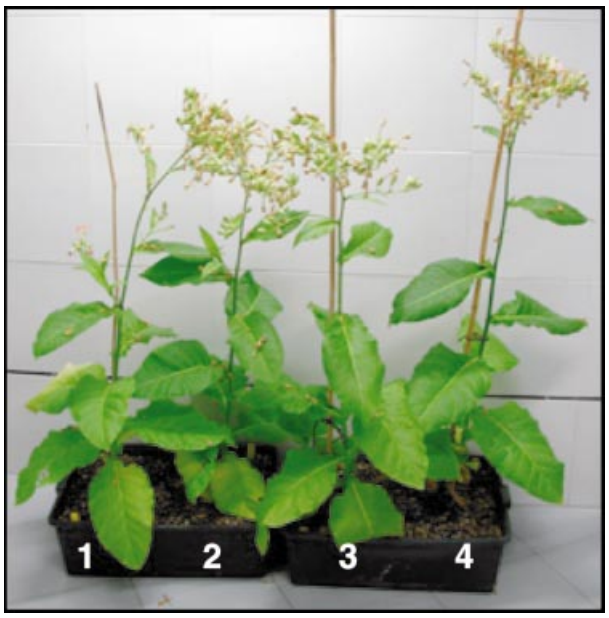

Figure 5 Plant T_1 phenotypes. 1,2: untransformed plants; 3: plant transformed with pLDAsdHSA; 4: plant transformed with pLDApsbAHSA.

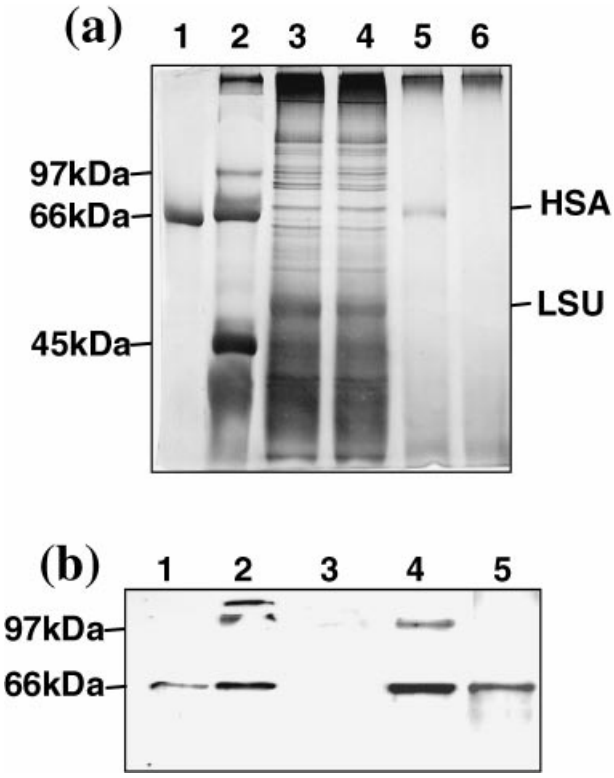

Figure 6 HSA extraction from inclusion bodies. (a) Silver stained SDS-PAGE gel showing 1: 500 ng pure HSA; 2: molecular weight marker; soluble fraction obtained after centrifugation of pLDApsbAHSA transformed plant extract (lane 3) or untransformed plant extract (lane 4); 5: HSA after solubilization from the pellet; 6: proteins from untransformed plant, which followed the same process as the proteins of lane 5. Amounts of protein loaded per well were 10 μg in lanes 3 and 4, 550 ng in lane 5 and 450 ng in lane 6. (b) Chemiluminiscent immunoblot detection of protein extracts. 1: 40 ng pure HSA; 2: HSA from a plant transformed with pLDApsbAHSA during the solubilization process, showing mono, di and trimeric forms; 3: proteins from an untransformed plant that followed the same process as the proteins for lane 2; 4: same HSA from lane 2 but in a more advanced stage of solubilization; 5: completely monomerized HSA after the end of the solubilization treatment (the sample of this lane corresponds with lane 5 in (a)).

One of the primary goals of this study was to develop a more efficient expression system for human serum albumin, an important human therapeutic protein that is highly susceptible to degradation. Expression of HSA in mature plants under the translational control of SD sequence resulted in very low levels of HSA accumulation, probably due to excessive proteolytic degradation and poor rates of translation. However, when expressed under the control of *psbA* promoter and 5′UTR, up to a 500-fold increase in HSA accumulation was observed in mature plants compared to other regulatory sequences tested. HSA was observed to form large inclusion bodies, resulting even in a noticeable increase in the size of transgenic chloroplasts and presumably offering protection to HSA from proteolytic degradation. Inclusion bodies facilitated purification of HSA from other cellular proteins. The HSA molecule has a chemical and structural function rather than an enzymatic activity, therefore complex studies are necessary to fully demonstrate the functionality of the molecule (see Dodsworth *et al*., 1996; Ohtani *et al*., 1998; Petersen *et al*., 2000; Tarelli *et al*., 1998; Watanabe *et al*.,

2001a,b). Such functional studies using *in vitro* refolded HSA are in progress.

Experimental procedures

Chloroplast expression vectors

pLDAsdHSA was constructed by inserting the *HSA* 1.8 kb *Eco*RI/*Not*I fragment into the multiple cloning site of the pLD vector (Daniell *et al*., 1998; Daniell *et al*., 2001b; De Cosa *et al*., 2001; Guda *et al*., 2000; Kota *et al*., 1999). This fragment contains the mature HSA coding sequence preceded by a Shine-Dalgarno (GGAGG) and it has an ATG as the initiation codon. These sequences were introduced by using the primer: 5′-GGAGGCAACCATGGATGCACACAAGAGTGAAGG-3′. For the pLDApsbAHSA vector, the 204 bp sequence including the promoter and the *psbA* 5′UTR, was amplified by PCR using tobacco DNA as template. The following primers were used: 5′-CCGTCGACGTAGAGAAGTCCGTATT-3′ and 5′-GCCCATGGTAAAATCTTGGTTTATTTA-3′. The fusion with the *HSA* gene was made at the *Nco*I site placed at the 3′ end of the *psbA* 5′UTR and then inserted into the pLD vector as a *Eco*RI/*Not*I fragment. Before proceeding with the bombardment, vectors were tested by Western blot analysis in *E. coli.*

Bombardment and regeneration

Sterile tobacco (cv. Petit Havana) leaves were bombarded using the Bio-Rad PDS-1000/He biolistic device as described previously (Daniell, 1997). Bombarded leaves were subjected to two rounds of selection on the RMOP medium containing 500 μg/mL of spectinomycin to regenerate transformants (Daniell, 1997). After regeneration, plants were rooted on 500 μg/mL of spectinomycin (Daniell *et al*., 2001b) and transferred to pots in growth chambers. Photoperiod was 16 h light and 8 h dark.

PCR and Southern blot analysis

PCR was used to analyse integration of different cassettes in the transformed plants as described (Daniell *et al*., 2001b,c; De Cosa *et al*., 2001; Kota *et al*., 1999). For Southern blot analysis, total DNA was extracted from leaves of transformed and untransformed plants (Qiagen Dneasy Kit). Total DNA (5 μg) was digested with *Bam*HI, electrophoresed on 0.7% agarose gels and transferred to nylon membranes (Duralon-UV Stratagene). The template for probing flanking sequences was a 0.81 kb *Bgl*II/*Bam*HI fragment and for *HSA* a 0.75 kb *Nco*I fragment. The probes were labelled with ^{32}P-dCTP using the oligolabelling procedure (Ready To Go, Amersham). Probes were hybridized to the membranes following the QUICK-HYB protocol (Duralon-UV, Stratagene).

Northern blot analysis

Total RNA was extracted from leaves of transformed and untransformed plants (Rneasy Plant Kit, Qiagen). RNA 2.5 μg was electrophoresed on 1.2% agarose/formaldehyde gels and then transferred to nylon membranes (Stratagene). A 0.21 kb *Xba*I/*Pst*I fragment of the 3′*psbA* gene was used as probe and labelled with ^{32}P-dCTP using the oligolabelling procedure (Amersham).

HSA quantification

The ELISA Human Albumin Quantification Kit (Bethyl Laboratories) was used. Transformed and untransformed leaves (100 mg) from potted plants grown under a 16 h photoperiod were ground in liquid nitrogen, resuspended in 700 μL of 50 mM NaOH and analysed following the manufacturer's protocol. Transgenic leaf extracts were diluted to fit in the linear range of the provided HSA standard. Absorbance was read at 450 nm. The DC protein assay (Bio-Rad) was used to determine total solubilized protein.

SDS-PAGE and immunoblot analysis

Transformed and untransformed leaves (100 mg) were ground in liquid nitrogen and resuspended in 200 μL of protein extraction buffer (200 mM Tris-HCl pH 8.0, 100 mM NaCl, 400 mM Sucrose, 14 mM βME, 0.05% Tween20, 0.1% SDS, 10 mM EDTA, 2 mM PMSF). Leaf extracts were boiled in sample buffer (Bio-Rad) and electrophoresed in a 10% polyacrylamide gel. Separated proteins were stained with Coomassie Brilliant Blue G-250 or transferred to a nitrocellulose membrane for immunoblotting. The primary antibody (rabbit anti-HSA, Nordic Immunology) was used at 1 : 10 000 dilution, and the secondary antibody (alkaline phosphatase conjugated mouse antirabbit, Sigma or goat antirabbit HRP conjugated, Southern Biotechnology) at 1 : 15 000. Alkaline phosphatase colour development reagents, BCIP/NBT, in AP Color Development Buffer (Bio-Rad) or the ECL kit (Amersham) were used for detection.

Solubilization of inclusion bodies

Soluble proteins were removed with a first extraction in 0.2 M NaCl, 25 mM Tris-HCl pH 7.4, 2 mM PMSF and 0.1% Triton

X-100. After centrifugation for 60 min at 20 000 ***g***, the pellet was solubilized for 16 h at 4 °C in 6 M Gu-HCl, 0.1 M βME and 0.25 mM Tris-HCl pH 7.4. After centrifugation for 60 min at 20 000 ***g***, the supernatant was then slowly diluted 100-fold in 100 mM NaCl, 50 mM Tris-HCl pH 8.5 and 1 mM EDTA for 24 h at 4 °C. Fractions were electrophoresed in a SDS-PAGE 10% gel and silver stained with Bio-Rad reagents and protocol.

Transmission electron microscopy and immunogold labelling

Seedlings and mature leaves from untransformed and transgenic plants were analysed. Fixation and immunogold labelled electron microscopy were performed as described by Vrekleij and Leunissen (1989). Sections were first blocked, incubated for 1 h with a goat antihuman albumin polyclonal antibody (Nordic Immunology; dilution range from 1 : 1000 to 1 : 10 000) and then incubated for 2 h with a rabbit antigoat IgG secondary antibody conjugate to 10 nM gold diluted 1 : 40 in blocking solution. Sections were examined in a Zeiss EM 10 transmission electron microscope at 60 kV.

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