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Production of Human Serum Albumin in Chloroplast-Transformed Tobacco Plants

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Abstract

Human serum albumin (HSA) is the most abundant protein in plasma and is the most often used intravenous protein in many human therapies. However, HSA is currently extracted only from plasma because commercially feasible recombinant expression systems are not available. This study attempted to develop an efficient system for recombinant HSA production by chloroplast transformation of tobacco. A HSA cDNA was isolated from a cDNA library constructed with human liver tissue. Chloroplast transformation vectors were constructed by introducing various regulatory elements to HSA regulatory sequences. Vectors were delivered by particle bombardment into leaf explants and chloroplast-transformed plants were subsequently regenerated into whole plants. Southern blot analysis confirmed that the HSA cDNA was incorporated between rps12 and orf70B of the chloroplast genome as designed. Western blot analysis revealed that hyper-expression and increasing the stability of HSA were achieved by modification of the regulatory sequences using the psbA5'UTRs in combination with elements of the 14 N-terminal amino acids of the GFP and the FLAG tag. However, only plants transformed with the vector containing all of these elements were able to accumulate HSA.

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Introduction

Human serum albumin (HSA) is the most abundant protein in plasma and is the most often used intravenous protein in manyhuman therapies. HSA is produced in the liver and rapidly secreted to the extracelluar space. Hepatocytes initially synthesize albumin as a prepro-protein of 609 amino acids, and subsequent posttranslational processes give rise to a mature monomeric protein with 585 amino acids (Dugaiczyk et al. 1982). The annual worldwide needs over 500 tons, representing a market value of more than $ 1.5 billion (Millan et al. 2003). However, HSA is currently extracted only from plasma because commercially feasible recombinant expression systems are not available. Although HSA cDNA has been expressed heterologously in *E. coli* (Latta et al. 1987), *Saccharomyces cerevisiae* (Quirk et al. 1989), *Pichia pastoris* (Ohtani et al. 1998, Watanabe et al. 2001), no system is yet commercially feasible.

On the other hands, plants as biofactories for the production of proteins are employed bya number of groups for the synthesis of edible vaccines, pharmaceuticals. Especially, chloroplast genetic engineering offers several unique advantages over nuclear transformation, which includes high expression levels of the recombinant proteins, multi-gene engineering in a single transformation event and transgene containment by maternal inheritance expression, as well as a lack of positional and pleiotropic effects and undesirable foreign DNA. DeCosa et al. (2001) reported that *Bacillus thuringiensis* (Bt) cry2Aa2 protein was produced in transgenic tobacco chloroplasts up to 46% of the total leaf protein.

Sijmons et al. (1990) firstly tried to express HSA in tobacco by nuclear transformation, but very low expression levels were attained (0.02% TSP). Recently, Millan et al.(2003) have reported that modification of regulatory

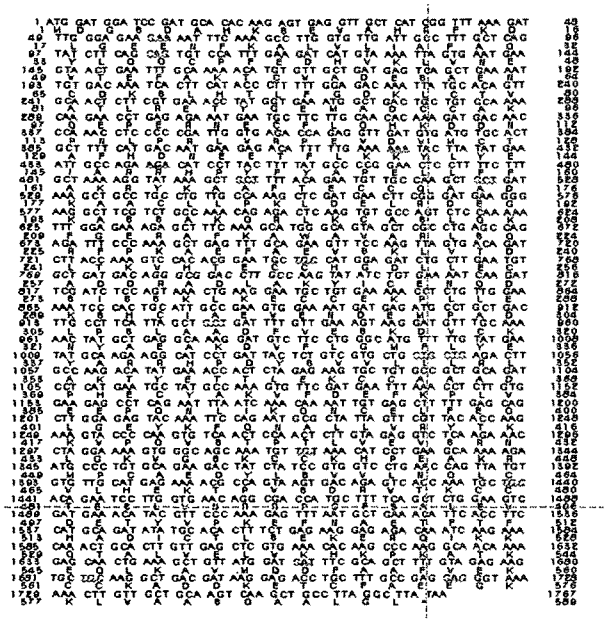

Figure 1. The nucleotide and deduced amino acid sequences of a HSA cDNA.
This clone contains the mature HSA coding sequence preceded by the ribosomal binding site and it has an ATG as the initiation codon.

sequences using chloroplast untranslational regions resulted in hyper-expression of HSA (11% TSP). However, HSA produced in transgenic chloroplasts formed inclusion bodies. This study attempted to develop an efficient system for HSA production by adding various regulatory elements to the upstream of a HSA cDNA. In addition, a sequence for the FLAG tag that facilitates purification of recombinant HSA proteins from transformed chloroplastswas also introduced into a vector.

Materials and Methods

Plant Material

Tobacco (*Nicotiana tabacum* L. cv. Samsun) plantlets were maintained in flasks containing Murashige and Skoog (1962) basal medium. Leaf blades (approximately 3 cm long) were excised and were subjected to particle bombardment.

HSA cDNA Cloning and Construction of Chloroplast Transformation Vectors

The HSA cDNA was amplified by RT-PCR using cDNA library constructed with human liver. The ATG start codon sequence and ribosomal binding site (RBS) were introduced in PCR primers (5'-GAAGGAGATATACCCATGG-ATGCACACAAGAGTGAGGT-3', 5'-TTATAAGCCTAAGGC-AG-3'). The PCR fragment was cloned into the EcoRV sites of pBluescript KS vector and confirmed by sequencing (Fig. 1). pTRH vector series was constructed by inserting the HSA EcoRV fragments into the multicloning site of the pTR vector containing the psbA5'UTR sequence, a sequence encoding the first 14 amino acids of GFP, and the FLAG tag sequence (Fig. 2). These elements were incorporated singly or in combination into the 5'-regulatory sequence of the HSA cDNA in vectors.

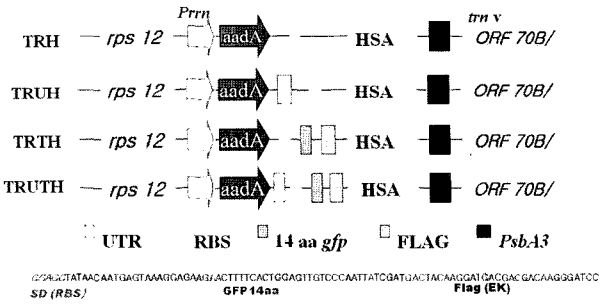

Figure 2. Chloroplast transformation vectors containing various regulatory sequences.
The aminoglycoside 3' adenylyltransferase (aadA) gene for resistance to spectinomycin and the HSA gene driven by the constitutive promoter of the rRNA operon (*Prrn*).

Particle Bombardment and Regeneration of Chloroplast-Transformed Plants

Leaf blades were placed abaxial side up onto filter paper discs on MS medium supplemented with 4.44 μM 6-benzyladenine and 0.54 μM α-naphthalaneacetic acid in plastic Petri dishes (37 x 15 mm). Gold particles (0.6 μM in diameter) coated with plasmid DNA (pTRH series) were bombarded into leaf blades using the particle delivery system PSD1000/He (BioRad). After 48 hrs of incubation at 25℃ in the dark, leaf blades were cut into segments (5 x 5 mm) and were placed adaxial side up on selection medium (RMOP containing 500 mg/l spectinomycin dihydrochloride, Daniell, 1997). Spectinomycin-resistant shoots obtained after 5-6 weeks were cut into segments (3 x 3 mm) and were placed onto plates containing the selection medium.

PCR and Southern Blot Analysis

Total genomic DNA from putative transgenic and wild-type plant was extracted using the DNeasy plant DNA Isolation Kit (Qiagen). PCR was carried out using the chloroplast flanking sequence (P1 and P2). After denaturation for 5 min at 94℃, samples were carried through 30 cycles using the following PCR conditions; 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 120 s.

For southern blot analysis, genomic DNA (10℃g) was digested with *Eco*RV that has no cut in the internal HSA coding region. Digested DNA was separated by electrophoresis on a 0.8% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeledchloroplast flanking

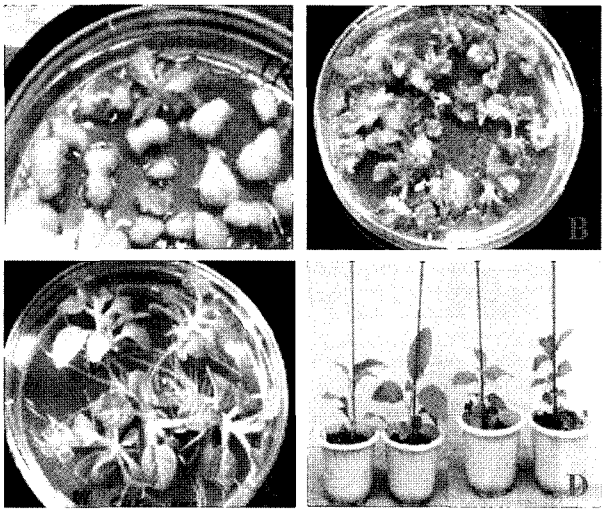

Figure 3. Production of chloroplast transformants. A, Adventiitous shoots formed on leaf explant in first round selection; B, Adventiitous shoots formed on leaf explant in second round selection; C, Root induction of chloroplast transformants; D, Chloroplast transformants grown in soil.

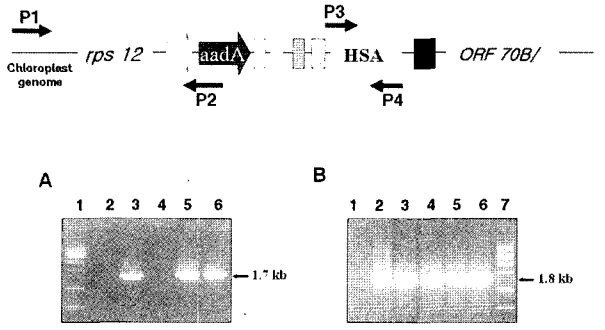

Figure 4. PCR analysis of wild-type plant and putative chloroplast transformants using two primer sets: (A) P1-P2 and (B) P3-P4. (A) Lane 1: 1 kb ladder Lane 2: wild-type plant Lane 3-5: putative transformants. (B) Lane 1: wild-type plant; Lane 2-5: putative transformants Lane 6: control plasmid Lane 7: 1 kb ladder.

region used for homologous recombination following standard procedures (Sambrook et al. 1989).

Western Blot Analysis

Total protein from transformed and untransformed tobacco leaves extracted with 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, 2 mM PMSF). Total protein (20 μg) was separated by a 10% SDS- PAGE and transferred to a PVDF membrane for immunoblotting. The primary antibody (rabbit anti-HSA, Sigma) was used at 1:10,000 dilution, and the secondary antibody (goat anti-rabbit HRP conjugated, Sigma) at 1:15,000. Detection was performed with the ECL kit (Amersham) as described in the kit protocol.

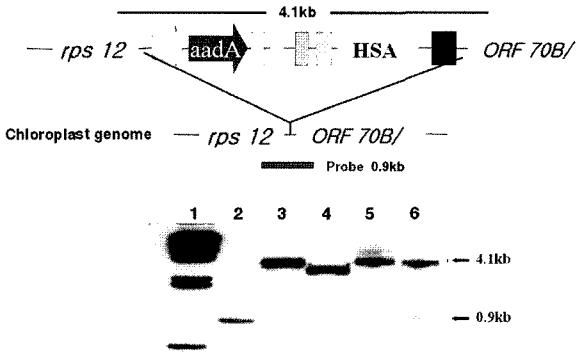

Figure 5. Southern blot analysis of chloroplast transformants. Plant DNA was digested with *Eco*RV and hybridized with a 0.9 kb probe, which contained the chloroplast flanking sequences used for homologous recombination. Lane 1: λ*Hin*dIII size marker Lane 2: wild-type plant Lanes 3-6: transformants.

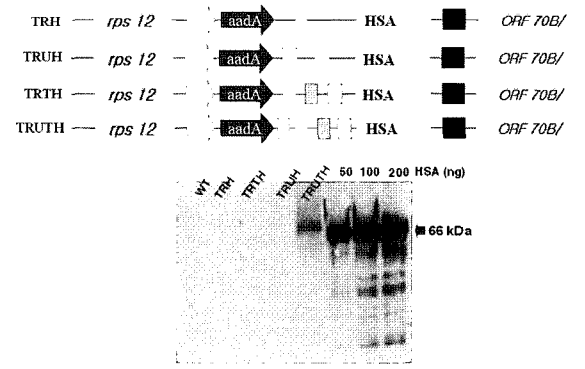

Figure 6. Western blot analysis of chloroplast transformants. Total soluble protein (20 μg) was separated by SDS-12% PAGE with HSA standards (50, 100 and 200 ng), which also contained 20 μg wild-type total soluble protein.

Results and Discussion

Putative transformants selected on spectinomycin-containing medium were grown to maturity in a greenhouse (Fig. 3). Putative chloroplast transformants produced an extra PCR band of 1.8 kb as expected (Fig. 4). To verify site-specific integration, southern blot analysis was carried out with the probe containing the flanking chloroplast border sequence of rps12 and orf70B (Fig. 5). A 0.9 kb and a 4.1 kb fragment were obtained from wild-type and transformed plants, respectively, when total plant DNAs digested with *Eco*RV were subjected to electrophoresis, indicating that the aadA and HSA genes were incorporated between rps12 and orf70B in the chloroplast genome.

Many human proteins including serum albumin are highly susceptibleto proteolytic degradation (Millan et al. 2003). Therefore, it is required to direct hyper-expression at the

transcription and translation of the transgene and stabilization of recombinant proteins in the chloroplast for their high accumulation. In this study, elements of the psb5'UTR, the N-terminal GFP, and the FLAG tag were introduced singly or in combination into the 5' regulatory sequence. As it turned out, only plants carrying all of the regulatory elements were able to accumulate HSA (Fig. 6). However, when either one of these elements was lacking, plants accumulated no HSA. Among three of theses sequences, the psb5'UTR sequence was prerequisite to accumulation of HSA. However, we do not know whether both elements of the N-terminal GFP and the FLAG tag are necessary for accumulation of HSA. It is because all possible combination of the regulatory elements was not tested.

The FLAG tag sequence was introduced into vectors to facilitate purification of recombinant HSA proteins from chloroplast transgenic tobacco. However, the possibility of enhanced stability of HSA by FLAG tag is not excluded in this study. The exact expression level of HSA was not able to be determined because the level was much lower than the lowest level of reference HSA in western blot analysis (Fig. 6). In this study,the FLAG tag sequence was introduced to the chloroplast genome for the first time. Further study is needed to establish the effective purification system using the FLAG affinity gels.

Acknowledgements

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References

De Cosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Overexpression of the Bt *cry2Aa2* operon in chloroplasts lead to formation of insecticidal crystals. Nature Biotechnol 19: 71-74

Dugaiczyk A, Law SW, Dennison OE (1982) Nucleotide sequence and encoded amino acids of human serum albumin mRNA. Proc Natl Acad Sci USA 79: 71-75.

Eibl C, Zou Z, Beck A, Kim M, Mullet J, Koop HU (1999) *In vivo* analysis of plastid *psbA*, *rbcL* and *rpl32* UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. Plant J 19: 333-345

Jeong WJ, Jeong SW, Woo JW, Choi DW, Liu JR (2004) Dicistronic expression of the green fluorescent protein and antibiotic resistance genes in the plastid for tracking and selecting plastid-transformed cells in tobacco. Plant Cell Rep 22: 747-751

Latta L, Knapp M, Sarmientos P, Brefort G, Becquart J, Guerrier L, Jung G, Mayaux J(1987) Synthesis and purification of mature human serum albumin from *E. coli*. Bio/Technology 5: 1309-1314

Millan AF, Mingo-Castel A, Miller M, Daniell H (2003) A chloroplast transgenic approach to hyper-express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnol J 1: 71-79.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473-497

Ohtani W, Ohda T, Sumi A, Kobayashi K, Ohmura T (1998) Analysis *Pichia pastoris* components in recombinant human serum albumin by immunological assays and by HPLC with pulsed amperometric detection. Anal Chem 70: 425-429

Quirk AV, Geisow MJ, Woodrow JR, Burton SJ, Wood PC, Sutton AD, Johnson RA, DodsworthN (1989) Production of recombinant human serum albumin from *Saccharomyces cerevisiae*. Biotechnol Appl Biochem 11: 273-287

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning A Laboratory Mannual 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PTJ, Staub JM, Nehra NS (1999) Stable chloroplast transformation in potato use of green fluorescent protein as a plastid marker. Plant J 19: 209-216

Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ, Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. Bio/Technology 8: 217-221

Watanabe H, Yamasaki K, Kragh-Hansen U, Tanase S, Harada K, Suenaga A, Otagiri M (2001) *In vitro* and *in vivo* properties of recombinant human serum albumin from Pichia pastoris purified by a method of short processing time. Pharm Res 18: 1775-1781

Ye GN, Hajdukiewicz PTJ, Broyles D, Rodriguez D, Xu CW, Nehra NS, Staub JM (2001) Plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. Plant J 25: 261-270