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PRODUCTION OF CORRECTLY PROCESSED HUMAN SERUM ALBUMIN IN TRANSGENIC PLANTS

Peter C. Sijmons, Ben M. M. Dekker, Barbara Schrammeijer, Theo C. Verwoerd, Peter J. M. van den Elzen and André Hoekema

Mogen International NV, Einsteinweg 97, 2333 CB Leiden, The Netherlands.

We have used a modified CaMV 35S promoter to direct the expression of chimaeric genes encoding human serum albumin (HSA) in transgenic potato and tobacco plants. To secrete the protein, either the human prepro-sequence or the signal sequence from the extracellular tobacco protein PR-S was used. We demonstrate secretion of HSA with both types of signal sequences in transgenic leaf tissue and in suspension cultures. HSA produced in transgenic potato plants was purified to chromatographic homogeneity. N-terminal amino acid sequence analysis revealed that the processing of the precursor protein was dependent on the type of signal sequence. Expression of the human preproHSA gene lead to partial processing of the precursor and secretion of proHSA. Fusion of HSA to the plant PR-S presequence resulted in cleavage of the presequence at its natural site and secretion of correctly processed HSA that is indistinguishable from the authentic human protein.

Gene transfer to plants has become routine for several plant species, facilitated by a variety of procedures[1]. This development offers the opportunity for genetic modification of crops. Recent examples are the introduction of genes confirming disease and insect resistance[1]. Plant genetic engineering also has potential for the production of valuable compounds. For the expression of recombinant proteins, plants may be a suitable alternative for microbial expression systems, as the production of plant biomass is inexpensive. This aspect becomes increasingly important in cases where large amounts of protein need to be produced. Examples of the expression of valuable proteins in plants now includes relatively small peptides[2,3] as well as antibodies[4]. To explore the potential of plants to produce commercially interesting proteins, we set out to express the 66.5 kD HSA protein (human serum albumin) in potato plants.

In man HSA is initially synthesized as prepro-albumin by the liver and released from the endoplasmatic reticulum after removal of the aminoterminal prepeptide of 18 amino acids[5]. The pro-albumin is further processed in the Golgi complex where the 6 residues of the propeptide are cleaved by a serine proteinase[6]. This results in the secretion of the mature polypeptide of 585 amino acids. The expression of the human preproHSA gene has been demonstrated in various microbial expression systems[7]. In *Saccharomyces cerevisiae* the protein was secreted, but it remained associated with the cells in culture. HSA produced in *E. coli* required additional *in vitro* processing to yield the mature protein[8]. In *Bacillus subtilis*, HSA could be secreted using bacterial signal peptides[9].

Whether human signal sequences are recognized by the plant secretory machinery is unknown. The signal for targeting of a protein is usually an N-terminal peptide that is removed from the precursor protein by a specific peptidase. Plant proteins destined for secretion have been shown to contain such N-terminal signal sequences[10–12]. In this report we demonstrate that the preprosequence of HSA can function in protein targeting across the plant plasma membrane. However, biochemical analysis of the recombinant protein purified from such plants shows that processing is incomplete. We describe a hybrid plant/human precursor that is properly processed to yield mature HSA in transgenic plants.

RESULTS

Chimaeric gene constructs. The structure of the chimaeric genes for the expression of various forms of HSA in transgenic plants is depicted in Figure 1. All constructs were placed in the plant expression cassette vector pMOG18. In this vector the inserted gene is placed under the control of a CaMV 35S promoter fragment[13] into which a duplicated enhancer sequence[14] was introduced (nucleotides -343 to -90 relative to the transcription start site[13]). Duplication of the enhancer has been shown to increase promoter activity[14]. The promoter fragment was followed by a synthetic leader sequence derived from alfalfa mosaic virus RNA4[15] with an ATG initiation codon in the context of a NcoI-site. It has been reported that mRNA translation can be enhanced by this viral leader sequence[16]. All fusions of this promoter fragment to the coding sequences studied were at the NcoI-site. One construct contained the wild type HSA coding sequence including its natural prepro-sequence[17] (pMOG236). Two other constructs contained a fusion of mature HSA with the presequence from the extracellular PR-S protein from tobacco. One of these (pMOG250) had an exact fusion between the C-terminal amino acid of the PR-S presequence (Ala) and the N-terminal amino acid of mature HSA (Asp). In the other (pMOG249) an extra Ala-codon (the first codon of mature PR-S) preceding the Asp-codon of HSA was included, preserving the natural Ala-Ala cleavage site of the PR-S precursor. This construct was made because the minimal structural requirements for cleavage of the plant pre-sequence are not known. As a control, a construct was made encoding mature HSA lacking a signal sequence (pMOG285). The four genes were subcloned as EcoRI-HindIII fragments in the binary vector pMBV4 carrying a chimaeric NOS-NPTII gene for

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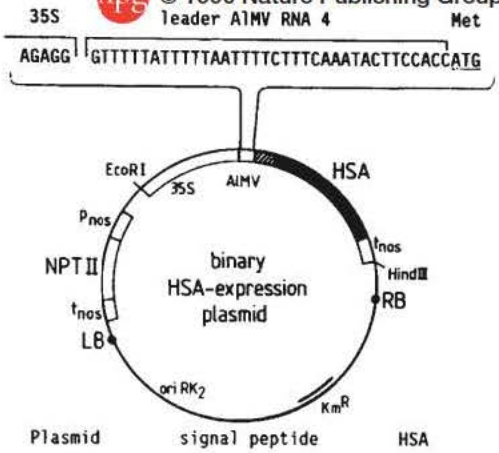

Plasmid	signal peptide	HSA
pMOG236	MKWVTFISLLFLFSSAYS RGVFRR (pre / pro)	DAHK...
pMOG249	MNFLKSFPFYAFLCFGQYFVAVTHAA (PR-S pre+A)	DAHK...
pMOG250	MNFLKSFPFYAFLCFGQYFVAVTHA (PR-S pre)	DAHK...
pMOG285		MDAHK...

FIGURE 1 Schematic representation of binary plant expression vector, and N-terminal sequences of the HSA-fusions. The fusions were verified by determination of the nucleotide sequences. The striped box represents the signal sequences, mature HSA sequence is shown in black. The amino acid sequences of the various fusion proteins are shown below the diagram. The extra alanine residue preceding mature HSA in construct pMOG249 is indicated with a star. The first four N-terminal amino acids of mature HSA are in bold. Abbreviations: LB/RB=left/right T-DNA border sequence; p_{nos}/t_{nos}=nopaline synthase promoter/terminator sequence. The translational start codon is underlined.

selection of transformed plants, and the resulting plasmids were mobilized into *Agrobacterium*[18].

Plant transformation and expression analysis. pMOG236, pMOG249 and pMOG250 were used for transformation[19] of 3x 400 potato tuber discs (*Solanum tuberosum* cv. Désiree). A total of 75 kanamycin-resistant plants was obtained, these were multiplied axenically, transferred to soil and grown to maturity. Transformation was confirmed by NPTII-assays of leaf material as described[20] (not shown). The expression level of the various HSA constructs in the transgenic plants was assayed using immunoblotting of total leaf protein extracts. The 66.5 kD HSA protein was detected in 48 potato lines, with expression levels up to 0.02% of total soluble leaf protein, using pure HSA as a standard (Fig. 2). The HSA-mRNA levels were measured on leaves of the same plants. By Northern analysis the 2 kb HSA-transcript was detected in 68 transgenic lines. To allow quantification of the HSA mRNA-levels, T7-transcripts made *in vitro* of the preproHSA-gene preceded by the alfalfa mosaic virus (AlMV) RNA4 leader were included as standards (Fig. 2). The amount of HSA-mRNA varied among plants to up to 0.025% of the total mRNA. In most plants we found a positive correlation between the HSA-mRNA levels and the protein levels (not shown). HSA-protein was detected in all parts of the transgenic potato plants assayed including leaf, stem, root and tuber tissue. Expression was highest (ca. 5-fold difference) in stems and top leaves. Despite the presence of the HSA protein in most tissues, almost all transgenic plants were phenotypically normal.

Secretion of HSA. To determine whether HSA was secreted by the potato plant cells, we purified extracellular fluid from fully developed leaves. The purity of the extracellular fluid was monitored through measurement of the specific activities of a cytosolic marker enzyme (glucose-6P dehydrogenase) and an extracellular marker enzyme (peroxidase)[21] as shown in Table 1. Levels of HSA were compared in purified extracellular protein and in total soluble protein by SDS-PAGE and Western blotting. For all gene constructs, the specific HSA-concentration in the extracellular fluid was higher than in the total extract, indicating that HSA was secreted (Fig. 3). To obtain further evidence for secretion of HSA by plant cells, we studied the expression and targeting of HSA in transgenic tobacco suspension cell lines. Transgenic plants were made containing the HSA constructs. The tobacco plants were screened for expression of the chimaeric gene and the highest expressor, carrying the pMOG249 construct, was selected for initiation of a cell suspension line. Once a rapidly growing homogenous suspension culture was obtained, both the culture medium and the cell pellet were analyzed for the presence of HSA. HSA accumulated in the culture medium (0.25 µg HSA/mg protein) when compared with the cellular HSA concentration (Fig. 3). We concluded that the signal sequence facilitated secretion of HSA from the tobacco suspension cells.

As a control experiment, pMOG285 encoding Met-HSA without a signal sequence was introduced into tobacco. Although HSA-mRNA could be detected (Fig. 2), HSA-protein could not be found after Western-blotting of 30 transgenic plants, either in total leaf extracts or in leaf extracellular fluid. Partial purification of the total soluble extracts with acetone precipitation and anion-exchange chromatography did not yield a positive HSA signal with anti-HSA. Possibly, unsecreted HSA is not stable in the cytoplasm.

Structural analysis of HSA. As no characteristic enzymatic properties are known for HSA, the chemical nature of the protein was studied by comparing the chromatographic behaviour of commercial HSA and HSA from plant origin using various fractionation techniques. Total homogenates were prepared from transgenic potato stem and leaf tissues. The extracts were incubated with antiHSA coupled to Reactigel spheres to recover recombinant HSA. The protein extracted from potato plants was analyzed by FPLC-anion exchange chromatography, FPLC-gel filtration and SDS-PAGE, and compared with natural HSA. We were not able to detect differences between the recombinant plant protein and natural HSA (Fig. 4a). These data suggest that cleavage of the precursor proteins had taken place in the plant. For a more detailed analysis we determined the N-terminal amino acid sequence of HSA from plants transformed with the

TABLE 1 Marker enzyme activities after isolation of extracellular fluid (n=5).

Marker Enzyme	Extracellular Fluid	Total Extract
Peroxidase (units / µg protein)	2.6±0.9	0.1±0.03
Glu6PdH (nmole NADPH/min/mg protein)	0.3±0.4	10.5±4.1

TABLE 2 N-terminal sequence analyses of purified HSA samples from transgenic potato plants.

Construct	Signal	N-terminal Sequence
mature HSA		D A H K S E...
pMOG236	Human prepro	R G V F R R D A H K S E...
pMOG249	PR-S + A	A*D A H K S E...
pMOG250	PR-S	D A H K S E...

Underlined sequences were actually determined, * indicates blocked N-terminal residu and presumed presence of this residu.

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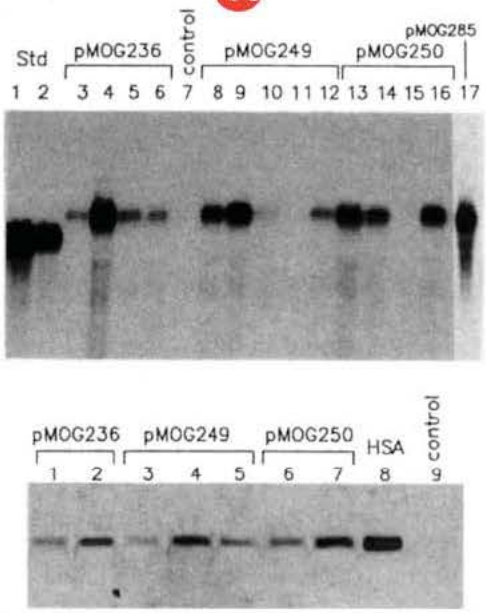


FIGURE 2 Northern (top) and Western (bottom) analysis of HSA-transgenic plants for levels of HSA mRNA and HSA protein. **Top panel** lanes 1 and 2; 250 and 100 pg T7-transcripts of the HSA gene made *in vitro* were used as standards[38]. These RNAs migrate somewhat faster than the signals in the plants due to the absence of a polyA-tail. Lanes 3–16, 10 μg of total RNA from leaves of individual transgenic potato plants containing the various constructs was loaded as indicated. As a probe a uniformly ^{32}P-labeled fragment containing the entire HSA-coding sequence was used. Lane 17 (10 μg total RNA pMOG285 tobacco) was taken from a different experiment. **Bottom panel,** 50 μg of total soluble leaf protein from individual transgenic potato plants was loaded as indicated in lanes 1–7. Lane 8, 15 ng standard HSA added to control plant extract. Control potato plants were transformed with a binary vector lacking any HSA-sequences.

three signal constructs. HSA was purified to chromatographic homogeneity from the transgenic plants (Fig. 4a). HSA from pMOG236 plants, carrying the human prepro signal sequence, still contained the 6 amino acid long prosequence at the N-terminus (Table 2). Evidently, only the 18 amino acid long presequence was cleaved during the secretion process. The chimaeric gene, encoding an exact fusion between the presequence from the tobacco PR-S protein and HSA (pMOG250), yielded mature HSA with the correct N-terminal amino acid, aspartate (Table 2). HSA isolated from plants transformed with pMOG249, which had the first Ala-codon of mature PR-S preceding HSA, was shown to be blocked for Edman degradation at the N-terminus. We presume that this protein consists of mature HSA preceded by Ala. In order to substantiate this, a further analysis with CNBr-cleavage was done with HSA purified from pMOG249 potato plants. SDS-PAGE of the CNBr-digest yielded identical patterns for recombinant pMOG249-HSA and standard HSA (Fig. 4b). As HSA was secreted both from potato leaf cells and tobacco suspension cells (Fig. 3), we assume that the alanine residue was still present after processing. Proteins with alanine at the N-terminus are known to be preferentially blocked[22].

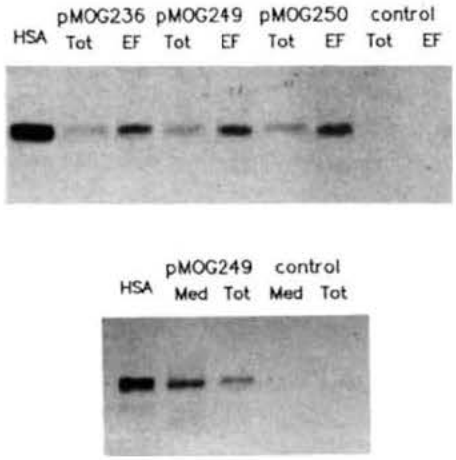
DISCUSSION

The results presented here show that a human secretory signal facilitates targeting of a protein outside the plant cell. Signal sequences of secreted proteins generally contain a positively charged N-terminus and a central hydrophobic region as a common pattern[23,24]. Although this pattern is also present in the human and plant signal sequences compared in this study, only the plant-derived signal generates the authentic mature protein in plants. The human prepro-sequence gives rise to proper targeting to the extracellular space, but yields an intermediate precursor protein. Indeed serine proteinases, necessary to remove the prosequence, are rare in plants[25]. Further, this result proves the assumption[6,26] that removal of the prosequence is not essential for secretion.

The analysis of the identity of plant-borne HSA is hampered by the absence of a characteristic enzymatic activity of the protein. Based on the different criteria used to compare it with commercial HSA we concluded that the proteins are indistinguishable. A more precise analysis of the correct folding of the molecule, including the formation of the 17 disulfide bonds in the mature protein and the ligand binding activities[27,28], will be needed to determine whether the proteins are in fact identical.

The availability of expression systems for the production of valuable recombinant proteins still is an important issue in biotechnology. The expression of HSA has already been reported in various microbial systems[7,8]. However, compared to the successful production of smaller human proteins like insulin, human growth hormone and various interferons, it has proven to be difficult to obtain useful levels of secreted HSA in these systems. The present report describes the potential to exploit transgenic crop plants as a source of secreted recombinant proteins. We have addressed the question whether secretion and proper processing of a foreign protein can be obtained in plants. Our results demonstrate that recombinant proteins that need a membrane passage to acquire their biological function can be produced in plants or plant cells. The starch potato in particular may be an interesting host to consider for the production of heterologous proteins. Wet milling processes used in the starch industry to obtain starch from tubers are compatible with the separation of high value proteins in the liquid fraction of the process. This fraction is essentially a waste product. As such, transgenic starch tubers would constitute an

FIGURE 3 Secretion of HSA from transgenic potato leaves and tobacco suspension cells. **Top panel:** Western analysis of extracellular fluid and total extracts from transgenic potato leaves. Lane 1, total soluble leaf protein +4 ng HSA added as standard. Lane 2–8, samples alternating from a total extract (Tot) and from extracellular fluid (EF) isolated from pMOG236, pMOG249, pMOG250 and control potato leaves as indicated (12 μg protein/lane). To highlight the difference between total extracts and extracellular fluid, the peroxidase staining procedure was stopped earlier compared to Fig. 2. **Bottom panel:** Western analysis of culture medium (Med) and cellular extract (Tot) of tobacco suspension cells from transgenic pMOG249 and a control cell line as indicated. Lane 1, total cellular protein +2 ng HSA added as standard (4 μg protein/lane).



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inexpensive source of biomass for protein production. At laboratory scale, reconstitution experiments with HSA added to grinded untransformed tubers, did not show any HSA degradation (data not shown), indicating that during the industrial process, HSA is stable.

More studies are needed to determine the possibility of producing HSA at high levels. Comparison of the promoter from a tuber-specific class I patatin gene, using the GUS gene as a reporter, showed a five-fold increase relative to the CaMV 35S promoter[29]. Further optimization of the expression levels to 1-2% of tuber protein may be a realistic target[4]. With an average potato yield of 50 tons/ha in The Netherlands, approximately 12 kg HSA could be produced per hectare. As the majority of production costs would be covered through the concomitant production of starch, the process may become economically feasible.

EXPERIMENTAL PROTOCOL

Plasmid constructions. The preproHSA gene was obtained from plasmid pCR206 (from Genentech, Inc.) on a 1.9 kb EcoRI-ClaI fragment. The coding region on this fragment was derived from the cDNA clones described by Lawn et al.[17] On plasmid pCR206, the ATG initiation codon immediately follows the EcoRI-site[7], the ClaI-site was 3′ to the HSA stop codon.

Construction of pMOG18. The expression cassette from plasmid ROK1[30] was cloned into the polylinker of pUC18[31] and was modified to contain the CaMV 35S promoter with a duplicated enhancer sequence[14], followed by the 5′ AlMV RNA4 leader sequence[15,16], the gene for β-glucuronidase[32] and the nopaline-synthase terminator[33]. The gene for β-glucuronidase was replaced by the HSA gene constructs as described below.

Construction of pMOG236. To allow the preproHSA gene to be inserted into the plant expression vector pMOG18 the synthetic linker 5′-C **ATG** AAG TGG GTA ACC CTCGAGATCGATG-3′ was placed in pMOG18, with a 5′ end compatible with the NcoI-site located behind the promoter/leader fragment and spanning the first 5 codons (shown as triplets) of preproHSA including the BstEII-site (underlined) located 9 nucleotides into the prepro sequence. In the resulting vector the entire preproHSA-gene was assembled by subcloning the 1864 bp BstEII-ClaI fragment from pCR206 into the BstEII- and ClaI-site provided in the linker.

Construction of pMOG249/250. For the fusions of the mature HSA coding sequence to the PR-S signal sequence[34], a silent mutation was created via site directed mutagenesis[35] procedures at the codons 9-11 of mature HSA changing the sequence CAT CGG TTT to CAT CGA TTT to create a ClaI-site. To provide the PR-S signal sequence the synthetic linker, 5′-C **ATG** AAA CTT CCT CAA AAG CTT CCC CTT TTA TGC CTT CCT TTG TTT TGG CCA ATA CTT TGT AGC TGT TAC GCATGCTCGAGATCGATG-3′ was placed in vector pMOG18, with a 5′ NcoI-compatible end and spanning the 24 amino acid long PR-S signal sequence (shown in triplets) up to a SphI-site (underlined) at the C-terminal Alanine codon GCA. In the resulting pMOG18/prePR-S vector the first 9 codons of mature HSA were provided by the synthetic SphI-ClaI fragment 5′-CT GAT GCA CAC AAG AGT GAG GTT GCT CAT-3′ to create an exact fusion (pMOG250). With the same fragment, but starting 5′ CT **GCC** GAT GCA . . ., a fusion to mature HSA was made with the first alanine codon of mature PR-S (**GCC**) still present (pMOG249). Finally the mutagenized mature HSA gene was introduced as a 1474 bp ClaI fragment in both vectors.

Construction of pMOG285. For the expression of the mature HSA-gene the sequence CGT CGA GAT at the cleavage site of the mature HSA coding sequence was mutagenized to CGC ATG GAT. This created an NcoI-site (underlined) and an ATG initiation codon for the expression of Met-HSA. The mutagenized gene was directly placed in vector pMOG18. The four constructs described above were all transferred to a binary vector as EcoRI-HindIII fragments, EcoRI preceding the CaMV promoter and HindIII 3′ proximal to the NOS-terminator. The binary vector pMOG23 used in this study is a modified version of plasmid Bin19[33]. Between the left and right T-DNA border fragments it contains the chimaeric 5′NOS-NPTII-3′NOS gene[33] and a multiple cloning site for the insertion of expression cassettes assembled in vectors such as pMOG18.

Plant transformation. Potato tuber discs (*Solanum tuberosum* cv. Désiree) were transformed as described[19] using the *Agrobacterium* binary vector system. The transgenic plants were selected on

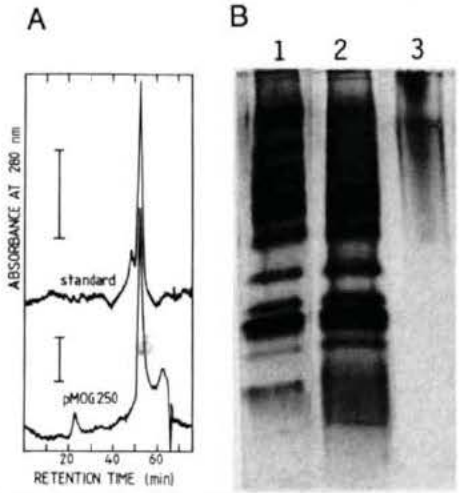

FIGURE 4 Analysis of purified HSA. **(a)** FPLC-gelfiltration profiles of purified HSA for human and plant origin on a HR10/30 Superose 6 column. The upper graph represents 10 μg standard HSA, the lower graph is purified HSA from pMOG250 potato plants (bar is 0.001 AU). HSA purified from pMOG236 and pMOG249 plants yielded identical Rf values. **(b)** CNBr digestion-patterns of HSA purified from pMOG249 potato plants as compared to HSA from human origin. Lane 1 contained 2.5 μg standard HSA, lane 2 contained 5 μg HSA from pMOG249 plants, lane 3 contained 2.5 μg undigested HSA.

media containing 100 mg/l kanamycin, multiplied axenically by meristem cuttings, transferred to soil and grown to maturity. The chimaeric HSA genes were also introduced into tobacco plants (*Nicotiana tabacum* cv. SR1) using standard leaf disc transformation procedures[36]. For the initiation of suspension cultures leaf segments of a transgenic plant expressing pMOG249 was placed on callus-inducing MS medium[37] containing 30 g/l sucrose, 2.0 mg/l NAA, 0.1 mg/l kinetin, 100 mg/l cefotaxim, 50 mg/l vancomycin and 50 mg/l kanamycin. Media were solidified with 0.8% Daichin-agar. Friable callus was then transferred to liquid medium of the same composition omitting kanamycin. After one week in suspension culture, cells were transferred to liquid MS medium with 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 100 mg/l cefotaxim and 50 mg/l vancomycin. The medium was renewed every 7 days for 6 weeks with the addition of 10 mg/l cysteine and a stepwise increase of kanamycin to a final concentration of 50 mg/l. The antibiotics were omitted after the 4th week.

HSA-expression analysis. Total RNA was isolated[38] from plants that had been grown in soil for 3 weeks. HSA transcripts were analyzed with Northern blotting. T7-transcripts[39] were made *in vitro* of the preproHSA-gene as follows. The synthetic leader sequence of AlMV RNA4[15] with a NcoI-site at the 3′ end (containing the ATG start codon) was cloned in T7 vector pBluescript (Stratagene Inc.). The entire preproHSA coding sequence was then cloned in this T7/AlMV vector as a NcoI-BamHI fragment from plasmid pMOG236. The T7 transcripts made *in vitro* were used as standards to quantify the HSA-mRNA levels. Soluble protein was extracted from 3×3 mm leaf segments from plants that had been grown in soil for 3 weeks. The tissue was homogenized in 50 μl 50 mM NaPi pH 7.0, 0.1% TritonX100, 1 mM Na_2EDTA and centrifuged. The protein content of the supernatant was determined[40] and 50 μg protein per lane was loaded on 1 mm thick 7.5–15% gradient SDS-PAGE, electroblotted onto nitrocellulose in 25 mM Tris, 192 mM glycine pH 8.3. The blot was air dried and probed for 2 h with commercial HSA goat-antiserum (Serva-lab) in 1.25% blotto, 0.2% Tween 20, 0.1% Triton X100 in 1× phosphate buffered saline (PBS), washed 3 times in the same buffer without blotto, followed by a 2 h probing with rabbit-anti-goat-peroxidase conjugate as above. After extensive washing, staining was done in 50 ml 1× PBS, 100 μl H_2O_2 and 0.5 ml *o*-Dianisidine-saturated MetOH.

Assays for secretion of HSA in plants. Secretion of HSA was studied both in intact leaves and in suspension cultures. To obtain extracellular fluid, leaves were carefully cut into 1 cm wide longitudinal sections, rolled in parafilm, mounted in a 10 ml syringe and vacuum-infiltrated twice for 2 min. in 5 mM Hepes, pH 6.3, 50 mM NaCl. Adhering fluid was removed at 200×g and

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the extracellular fluid was collected by centrifugation (10 min, 600×g). The fluid was immediately assayed for marker enzyme activity and protein content followed by cold acetone precipitation. Total soluble protein samples were prepared from the same plants as described above. Samples were dissolved in sample buffer and analyzed with Western blotting as described above. The culture medium and suspension cells that were initiated from HSA-transgenic tobacco were analyzed in the 10th week after initiation when a finely dispersed culture of primarily intact cells was obtained. The culture medium was cleared of cells and debris by centrifugation. Suspension cells were washed twice with medium and the final pellet was homogenized in PBS and centrifuged. Equal protein samples from both fractions were lyophilized and analyzed with Western blotting.

Purification of HSA from transgenic plants. For the extraction and purification of potato HSA, 1000 g of stem and leaf tissue from the highest expressors was homogenized in 1000 ml cold PBS, 0.6% polyvinylpolypyrrollidone, 0.1 mM PMSF and 1 mM EDTA. The homogenate was clarified by filtration, centrifuged and the supernatant incubated for 4 h with 1.5 ml polyclonal anti-HSA coupled to Reactigel spheres (Pierce Chem) in the presence of 0.5% Tween 80. The complex HSA-antiHSA-Reactigel was collected and washed with 5 ml 0.5% Tween 80 in PBS. HSA was desorbed from the Reactigel complex with 2.5 ml of 0.1 M glycine pH 2.5, 10% dioxane, immediately followed by a buffer exchange with Sephadex G25 to 50 mM Tris pH 8.0. The sample was then loaded on a HR5/5 MonoQ anion exchange-column (Pharmacia) and eluted with a linear NaCl gradient (0-350 mM NaCl in 50 mM Tris pH 8.0 in 20 min at 1.0 ml/min). Fractions containing the concentrated HSA (at 290 mM NaCl) were lyophilized and applied to a HR10/30 Superose 6 column (Pharmacia) in PBS at 0.3 ml/min. The HSA peak was directly spotted onto prewetted PVDF membrane for sequence analysis or for CNBr-digestions.

HSA structural analysis. N-terminal amino acid sequence analysis was done via automated Edman degradation on an Applied Biosystems 477A Protein sequencer by Eurosequence, Groningen, The Netherlands. For CNBr digestion, HSA isolated from pMOG 249 plants was desorbed[41] from the PVDF membrane and precipitated with cold acetone. The resulting pellet was dried and dissolved in 10 µl formic acid with 450 nmole CNBr/µg HSA, incubated for 24 h at room temperature and analyzed on Ag-stained 20–27% gradient SDS-PAGE[42].

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