Production of recombinant Human Serum Albumin in transgenic plants and plant cells

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Annelerin en güzeline

While the work for this thesis was being completed, my mother passed away.

I will always be thankful for my mother's encouragement and unconditional love.

This thesis is dedicated with love to the memory of

Mübeccel YÜCELT MAVITUNA

01.12.1934-27.11.2001

She was a wonderful daughter, sister, mother and best friend to all who knew her.....

I Introduction

Over the past decades manipulation of DNA *in vitro* has developed from the transfer of genetic information between prokaryotic organisms to a technology, which facilitates efficient and controlled production of proteins in foreign hosts. Recombinant proteins have been gaining significance as research tools and therapeutics in medicine, yet the supply of many eukaryotic proteins, which have potential clinical or industrial use, is often limited by their low natural availability. So far, various expression systems are established and exploited commercially. The recombinant protein production systems utilized today range from prokaryotes such as *Escherichia coli* (*E. coli*) and *Bacillus subtilis* to eukaryotes such as yeast, fungi, mammalian and insect cell cultures, animals and plants.

The expression of heterologous genes in bacteria is the simplest and most inexpensive way available for obtaining large amounts of desired proteins for research, industrial and clinical research purposes. *E. coli* is the most widely used host for the production of recombinant proteins because it is such a well-characterized system. Due to availability of various strong promoters and techniques, the efficient production of recombinant proteins in *E. coli* has become a routine matter. Furthermore, *E. coli* can readily be grown to high cell density and this has led to the development of inexpensive high-yield fermentation processes for the production of therapeutic and industrial proteins on a large scale (Jeong and Lee 1999). Expression of human insulin in bacteria was successful in 1982 and it became the first protein to be approved for therapeutic use (Brousseau *et al.* 1982).

While recombinant proteins expressed in the cytoplasm of bacteria are often insoluble and therefore inactive, soluble and bioactive proteins can frequently be obtained from eukaryotic cells. Furthermore eukaryotic cells have the capacity to carry out posttranslational modifications, such as glycosylation, phosphorylation on tyrosine, serine and threonine residues or the addition of fatty acids (Geisse *et al.* 1996). Many important pharmaceutical proteins are currently produced by using mammalian and insect cells. Mammalian expression systems comprise a plethora of different cell lines used for protein production. Most commonly, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells and myeloma cell lines are employed as hosts for the

establishment of stable transfectants (Chu and Robinson 2001). The baculovirus expression system on the other hand has been used extensively for the expression of recombinant proteins in insect cells (Poul *et al.* 1995). Recently, recombinant baculovirus vectors engineered to contain mammalian cell-active promoter elements have been used successfully for transient and stable gene delivery in a broad spectrum of primary and established mammalian cells (Kost and Condreay 2002) as well. These production systems have the advantage of carrying out the authentic post-translational modifications, but the cost of setting-up and running industrial scale mammalian cell cultures can be prohibitive and difficult to scale up.

Transgenic animals offer an alternative for producing recombinant proteins. A number of proteins, including biopharmaceuticals, have been produced in the milk of transgenic mice, pigs, sheep, and dairy cattle at concentrations of up to ten grams per liter (Velander *et al.* 1992; Devinoy *et al.* 1994). Latest experiments have demonstrated that the expression of recombinant proteins in egg white of transgenic chickens is a new alternative for the animal expression systems (Harvey *et al.* 2002). But there are potential social and ethical barriers associated with using animals as protein production systems. In addition, gene transfer to domestic mammals such as sheep, goat, pigs and cows is not a trivial process, and scale up is slow. There is generally a long development phase before protein production begins and there are many regulatory processes that have to be fulfilled (Echelard 1996).

I.1 Molecular Farming

Plants have been proven as versatile expression systems for many forms of different recombinant proteins. Molecular farming is the production of pharmaceutically important and commercially valuable proteins in plants (Schillberg *et al.* 2002; Fischer *et al.* 2003). The tools of plant biotechnology that have been developed to improve agronomics via herbicide, insect and virus resistance or to alter seed and fruit traits are now being applied to generate new protein products for food, feed and pharmaceutical industry. Plant expression systems are attractive because they offer advantages over the classical expression systems based on bacterial, microbial and animal cells:

- Plant cells have a higher eukaryote protein synthesis pathway, similar to animal cells with minor differences in protein glycosylation (Cabanes-Macheteau *et al.* 1999) whereas bacteria cannot perform most of the important mammalian post-translational modifications. When differences between plant and mammal post-translational processing do represent a problem, it may be possible to engineer plants with altered protein maturation pathways (Bakker *et al.* 2001).

- Recombinant proteins can also be directed into plants intracellular compartments such as vacuole, endoplasmic reticulum or protein bodies, where they are more stable (Conrad and Fiedler 1998) They can be expressed in definite compartments like chloroplast (Staub *et al.* 2000; Daniell *et al.* 2001) or even targeted to storage organs such as seeds, where they remain stable for months even at ambient temperatures, reducing the costs of distribution and storage (Stöger *et al.* 2000).

- Plant systems are more economical than industrial facilities using bioreactors for cultivation of cell systems.

- The technology for harvesting and processing plants and plant products on a large scale is already available.

- The purification requirement can be eliminated when the plant tissue containing the recombinant protein is used as a food.

- Concerns about contamination of expressed proteins with human or animal pathogens (HIV, hepatitis viruses) or the co-purification of blood borne pathogens and oncogenic sequences, are entirely avoided by using plants.

Molecular farming in plants can be accomplished by stable or transient expression of a recombinant protein in plants. Transient gene expression can rapidly provide large amounts of recombinant proteins for detailed structural and functional characterisation. Both for transient and stable transformation, several aspects (for example the choice of the host plant or the plant organ where expression of the protein is targeted) may influence the details of the approach but in general the first step of the process consists in adapting the gene of interest for expression (Figure I.1). Depending on the chosen plant species, the adapted gene is introduced into plant cells, using well established techniques like transformation by *Agrobacterium tumefaciens* based gene transfer

(Horsch *et al.* 1985), transformation by biolistics (Christou 1995) or by inserting the transgene into a plant virus, which then enters the plant via the normal infection route (Kumagai *et al.* 2000). In contrast to transient expression systems, stable transformation is characterized by integration of the target gene into the host plant genome. Marker-based selected transgenic plants are regenerated and selected for the presence of the recombinant protein. The transgenic plants are then analysed to determine the level of expression of the recombinant protein. Only those plants that produce satisfying amounts of the recombinant protein are kept in the process. After a passage and multiplication in the greenhouse, the offspring of the selected transgenic plants can be grown in the field to assure large scale production of the necessary biomass. The newly produced biomass is harvested using existing agricultural tools and the recombinant protein is isolated and purified from the production plant.

As an alternative to whole plants, plant suspension cell cultures can be used for recombinant protein production (James *et al.* 2000). Plant cells maintained in liquid medium under gentle agitation form cell suspension cultures. The cells can be transformed by any of the methods, except viral inoculation, discussed above, resulting in cell lines that continuously produce a recombinant protein. When clinical use of recombinant proteins is intended, their production under defined, controllable and sterile conditions with straightforward purification protocols as can be done in flasks or fermenters may be advantageous (Fischer *et al.* 1999). Suspended plant cells have been used in several recent studies producing a variety of foreign proteins (Doran 2000). These include recombinant antibodies and antibody fragments (Fischer, Liao and Drossard 1999; Fischer *et al.* 1999), proteins of therapeutic value such as interferon-2 and 4 (Magnuson *et al.* 1998) and human antitrypsin (Terashima *et al.* 1999; Huang *et al.* 2001). The most commonly used host species for protein synthesis in suspension cultures is tobacco (James, Wang *et al.* 2000; Liu *et al.* 2001). For molecular farming purposes, the expense of setting up and maintaining suspension cells in shaker flasks or fermenters may be prohibitive. Recent experiments demonstrated that production of commercially important enzymes like ß-glucuronidase and invertase is feasible but unlikely to be competitive, since scaling up is too expensive when compared to the minimal cost of agriculture (Doran 2000).

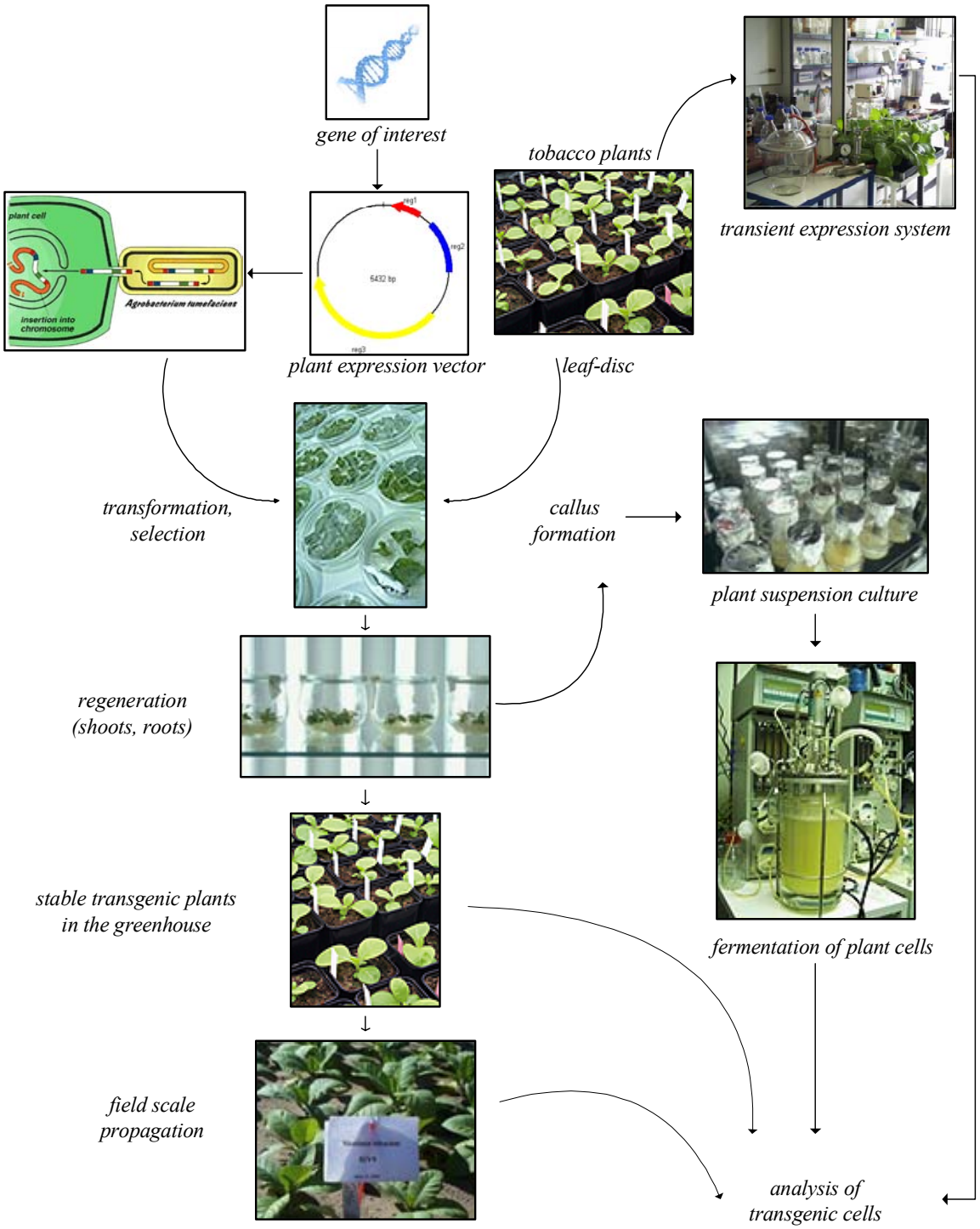

Figure I.1 Schematic presentation of the production of a recombinant protein of therapeutic interest in transgenic tobacco plants, suspension cell cultures and transiently transformed tobacco leaves**.**

I.1.1 Choice of the host plant

Crops currently being used to express recombinant proteins include cereals (rice, wheat, maize), legumes (pea, soybean, alfalfa) and fruit and root crops (tomato, potato). (Fischer, Twyman and Schillberg 2003). For practical reasons, tobacco is very often used to test the feasibility of producing a given recombinant protein in transgenic plants. The transformation of tobacco plants with *A. tumefaciens* based binary vectors is well established and easy to perform (Horsch, Fry *et al.* 1985). In addition it has been shown that tobacco plants can correctly process complex human proteins (Cramer *et al.* 1996). Tobacco as a non-food and feed crop generally has the greatest biomass yields per hectar because it can be cropped several times a year (Bernstein, Lowe and Sheen 1982; Cramer, Boothe and Oishi 1999). A single transgenic tobacco plant can produce up to a million seeds, thereby facilitating rapid scale up to substantial acreage. One of the two major disadvantages of tobacco in a molecular farming approach is that the large scale extraction and purification from plant leaves either has to be reduced to a very short period during the year, at harvest season, or rather cost intensive storage conditions for the fresh biomass have to be developed. The second disadvantage encountered is the presence of the nicotine and other toxic alkaloids that have to be eliminated during the purification process. From this point of view alternative plants and plant organs, like the seeds of cereals (Stöger, Vaquero *et al.* 2000), potato tubers (Park and Cheong 2002) and seeds of beans or peas (Perrin *et al.* 2000) appear to be more suitable targets for the production of recombinant proteins. Potato tubers would seem an organ of choice since the same standard processes used in the starch industry may be adapted with little modifications to separate proteins. Also, storage of tubers or seeds is easy so that a continuous production from stored biomass can be assured throughout the year.

I.1.2 Plant expression cassette design

Promoters

Transgene expression in plants is often performed through the use of numerous constitutive, inducible and tissue-specific promoters available. The most commonly used constitutive promoters are of viral origin such as the cauliflower mosaic virus 35S (CaMV 35S) promoter that drives very high levels of transcription in most tissues of the

plant (Benfey, Ren and Chua 1990). Further increase of the activity could be achieved by modification of the enhancer region (Rathus, Bower and Birch 1993). Although the constitutive promoters of viral origin can drive very high levels of expression, in some cases deleterious effects such as gene slicing via cosuppression have been reported (Koosha, Muller and Davis 1989). This phenomenon may be less common in the case of constitutive promoters of plant origin such as maize ubiquitin-1 promoter (*ubi-1*) (Christensen and Quail 1996) and rice actin-1 promoter (*act-1*). These constitutive promoters facilitate widespread expression in the plant. However this is not always advantageous since accumulation of recombinant proteins in vegetative organs may adversely affect plant growth and regeneration. Rather than overexpressing a transgene in all tissues, another option is to target expression of the transgene to specific organs such as leaves, tubers or seeds. Promoters like sps1 promoter (Chavez-Barcenas *et al.* 2000) for leaf specific expression or patatin promoter (Park and Cheong 2002) for tuber-specific expression have been identified and well characterized. A further advantage of this option is that it enables the production of the desired product in easily harvested tissues. In certain cases, it may be also advantageous for protein stability and/or downstream processing if recombinant proteins are expressed by the use of inducible promoters. A wound-inducible defence gene derived promoter (Cramer, Weissenborn *et al.* 1996; Hansen *et al.* 1997) is an inducible promoter example for recombinant protein expression in plants. The *MeGA*™ (mechanical gene activation) system from CropTech is using a wound-inducible HMG2 (hydroxy-3-methylglutaryl CoA reductase) promoter. When transgenic tobacco plants containing the *MeGA*™ expression cassette are subjected to mechanical stress by shearing or shredding after harvesting, gene expression occurs and recombinant protein is synthesized usually within a 24-hour period.

Transcription terminator

In addition to the promoters, further factors to be considered in design of a plant expression cassette relates to the type of polyadenylation signal or transcription terminator to be used. The most commonly used transcription terminator/poly(A) signals are derived from either the CaMV 35S RNA gene or the nopaline synthase (*nos*) gene from *A. tumefaciens*. This has been used successfully in a wide variety of species that include dicot and monocot plants (Gleave 1992). However, there are indications

that some plant-derived transcription terminators are more effective such as the 3` noncoding regions of the Me1 gene (Ali and Taylor 2001) and the wheat histone H3 gene (Ohtsubo and Iwabuchi 1994). The use of the Me1 3 noncoding region has led to a generalized enhancement of several fold in gene expression from promoters of different classes (Ali and Taylor 2001) without any alteration in the expression pattern of the promoters.

Targeting signals

Exploiting the innate protein sorting and targeted mechanisms that plant cells use to target host proteins to organelles can increase accumulation levels of recombinant proteins in plants. Protein targeting is important for three reasons in molecular farming. Firstly, the destination of a protein affects its stability, which in turn affects the final yield of the protein because total yield reflects not only the rate of production but also that of degradation. Secondly, the destination of the protein is important if it needs to be modified. Glycoproteins, for example, must be targeted to the secretory pathway since glycans are added to proteins in the endoplasmic reticulum (ER) and golgi apparatus of plant cells (Horvath *et al.* 2000). Thirdly, protein targeting may be essential for plant survival. Proteins accumulating in the cytosol may be toxic, but the same protein accumulating to the same or greater levels in the chloroplast or vacuole is often tolerated because in this case they do not interfere with endogenous metabolism.

Specific amino acid sequences required for targeting of proteins to particular organelles and for retention of proteins in organelles have been identified. Targeting signals can be used to direct the protein for secretion (Voss *et al.* 1995; Magnuson, Linzmaier *et al.* 1998) or to intracellular organelles (ER, chloroplast, vacuole) (Moloney and Holbrook 1997). For example, for retrieval of secreted proteins to the ER, an H/KDEL sequence near the carboxyl terminus specifies that a protein should remain in the ER, where the nascent glycoprotein is retained in its high mannose form, identical between plants and mammals. Targeting proteins for secretion to the intercellular space beneath the cell wall (apoplast) has advantages for downstream processing and also leads to significant levels of expression. But in case of naturally secreted proteins, ER retention can give 10-100 fold higher yields, presumably due to favourable molecular environment in the ER lumen and the presence of chaperones (Conrad and Fiedler 1998).

Optimization of expression by codon usage

In plants, as in other eukaryotes, most synonymous codons of the genetic code are not used with equal frequency but instead some codons are preferred, whereas others are rare (van Hoof and Green 1997). Accordingly, the choice of codons has been suggested to influence the rate of translation and mRNA degradation. One possible mechanism by which rare codons can effect the rate of translation is that a ribosome may pause when encountering a rare codon, because it may take longer for a rare isoaccepting t-RNA to enter the A-site of the ribosome (Murray, Lotzer and Eberle 1989). A shortage of a specific charged tRNA in bacteria can cause the ribosome to pause at codons translated at that tRNA. As few as one (Hsu, Harms and Umbarger 1985) or two codons (Yanofsky 2000) for which little charged tRNA are available can cause ribosomal pausing in bacteria. Especially in cases were rare codons are present at the 5'-end of the mRNA or in clusters expression levels are low and truncated protein products are found (van Hoof and Green 1997). In addition, it became clear that the pattern of relative use of synonymous codons was shown to differ between the taxonomic groups, primarily in the use of G + C in the degenerate third base (Murray, Lotzer *et al.* 1989). Human genes for example possess a GC content of 52.5%, where genes of potato plants contain only 42.9%, tobacco plants 43.5% and wheat plants 55.6%.

Different technically complex strategies have been used to palliate these problems. The first is site directed mutagenesis studies to partially modify the coding sequence of the gene of interest. Insecticidal toxin genes from *Bacillus thrungiensis* (*B.t*) when expressed in plants without modification could only accumulate to minimal amounts (Koziel, Carozzi and Desai 1996). Codons that inhibit efficient plant gene expression were selectively removed throughout the coding sequence by site directed mutagenesis to partially modify the gene without changing the amino acid sequence (Perlak *et al.* 1991). Among the most highly expressing tobacco and tomato plants, transgenic plants with the partially modified (3% nucleotide difference) cryIA(b) gene had a 10-fold higher level of insect control protein and plants with the fully modified (21% nucleotide difference) cryIA(b) had a 100-fold higher level of recombinant cryIA(b) insect control protein compared with the wild-type gene. The second approach is complete codon engineering by replacing codons that are rarely found in the plant genes with more favourable codons throughout the whole gene. The use of partially or fully modified

synthetic gene has the advantage to help solving all the problems resulting from inappropriate codon usage and from other factors that could interfere with efficient expression. Using this approach, a fully synthetic gene coding for the winter flounder antifreeze protein (AFP) in corn (Georges, Saleem and Cutler 1990) and porcine alpha-lactalbumin gene in the kernels of transgenic maize (Yang *et al.* 2002) were expressed. However, in both of these studies a comparison for the expression level of the authentic and plant codon optimised gene was not carried. Adang *et al.* compared plant codon optimized cryIIIA delta-endotoxin gene of *B.t.* with native gene expression by electroporation of dicot (carrot) and monocot (corn) protoplasts. CryIIIA-specific RNA and protein was detected in carrot and corn protoplasts only after electroporation with the rebuilt gene (Adang *et al.* 1993). On the other hand, it was demonstrated that plant codon optimization of the human interleukin 4 gene for expression in tobacco plants did not improve the level of the optimized protein when compared to the authentic protein (Dr. Nicole Raven, Institut für Biologie VII, personal communication).

I.1.3 Plant Transformation

Plant transformation involves the transfer of genetic information from the natural source of the desired protein into a suitable plant expression host. The most common methods used are viral vectors (Porta and Lomonossoff 1996; Scholthof, Scholthof and Jackson 1996; MacFarlane and Popovich 2000; Toth *et al.* 2001), biolistic transformation (Christou 1995; Drakakaki, Christou and Stöger 2000; Lepri *et al.* 2001), protoplast transformation (Fromm, Taylor and Walbot 1985; Terzaghi and Cashmore 1997; Newell 2000) and *Agrobacterium* mediated gene transfer (Horsch, Fry *et al.* 1985; Hansen, Shillito and Chilton 1997; Zupan *et al.* 2000; Zupan, Ward and Zambryski 2002).

Agroinfiltration

In agroinfiltration, agrobacteria carrying the expression vector are delivered into leaf tissue by vacuum infiltration. Bacterial proteins then catalyse the transfer of the gene of interest into the host cells and protein expression can be detected 24 hours after infiltration. As in conventional methods for generating transgenic plants, genes of interest are cloned into binary vectors that are transferred into *Agrobacterium* strains. A

bacterial suspension is then used for vacuum infiltration of the leaves (Kapila *et al.* 1996; Vaquero *et al.* 1999) and no selection method to identify transformed cells is required since the leaf tissue is only used for transient protein production. In agroinfiltration the transferred DNA does not integrate into the host chromosome but is present in the nucleus, where it is transcribed and this leads to transient expression of the gene of interest (Kapila, De Rycke *et al.* 1996). Agroinfiltration is rapid and yields sufficient quantities of protein for initial characterization of protein stability and function. A major advantage is that multiple genes present in different populations of agrobacteria can be simultaneously expressed. Thus the assembly of complex multimeric proteins can be tested in plants (Vaquero, Sack *et al.* 1999). A further advantage is that agroinfiltration can be scaled up to produce milligrams of recombinant protein and may be even more suitable for pre-clinical trials without the need for the production of stably transformed plants.

Expression in stably transformed plants

Initial structural and functional characterization of a recombinant protein can be performed by transient expression in leaves. However, when long-term production of complex molecules is anticipated, stable transformation is a prerequisite. Expression levels can be optimised in stable transgenic plants, where the quantity of recombinant protein obtained will only be ultimately limited by the harvested acreage. In addition accumulation levels in stably transformed plants increased in following progenies (T1 and T2) (Zimmermann *et al.* 1998; Hood, Woodard and Horn 2002). There are two major disadvantages to the use of stably transformed plants. The first is the timescale and cost development, which reflects the need for greenhouse space in the several months required for regeneration of the primary transformants. The second is the fact that integrated nuclear transgenes are exquisitely sensitive to their chromatin environment, resulting in a highly variable transgene expression level.

I.2 Production of therapeutic proteins in plants

The production of recombinant proteins for therapeutic use in transgenic plants has become an alternative to the isolation of these molecules from natural or other

recombinant sources. Plant "bioreactors" may offer the possibility of an inexpensive large scale production of high volumes of recombinant proteins with increased safety concerning contaminations with human pathogens.

Proteins derived from human plasma have become critically important therapeutic products since their introduction in the 1940s. In the last 20 years, the development in molecular biology has paved way for alternatives to the natural blood products. Today, transgenic plants have been generated that express proteins such as, interferon-2 and 4 (Magnuson, Linzmaier *et al.* 1998), human α and β hemoglobin (Dieryck *et al.* 1997), human α-1-antitrypsin (AAT) (Terashima, Murai *et al.* 1999) and two of the most expensive drugs: glucocerebrosidase (Cramer, Boothe *et al.* 1999) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Lee *et al.* 1997; James, Wang *et al.* 2000).

Human α-1-antitrypsin (AAT), a protein of therapeutic potential in cystic fibrosis, liver disease, and haemorrhages is one of the most abundant human protease inhibitor found in the blood. Recently it was produced in rice suspension culture in a 5 L bioreactor with extracellular concentrations of 51 and 40 mg active rAAT/L within 1.7 and 2.5 days respectively (Trexler, McDonald and Jackman 2002). Another protein for transgenic expression in plants is glucocerebrosidase. Gaucher's disease is a recessively inherited lysosomal storage disorder resulting from deficiencies of lysosomal hydrolase glucocerebrosidase enzyme (Cramer, Boothe *et al.* 1999). A drug developed from enzyme purified from human placentas is highly effective in reducing clinical symptoms. However, 10–12 tons/year of placentas are required to produce enough glucocerebrosidase for the average type I Gaucher's patient, making it one of the world's most expensive drugs (Giddings *et al.* 2000). Glucocerebrosidase production in transgenic tobacco was patented by Cramer and colleagues in 1999. Their studies strongly support the future commercial viability of transgenic plants for the production of glucocerebrosidase, and of other lysosomal enzymes, for enzyme replacement therapy. Another example for molecular farming of pharmaceutical protein in plants is hirudin, an anticoagulant used to treat thrombosis. It was originally isolated from the leech *Hirudo medicinalis* but is now mostly produced by recombinant bacteria and yeast. Thus far, oilseed rape, tobacco, and Ethiopian mustard have been engineered to produce hirudin, but purification from seed has proved to be expensive (Parmenter *et al.*

1995). The development of transgenic plants in which hirudin genes are fused to oleosin genes is likely to make purification easier and cheaper. The fusion construct is designed to contain an endoprotease recognition site between the two genes, enabling the eventual cleavage of the recombinant fusion protein and the recovery of the hirudin. Oleosin–hirudin fusion proteins are isolated with oil bodies, from which they can be easily separated by flotation centrifugation. This method of purification ensures that hirudin in the plants only becomes active after harvesting, thus limiting environmental exposure. Oilseed rape transgenic for hirudin is now grown commercially in Canada by SemBioSys (Calgary, Canada) (Giddings, Allison *et al.* 2000).

I.2.1 Human Serum Albumin

Human serum albumin (HSA) is a non glycoprotein, one of the few secreted proteins which lacks carbohydrate and is the most abundant protein component of the blood plasma, the normal concentration being 42g/L (Peters 1985). It contributes 80% of the colloid osmotic pressure that provides the driving force to retain the fluid within blood vessels. In addition to the osmotic function, HSA provides a high-capacity reservoir to stabilize the concentration of free ligands (Kragh-Hansen 1990).

The mature HSA is a 66.5 kDa single chain protein of 585 amino acids that is produced in the liver. The complete nucleotide sequence of HSA was published in 1982 (Dugaiczyk, Law and Dennison 1982). The protein contains a total of 17 disulphide bridges which are positioned in a repeating series of five loop-link-loop structures centered around eight sequential Cys-Cys pairs. In addition, the protein has one thiol group and one single tryptophan. The high total charge, potentially about 185 ions per molecule at pH 7, aids its solubility and the many disulfide bonds, a feature of most extracellular proteins, contribute to its stability. The translation of the albumin RNA starts with a signal peptide of the type commonly found on proteins destined for secretion. It is synthesized in the liver as a precursor, preproalbumin (Dugaiczyk, Law *et al.* 1982). The 18 amino acid prepeptide, or signal sequence is apparently removed upon secretion of proalbumin, into the lumen of ER. The unusual feature of albumin biosynthesis is the occurrence of proalbumin, which carries the basic hexapeptide Arg-Gly-Val-Phe-Arg-Arg at the amino terminus of the polypeptide chain. The propeptide

does not appear to effect ligand binding or other properties of albumin, and its purpose may be to guide the new polypeptide chain through the cytoplasm to the Golgi complex for proteolytic processing and secretion (Lawn *et al.* 1981).

Currently human serum albumin (HSA) is prepared by fractionation of donated blood plasma and is used in plasmaphoresis, fluid replacement, the treatment of burns, traumatic shock, diuretic-resistant oedema and for some groups of surgical patients (Goodey 1993).

The characteristic binding locations and chemistry for a selection or representative biological and pharmaceutical ligands for HSA were determined. The protein has been described as the major colloid that retains fluid in the vascular system, acting as a tramp streamer by dithering (a mixed cargo of metabolites around various organs (Kragh-Hansen 1990). Of these, long chain fatty acids are quantitatively the most abundant, with the normal loading being approximately two fatty acids per molecule of albumin (Petitpas *et al.* 2001). HSA also binds bilirubin (Petersen *et al.* 2000; Weisiger *et al.* 2001), amino acids, numerous drugs (Ozer and Tacal 2001; Petitpas *et al.* 2001) and heavy metals and is implicated in the transfer of many ligands across organ-circulatory interfaces such as liver, intestine, kidney and brain. The molecule has been succinctly described as the protein that makes blood thicker than water.

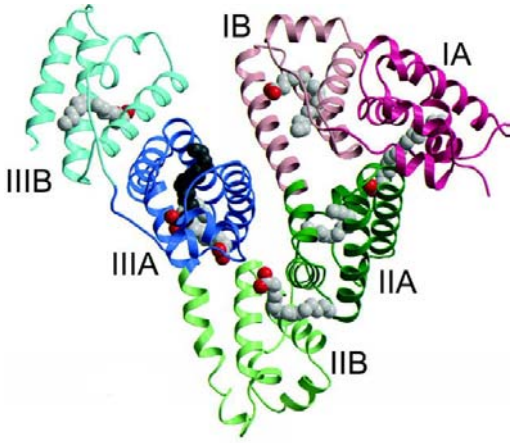

Figure I.2 The 3 dimensional structure of HSA. The amino terminus of the molecule is on the right. Two of the homologous domains form a compact structure on the right; IA, IB, IIA and IIB. The third domain is more extended, from top left to the bottom of the structure (IIIA and IIIB).

Carter and co-workers reported the first crystal structure of HSA at low resolution in 1990 (Carter and He 1990) and its redefined structure was published by the same group

(He and Carter 1992) (Figure 1.2). The structure composed of three domains I, II and III, which confer to the protein a heart shaped molecule. Each domain is a product of two subdomains that possess common structural motifs. The principal regions of ligand binding to human serum albumin are located in hydrophobic cavities in subdomains IIA and IIIA, which exhibit similar chemistry. The subdomains share a number of common features, such as the hydrophobic face, the cluster of basic amino acid residues, and proline residues at the tips of the long loops. However, it is known that each subdomain is also unique and probably exhibits a certain degree of binding specificity (Gelamo *et al.* 2002). More recently a new triclinic crystal form of native or *Pichia pastoris* expressed recombinant HSA (rHSA) was obtained at the highest resolution level (2.5 Å) reported so far (Sugio *et al.* 1999). The structures of pool plasma HSA (pHSA) and rHSA have a root mean square (r.m.s) deviation of 0.24 °A over all C-alpha atoms, which implies that two of the proteins are virtually identical.

I.2.2 Recombinant HSA expression

HSA is the most abundant protein in human plasma involved in the maintenance of a normal osmolarity and also in the transport of hydrophobic molecules. This protein has a big market requirement in that it can be used as replacement fluid during septic or traumatic shock, to compensate for blood loss, and to treat burn victims. At present, HSA is largely produced (300 tons/year) by conventional techniques involving fractionation of plasma obtained from blood donors (Saliola *et al.* 1999). It would be a great advantage to be able to use genetic engineering to obtain rHSA in good yield and at lower cost, with no danger of contamination by human pathogens. For this reason, great efforts have been dedicated to the production of this protein on a large scale by transgenic organisms. To date, expression of rHSA has been studied in *E.coli*, (Lawn, Adelman *et al.* 1981; Latta *et al.* 1990) *Bacillus subtilis* (Saunders *et al.* 1987), *Saccharomyces cerevisiae* (Quirk *et al.* 1989; Sleep, Belfield and Goodey 1990; Okabayashi *et al.* 1991; Kang *et al.* 2000), *Kluyveromyces lactis* (Saliola, Mazzoni *et al.* 1999), *Pichia pastoris* (Ohi *et al.* 1998; Ohtani *et al.* 1998) and potato (*Solanum tuberosum*) (Sijmons *et al.* 1990; Farran *et al.* 2002). (Table I.1).

Table I.1 Recombinant HSA expression in different heterologous organisms. Expression level in the table represents the highest level of rHSA produced in this host organism.

Organism	Expression level	Reference
Escherichia coli	7% of total bacterial protein	(Latta, Philit *et al.* 1990)
Saccharomyces cerevisiae	85 mg/L	(Okabayashi, Nakagawa *et al.* 1991)
Pichia pastoris	270 mg/L	(Ohtani, Nawa *et al.* 1998)
Kluyveromyces lactis	1 g/L	(Saliola, Mazzoni *et al.* 1999)
Solanum tuberosum	0.2% of total soluble protein	(Farran, Sanchez-Serrano *et al.* 2002)

Since rHSA has to be priced competitively to make any impact on the HSA market, production costs must be kept to an absolute minimum. Therefore, any production process involving the use of expensive media of the type used in mammalian cell culture is effectively excluded from consideration. Another constraint is the high number of disulfide bridges and the size of the protein (66.5 kDA), which in terms of heterologous expression considered to be large. Attempts to produce large quantities of such proteins in an intracellular form in recombinant organisms often results in the accumulation of the protein in an insoluble denatured form in inclusion bodies. In such cases the disulfide bonds are usually formed incorrectly or not at all.

rHSA has been expressed as insoluble aggregates in the cytoplasm of *E.coli* (Lawn, Adelman *et al.* 1981). However, there are distinct disadvantages in producing HSA in *E.coli*. For example, conversion of the insoluble product to the soluble form, which is frequently inefficient in *vitro*, is both time-consuming and expensive. Besides, HSA derived from *E.coli* may be contaminated by endotoxin, which causes an adverse reaction in patients (Okabayashi, Nakagawa *et al.* 1991). Therefore an early decision was to secrete the protein. The key advantages of such a route are that the secreted protein is, by definition, soluble and will not require disruption of the host organism prior to protein purification (Goodey 1993). Saunders and his co-workers have studied the secretion of rHSA from *Bacillus subtilis* using bacterial signal sequences (Saunders, Schmidt *et al.* 1987). It was found that the efficiency of processing of the leader peptides is inversely correlated with the productivity.

One problem with rHSA accumulation that has been observed is that a proportion of the secreted rHSA molecules are shorter than the full-length protein, the nature and amount of these truncated molecules vary according to the producer organism, growth regime leader sequence adopted (Sleep, Belfield *et al.* 1990). These molecules may be the result of proteolytic degradation of the full-length protein or incomplete translation of the mRNA. *Saccharomyces cerevisiae* has long been known to secrete few or no extracellular proteases, however investigations carried out in recent years have begun to reveal that extracellular proteolysis represents one of the most significant barriers to the secretory production of heterologous proteins in this host organism. For example, the main problem in the production of rHSA in *S. cerevisiae* was found to be the extracellular proteolytic cleavage of the recombinant protein during prolonged culture (Kang, Choi *et al.* 2000). In another study, rHSA expressed in the yeast *S. cerevisiae* accumulated in the insoluble fraction in a denatured form (Quirk, Geisow *et al.* 1989). Extraction of the product requires cell breakage, solubilization, reduction and renaturation prior to purification (Goodey 1993). Such downstream processing would not be practical at the scale envisaged for rHSA and would certainly not be cost effective.

It was reported that the electrophoretic karyotype of *S. cerevisiae* may not necessarily be stable. For example, during serial cultivation of *S. cerevisiae* wild strain, modifications of electrophoretic karyotype were observed at 55 generations (Longo and Vezinhet 1993). *Pichia pastoris* is frequently used as an efficient host for heterologous gene expression, the high stability of chromosomal DNA in large-scale fermentation is required for stable production, especially for pharmaceutical products. From this point of view, the gene stability of a *P. pastoris* recombinant strain expressing rHSA after long-term serial cultivation under the conditions of vegetative and non- selective growth was investigated (Ohi, Okazaki *et al.* 1998). Isolates from 83 generations showed HSA production, suggesting that elimination of HSA expression constructs hardly occurred during serial cultivation. In addition, the cells of each generation produced the almost same amount of HSA, approximately 110 μg/ml. Thus, *P. pastoris* was found to have superior gene stability; therefore, it is regarded as a suitable host for heterologous gene expression even on an industrial scale.

Kluyveromyces lactis is another promising organism that can be used as an alternative host due to its good secretory properties. Under the control of the KlADH4 promoter *K. lactis* cells grow in complex and defined media resulting in a high biomass production (70 to 80 g dry-cell weight/L), and a significant amount of rHSA production was observed in fed-batch cultures (1 g/L) when compared to batch cultures (0.2 to 0.3 g/L) (Saliola, Mazzoni *et al.* 1999).

Sijmons *et al*. (1990) have shown that rHSA can be produced in leaves of transgenic tobacco and potato and tobacco suspension cells. The transgenic constructs contained either the authentic HSA signal peptide or a preseqeunce from the extracellular PR-S protein from tobacco fused to the coding region of HSA. The secreted protein was either isolated from leaf tissue or from plant cell suspension cultures. Chromatographically purified rHSA was analysed by N-terminal amino acid sequencing. The recombinant protein containing the human signal peptide was not correctly processed, while the construct with the plant specific prepropeptide allowed the production of correctly cleaved rHSA in transgenic plants, and transgenic tobacco suspension cell lines. Plant specific signal sequence facilitates secretion of 0.25µg rHSA/mg protein into the culture medium. However, expression levels were, too low for most commercial applications being 0.02% of the total soluble protein in shoot and young leaves, and five times lower in roots and tubers.

Recently Farran and his co-workers studied expression of rHSA in tubers of transgenic potato plants (Farran, Sanchez-Serrano *et al.* 2002). HSA gene was cloned under the B33 promoter of patatin (a specific tuber promoter). The rHSA amino terminal was fused to a tuber signal peptide (protease inhibitor II) and *Agrobacterium* mediated gene transfer to potato leaves was performed. rHSA was detected in the tubers of transgenic potato plants, the highest accumulation level reaching to 0.2% of the tuber soluble protein. This higher level than the previous report by Sijmons *et al.* (1990) was attributed to the differences in the expression cassettes used for transformation.

I.4 Aim of the Study

Plant molecular biotechnology is receiving increasing attention because it is a source for innovation for the agriculture, food, chemical and pharmaceutical industry. Besides

genetic engineering is conventionally used for transferring beneficial traits to plant, a recent emerging field is the production of recombinant proteins in plants. The increasing demand for pharmaceutically safe and active human proteins can no longer be met by merely purifying from original sources as the supply of human blood, placenta or other tissues and organs as starting material for the purification of proteins is limited. In addition, several examples have shown in the past that the extraction of proteins from human tissue is not free of risk of contamination with pathogens such as HIV, Hepatitis B virus or prions and thus may lead to the transmission of infectious diseases to the treated patients. In the future, demand for existing biopharmaceuticals (e.g., erythropoietin, insulin and human serum albumin), as well as new therapeutic proteins discovered through genomics, is expected to rise considerably. Therefore, it is prudent to evaluate alternative transgenic production systems and determine how the future availability of safe recombinant biopharmaceuticals can be ensured in a cost-effective manner. Plants as bioreactor offer the possibility of an inexpensive large-scale production of high volumes of recombinant proteins with increased safety concerning contaminations with human pathogens. Due to increasing demand on albumin, 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, hepatitis that may be transmitted during infusion of contaminated blood derived products.

The primary task of this thesis was the establishment of an expression system for the production of rHSA that is used in fluid replacement, the treatment of burns, traumatic shock and for some groups of surgical patients. Aim of this study will be achieved by the following approaches:

⇒ Plant codon optimization: Since codon usage pattern of human proteins does not match the pattern found in plants, HSA needs to be codon optimised for optimal expression in the foreign host. A new strategy will be developed for the assembly of a semi-synthetic plant codon optimised HSA using PCR.

⇒ Expression of rHSA in different organisms: In order to evaluate different expression systems in terms of their efficiencies for molecular farming purposes, rHSA will be expressed in bacteria, tobacco, wheat plants and tobacco suspension cells. Protein accumulation endoplasmic reticulum (ER) using a KDEL retention signal.

⇒ Purification of rHSA from transgenic tobacco leaves: An efficient purification method will be needed to establish an efficient protocol in order to obtain better or equal to the quality of the existing HSA obtained by blood fractionation.

A schematic overview of the thesis is presented in Figure I.3.

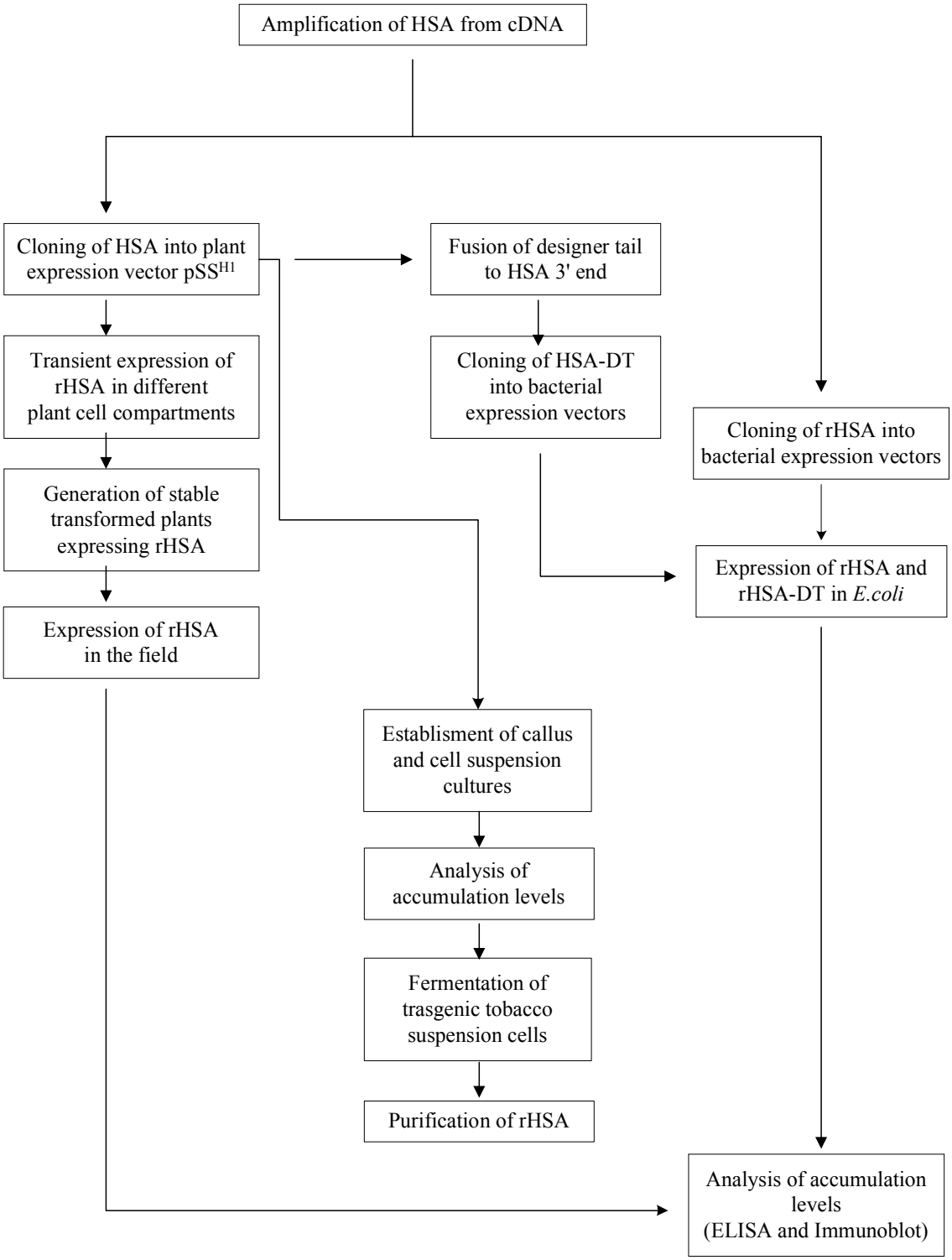

Figure I.3 Schematic presentation of the thesis work packages.

II Materials and Methods

II.1 Materials

II.1.1 Chemicals and consumables

Laboratory chemicals used throughout the work were purchased from the following companies: Bio-Rad (München), Boehringer (Mannheim), Fluka (Neu-Ulm), Gibco BRL (Eggenstein), ICN (Eschwege), Merck (Darmstadt), MWG-Biotech (Ebersberg), Pharmacia (Freiburg), Pierce (Rockford, IL, USA), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg), Sigma (Deisenhofen). The consumables were purchased from: Biozym (Hess, Oldendorf), Eppendorf (München), Greiner (Solingen), Kodak (Stuttgart), Millipore (Eschborn), Nunc (Biebrich), Roth (Karlsruhe), Schott Glaswerke (Mainz), Serva (Heidelberg), USB/Amersham (Braunschweig), Whatman (Bender & Hobeim, Bruchsal) and Zeiss (Oberkochen).

II.1.2 Enzymes and reaction kits

Restriction enzymes either from New England Biolabs (Schwalbach) or Gibco BRL were used for DNA digestion. Expand^{TM} high fidelity DNA *Taq* polymerase from Boehringer Mannheim was used for PCR amplification (II.2.3.6). DNA *Taq* polymerase from Gibco BRL was used for identification of recombinant bacteria by PCR (II.2.3.6.1).

The following kits were used:

QIAprep spin Miniprep kit	Qiagen (Hilden)
QIAquick gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
QIAquick plasmid Midi kit	Qiagen
QIAquick plasmid Maxi kit	Qiagen
Thermosequenase Cycle Sequencing Kit	USB/Amersham (Braunschweig)
PCR 2.1 TOPO Cloning Kit	Invitrogen

II.1.3 Media stock solutions and buffers

All buffers, media and solutions were prepared according to standard protocols, as described (Sambrook, Fritsch and Maniatis 1996; Coligan, Kruisbeek and Margulies 1998) except special buffers listed at the end of the corresponding method description. Heat-sensitive additives, such as antibiotics, were prepared as stock solutions, filter-sterilized (pore size; 0.2µm) and added to the medium after cooling to 50°C. Plant cell culture media and chromatography buffers were prepared with ultra pure water.

II.1.4 Antibodies and substrates

Mouse anti-human serum albumin (HSA) monoclonal antibody (Sigma) was used as the primary antibody and alkaline phosphatase (AP) conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA; distributor: Dianova, Hamburg) as secondary antibody for detection of HSA by immunoblot (II.4.8). AP conjugated rabbit anti chicken (Fc) (Dianova, Hamburg) as primary antibodies and pNPP (Sigma) as substrate tablets were used in the titer determination of polyclonal antibodies from chicken egg yolk (II.4.9.1). Polyclonal affinity purified goat-anti-HSA antibody (NatuTec, Frankfurt) was used for coating of ELISA plates (II.4.9.2). Peroxidase conjugated IgG fraction of rabbit anti-HSA antibody (Rockland, USA) was used as the primary antibody. For detection of peroxidase, ABTS substrate tablets and buffer were purchased from Boehringer Mannheim.

II.1.5 Plasmid Vectors

II.1.5.1 pHSA36 and pHSA206

Human serum albumin cDNA that was obtained from *in vitro* translation of human liver mRNA was cloned into two pBR322 plasmid vectors; (Dugaiczyk, Law *et al.* 1982) pHSA36 and pHSA206. These two plasmids share 0.15 kilo base of homologous DNA. Together they encode the entire sequence of HSA, starting with the CTT codon for Leu at position –10 of the prepeptide and extending into the 3' untranslated region of polyA.

Plasmids pHSA36 and pHSA206 were a kind gift from Prof. Dr. Kreuzaler (RWTH Aachen, Institute for Biology I).

II.1.5.2 PCR 2.1-TOPO

PCR 2.1-TOPO (Invitrogen) has a size of 3.9 kb and contains ampicillin and kanamycin resistance markers, lacZ reporter gene, T7 promoter, *Eco*RI sites flanking the PCR insertion site, and the f1 origin of replication. The vector has been engineered to be a linearized plasmid with 3' deoxythymidine (T) overhangs that is activated by being covalently bonded to topoisomerase I. The 3' A overhangs of the PCR product complement the 3' T overhangs of the vector and allow for fast ligation with the already present topoisomerase I. PCR 2.1-TOPO was used for cloning the plant codon optimised HSA after SOE PCR amplification.

II.1.5.3 pGEM-3(zf)+

pGEM-3(zf)+ (Promega, GenBank: X65306) has a size of 3.2 kb and contains the origin of replication of the filamentous phage f1. The pGEM-3Zf(+) vector contains SP6 and T7 RNA polymerase promoters flanking a region of multiple cloning sites within the alpha-peptide coding region of beta-galactosidase. pGEM-3zf was used for cloning and amplification of foreign DNA in *E.coli* and thereby functioned as an intermediate vector before cloning the plant expression cassettes into plant expression vector pSSH1 (Voss, Niersbach *et al.* 1995).

II.1.5.4 pUC18

For cloning and amplification of foreign DNA in *E.coli* high copy number pUC18 vector was used (Yanisch-Perron, Vieira and Messing 1985). pUC18 is a derivative of pBR322 vector with a size of 2.7 kb. The plasmid encodes resistance to ampicillin and has a multiple cloning site within the lacZ alpha-fragment. Inserts cloned into this site disrupt beta galactosidase activity and resulting in white colonies on X-Gal/IPTG plates.

II.1.5.5 pET 21d(+)

The pET21a(+) vector (Novagen, Madison WI, USA) has a size of 5.4 kb and contains the origin of replication of the filamentous phage f1. It carries T7 promoter plus a C-terminal his tag sequence with ampicillin resistance gene as a selectable marker.

pET21a(+) was used for the expression of human serum albumin (HSA) in the cytoplasm of *E. coli* and his tag sequence allowed the purification of the recombinant protein via immobilized metal affinity chromatography (II.6.1).

II.1.5.6 pET 22b(+)

The pET22b(+) vector (Novagen) has a size of 5.5 kB and contains the origin of replication of the filamentous phage f1. It carries a T7 promoter an N-terminal *pelB* signal sequence for periplasmic localization, plus C-terminal his tag sequence with ampicillin resistance gene as a selectable marker. pET22b(+) was used for the periplasmic expression of HSA and his tag sequence allowed the purification of the recombinant protein via immobilized metal affinity chromatography (II.6.1).

II.1.5.7 pSSH1

For transformation of *Agrobacterium tumefaciens* and transient and stable expression of rHSA in plants, plant expression vector pSSH1 (Voss, Niersbach *et al.* 1995) was utilized. Vector pSSH1 (10.3 kB, Amp^R, $Carb^R$ and Km^R) is a derivative of the binary vector pPCV002 (Koncz and Schell 1986)and contains the Cauliflower Mosaic Virus (CaMV35S) expression cassette from pRT101 (Töpfer *et al.* 1988)with a duplicated 35S enhancer region (Kay *et al.* 1987) and the termination and polyadenylation signal of the CaMV35S.

II.1.5.8 pAL76

For particle bombardment of wheat, plant expression vector pAL76 was used. It contains the ubiquitin 1 promoter and intron 1 from maize (Christensen and Quail 1996)and the nos terminator. Vector pAL76 was a kindly provided by Dr. Eva Stöger (RWTH Aachen, Institute for Biology VII).

II.1.6 Oligonucleotides

All primers used in this study were synthesized and HPLC purified by MWG-Biotech. Stock solutions of 100 pmol in autoclaved and filter sterilized (0.2µm) bidestilled water was prepared and stored at -20°C.

- **Primers used for SOE-PCR to combine two overlapping HSA cDNA fragments derived from the recombinant plasmids pHSA36 and pHSA206 (III.1).**

HSAforwardnew:	5'- GGC GAA TTC CAT GGA TGC ACA CAA GAG TGA GGT TGC-3' (36 mer)
HSAbackward:	5'-GCG AAG CTT GTC GAC TAA GCC TAA GGC AGC TTG ACT TG-3' (38 mer)
SOE-HSA-For:	5'-GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG-3' (30 mer)
SOE-HSA-Back:	5'-CGG GGC ATA AAA GTA AGG ATG TCT TCT GGC-3' (30 mer)

- **Primers used for DNA sequencing (II.2.3.7). Primers were labelled at the 5'end with IRD 700 or IRD 800.**

Universe:	5'-GTT GTA AAA CGA CGG CCA GT-3' (20 mer)
Reverse:	5'-ACA CAG GAA ACA GCT ATG AC-3' (20 mer)
PSS 5':	5'-ATC CTT CGC AAG ACC CTT CCT CT-3' (23 mer)
PSS 3':	5'-AGA GAG AGA TAG ATT TGT AGA GA-3' (23 mer)
HSAseqf1:	5'- GAC TCA AAT GTG CCA GTC TCC- 3'(21 mer)
HSAseqf2:	5' GTT ACA CCA AGA AAG TAC CCC AAG- 3' (24 mer)
HSAseqb1:	5'- GGT GTA ACG AAC TAA TAG CG-3' (20 mer)
HSAseqb2:	5'- GGC ACA TTT GAG TCT CTG TTT GG-3' (23 mer)
Modseqf1:	5'- AGG CTT CCT CTG CCA AAC AGA GG-3' (23 mer)
Modseqb1:	5'- TTG AGC CTC TGT TTG GCA GAG G-3' (22 mer)
Modseqf2:	5'- ACC TCT TGT GGA AGA GCC TCA G-3' (22 mer)
Modseqb2:	5'- CTG AGG CTC TTC CAC AAG AGG-3' (21 mer)

- **Primers used for SOE-PCR to optimize codon usage of HSA cDNA (III.2)**

In order to amplify plant codon optimised HSA via SOE PCR, 27- 83 nucleotides long 32 different primers were used as the building blocks. HSAmod1-16 as forward primers

and HSAmod7-32 as backward primers were used in the reaction mix. Forward and backward primers have complementary sequences differing in length of 21-24 nucleotides. Sequences of HSAmod1- HSAmod32 are listed in the (VIII.2).

II.1.7 Biological Materials

II.1.7.1 *Escherichia coli* strains

Throughout this work, the following *E.coli* strains were used as recipient of foreign DNA for the propagation and isolation of plasmid DNA and for protein expression.

Strain	Source	Genotype
DH5α	(Ausubel, Brent and Kingston 1994)	endA1hsdR17($r_k^-m_k^+$)supE44thi-IrecA 1gyrA(Nal^r) relA1D (lacIZYA-argF) U169 deoR (phi80dlacD(lacZ)M15)
BL21(DE3)	Stratagene	F^- ompT gal [dcm] (Adang, Brody *et al.*) hsdSB (rB^- mB^-; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene

II.1.7.2 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain GV3101 carrying the helper plasmid pMP90RK (GM^R, KM^R, Rif^R) (Koncz and Schell 1986) was used for Agrobacterium-mediated gene transfer.

II.1.7.3 *Plants*

N. tabacum L. cv. Petite Havana SR1, Badische Geudertheimer K.31, Virginia ITP, Adonis Schwester, Barley Jupiter, Barley Saturn, Korso, Nicotine free tobacco (NFT) 51 and Maryland Mammoth were used for transient expression by agrobacterial vacuum infiltration (II.2.2.5). Petite Havana SR1 plants were also used for the generation of stably transformed plants (II.2.2.6).

N. tabacum L. cv. Bright Yellow 2 (BY-2) suspension cells were used for stable expression of recombinant HSA (II.2.2.4).

Zucchini, *Cucurbita maxima cv.* Diamant 103 and bean, *Phaseolus vulgaris cv.* Marona were used for transient expression of rHSA by agrobacterial vacuum infiltration (II.2.2.5).

II.1.7.4 Animals

Polyclonal IgY-antisera were raised in adult, female, brown „Leghorn"-chicken under approval of the "Regierungspräsidium des Landes NRW" (RP-Nr.: 23.203.2 AC 12, 21/95).

II.1.8 Chromatograhpy columns matrix and membranes

Immobilon^{TM}-P transfer membrane (PVDF) (0.45µm) from Millipore, Hybond^{TM}-C nitrocellulose membrane (0.45µm) from Amersham Life Science (Braunschweig, Germany) and Whatman no.1 paper from Whatman were used in the analysis. 1.4 ml of Source 30S matrix (Pharmacia) as cation exchange medium in BioCart5 column (Kronlab, Sinsheim) was utilized for ion exchange chromatography.

II.1.9 Equipment

1. Cameras: MP4 (Polaroid, Cambridge, MA, USA). E.A.S.Y 429K camera (Herolab, Wiesloch).
2. Centrifuges: Avanti^{TM} 30 and Avanti^{TM}J-25 (Beckman, California, USA), Biofuge A (Heraeus, Hanau), Sigma 3-10 and Sigma 4-10 (Sigma, St. Louis, Missouri, USA), RC5C and RC5B plus (Sorval instruments, Du Pont, Bad Homburg). Rotors: F0650, F2402H, JLA 10.500 and JA 25.50 (Beckman), #1140 and #11222 (Sigma), RLA-300, SS-34 and GS-3 (Du Pont).
3. Chromatography equipment: ÄKTA explorer 10xT (Pharmacia) or an Äkta Purifier 100 chromatography system (Pharmacia) with cation exchange columns, evaluation and documentation software (Pharmacia).
4. DNA gel electrophoresis apparatus: wide mini and mini cells for DNA agarose electrophoresis (BioRad, München) and power supplies (BioRad).

5. DNA-sequencing machine: LI-COR IR2-4200 Sequencer (LI-COR, Bad Homburg) and Base ImageIR^{TM} 4.0 software (LI-COR).
6. Electroporation apparatus: Gene pulser^{TM}, Pulse controller unit, Extender unit (BioRad) and 0.2 cm or 0.4 cm cuvettes (BioRad).
7. Innova^{TM} 4340 incubator shaker (New Brunswick scientific, Nürtingen).
8. PCR Thermocyclers: Primus and Primus 96 plus (MWG Biotech).
9. Photometers: Spectrophotometer Uvikon 930 (Kontron, Neufahrn) and Cuvettes # 67.742 (Sarstedt, Nürnbrecht), multi-channel spectrophotometer Spectromax 340 (Molecular Devices, Sunnyvale, California).
10. Probe sonicator (Braun, Melsungen).
11. Protein gel electrophoresis equipment: Mini PROTEAN II^{TM} from BioRad. Phast system: separation and control unit and the development unit for Phast protein gels (Pharmacia). Gel Air Dryer (BioRad).
12. BioRad Protean IEF cell.
13. UV-Transilluminators: wavelength 302nm and UVT-20M (Herolab). UV-chamber (Bio-Rad).
14. BioRad He2000 particle bombardment system.
15. Biobench 7, Applikon, Schiedam, The Netherlands.
16. Shaker: Innova 4430 (New Brunswick Scientific GmbH, Nürtingen).
17. Microwave: Micromat (AEG, Frankfurt).
18. Incubator: WTB binder (Tutlingen, Germany).
19. Balance: Sartorius BP 610, 1202 MP and BP 121 S (Santorius, Göttingen).
20. Vortex: K-550-GE (Bender and Hobein AG, Zürich).
21. Vacuum exicator (Glasswerk Wertheim).

II.2 Molecular Biology Methods

All work with recombinant DNA and genetically modified organisms was performed in accordance with "S1" safety regulations and was approved by the "Regierungspräsidium des Landes NRW" [(AZ 521-K-1-8/98: AI3-04/1/0866/88 (S1) and 55.8867/-4/93 (greenhouses)].

II.2.1 Transformation, selection and characterisation of recombinant bacteria

II.2.1.1 Growth and maintenance of *Escherichia coli*

E. coli cells were grown at 37°C either in LB (amp) medium in a shaker incubator at 225 rpm, or on agar-solidified 1.5% (w/v) LB plates. For long-term storage, 600µL of an overnight (o/n) liquid culture was mixed with an equal volume of 40% (v/v) glycerol and stored at –80°C°.

LB medium	
Peptone	1 % (w/v)
Yeast extract	0.5 % (w/v)
NaCl	0.5% (w/v)
	pH 7.4

LB (amp) medium; LB medium +100 µg/ml Ampicillin.

II.2.1.2 Growth and maintenance of *Agrobacterium tumefaciens*

A. tumefaciens cells were grown in YEB medium after adding 100µg/ml rifampicin and 25µg/ml kanamycin at 28°C in a shaker incubator at 200 rpm. For selection of recombinant bacteria 100µg/ml carbenicillin was added to the growth medium. For long-term storage 600µL of an o/n liquid culture was mixed with an equal volume of glycerol stock media (GSM) and stored at –80°C°.

YEB medium	
Nutrient broth or beef extract	0.5% (w/v)
Yeast extract	0.1% (w/v)
Peptone	0.5% (w/v)
Sucrose	0.5% (w/v)
	pH 7.4

2 mM $MgSO_4$, 25µg/ml kanamycin, 100µg/ml rifampicin and 100µg/ml carbenicillin were added after autoclaving and cooling.

GSM	
Glycerol	50% (v/v)
$MgSO_4$	100 mM
Tris-HCl	25 mM
	pH 7.4

II.2.1.3 Preparation of competent *E. coli* cells for heat-shock transformation

E. coli strain DH5α competent cells were prepared for $RbCl_2$-mediated heat shock transformation. A single bacterial colony was inoculated in 5 ml of LB broth and cultured at 37°C o/n. 0.5 ml of fresh o/n culture was transferred into 100 ml of LB broth. The cells were cultured at 37°C for 3-4 hours until the OD_{600nm} reached 0.4-0.5 and then transferred to an ice-cold tube. After placing on ice for 10 min, the cells were recovered by centrifugation (2,000g/4°C/10 min). The pellets were gently dissolved in 30 ml ice-cold TfB-I solution and stored on ice for 10 min. The cells were recovered by centrifugation as described above and resuspended in 4 ml ice-cold TfB-II. 200 µl-aliquots of the suspension were dispensed into prechilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C.

	TfB-I	*TfB-II*
RBCl	100 mM	10 mM
$MnCl_2$	75 mM	-
$CaCl_2$	10 mM	75 mM
LiCl	0.5 mM	-
K acetate	35 mM	-
glycerol	15% v/v)	15 % (v/v)
MOPS	-	10 mM
	pH 5.8	pH 6.8

II.2.1.4 Transformation of *E. coli* by heat-shock

As soon as the competent cells (II.2.1.3) were thawed, plasmid DNA (II.2.3.1) or ligation products (II.2.3.5) were diluted 1:5 in sterile dH_2O and mixed gently with the competent cells then stored on ice for 30 min. The cells were incubated for 90 seconds at 42°C and placed on ice for 2 min. 800 µl of SOC medium were added to the

eppendorf tubes and transformed cells were incubated at 37°C for 45 min. 200 µl of cells were plated onto a LB-agar plate supplemented with appropriate antibiotics and incubated at 37°C overnight.

SOC medium

Bacto tryptone	1% (w/v)	
Yeast extract	0.5% (w/v)	
NaCl	10 mM	
KCl	2.5 mM	
autoclaved and added;		
sterile $MgCl_2$	1 M	10 ml
sterile $MgSO_4$	1 M	10 ml
sterile glucose	1 M	20 ml
filled up to 1 L and filter sterilized.		

II.2.1.5 Preparation of competent *E. coli* cells for electroporation

E. coli strain DH5α or BL21λDE3 competent cells were prepared for electroporation as described (Dower, Miller and Ragsdale 1988). Briefly, a single colony was cultured in LB-broth at 37°C until the mid-log phase (OD_{600nm} = 0.5-0.8); the cells were placed on ice and harvested by centrifugation (3,000g/4°C/10 min). Cells were washed three times with sterile water and suspended in ice-cold 10% (v/v) glycerol to a 300-fold concentration from the original culture volume (at $>10^{10}$ cells/ml). 40 µl aliquots were stored at -80°C.

II.2.1.6 Transformation of *E. coli* by electroporation

Electrocompetent cells (II.2.1.5) were thawed on ice and 40 µl of the cells were mixed with 1 pg to 300 ng of DNA in sterile dH_2O. The cell/DNA mixture was transferred into a prechilled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 µF, 2.5 kV, 200 Ω), the cells were diluted in 1 ml of SOC medium and incubated at 37°C with shaking for 1 h. Finally; 50-200 µl of the cells were plated onto LB-agar containing appropriate antibiotics and incubated at 37°C o/n.

II.2.1.7 Preparation of competent *A. tumefaciens* cells for electroporation

A single colony of *A. tumefaciens* grown on a YEB-agar containing 100µg/ml rifampicin (Rif) and 25 µg/ml kanamycin (Km) (YEB-Rif-Km) was inoculated in 5 ml of YEB-Rif-Km medium in a 100 ml Erlenmeyer flask and incubated at 28°C for two days with shaking (250 rpm). One ml of the culture was transferred into 100 ml of YEB-Rif-Km medium and cultivated at 28°C for 15-20 h with shaking (250 rpm) until the OD_{660} reached 1-1.5. The cells were chilled on ice for 15 min and spun down by centrifugation (4,000g/4°C/5 min). The culture medium was decanted and the cells were washed three times with 10 ml of dH_2O by centrifugation and resuspended in 500 µl of sterile 10% (v/v) glycerol. 45 µl-aliquots of the suspension were dispensed into prechilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C.

II.2.1.8 Transformation of *Agrobacterium tumefaciens* by electroporation

0.2-1.0 µg of plasmid DNA (II.2.3.1) in sterile dH_2O was added to a thawed aliquot of *A. tumefaciens* electrocompetent cells (II.2.1.7) and placed on ice for 3 min. The cell/DNA mixture was transferred into a prechilled electroporation cuvette (0.2 cm) and assembled into a safety chamber. After application of the pulse (25 µF, 2.5 kV, 200 Ω), the cells were diluted in 1 ml of YEB medium in a 4.0-ml tube and incubated at 28°C with shaking (250 rpm) for 1 h. Finally, 50-100 µl of the cells were plated on YEB-agar containing 100µg/ml rifampicin (rif), 25µg/ml kanamycin (Km) and 100µg/ml carbenicillin (carb) (YEB-Rif-Km-Carb) and incubated at 28°C for 2-3 days. As a negative control, dH_2O was used to transform competent *Agrobacterium* cells.

II.2.2 Transformation of plants and suspension cultures

II.2.2.1 Growth and maintenance of *Nicotiana tabacum*

Tobacco plants were grown in a greenhouse in ED73 standard soil (Patzer, Sinntal-Jossa) with 0-30% (v/v) sand under supplementary illumination of 10 000 Lux (plus the sun light), 70-90% humidity and 16h photoperiod) at 24°C (or higher depending on the

outside temperature). To prevent pollination from other plants, flowers were covered with plastic bags with micro pores. Mature, dried seeds were stored in paper bags at RT.

II.2.2.2 Growth and maintenance of *Nicotiana tabacum* cv. BY-2 cells

N. tabacum cv. BY-2 suspension cells and calli were grown in MSMO medium. Liquid medium was adjusted to pH 5.2, while medium solidified with 0.8% (w/v) agar was adjusted to pH 5.8. Both suspension cells and calli were cultivated at 26°C in the dark. Suspension cells were sub-cultured weekly using a 2% (v/v) inoculum while pinpoint-sized clumps of callus cells were transferred to fresh plates monthly.

MSMO medium	
MSMO salts	4.4 g/L
thiamin/HCl	0.4 mg/L
2,4-D	0.2 mg/L
KH_2PO_4	0.2 g/L
sucrose	30 g/L
	pH 5.2

II.2.2.3 Preparation of recombinant *Agrobacterium. tumefaciens*

A single colony of a positive clone was checked by *Agrobacterium*-control PCR (II.2.3.6.1) and inoculated in 5 ml of YEB medium and cultivated at 28°C for 2-3 days with shaking at 250 rpm. One ml was transferred into 100 ml of YEB-Rif-Km-Carb medium (II.2.1.2) and cultivated o/n at 28°C with shaking at 250 rpm. The following day the cells were pelleted by centrifugation at 5,000 g for 10 min at 15°C and transferred into 250 ml of induction medium in a 500-ml flask and cultivated o/n at 28°C with shaking at 250 rpm. Bacterial cells were centrifuged (4,000g/15-25°C/15 min) and resuspended in 50 ml of MMA solution and kept for 2 h at RT. The cell suspension was diluted to an OD_{600nm} of 2.4 and 100 ml of the diluted cell suspension was used for vacuum infiltration of plant leaves (II.2.2.5).

Induction medium	
YEB medium (pH 5.6)	
MES	10 mM
	pH 5.6

2 mM $MgSO_4$, 25µg/ml kanamycin, 100µg/ml rifampicin, 100µg/ml carbenicillin, and 20µM acetosyringone were added after autoclaving and cooling.

MMA Buffer	
MS salts	0.43% (w/v)
MES	10 mM
Sucrose	2% (w/v)
	pH 5.6

200µM acetosyringone was added after autoclaving and cooling.

II.2.2.4 Transformation of *Nicotiana tabacum* cv. BY-2

N. tabacum cv. BY-2 cells were transformed by co-cultivation with recombinant A. *tumefaciens*. Three ml of a 3 days old BY-2 culture in the log phase of growth were mixed in an empty petri dish (5cm) with 150µl of an o/n culture of recombinant *A. tumefaciens*. After incubation for 3 days in the dark at 24°C, the cells were transferred to centrifuge tubes and sedimented (3min/100g/RT). The supernatant was removed and the pelleted cells washed with 10ml MS medium (II.2.2.6). After three cycles of sedimentation and washing in MS, the cells were resuspended in an equal volume of MS medium and 200µl aliquots were plated on MS agar supplemented with 75µg/ml kanamycin and 250µg/ml cefotaxime. The plates were incubated in the dark at 24°C. Pinpoint-sized clumps of kanamycin-resistant cells were visible after 20-25 days and were transferred to fresh plates containing the same medium and cultivation continued until sufficient cell material for screening by immunoblot (II.4.8) was available.

II.2.2.5 Vacuum infiltration of intact plant leaves

For transient expression of recombinant proteins in different *N. tabacum* cultivars and edible plants like zucchini and bean; young leaves (4 leaves for each construct) were placed in 100 ml of recombinant *A. tumefaciens* (II.2.2.3) suspension in a Weck glass and a continuous vacuum (60-80 mbar) was applied for 15-20 min. The vacuum was

released rapidly and the leaves were briefly rinsed in tap water and kept with adaxial side upward on wet Whatman paper. After incubating the leaves at 22°C with a 16 h photoperiod for 72 h in the plastic tray that was sealed with saran wrap, the central vein of the leaves were removed. Remaining of the leaves were weighed, frozen in liquid nitrogen and stored at -80°C until analysis (II.4.2).

II.2.2.6 Stable transformation of tobacco plants

Stable transformation of *N. tabacum* was performed with the help of Dr. Flora Schuster (RWTH Aachen, Institute für Biology VII). Transgenic *N. tabacum* cv. Petite Havana SR1 was generated by leaf disc transformation using recombinant *A. tumefaciens* (II.2.1.8) and transgenic T_0 plants were regenerated from transformed calli (Fraley *et al.* 1983; Horsch, Fry *et al.* 1985). Briefly, wild type plants were grown on MS medium in Weck glasses and the youngest leaves (length up to 6 cm) were used for transformation. The agrobacteria suspension was prepared as described (II.2.2.3) and the OD_{600} was adjusted to at least 1.0 after dilution in MMA buffer. The leaves were cut into 8-10 pieces (without the central vein) and transferred into Weck glasses containing 50-100 ml of recombinant agrobacteria suspension and incubated for 30 min at RT. The leaf pieces were then transferred onto sterile pre-wetted Whatman filters in petri dishes, closed with saran wrap and incubated at 26-28°C in the dark for two days. Following washing with distilled water containing 100µg/ml kanamycin, 200µg/ml claforan and 200µg/ml Betabactyl (Ticarcillin/Clavulanic acid, 25:1), leaf pieces were transferred onto MS II-plates and incubated at 25°C in the dark for one week and with a 16 h photoperiod for 2-3 weeks.

After shooting, the shoots were removed, transferred onto MS-III-plates and incubated at 25°C with a 16 h photoperiod for 10-14 days until roots developed. The small plants were transferred into Weck glasses containing MS-III medium and incubated at 25°C in 16 h light rhythm for 2 weeks until they were transferred into soil. Young leaves from regenerated transgenic plants were used for extraction of total soluble proteins (II.4.2) and for analysis of recombinant protein accumulation by immunoblot (II.4.8) and ELISA (II.4.9).

MS medium	
MS-salts	0.43% (w/v)
Myo-Inosite (SERVA)	0.1% (w/v)
Sucrose	2% (w/v)
Thiamin-HCl	0.4 mg/l
A. bidest	add to 1000 ml

The pH was adjusted to 5.8 with 1 N NaOH (for preparation of solid medium, 0.8% (w/v) agar were added), autoclaved and 500 µl of vitamin solution I were added upon cooling to 55°C.

MS-II medium	
MS medium supp. with	
BAP (in DMSO)	1 mg/l
Kanamycin	100 mg/l
Claforan	200-500 mg/l
Betabactyl	200-250 mg/l

MS-III medium	
MS medium supp. with	
Kanamycin	100 mg/l
Claforan	200-500 mg/l
Betabactyl	200-250 mg/l

Vitamin solution I	
Glycine	0.4% (w/v)
Nicotinic acid	0.1% (w/v)
Pyridoxine	0.1% (w/v)

Filter sterilized and stored at 4°C.

II.2.2.7 Bombardment of wheat embryos

Immature wheat (*Triticum aestivum* L. cv. Bobwhite) embryos were used as explants for the delivery of the HSA-KDEL gene integrated in pal76 vector (II.1.5.8). DNA for particle bombardment was prepared by mixing plasmids containing the HSA-KDEL gene with the selectable marker in equimolar ratios not exceeding 15 µg of total DNA and 2.5 mg of gold particles (0.7 µm). Coating of DNA and bombardment of wheat embryos in a BioRad He2000 apparatus at 900psi Helium pressure was done by Dr. Eva Stöger (RWTH Aachen, Institute for Biology VII). Following selection of wheat on PPT (2.5 mg/l), PPT resistant plant material was recovered.

II.2.3 Recombinant DNA techniques

II.2.3.1 Isolation of plasmid DNA

For simultaneous screening of numerous recombinant clones, TELT method was used to prepare plasmid DNA on mini-scale. To prepare DNA for nucleotide sequence analysis, Qiagen plasmid "DNA Mini prep kit" or "Midi prep kit" was used according to manufacturer's instructions.

II.2.3.1.1 TELT method

The TELT method was performed as described (Holmes and Quigley 1981). Briefly, recombinant *E. coli* cells (II.2.1.3) from 3.0 ml overnight cultures were harvested by centrifugation (13,000g/RT/30 sec). The cell pellet was resuspended in 100 µl of TELT buffer followed by vigorous vortexing with the same volume of PCI (phenol/chloroform/isoamyl alcohol, 25:24:1) and centrifugation (13,000g/RT/5 min). The clear supernatant was transferred into an Eppendorf tube and plasmid DNA was precipitated using 2.5 volumes of ice-cold 100% (v/v) ethanol. The DNA pellet was washed twice with 70% (v/v) ethanol and air-dried for 10 min at room temperature. Finally plasmid DNA was dissolved in 30 µl of sterile distilled water.

TELT Buffer

Tris-HCl (pH 8.0)	50 mM
EDTA	62.5 mM
LiCl	2.5 mM
Triton X-100	4% (v/v)

TELT buffer was stored at 4°C.

II.2.3.2 Restriction enzyme digestion and precipitation of DNA

Restriction enzyme digestions were carried in the buffer supplied with the enzyme and in accordance with the supplier's recommendations for temperature and duration of the digestion. 1 µg DNA was mixed with 1-2 units of the appropriate enzyme and incubated for 1-3 hours at the enzyme's optimal temperature. For double digestions, combination of the two enzymes at the most suitable buffer was carried. If combination of the two enzymes were not possible, DNA was digested and purified via the PCR purification kit

(Qiagen) prior to the digest with the second enzyme. Reactions were stopped by freezing at –20°C until further processing.

For precipitation of plasmid DNA, DNA solution was mixed with 0.1 volumes of 0.3 M NaAc (pH 5.2) and 2.5 volumes of absolute ethanol. After storing this mix at -20°C for 30 min, DNA was pelleted via centrifugation (10 min/13000g/4°C). Subsequently, the DNA pellet was washed with 70% ethanol, air dried at room temperature and finally suspended in 10 mM Tris-HCl (pH 8.5).

II.2.3.3 Agarose gel electrophoresis

Analytical as well as preparative gel electrophoresis of double-stranded DNA fragments was performed as described (Sambrook, Fritsch *et al.* 1996)in 0.8-1.5% agarose gels supplemented with ethidium bromide (0.1µg/ml). As marker, a defined amount of *Pst*I digested Lambda DNA or 100bp ladder (New England Biolabs) was included for determination of fragment size and estimation of concentration. Bands were visualized using a UV transilluminator at 302nm. For the documentation of the bands, the video documentation system of the Herolab Company together with the Software "Easy Quant" and was employed. In preparative electrophoresis, the desired fragment was excised with a sterile scalpel directly on the transilluminator and the DNA was extracted from the agarose gel using the Qiaex gel extraction kit (Qiagen). The yield of the extraction was verified by analytical electrophoresis.

II.2.3.4 Determination of the DNA concentration

The concentration of the isolated and purified DNA was determined by spectrophotometric measurement of concentration by measuring the absorbance of the solution at 260 nm, the wavelength at which both DNA and RNA absorb maximally, 50µg/ml DNA gives an absorbance of 1 at 260 nm (Sambrook, Fritsch *et al.* 1996).

II.2.3.5 Ligation of DNA fragments

DNA ligations were carried out in a total volume of 20µl. The insert: vector molar ratio was adjusted to 3:1 for sticky ends in 1 x T4 ligase buffer (New England Biolabs) and 0.1U of T4 DNA ligase was added. The reactions were incubated o/n at 16°C. Ligated DNA was precipitated, washed with 70% (v/v) ethanol at RT, resuspended in 10µl of sterile ultrapure water and stored at –20°C until transformation.

II.2.3.6 PCR amplification

Polymerase chain reaction (PCR) offers a fast and convenient method of amplifying a specific DNA segment. In this technique, a denaturated (strand-separated) DNA sample is incubated with *Taq* DNA polymerase, dNTPs and two oligonucleotide primers whose sequences flank the DNA segment of interest so that they direct the DNA polymerase to synthesize new complementary strands. Multiple cycles of the process allows amplification of the genes of interest (Saiki *et al.* 1988). The reactions were performed in 0.2 ml PCR reaction tubes (Biozym Diagnostik GmbH, Oldendorf) using DNA thermal cycler (MWG). PCR reactions were carried out in a total volume of 50 µl as described below:

Components	*Volume*	*Final concentration*
10X PCR buffer	5 µl	1X
50 mM $MgCl_2$	1.5 µl	1.5 mM
10 mM dNTP mixture	1 µl	0.2 mM each
10 pmol forward Primer	0.5-1 µl	5-10 pmol
10 pmol backward primer	0.5-1 µl	5-10 pmol
Template DNA	0.5-5 µl	10-100 ng
Taq DNA polymerase (5U/µl)	0.25 µl	1.25 units
dH_2O	added to 50 µl	

PCR Conditions			
Initial denaturation	95° C	5 min	
Denaturation	95° C	30 sec	25-30 cycles
Primer annealing	Tm	30 sec	
Primer extension	72° C	45 sec	
Final Extension	72° C	7 min	
	4° C	Continuously	

The annealing temperature was adapted according to T_m value of primers. The optimal annealing temperature (T_m) of the primers was experimentally optimised or calculated by the empirical formula (Wu *et al.* 1991):

$$T_m = \{22 + 1.46[2X(G+C)+(A+T)]\}$$

PCR products were resolved on a 1-1.2% (w/v) agarose gel to check the amplification and integrity of the amplified product (II.2.3.3).

II.2.3.6.1 Colony check PCR

For rapid identification and verification of the foreign gene in recombinant bacteria, control-PCR was carried out as described (Jesnowski, Naehring and Wolf 1995). Isolated single colonies were picked using sterile tips and suspended in 13 µl of sterile dH_2O in 1.5-ml Eppendorf tubes. The suspension was boiled for 10 min and cell debris was sedimented by centrifugation (13,000g/RT/5 min). Ten µl of the supernatant was used for PCR reaction in a total volume of 25 µl by adding 2.5 pmol each of the forward and backward primer and 25 amplification cycles were performed. PCR products were resolved on a 1.2% (w/v) agarose gel (II.2.3.3).

II.2.3.6.2 SOE-PCR amplification

The gene splicing by overlap extension (SOE) PCR technique was used to generate HSA cDNA by combining two overlapping HSA fragments (III.1) and to synthesize the semi-synthetic HSA gene (III.2). Equimolar amounts of overlapping fragments were mixed in a total volume of 50µl by adding 10 pmol of each dNTP, 1 unit of Expand™ high fidelity *Taq* DNA polymerase and the corresponding buffer according to manufacturer's instruction (Roche). PCR amplification was carried according to the following table:

PCR Conditions

Initial denaturation	94° C	1 min	
Denaturation	94° C	1 min	5 cycles
Primer annealing	50° C	2 min	
Primer extension	65° C	2 min	

Ten µl of the PCR product from the above reaction was added to 10 pmol of each dNTP, 1 unit of Expand™ high fidelity *Taq* DNA polymerase and polymerase buffer in a total volume of 50 µl. This cocktail was used for a subsequent round of PCR.

PCR Conditions

Initial denaturation	94° C	2 min	
Denaturation	94° C	1 min	25 cycles
Primer annealing	60° C	1.5min	
Primer extension	65° C	2 min	
Final Extension	65° C	10 min	
	4° C	Continuously	

II.2.3.7 DNA sequencing

The correct nucleotide sequence of cDNA fragments and expression constructs was verified by DNA sequencing using a "Thermosequenase sequencing kit" (USB/Amersham) and an LICOR 4200 IR2 automated DNA sequencer (MWG) using fluorescently labelled primers. Extension reactions were terminated by incorporation of dNTPs.

As described earlier, plasmid DNA was isolated (II.2.3.1) using one of the kits from Qiagen. After determination of the DNA concentration (II.2.3.4), the purity and integrity of the plasmid preparation was checked on an agarose gel (II.2.3.3) and was added as the template plasmid DNA into the sequencing reactions. For the PCR reactions, 1.5µl of the respective termination mix (1.25mM of each dNTP plus 1.25 mM of one of the four ddNTPs) were pipetted into PCR reaction tubes. 4.5µl of sequencing master mix (2 µl 10 x *Taq* DNA polymerase buffer, 2U *Taq* polymerase, 1µg plasmid-DNA, 2.5 pM of each of the respective primers, 1µl DMSO, ultrapure H_2O) were added to each of the tubes and overlaid with a drop of PCR oil. After incubation in a PCR thermocycler (initial denaturation 4min/94°C, followed by 35 cycles of annealing (20sec/50°C), extension (30s/72°C) and denaturation (20s/95°C) the reactions were stopped by adding 3 µl of stop buffer to each tube. The products were frozen at –20°C and resolved on a denaturing (6M urea) polyacrylamide gel (4.6–6.0%). For evaluation of sequencing data DNA analysis software package was used according to manufacturer's instructions.

II.3 Expression of recombinant proteins

II.3.1 Bacterial expression

Recombinant HSA and the fusion protein HSA-Designer Tail (III.3) were expressed in the cytoplasm and periplasm of *E.coli* using different bacterial vector systems. A general protocol for expression of proteins in *E.coli* is as follows: A single colony of *E. coli* strain BL21 (Invitrogen, Leek, the Netherlands) harbouring recombinant plasmid DNA was inoculated in 10 ml of 2YT medium containing 1% (w/v) glucose and 100 µg/ml ampicillin (LBGA) and cultivated o/n at 37°C. Five ml of the o/n culture was

transferred into 400 ml of LBGA medium and cultured at 37°C to OD_{600} of 0.5-1.0. Expression of recombinant proteins was induced for 3-5 h at 28°C-30°C by addition of IPTG to a final concentration of 1.0 mM. Cells were harvested by centrifugation (4,500 rpm/4°C/15 min) and processed either for cytoplasmic or periplasmic extraction of the proteins from *E.coli* (II.4.1).

II.3.2 Expression of rHSA in the field

The field trial site was located on the AAFC-Delhi Research Station, 1 NTR, lot 40, Township of Middleton, Region of Haldimand-Norfolk, Delhi, Ontario and field tests were performed by Dr. Jim Brandle (Agriculture and AgriFood Canada). The trial was transplanted by hand with greenhouse seedlings. One transgenic line, one untransformed control was evaluated within a randomised complete block design with four replications. The planting density was approximately 111,000 plants/ha and plots had four rows with 32 plants in each plot. Approximately 30 days following transplantation, the plots were harvested and the plant material bulked for evaluation of dry matter yield and soluble protein concentration.

II.4 Protein analysis

II.4.1 Isolation of total soluble proteins from *E. coli*

For bacterial expression and purification, HSA constructs were subcloned into a prokaryotic vector for cytosolic expression and transformed into *E. coli* Bl21λDE3 strain. Cells from induced cultures (II.3.1) were harvested by centrifugation (4,500 rpm/4°C/15 min) and washed with 80 ml of TEN buffer. The pellet was resuspended in 10 ml of ice-cold TEN buffer containing 10 mg/l lysozyme. After 30 min on ice the suspension was sonicated to lyse the cells (120 W, 5 x 30sec with 1 min break interval). The cell debris was pelleted by centrifugation (12,000g/4°C/30 min).

For periplasmic extraction, pelleted cells (II.3.1) were resuspended in 10 ml of resuspension buffer (30 mM Tris-HCl, 20% (w/v) sucrose, pH 8.0) on ice. EDTA was

added to a final concentration of 1 mM. The suspension was incubated on ice with gentle agitation for 10 min. After centrifugation (8,000g/4°C/20 min), the supernatant (S1) was collected. The pellet was resuspended in 9 ml of cold 5 mM $MgSO_4$, stirred for 8-9 min at 4°C and EDTA to a final concentration of 1 mM was added to the mix. After stirring the suspension for additional 3 min, the suspension was centrifuged (8,000g/4°C/20 min). The supernatant (S2), known as the osmotic shock fraction, was mixed with the first supernatant (S1) and dialysed against PBS prior to IMAC affinity purification (II.6.1).

TEN Buffer	
Tris-HCl	10 mM
EDTA	1 mM
NaCl	0.14 mM
	pH 7.4

II.4.2 Isolation of total soluble proteins from plant leaves

For extraction of total soluble proteins, frozen leaves were ground in liquid nitrogen to a fine powder with a mortar and pestle. Total soluble proteins were extracted using 2 ml of extraction buffer per gram leaf material. Cell debris was removed by centrifugation (16,000g/4°C/30 min) and the supernatant was used for expression analyses by SDS PAGE, immunoblot and ELISA (II.4.8 and II.4.9).

Extraction Buffer	
Tris-HCl	200 mM
EDTA	5 mM
DTT	0.1 mM
Tween 20	0.1% (v/v)
	pH 7.5

For isolation of protein prior to two dimensional gel electrophoresis (II.4.6.2), 2 g of fresh transgenic leaf was ground in liquid nitrogen to a fine powder. The powder was mixed with 2.5 volumes of 10 % (v/v) trichloro acetic acid (TCA) solution in acetone. After storing this mix at -20°C for 60 min, soluble proteins were pelleted via centrifugation (10 min/13000g/4°C). Subsequently, the protein pellet was washed with 2.5 volumes of 99% acetone; air dried at room temperature and stored at -20°C until used.

II.4.3 Isolation of total soluble proteins from tobacco suspension cultures

For extraction of total soluble proteins from *N. tabacum* cv BY-2 suspension cultures, a 5 ml aliquot of the culture was centrifuged (10min/5000rpm/4°C). The supernatant (2-3ml) removed after centrifugation was further analysed by ELISA (II.4.9) and/or immunoblot (II.4.8). For the extraction of intracellular proteins, 2 ml of extraction buffer (II.4.2) was added to 1g (wet weight) of cells and sonicated on ice until the membranes were disrupted (three times 2 min bursts separated by 1 min). After sonication the sample was spun at 10.000rpm for 10 min to collect total soluble proteins supernatant for ELISA or immunoblot analysis.

II.4.4 Isolation of total soluble proteins from wheat seeds

Extraction of the TSP from dry wheat seeds were done by squashing them between glass plates and grinding to a fine powder in a mortar. Citrate buffer (pH 6) with 100 mM NaCl was used in a proportion of 1:3 (3 ml/g). TSP extract was analysed by ELISA or immunoblot analysis.

II.4.5 Quantification of total soluble proteins

Bradford assay, (Bradford 1976) using BSA as a standard, was used for quantification of protein concentration. The protein solution was diluted in the buffer used for solubilization of the protein of interest and BSA was serially diluted in the same buffer. Ten µl of each dilution was transferred into the wells of a low-protein-binding 96-well microtiter plate (Greiner, Solingen). Ten µl of the buffer was used as a blank. 200 µl of filtered Bradford colour reagent were added to each well, mixed with the proteins and incubated at RT for 10 min followed by the measurement of OD_{595nm}. For each dilution, measurements were performed in duplicate and the average was taken for the calculation of the protein concentration.

II.4.6 Gel electrophoresis of proteins

II.4.6.1 One-dimensional gel electrophoresis

Proteins were electrophoretically separated by discontinuous SDS-PAGE as described. (Laemmli 1970) (Stacking gel: T = 4%, C =2.6%, pH 6.8; separating gel: T=12%, C = 2.6%, pH 8.8). Before loading, proteins were denaturated and solubilized in 1x SDS sample buffer in a water bath at 100°C for 10 min. Separated proteins were revealed by gel staining with Coomassie brilliant blue (II.4.7.1), or transferred onto nitrocellulose membrane for immunoblot analysis (II.4.8).

SDS-PAGE running buffer pH 8.3	
Tris-HCl	125 mM
Glycine	960 mM
SDS	0.5 % (w/v)

10x SDS Sample Buffer pH 8.3	
Tris-HCl	0.35 M
DTT	0.6 M
SDS	10.3 % (w/v)
Bromophenolblue	0.05 % /w/v)
Glycerol	36 % (w/v)

II.4.6.2 Two-dimensional gel electrophoresis

The principle of high resolution 2-D gel electrophoresis was introduced by O´Farrell (O'Farrell 1975). The method separates proteins according to the physico-chemical parameters, charge (isoelectric point) in the first dimension and size (molecular weight) in the second dimension. The BioRad Protean IEF cell was used to perform isoelectric focusing of protein-containing samples using immobilized pH-gradient gel strips (Ready Strip IPG strips, BioRad). Since IPG strips are dehydrated, for passive rehydration, strips were placed in contact with the protein sample diluted in rehydration/sample buffer. The strips were covered with mineral oil to prevent evaporation and left overnight on the bench at RT according to manufacturers instructions. Prior to SDS-PAGE the IEF strips were equilibrated in an equilibration solution supplemented with 4.8% (v/v) iodoacetamide to prevent vertical gel spot

streaking. Samples were analysed in the first dimension on a pH 5-8 or 3-10 gradient and further separated in the second dimension on a 10% or 12% SDS PAA gel (II.4.6.1).

Rehydration /Sample Buffer	
Urea	9 M
Chaps	4 % (w/v)
Ampholyte (pH 3-10)	0.4 % (v/v)
DTT	1 % (65 mM)

II.4.7 Staining of protein gels

II.4.7.1 Coomassie brilliant blue staining

Expressed and purified recombinant HSA separated on one-dimensional SDS-PAA gels were visualized by Coomassie brilliant blue staining. Proteins were detected after incubating the gel for 30 min in Coomassie staining solution at RT under constant rocking. Coomassie staining was removed by destaining solution until the protein bands was clearly visible. The gel was dried between cellophane sheets for documentation purposes.

Coomassie staining solution	
Coomassie brillant blue G-250	0.25% (w/v)
Methanol	50% (v/v)
Glacial acetic acid	9% (v/v)

Coomassie destaining solution	
Methanol	10% (v/v)
Glacial acetic acid	10% (v/v)

II.4.7.2 Silver Staining

For higher sensitivity and to detect small amounts of proteins, two-dimensional gels were silver stained according to the "Silver staining protocol for proteins" from Pharmacia. Initial fixation step was carried out overnight in order to reduce background staining of polyacrylamide gels. The gels were sensitized by 30 min incubation in prestain solution, and subsequently rinsed three times with bidistilled water for 5 min each. After rinsing, the gels were submerged in 0.1% (w/v) silver nitrate solution for 20 min. The silver nitrate solution was discarded, gels were rinsed twice with water for 1

min and then developed in sodium carbonate solution containing formaldehyde. After the desired intensity of staining was achieved (3-5 min), the development was terminated by discarding the reagent, followed by washing the gels with water. The development process was stopped with an aqueous EDTA solution. Silver-stained gels were stored in a solution of 1% (v/v) acetic acid at 4°C until analyzed.

Fixation solution	
Ethanol	40% (v/v)
Glacial acetic acid	10% (w/v)

Prestain solution (Sensitizing)	
Ethanol	30% (v/v)
Glutardialdehyde	25% (w/v)
Sodium thiosulphate	5% (w/v)
Sodium acetate	6.8% (w/v)

Silver nitrate solution	
Silver nitrate	2.5% (w/v)
Formaldehyde (37%) (w/v)	0.04% (v/v)

Developing solution	
Sodium carbonate	2.5% (w/v)
Formaldehyde (37%) (w/v)	0.02% (v/v)

Stop solution	
EDTA- $Na_2.2H_2O$	1.46% (w/v)

II.4.8 Immunoblot

Electrophoretically separated proteins were transferred from a SDS-PAA gel (II.4.6.1) to a Hybond^{TM}-C nitrocellulose membrane (0.45 µm) in the presence of transfer buffer. The membrane was blocked with PBS buffer containing 3% (w/v) skim milk (MPBS) and blotted proteins probed with a primary antibody (II.1.4) that reacted specifically with antigenic epitopes displayed by the target protein attached to the membrane. The bound antibody was detected by addition of an appropriate secondary polyclonal antibodies (II.1.4) coupled to alkaline phosphatase (AP). Both primary and secondary antibodies were diluted in MPBS. The target protein was finally revealed by addition of substrate NBT/ BCIP (BioRad).

The following is a general protocol for the Immunoblot analysis.

Table II.1 Illustration of the steps for Immunoblot. All analysis steps were performed in 10-20 ml of solution on a shaker at room temperature.

Con.= Concentration Dil.= Dilution
Inc.= Incubation Temp. = Temperature
RT = room temperature o/n= over night
GAM^{AP} = AP conjugated goat anti mouse antibody
NBT= nitro blue tetrazolium chloride BCIP=5-bromo-4-chloro-3-indolylphosphate

	Step	Con. /Dil.	Inc. (min)	Temp (°C)
	Blocking	5% (w/v) MPBS	30	RT
	Primary antibody		60	RT
	mouse anti-HSA	1:5000 in PBS		
or	mouse anti-his6	1:2500 in PBS		
or	mouse anti-cmyc	1:3000 in PBS		
	Secondary antibody GAM^{AP}	1:5000 in PBS	60	RT
	NBT/BCIP		2-10	RT

To remove unspecifically bound antibodies the membrane was washed 3 times for 10 min with PBST between each step. Membrane was equilibrated with AP buffer prior to substrate addition.

PBS (Phosphate buffered saline) (pH 7.3)	
NaCl	137 mM
KCl	2.7 mM
$Na_2HPO_4.2H_2O$	8.1 mM
KH_2PO_4	1.5 mM

PBST (pH 7.3)	
PBS	1x
Tween-20	0.05% (w/v)

MPBS (Blocking buffer) (pH 7.3)	
PBS	1x
Skim milk	3% (w/v)

Transfer buffer (pH 8.3)	
Tris-HCl, pH 8.3	25 mM
Glycine	192 mM
Methanol	20 % (v/v)

AP buffer (pH 9.6)	
Tris-HCl, pH 9.6	100 mM
NaCl	100 mM
$MgCl_2$	5 mM

II.4.9 Enzyme Linked Immunosorbent Assay (ELISA)

The Enzyme Linked Immunosorbent Assay (ELISA) is a rapid and sensitive method for detection of antigens (Ag) or antibodies (Ab) in a wide variety of biological samples. Many variations in the methodology of the ELISA have evolved since its development in the 1960s but the basic concept is still the immunological detection and quantification of single or multiple Ag or Ab in a given sample.

II.4.9.1 Determination of IgY titer in egg yolk

For titer determination of polyclonal antibodies from chicken egg yolk an indirect ELISA assay was performed. ELISA plates were coated with HSA (1µg/mL in coating buffer). After blocking of free binding sites by incubation with blocking buffer, serial dilutions of the yolk (1:500 – 1:256,000 in PBS) were added and bound IgY was detected using Fc-specific RAC^{AP} antibodies and PNPP substrate tablets (Sigma). The quantity of bound antibodies was photometrically determined at E_{405nm}.

Table II.2 Illustration of the steps for titer determination of chicken egg yolk. All the steps were performed in 10-20 ml of solution on a shaker at room temperature.

Con.= Concentration Dil.= Dilution Vol.= Volume
Inc.= Incubation CB= Coating buffer Temp= Temperature
o/n= over night SB= Sample buffer BSA= Bovine Serum Albumin
RAC^{AP} = AP conjugated rabbit anti chicken antibody
AP= alkaline phosphatase EMA-P= Buffer for enzyme labelled antibody
RT= Room temperature PNPP= p-nitrophenyl phosphate

Step	Con. /Dil.	Vol. (µl)/well	Inc. (min)	Temp (°C)
Coating	1µg/ml in CB	100	60	37
Blocking	1% (w/v) BSA in PBST	300	o/n	4
Sample	Dil. in PBS	100	120	37
RAC^{AP}	1:5000 in EMA-P	100	60	37
PNPP	1 mg/ml in SB	100	15-60	RT

Between each step wells were washed three times with 1x PBST.

Coating buffer (pH 9.6)	
Na_2CO_3	15 mM
$NaHCO_3$	35 mM
Na-azid	0.02% (w/v)

EMA-P buffer (pH 7.4)	
PVP (20-30 kDa)	2% (w/v)
BSA	0.2% (w/v)
Na-azid	0.02% (w/v)
Tween-20	0.05% (w/v);
1xPBS pH 7.4	

Sample buffer (pH 9.8)	
Diethanolamin	0.1 M
$MgCl_2$	1 mM

II.4.9.2 Detection of recombinant HSA in bacteria and plant

For detection of bacterial and plant-expressed recombinant HSA and HSA-DT fusion proteins, an indirect ELISA was used. Goat-anti-HSA antibody (Bachem) was coated on microtiter ELISA plates. After blocking of free sites by incubation with blocking buffer, standards and samples were added in replicates of three. Recombinant HSA was detected using peroxidase-conjugated rabbit anti-HSA antibody (Rockland) and ABTS substrate tablets dissolved in ABTS buffer (Boehringer Mannheim). The adsorption was read at OD_{690nm}.

The following schema was followed for ELISA;

Table II.3 Illustration of the steps for HSA quantification in bacteria and plant. All the steps were performed in 10-20 ml of solution on a shaker at room temperature.

Con.= Concentration Dil.= Dilution Vol.= Volume
Inc.= Incubation Temp= Temperature RT = room temperature
o/n= over night
α-GHSA = Goat anti-HSA antibody
α-RHSA^P = peroxidase-conjugated rabbit anti-HSA antibody
ABTS = 3-ethylbenzthiazoline-6-sulfonic acid

Step	Con. /Dil.	Vol. (µl)/well	Inc. (min)	Temp (°C)
α-GHSA	1:500 in TBS	200	o/n	4
Blocking	3% (w/v) MTBS	200	30	RT
Sample	Dil. in TBS	100	60	RT
α-RHSA^P	1:20000 in TBS	100	60	RT
ABTS	1 mg/ml in ABTS buffer	100	1-10	RT

Between each step wells were washed three times with washing buffer.

TBS buffer (pH 8.0)	
Tris-HCl, pH 8.0	0.05 mM
NaCl	0.138 M
KCl	2.7 mM

Washing buffer (pH 8.0)	
TBS buffer	
Tween-20	0.05 %(v/v)

MTBS (Blocking buffer) (pH 8.0)	
TBS buffer	
non-fat dry milk	3 % (w/v)

II.5 Production of IgY anti sera

II.5.1 Immunization of chicken

The treatment and maintenance of laboratory animals was approved by the "Regierungspräsidium des Landes NRW" (RP-Nr.: 23.203.2 AC 12, 21/95) and supervised by Dr. Hirsch who is responsible for biological safety at the Institute for Biology VII, RWTH Aachen. Initial chicken immunizations were performed with 100µg of pure HSA (Sigma, Catalogue number: A1653) in PBS emulsified with an

equal volume of complete Freund's adjuvant (GibcoBRL). Two 500µl aliquots were injected at two different places of pectoral muscle. Three and six weeks after the initial immunization, injections were repeated as above, except that incomplete Freund's adjuvant (GibcoBRL) was used. Two weeks after the third immunization, eggs from the immunized chicken were collected and the specific IgY antibody titer determined by ELISA (II.4.9.1).

II.5.2 Isolation of chicken polyclonal antibodies from egg yolk

IgY antibodies were purified from egg-yolk by repeated precipitation with PEG-6000 as described. (Polson *et al.* 1985). All centrifugations were at 20,000x g for 20 min at 4°C. The yolk sacs were collected, washed with cold tap water, the yolk volume determined and four volumes of cold PBS added. 3.5% (w/v) of solid PEG-6000 was added and stirred until dissolved. After centrifugation the supernatant was filtered through Miracloth and Whatman 3M paper, 8.5% (w/v) of PEG-6000 was added and re-centrifuged. The pellet was resuspended in 2.5-fold of the original yolk volume of PBS, treated with 12% (w/v) PEG-6000 and recentrifuged. The pellet was resuspended in 0.25-fold the original yolk volume of PBS containing 0.02% (w/v) NaN_3, treated with 50% (v/v) ice-cold ethanol and centrifuged again to remove all PEG-6000. The supernatant containing the purified IgY antibodies was filter-sterilized and stored in aliquots at –20°C. An indirect ELISA was performed in order to determine the concentration of polyclonal antibodies in egg yolk (II.4.9.1).

II.6 Recombinant protein purification

II.6.1 Immobilized Metal Affinity Chromatography (IMAC)

His-6 tagged secreted recombinant HSA was affinity purified by IMAC. Collected periplasmic fractions from *E.coli* suspension were dialysed o/n against PBS (II.4.1). Ni-NTA-agarose matrix was added in a disposable column, charged with 5 column volumes (CV) of 50 mM $NiSO_4$ and washed with 10 CV of 0.5 M acetic acid before equilibration with 10-20 CV of binding buffer (1x PBS, 10 mM imidazol; 500 mM

NaCl). Imidazol and sodium chloride were added into the dialyzed periplasmic fraction (II.4.1) to a final concentration of 10 mM and 500 mM, respectively. The sample was passed twice through the pre-equilibrated Ni-NTA matrix. The column was washed with 20 volumes of wash buffer (1x PBS, 30 mM imidazol; 500 mM NaCl) and the proteins were eluted in three fractions with 700 µl of elution buffer (1x PBS, 250 mM imidazol pH 4.5). Collected fractions were immediately dialysed against PBS (pH 7.4) to remove imidazol and salt. To control the yield and purity of dialyzed recombinant HSA, 5 µl were separated by SDS-PAGE (II.2.4.6.1).

II.6.2 Cation Exchange Chromatography

Total soluble proteins were extracted using 2 ml of extraction buffer (Acetate buffer; pH: 4.5+5 mM EDTA) per gram leaf material. Cell debris was removed by centrifugation (16,000g/4°C/30 min) and the supernatant was used for cation exchange chromatography (CEX). Conventional chromatography in packed bed columns was performed by Dr. J. Drossard (Institute for Biology VII, RWTH Aachen) using a Äkta Explorer 10XT or a Äkta Purifier 100 chromatography system (Pharmacia) with cation exchange columns. Chromatography was performed using either a 1.4 ml Source 30S (Pharmacia) cation exchange medium in BioCart5 column (Kronlab, Sinsheim) or a XK50/20 cation exchange column filled with 120 ml of Seppharose Big Beads equilibrated with the respective loading buffer (20 mM acetate pH 4.0) with a linflow of 300 cm/h.

III Results

The objective of the present work was production of recombinant human serum albumin (rHSA) in transgenic organisms. Therefore HSA cDNA was cloned into different bacterial and plant expression vectors. Initial attempt was to express rHSA in *E. coli*. After confirmation of bacterial expression, different plant species were evaluated for high level production of rHSA. Transient expression of rHSA was carried via *Agrobacterium* mediated gene transfer using tobacco leaves and leaves from edible plants like zucchini and bean leaves. The same expression cassettes developed for agroinfiltration were used to generate stable transgenic tobacco plants. High level expressors were selected and evaluated for several generations in the greenhouse. Moreover T2 generation was analysed to confirm rHSA accumulation in the field. In addition, stable transformation of wheat was performed by bombarding wheat embryos using a HSA expression construct downstream to ubiquitin promoter. Stable expression of rHSA in transgenic tobacco suspension cells was performed. Secreted rHSA to the BY2 culture medium was analysed and stability of the secreted protein was improved by addition of stabilizing agents. For downstream processing of the transgenic tobacco plants producing rHSA, cation exchange chromatography was experienced. Conditions for the partial purification of rHSA from tobacco leaves were optimised.

III.1 Production of polyclonal anti-HSA serum

To detect rHSA accumulation in protein extracts of different production organisms polyclonal antibodies against HSA were prepared. For the generation of these antibodies, chicken were immunized by injecting 100µg of commercial HSA (Sigma) (II.5.1). Two weeks after the third boost, polyclonal chicken IgY antibodies were purified from chicken egg yolk (II.5.2). IgY was purified by repeated PEG precipitation (Polson, Coetzer *et al.* 1985) with an average yield of 8 mg IgY per ml yolk, determined by spectrophotometry. As shown in Fig III.1A (lane 1) the purification of IgY antibodies resulted in some weak additional protein bands visible at ~40kDa. It was not determined if these bands were contaminants or IgY degradation products produced during purification and storage. The antibody titer referring to the highest dilution at which antigen-specific binding is visible by an E_{405nm} of 0.2 after 1 hour substrate induction (II.4.9.1). The titer of HSA specific antibodies in egg yolk was determined to be 1: 130,000 by indirect ELISA (Figure III.1B).

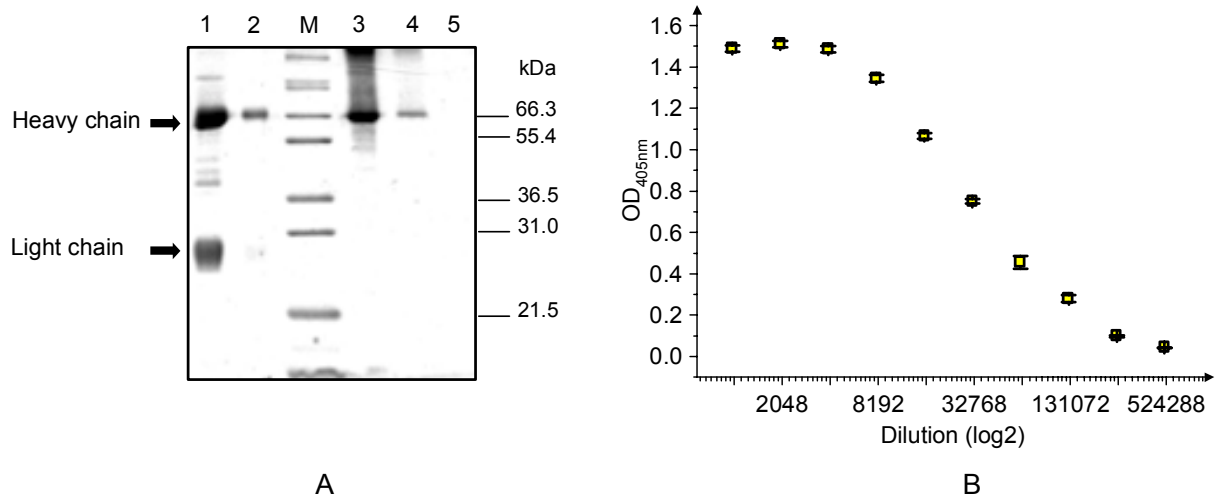

Figure III.1 SDS PAGE analysis (A) and titer determination (B) of polyclonal antibodies purified from chicken egg yolk. **A**: 3 µl of purified IgY (II.5.2) per lane was separated on a 12% SDS-PAGE gel (II.4.6.1). The gel was stained with Coomassie Brilliant Blue after electrophoresis as described (II.4.7.1). 1: 1:10 diluted IgY; 2: 1:100 diluted IgY; M: Molecular weight marker (Mark 12, Novex); 3: 10 µg BSA; 4: 1 µg BSA; 5: 0.1 µg BSA. B: Determination of polyclonal antibody titer by ELISA. Commercial HSA (Sigma) (1µg/mL in coating buffer) was coated onto a microtiter plate. Serial dilutions of purified sera were added. Bound antibodies were detected by addition of GACAP polyclonal antibody (1:5000). ELISA readings were performed at OD405nm after 1 h min incubation with pNPP substrate at 37°C.

Polyclonal anti-HSA antibodies from chicken egg were intended to use in the immunoblot (II.4.8) analysis of rHSA accumulation in different host organisms. However, initial experiments (data not shown) showed that the specificity of the generated polyclonal serum was not sufficient to obtain reproducible results. Therefore the polyclonal serum was replaced by highly specific commercially available monoclonal antibodies in the ongoing work (II.1.4).

III.2 Construction of HSA expression constructs

III.2.1 Cloning of rHSA

The full length mRNA encoding HSA is 2163 nucleotides (nt) long, with a short 5' untranslated region (UTR) of 46 nt and a 3' UTR of 207 nt (Lawn, Adelman *et al.* 1981). The HSA coding sequence is 1827 bp long encoding a 609 amino acid preproalbumin (Dugaiczyk, Law *et al.* 1982). The 18 amino acid signal sequence is apparently removed upon secretion of preproalbumin into the lumen of the endoplasmic reticulum. The six amino acid pro sequence

is subsequently removed in the Golgi apparatus (Lawn, Adelman *et al.* 1981). Finally the mature HSA protein, 585 amino acids in length is released into the blood.

The HSA cDNA was obtained from *in vitro* transcription of human liver mRNA and two overlapping fragments were separately cloned into pBR322 plasmids resulting in pHSA36 and pHSA206 (Dugaiczyk, Law *et al.* 1982). These two plasmids share 0.15 kb of homologous DNA (Figure III.2). Together they encode the entire sequence of HSA, starting with the CTT codon for Leu at position –10 of the prepeptide and extending into the 3' untranslated region of polyA (II.1.5.1).

In order to combine the two HSA cDNA inserts by SOE PCR (II.2.3.6.2), four primers (II.1.6) were designed (Figure III.2A). The primer design enabled deletion of the leader and hexapeptide of authentic HSA and introduction of *Eco*RI/*Nco*I sites at the 5' and *Sal*I/*Hind*III sites at the 3' end for subsequent subcloning into expression vectors.

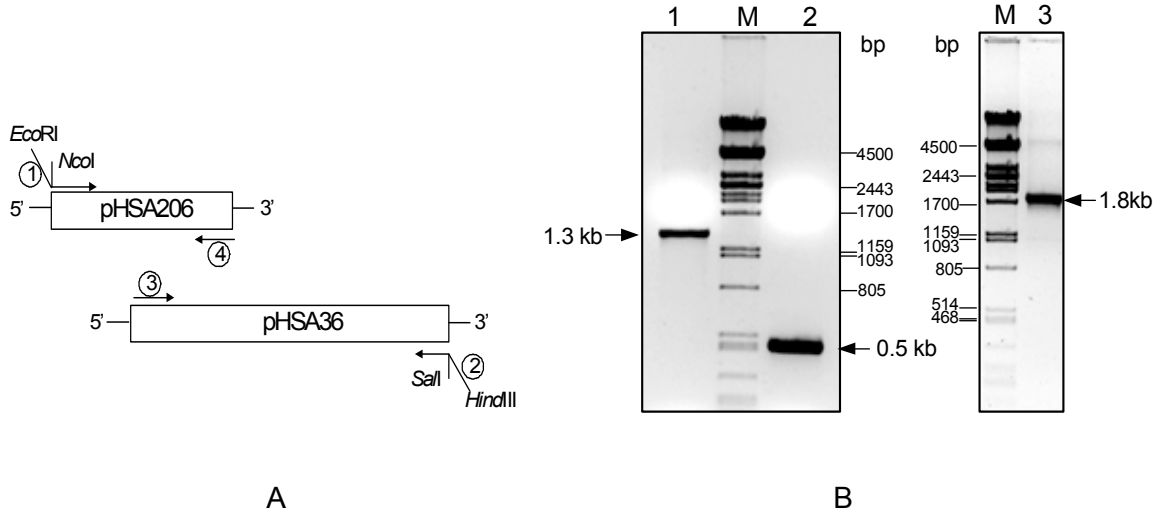

Figure III.2 Diagram of overlapping cDNA inserts (A); Analysis of SOE PCR (II.2.3.6.2) by agarose gel electrophoresis (B).A: Plasmids pHSA36 and pHSA206 in pBR322 are illustrated together with the overlapping sequence and the primers used for amplification of HSA cDNA (①: HSAforwardnew;② : HSAbackward;③ : HSAfor; ④ :HSAback). B: (1) 1.3 kb HSA cDNA fragment (2) 0.5 kb HSA cDNA fragment. (3) The complete cDNA of HSA, 1.8 kb. 5 µl of the amplified DNA was separated on 1.2% (w/v) agarose gel (II.2.3.3). M: PstI digested λ DNA.

The 1.3 kb HSA cDNA fragment was amplified from pHSA36 using the primers HSAfor and HSAbackward. The 0.5 kb HSA cDNA fragment was amplified from the template pHSA206 using the HSAforwardnew and HSAback primers. These two fragments were combined by SOE PCR (II.2.3.6.2) to obtain the complete rHSA cDNA. The 1.8 kb SOE PCR product was gel purified (II.2.3.3) digested with *Eco*RI/*Hind*III (II.2.3.2) and cloned into the

*Eco*RI/*Hind*III site of the pGEM-3zf vector (II.1.5.3). Sequencing was done using the forward and backward primers specific for the vector DNA and/or specific for HSA (II.1.6). Nucleotide sequence of rHSA cDNA was identical to the sequence published by Dugaizyck *et al.* (1982).

III.2.2 Construction of plant codon optimized rHSA

The codon usage pattern applied in different organisms varies. Presence of high contents of "rare" codons that are used at low frequencies in a host organism often leads to low levels of foreign protein expression or truncated products because of the premature termination in protein translation (Chen and Inouye 1990; Hannig and Makrides 1998). To overcome this problem, a novel plant codon optimised semi-synthetic gene for high-level HSA expression was generated. The frequencies of amino acid coding sequences (CDSs) for 8792 different organisms were compiled from the taxonomical divisions of the GenBank DNA sequence database (Nakamura, Gojobori and Ikemura 2000), source: http://www.kazusa.or.jp/codon/. By the help of this codon usage database it was possible to replace the codons that are poorly (less than 1%) presented in the tobacco, wheat and rice genome by the codons that are preferred.

The albumin cDNA consists of 585 amino acids and the gene contains 18.3% rare codons for tobacco, wheat and rice plants expression that are distributed throughout the entire sequence. Distribution of rare codons in HSA sequence is illustrated in Appendix VIII.3. The DNA sequence of the rHSA was altered to match the plant preferred pattern of codon usage by codon replacement (VIII.4), while maintaining the identical amino acid sequence for expression of the recombinant protein. The plant codon optimised HSA was synthesized via PCR. The final product after PCR assembly was 1755 bp long, 1138 of which are made of the primers used for the amplification. This corresponds to 65% of the total sequence.

To assemble the plant codon optimised HSA, 32 different primers (VIII.2) differing in length were designed. They did not cover all the HSA sequence for a complete synthetic gene rather only parts of the sequence were modified and only for those parts primer addition was required. The reason for this strategy was to reduce the complexity and cost of the PCR assembly for amplification of a totally synthetic gene, which would require at least 50 primers. Since only the regions with rare codons were replaced, the rest of the sequence was

kept as the authentic HSA gene sequence. The length of the primers varied between 27 nt and 83 nt. For the amplification of the plus strand as well as the minus strand consisting of 16 primers were used. The primers were organized in such a way that, upon assembly, complementary regions in the primers overlapped between 21 and 24 nt (Figure III.3).

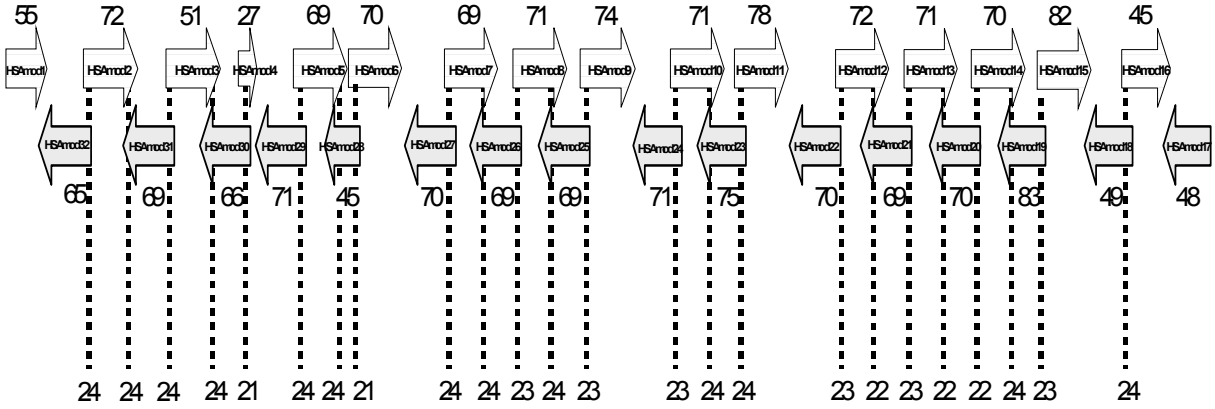

Figure III.3 Schematic representation of primer arrangement to generate the plant codon optimised rHSA. HSAmod1-16; forward primer (VIII.2); HSAmod17-32; backward primer; lengths of the oligos in base pair (bp) are indicated above or below the arrows; numbers under the dotted lines indicate length of the complementary overlapping sequence.

To avoid amplification of the authentic HSA only via the primers 1 and 17 by PCR, the template cDNA was digested (II.2.3.2) into three smaller fragments. Using *ApaL*I, a template of 400 bp (T400bp) and using *Nco*I-*Sal*I restriction enzyme combinations two templates of 800 bp (T800bp) and 1000 bp (T1000bp) were extracted from the agarose gel (II.2.3.3). Twelve reactions were prepared in the presence/absence of these sub-fragments together with the corresponding equimolar primer combination. A schematic representation of the PCR assembly is illustrated in Figure III.4A. The PCR reaction was performed under the following condition: an initial denaturation for 3 min at 94°C followed by 20 cycles of denaturation at 94°C for 1 min, annealing 55°C for 90 seconds, extension 72°C for 2 min and a fill-in cycle of 72°C for 10 min. PCR products from the assembly were separated on a 1.2% (w/v) agarose gel and illustrated in Figure III.4B.

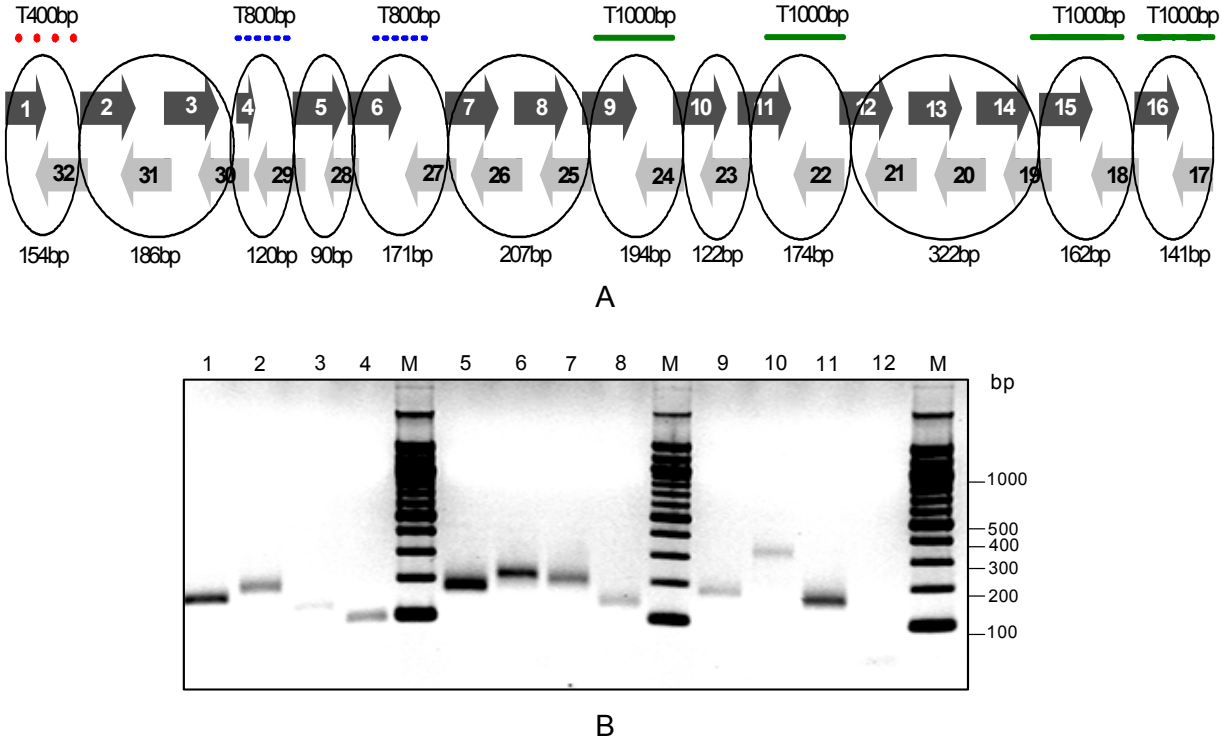

Figure III.4 Schematic representation of PCR amplification of plant codon optimised rHSA (A); Analysis of PCR products by agarose gel electrophoresis (B). A. Twelve fragments using the template T400bp, T800bp or T1000bp or without any template were PCR amplified (II.2.3.6.2) using HSAmod primers 1-32. The primers used for each PCR reaction is indicated by the circles and the length of the amplified PCR product is shown in bp. B. 5 µl of the amplified DNA differing in length (between 90- 322 bp) were separated on a 1.2% (w/v) agarose gel and visualised by agarose gel electrophoresis (II.2.3.3). PCR product of 1: 154 bp; 2: 186 bp; 3: 120 bp; 4: 90 bp; 5: 171 bp; 6: 207 bp; 7: 194 bp; 8: 122 bp; 9: 174 bp; 10: 322 bp; 11: 162 bp; 12: 141 bp. M: 100 bp ladder.

Three contiguous overlapping fragments were SOE PCR (II.2.3.6.2) amplified by mixing four of the twelve fragments in each reaction (Figure III.5A). Fragments 1 to 4, 5 to 8 and 9 to 12 were separately pooled after purification from the gel (II.2.3.3) and used as templates in the SOE PCR-I.1 reaction. The three samples were annealed using the following condition: an initial denaturation for 3 min at 94°C followed by 5 cycles of denaturation at 94°C for 1 min, annealing 55°C for 90 seconds, extension 72°C for 2 min and a fill-in cycle of 72°C for 10 min. From these reactions 10µl each was taken and the combined fragments were amplified in three separate reactions (SOE PCR-I.2) using the primers 1 and 28 for 481bp fragment, primers 6 and 23 for 624bp fragment and primers 11 and 17 for 729bp fragment (Figure III.5A). Amplification scheme was the same as for the SOE PCR-I.1 except that amplification was performed for 25 cycles and annealing was at 60°C for 2 min. The SOE PCR-I.2 resulted in three fragments (F481 bp, F624 bp and F729 bp), which were separated on an agarose gel (Figure III.5B).

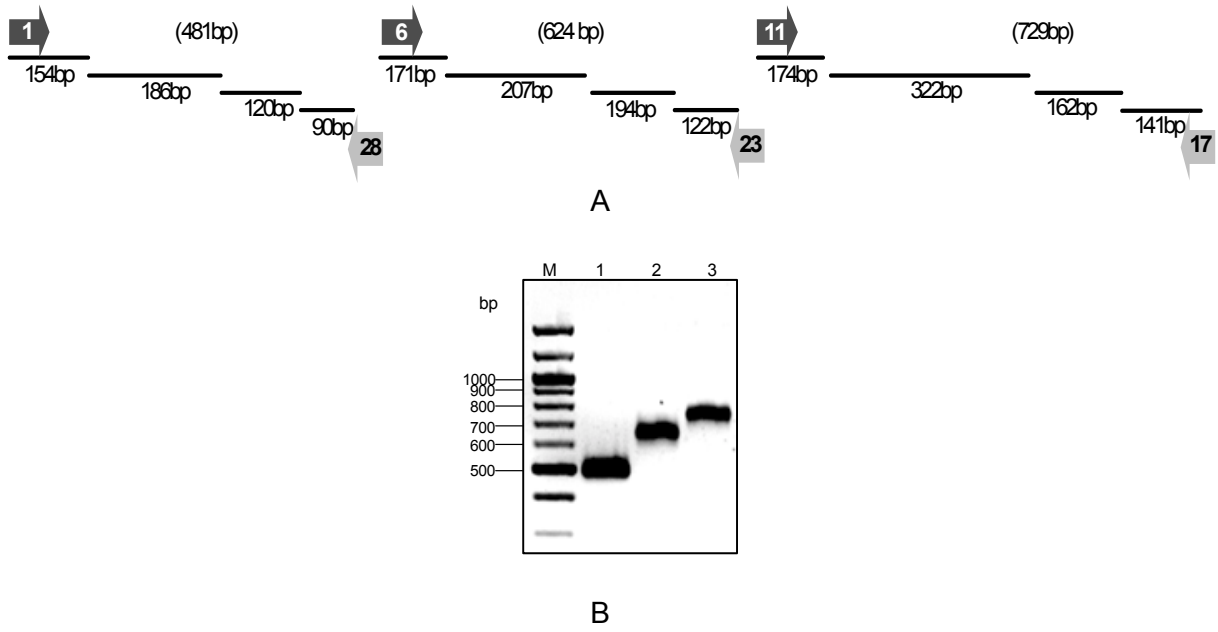

Figure III.5 Schematic representation of fragment assembly by SOE PCR-I (A); Analysis of SOE PCR products by agarose gel electrophoresis (B). A. Three contiguous overlapping fragments were amplified by mixing twelve fragments in a set of four. Primers 1 and 28 were used for the amplification of the 480bp fragment, primers 6 and 23 for the 624 bp fragment and primers 11 and 17 for the 729 bp fragment. The length of the assembled fragments and the final products are indicated in bp. The primers used for amplification in SOE PCR-I.2 are shown in arrows. B. 5 µl of the amplified DNA fragments obtained SOE PCR-I.2 were separated on a 1.2% (w/v) agarose gel (II.2.3.3) and visualised. 1: 481 bp fragment; 2: 624 bp fragment; 3: 729 bp fragment. M: 100 bp ladder

The resulting fragments from the SOE PCR-I were gel purified (II.2.3.3) and used as templates for a second round of SOE PCR (SOE PCR-II). The 481 bp, 624 bp and 729 bp fragments were diluted 1:100 and added to a reaction tube. The SOE PCR-II.1 was performed under the following condition: an initial denaturation for 3 min at 94°C followed by 5 cycles of denaturation at 94°C for 1 min, annealing 38°C for 10 min, extension 72°C for 2 min and a fill-in cycle of 72°C for 10 min. 20µl of the assembled final PCR fragment was then amplified in SOE PCR-II.2 using primers 1 and 17 (Figure III.6A). Amplification procedure was the same as the initial SOE PCR-I.2 except that amplification in the previous annealing was performed at 60°C and in SOE PCR II.2 at 55°C for 2 min. The final PCR product containing the full-length codon optimised HSA cDNA was separated on an agarose gel (Figure III.6B) demonstrating that a fragment of the expected size (1.8 kb) was amplified.

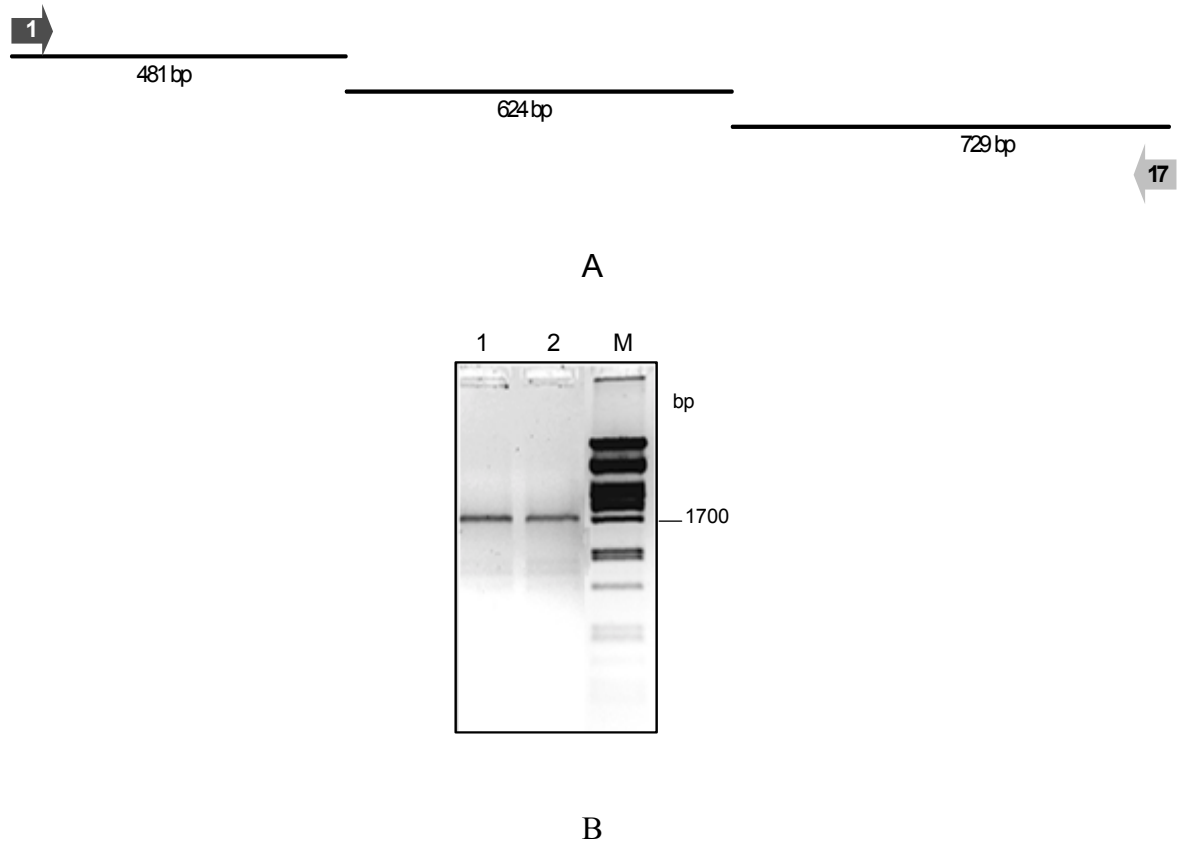

Figure III.6 Schematic representation of fragment assembly by SOE PCR-II (A); Analysis of SOE PCR products by agarose gel electrophoresis (B). A. Final plant codon optimized SOE PCR product was amplified by using three contiguous overlapping fragments (481 bp fragment, 624 bp fragment and 729 bp fragment) with primers 1 and 17. The length of the final products is indicated in bp. The primers used for amplification in SOE PCR-I.2 are shown in arrows. **B.** 5 µl of the amplified DNA fragment obtained by the SOE PCR-II.2 was separated on a 1.2% (w/v) agarose gel (II.2.3.3) (lane 1 and lane 2). M: *Pst*I digested λ DNA.

The plant codon optimised rHSA (1.8 kB) fragment was gel purified (II.2.3.3), digested with *Eco*RI and cloned into the *Eco*RI site of the TOPO TA vector (II.1.5.2). Fifty independent clones were sequenced with primers specific for vector DNA and/or specific for the plant codon optimised HSA (II.1.6). Sequence alignment showed that as many as 35 mutations per clone, which include insertions, deletions or base changes were detectable in the nucleotide sequence. Analysis of the sequence revealed that the mutations were exclusively located in the regions covered by the 32 primers used for the assembly and amplification, but not in the template regions. This demonstrates that the observed mutations are mainly introduced by the primers indicating the bad quality of the used oligonucleotides.

The clones 13 and 31 having the least errors still contained 19 and 20 mutations respectively. The representative schema for the location of the mutations in the sequence of the clones 13 and 31 is given in Figure III.7.

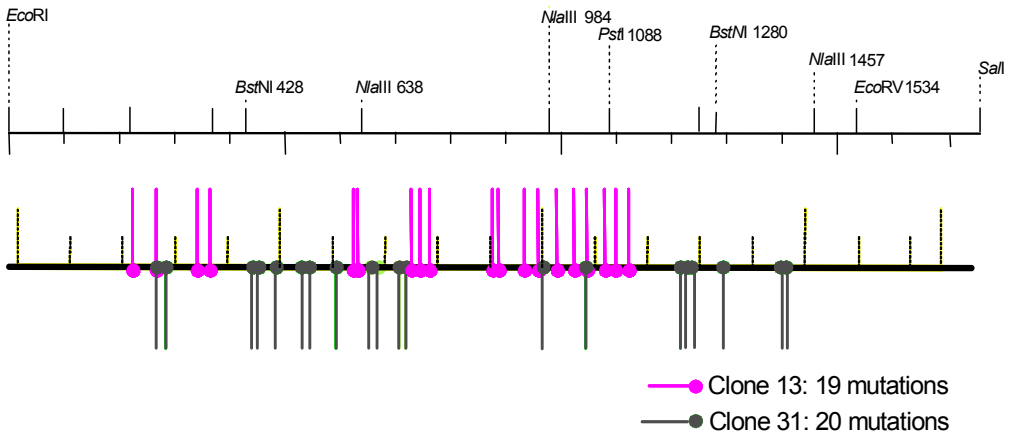

Figure III.7 Schematic representation of the mutations in the plant codon optimised rHSA. Arrows with the pink colour indicate the location of mutations in clone 13, and arrows with the grey colour in clone 31.

It has been demonstrated that it is possible to amplify a semi-synthetic gene by the help of the proper primer combination and by optimising the conditions for PCR amplification. Even though the assembly of the final PCR product with the expected molecular weight (Figure III.6B) was successful, incorporation of mutations by primers in the sequence of the plant codon optimised gene required additional experiments to remove these errors. Mutations in the final HSA cDNA could be eliminated by different approaches like *in vitro* site directed mutagenesis or combining smaller fragments possessing the correct sequence by a restriction enzyme digestion approach. However, the elimination of a minimum of 19 mutations would be labour intensive and time consuming and therefore efforts to generate a corrected plant codon optimised gene were undertaken. Instead the work was focused on the improved rHSA production parameters using the original HSA cDNA sequence.

III.2.3 Generation of HSA expression constructs

III.2.3.1 Bacterial expression constructs

HSA is one of the secreted proteins that do not require glycosylation and it was confirmed previously that HSA is accumulating in cytoplasm of *E. coli* (Lawn, Adelman *et al.* 1981). In

this thesis, two approaches for expression of HSA in *E. coli* were examined. The HSA cDNA was cloned into the vectors pET21d (II.1.5.5) and pET22b (II.1.5.6) via *Nco*I and *Sal*I restriction enzyme sites. Both expression vectors contain the T7 promoter, pBR322 origin, ampicillin resistance gene as selective marker and a C-terminal his-tag sequence for purification. The pET22b vector (II.1.5.6) carries additionally a *pel*B leader sequence for targeting the heterologous expressed protein to the periplasm. Recombinant plasmids pET21d-HSA (cytoplasmic expression) and pET22b-HSA (periplasmic expression) were transformed separately into *E. coli* strain BL21λDE3 and used for bacterial expression (III.4.1).

III.2.3.2 Plant expression constructs

For generation of the HSA-APO construct enabling secretion of rHSA into apoplastic space of plant cells, HSA in the pGEM-3zf vector (III.2.1) was digested with *Sal*I and partially with *Nco*I. Subsequently, the hemoglobin gene (HEME) in the HEME-APO construct was replaced by the HSA cDNA in the pUC18 vector containing chalchone synthase 5' untranslated region (CHS) and the plant codon optimised signal sequence (LPL*) at the 5' and cmyc and his6 tag at the 3' end. Finally the HSA cassette was cut from HSA-APO in pUC18 including CHS and LPL* via *Eco*RI and *Sal*I enzymes and cloned into the pSSH1 vectors containing either cmyc-his6 (HSA-APO) or KDEL tag (HSA-KDEL) at the 3' terminus. The resulting HSA-APO and HSA-KDEL constructs in pSSH1 plant expression vector were transformed into *E. coli* and DNA from independent clones was sequenced (II.2.3.7) in order to verify the expected sequence.

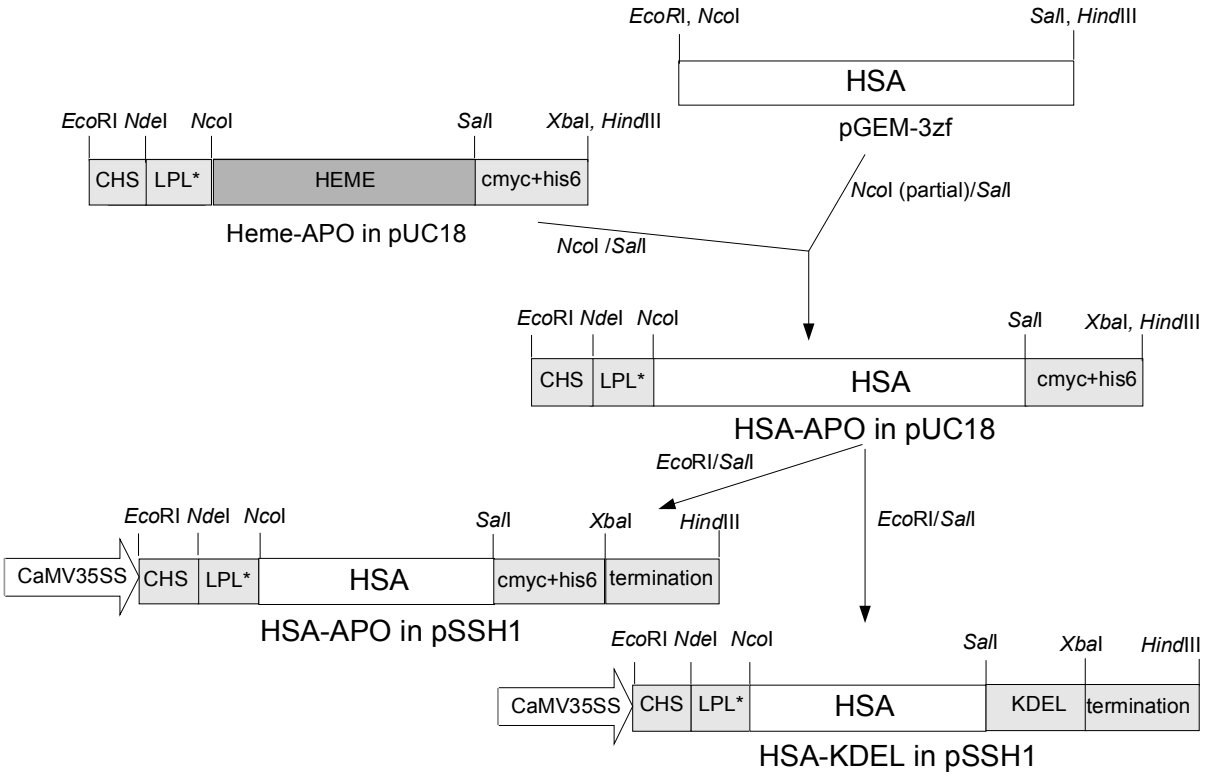

Figure III.8 Construction of pSSH1 plant expression vectors HSA-APO and HSA-KDEL. CHS: 5' untranslated region of chalcon synthase; LPL*: plant codon optimized leader peptide derived from murine light chain of TMV-specific rAb24; HEME: hemoglobin; CaMV35SS; double enhanced 35S promoter from CaMV; c-myc: myc epitope; His6: his-6 tag; KDEL: ER retention signal; termination: termination signal of CaMV 35S.

The constructs HSA-APO and HSA-KDEL in pSSH1 were transformed into *A. tumefaciens* strain GV3101 carrying the helper plasmid pMP90RK (GM^R, KM^R, Rif^R) by electroporation (II.2.1.8). Seven independent recombinant colonies from each transformation were screened for the presence of an insert by PCR (II.2.3.6.1). Recombinant *Agrobacterium* cultures of each construct were prepared from single colonies and used for transient expression (III.4.2), stable transformation of *N. tabacum* (III.4.3) and stable transformation of BY2 tobacco suspension cells (III.4.5).

In addition to the constructs HSA-APO and HSA-KDEL in pSSH1 for tobacco, zucchini and bean transformation, HSA-KDEL in pAL76 vector was also generated for expression of rHSA in wheat. HSA-KDEL cassette from HSA-KDEL in pUC18 including CHS and LPL* at the 5' end and KDEL tag at 3' end was digested via *Eco*RI and *Hind*III enzymes and cloned into the pAL76 vector which contains ubiquitin 1 promoter and intron 1 from maize and nos termination signal (II.1.5.8). The resulting HSA-KDEL construct in pAL76 plant expression vector was transformed into *E. coli* and DNA from independent clones was

sequenced (II.2.3.7) in order to verify the expected sequence. HSA-KDEL in pAL76 vector was used for bombardment of immature wheat embryos (*Triticum aestivum* L. cv. Bobwhite).

III.3 Expression of rHSA

III.3.1 Bacterial expression

To achieve rHSA production in bacteria plasmids pET21d-HSA (cytoplasmic expression) and pET22b-HSA (periplasmic expression) were transformed separately into *E. coli* strain BL21λDE3 and expression was induced by 1 mM IPTG for 4 hours (II.3.1). For cytoplasmic extraction *E. coli* cells transformed with the pET21d-HSA vector DNA were suspended in TEN buffer and sonicated (II.4.1). For periplasmic extraction the pET22b-HSA cell pellet was resuspended in Tris-Sucrose solution and supernatant was collected after centrifugation (II.4.1). Subsequent to immobilized metal affinity chromatography (IMAC) purification exploiting the His-tag for binding to Ni-NTA matrix (II.6.1), cytoplasmatically and periplasmatically expressed rHSA was analysed by ELISA (data not shown), SDS-PAGE (Figure III.9A) and immunoblot (Figure III.9B).

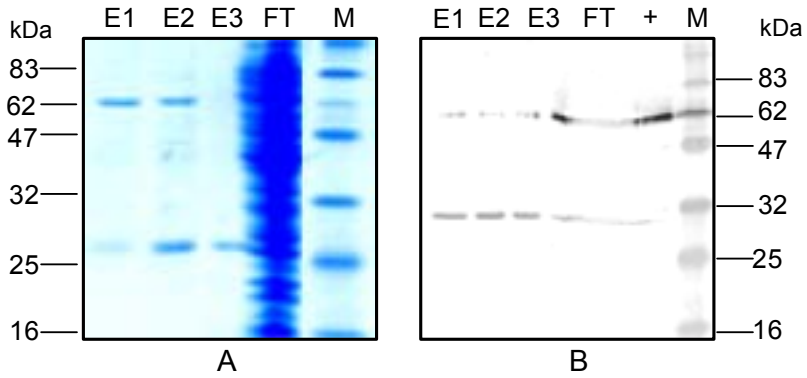

Figure III.9 SDS-PAGE (A) and Immunoblot analysis (B) of purified rHSA from *E. coli* BL21λDE3 cytoplasm. Affinity purified rHSA was resolved on 12% (w/v) SDS-PAGE (II.4.6.1) then stained with Coomassie brilliant blue (II.4.7.1) (**A**) or blotted onto nitrocellulose membrane followed by immunoblot analysis (**B**) (II.4.8). Anti HSA monoclonal antibody (Sigma) was used as the primary antibody followed by GAM^{AP} as secondary antibody. Immunoblot was developed by addition of NBT/BCIP substrate. E1-E3: 5 µl of the first, second and third elution fractions of rHSA; FT: Flow through; +: 50 ng HSA (Sigma); M: Molecular weight protein marker.

The level of rHSA expressed in the periplasm of *E. coli* was below the threshold level for immunoblot analysis and ELISA (data not shown). For cytoplasmic expression two proteins with the same intensity migrating at around 62 kDa and 27 kDa were observed on a SDS

PAA gel (Figure III.9A). The 27 kDa band on the SDS PAA gel might be a co-purified bacterial protein after affinity purification. The 62 kDa band on the SDS PAA gel corresponded approximately to the expected size of rHSA (66 kDa). Immunoblot analysis of the same samples (Figure III.9B) with anti-HSA monoclonal antibody however showed that these bands were co-purified bacterial proteins and only a small fraction of the band at 62 kDa was rHSA. In addition, a significant amount of rHSA was also present in the flow through (Figure III.9B lane FT), indicating that binding conditions to the Ni-NTA matrix needs to be further optimised. Another lower band (at around 30 kDa), which was recognized by the monoclonal anti-HSA antibody, is most probably a truncated form of rHSA. The yield of affinity purified cytoplasmatically expressed rHSA was determined by ELISA (data not shown) to be 0.002 mg/L bacterial culture.

rHSA could only be detected in the cytoplasm of *E.coli*. The periplasmic environment did not allow accumulation of this protein to a detectable amount. After confirmation of the bacterial expression, the next step was to test the rHSA accumulation in the plant system.

III.3.2 Transient expression of rHSA in plant leaves

Transient gene expression in plants has several advantages over the generation of stably transformed transgenic plants. Regenerating transgenic plants from transformed cells is both labour intensive and time consuming. In contrast, transient expression systems allow the rapid evaluation and improvement of plant expressed proteins. They present a feasible method for testing accumulation of the foreign protein *in vivo* before progressing to develop stably transformed plants (Kapila, De Rycke *et al.* 1996). Therefore, rHSA was transiently expressed in leaves of different plant species to confirm product integrity and to screen for the best expression host and construct before generation of transgenic plants.

III.3.2.1 Transient expression in *N. tabacum* cv. Petite Havana SR1 leaves

Recombinant Agrobacteria carrying the HSA-APO and HSA-KDEL constructs (III.2.3.2) were used for vacuum infiltration of tobacco leaves (II.2.2.5). Four to six leaves were infiltrated with recombinant agrobacteria (II.2.2.5). Accumulation of rHSA in tobacco leaves was analysed by immunoblot 72 hours after vacuum infiltration. The identity and integrity of

the expressed proteins were confirmed by immunoblot analysis (II.4.8) using either specific anti-cmyc or anti-KDEL antibodies. Immunoblot analysis revealed a distinct band of approximately 66 kDa, corresponding to the molecular weight of HSA for both apoplastic and ER constructs (Figure III.10).

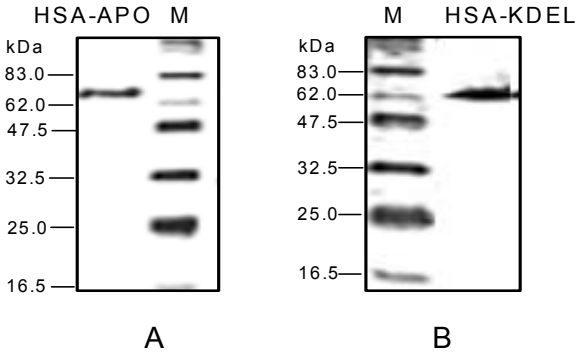

Figure III.10 Immunoblot analysis of HSA-APO (A) and HSA-KDEL (B) transiently expressed in *N. tabacum* cv. Petite Havana SR1 leaves. Total soluble leaf proteins were extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.4.2), separated by 12% (w/v) SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immuno detection was carried out with anti-c-myc antibody (1:5000) (A) or anti KDEL antibody (1:500) (B), followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. 5 µl of TSP extracted from tobacco leaves transformed with HSA-KDEL (A) and HSA-APO (B) M: prestained protein marker.

Due to unavailability of anti HSA antibody at that point of the project, two different detection antibodies (anti-cmyc and anti-KDEL) were selected to confirm the accumulation by immunoblot. Considering the reduced sensitivity of the anti-KDEL detection antibody immunoblot analysis demonstrated that higher rHSA levels were obtained when the protein accumulated in the ER.

III.3.2.2 Alternative *N. tabacum* cultivars

In addition to *N. tabacum* cv. Petite Havana SR1, eight additional tobacco varieties were evaluated in their performance of expressing rHSA in tobacco leaves. These varieties include Gead Korso, Barley Junifer, Adonis Schwester, Barley Saturn, Virginia, Badische Geudentheimer, Maryland Mammoth and Nicotine free tobacco. For comparing transient expression, the HSA-APO construct (III.2.3.2) was chosen that allows secretion of recombinant HSA into the apoplast. Accumulation of rHSA in tobacco leaves was analysed by immunoblot and ELISA 72 hours after vacuum infiltration. Immunoblot analysis (data not shown) revealed a distinct band of approximately 66 kDa, corresponding to the molecular

weight of rHSA for the apoplastic construct in all cultivars analysed. Furthermore in several samples including the negative control (wild type extract of SR1), another band at around 32 kDa, was recognized by the monoclonal anti-HSA antibody. The presence of this smaller band in most of the plant extracts might be an indication of a plant protein reacting unspecifically with the anti-HSA antibody. According to the immunoblot results, the level of expression of rHSA varied considerably between the tobacco varieties. Highest level expression was observed in Adonis Schwester, Barley Saturn and Maryland Mammoth (data not shown).

To quantify the amount of rHSA ELISA was performed and it confirmed the results of the immunoblot analysis. In the varieties Adonis Schwester, Barley Saturn and Maryland Mammoth the accumulation levels were 5-10 times higher than the other tobacco cultivars tested (Figure III.11). Highest accumulation level was 10 µg rHSA per g leaf material in Adonis Schwester and Barley Saturn, the lowest level (1 µg rHSA per g leaf material) was observed in transiently transformed leaves of Barley Jupiter.

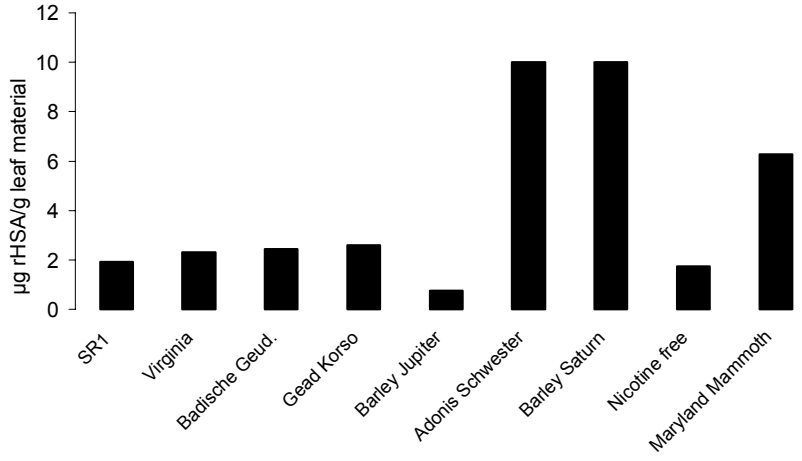

Figure III.11 Determination of rHSA accumulation levels in transiently transformed tobacco cultivars by ELISA. Goat anti-HSA antibody (1:500 diluted in TBS) was coated to ELISA plates (II.4.9.2). After blocking with MTBS, 100 µl of the serially diluted plant extracts (II.4.2) were added to the coated plates. Bound rHSA was detected by addition of peroxidase-conjugated rabbit anti-HSA antibody (1: 20000). ELISA readings ($OD_{690\ nm}$) were performed after 1-10 min incubation with ABTS substrate solution buffer (II.4.9.2).

In addition to the advantage of an observed higher level of accumulation, Adonis Schwester, Barley Saturn and Maryland Mammoth also have higher leaf biomass in comparison with the SR1 tobacco and they are widely grown in the fields. Therefore these varieties could be an alternative to the present model tobacco variety SR1. A disadvantage with Adonis Schwester

and Barley Saturn varieties is the unavailability of seeds from the adult plants for the new generation.

Table III.1 Production levels of rHSA in plants.

HSA-APO; plant expression construct carrying double enhanced 35S promoter from CaMV(CaMV35SS), 5' untranslated region of chalcon synthase (CHS) and plant codon optimized leader peptide derived from murine light chain of TMV-specific rAb24 (LPL*) at 5' end and cmyc and his6 epitopes (cmyc/his6) for detection and purification at 3' end. HSA-KDEL; plant expression construct carrying double enhanced 35S promoter from CaMV(CaMV35SS), 5' untranslated region of chalcon synthase (CHS) and plant codon optimized leader peptide derived from murine light chain of TMV-specific rAb24 (LPL*) at 5' end and KDEL retention signal of proteins in the endoplasmic reticulum (ER) at 3' end.

plant species	localisation	cultivation	expression system	maximum accumulation
N. tabacum cv. Petite Havana SR1	apoplast	greenhouse	transient	2 µg/g leaf material
N. tabacum cv. Virginia	apoplast	greenhouse	transient	2.3µg/g leaf material
N. tabacum cv. Badische Geudentheimer	apoplast	greenhouse	transient	2.4µg/g leaf material
N. tabacum cv. Gead Korso	apoplast	greenhouse	transient	2.6µg/g leaf material
N. tabacum cv. Barley Jupiter	apoplast	greenhouse	transient	0.76µg/g leaf material
N. tabacum cv. Adonis Schwester	apoplast	greenhouse	transient	10µg/g leaf material
N. tabacum cv. Barley Saturn	apoplast	greenhouse	transient	10µg/g leaf material
N. tabacum cv. Nicotine free	apoplast	greenhouse	transient	1.7µg/g leaf material
N. tabacum cv. Maryland Mammoth	apoplast	greenhouse	transient	6.3µg/g leaf material

III.3.2.3 Transient expression of rHSA in zucchini and bean leaves

In order to test the feasibility of other plant varieties for the transient expression of rHSA, expression of the HSA-APO construct was analysed in zucchini and bean leaves. Accumulation of rHSA in leaves was analyzed by immunoblot (data not shown) and ELISA 72 hours after vacuum infiltration (Figure III.12).

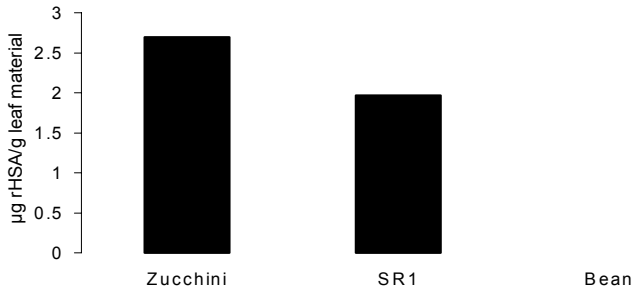

Figure III.12 Determination of rHSA accumulation levels in transiently transformed zucchini, SR1 and bean leaves by ELISA. Goat anti-HSA antibody (1:500 diluted in TBS) was coated to ELISA plates (II.4.9.2). After blocking with MTBS, 100 µl of the serially diluted plant extracts (II.4.2) were added to the coated plates. Bound rHSA was detected by addition of peroxidase-conjugated rabbit anti-HSA antibody (1: 20000). ELISA readings (OD $_{690\ nm}$) were performed after 1-10 min. incubation with ABTS substrate solution buffer (II.4.9.2).

Transient expression of rHSA in the apoplasm of bean extracts was neither detected in the immunoblot (data not shown) nor in ELISA. However, 2.65 µg rHSA per g leaf material was detected in zucchini which is slightly higher than the model host tobacco species SR1 (2 µg HSA per g leaf).

This experiment has proven that other plant species could be also used for production of rHSA. The transient expression experiments have shown that highest rHSA accumulation was obtained in tobacco. Although tobacco cultivars Adonis Schwester, Barley Saturn and Maryland Mammoth seemed to be most suitable, Petit Havana SR1 was used for generation of stable transformed plants since only for this cultivar reliable transformation protocols and experience in crossing and breeding were available.

III.3.3 Stable expression of rHSA in transgenic tobacco plants

III.3.3.1 Transgenic tobacco plants producing HSA-APO

Fourty independent transgenic *N. tabacum* cv. Petite Havana SR1 lines were generated by leaf disc transformation using recombinant *A. tumefaciens* (II.2.1.8) carrying the HSA-APO construct. Transgenic T_0 plants were regenerated from transformed calli. Young leaves from regenerated transgenic plants were used for extraction of total soluble proteins (II.4.2) and for analysis of recombinant protein accumulation by immunoblot (II.4.8).

Immunoblot analysis revealed that 32 out of 40 T_0 analysed plants accumulated apoplastic rHSA (Figure III.13). A clear band with an identical relative mobility to commercial HSA was detected. Sometimes additional immuno-positive bands of a smaller molecular weight were observed both in the untransformed (negative control tobacco SR1) and transformed lines (Figure III.13). Most likely these are degradation products or plant derived proteins cross-reacting with the detection antibody.

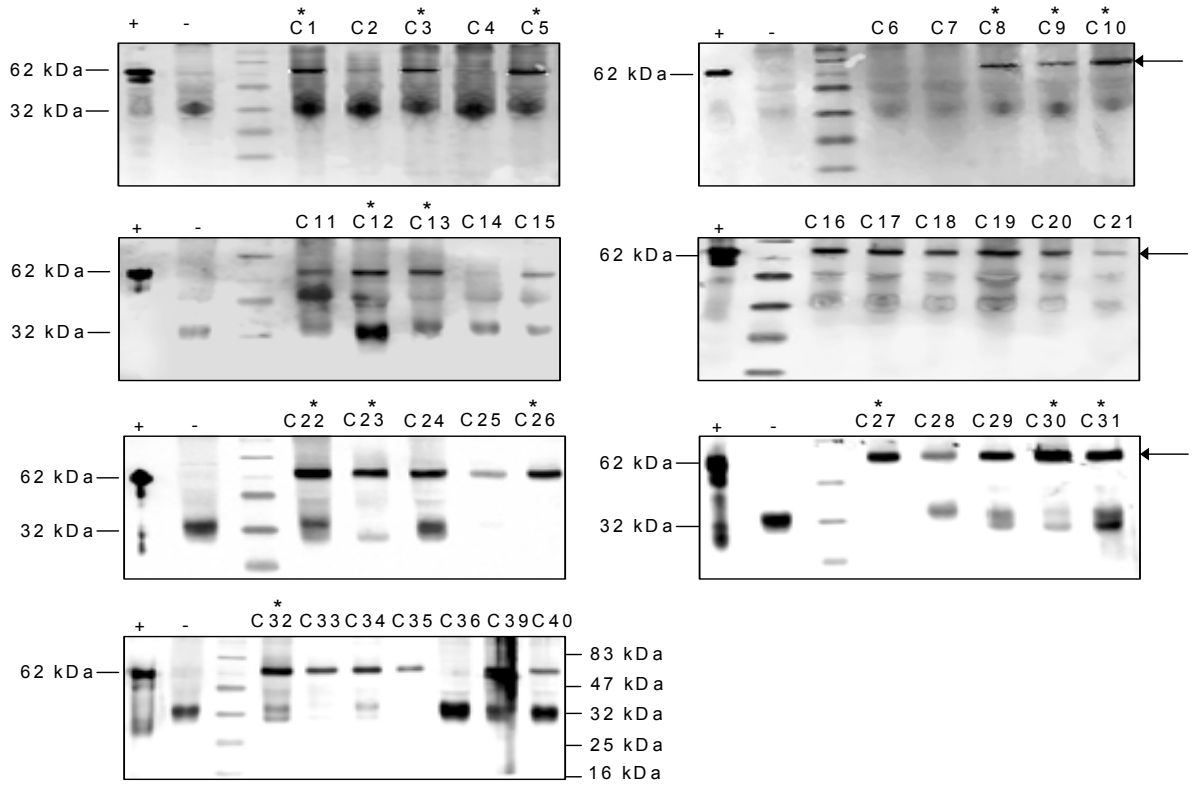

Figure III.13 Screening of stable transgenic tobacco plants expressing HSA-APO. Total soluble leaf proteins were extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.4.2), separated by a 12% (w/v) SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immuno detection was carried out with anti-HSA antibody (1:5000) followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. C1-C40: 5 µl of TSP extracted from transgenic HSA-APO tobacco leaves; M: prestained protein marker; -: 5 µl of wild type plant extract +: 50 ng of HSA (Sigma). Tobacco lines selected for the establishment of homozygous lines are indicated by a *.

The lines with the highest level of apoplastic rHSA (C1, C3, C5, C8, C9, C10, C12, C13, C22, C23, C26, C27, C30, C31, and C32) were selected and self pollinated for establishment of homozygous lines. From these fifteen lines seeds were collected and three of these lines (C10, C22 and C30) with the highest accumulation were further analysed in the following generations and results are illustrated in III.3.3.3.

III.3.3.2 Transgenic tobacco plants expressing HSA-KDEL

Fourty independent transgenic lines were generated by leaf disc transformation using recombinant *A. tumefaciens* (II.2.1.8) carrying HSA-KDEL construct. From regenerated T_0 transgenic plants young leaves were collected and used for analysis of recombinant protein accumulation by immunoblot (II.4.8).

Immunoblot analysis revealed that 37 out of 40 T_0 analysed plants were positive for rHSA accumulation (Figure III.14) and levels vary between siblings. Recombinant HSA migrated according to its predicted molecular weight of 66 kDa. As it was observed in the immunoblots of transgenic HSA-APO plants (Figure III.13), immuno-positive band of a smaller protein migrating at around 30 kDa was also observed in the extracts of HSA-KDEL plants (Figure III.14).

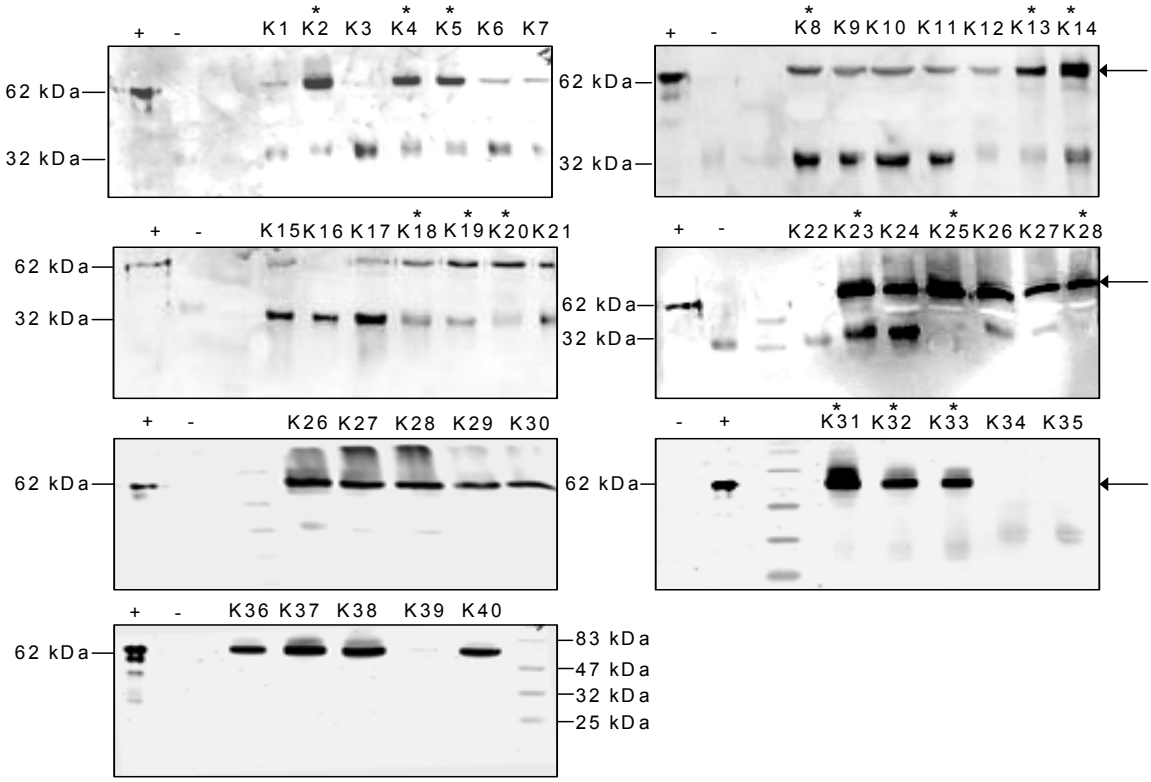

Figure III.14 Screening of stable transgenic SR1 plants expressing HSA-KDEL. Total soluble leaf proteins were extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.4.2), separated by a 12% (w/v) SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immuno detection was carried out with anti-HSA antibody (1:5000) followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. K1-K40: 5 µl of TSP extracted from transgenic HSA-KDEL tobacco leaves; M: prestained protein marker; -: 5 µl of wild type plant extract +: 50 ng of HSA (Sigma). Tobacco lines selected for the establishment of homozygous lines are indicated by a *.

The lines with the highest levels (K2, K4, K5, K8; K13, K14, K18, K19, K20, K23, K25, K28, K31, K32 and K33) were selected and self pollinated for establishment of homozygous lines. From these fifteen lines seeds were collected and three of these lines (K2, K31 and K32) with the highest accumulation were analysed in the next generation and results are illustrated in III.3.3.3.

In general, immunoblots are not as amenable as ELISA to determine the level of accumulation of a foreign protein. However, band intensities are usually compared with known amount of a standard protein to get a rough estimate of the level of accumulation. In the immunoblots of HSA-APO (Figure III.13) and HSA-KDEL (Figure III.14), the intensity of the rHSA bands was comparable. Using this rough estimation, although variation between different siblings are observed, in general rHSA levels were higher in the HSA-KDEL producing plants when compared to the HSA-APO plants.

III.3.3.3 Analysis of rHSA accumulation in the T_1 generation

From transgenic T_0 plants 15 lines for each construct (HSA-APO and HSA-KDEL) were selfed and their seeds were collected. Transgenic T_0 plants with the highest accumulation of rHSA (C10, C22 and C30 for HSA-APO, K2, K31 and K32 for HSA-KDEL) were selected for further analysis. Seeds of these lines were germinated in the greenhouse and, young leaves of the T_1 plants were collected. Total soluble protein (TSP) from leaves of nine plants per line was extracted for Bradford protein determination and recombinant protein accumulation analysis by immunoblot. Bradford total protein quantification revealed that TSP content varied between 11.6-19.84 mg per g leaf (data not shown). Accumulation of the recombinant protein in tobacco leaves was analysed by immunoblot using crude extracts of total soluble leaf protein. Immunoblot experiments revealed that 7 out of 9 carrying the HSA-APO construct and 9 out of 9 plants with integrated HSA-KDEL construct were positive (data not shown). Recombinant HSA migrated according to its predicted molecular weight of 66 kDa.

The T_1 progeny was analysed for a 3:1 Mendelian segregation ratio on kanamycin selection plates (II.2.2.6), indicating the events of T-DNA integration at different genetic loci. Results were tabulated as the ratio of tolerant to susceptible seedlings and tested if they segrated in a 3:1 ratio. Chi-square values are calculated according to the following formula:

$$\chi^2 = \sum \frac{(\text{Observed Value} - \text{Expected Value})^2}{(\text{Expected Value})}$$

Table III.2 Segregation analysis of the transgenic T_1 lines expressing rHSA either in the ER (KDEL 2, 31, 32) or in the apoplast (APO 30, 22, 10).

Transgenic	Resistant	Susceptible	Chi square*
HSA-APO			
APO-30	55	18	0.0045
APO-22	46	16	0.0215
APO-10	36	10	0.26
HSA-KDEL			
KDEL-2	32	7	1.0256
KDEL-31	27	9	0
KDEL-32	73	20	1.0533

(* Values less than 3.83 indicate a good fit to a 3 resistant: 1 susceptible ratio at the P = 0.05 level of probability with degree of freedom = 1.) Number of resistant and susceptible plants is tabulated and Chi-square values are calculated.

Chi-square values of less than 3.84 fit a 3:1 segregation ratio and indicate a single dominant Mendelian locus with 95% of confidence. Mendelian segregation analyses indicate that more likely single integration of the transgenes (HSA-APO or HSA-KDEL) into the chromosomal DNA of all analysed transgenic line has taken place.

III.3.3.4 Analysis of rHSA accumulation in homozygous T_2 lines

To obtain individuals homozygous lines, seeds from T_1 plants were collected and from five positive T_1 lines, at least 15 T_2 plants were grown until young leaves for analysis were available. All seeds from that parent for which no recessive segregants were seen should be homozygous, and it should be possible to propagate this line indefinitely with no loss of the transgene due to segregation. Seeds from transgenic lines were collected, grown in greenhouse and leaf extracts were analysed until the homozygous generation could be obtained. Total soluble protein was extracted from leaves of homozygous T_2 lines producing HSA-APO and HSA-KDEL and used for immunoblot analysis. It revealed that rHSA migrated according to its predicted molecular weight of 66 kDa. As in the transiently

expressed rHSA and stably transformed T_0 and T_1 generations a second band at around 30 kDa was observed. Most likely these are plant derived proteins unspecifically interacting with the detection antibody or degradation products.

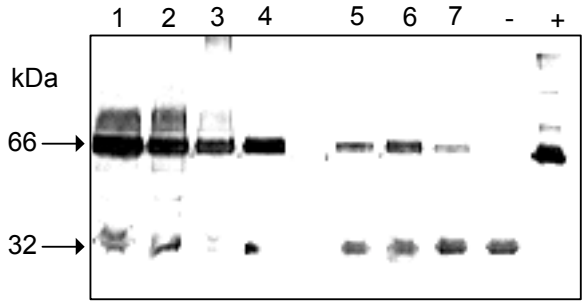

Figure III.15 Screening of stable transgenic T_2 plants producing HSA-APO and HSA-KDEL. Total soluble leaf proteins were extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.4.2), separated by 12% (w/v) SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immunodetection was carried out with anti-HSA antibody (1:5000) followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. 5 µl of total soluble protein extracted from T_2 transgenic tobacco leaves were separated. 1-4) HSA-KDEL 2/12-1, 31/7-1, 32/4-2, 32/4-3**;** 5-7) HSA-APO 22/5-5, 22/5-6, 30/1-1; -: 5 µl of wild type plant extract +: 50 ng of HSA (Sigma).

A detailed comparison of HSA-APO and HSA-KDEL accumulation levels was performed by ELISA in T_2 lines. The HSA-APO protein accumulated to levels of 1.8 to 6 µg rHSA per g leaf and HSA-KDEL to 5 up to 100 µg HSA per g leaf material (Figure III.16).

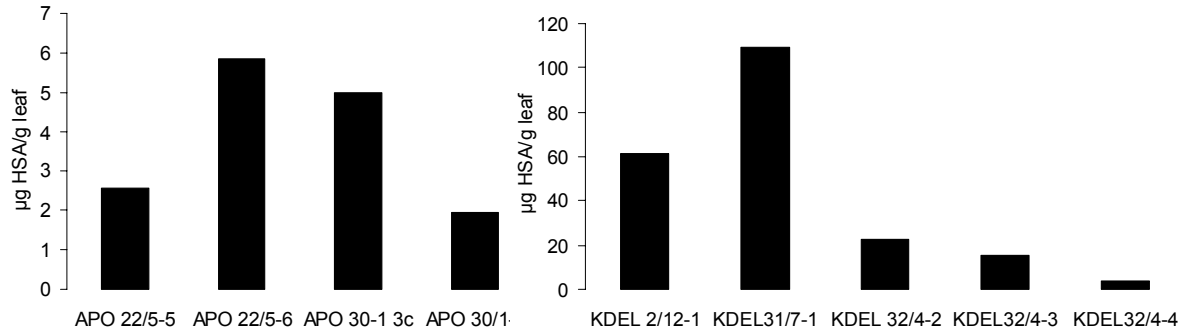

Figure III.16 Determination of rHSA accumulation levels in homozygous T_2 lines by ELISA. Goat anti-HSA antibody (1:500 diluted in TBS) was coated to ELISA plates. After blocking with MTBS, 100 µl of the serially diluted plant extracts were added to the coated plates. Bound rHSA was detected by addition of peroxidase-conjugated rabbit anti-HSA antibody (1: 20000). ELISA readings ($OD_{690\ nm}$) were performed after 1-10 min. incubation with ABTS substrate solution buffer (II.4.9.2).

The accumulation level in the homozygous transgenic T_2 line HSA-KDEL 31/7-1 was 100 µg rHSA per g leaf material and this is the highest level observed among the lines tested. This transgenic line was further propagated for downstream processing (III.4) of rHSA from transgenic leaf material. Highest apoplastic rHSA level of 5.86 µg rHSA per g leaf material was detected in the homozygous T_2 line HSA-APO 22/5-6. The seeds from this line were collected and send to Canada to perform a field trial (III.3.3.5).

III.3.3.4.1 Analysis of plant derived rHSA with 2-D SDS PAGE

The gel electrophoretic analysis of proteins in two subsequent dimensions, first according to their pI and then according to their molecular weight, is still the most common and successful technique for the comparative separation of a heterogeneous protein mixture. Despite its limitations such as the incomplete separation of very acidic or very basic proteins, its resolution in the analysis of complex mixtures is still unrivalled. For a more detailed investigation of rHSA accumulation in the ER of the plant cells two dimensional gel electrophoresis was performed.

Total soluble proteins from transgenic leaf tissue accumulating rHSA in the ER were isolated as described (II.4.2). TCA precipitated soluble proteins were diluted in the rehydration/sample buffer (II.4.6.2) and prior to two dimensional gel electrophoresis analysed by SDS PAGE and immunoblot. As it is visualised in Figure III.17, the protein migrated in a higher relative mobility than the authentic HSA which is most probably due to presence of high concentration of Urea (9 M) in the rehydration buffer used to dilute the extract.

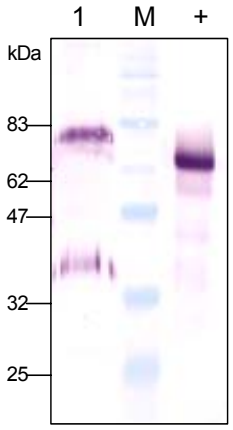

Figure III.17 Immunoblot analysis of transgenic HSA-KDEL T_4 tobacco leaves. TSP was extracted by mixing ground leaf powder with 10% (v/v) Trichloroacetic acid (TCA) solution in Acetone (II.4.2) TCA precipitated leaf proteins were homogenized in the presence of rehydration buffer (II.4.6.2) separated SDS PAGE and blotted onto a nitrocellulose membrane (II.4.8). Immunodetection was carried out with anti-HSA antibody (1:5000) followed by 1 hr incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. 1) 5 µl of TSP extracted from T_4 transgenic tobacco leaves; M) prestained protein marker; +: 100 ng of HSA (Sigma).

Protein extracts derived from leaves of *N. tabacum* SR1 wild type and HSA-KDEL transgenic line were diluted in rehydration/sample buffer (II.4.6.2) and then separated on pH 3-10 gradient immobilized pH-gradient gel strips in a BioRad Protean IEF cell (II.4.6.2). The 10% SDS PAA gels were subsequently stained with silver (II.4.7.2). Since silver staining is 10-100 folds more sensitive than Coomassie staining, plenty of plant proteins could be detected on the silver stained gels (data not shown). Abundance of other plant proteins complicated the localization of rHSA with the expected molecular weight and pI point. Therefore the two dimensionally separated proteins were immunoblotted on nitrocellulose membranes. Immunodetection was carried out with an anti-HSA monoclonal antibody. The results of the two-dimensional gel electrophoresis of rHSA accumulation in the ER correlated with those obtained after an initial one dimensional gel electrophoresis and immunoblotting of plant extracts (Figure III.18). Relative mobility and theoretical pI point of authentic HSA (5.3) is found to be similar to rHSA extracted from transgenic leaves. Both using the wild type SR1 control extract and transgenic plant extract another protein at around 30 kDa was observed. This protein was also visualised in the one-dimensional SDS PAGE profile (Figure III.17) and is most probably a degradation product.

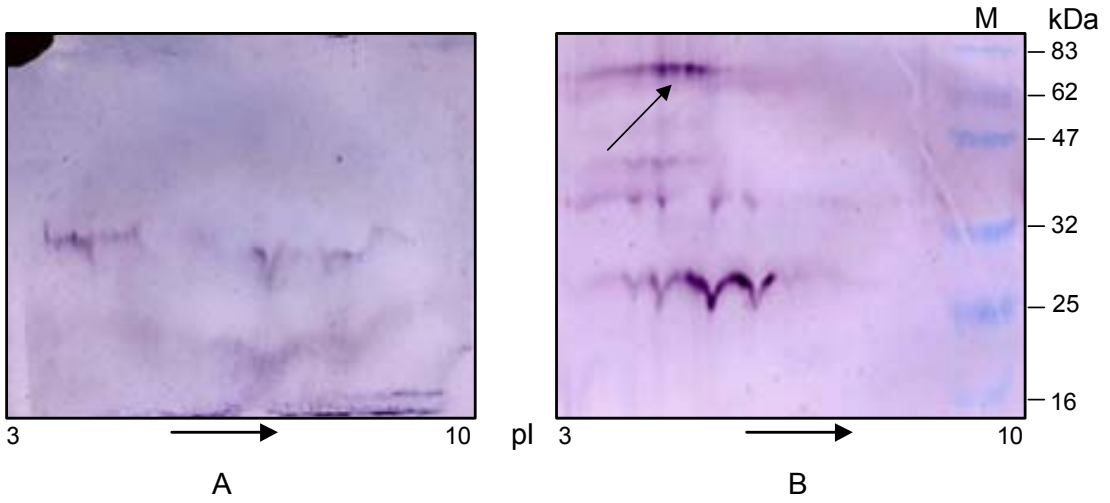

Figure III.18 Comparative two dimensional gel electrophoresis and Immunoblot analysis of wild type (A) and transgenic (B) T4 stable tobacco expressing rHSA in ER. TSP was extracted by mixing ground leaf powder with 10% (v/v) Trichloroacetic acid (TCA) solution in Acetone (II.4.2) TCA precipitated leaf proteins were homogenized in the presence of rehydration buffer separated by 2-D SDS PAGE (II.4.6.2) and blotted onto a nitrocellulose membrane (II.4.8). Immunodetection was carried out with anti-HSA antibody (1:5000) followed by 1 hr incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. 5 µl of TSP extracted from the leaves of A) Wild type SR1; B) HSA-KDEL; M) prestained protein marker.

III.3.3.5 Field trials of transgenic tobacco plants producing rHSA

Greenhouse observations of the transgenic plants at different cultivation stages indicated that the general phenotypic characteristics of the plants were similar to wild type plants. Rarely negative phenotypic changes are observed in some agronomic characteristics of plants upon transformation and regeneration. These changes are often caused by the regeneration procedure and tend to create reduced fitness (Dr. Jim Brandle, personal communication). Such possible changes in fitness can be determined in transgenic plants after greenhouse observations and field testing has taken place. Furthermore, amount of rHSA obtained in the greenhouse plants and field grown plants could be compared to get an idea for the performance of the transgenic plants under field conditions.

The seeds from the T_2 line HSA-APO-22/5-6 were used for the field trial. The site was located in Canada and field tests were performed by Dr. Jim Brandle (Agriculture and AgriFood Canada) (II.3.3). Trial area was approximately 15 m^2. Around 30 days following transplantation of greenhouse seedlings, the plots were harvested and the plant material bulked for evaluation of dry matter yield and soluble protein concentration. The accumulation

level of rHSA secreted to the apoplastic space of transgenic tobacco plants under field conditions are presented in Table III.3.

Table III.3 Analysis of rHSA accumulation in the transgenic HSA-APO-22/5-6 in the field. Leaf yield and TSP amount were determined in transgenic plants for the 15 m^2 trial area and converted to one hectare (1 hectare ~ 10,000 m^2). TSP: Total soluble protein; kg f.w/ha: kg fresh weight per hectare.

Line	Leaf yield (kg f.w./ha)	TSP (kg/ha)	HSA (%TSP)	HSA (kg/ha)
HSA-APO-22/5-6	1563	13.55	0.052	0.704

Total protein quantification by Bradford revealed that TSP content was 8.6 mg per g leaf material. The amount of rHSA accumulation in the field was estimated as 0.052% of TSP by immunoblot (data not shown). A total of 1563 kg fresh transgenic leaf with a TSP of 13.55 kg/ha was estimated to be harvested from one hectare of field area. Recombinant HSA account for 0.052% of this protein content. Approximately 700 g of rHSA per hectare is a rough estimate for the level of rHSA production in transgenic plants grown in the field.

Transgenic plants accumulated 4.472µg rHSA per g leaf material under field condition. This level is in good agreement with the homozygous T_2 transgenic plants grown in the greenhouse producing HSA-APO to levels of an average 5 µg rHSA per g leaf material (Figure III.16).

III.3.4 Production of rHSA in transgenic wheat seeds

Wheat seeds are used in order to exploit the production of rHSA in these protein rich storage organs that can be stored almost indefinitely. Immature wheat (*Triticum aestivum* L. cv. Bobwhite) embryos were used as explants for the bombardment (II.2.2.7) of the HSA-KDEL cDNA integrated in the pAL76 expression vector (II.1.5.8). Following selection of the wheat on phosphinotricin (PPT), PPT resistant plant material was recovered. The efficiency of transgenic plant recovery was around 1%. Transgenic lines were then analysed for rHSA accumulation.

Total soluble proteins from dry wheat seeds (II.4.4) were analysed by ELISA and immunoblot. Using both procedures 80-100 µg rHSA per g seed material was detected in samples of pooled T_4 wheat seeds (data not shown). Immunoblot analysis revealed a distinct

band of approximately 66 kDa, corresponding to the molecular weight of HSA (Figure III.19). In addition, two other lower bands at around 45 and 30 kDa were recognized by the monoclonal anti-HSA antibody. These bands were also visible in the immunoblot analysis of various plant protein extracts (see Figure III.14, III.19. III.20 and III.21), which might be an indication of degradation during extraction of the recombinant protein from different plant species.

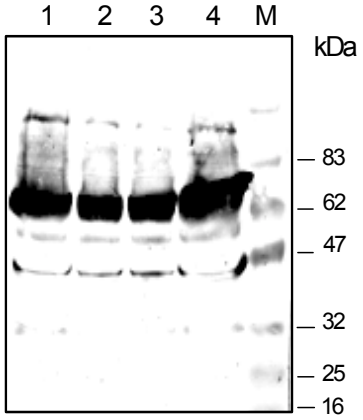

Figure III.19 Immunoblot analysis of the ER targeted rHSA extracted from transgenic wheat kernels. Total soluble proteins from wheat kernels was extracted (II.4.4) and 15 µl of the extract was separated by 12% (w/v) SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immuno detection was carried out using an anti-HSA antibody (1:5000) followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection. 1-4: 15 µl of total soluble proteins extracted from individual wheat kernels accumulating rHSA in the ER; M: prestained protein marker.

It is clearly demonstrated that wheat seeds are an alternative production system for molecular farming of rHSA. High accumulation levels in the seeds as much as 100 µg rHSA/g seed could be achieved. These values correspond to the rHSA level obtained in the transgenic tobacco plants using the same ER targeting construct.

III.3.5 Production of rHSA in transgenic tobacco suspension cells

Intact plants and their seeds are not the only expression system available for plant based molecular farming. Plant suspension cells are a model system that can be easily transformed and cultivated on a large scale in fermenters (Fischer, Emans *et al.* 1999). In this study, in addition to tobacco, zucchini, bean and wheat seeds, tobacco suspension cell culture was also evaluated in terms of expression and secretion of rHSA. *N. tabacum* cv. BY-2 cells were transformed by co-cultivation with recombinant A. *tumefaciens* carrying HSA-APO and HSA-KDEL constructs (III.2.3.2). Pinpoint-sized clumps of kanamycin-resistant cells were

screened for expression of the chimeric gene (data not shown) and highest expressors, for the HSA-APO and HSA-KDEL construct were selected for the initiation of cell suspension lines. Once a rapidly growing homogeneous suspension culture was obtained, both the culture medium and the cell pellet were analysed for the presence of rHSA.

After screening kanamycin resistant cells for highest expressors, two cell lines for HSA-APO (HSA-APO29, HSA-APO16) and one for HSA-KDEL (HSA-KDEL12) were selected. Suspension cells were sub-cultured weekly using a 2% (v/v) inoculum. Samples were taken aseptically from 3, 5, 7 and 10 days homogenously grown cultures after subculturing to determine the optimal time point for cell harvest in respect to rHSA accumulation. Fresh cell weight (FCW) was determined by pelleting the cells taken from 1 ml sample culture. No significant difference in terms of FCW between the transgenic lines was observed. FCW on day 3 was approximately 0.18 g per ml suspension culture, reaching to 0.54 g per ml on day 5 and remaining constant at 0.55 g per ml after 7 and 10 days of growth (data not shown).

Recombinant protein accumulation was measurable after 3 days of subculturing. For the cell line HSA-APO16, the highest accumulation level of 0.811 µg per g of cells was determined on day 5 and highest accumulation in the culture medium (0.494 µg per ml) on day 10. No secretion of rHSA into the culture medium was detected when cell line HSA-KDEL12 was analysed during these 10 days. For HSA-APO29, the highest accumulation level of 1.87 µg per g of cells was determined on day 7 and the highest accumulation in the culture medium 1.84 µg per ml was obtained on day 10 using the same cell line (Figure III.20).

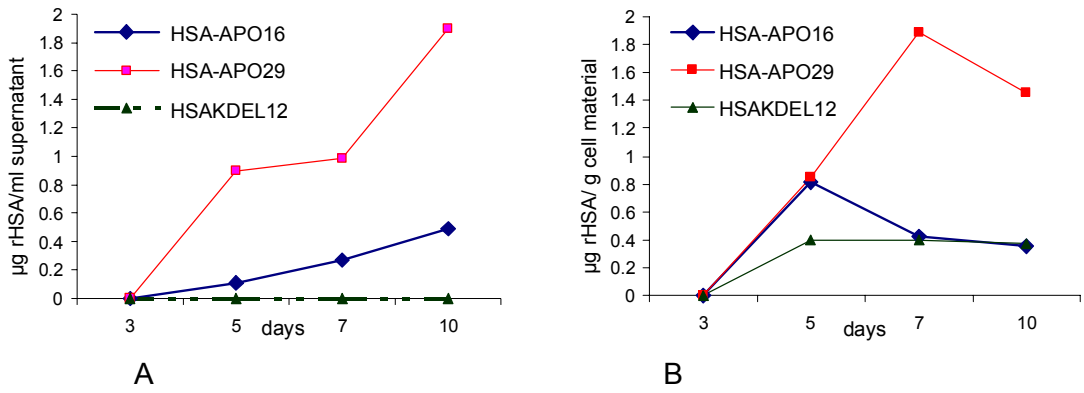

Figure III.20 Determination of rHSA levels in the culture medium (A) and in BY2 cells (B) by ELISA. Goat anti-HSA antibody (1:500 diluted in TBS) was coated to ELISA plates (II.4.9.2). After blocking with MTBS, 100 µl of the serially diluted plant extracts (II.4.2) were added to the coated plates. Bound rHSA was detected by addition of peroxidase-conjugated rabbit anti-HSA antibody (1: 20000). ELISA readings (OD $_{690\ nm}$) were performed after 1-10 min. incubation with ABTS substrate solution buffer (II.4.9.2).

Expression of rHSA in the transgenic BY2 cells and secretion to the supernatant were also compared at different stages of growth in the suspension cells by immunoblot. In addition to the expected size of rHSA with variable accumulation levels of HSA-APO and HSA-KDEL at 3, 5, 7 and 10 days, degradation bands at around 30 kDa were also observed (data not shown). As shown in ELISA (Figure III.20), and immunoblot highest levels of rHSA accumulation and secretion was observed using HSA-APO29 cell line. Therefore this line was chosen for the further experiments with the transgenic BY2 suspension cells.

III.3.5.1 Analysis of rHSA secreted to the culture medium

HSA-APO29 BY2 cell suspension culture was grown for 7 days. After separating the cells from the culture medium the amount of secreted rHSA was determined to be 1-1.5 µg per ml culture supernatant. Bradford analysis of the total soluble proteins in the supernatant revealed the amount in the medium to be 0.3-0.5 mg per ml. Therefore the amount of recombinant HSA secreted to the medium is in the range of 3-5 µg per mg total secreted protein, which is at least 15 times more than the secretion level obtained by Sijmons *et al.* (1990).

III.3.5.2 Improving rHSA stability in the culture medium

Due to the occurrence of unfavourable environmental conditions, the loss of proteins due to product instability during plant cell culture and subsequent purification significantly influences product yields. Protein stability following secretion can be improved with the addition of appropriate chemical agents (or stabilizers) to the growth media or storage solution (James and Lee 2001). By using this strategy, protein yields can be increased significantly with the addition of inexpensive chemical agents. Polyvinyl pyrrolidone (PVP) (Magnuson *et al.* 1996), DMSO (Wahl, An and Lee 1995), gelatine (Lee *et al.* 2002) have been used to protect extracellular proteins in transgenic plant cell and organ cultures.

In order to test the effect of additional compounds to transgenic BY2 cultures, HSA-APO29 suspension cell culture was cocultivated starting from the first day of subculture in the presence of 10 mM glutamine or PVP 40.000 (0.8 g/L). A third batch of culture was initiated to investigate the effect of increasing the sucrose concentration in the MS medium from 3% to 5%. Use of glutamine or increasing the sucrose content in the suspension culture media

resulted in a decrease in the amount of secreted rHSA when compared to the untreated control. In contrast, the level of secreted protein was increased by 80% upon addition of PVP 40.000 (Figure III.21).

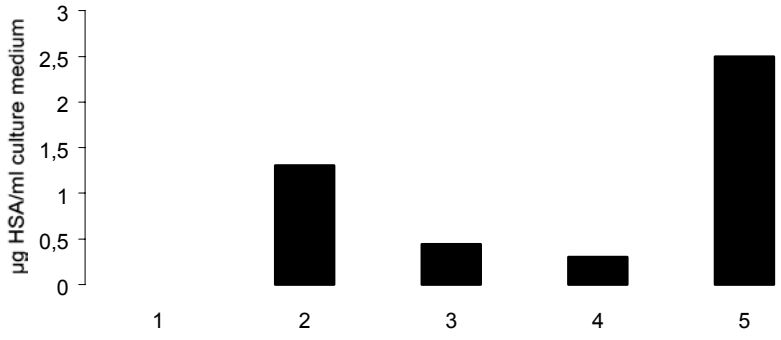

Figure III.21 rHSA accumulation in the culture medium of the HSA-APO29 cells grown in the absence or presence of stabilizing agents. Goat anti-HSA antibody (1:500 diluted in TBS) was coated to ELISA plates. After blocking with MTBS, 100 µl of the serially diluted cell culture supernatants separated from the suspension cells grown with or without stabilising agents were added to the coated plates. Bound rHSA was detected by addition of peroxidase-conjugated rabbit anti-HSA antibody (1: 20000). ELISA readings (OD $_{690nm}$) were performed after 1-10 min. incubation with ABTS substrate solution buffer (II.4.9.2). 1) Wild type BY2 suspension; 2) HSA-APO29; 3) HSA-APO29+10 mM glutamine; 4) HSA-APO29+50 g /L sucrose; 5) HSA-APO29+PVP 40.000 (0.8 g/L)

PVP with different molecular weights were tested by adding PVP to the HSA-APO29 culture from the first day of subculture. The working concentration was kept at 0.8 g/L. In the experiments, PVP (10.000, 40.000 and 360.000) alone or mixed with wild type BY2 suspension cultures were used as a negative control where commercial HSA mixed with PVPs were used as a positive control.

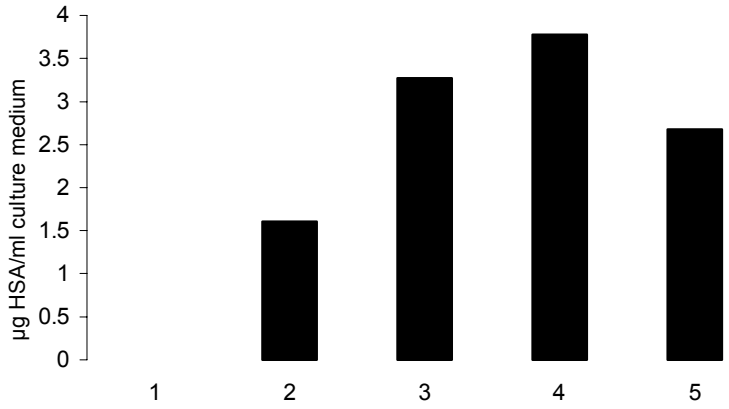

Figure III.22 rHSA accumulation in the culture medium of the HSA-APO29 cells grown in the absence or presence of PVP with varying molecular weight. Goat anti-HSA antibody (1:500 diluted in TBS) was coated to ELISA plates. After blocking with MTBS, 100 µl of the serially diluted cell culture supernatants separated from the suspension cells grown with or without PVP were added to the coated plates. Bound rHSA was detected by addition of peroxidase-conjugated rabbit anti-HSA antibody (1: 20000). ELISA readings (OD $_{690nm}$) were performed after 1-10 min. incubation with ABTS substrate solution buffer (II.4.9.2). 1) Wild type BY2 suspension; 2) HSA-APO29; 3) HSA-APO29+PVP 10.000; 4) HSA-APO29+PVP 40.000; 5) HSA-APO29+PVP 360.000.

The presence of PVP increased the level of accumulated rHSA in the culture medium at least 2-fold from 1.5 µg HSA per ml to 3.0 µg HSA per ml independent of the molecular weight of PVP tested. The highest level of secretion (3.75 µg rHSA per ml culture medium) was achieved using PVP 40.000 (Figure III.22).

The ELISA results were also confirmed by immunoblot analysis of the secreted rHSA (Figure III.27). Although there is an increased recovery of secreted rHSA by using PVP 40.000, two lower bands (at around 25 and 32 kDa) were recognized by the monoclonal anti-HSA antibody as well. These smaller truncated forms of rHSA were present in the supernatant of suspension culture cocultivated in the presence or absence of PVP. This was an indication that additional PVP might result in enhanced recovery of the product from the suspension culture but did not lead to an increased stability of the recombinant protein.

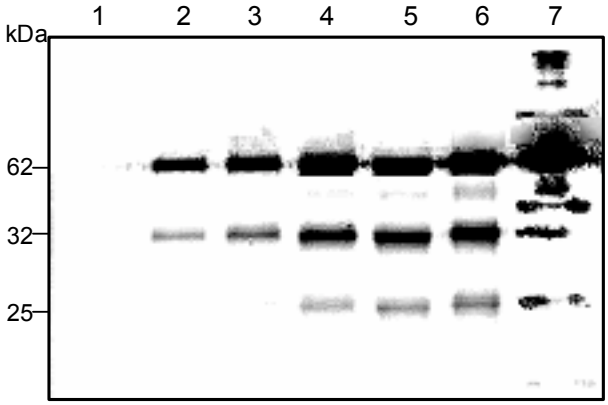

Figure III.23 Immunoblot analysis secreted rHSA from HSAcmyc29 cells grown in the presence or absence of PVP with varying molecular weight. The supernatant of BY2 suspension cells was separated by centrifugation, 10 µl was separated by SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immunodetection was carried out with anti-HSA antibody (1:5000) followed by 1 hr incubation with GAMAP (1:5000) and NBT/BCIP detection. 1) Wild type; 2) HSA-APO29; 3) HSA-APO29; 4) HSA-APO29+PVP 10.000; 5) HSA-APO29+PVP 40.000; 6) HSA-APO29+PVP 360.000 7) Prestained protein marker + HSA (100 ng Sigma).

III.4 Downstream processing of plant produced rHSA

Highly efficient purification schemes are a prerequisite for the isolation of recombinant protein from a foreign host organism. Although established protocols are available for purification of recombinant proteins produced by animal or microbial sources, there is little data available on the purification of recombinant proteins from plants, plant suspension culture cells, leaves or seeds (Moloney and Holbrook 1997).

Ion exchange chromatography (IEC) is the reversible interaction between charged molecules and oppositely charged chromatographic column. In cation exchange, negatively, and in anion exchange positively charged columns are coupled to the column medium. Elution is performed by change in the salt concentration or pH gradient. The advantages of IEC lie in the reasonably priced media and buffer costs and the simplicity and robustness of the method (Drossard, 2000). Cation exchange chromatography (CEX) as the capture step for rHSA purification was chosen due to the pI point of around 5.3 and also in order to avoid co-purification of nucleic acids, which are contaminating the protein extracts.

III.4.1 Optimum pH for binding of rHSA to CEX matrix

First experiments were done in a small scale purification system that was designed to permit scale up without major modifications. Therefore, chromatography media with identical functional groups were chosen for both small and large scale applications, no expensive reagents were added and simple inexpensive chromatography buffers were selected. For the same reason, no preconcentration steps like ammonium sulphate precipitation were included in the protocol.

To determine the best binding conditions to the cation exchange column, 100 µl of 10 mg per ml commercially available HSA (Sigma) in mQ water was used in the initial experiments. Chromatography was performed using a 1.4 ml Source 30S (Pharmacia) cation exchange medium in a BioCart5 column (Kronlab, Sinsheim) equilibrated with the respective loading buffer (acetate buffer pH 4.0-5.0). Samples of 100 µl were injected and bound material eluted using a salt gradient (0-1 M NaCl).

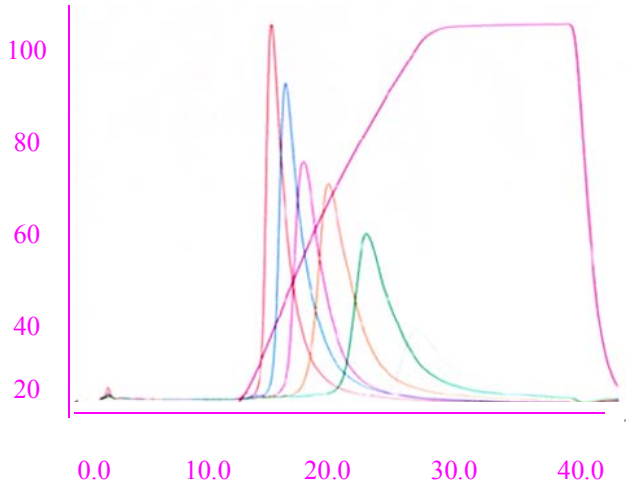

Figure III.24 Cation exchange chromatography of commercially available HSA. Column: BioCart5; medium: 1.4 ml Source 30S; feed: 100 µl of 10 mg per ml HSA (Sigma) dissolved in mQ water; loading buffer: acetate buffer shown in colored lines, first line in the orange colour represents acetate buffer pH 5.0, blue pH 4.8, red 4.6, small orange 4.4 and green 4.2; elution buffer: salt gradient (0-1 M NaCl).

The chromatogram is illustrated in Figure III.24. Binding profile of authentic HSA to the cation exchange column suggested that binding of HSA to the medium is pH dependent and optimum pH lies is in the range of pH 4.4 to pH 4.6. Therefore, extraction of the rHSA from transgenic leaves should be carried in this pH range, in order to assure optimum binding of protein to the chromatography matrix. In the previous experiments Tris buffer at pH: 8.0 (50

mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% (v/v) Tween 20 + 10 mM DTT) for extraction of the rHSA was taken.

III.4.2 Purification of rHSA from transgenic leaves

III.4.2.1 Optimisation of extraction buffer

In order to evaluate different buffer compositions for extraction of rHSA from transgenic tobacco plants prior to purification, total soluble protein from 1 g of transgenic leaf material (HSAKDEL31) was extracted in the extraction buffers 1, 2, 3 and 6 in the following list. After extracting total soluble proteins with these buffers pH was adjusted to 4.0 in 4, 5, 7 and 8. The typical buffer used for extracting rHSA from transient and stable transformed plants was buffer 3.

1. Acetate buffer (pH 4.0) + 5 mM EDTA
2. Acetate buffer (pH 4.0) + 5 mM EDTA + Complete^{TM} protease inhibitor (ROCHE)
3. 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% (v/v) Tween 20 + 10 mM DTT
4. 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% (v/v) Tween 20 + 10 mM DTT with a pH shift to 4.0
5. 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% (v/v) Tween 20 + 10 mM DTT with a pH shift to 4.0 + Complete^{TM} protease inhibitor (ROCHE)
6. PBS + 100 mM NaCl at pH 6.0
7. PBS + 100 mM NaCl at pH 6.0 with a pH shift to 4.0
8. PBS + 100 mM NaCl at pH 7.0 with a pH shift to 4.0 + Complete^{TM} protease inhibitor (ROCHE)

EDTA and Complete^{TM} protease inhibitor (ROCHE) were added only for small scale test purposes in order to check if these additives are playing any role for increasing the stability of the protein during extraction protocol. The amount of rHSA extracted from stable transgenic plants using different buffers was evaluated by ELISA (Figure III.25).

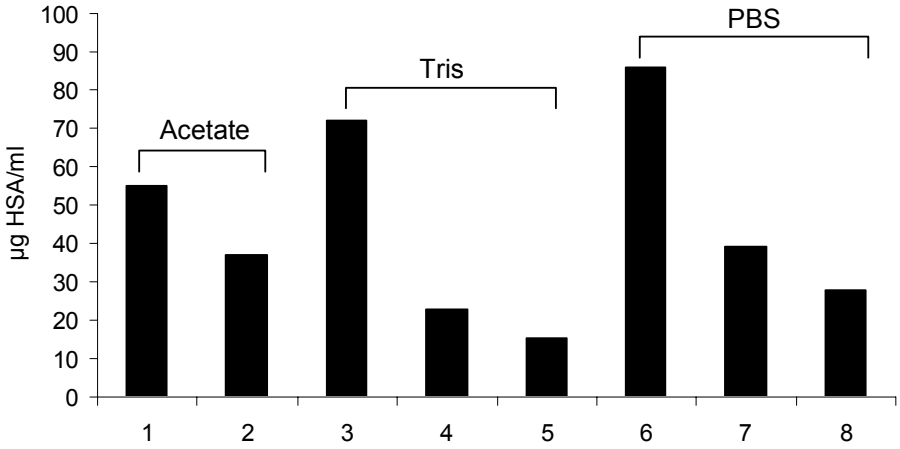

Figure III.25 Determination of rHSA in tobacco leaves using different plant extraction buffers. Goat anti-HSA antibody (1:500 diluted in TBS) was coated to ELISA plates. After blocking with MTBS, 100 µl of the serially diluted plant extracts were added to the coated plates. Bound rHSA was detected by addition of peroxidase-conjugated rabbit anti-HSA antibody (1: 20000). ELISA readings (OD_{690nm}) were performed after 1-10 min. incubation with ABTS substrate solution buffer (II.4.9.2). 1) Acetate buffer (pH 4.0) + 5 mM EDTA 2) Acetate buffer (pH 4.0) +5 mM EDTA + complete™ protease inhibitor (ROCHE) 3) 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% Tween 20 + 10 mM DTT 4) 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% Tween 20 + 10 mM DTT with the pH shift to 4.0 5) 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% Tween 20 + 10 mM DTT with the pH shift to 4.0 + complete™ protease inhibitor (ROCHE) 6) PBS+100 mM NaCl at pH 6.0 7) PBS + 100 mM NaCl at pH 6.0 with the pH shift to 4.0 8) PBS + 100 mM NaCl at pH 7.0 with the pH shift to 4.0 + complete™ protease inhibitor (ROCHE).

In Figure III.25 it is clearly shown that PBS buffer with 100 mM NaCl at pH 6.0 is the best buffering system for rHSA extraction from transgenic leaves. However, the optimum pH for binding of HSA to the cation exchange matrix was determined to be between pH 4.4-4.6 (see III.24). The protein solutions therefore have to undergo an acidic pH shift for optimum binding to cation exchange matrix. As it is indicated in Figure III.25 column 4, 5, 7 and 8, after this pH shift there is a drastic decrease in the amount of rHSA that could be detected by ELISA. On the other hand using acetate buffer at pH 4.0 together with EDTA as a chelating agent (Figure III.25 column 1) was also effective for the extraction of the rHSA from transgenic leaves and it has the advantage of being closer to the optimum pH for binding to the cation exchange matrix. That is the reason why acetate buffer at pH 4.0 + 5 mM EDTA was the buffer of choice for the extraction of rHSA from transgenic leaf material.

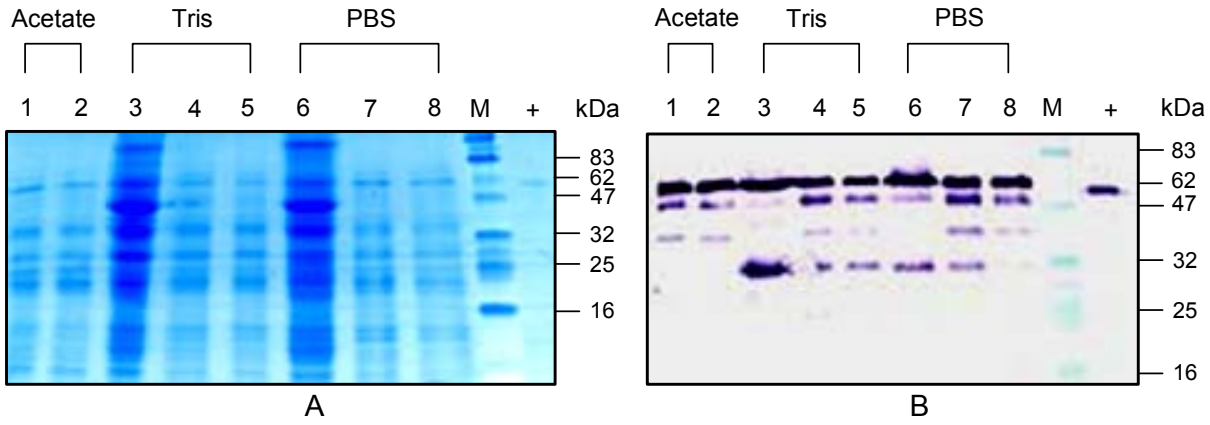

Figure III.26 SDS PAGE (A) and immunoblot analysis (B) of rHSA extracted from transgenic HSAKDEL31 N. Tabacum SR1 leaves using different buffers. TSP was extracted by grinding tobacco leaf tissue in two volumes extraction buffer (II.4.2), separated by 12% (w/v) SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immunodetection was carried out with anti-HSA antibody (1:5000) followed by 1 hr incubation with GAM^{AP} (1:5000) and NBT/BCIP detection. 1) Acetate buffer (pH 4.0) + 2 mM EDTA 2) Acetate buffer (pH 4.0) + 2 mM EDTA + complete^{TM} protease inhibitor (ROCHE) 3) 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% Tween 20 + 10 mM DTT 4) 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% Tween 20 + 10 mM DTT with the pH shift to 4.0 5) 50 mM Tris-HCl (pH 8.0) + 100 mM NaCl + 5 mM EDTA + 0.1% Tween 20 + 10 mM DTT with the pH shift to 4.0 + complete^{TM} protease inhibitor (ROCHE) 6) PBS + 100 mM NaCl at pH 6.0 7) PBS + 100 mM NaCl at pH 6.0 with the pH shift to 4.0 8) PBS + 100 mM NaCl at pH 7.0 with the pH shift to 4.0 + complete^{TM} protease inhibitor (ROCHE); M: prestained protein marker; +. 100 ng HSA (Sigma).

As shown in Figure III.26A, many other plant proteins are visible on the SDS PAGE after extraction with buffer 3 (50 mM Tris-HCl (pH 8.0) + 100 mM NaCl+ 5 mM EDTA+ 0.1% (v/v) Tween 20+ 10 mM DTT) and buffer 6 (PBS + 100 mM NaCl at pH 6.0). Whereas using 0.1 M acetate buffer at pH 4.0 (lanes 1 and 2) most of the plant proteins could be precipitated prior to purification. Differences in the amount of extracted rHSA from transgenic leaves observed in ELISA (Figure III.30) could not be confirmed by immunoblot (Figure III.26B). The intensity of the bands at the expected size of rHSA (66kDa) looked similar and different additional immuno-positive bands of a smaller molecular weight were detected by the anti-HSA antibody. Addition of Complete^{TM} protease inhibitor (ROCHE) was not helpful to avoid degradation (Figure III.26B lane 2, 5 and 8), indicating that this protease inhibitor is not effective to prevent the action of an unknown protease in the plant cells.

III.4.2.2 Influence of freezing leaves prior to extraction of rHSA

In order to analyse conditions that avoid or reduce the degradation of the rHSA isolated from transgenic leaves two different tobacco leaves batches were taken for extraction. In one set

the leaves were collected from greenhouse and used immediately for extraction, in the second set the leaves that were collected, frozen in liquid N_2 and stored for 6 months at –80°C were taken for extraction. Furthermore as a control the commercial HSA was mixed with the wild type tobacco extract. This was done to examine if the above mentioned unknown protease in the plant cell is also able to degrade the commercial HSA.

All samples were extracted in acetate buffer (pH 4.0) plus 5 mM EDTA. The transgenic HSAKDEL31 tobacco leaves were used for these experiments. As a control SR1 wild type plant leaves were extracted in the same buffer and during extraction, the extracts were supplemented with the 200 ng of commercially available HSA (Sigma).

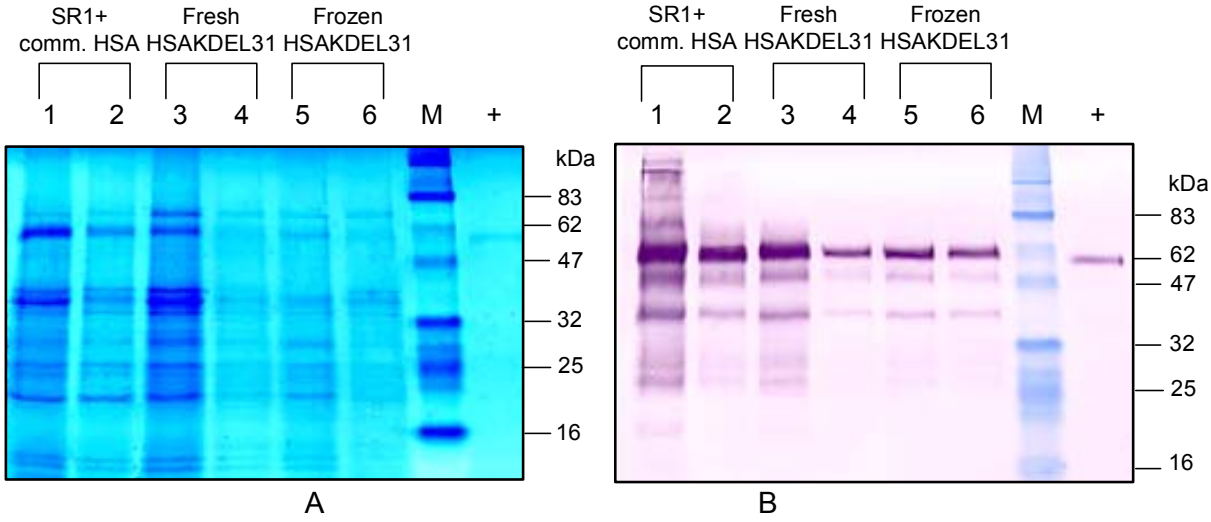

Figure III.27 SDS PAGE (A) and immunoblot analysis (B) of rHSA extracted from fresh and frozen transgenic HSAKDEL31 leaves. Total soluble proteins were extracted by grinding tobacco leaf tissue in two (1, 3 and 5) or four volumes (2, 4 and 6) of extraction buffer (acetate buffer pH 4.0+ 5 mm EDTA), separated by SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immuno detection was carried out with anti-HSA antibody (1:5000) followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. 1). Wild type (SR1) 1:2 diluted extract +200 ng HSA (Sigma); 2) Wild type (SR1) 1:4 diluted extract+ 200 ng HSA (Sigma) 3) Fresh HSAKDEL31 1:2 diluted extract; 4) Fresh HSAKDEL31 1:4 diluted extract; 5) -80°C frozen HSAKDEL31 1:2 diluted extract; 6) -80°C frozen HSAKDEL31 1:4 diluted extract; M) prestained protein marker; +) 100 ng HSA (Sigma).

In Figure III.27A on the Coomassie stained SDS PAA gel much less total soluble proteins were visible in lane 5 (frozen transgenic leaf extract) when compared with the fresh leaf extracts (lane 3). On the immunoblot (Figure III.27B), in addition to the expected size of rHSA (66kDa), additional immuno-positive bands almost in all the lanes including the control (wild type SR1 extract +200 ng HSA) was recognized by the monoclonal anti-HSA antibody. The presence of these smaller bands in all of the plant extracts investigated in this study is an

indication of a degradation process. Presence of similar degradation bands in the wild type tobacco extract supplemented with commercial HSA showed that this degradation is taking place during total soluble protein extraction.

Dilution of the extract in 1:2 or 1:4 resulted in the dilution of the rHSA but not in the reduction of the degradation products, which are equally well diluted (Figure III.27B). Using fresh leaves collected from greenhouse and directly processed for extraction, resulted in a more efficient extraction of rHSA (Figure III.27B lane 3 and 4) when compared to frozen leaves (lane 5 and 6). But again the degradation of the recombinant protein could not be lessened or avoided.

III.4.2.3 Small scale purification of rHSA from transgenic tobacco leaves

After determining the optimum pH for binding of rHSA to the cation exchange matrix (Figure III.24) and the optimum extraction conditions (III.4.2.1 and III.4.2.2), first experiments were done in a small scale purification system that was designed to permit scale up without major modifications. Fresh transgenic leaves from line HSAKDEL31 were extracted in two volumes of acetate buffer (pH 4.5). The extract was divided into two and one part of the protein isolate was heated at 60°C for 30 min, where the other part was used for chromatography directly after extraction. The aim of this heat treatment was to remove plant proteins that are interacting with the matrix prior to chromatography. CEX was performed using a 1.4 ml Source 30S (Pharmacia) cation exchange medium in BioCart5 column (Kronlab, Sinsheim) equilibrated with the respective loading buffer (20 mM acetate pH 4.5). A sample of 20 ml was used as feed and bound material was either eluted using a salt gradient (0-1 M NaCl) (Ea in Figure III.28A) or a salt gradient and a pH shift to 8.0 (Eb in Figure III.28A).

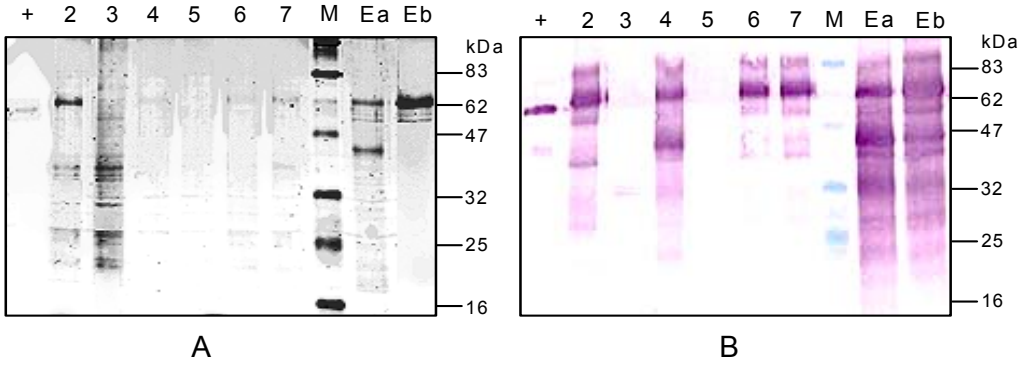

Figure III.28 SDS PAGE (A) and immunoblot analysis (B) of rHSA after partial purification by CEX. Total soluble proteins were extracted by grinding tobacco leaf tissue in two volumes of extraction buffer (acetate buffer pH 4.5), separated by SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immuno detection was carried out with anti-HSA antibody (1:5000) followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. +) 100 ng HSA (Sigma) 2) HSAKDEL31 transgenic leaves extracted in acetate buffer (pH 4.5); 3) SR1 leaves extracted in acetate buffer (pH 4.5); 4) protein extract #2 heated at 60°C for 30 min 5) protein extract #3 heated at 60°C for 30 min; 6) Flow through (salt gradient); 7) Flow through from heated extract (salt gradient and pH shift to 8.0); 8) M: prestained protein marker; 9) Ea) Eluate (salt gradient); 10) Eb) Eluate (salt gradient and pH shift to 8.0)

As shown by SDS PAGE analysis (Figure III.28A), there is a huge decrease in the amount of plant protein when the leaf extract was heated at 60 °C for 30 min. Amount of rHSA seemed to be similar both in the heated and non-heated samples (Figure III.28B lanes 2 and 4 respectively). However, ELISA results showed that almost one half of the rHSA was lost when the extract was heated (Table III.4, initial feed). Although, heating the extract resulted in the reduction of the contaminating plant protein concentration, it resulted in a huge loss of the rHSA in the initial feed prior to purification. Consequently extract heating was not included into the extraction protocol.

In addition, ELISA results confirmed that elution of rHSA from the cation exchange matrix was also a very important step to optimise. Salt gradient alone or in combination with a pH shift to 8.0 were investigated during the elution step. As illustrated in the Table III.4, using salt gradient and a pH shift to 8.0 was more effective (115 µg rHSA per ml eluate) for elution of rHSA than using salt gradient alone (62 µg rHSA per ml eluate).

Table III.4 Analysis of CEX results after partial purification of rHSA from transgenic leaf material by ELISA. 10 g of transgenic HSAKDEL31 leaves were extracted in two volumes of acetate buffer (pH 4.5). Flow through a: Flow through of the CEX from HSAKDEL transgenic leaf extract; Flow through b: Flow through of the CEX from HSAKDEL transgenic leaf after heating the extract at 60 °C for 30 min.

	rHSA (µg/ml)	Total volume (ml)
Initial feed	45	20
Initial feed heated at 60 °C for 30 min	25	20
Flow through a	22	40
Eluate a (salt gradient)	62	0.5
Flow through b	27	40
Eluate b (salt gradient and pH shift to 8.0)	115	0.5

ELISA results also showed that almost half of the rHSA available in the initial feed is still in the flow through a (Table III.4). Further optimisation of the protocol was necessary for efficient binding of rHSA to the cation exchange matrix.

For this purpose, a new batch of fresh transgenic leaves from line HSAKDEL 31 were extracted in acetate buffer (pH 4.5) plus 5 mM EDTA. EDTA was added to the sample to inhibit rHSA fragmentation observed in the previous eluates (Figure III.28B, lane Ea and Eb). Linflow was reduced from 600 cm/h to 300 cm/h in order to increase the contact time of the recombinant protein with the matrix. CEX was performed using a 1.4 ml Source 30S (Pharmacia) cation exchange medium in BioCart5 column (Kronlab, Sinsheim) equilibrated with the respective loading buffer (20 mM acetate pH 4.5) and with a linflow of 300 cm/h. 40 ml of sample was used as feed and bound material was eluted using a salt gradient (0-1 M NaCl) and a pH shift to 8.0. The chromatogram is illustrated in Figure III.29.

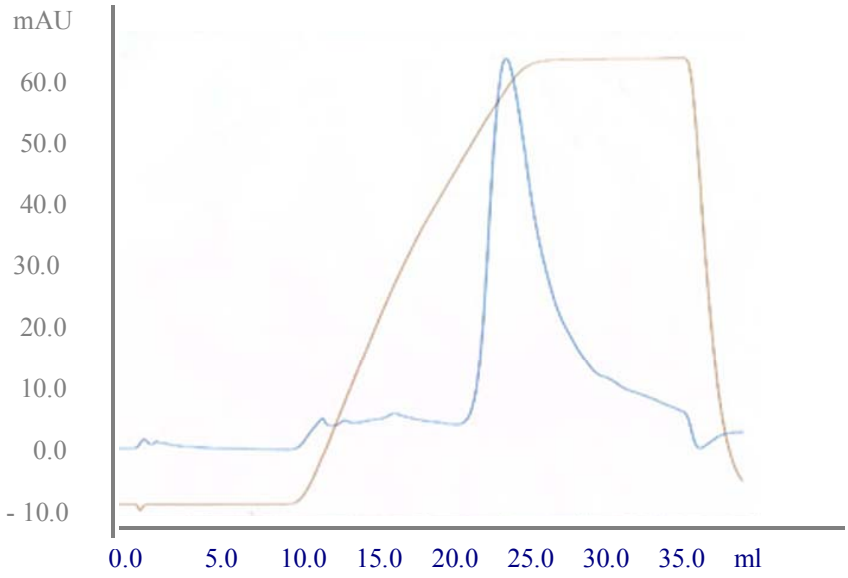

Figure III.29 Cation exchange chromatography of rHSA from transgenic tobacco plants. Column: BioCart5; medium: 1.4 ml Source 30S; feed: 40 ml of homogenate prepared by extraction transgenic tobacco leaves in acetate buffer (pH 4.5) plus 5 mM EDTA; loading buffer: acetate buffer; linflow of 300 cm/h. elution buffer: salt gradient (0-1 M NaCl) and a pH shift to 8.0.

A clear band with an identical relative mobility to commercial HSA was clearly visualised both on the SDS PAGE gel (Figure III.30A) and immunoblot (Figure III.30B). Also the 30 kDa degradation product was visible indicating that it is co-purified or that degradation occurs also during purification.

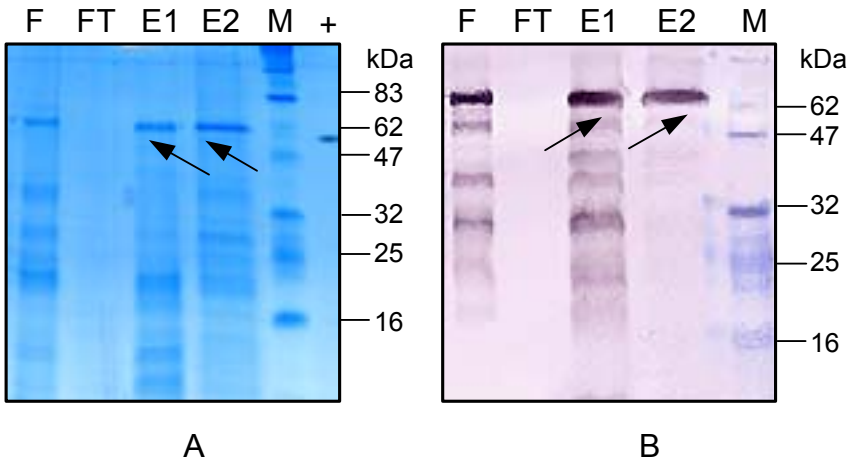

Figure III.30 SDS PAGE (A) and immunoblot analysis (B) of rHSA after partial purification via CEX. Total soluble proteins were extracted by grinding tobacco leaf tissue in two volumes of acetate buffer pH 4.5 plus 5 mM EDTA, separated by SDS-PAGE (II.4.6.1) and blotted onto a nitrocellulose membrane (II.4.8). Immuno detection was carried out with anti-HSA antibody (1:5000) followed by 1 h incubation with GAM^{AP} (1:5000) and NBT/BCIP detection for 5 min at RT. F) Initial feed FT) Flow through; E1) Eluate 1; E2) Eluate 2; M: prestained protein marker; +) 100 ng HSA (Sigma)

Compared to the previous run (see Table III.4), no rHSA was observed in the flow through, which shows that binding to the cation exchange matrix was optimized. Concentration of the rHSA was determined by ELISA as described (II.4.9.2) and tabulated in Table III.5.

Table III.5 Analysis of cation exchange chromatography results after purification of rHSA from transgenic leaf material by ELISA. 10 g of transgenic HSAKDEL31 leaves were extracted in two volumes of acetate buffer (pH 4.5) plus 2 mM EDTA. Bound material was eluted using a salt gradient (0-1 M NaCl) and a pH shift to 8.2

	rHSA (µg/ml)	Total volume (ml)
Initial feed	52	20
Flow through	0	40
Eluate 1	222	2
Eluate 2	186	0.5

In the initial feed of 20 ml, approximately 1 mg of rHSA was determined by ELISA. 537 µg, around 50 % of this protein could be obtained in the eluates after CEX.

III.4.2.4 Large scale purification of rHSA from transgenic tobacco leaves

Initial experiments were performed with 10 g of transgenic leaf material in order to determine the most suitable feed material, extraction buffer and flow rate for the optimised chromatography conditions. A scale up is necessary in order to check if these conditions are also applicable for large scale downstream processing of the recombinant protein. After completing the small scale pilot experiments with the stable transgenic leaf material, 700 g of transgenic leaf material was extracted in the 2 volumes of acetate buffer pH 4.5 plus 5 mM EDTA and used for CEX. 1400 ml of the clarified leaf extract was loaded as initial feed on a CEX matrix. Chromatography was performed using a XK50/20 cation exchange column filled with 120 ml of Seppharose Big Beads equilibrated with the respective loading buffer (20 mM acetate pH 4.5). CEX was performed with a flow rate of 33.5 ml/min (100 cm/h). Bound material was eluted using a salt gradient step 0-1 M NaCl and a pH shift to 8.0. The eluted fractions were collected and analysed for the presence of rHSA by SDS-PAGE (Figure III.31) and ELISA (Table III.6).

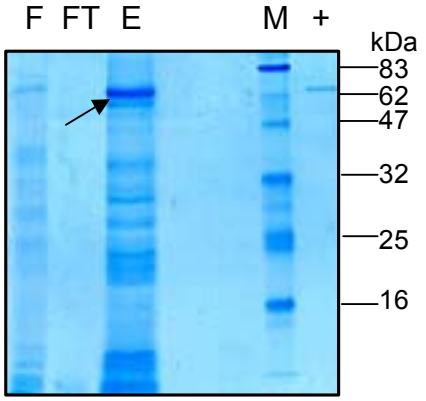

Figure III.31 SDS PAGE of rHSA after partial purification via CEX. Total soluble proteins were extracted by grinding (II.4.2) tobacco leaf tissue in two volumes of extraction buffer (acetate buffer pH 4.5 plus 5 mM EDTA), separated by 12 % (w/v) SDS-PAGE (II.4.6.1). F) Initial feed for CEX; FT) Flow through; E1) Eluate 1; E2) Eluate 2; M: prestained protein marker; +) 100 ng HSA (Sigma).

Partial purification of the rHSA was achieved by using cation exchange chromatography. Coomassie stained SDS PAGE showed that other plant proteins were still present after this first step of chromatography indicating that large scale conditions have to be optimised. ELISA was performed with the samples taken before, during and after purification in order to determine the concentration of the protein at different stages of purification (Table III.5).

Table III.6 Analysis of CEX results after partial purification of rHSA from transgenic leaf material.

	rHSA (µg/g)	Total volume (ml)	% Recovery
Initial feed	81.6	1400	57
Flow through	0	1200	0
Eluate	370	120	22

From 700 g of transgenic leaf material 22 mg of rHSA could be purified using a single step cation exchange chromatography.

IV Discussion

IV.1. The variability of the HSA cDNA

The human serum albumin gene is present as a single copy (Takahashi *et al.* 1987) coding for 585 amino acids (Peters 1985). Sequences for HSA have been published by Meloun *et al.* (1975), Lawn *et al.* (1981) and Minghetti *et al.* (1986). These published protein sequences of HSA have the same length but differ in about 20 amino acids. In this work, HSA cDNA was amplified from two overlapping cDNA fragments (Dugaiczyk *et al.* 1982) and sequence was verified. Among the published reports of HSA sequences, the Dugaiczyk sequence is most similar to that of Minghetti *et al.* (1986), with only one amino acid change (Glu to Gly) at position 294.

At all discrepant positions the nucleotide sequencing has been carefully confirmed and it is unlikely that DNA sequencing errors are the cause of these reported differences. The possibility of artefacts introduced by cDNA cloning cannot be ruled out. However, other likely explanations exist for the amino acid sequence differences among various reports. These include changes in amidation that occurs either *in vivo* or during protein sequencing (Lawn *et al.* 1981). Polymorphism in HSA may also account for differences in the sequence. About 100 variant forms of the albumin molecule (alloalbumins) have been described (Nicholson *et al.* 2000) as well as two mutations that cause nearly 30 cases of analbuminaemia have been detected by protein electrophoresis (Weitkamp *et al.* 1973). Most alloalbumins have a single amino acid substitution, resulting from a point mutation; a few alloalbumins have a truncated and altered carboxyl terminus (Madison *et al.* 1991; Watkins *et al.* 1994). Therefore, rather than cloning artefacts or sequencing errors, changes observed in this work and some of the published sequences are more likely genetic variants of HSA present in the nature.

IV.2 Construction of the plant codon optimized HSA

Heterologous genes containing high levels of rare codons that are used in low frequency in the host organism often show low levels of recombinant protein expression or truncated products because of premature stops in protein translation (Chen *et al.* 1990; Hannig *et al.*

1998; Kang *et al.* 2004). Many instances of poor expression of foreign genes in plants have been investigated, and several post-transcriptional events that led to low levels of expression, such as improper codon usage, (Perlak *et al.* 1991) abnormal splicing, (Haseloff *et al.* 1997) premature polyadenylation (Jarvis *et al.* 1997) and mRNA instability (Murray *et al.* 1989) have been described. The authentic HSA cDNA contains 18.3 % rare codons, dispersed over the entire sequence. Therefore, it was decided to adapt the codon usage in the rHSA sequence to the one in wheat and rice plants to optimise the plant expression.

The protein coding sequence can be optimised by various strategies like oligonucleotide ligation, (Heyneker *et al.* 1976) self priming PCR (Dillon *et al.* 1990) or cloning of large (approx. 100 bp long) inserts into a plasmid using a technique of oligo-directed double-strand break repair (the *Fok*I method) (Mandecki *et al.* 1988). A particularly attractive method, due to its inherent simplicity, is assembly PCR (Stemmer *et al.* 1995). This involves the generation of overlapping oligonucleotides which, when assembled, form the template for the gene of interest. The oligonucleotides are repetitively extended by PCR to assemble the full-length gene.

Based on a similar assembly PCR approach, a semi-synthetic HSA gene was amplified. Authentic HSA cDNA was digested in three smaller fragments in order to avoid amplification of the template HSA by the two external primers. These three templates, T400, T800 and T1000 were used in the assembly PCR together with 32 different primers designed for the exchange of rare codons in wheat and rice. Primers did not cover the complete synthetic gene rather only the modified sequence fragments. In this way complexity of the PCR could be reduced and only the regions with the rare codons were replaced while the rest was kept as authentic HSA sequence. After four rounds of PCR, a fragment of the expected size of 1.8 kb PCR was assembled and the product was subsequently purified and cloned. A pre-screen of the received clones by analysing a characteristic enzymatic activity of the expressed protein was hampered due to the absence of such an activity test for HSA. In order to identify a PCR assembled semi-synthetic HSA cDNA, 50 individual clones were sequenced either using HSA or vector specific primers. Computer-based sequence alignment of these clones revealed that the rHSA was successfully assembled in the right order. However, as many as 35 mutations including deletions, insertions and base changes were detected. Clones 13 and 31 having the lowest error rate still contained 19 and 20 base pair exchanges, respectively. Analysis of the plant codon optimised HSA showed that the mutations were exclusively in the primer regions of the final product. Therefore the observed mutations in the sequence are

more likely due to bad quality of the primers used in the assembly. The frequencies of the nucleotide errors are largely dependent on the quality of the nucleotides, rather than the fidelity of the polymerase (Hoover *et al.* 2002). The primers were synthesized and purified using standard procedures by MWG Biotech and delivered in the lyophilised form.

One approach to remove these mutations is to use site directed mutagenesis (Ishii *et al.* 1998). *In vitro* site-directed mutagenesis is an invaluable technique for the modification of a given sequence. It involves **(1)** cloning the DNA of interest into a plasmid vector, **(2)** denaturing the plasmid DNA to produce single strands, **(3)** annealing synthetic oligonucleotide with desired mutation (point mutation, deletion, or insertion) to the target region, **(4)** extending the mutant oligonucleotide using a plasmid DNA strand as the template and **(5)** propagating the heteroduplex by transformation in *E. coli*. In theory, after propagation, about 50% of the transformants will carry a heteroduplex in which the desired mutation is incorporated. Although some commercial mutagenesis kits guarantee a mutation efficiency of 90 % like for example with Stratagene's QuikChange® site-directed mutagenesis kit, it suffers from the following disadvantages; (i) in general, mutations are introduced at only one site at a time (Kirsch *et al.* 1998); introduction of more than one mutation at different sites takes longer, since it requires a transformation and a DNA preparation step between consecutive rounds of mutagenesis; (ii) two complementary mutagenic oligonucleotide primers are needed for a mutation site. To correct clones 13 and 31 comprising 19 and 20 mutations respectively, 40 mutagenic oligonucleotide primers are required and mutations should have been eliminated once at a time with following transformation and DNA preparations steps. The final products should have been sequenced for verification of the sequence changes. The accomplishments of these mutagenesis experiments were out of the scope of this present work and therefore the efforts to generate a synthetic plant codon optimized human serum albumin cDNA were stopped.

In the retrospective, the assembly of the full-length HSA cDNA could have been modified. To reduce the possibility of errors during oligonucleotide synthesis, the oligonucleotides should be rather short, yet they must still be long enough to provide stable priming overlaps. In this work, the primer length for assembly of the semi-synthetic HSA cDNA varied between 27 nt and 83 nt and they overlapped between 21 and 24 nt (Figure III.3). While assembly PCR approach is simple in principle, in practice numerous complications can lead to errors in the synthesis (Hoover *et al.* 2002). For example, a 1.1-kb fragment containing the TEM-1 beta-lactamase-encoding gene (*bla*) was assembled in a single reaction from a total of 56

oligos, each 40 nucleotides (nt) in length. Five silent point mutations were introduced creating five new restriction sites, to differentiate the synthetic gene from the naturally occurring *bla* gene in pBR322. At the end of assembly the *bla* gene was sequenced and three additional point mutations were detected (Stemmer *et al.* 1995). Insertion of additional restriction sites into the sequence enables the removal of mutations by exchanging the mutated against the expected sequences. Larger genes can be divided in subfragments that are assembled separately. After sequence verification of the cloned subfragments, they can be assembled to the full-length construct. It may be an advantage to design the oligonucleotide sequences to generate unique restriction sites within the assembled gene to allow subsequent manipulations by recombinant DNA techniques. This way it could be possible to exchange the smaller subfragments containing mutated sequence with a subfragment containing the expected sequence. By combination of these two parameters, the nucleotide sequence of a major industrial lipase, *lip1* gene was first subdivided into four separately synthesized segments of ca. 400 bp. Unique restriction sites were strategically positioned throughout the gene to facilitate the analysis of primary subclones containing the four blocks and the construction of the gene by their assembly. The final codon optimised version of the gene was 1688 bp in length and had a 77 % nucleotide sequence identity with the natural gene using *P. pastoris* cells, the synthetic gene was functionally overexpressed, allowing for the first time to produce recombinant *lip1* of high purity at a level of 150 U/mL culture medium (Brocca *et al.* 1998).

In plant system, several examples of codon optimization strategies are present. The gene encoding green fluorescent protein (GFP) from *Aequorea victoria* was resynthesized to adapt its codon usage for expression in plants by increasing the frequency of codons with a C or a G in the third position from 32 to 60 %. A comparison of GFP production in transgenics with the wild type and the synthetic *gfp* gene under the control of the enhanced CaMV 35S promoter showed that the large-scale alterations in the *gfp* gene increased the frequency of high expressors in the transgenic population but hardly changed the maximum GFP concentrations. The authors attributed this to a reduced regeneration capacity of transformed cells with higher GFP concentrations (Rouwendal *et al.* 1997). Similarly, another marker gene, luciferase was also codon optimized for high level expression in plants (Lonsdale *et al.* 1998). In tobacco cells, a codon modified form of the luciferase gene (luc+) available from Promega, shows no significant enhancement of activity over that of the wild-type gene, although there are 53- to 59- and 14- to 23-fold enhancements of activity in wheat scutellum

and Black Mexican Sweet (BMS) maize cells, respectively. Lonsdate *et al*., (1998) attributed these differences to the different tRNA pools present in different tissues and species.

Recently, the coding sequence of the cholera toxin B subunit (CTB) was optimized, based on the modification of codon usage to that of tobacco plant genes and the removal of mRNA-destabilizing sequences (Kang *et al.* 2004). The recombinant CTB protein constituted approximately 1.5 % of the total soluble protein in transgenic tobacco leaves. This level of CTB production was approximately 15-fold higher than that in tobacco plants that were transformed with the bacterial CTB gene.

These studies in heterologous expression hosts have shown that codon usage can influence the expression of foreign proteins. However, in general the increase in protein levels that may be obtained by codon optimization is relatively modest and it is in a few fold range. In conclusion, mindful attention to cryptic features like codon bias changes to correctly match the tRNA complement of the new host, investigation of accumulating sequence motifs that would be recognized as premature polyadenylation signals or cryptic intron splice sites in plants can lead to improvement of these strategies. Similarly, consideration of mRNA secondary structures that might hinder expression or RNA stability in the transgenic host can lead to development of modified trangenes whose expression increases several hundred folds.

IV.3 Production of recombinant HSA in bacteria and plant cells

HSA is a monomeric globular pre-protein whose mature form consists of a single polypeptide chain of 585 amino acids (66.5 kDa with 17 disulfide bonds). It is highly susceptible to proteolytic degradation in recombinant systems and is expensive to purify. Estimates by industry suggest that the cost-effective yield for pharmaceutical production in plants is 0.1 mg of rHSA per g of fresh weight (Fernandez-San Millan *et al.* 2003). 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 setup cost (Daniell 2003).

The increasing production of proteins in heterologous hosts through the use of recombinant DNA technology has brought the problem of proteolytic degradation into focus; heterologous proteins appear to be more prone to proteolysis. Recombinant proteins are often regarded by a

cell as foreign, and therefore are degraded much faster than most endogenous proteins. Proteolytic stability of recombinant proteins is a significant factor influencing the final yield. In this work bacterial and plant production platforms were evaluated for the expression of rHSA.

IV.3.1 Bacterial expression

HSA is characterized by a low content of tryptophan and methionine and a high content of cysteine and the charged amino acids, aspartic and glutamic acids, lysine and arginine. In this work, two different constructs were used for expression of rHSA in the cytoplasm or periplasm of *E. coli*. Expression of rHSA was achieved in the cytoplasm but in very low amounts. The concentration of the affinity purified cytoplasmic rHSA was determined to be 0.002 mg per liter bacterial culture (III.3.1). In nature, the presence of 17 disulphide bridges is the unique feature of the albumin molecule (Carter *et al.* 1990). HSA cDNA was cloned into the pET21d vector, allowing expression in the cytoplasm. It is a reducing environment and due to this reason it might be difficult to ensure proper disulphide bond formation.

The change of compartment can lead to better protein folding resulting in higher expression levels. Therefore, the second approach was to secrete HSA into the periplasm by means of the *pelB* leader sequence. Secretion of the target protein to the periplasm has a number of distinct advantages like the oxidizing environment of the periplasm allows for the formation of disulfide bonds, which does not occur in the reducing environment of the cytoplasm. Also, the periplasm contains two foldases that catalyze the formation and isomerization of disulfide bonds. Since the periplasm contains only 4 % of the total cellular proteins, the target protein is effectively concentrated and can be purified with less contaminant. In addition protein degradation in the periplasm is also less extensive in the periplasm (Talmadge *et al.* 1982). However, capacity for secretion and post-translational modification are usually limited {Hannig *et al.* 1998} and not all expressed protein are secreted into the periplasm but also found in the medium, the cytoplasm or the cytoplasmic membrane.

To our knowledge there is no published report describing high level expression of rHSA in *E. coli*. For a successful bacterial expression system either in *E. coli* or in another strain, optimization of culture conditions must be carried out. The expression of the recombinant protein can be optimized by doing a time course of induction, altering induction temperature or screening growth media. For each test, the recombinant protein should be analyzed by

ELISA, SDS-PAGE and/or Western blot to determine optimal conditions for protein production. Instead of optimizing conditions for bacterial expression, the thesis was focused on the expression of rHSA in plant cell and suspension cultures.

IV.3.2 Expression of rHSA in plants

Molecular farming in plants has the potential to provide virtually unlimited quantities of recombinant proteins for use as diagnostic and therapeutic tools in health care and the life sciences (Twyman *et al.* 2003). Plants produce a large amount of biomass and protein production can be increased using plant suspension cell culture or by the propagation of stably transformed plant lines in the field.

In this work, we have used nuclear transformation of various plant species to enable targeting of recombinant HSA to the secretory pathway, which should ensure proper folding and formation of the disulfide-bridges. Transgenic tobacco and wheat plants as well as tobacco suspension cells were generated using appropriate targeting signals to achieve subcellular HSA localisation in the plant cell apoplast or ER. Transient gene expression system offer several advantages over analyses of stable expression, for example initiation of gene expression, and synthesis of the protein can be analyzed within a very short time and is not affected by position effects (Vaquero *et al.* 1999). The results can be evaluated before initiating stable transformation of plants and experimental procedure does not require sophisticated equipment and is inexpensive. Therefore this system is suitable for the development and initial characterization of new recombinant protein.

Tobacco is a non-food and non-feed crop and an ideal choice for the production of therapeutic proteins because of its relative tractability to genetic manipulation and an impending need to explore alternate uses for this crop. Tobacco is an excellent biomass producer (in excess of 40 tons of leaf fresh weight/acre, based on multiple cuttings per season) and a prolific seed producer (up to one million seeds produced per plant), thus hastening the time in which a product can be scaled up and brought to market. Tobacco is widely used as a model system to test the suitability of plant-based expression systems for production of therapeutic proteins and other transgenes (Daniell 2003). More transgenes have been introduced into the tobacco chloroplast or nuclear genome than all other crop species combined. Both nuclear and chloroplast genomes of tobacco have been transformed with relative ease. Tobacco is a self-

pollinating crop and there are no known wild or cultivated relatives in North America. Several studies have established that transgenes are maternally inherited when introduced via the chloroplast genome in tobacco. In addition, both male sterility and seed sterility techniques have been developed already for tobacco. Thus, advanced technology is readily available for containment of gene flow through pollen and seeds.

In this study, eight different tobacco cultivars were evaluated by transient expression and the maximum accumulation levels obtained was compiled in Table III.1. For six of the cultivars tested, expression levels varied between 0.76 to 6.3 µg per g leaf material. For the cultivars Adonis Schwester and Barley Saturn, accumulation levels of 10 µg per g leaf material were achieved in the apoplasm. Although leaf size in these varieties is bigger than the model Petit Havana SR1 tobacco plants, which is an advantage for molecular farming purposes, a disadvantage of these cultivars is the unavailability of the seeds for the next generation which will be a major drawback for the stable transformation.

Clearly, transient expression is a prerequisite to stable transformation because it allows both the expression vectors and protein stability to be tested. For stably transforming tobacco plants, HSA gene was cloned into a binary vector that can be moved between *E. coli* and *A. tumefaciens*. Transformation was followed by selection for cells with stably integrated copies of the rHSA by following a selectable marker resistance gene that is introduced in the expression vector. Using plant extracts from stably transformed tobacco plants, correct size of the recombinant protein was confirmed by immunoblot analysis and the quantity of protein was estimated by ELISA. Maximum accumulation level of 100 µg per gram plant material (0.5–1 % of TSP) was obtained when the recombinant protein was targeted to the ER. These levels are at least 2.5 fold higher than those reported in the literature for nuclear transformation of potato by Farran *et al.* (2002). By including the appropriate signal peptide sequence or fusion responsible for directing expression and deposition, it is possible to target recombinant proteins to the lumen of the ER, vacuole or other cellular compartments. These type of targeting signals can be used to protect the foreign protein from proteolytic degradation, preserve their integrity and to increase accumulation levels (Moloney *et al.* 1997). In this work, usage of KDEL retention signal boosted the production of rHSA in tobacco plants. Although it is conceivable that the different transcripts are translated with different efficiencies, a more plausible explanation for the differences in the protein yield is that rHSA is more stable, or more rHSA is correctly folded in the ER than in the apoplast, as reported for the scFv expression in other plant species (Artsaenko *et al.* 1995). Schouten *et al.*

(1997) have demonstrated the addition of a C-terminal KDEL peptide increased cytosolic expression level of a scFv fragment. Maximum expression levels of up to 0.2% of the total soluble protein were measured, whereas the same scFv fragments without KDEL did not show any accumulation in the cytosol of transgenic plants. They suggested that short polypeptides such as KDEL sequence may protect the scFv antibody from proteolytic degradation (Schouten *et al.* 1997).

Besides different tobacco varieties three edible plants, zucchini, bean and wheat were also used for expression of rHSA (Table III.1). In bean leaves, accumulation levels were below detection levels for ELISA and western blot. In zucchini and in model tobacco SR1, transient accumulation levels of 2.65 µg per g and 2 µg per g leaf material were achieved, respectively. Besides intact tobacco plants, immature wheat embryos were also used for bombardment of HSA-KDEL construct containing ubiquitin promoter. Expression levels in dry wheat seeds were determined as 100 µg per gram seed. The use of transgenic wheat seeds enables the storage of rHSA in seeds for long time periods. We have stored transgenic wheat seeds for more than 12 months without significant loss of product yield. Stöger *et al.* (2000) investigated the expression of a construct containing scFvT84.66 tagged with a C-terminal KDEL sequence in wheat and rice (Stöger *et al.* 2000). The highest expression levels of 29 µg per g in leaves and 32 µg per g in seeds were detected using ubiquitin promoter. In our study, although the expression levels were much higher than this, it should be possible to enhance production levels further, for example by testing a range of alternative promoters, including seed-specific promoters.

In addition to these studies using the plants grown in the greenhouse, in year 2000 a field trial has been performed in Canada using transgenic *N. tabacum* cv. Petite Havana SR1 plants producing rHSA accumulating in the plant apoplast. The analyses revealed that similar levels were obtained when compared to plants cultivated in the greenhouse demonstrating that these transgenic plants are suitable for production of rHSA in the field at large scale. In the literature, performance comparison of greenhouse and field grown plants were done mainly for herbicide resistance transgenic plants. Ramachandran *et al.* (1998) evaluated the performance of selected transgenic herbicide resistance canola lines under greenhouse and field conditions. Tested transgenic lines provided high levels of resistance against diamondback at all growth stages under greenhouse conditions. Transgenic canola also provided excellent control of diamondback moth under field conditions both as a pure stand and in mixtures (Ramachandran *et al.* 1998).

Sijmons *et al.* made the first reported attempt to express rHSA in transgenic plants in 1990, but very low expression levels were obtained (0.02% of TSP). Later in 2002, Farrran *et al.* showed that rHSA was accumulated in the tubers of transgenic potato plants with the highest accumulation level reaching to 0.2% of the tuber soluble protein. Recently, Fernandez-San Millan *et al.* (2003) reported a more efficient method of rHSA production via a novel approach, chloroplast genetic engineering. Modification of HSA regulatory sequences using chloroplast untranslated regions (UTRs) resulted in rHSA accumulation of up to 11.1% of TSP, compensating for excessive proteolytic degradation. This is 500-fold higher than previous reports on rHSA expression upon nuclear transformation (Daniell 2003). However, rHSA accumulated in inclusion bodies, which facilitated purification, but solubilization with Gu-HCl and subsequent refolding was necessary for extraction. No data for functionality of the resolubilised HSA has been reported so far. Since HSA has 17 disulfide-bridges resolubilisation under reducing conditions and subsequent oxidative refolding is not expected to be capable of producing high quantities of correctly folded and biologically active HSA. This is evident from the fact that all platforms currently used for production are eucaryotic systems and this is required to ensure proper disulfide-bridge formation. Therefore, chloroplast expression with equal properties to procaryotic production systems seems not to be a viable option for HSA production.

Estimates by industry suggest that the cost-effective yield for pharmaceutical production is 0.1 mg of HSA per g of fresh weight (Fernandez-San Millan *et al*. 2003). In this study the highest expression level 100 µg rHSA per g leaf material could be achieved in stably transformed tobacco plants and 100 µg rHSA per g in wheat seed, in bombarded wheat embryos. Two different technologies were used in order to generate these transgenic plants. *Agrobacterium* mediated gene transfer has a restricted host range and does not efficiently infect monocots, where for monocots, biolistic delivery of genes is the method in use (Christou 1995).

In conclusion, new techniques for producing transgenic plants will improve the efficiency of the process and will help resolve some of the environmental and health concerns. Among the expected changes are the following:

- More efficient transformation that is a higher percentage of plant cells will successfully incorporate the transgene.
- Better marker genes to replace the usage of antibiotic genes

- Better control of gene expression through more specific promoters, so that the inserted gene will be active only when and where needed.
- Transfer of multi-gene DNA fragments to modify more complex traits.

IV.3.3 Expression of rHSA in plant cell suspension cultures

Culture cells are convenient for the controlled production of heterologous proteins, especially for pharmacological application. Although most interest during the past 15-20 years has focused on microbial and animal cell cultures, plant cells are now considered to be useful systems for large-scale protein production (Lee *et al.* 2002). Plant cell cultures have several advantages compared with microbial and animal cell cultures. They have a reduced risk of mammalian viral contaminants, oncogenes and bacterial toxins, and they can also produce correctly folded proteins and assemble multimeric proteins such as antibodies (Fischer *et al.* 1999).

In this thesis, production of rHSA in plant cells followed by secretion into the culture medium was achieved using transgenic BY2 suspension cell culture. Accumulation levels in the cells varied between 0.4-1.87 µg per g of cells and secretion into the culture supernatant was 0.494-1.84 µg per ml culture medium.

The human milk protein sCD14 was expressed in tobacco BY2 plant cell cultures. Tobacco cells were transformed with either the native human signal peptide (SP) or a plant SP, under the control of the CaMV35S promoter. The protein was detected by western blot analysis at an estimated level of 5 µg per L in a non-soluble fraction of the culture medium (Girard *et al.* 2004).

Expression of rHSA in tobacco suspension culture and secretion by the cultured plant cells into the culture medium was previously shown by Sijmons *et al.* (1990). The construct used for transformation of the tobacco suspension cells contained a fusion of mature HSA with the presequence from the pathogenesis related signal (PR-S) protein from tobacco, which is used as the signal sequence for secretion of HSA across the plant plasma membrane. Recombinant HSA was found to be correctly processed and indistinguishable from the authentic human protein. It was detected in the culture medium at a level of 0.25 µg per mg protein when compared with the cellular HSA concentration in the immunoblot and appeared to represent the majority of the synthesized protein, suggesting almost all of it was secreted (Sijmons *et al.*

1990). The highest rHSA level reported in this work, 1.87 µg per ml culture supernatant is higher than the previously described report of Sijmons *et al*., although in both of the works same CaMV35SS promoter was used. The expression cassettes used differ in two ways (i) tobacco PR-S signal peptide versus plant codon optimized leader peptide derived from murine light chain of TMV (ii) NOS terminator versus termination signal of CaMV35S. Sijmons *et al* in his work compared usage of human and plant signal sequences and found that 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. Therefore these results prove that it was necessary to delete the leader and hexapeptide of authentic HSA prior to cloning into expression vectors.

Foreign proteins produced in plant cell cultures are harvested from plant biomass or from culture supernatant. Despite the dilution of the secreted protein to low concentrations in a relatively large liquid volume, plant cell culture of foreign protein can be advantageous. However, accumulation of the protein in the cell and in the culture medium depends largely on its stability in the extracellular environment and this is of particular importance for proteins with complicated structures like HSA.

During the Second World War, polyvinylpyrrolidone (PVP) was used to dilute the limited blood supply and therefore PVP was analysed as a stabilizer in the tobacco suspension cultures secreting rHSA. PVP with varying molecular weights were added to the HSA-APO29 culture from the first day of subculture. Although 2.5 fold more secretion of rHSA in to the culture medium could be achieved, increase in the stability of the protein was not observed. Smaller truncated forms of rHSA were present in the supernatant of suspension culture cocultivated in the presence or absence of PVP (III.3.5.2). Change in working concentration (0.8 g/L) or addition of PVP at different time intervals was not investigated. The use of protein stabilizing agents such as PVP has been shown to improve antibody production in suspension cultures, suggesting that the medium is the site of antibody degradation (La Count *et al.* 1997). Similarly, studies with bioactive hGM-CSF production in tobacco suspension culture shown that, accumulation of hGM-CSF was drastically reduced during the exponential phase of cell growth. In order to improve the yield of hGM-CSF in tobacco suspension cultures three different polymer stabilizing agents were tested. Addition of PVP (0.05%) and PEG (2%) could not stabilize hGM-CSF and all hGM-CSF was degraded in 1 day. Following gelatine (2%) addition, about 90% of hGM-CSF remained stable up to day 5, however most of the protein was degraded rapidly (Lee *et al.* 2002).

In conclusion, a great advantage of plant cell suspension cultures is that recombinant protein can be produced under certified conditions (GMP, GLP) (Torres *et al.* 1999). Recent improvement in the design of novel promoters and gene targeting will lead to significant improvements in recombinant product yields in plant cell lines that are devoid of noxious compounds. However, media optimisation, improvement of nutrient supply, (Sato *et al.* 1996) feeding of precursors or elicitors, (Yukimune *et al.* 1996) improved fermenter design and agitation conditions (Böhme *et al.* 1997) need to be evaluated to obtain higher productivity during extended use of plant cell suspension cultures in recombinant protein production.

IV.4 Production systems for rHSA, system of choice

Recombinant HSA production has been examined in bacteria, yeast, plant cells and transgenic animals. Among these systems, rHSA production by *Sacharomyces cerevisiae*, *Pichia pastoris* and *Kluyveromyces lactis* are being followed up to develop a commercially viable technology. Amersham Pharmacia Biotech invented a manufacturing process to make HSA by genetic manipulation of *P. pastoris* on a commercial scale. Application for government approval of the process was lodged in 1997. The application was made under the brand name Albrec. Typical production levels in yeast cultures are approximately 150 mg per L.

Yet the rHSA production in plants remains a reasonable strategy in view of the high production volume with low cost (Giddings *et al.* 2000; Hood *et al.* 2002; Fischer *et al.* 2003). The advantages of intact plants lie in the fast biomass build-up, the low cultivation costs and the easy storage and distribution of transgenic seed material. In this work, different transgenic plants and tobacco suspension cultures were tested in terms of their performance in expressing a recombinant human protein. Among these systems, tobacco and wheat were determined to be the best producers for rHSA. In stably transformed tobacco plants, expression levels reached 100 µg rHSA/g leaf material and in bombarded wheat embryos 100 µg rHSA/g wheat seed. Consequently, these two alternative plants represent a viable alternative to mammalian and prokaryotic expression systems for the production of rHSA.

IV.5 Downstream processing

HSA has been used clinically to treat a number of diseases with high dosage. It is currently used in greater volume than any other biopharmaceutical solution that is available (Matejtschuk *et al.* 2000). Extremely pure product is required in large-scale production. Albumin is now predominantly derived from human plasma, although both time-expired blood and in some countries, placental material have been used as sources in the past.

The classic cold ethanol precipitation method developed and it has been used for the production of albumin from human plasma since 1946 (Cohn *et al.* 1946). Since then, some pharmaceutical providers have chosen to supplement this process with additional purification steps (Adcock *et al.* 1998). However, chromatographic purification of HSA has been increasingly studied in the last few years. Application of chromatography, especially ion exchange, affinity, and size-exclusion, has opened a new area in the production of pHSA (plasma derived HSA). Ion exchange chromatography and hydrophobic chromatography play a central role in the purification scheme. Integration with other chromatographic techniques such as size-exclusion, metal chelate, and affinity gives improved purification results.

In this work, a single cation exchange chromatography step resulted in removal of most of the plant proteins. The purification of HSA is challenging since this protein binds to various compounds which makes it difficult to separate contaminants during downstream processing. According to our results (III.4.1), the optimum pH lies in the range of pH 4.4 to 4.6 for cation exchange chromatography using commercially available HSA, the optimum buffer is determined as 0.1 M acetate buffer at pH 4.0 for extraction of rHSA from transgenic tobacco leaves. Besides, using fresh leaves collected from greenhouse and directly processed for extraction resulted in higher amounts of rHSA when compared with the shock frozen leaves. In order to remove contaminating plant proteins, heating of the extract was also evaluated however a huge loss in the recombinant protein was observed and therefore this step was not pursued any further.

In order to verify both a correct processing of the rHSA signal peptide and an N-terminal end identical to native HSA, Farran *et al.* (2002) partially purified rHSA from fresh transgenic potato tubers. Extraction scheme included homogenization in phosphate buffer (pH 7.4), an initial purification using DEAE Seppharose FF column, a linear gradient salt elution, desalting using HiPrep column and affinity purification using Blue Seppharose FF column.

Partial purification achieved this way was sufficient for amino terminal sequencing of the rHSA.

In this study, a single step chromatography was performed by using 700 g of transgenic tobacco leaf material. 22 mg of rHSA could be partially purified. Additional steps like salt precipitation and affinity purification using rHSA itself or different tags must be added to the purification protocol in order to achieve a higher level of purification. Time and cost investments are two important factors to consider in the protocol. It is known that traditional purification of biopharmaceuticals using columns accounts for 30% of the production cost and 70% of the setup cost (Daniell 2003).

Recombinant HSA produced in *P. pastoris* yielded up to 150 mg per L broth of culture supernatant. The rHSA was isolated and purified by hollow-fiber ultrafiltration, Phenyl-Seppharose hydrophobic chromatography and antibody-immunoadsorbent chromatography. And finally rHSA was purified to electrophoretic purity (Qiu *et al.* 2000). Kobayashi *et al.* (1998) used *Pichia* host system as well. Fermentation process involved methanol feeding for the secretion of rHSA into the culture broth. After optimizing the physiological and environmental aspects of the fermentation, the production level of rHSA exceeded 10 g rHSA per L (Kobayashi *et al.* 1998). The purification process employed STREAMLINE™ expanded bed adsorption for direct capture of the target product. Recombinant HSA was adsorbed to the column, while yeast cells and impurities passed through.

A combined method, whereby chromatographic purification steps supplement the cold ethanol fractionation process has been adopted by a number of manufacturers for blood plasma derived HSA. Addition of a single anion exchange chromatography by preparation of the product Zenalb® markedly improved the quality of the product. Similarly, using transgenic tobacco plants different polishing approaches like gel filtration or anion exchange chromatography needs to be further investigated. Optimum design of these steps will lead to a recombinant product which can then be tested for various clinical tests.

IV.6 Conclusions and future perspectives

Plants have many advantages compared with traditional systems for the molecular farming of pharmaceutical proteins. These include the low cost of production, rapid scalability, the absence of human pathogens and the ability to fold and assemble complex proteins accurately (Ma *et al.* 2003).

In this work it could be shown that rHSA can be produced in different organisms including bacteria, plants and plant cell suspension cultures with varying production levels. Estimates by industry suggest that the cost-effective yield for pharmaceutical production in plants is 0.1 mg of rHSA per g of fresh weight (Fernandez-San Millan et al. 2003). This level was achieved when rHSA was expressed stably in the ER of *N. tabacum* cv. Petite Havana SR1 plants. In recent experiments we have transformed the tobacco cultivar Maryland Mammoth that produces more biomass per hectare than Petite Havana SR1. Transient expression analyses showed that rHSA levels were comparable to that obtained in the cultivar Petite Havana SR1. Currently, stable transformation of this line is ongoing. These levels could be improved by selfing and crossing elite plant lines. It has been demonstrated that recombinant protein levels can be significantly increased using these breeding procedures (Zimmermann et al. 1998; Hood et al. 2002). In addition to tobacco plants, wheat seeds are determined as an alternative high- expression system and further advantage is the easy storage of the seed kernels for prolonged time. Transgenic wheat seeds accumulating rHSA were stored for more than 12 months without the significant loss of product yield.

Recent approvals for the commercialization of GM crops containing antibiotic resistance markers have raised concern in Europe about the risk of spreading antibiotic resistance genes to previously unsusceptible microbes and hence making them resistant to antibiotics used. As a consequence governments in the European Union have recommended “phasing out” GM crops containing antibiotic resistance markers by 2005 (web site http://biosafety.ihe.be/ARGMO/Documents/EFB_AntibioticRM_English.pdf). Therefore in order to commercialise rHSA expressed in a host organism, marker free crops should be generated. For this purpose design of a marker free system has been carried out and a project for the expression of this protein is ongoing.

In this thesis it could be also shown that an alternative production technology is the use of plant cells followed by secretion into the culture medium. Fermentations of transgenic tobacco cell lines were performed at 5 L working volume and in batch fermentation. It has been shown that the system is productive for more than 30 days. Improvement of the batch and continued fermentation system parameters are in progress in order to have superior accumulation of rHSA in the culture medium.

Independent of the expression system of choice, downstream processing of the protein should be carried in the most cost-effective way with highest percent recovery. Experiments carried out during this study proved the importance of optimising the purification conditions. It has

been shown that with proper extraction conditions, a single cation exchange chromatography step led to removal of most of the plant proteins.

Further improvement in the purification strategies is necessary in order to efficiently purify rHSA from the host organism. Purified rHSA can be than evaluated for structural identity and function by comparing its biochemical properties to the human counterpart. Biochemical characterization could be done using by fluorometric methods or by surface plasmon resonance technology in order to study known interactions between various drugs (e.g. warfarin, salicylate ion, and bilirubin) and rHSA.

V Summary

Human serum albumin (HSA) is the most abundant protein in the circulatory system. The principal function of HSA is to transport fatty acids, but it is also capable of binding a great variety of metabolites and drugs. The potential of HSA in various applications, like usage as a plasma expander, stabilizing agent, drug delivery protein and as an adjunct in insulin action prolongation demands a great supply of pure HSA. Presently HSA is manufactured from the blood pooled from commercial blood donors. There is no guarantee regarding contamination of the product by viral pathogens and prions, causing dreadful diseases. The annual world need exceeds 500 tons, representing a market value of more than 1.5 billion US$ (Fernandez-San Millan *et al.* 2003). Considering the amount of plasma expanders required in critically ill patients, the use of albumin represents a significant cost (Nicholson *et al.* 2000).

Such a huge demand can not longer be met by merely purifying HSA from original sources, as the supply of human blood is limited. Alternative ways to obtain HSA are 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/by infusion of products derived of contaminated blood.

The initial attempt was to generate a novel plant codon optimised semi-synthetic HSA gene by PCR amplification. The strategy was to eliminate the rare codons that are used at low frequencies in the plant host system resulting in an optimized codon usage pattern for high-level expression of recombinant HSA (rHSA). Sequence analysis revealed the presence of high number mutations in the final product, including insertions and deletions of several bases which could not be eliminated.

The main focus of the work was to establish an alternative expression system for the production of the authentic HSA cDNA. Further analysis included expression and characterization of rHSA in *E. coli*, transient expression in leaves of tobacco, zucchini and bean, stable expression in (intact) tobacco and wheat plants and tobacco cell suspension culture. For bacterial expression of rHSA, cytoplasmic and periplasmic expression vectors were generated. After verifying expression in *E. coli*, purification of the recombinant protein was carried out. Tobacco, zucchini and bean host systems were assessed for expression of rHSA. No difference in transient accumulation levels were observed when the protein was either secreted to the apoplast or targeted to endoplasmic reticulum (ER). Further analysis

included stable transformation of tobacco plants via *Agrobacterium* mediated gene transfer. Accumulation of rHSA in the ER and secretion into the apoplast were confirmed by immunoblot analysis. In parallel to the expression studies in intact plants, tobacco suspension cultures were initiated and both intracellular and secreted protein could be detected in batch culture of the transgenic BY2 cells. Protein stability following secretion could be improved with the addition of appropriate chemical agents (stabilizers) to the growth media. The results achieved by all expression systems and their comparison were performed.

Finally downstream processing of the rHSA from transgenic tobacco leaves was investigated. Conditions for an efficient extraction of the recombinant protein from plant system were optimised and cation exchange chromatography to partially purify rHSA was carried out.

VI References

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VII Appendices

VII.1 Abbreviations

ABTS	2,2'-Azino-di-(3-ethylbenzthiazoline sulphonate) diamonium salt
Ab	Antibody
Ag	Antigen
Amp	Ampicillin
AP	Alkaline phosphatase
BCIP	5-Bromo-4-Chloro-3-Indolyl-Phosphat
bp	Base pair
BSA	Bovine serum albumin
CaCl2	Calcium chloride
CaMV	Cauliflower mosaic virus
Carb	Carbenicillin
cDNA	Complementary DNA
CHS	Chalchone synthase
CI	Chloroform/isoamyl alcohol (24:1)
Cys	Cysteine
cv.	Cultivar
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleoside triphosphate
DTT	1, 4-dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
ER	Endoplasmic reticulum

EtOH	Ethanol
EtBr	Ethidium bromide
Fc	Fragment crystalline
g	Relative centrifugal force (RCF)
GAM	Goat anti-mouse antibody
GAM^{AP}	Alkaline phosphatase conjugated goat anti mouse antibody
GAR	Goat anti-rabbit antibody
GSM	*Agrobacteria* glycerol stock media
h	Hour(s)
HCl	Hydrochloric acid
his	Histidine
HSA	Human serum albumin
IEF	Isoelectric focusing
IPTG	Isopropyl B-D-thiogalactopyranoside
IMAC	Immobilized metal ion affinity chromatography
iv	intravenous
kb	Kilobase pair
kDa	Kilodalton
KDEL	KDEL (Lys-Asp-Glu-leu) ER retention signal
Km	Kanamycin
λ	Lambda
L	Liter
μ	Micro
m	Mili
M	Molar
$MgSO_4$	Magnesium sulphate
MES	2-(N-morpholino)-ethanesulphonic acid
min	Minute(s)

MPBS	Non-fat skim milk powder in PBS
MgSO4	Magnesium sulphate
MOPS	3-(morpholino)propanesulfonic acid
mRNA	Messenger RNA
Mw	Molecular weight
N	nano
nd.	not detectable
nt	nucleotide
NaCl	Sodium chloride
NBT	p-nitro blue tetrazolium chloride
Ω	Ohm
OD	Optical density
o/n	Overnight
ORF	Open reading frame
Ori	Origin of replication
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	0.1% (v/v) Tween-20 in PBS
PCI	Phenol/chloroform/isoamyl alcohol (25:24:1)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	A logarithmic measure of hydrogen ion concentration
pNPP	para-nitrophenyl phosphate
poly A	Polyadenylation signal
PVP	Polyvinyl pyrrolidone
RAC	rabbit anti chicken antibodies
rHSA	recombinant human serum albumin

rif	Rifampicilin
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Rounds per minute
RT	Reverse transcription
sec	second(s)
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA
TEMED	N, N, N, N-tetramethylene-ethylenediamine
Tris	Tris (Hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan monolaureate
UTR	untranslated region
UV	Ultraviolet
Vol.	volume
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight

VII.2 List of primers used for the amplification of plant codon optimised HSA

HSAmod1-16 as forward primers and HSAmod7-32 as backward primers were used in the reaction mix. Forward and backward primers have complementary sequences differing in length of 21-24 nucleotides. Schematic representation of arrangement of primers for SOE PCR of plant codon optimised HSA are given in Figure III.2.

HSAmod1

5’ –GCG GAA TTC CAT GGA TGC ACA CAA GAG CGA GGT TGC TCA CCG CTT TAA AGA TTT G –3’

HSAmod2

5’ –GTG AAA TTG GTG AAT GAA GTC ACT GAA TTT GCA AAA ACC TGC GTG GCT GAT GAG TCC GCT GAA AAT TGC GAC –3’

HSAmod3

5’ –GGA GAC AAA TTG TGC ACC GTT GCA ACT CTT CGC GAA ACC TAC GGT GAA ATG –3’

HSAmod4

5’ –CAA GAA CCT GAG AGG AAT GAA TGC TTC –3’

HSAmod5

5’ –GCT TTT CAC GAC AAT GAA GAG ACC TTT TTG AAA AAA TAC TTG TAC GAA ATT GCC AGG AGG CAC CCT TAC –3’

HSAmod6

5’ –TTT TAC GCC CCG GAA CTC CTT TTC TTT GCT AAA AGG TAC AAA GCT GCT TTT ACC GAA TGC TGC CAA GCT G –3’

HSAmod7

5’ –CTC AAA TGC GCC AGC CTC CAA AAA TTT GGA GAA AGG GCT TTC AAA GCA TGG GCA GTG GCT CGC CTG AGC –3’

HSAmod8

5’ –TTG CAG AAG TTT CCA AGT TGG TGA CCG ATC TTA CCA AAG TCC ACA CCG AAT GCT GCC ACG GAG ATC TGC TT –3’

HSAmod9

5’ –ACC TTG CCA AGT ACA TCT GCG AAA ATC AGG ATT CCA TCT CCA GCA AAC TGA AGG AAT GCT GCG AAA AAC CTC TG –3’

HSAmod10

5’ –GCA AAA ACT ACG CTG AGG CAA AGG ATG TCT TCC TGG GCA TGT TTT TGT ACG AAT ACG CAA GGA GGC ACC CT –3’

HSAmod11

5’ –CTT GCC AAG ACC TAC GAA ACC ACT CTG GAG AAG TGC TGC GCC GCT GCA GAT CCT CAC GAA TGC TAC GCC AAA GTG TTC –3‘

HSAmod12

5’ –GAG AGT ACA AAT TCC AGA ATG CGC TCT TGG TTC GCT ACA CCA AGA AAG TGC CCC AAG TGT CCA CTC CAA CTC –3’

HSAmod13

5’ –GAA AAG TGG GCA GCA AAT GCT GCA AAC ACC CTG AAG CAA AAA GGA TGC CCT GCG CAG AAG ACT ACC TCT CC –3’

HSAmod14

5’ –GCA CGA GAA AAC CCC AGT GAG CGA CAG GGT CAC CAA ATG CTG CAC CGA GTC CTT GGT GAA CAG GCG CCC A –3’

HSAmod15

5’ –CCC AAA GAG TTT AAT GCT GAA ACC TTC ACC TTC CAC GCA GAT ATC TGC ACC CTT TCT GAG AAG GAG AGG CAA ATC AAG AAA C –3’

HSAmod16

5’ –AAA GCT GTT ATG GAT GAT TTC GCA GCT TTT GTG GAG AAG TGC TGC –3’

HSAmod17

5’ –GCC TTC GAA CAG CTG CAA GCC CAA GGC AGC TTG GCT TGC AGC AAC AAG –3’

HSAmod18

5’ –TGC GAA ATC ATC CAT AAC AGC TTT CAG TTG CTC TTT GGT TGC CTT GGG C –3’

HSAmod19

5’ –GTT TCA GCA TTA AAC TCT TTG GGA ACG TAG GTT TCA TCG ACT TCC AGA GCG GAA AAG CAT GGG CGC CTG TTC ACC AAG GAG TC –3’

HSAmod20

5’ –GCT CAC TGG GGT TTT CTC GTC CAA CAC GCA CAA CTG GTT CAG GAC CAC GGA GAG GTA GTC TTC TGC GCA G –3’

HSAmod21

5’ –GCA GCA TTT GCT GCC CAC TTT TCC GAG GTT CCT GGA GAC CTC CAC AAG AGT TGG AGT GGA CAC TTG GGG –3’

HSAmod22

5’ –CGC ATT CTG GAA TTT GTA CTC TCC AAG CTG CTT AA AAG CTC GCA GTT TTG TTT GAT CAA ATT CTG AGG C –3’

HSAmod23

5’ –AGT GGT TTC GTA GGT CTT GGC AAG CCT CAG CAG CAG CAC GAC AGA GTA ATC AGG GTG CCT CCT TGC GTA TTC GTA –3’

HSAmod24

5’ –CTT TGC CTC AGC GTA GTT TTT GCA AAC ATC CTT GCT TTC AAC AAA ATC AGC AGC CAA GGA AGG CAA GTC AG –3’

HSAmod25

5’ –TTC GCA GAT GTA CTT GGC AAG GTC CGC CCT GTC ATC AGC GCA TTC AAG CAG ATC TCC GTG GCA GCA TTC –3’

HSAmod26

5’ –CAC CAA CTT GGA AAC TTC TGC AAA CTC AGC TTT GGG AAA CCT CTG GCT CAG GCG AGC CAC TGC CCA TGC –3’

HSAmod27

5’ –TTT TTG GAG GCT GGC GCA TTT GAG CCT CTG TTT GGC AGA GGA AGC CTT CCC TTC ATC GCG AAG TTC ATC G –3’

HSAmod28

5’ –AAG GAG TTC CGG GGC GTA AAA GTA AGG GTG CCT CCT GGC AAT TTC –3’

HSAmod29

5’ –GGT CTC TTC ATT GTC GTG AAA AGC AGT GCA CAT CAC ATC AAC CTC TGG CCT CAC CAA GCG GGG GAG GTT TG –3’

HSAmod30

5’ –GCA TTC ATT CCT CTC AGG TTC TTG TTT TGC GCA GCA GTC AGC CAT TTC ACC GTA GGT TTC GCG AAG –3’

HSAmod31

5’ –TGC AAC GGT GCA CAA TTT GTC TCC AAA AAG GGT GTG AAG AGA TTT GTC GCA ATT TTC AGC GGA CTC ATC –3’

HSAmod32

5’ –AGT GAC TTC ATT CAC CAA TTT CAC GTG ATC TTC AAA TGG GCA CTG CTG AAG GTA CTG AGC AAA GG –3’

VII.3 Rare codon distribution in HSA sequence

1 ATG AAG TGG **GTA** ACC TTT ATT TCC CTT CTT TTT CTC TTT AGC **TCG** GCT **TAT** TCC AGG
GGT GTG TTT **CGT CGA** GAT GCA CAC AAG **AGT** GAG 90

91 GTT GCT **CAT CGG** TTT AAA GAT TTG GGA GAA GAA AAT TTC AAA GCC TTG GTG TTG ATT
GCC TTT GCT CAG **TAT** CTT CAG CAG **TGT** CCA TTT 180

181 GAA GAT **CAT GTA** AAA **TTA** GTG AAT GAA **GTA** ACT GAA TTT GCA AAA **ACA TGT GTA**
GCT GAT GAG **TCA** GCT GAA AAT **TGT** GAC AAA **TCA** CTT 270

271 **CAT** ACC CTT TTT GGA GAC AAA **TTA** TGC **ACA** GTT GCA ACT CTT **CGT** GAA ACC **TAT**
GGT GAA ATG GCT GAC TGC **TGT** GCA AAA CAA GAA CCT 360

361 GAG **AGA** AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA AAC CTC CCC **CGA**
TTG GTG **AGA** CCA GAG GTT GAT GTG ATG TGC ACT GCT 450

451 TTT **CAT** GAC AAT GAA GAG **ACA** TTT TTG AAA AAA TAC **TTA TAT** GAA ATT GCC **AGA**
AGA CAT CCT TAC TTT **TAT** GCC CCG GAA CTC CTT TTC 540

541TTT GCT AAA AGG **TAT** AAA GCT GCT TTT **ACA** GAA **TGT** TGC CAA GCT GCT GAT AAA GCT
GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT **CGG** 630

631GAT GAA GGG AAG GCT **TCG** TCT GCC AAA CAG **AGA** CTC AAA **TGT** GCC **AGT** CTC CAA
AAA TTT GGA GAA **AGA** GCT TTC AAA GCA TGG GCA GTG 720

721 GCT CGC CTG AGC CAG **AGA** TTT CCC AAA GCT GAG TTT GCA GAA GTT TCC AAG **TTA** GTG
ACA GAT CTT ACC AAA GTC CAC **ACG** GAA TGC TGC 810

811 **CAT** GGA GAT CTG CTT GAA **TGT** GCT GAT GAC AGG GCG GAC CTT GCC AAG **TAT** ATC
TGT GAA AAT CAG GAT **TCG** ATC TCC **AGT** AAA CTG AAG 900

901 GAA TGC **TGT** GAA AAA CCT CTG TTG GAA AAA TCC CAC TGC ATT GCC GAA GTG GAA
AAT GAT GAG ATG CCT GCT GAC TTG CCT **TCA TTA** GCT 990

991 GCT GAT TTT GTT GAA **AGT** AAG GAT GTT TGC AAA AAC **TAT** GCT GAG GCA AAG GAT
GTC TTC CTG GGC ATG TTT TTG **TAT** GAA **TAT** GCA **AGA** 1080

1081 AGG **CAT** CCT GAT TAC TCT GTC GTG CTG CTG CTG **AGA** CTT GCC AAG **ACA TAT** GAA
ACC ACT **CTA** GAG AAG TGC **TGT** GCC GCT GCA GAT CCT 1170

1171 **CAT** GAA TGC **TAT** GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT GTG GAA GAG CCT CAG AAT **TTA** ATC AAA CAA AAC **TGT** GAG CTT TTT AAG 1260

1261 CAG CTT GGA GAG TAC AAA TTC CAG AAT GCG **CTA TTA** GTT **CGT** TAC ACC AAG AAA **GTA** CCC CAA GTG **TCA** ACT CCA ACT CTT **GTA** GAG GTC 1350

1351 **TCA AGA** AAC **CTA** GGA AAA GTG GGC AGC AAA **TGT TGT** AAA **CAT** CCT GAA GCA AAA **AGA** ATG CCC **TGT** GCA GAA GAC **TAT CTA** TCC GTG GTC 1440

1441 CTG AAC CAG **TTA TGT** GTG TTG **CAT** GAG AAA **ACG** CCA **GTA AGT** GAC **AGA** GTC **ACA** AAA TGC TGC **ACA** GAG TCC TTG GTG AAC AGG **CGA** CCA 1530

1531 TGC TTT **TCA** GCT CTG GAA GTC GAT GAA **ACA** TAC GTT CCC AAA GAG TTT AAT GCT GAA **ACA** TTC ACC TTC **CAT** GCA GAT **ATA** TGC **ACA** CTT 1620

1621 TCT GAG AAG GAG **AGA** CAA ATC AAG AAA CAA ACT GCA CTT GTT GAG CTT GTG AAA CAC AAG CCC AAG GCA **ACA** AAA GAG CAA CTG AAA GCT 1710

1711 GTT ATG GAT GAT TTC GCA GCT TTT **GTA** GAG AAG TGC TGC AAG GCT GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG GGT AAA AAA CTT GTT 1800

1801 GCT GCA **AGT** CAA GCT GCC **TTA** GGC **TTA** TAA ***

VII.4 Comparison of authentic and plant codon optimised HSA sequence

The sequence represented as HSA illustrates the original HSA sequence, whereas HSAmod represents the sequence that was designed in order to optimize the codon usage for expression in tobacco, wheat and rice plants.

```
HSA         gatgcacacaagagtgaggttgctcatcggtttaaagatttgggagaagaaaatttcaaa
            ||||||||||||||| |||||||||||  || ||||||||||||||||||||||||||||
HSAmod.     gatgcacacaagagcgaggttgctcaccgctttaaagatttgggagaagaaaatttcaaa

HSA         gccttggtgttgattgcctttgctcagtatcttcagcagtgtccatttgaagatcatgta
            |||||||||||||||||||||||||||||| |||||||||||| |||||||||||||| ||
HSAmod.     gccttggtgttgattgcctttgctcagtaccttcagcagtgcccatttgaagatcacgtg

HSA         aaattagtgaatgaagtaactgaatttgcaaaaacatgtgtagctgatgagtcagctgaa
            ||||| |||||||||||| |||||||||||||||| || || |||||||||||| ||||||
HSAmod.     aaattggtgaatgaagtcactgaatttgcaaaaacctgcgtggctgatgagtccgctgaa
```

```
HSA         aattgtgacaaatcacttcatacccttttttggagacaaattatgcacagttgcaactctt
            ||||| |||||||| ||||| |||||||||||||||||||| ||||| ||||||||||||
HSAmod.     aattgcgacaaatctcttcacacccttttttggagacaaattgtgcaccgttgcaactctt

HSA         cgtgaaacctatggtgaaatggctgactgctgtgcaaaacaagaacctgagagaaatgaa
            || |||||||| |||||||||||||||||||| |||||||||||||||||||| ||||||
HSAmod.     cgcgaaacctacggtgaaatggctgactgctgcgcaaaacaagaacctgagaggaatgaa

HSA         tgcttcttgcaacacaaagatgacaacccaaacctcccccgattggtgagaccagaggtt
            |||||||||||||||||||||||||||||||||||||||||| |||||||| |||||||||
HSAmod.     tgcttcttgcaacacaaagatgacaacccaaacctcccccgcttggtgaggccagaggtt

HSA         gatgtgatgtgcactgcttttcatgacaatgaagagacatttttgaaaaaatacttatat
            |||||||||||||||||||||| |||||||||||||| |||||||||||||||||| ||
HSAmod.     gatgtgatgtgcactgcttttcacgacaatgaagagacctttttgaaaaaatacttgtac

HSA         gaaattgccagaagacatccttacttttatgccccggaactccttttctttgctaaaagg
            |||||||||||| || || |||||||||||| |||||||||||||||||||||||||||||
HSAmod.     gaaattgccaggaggcacccttacttttacgccccggaactccttttctttgctaaaagg

HSA         tataaagctgcttttacagaatgttgccaagctgctgataaagctgcctgcctgttgcca
            || |||||||||||||| ||||| |||||||||||||||||||||||||||||||||||
HSAmod.     tacaaagctgcttttaccgaatgctgccaagctgctgataaagctgcctgcctgttgcca

HSA         aagctcgatgaacttcgggatgaagggaaggcttcgtctgccaaacagagactcaaatgt
            |||||||||||||||| |||||||||||||||| |||||||||||||| ||||||||
HSAmod.     aagctcgatgaacttcgcgatgaagggaaggcttcctctgccaaacagaggctcaaatgc

HSA         gccagtctccaaaaatttggagaaagagctttcaaagcatgggcagtggctcgcctgagc
            ||||| |||||||||||||||||| |||||||||||||||||||||||||||||||||||
HSAmod.     gccagcctccaaaaatttggagaaagggctttcaaagcatgggcagtggctcgcctgagc

HSA         cagagatttcccaaagctgagtttgcagaagtttccaagttagtgacagatcttaccaaa
            ||||| |||||||||||||||||||||||||||||||||| ||||| ||||||||||||
HSAmod.     cagaggtttcccaaagctgagtttgcagaagtttccaagttggtgaccgatcttaccaaa

HSA         gtccacacggaatgctgccatggagatctgcttgaatgtgctgatgacagggcggacctt
            |||||||| |||||||||| |||||||||||||||| ||||||||||||||||||||||
HSAmod.     gtccacaccgaatgctgccacggagatctgcttgaatgcgctgatgacagggcggacctt

HSA         gccaagtatatctgtgaaaatcaggattcgatctccagtaaactgaaggaatgctgtgaa
            |||||||| ||||| |||||||||||||| |||||||| |||||||||||||||| |||
HSAmod.     gccaagtacatctgcgaaaatcaggattccatctccagcaaactgaaggaatgctgcgaa

HSA         aaacctctgttggaaaaatcccactgcattgccgaagtggaaaatgatgagatgcctgct
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HSAmod.     aaacctctgttggaaaaatcccactgcattgccgaagtggaaaatgatgagatgcctgct

HSA         gacttgccttcattagctgctgattttgttgaaagtaaggatgtttgcaaaaactatgct
            |||||||||||| || |||||||||||||||||| |||||||||||||||||| |||
HSAmod.     gacttgccttccttggctgctgattttgttgaaagcaaggatgtttgcaaaaactacgct

HSA         gaggcaaaggatgtcttcctgggcatgtttttgtatgaatatgcaagaaggcatcctgat
            |||||||||||||||||||||||||||||||| ||||| ||||| ||||| ||||||
HSAmod.     gaggcaaaggatgtcttcctgggcatgtttttgtacgaatacgcaaggaggcaccctgat

HSA         tactctgtcgtgctgctgctgagacttgccaagacatatgaaaccactctagagaagtgc
            |||||||||||||||||||||| |||||||||| || |||||||||| |||||||||
HSAmod.     tactctgtcgtgctgctgctgaggcttgccaagacctacgaaaccactctggagaagtgc
```

```
HSA         tgtgccgctgcagatcctcatgaatgctatgccaaagtgttcgatgaatttaaacctctt
            || |||||||||||||||| |||||||| ||||||||||||||||||||||||||||||
HSAmod.     tgcgccgctgcagatcctcacgaatgctacgccaaagtgttcgatgaatttaaacctctt

HSA         gtggaagagcctcagaatttaatcaaacaaaactgtgagcttttaagcagcttggagag
            |||||||||||||||||||| |||||||||||||| ||||||||||||||||||||||||
HSAmod.     gtggaagagcctcagaatttgatcaaacaaaactgcgagcttttaagcagcttggagag

HSA         tacaaattccagaatgcgctattagttcgttacaccaagaaagtaccccaagtgtcaact
            |||||||||||||||||||| || ||||| |||||||||||||| |||||||||| |||
HSAmod.     tacaaattccagaatgcgctcttggttcgctacaccaagaaagtgccccaagtgtccact

HSA         ccaactcttgtagaggtctcaagaaacctaggaaaagtgggcagcaaatgttgtaaacat
            |||||||||| |||||||| || ||||| |||||||||||||||||||| || |||||
HSAmod.     ccaactcttgtggaggtctccaggaacctcggaaaagtgggcagcaaatgctgcaaacac

HSA         cctgaagcaaaaagaatgccctgtgcagaagactatctatccgtggtcctgaaccagtta
            |||||||||||||| |||||||| |||||||||| || ||||||||||||||||||||
HSAmod.     cctgaagcaaaaaggatgccctgcgcagaagactacctctccgtggtcctgaaccagttg

HSA         tgtgtgttgcatgagaaaacgccagtaagtgacagagtcacaaaatgctgcacagagtcc
            || |||||||| |||||||| ||||| || ||||| ||||| |||||||||| ||||||
HSAmod.     tgcgtgttgcacgagaaaaccccagtgagcgacagggtcaccaaatgctgcaccgagtcc

HSA         ttggtgaacaggcgaccatgcttttcagctctggaagtcgatgaaacatacgttcccaaa
            |||||||||||||| |||||||||| |||||||||||||||||| ||||||||||||
HSAmod.     ttggtgaacaggcgcccatgcttttccgctctggaagtcgatgaaacctacgttcccaaa

HSA         gagtttaatgctgaaacattcaccttccatgcagatatatgcacactttctgagaaggag
            |||||||||||||||| |||||||||| |||||||| ||||| ||||||||||||||||
HSAmod.     gagtttaatgctgaaaccttcaccttccacgcagatatctgcaccctttctgagaaggag

HSA         agacaaatcaagaaacaaactgcacttgttgagcttgtgaaacacaagcccaaggcaaca
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
HSAmod.     aggcaaatcaagaaacaaactgcacttgttgagcttgtgaaacacaagcccaaggcaacc

HSA         aaagagcaactgaaagctgttatggatgatttcgcagcttttgtagagaagtgctgcaag
            |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
HSAmod.     aaagagcaactgaaagctgttatggatgatttcgcagcttttgtggagaagtgctgcaag

HSA         gctgacgataaggagacctgctttgccgaggagggtaaaaaacttgttgctgcaagtcaa
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
HSAmod.     gctgacgataaggagacctgctttgccgaggagggtaaaaaacttgttgctgcaagccaa

HSA         gctgccttaggcttataa
            |||||||| ||||| |||
HSAmod.     gctgccttgggcttgtaa
```

VII.5 Figures

VII.6 Tables

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Lebenslauf

Persönliche Daten

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1989-1993	Studium der Biologie an der Orta Dogu Teknik Universität in Ankara Türkei. Abschluß: Bachelor of Science.
1993-1996	Studium der Biotechnologie an der Orta Dogu Teknik Universität in Ankara Türkei. Abschluß: Master of Science. Thema der Arbeit: “Effect of auxin-like plant growth regulators on salt stressed wheat varieties”
1996-1998	Promotion am der Orta Dogu Teknik Universität in Ankara, Türkei.
Seit 1998	Wechsel für Promotion am Institut für Biologie VII (Molekulare Biotechnologie) der RWTH Aachen unter Leitung von Prof. Dr. Rainer Fischer.

Berufstätigkeit

1994-1998	Research assistant in Biochemistry und Teaching assistant in Genetics Laboratory an der Orta Dogu Teknik Universität in Ankara, Türkei.
1998-2002	Wissenschaftliche Mitarbeiterin am Institut für Biologie VII (Molekulare Biotechnologie) der RWTH Aachen.
seit 2003	Wissenschaftliche Angestellte am Institut für Klinische Chemie und Pathobiochemie sowie Klinische-Chemisches Zentrallaboratorium der Universitätsklinikum Aachen.