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Development of New Platforms for Efficient Production and Purification of Recombinant Protein in Higher Plants

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DEVELOPMENT OF NEW PLATFORMS FOR EFFICIENT PRODUCTION AND PURIFICATION OF RECOMBINANT PROTEIN IN HIGHER PLANTS

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ABSTRACT

The world-wide demand for recombinant proteins continuously increases as new medical and industrial applications are developed. Higher plants have the potential to help meet this rising demand as green bioreactors. A major hurdle, however, is low recombinant protein yields in higher plants and, as with many production systems, high cost associated with downstream purification and the production of short peptides. The goal of this research was to address each of these problems to further increase the utility of plant bioreactors.

The short antigenic peptide 277 (p277) from heat shock protein 60, which has the potential for use in the prevention of type 1 diabetes, was fused to an known adjuvant, the non-toxic B subunit of cholera toxin (CTB). The fused CTB-p277 retained the ability to form a homo-tetramer, and bound to GM_1 ganglioside, allowing for the oral delivery of the fusion protein to induce mucosal tolerance and prevention of diabetes.

Protein yield increases can be achieved by either increasing overall plant yield or increasing the relative accumulation of the desired protein. Using RNA interference, a knockdown of *Solanum tuberosum* plastidic ATP/ADP transporter, involved in energy transport into heterotrophic plastids, resulted in a 30% increase in tuber biomass, as well as a two-fold increase in soluble protein content. In addition, expression of a monoclonal antibody in the knockdown line produced double the concentration of antibody per soluble protein compared to wild-type. Taken together, this is a 4-fold yield increase compared to wild-type.

Protein purification accounts for a significant portion of its production cost. We developed the recombinant production of soybean agglutinin (SBA) for potential use as an

affinity tag. SBA was purified to high quality using an agarose-N-acetyl-D-galactosamine column, resulting in a 1-step purification process. Recombinant SBA performed identically to native SBA during *in vitro* assays, including agglutination of red blood cells (RBC). A fusion of SBA with green fluorescent protein (GFP) resulted in SBA-GFP that retained its *in vivo* fluorescence, purification through a 1-step process as well as the ability to agglutinate RBC and *in vitro* fluorescence of the agglutinated cells.

Keywords: ATP/ADP transporter, soybean agglutinin, p277, heat shock protein 60, green bioreactor, cholera toxin b subunit, recombinant protein production, higher plants, tobacco, potato

CO-AUTHORSHIP

The following thesis contains materials from previously published manuscripts as well as manuscripts submitted for publication, which are co-authored by Reynald Tremblay, Xiaofeng Wang, Hong Diao, Mary Feng, Rima Menassa, Anthony Jevnikar, Norman Hüner and Shengwu Ma

Xiaofeng Wang assisted with the GM1-ganglioside ELISA to confirm CTB function (Chapter 5, figure 5.5b)

Hong Diao generated the wild-type potato plants expressing the monoclonal antibody and assisted in quantification of protein expression (Chapter 2, figure 2.6d,e)

Mary Feng provided assistance for the imaging of hemagglutination (Chapter 3, figure 3.6a,b)

Rima Menassa and Anthony Jevnikar provided insight and assisted with the preparation of the manuscripts (R.M. Chapter 3, A.J. Chapters 2-5)

My supervisors Norman Hüner and Shengwu Ma provided insight and assisted with the preparation of the manuscripts (N.H. Chapters 2-4, S.M. Chapters 2-5)

DEDICATION

To my loving wife Laura, who has walked this journey with me and supported me throughout my life, and my son Jacob, who makes it all worthwhile.

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AATP1	ATP/ADP transporter
AGPase	ADP-glucose pyrophosphorylase
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
CONA	concanavalin A
CT	cholera toxin
CTB	cholera toxin β-subunit
CTB-p277	cholera toxin β-subunit fused with peptide 277 of heat shock protein 60
DAI	days after inoculation
EAE	experimental allergic encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ELP	elastin-like polypeptide
Fab	fragment-antigen binding region
GAD65	glutamic acid decarboxylase 65
GAD67	glutamic acid decarboxylase 67
GFP	green fluorescent protein
GI	gastro-intestinal
GOI	gene of interest
His	Histidine
HIV	human immunodeficiency virus
HRP	horseradish peroxidase

HSP60	heat shock protein 60
IgG	immunoglobulin
IL-4	interleukin 4
INS	insulin
MBP	myelin basic protein
MHC	major histone complex
Nb	*Nicotiana benthamiana*
NOD	non-obese diabetic
Nos	noplaline synthase
NTT1	nucleotide transporter 1
p277	peptide 277 fragment of heat shock protein 60
PKPI	potato kunitz-type proteinase inhibitor
RBC	red blood cells
riAATP1	RNAi inhibited ATP/ADP transporter
rSBA	recombinant soybean agglutinin
rSBA-GFP	recombinant soybean agglutinin fused with green fluorescent protein
SARS	severe acute respiratory syndrome
SBA	soybean agglutinin
scFv	single-chain variable fragment
SEM	standard error measurement
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
St	*Solanum tuberosum*

T1DM	type I diabetes mellitus
TBS-T	tris buffered saline with tween 20
TEV	tobacco etch virus
TSP	total soluble protein
UTL	untranslated leader
UTR	untranslated region
Wt	wild-type

CHAPTER 1

GENERAL INTRODUCTION

1.1 Plants as green bioreactors

There is ever increasing demands for recombinant proteins for use in biomedical research, industrial production and for academic investigation. Traditional recombinant production systems, such as both prokaryotic and eukaryotic cell culture, have limitations due in large part to scalability, drastically increasing the cost of production as demands rise. In addition, there are a number of host platform-specific limitations, including pathogen contamination (mammalian cell culture) and lack of post-translation modification (prokaryotic reactors). Plants may offer the solution as green bioreactors, offering a variety of advantages such as nearly unlimited scalability, from small scale trials in growth chambers to large open-field mass production, and all at relatively inexpensive cost. Additionally, as eukaryotes, plants are capable of a suite of post-translational modifications such as glycosylation and disulphide bridging, and have enhanced biosafety over production in mammalian cell culture systems, as there are no known cross-kingdom pathogens (Tremblay et al. 2010b). For biomedical therapeutics, plants also offer the potential for oral delivery of minimally processed tissue, drastically reducing the cost of treatments (Arakawa et al. 1998; Ma et al. 1997; Ploix et al. 1999). The range of products produced in plants to date is extensive, with everything from monoclonal antibodies to treat human diseases to cellulose digesting enzymes for biofuel production (McCormick et al. 1999; Hood et al. 2007; Floss et al. 2008; Strasser et al. 2008; Zhou et al. 2008; Rosales-Mendoza et al. 2011).

1.2 Plant bioreactors

1.2.1 Seed and fruiting body systems

A wide variety of plant reproductive structures have been used to generate and store recombinant proteins, including seeds in cereals and legumes, as well as fruits such as tomato

(Ma et al. 2003; Ramirez et al. 2007; Ludwig et al. 2004; Rosales-Mendoza et al. 2011; Cunha et al. 2010). Seeds are attractive bioreactors for a variety of reasons, including the potential for high accumulation in protein storage bodies and simplified long-term storage (Stoger et al. 2000). For therapeutic proteins, many seed and fruiting body expression platforms can be used directly for oral delivery with minimal processing, such as immunization against the Norwalk virus through oral delivery of tomato expressing the Norwalk capsid protein (Zhang et al. 2006).

Despite the above advantages, the use of plant reproductive tissue presents a major problem, namely the increased risk of environmental contamination. The production of pollen provides an opportunity for contamination of plants earmarked for human consumption, as well as the potential for outcrossing to wild relatives. This risk can be partially overcome through the use of male-sterile lines for the expression of recombinant proteins, preventing the spread of pollen containing the recombinant protein or its coding sequence (Feil et al. 2003). In fruit-based expression, the level of recombinant protein is typically low and non-uniform, creating difficulties in accurately producing doses for ingestion, although it is possible to reach uniformity through batch production and homogenization (Joensuu et al. 2008).

1.2.2 Potato

Potato (*Solanum tuberosum*) tubers have been used to accumulate a wide variety of recombinant proteins, including monoclonal antibodies, viral coat proteins, cytokines, plant lectins and insulin (Mason et al. 1996; Arakawa et al. 1998; De Wilde et al. 2002; Tremblay et al. 2010a; Richter et al. 2000; Carrillo et al. 2001; Yu and Langridge 2003; Ma et al. 2005). Potato offers a number of advantages as a bioreactor over other plants species. First and foremost is biomass accumulation, with nearly 30 tonnes per hectare of tubers commonly harvested, compared to 2 to 3 tonnes per hectare for grain species (Statistics Canada, 2010). While tuber

protein content is relatively low, the amount of biomass accumulation more than offsets this deficiency, with only soybean producing more protein per hectare on average. Secondly, recombinant proteins produced in tubers are stable. For example, following six months of cold storage, tubers had lost less than 10% of a recombinant monoclonal antibody (De Wilde et al. 2002). One proposed reason for this is tubers produce a significant quantity of proteinase inhibitors and are thought to stabilize native tuber proteins during extended dormancy (Weeda et al. 2009). Potato transformation is relatively simple, achieved by incubating *Agrobacterium* with either leaf or tuber discs (Bourque et al. 1987). In addition to these advantages, potato propagates vegetatively, drastically reducing the risk of environmental outcrossing, although do to the harvested biomass occurring below the surface, residual plants may overwinter in the field.

Potato tubers develop from the terminal ends of underground stems called stolons (See Figure 1.1). The forming tuber begins to enlarge along the transverse axis of the stolon, with an increase in the accumulation of protease inhibitors, as well as storage proteins, particularly patatin (Weeda et al. 2009). Large-scale starch accumulation occurs during the bulking phase, when sugars imported from the leaves into tuber parenchyma with starch-containing plastids called amyloplasts. The imported sugars are linked through 1,4-glycosidic bonds to form amylose, which are linear chains of starch, and can be subsequently branched through 1,6-glycosidic bonding, creating amylopectin.

As mentioned above, low protein concentration is a drawback to potato. While overall yield is high, the requirement to process 10 times the tissue to achieve comparable levels of

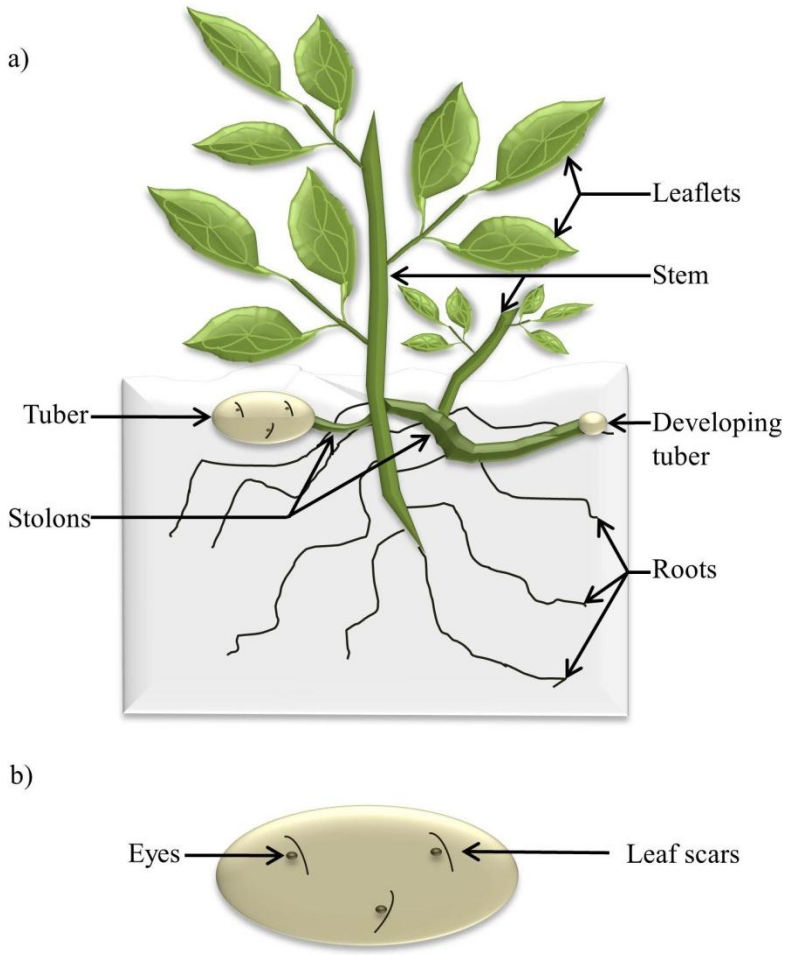

Figure 1.1: Diagram of a potato plant. a) Depiction of the aerial and below ground portion of a potato plant. Tubers develop at terminal ends of stolons, which are below ground stems that can give rise to aerial portions. b) Depiction of a potato tuber. Leaf scars subtend eyes, which are meristematic tissue that give rise to stolons following periods of dormancy to generate new plants.

protein yield of grains per hectare increases the post-production costs. As with all plant bioreactors that are based in food staples, there is also a risk of contamination of the food chain.

1.2.3 *Nicotiana* species

Two species of *Nicotiana*, *tabacum* (tobacco) and *benthamiana* (a close relative of tobacco), are routinely used for the production of recombinant proteins. Tobacco offers several advantages as a green bioreactor. First and foremost, tobacco is not part of the food supply chain, removing the risks for accidental contamination. Secondly, tobacco leaf-based bioreactors eliminate the requirement for pollination, and existing agricultural infrastructure is designed with leaf harvest in mind. In addition, it would offer current tobacco farmers an alternative crop destination point, from cancer-causing to therapeutic-producing tobacco. The equipment used for tobacco harvesting is specific to tobacco and so for farmers to switch to a different crop would require massive changes in infrastructure. Tobacco also has a high yield, with upwards of 100 tonnes per hectare of leafy green material per year. Known as the "white mouse" of the plant world, tobacco is also one of most heavily studied recombinant protein expression systems, with a variety of expression options readily available, including transient and stable expression (Tremblay et al. 2010b). Oral delivery is also a possibility, as low-nicotine low-alkaloid varieties, such as 81V9, have proven safe in animal feeding trials (Menassa et al., 2007)

1.3 Medically valuable protein production in green bioreactors

1.3.1 Antibodies

Recombinant antibodies are useful for a variety of therapeutic interventions and make up the bulk of protein therapeutics under development. Monoclonal antibody production requires the coordinated assembly of a heavy and light chain. The first plant-made antibody, an anti-

mouse IgG_1, was produced by expressing the heavy and light chains in two different plants and then crossing the two independent lines together (Hiatt et al. 1989). Since that time, a wide range of antibodies have been produced in plant bioreactors. The first plant-made antibody approved for human use, CaroRx^{Tm}, is a secretory IgA antibody that prevents the colonization of *Streptococcus mutans* and reduces the risk of tooth decay (Lehner et al. 1985; Ma et al. 1989; Ma et al. 1998). New approaches to the treatment of cancer utilize a patient's own antibodies to prevent further spread and reoccurrence. In clinical trials, patient idiotype-specific single-chain variable fragment (scFv) were isolated from the individual patients, sequenced and then ligated into a plant expression vector and subsequently transiently expressed in *N. benthamiana* and injected back into the patients (McCormick et al. 1999; McCormick et al. 2003; McCormick et al. 2008). Accomplished in only a few months after the initial biopsy, this demonstrates the utility of green bioreactors to respond to meet the demands for vaccines following the onset of new epidemics and their utility in treating diseases that require a more individualistic, tailored therapeutic.

1.3.2 Vaccines

A number of potential vaccines have been produced to date in transgenic plants, including foot and mouth disease, severe acute respiratory syndrome (SARS), cholera and human immunodeficiency virus (HIV) (Wu et al. 2003; Huang et al. 2005; Pogrebnyak et al. 2005; Jani et al. 2004; Marusic et al. 2007; Zhang et al. 2002). Typically this involves the production of a component of the infectious agent, be it a viral capsid protein fragment or bacterial cell surface protein. The effectiveness of these recombinant vaccines combined with the attractiveness of plant-made pharmaceuticals has led to increased commercial interest in recent years, including

the recent expansion of Medicago Inc., a Quebec City-based company that produces flu vaccines via transient expression in plants (D'Aoust et al. 2010).

1.3.3 Treatment of autoimmune disease

Autoimmune diseases are the result of the immune system's failure to distinguish self-proteins from foreign ones, resulting in an immune response against tissues/organs displaying those proteins. Type 1 diabetes mellitus (T1DM) is an autoimmune disease that results in the destruction of the insulin producing islet cells of the pancreas, resulting in the individual's loss of glycemic control (Ma et al. 1997). In T1DM, there are a number of potential proteins that become immune targets, with the two most characterized being insulin (INS) and glutamic acid decarboxylase 65 (GAD65) (Ma et al. 1997; Arakawa et al. 1998; Ma et al. 2004; Li et al. 2006a; Ruhlman et al. 2007). A potential therapy for T1DM is through the induction of oral tolerance, a natural process whereby the immune system is disarmed against ingested proteins, attenuating immune responses against the thousands of harmless dietary proteins that pass through the gut each day. Oral tolerance can be induced in non-obese diabetic (NOD) mice, a model for T1DM, by feeding plant tissue expressing GAD65 and an immunomodulatory cytokine, interleukin 4 (IL-4) (Ma et al. 2004). Other targets of the autoimmune response have been evaluated with similar success, including insulin (Arakawa et al. 1998). Research has also demonstrated that small antigenic peptides, those amino acid sequences that are targeted by the immune response, can also prove effective. The small size of these peptides, typically from 5 to 20 amino acids, typically necessitates fusion with a larger protein. One such example is the production of the B chain of insulin as a fusion with the non-toxic B subunit of cholera toxin (CTB), resulting in a large, stable homopentamer (Li et al. 2006a).

1.4 Plant transformation techniques

1.4.1 Particle Bombardment

Transformation via microparticle bombardment involves the coating of the desired expression cassette to a microparticle, typically gold or tungsten, and subsequent discharge of a high pressure burst of helium (Taylor and Fauquet 2002). The subsequent particle-cassette is hurled towards the target tissue and penetrates plant tissue. In the process, this causes significant damage to the surrounding tissue. Bombardment can perform a variety of plant transformations, including transient and stable nuclear, chloroplastic and mitochondrial expression (Taylor and Fauquet 2002). Biolistic delivery allows for the insertion of minimal expression cassettes, which are cassettes that contain the gene of interest plus transcription machinery and possibly a selection marker. A major advantage of this system is that a wide-range of plant species can be transformed through relatively standardized protocols that are typically difficult transform via other methods, resulting in transformation of plants such as palm trees, tobacco, tomato and rice (Parveez et al. 1997; Lutz et al. 2001; Zhou et al. 2008; Lee et al. 2006).

1.4.1.1 Plastid transformation

Plastid transformation via particle bombardment has the greatest potential for recombinant protein accumulation amongst all plant transformation techniques to date, with reports of recombinant protein accumulation ranging from under 1% to over 50% of soluble protein (Lentz et al. 2010; Li et al. 2006b; Ruhlman et al. 2007; Lenzi et al. 2008; Zhou et al. 2008; Soria-Guerra et al. 2009). There are several reasons for the high accumulation potential of plastids, including multiple copies of DNA per plastid, with mature chloroplasts containing between 50 to 100 copies of the plastid genome, and ~100 plastids per mature leaf cell (Maliga 2004; Grevich and Daniell 2005). This results in the potential for nearly 10,000 copies of the

DNA for the production of recombinant protein. In addition, the transformed gene is inserted into the plastid genome via homologous recombination, allowing for the optimization of insertion point to suit each expression cassette, something not routinely possible with plant nuclear genomic transformation. Plastid-based expression also offers a level of environmental containment, as plastids are maternally inherited and thus is unlikely out-cross through pollen spread to relative species or untransformed sister crops (Svab and Maliga, 2007).

There are, however, limitations to the use of plastid transformation. Firstly, with nearly 10,000 copies of DNA per cell, selection time is dramatically increased in order to reach a homoplastic state (point at which all copies of DNA contain the transgene). Second, plastids are prokaryotic in origin and thus are unable to perform eukaryotic post-translational modifications such as glycosylation, required for many glycoproteins to function properly and for increased stability.

1.4.2 *Agrobacterium*

Recombinant protein expression in plants is also routinely achieved by infecting plants with *Agrobacterium tumefaciens*, recently reclassified as *Rhizobium radiobacter*, a bacterium that causes crown gall disease in a variety of plants species (Young et al. 2001). *Agrobacterium* is able to insert a portion of its plasmid T-DNA into plants cells, which is then transported to the nucleus and the resulting gene sequences are expressed. Depending on the protocol used, this can result in the stable integration of the T-DNA into the plant nuclear genome or transient expression of multiple copies of non-integrated T-DNA (Sparkes et al. 2006). Originally limited to a relatively low number of species, particularly dicotyledonous plants, a wide-range of dicot and several monocot plants are now readily transformed via *Agrobacterium* transformation,

including tobacco, Arabidopsis, carrot, tomato, potato, cotton, rice, and corn (Rosales-Mendoza et al. 2011; Zhang et al. 2006; Wesley et al. 2001; Hood et al. 2007).

1.4.2.1 Stable nuclear transformation

Nuclear transformation via *Agrobacterium* results in the stable and heritable integration of the transgene into the host genome. There are a variety of advantages to stable nuclear integration. In the case of an agronomically advantageous trait such as resistance to the herbicides 2,4-D and BASTA, there is immense value to the resistance being heritable as opposed to transiently inducing resistance with each generation (Wright et al. 2010; Viegas and Notani 1993). Another advantage is the nearly unlimited scalability of stable integration with extremely minimal input following the initial transformation and characterization of a line. This means that an experiment can transfer from growth chamber to greenhouse or open fields simply by amplifying the initial characterized lines, without the need to continually transform each successive generation. There are, however, several major hurdles that have yet to be fully overcome with stable transformation. First and foremost are the typically low levels of recombinant protein expression, with accumulation typically less than 0.5% of soluble protein. Secondly is the risk of environmental spread of the transgene, as nuclear material contained within pollen could out-cross to either unmodified sister plants or to natural species in proximity to the open fields. Several groups have generated either male-sterile lines or lines that are capable of deleting the transgene in pollen, potentially limiting gene transfer as a major concern (Evans et al. 2005; Feil et al. 2003).

1.4.2.2 Transient expression

The use of *Agrobacterium* to transiently express recombinant proteins has a variety of applications from academic research to commercial and pharmaceutical protein production.

Transient expression has a number of advantages over stable integration, including typically higher protein accumulation levels (Sparkes et al. 2006). Further enhancement of accumulation during transient expression can be achieved by co-expressing a suppressor of the plant RNA silencing machinery through the expression of a viral p19 protein (Voinnet et al. 2003). The inclusion of p19 expression typically results in an increase of 50-times or greater accumulation of the protein of interest. Transient expression can also be accomplished in a significantly shorter period of time compared to stable integration, with typical induction experiments lasting between 3 and 7 days, followed by harvest and isolation, as opposed to typically having to wait 3 to 6 months before sufficient recombinant protein can be harvested from mature transgenic plants. For example, it was possible for researchers to isolate novel, patient-specific antibody sequences, clone them, transiently express them and harvest sufficient protein for re-inoculation in under 6 weeks, something inconceivable via stable nuclear integration, which would most likely have taken between 6 months to a year (McCormick et al. 1999; McCormick et al. 2008; McCormick et al. 2003). A major drawback associated with transient expression is the high input requirements, both in materials and personnel, compared to stable integration. This may create limitations for long-term production of proteins needed in large quantities, but as demonstrated for the production of patient-specific antibodies, there are still a variety of commercial and research applications.

1.5 Hurdles in plant bioreactors

As discussed above, plants offer a variety of advantages as bioreactors. There remains a variety of hurdles, some of which are unique to the type of expression system (transient versus stable, for example). Others are common to all bioreactors, such as the cost associated with purification. A third hurdle involves the generation of small protein fragments. As mentioned in

section 1.3.3, smaller protein fragments, or epitopes, can prove more effective in the induction of oral tolerance. However, it is extremely difficult to generate short peptides in plants.

1.5.1 Yield

The overall recombinant protein production capacity of a plant bioreactor is dependent on 1) the biomass yield in a given area, 2) the concentration of soluble protein produced by the individual plant and 3) the relative amount of the recombinant protein produced compared to all other proteins. Typically, bioreactor research tends to focus on the third point over the first two, as the majority of species used as bioreactors have already been selected for centuries or even millennia for maximum yield and quality. As a result, advances are typically expressed in increase in percent of soluble protein. For example, in work by Patel and colleagues in 2007, the authors demonstrated an increase of over 90-fold in Il-10 accumulation when Il-10 is fused with elastin-like polypeptide (ELP) and stably expressed via nuclear transformation. This is an example of fusion with a more stable protein partner that, in the case of ELP, is also able to compartmentalize accumulation within specialized protein storage organelles called protein bodies (Conley et al., 2009b). Organelle-specific localization is also another method for increasing accumulation, such as the inclusion of the c-terminal tag K/HDEL that results in endoplasmic reticulum retention (Tremblay et al. 2010b). The selection of the promoter for expression, what untranslated regions are used, as well as codon usage can all influence the accumulation of the protein of interest. The gains from these various techniques, however, have yet to consistently increase production in stably transformed nuclear lines to be truly competitive against other bioreactors. Chloroplast transformation can meet the demands for high-yield expression required to be truly competitive, but are incapable of producing glycoproteins, resulting in an excellent platform for a more limited number of proteins.

1.5.2 Purification

All bioreactors, regardless of host species or whether they are cell-culture or whole-organism based, require post-harvest protein purification. In cell culture systems, secretion to the media can reduce the complexity of purification, but as discussed in section 1.1, have limited scalability and are more expensive than whole-plant production. For proteins intended for medicinal use, or that are needed in ultrapure form, the downstream processing can account for upwards of 80% of the production costs (Walsh 2002). As such, a variety of genetic fusions have been used to decrease this cost. The inclusion of a repeat histidine tag (His), typically 6 amino acids long, is perhaps the most commonly used purification tag for laboratory use as it can be relatively inexpensive on small scales, requiring an immobilized gel such as agar or sephadex with bound nickel ions to facilitate purification. Under optimized conditions, His purification can reach nearly 95% purity in secretory bacterial systems, but in plants, rarely achieves high levels of purity (Terpe 2003). An alternative approach to affinity-based purification is through the use of ELP. ELP undergoes inverse phase transition with increasing temperature and/or salinity, resulting in a reversible change in solubility that can be repeated several times in order to remove contaminating proteins. For example, Conley and colleagues (2009a) demonstrated that ELP-fusions could be readily purified using inverse phase transition cycling as well as enhancing protein accumulation in plants. However, the purification was, at best, reported at 66% of soluble protein. One proposed explanation is the high quantities of Rubisco, which is involved in catalyzing the first step of carbon fixation in plants and is the most abundant protein in nature. The high levels of Rubisco found within green leaf tissue can cause problems with purification regardless of method used. An alternative possibility is that plants contain proteins that likewise

undergo phase transition under similar conditions and this may limit the utility of ELP for protein purification.

1.5.3 Generation of short peptides

In autoimmune disease, the immune response is typically directed against a limited portion of the targeted protein, short amino acid sequences called epitopes. For example, in T1DM, the β chain of insulin (InsB), 30 amino acids long, is a well characterized target of the immune response, while the α chain, a 20 amino acid fragment, is not a target (Weiner 1997). There are reasons why it is preferable to use only protein fragments containing epitopes, as there is a lower risk of negative side-affects than might be associated with full-length administration. For example, full-length INS may lead to the induction of hypoglycaemia if delivered orally (Li et al. 2006a). However, it is extremely difficult to produce short peptide sequences in plants, as evidenced by the literature where nearly all reports of short peptide generation are as fusion partners with larger, more complex proteins. For example, InsB has only been generated as a fusion with CTB (Li et al. 2006a).

1.6 ATP/ADP transporter

In tubers, energy must be imported into the amyloplasts through the plastidic ATP/ADP transporter (AATP1) (Tjaden et al. 1998). When *AATP1* is overexpressed, tubers accumulate greater quantities of starch, with one line producing 36% more starch compared to wild-type (Tjaden et al. 1998). In contrast, when *AATP1* is disrupted through the expression of full-length antisense *AATP1*, starch is reduced by over 40%. This decrease in starch is associated with a subsequent decrease in overall tuber biomass accumulation and morphology, with the most extreme lines in this experiment having tuber total weight per plant at ~70% of wild-type and producing more but smaller tubers with adventitious budding. In addition, there is a subsequent

increase in soluble sugars in the antisense lines (Geigenberger et al. 2001; Tjaden et al. 1998). The changes in tuber biomass and morphology are consistent with other studies that have knocked down or knocked out genes directly involved in starch biosynthesis, such as ADP-glucose pyrophosphorylase (Muller-Rober et al. 1992).

1.7 Soybean Agglutinin

Soybean agglutinin (SBA) is a 32 kDa homolectin found in soybean seeds. Lectins are proteins that bind to sugar moieties with varying affinity. SBA binds to *N*-acetyl-D-galactosamine, a glycan that can be found on the extracellular portions of cell membrane glycoproteins. The presence of these glycans on the cell surface of red blood cells allows for SBA to induce agglutination (clumping) of the cells, hence the protein name agglutinin. SBA accumulates to nearly 2% of the soluble protein in soybean seeds and is typically extracted from defatted soybean flour (Percin et al. 2009; Lindstrom et al. 1990). The affinity of SBA for its ligand allows for purification using affinity chromatography, with *N*-acetyl-D-galactosamine-bound agarose or sepharose columns allowing for the efficient and simple isolation of SBA from other soybean proteins, with typical recovery of high purity SBA ranging around 90% (Percin et al. 2009).

The makeup of surface exposed glycoproteins varies from cell to cell, allowing for the use of lectins, such as SBA, to be used to distinguish between cell types based on binding pattern. For example, SBA has been shown to bind to cell lines derived from two independent pancreatic cancers, but does not bind to healthy pancreas-derived cells (Nishimura et al. 1993). SBA binding patterns can help distinguish between stomach cancers with higher survivorship (SBA+) and those with higher mortality (SBA-) (Terashima et al. 1997). The ability of SBA to bind to particular cells in suspension has been adapted for use in clinical settings. SBA does not

bind strongly to healthy lymphocytes, but does to leukemic lymphocytes (Bakalova and Ohba 2003). This allows for the potential *in vitro* removal of leukemic cells via lectin chromatography and the return of the healthy lymphocytes to the body.

Recombinant SBA has been produced in a variety of systems, including monkey COS-1 cells, *E. coli* and tobacco plants (Lindstrom et al. 1990; Adar et al. 1997). The goals of these studies were to examine either the regulatory elements involved in SBA expression using tobacco as the platform (Lindstrom et al., 1990) or the involvement of glycosylation in tetrameric assembly by comparing non-glycosylated SBA (bacterial) versus glycosylated SBA (COS-1) (Adar et al., 1997). The absence of glycosylation had no impact on SBA's ability to induce agglutination, suggesting that glycans are not required. Glycosylation, however, is important for the stability of SBA (Sinha and Surolia 2005). COS-1 derived SBA appears to have similar characteristics to native SBA, binding to sugars in a similar pattern and appearing to have similar glycosylation, but it requires significantly more COS-1 SBA to induce agglutination of red blood cells compared to native or bacterial-produced SBA, suggesting the COS-1-made SBA does not retain native SBA binding affinity (Adar et al. 1997).

1.8 Cholera toxin β subunit

Cholera toxin is a secretory protein complex produced by the bacteria *Vibrio cholerae* and is the causal agent in cholera. The β subunit of cholera toxin (CTB) is a non-toxic protein that forms pentamers and is able to bind to GM_1 gangliosides, important for both the natural binding of cholera toxin as well as its potential use as a therapeutic adjuvant (Holmgren et al. 1993). Its ability to bind to GM_1 gangliosides offers the potential for CTB to function as an oral adjuvant, able to bind to epithelial linings of the intestine and be displayed to the peripheral immune system, resulting in the induction of tolerance (Sun et al. 2010). CTB fusions with

insulin, either full-length or the β-chain only, have proven effective in delaying the onset of T1DM in the non-obese diabetic (NOD) mouse model of the disease through oral administration (Bergerot et al. 1997; Arakawa et al. 1998; Ploix et al. 1999; Sadeghi et al. 2002; Ruhlman et al. 2007). There are, however, considerations in the use of CTB as an adjuvant where long-term administration is concerned. In mice fed potato expressing CTB-Insulin during a long-term study, there were detectable levels of mucosal anti-CTB antibodies, potentially neutralizing the ingested CTB-insulin over the long-term (Arakawa et al. 1998). While this is of concern for long-term administration, studies have shown that the use of CTB as an adjuvant can greatly reduce both the amount of protein needed to induce tolerance, as well as the number of doses required, potentially negating the need for long-term dosage (Sun et al. 2010).

1.9 p277

p277 is the name given for the 23 amino acids 437 to 460 of heat shock protein 60 (HSP60) that has been implicated in T1DM (Elias and Cohen 1994). Early work demonstrated that p277 was capable of inducing diabetes in standard mice when administered with carrier molecules (Elias et al. 1995). This study also demonstrated that following an immunological challenge with p277 and subsequent transient hyperglycaemia, NOD mice were resistant to the spontaneous development of T1DM, supporting p277 as a possible therapeutic in preventing diabetes. Follow-up work by the same group demonstrated that administration of p277 to NOD mice with either no or overt signs of hyperglycaemia resulted in a reduction in islet inflammation, a marker for the onset of T1DM in NOD mice (Elias and Cohen 1995). Subsequent phase II clinical trials of the modified form of the peptide (Diapep277 has the cysteine residues replaced with valine) have been completed and found that relatively few doses of Diapep277 is required to maintain c-peptide levels, a marker used for tracking the progression

of the disease, in newly diagnosed patients (Babad et al. 2010). Phase III clinical trials are currently underway. However, p277 is currently generated chemically, an expensive method for peptide generation, potentially limiting its deployment following clinical trials.

1.10 Research goals and objectives

The rising demand for recombinant proteins for academic, industrial and medical uses necessitates the implementation of more effective and efficient bioreactor production. While green bioreactors are beginning to be recognized as an inexpensive alternative to traditional cell culture-based systems, they are still limited by low yields for nuclear expressed glycoproteins. In addition, there is an increased demand for more efficient means of production and purification of recombinant proteins for both small and large scale production, as well as the development of more effective recombinant proteins destined for therapeutic use.

The goal of the research presented in this thesis is to create tools to enhance the effectiveness of plants as green bioreactors and their utility to the end user, reducing the high costs typically associated with production, those of yield and purification, and increasing the efficiency at each stage of the line. To achieve this goal, my research focused on three themes: 1) to increase the yield of soluble recombinant proteins made in transgenic plants, 2) to develop a simplified, rapid and high-purity recombinant protein recovery system and 3) to examine the feasibility of generating small therapeutic peptides in green bioreactors for the treatment of T1DM. To this end, the specific objectives of this project were:

1) To demonstrate the generation of a genetic fusion of CTB with p277 in stably transformed tobacco and determine if the CTB-p277 fusion remains stable and retains CTB pentamer formation, important for binding to GM_1 gangliosides.

2) To determine if a minor reduction in starch accumulation in potato tubers could increase overall yield. To this end RNA induced gene silencing of the *AATP1* gene, with the emphasis during initial screening focusing on biomass yield. The concentration of soluble proteins as well as the ability to accumulate a recombinant monoclonal antibody in the highest biomass accumulating knockdown line will also be examined to assess the overall improvement in recombinant protein production. Also assessed was the long-term viability of the tubers, as an advantage of tuber-based production is the ability to store recombinant proteins for extended periods of time.

3) Recombinant SBA was generated both transiently in *Nicotiana benthamiana* and stably in potatoes as a first-step towards generating a novel affinity tag system. The recombinant protein was compared with native SBA to determine if it retained its ligand specificity, ability to induce agglutination, similar glycosylation pattern and resistance to gastric passage.

4) To validate genetic fusions with recombinant SBA as a novel affinity tag. GFP was genetically fused to SBA and examined for both *in vivo* activity (fluorescence), as well as activity post-purification following the induction of agglutination of red blood cells. The fusion protein was compared to *N. benthamiana*-made recombinant SBA for the purpose of determining purification level and efficiency, as well as agglutination and ligand specificity.

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CHAPTER 2

EXPRESSION OF A FUSION PROTEIN CONSISTING OF CHOLERA TOXIN B SUBUNIT AND AN ANTI-DIABETIC PEPTIDE (P277) FROM HUMAN HEAT SHOCK PROTEIN IN TRANSGENIC TOBACCO PLANTS

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2.1 Introduction

Peptide p277 is composed of 24 amino acids from the sequence (437-460) of the human 60 kDa heat-shock protein (Hsp60), with the two cysteine residues of the native sequence replaced by valines (Elias and Cohen 1994). There is accumulating evidence to suggest that like glutamic acid decarboxylase (GAD), insulin or insulin B chain 9-23 peptide, p277 of the Hsp60 protein is an autoantigen serving as a target of T-cells in autoimmune diabetes in humans and NOD (non-obese diabetic) mice, and could therefore represent a potentially effective therapeutic agent for the treatment of autoimmune diabetes. Indeed, a single injection of 100μg of p277 in incomplete Freund's adjuvant can induce a shift in the cytokine profile of the spontaneous T cell response from a damaging Th1-type to anti-inflammatory Th2 type, leading to the permanent arrest of the β cell damage in NOD mice (Elias et al. 1997). Furthermore, p277 was shown to be effective even when the treatment was initiated in mice that were already clinically diabetic (Elias and Cohen 1995). The promising results in animal tests have paved the way for clinical trials for the treatment of Type 1 diabetes with peptide p277. A phase 1 human clinical trial showed that subcutaneous administration of p277 is safe, causing no adverse side effects in volunteers with longstanding Type 1 diabetes and with no detectable C-peptide (Cohen 2002), and phase 2 clinical trial in early onset Type 1 diabetes showed that treatment with p277 halted disease progression, prevented further destruction of the beta-cells, and prolonged patients' insulin production (Raz et al. 2001). Presently a phase III human clinical trial is in progress. Taken together, these results suggest that the human Hsp60 peptide p277 may hold promise for therapy against Type 1 diabetes.

Oral administration of autoantigens to induce oral immune tolerance represents one of the most desirable methods for the prevention and treatment of autoimmune diseases. In addition to

its simplicity and greater patient acceptance, the advantages of oral antigen therapy include increased specificity, as well as reduced toxic side effects associated with systemic therapy (e.g., by injection). Oral administration of autoantigens has been reported to be effective in suppressing disease development in animal models of autoimmunity, including the NOD mouse model of Type 1 diabetes (Weiner et al. 1994; Strobel and Mowat 1998). However, the effective use of oral tolerance in the treatment of human autoimmune disease may critically depend on the development of mucosal adjuvants and delivery systems to enhance its efficacy (Ma et al. 2004). Another serious limitation in the clinical application of oral tolerance strategies will be the potentially huge cost of producing autoantigens, particularly if repeated regular doses are required to maintain beneficial effects.

Chlolera toxin (CT), which is a major pathogenic agent produced by *Vibrio cholerae,* is a potent mucosal immunogen and adjuvant, and most of these activities are retained by the nontoxic B subunit of CT (CTB). This is in part due to its high binding affinity for the GM1-ganglioside receptor present in most cells of the body (Cuatrecassas 1973). CTB has been demonstrated to be an efficient oral adjuvant for mucosal vaccines (Bergquist et al. 1997; Czerkinsky et al. 1989). Furthermore, CTB has been shown to induce oral tolerance for linked antigens. For example, myelin basic protein (MBP) conjugated to CTB prevented or suppressed experimental allergic encephalomyelitis (EAE), when using lower concentrations of the conjugate than is usually necessary to induce tolerance with the protein alone (Sun et al. 2000). Oral administration of insulin or the B chain of insulin conjugated to CTB enhanced oral tolerance, and reduced the dose of antigen and the administration rate necessary for suppression of diabetes in NOD mice (Ploix et al. 1999; Sadeghi et al. 2002). Promising results against type II collagen-induced arthritis have been obtained by intranasal administration of low doses of type

II collagen-CTB conjugate (Tarkowski et al. 1999). These results indicate that mucosal administration of autoantigens conjugated to CTB may represent a useful future treatment approach for human autoimmune diseases, such as type I diabetes. At the present time, the mechanism underlying CTB's efficacy as a mucosal adjuvant or carrier molecule has not yet identified with certainty. It has been speculated that the CTB binding to GM1 increases the permeability of the membrane to the antigen (Nashar et al. 1996). CTB also induces the major histocompatibility complex (MHC) class II expression on B cells and significantly stimulates antigen presentation in macrophages (George-Chandy et al. 2001).

We have developed a new method for inducing oral tolerance, which is based on the use of transgenic plants such as potato and low-nicotine tobacco to express and deliver recombinant autoantigens such as murine GAD67 to the mucosal immune system (Ma et al. 1997). Transgenic plants offer several advantages for an oral tolerance strategy, not the least of which is their high production potential for relevant autoantigens with nearly unlimited scale up (Kusnadi et al. 1997). As protein purification costs can eliminate the economic advantage of any production system, an additional advantage of transgenic plants for oral tolerance is that plants can also become effective delivery systems without extensive purification. Plant expression also largely eliminates concerns regarding potential pathogens that could be transmitted to humans. Lastly, augmented immune responses to plant produced vaccines may suggest increased stability for plant expressed recombinant proteins to gastrointestinal degradation. Collectively these features make plants an ideal expression and delivery system for oral tolerance. More recently, we have demonstrated that in addition to synthesizing autoantigens, transgenic plants can be used to express immunoregulatory cytokines such as interleukin-4 (IL-4), and that combined oral administration of plant-derived human GAD65 and plant-derived IL-4 had a synergistic effect in

inducing robust oral immune tolerance in NOD mice, an animal model for human Type 1 diabetes (Ma et al. 2005; Ma et al. 2004).

The aim of the present study was to produce a fusion protein consisting of CTB and the peptide p277 (CTB-p277) in transgenic plants. Our long-term goal is to develop a plant-based oral peptide vaccine to treat Type 1 diabetes via the induction of oral tolerance. Here we demonstrate that the CTB-p277 fusion protein can be efficiently produced in transgenic tobacco plants. Moreover, the plant-derived fusion protein retains important functional characteristics of the native CTB, including pentamerization and GM1 ganglioside receptor binding. The production of transgenic plants expressing the human Hsp60 peptide p277 linked to mucosal adjuvant CTB offers an important first step towards the development of a safe, effective and inexpensive vaccine product for Type 1 diabetes

2.2 Materials and Methods

2.2.1 Plasmid construction

An oligonucleotide containing an engineered *Xho*1restriction site followed by the coding sequences for the peptide p277 was custom synthesized by commercial suppliers. The oligonucleotide was rescued by cloning into the *HincII* site of the pUC 19 vector to generate pUC19-p277. To create the CTB-p277 fusion protein, the p277 encoding sequence was isolated from pUC19-p277 as a *XhoI* and *BamHI* fragment and cloned into the same sites of pUC19-CTB. The plasmid pUC19-CTB contains the *ctx*B gene with its signal peptide replaced by a plant signal peptide (i.e., the signal sequence of peanut peroxidase) (Li et al. 2006a). The in-frame fusion of CTB with p277 was confirmed by sequencing analysis. The fusion gene was isolated as a *NcoI* and *XbaI* fragment and inserted into the same sites of pTRL2-GUS (Carrington and Freed 1990) to replace the GUS gene. The resulting expression cassette comprising the CaMV35S

promoter with a double enhancer sequence (2x35S) fused to a 5' untranslated tobacco etch virus (TEV) leader sequence, *ctx*B-p277, and the nonpaline synthase (NOS) terminator were excised as a *HindIII* fragment and cloned onto into the pBI101.1 binary vector to create pRT-CTB-p277. Plasmid pBI101.1 is a derivative of pBI 101 in which the GUS gene together with the NOS terminator was deleted. The pRT-CTB-p277 binary vector was transformed into *Argobacterium tumefaciens* strain LBA4404 by tri-parental mating (Lige et al. 2001).

2.2.2 Plant transformation

Nicotiana tabacum cv. 81V9 leaf discs were transformed by co-cultivation with *A. tumefaciens* LBA4404 containing the pRT-CTB-p277 binary plasmid as described by Horsch et al (1985). Transformed leaf discs were selected on callus inducing MS104 medium containing 100mg/l kanamycin. New shoots that developed from calli were transferred to magenta boxes containing MS medium with 50mg/l kanamycin and maintained.

2.2.3 PCR analysis of transgenic plants

Transgenic plant DNA was isolated from leaves of tobacco plants grown aseptically in Magenta boxes according to Ma et al. (2004). PCR was carried out using forward primer 5'-ATGGCACTTCCAATTAGCAAG-3' (corresponding to codons encoding the N'Terminal engineered signal peptide sequence of CTB) and reverse primer 5'-TTAGTCCTCATTTGCGGGTGTA AGGGA-3' (corresponding to codons encoding the C-terminal p277 amino acid sequence) to detect the presence of the *ctx*B-p277 fusion gene, or reverse primer 5'-CTCGAGCGGCCCCGGCCCA TTTGCCA-3' (corresponding to the C-terminal end of CTB) to detect the presence of the *ctx*B gene. Cycling conditions comprised an initial step at 94°C for 3 min followed by 30 cycles of 45 s at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension step at 72°C for 10 min.

2.2.4 RNA purification and RT-PCR analysis

Plant RNA was purified with RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. For RT-PCR, first-strand cDNA was generated in a 20-µl reaction containing 5 µg of total RNA, 0.5 µg of oligo$(dT)_{18}$ and 20 units of SuperScript™ II RNase H Reverse Transcriptase (Invitrogen). A total of 100 ng of RNA-derived first strand cDNA were then used for PCR reaction using the same primer pair utilized above for the detection of *ctx*B-p277 fusion gene. The polymerase used was *Taq* DNA polymerase (Amersham). The PCR cycling conditions were the same as above. RT-PCR products cloned into pUC-19 were sequenced to confirm full length *CTB-p277* expression.

2.2.5 Western blot analysis of transgenic pRT-CTB-p277 plants

Accumulation of the fusion protein in transgenic pRT-CTB-p277 plants was analyzed by Western blotting. Expanded leaves of tobacco plants were ground by mortar and pestle and resuspended in extraction buffer (200 mM Tris pH8.0, 100 mM NaCl, 400 mM Sucrose, 10mM EDTA, 14 mM ß-mercaptoethanol, 1 mM phenyl-methylsulfonyl fluoride, 0.05% v/v Tween-20, 2 µg/ml aprotinin, 2 µg/ml leupeptin). Samples were centrifuged for 10 min at 4 °C and the supernatant was collected. The concentration of total soluble protein (TSP) was measured according to the Bradford method using the Protein Assay dye (500-0006, Bio-Rad). Samples were boiled for 5 min or left untreated, separated by SDS-PAGE and blotted onto PVDF membrane (Millipore, Burlington, MA). The membrane was blocked overnight in 5% skim milk-TBST (20mM Tris, 150 mM NaCl, 0.01% v/v Tween20, pH7.6), then incubated for 2 h in 1:500 dilution (v/v) of rabbit anti-CTB primary antibody (C-3062, Sigma). The blot was then incubated for 1 h with goat anti-rabbit peroxidase-linked antibody (G-7641, Sigma). Antibody detection

was accomplished with SuperSignal® West Pico Chemiluminescent Substrate (34080, Pierce, Rockford, IL). Signals were visualized using a Lumi-imager (Roche Diagnostics).

2.2.6 Quantification of CTB-277 protein accumulation by ELISA

Quantitative ELISA (enzyme-linked immunosorbent assay) determined the level of CTB-p277 fusion protein accumulation in transgenic plants as described previously (Li et al. 2006a). In brief, triplicate serial dilutions of the plant protein extracts and a CTB standard (C-9903, Sigma) were bound to a 96-well microtiter plate overnight at 4°C. Background was blocked for 2 h in 5% (w/v) skim milk-PBST. Plates were washed with PBS, and then incubated with rabbit anti-cholera toxin antibody (C-3062, Sigma) at a dilution of 1:1000 for 2 h at 37 °C. Following wash with PBS, plates were incubated with goat anti-rabbit peroxidase-linked antibody (G-7641, Sigma) and developed with the Substrate Reagent Pack (DY999, R&D Systems). Optical density (OD) values were measured at 450 nm, with TSP from untransformed tobacco plants used for background subtraction, and compared to the standards to determine expression level.

2.2.7 GM1-ganglioside binding assay

The GM1-ELISA assay was performed to determine the binding capacity of plant-derived CTB-p277 fusion protein to GM1 ganglioside. Briefly, the microtiter plate coated overnight at 4^0C with monosialoganglioside-GM1 (Sigma G-7641) at a concentration of 3 μg/ml (in bicarbonate buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$, adjusted pH to pH 9.6) was blocked with 3% (w/v) fat-free milk powder in PBS and incubated at room temperature for 2 h, followed by three washes with PBST. The wells were loaded with protein isolated from transgenic plants, or bacterial CTB as a positive control. Plates were incubated overnight at 4^0C. After washing, the specific binding of plant-derived CTB-p277 to GM1 ganglioside was visualized by the addition of rabbit anti-cholera toxin antibody (Sigma C-3062; 1: 2000 dilution) followed by enzyme-

conjugated anti-rabbit IgG (1:5000 dilution) and enzyme substrate 3,3',5,5'-tetramethylbenzidine(TMB; Sigma) as for an ordinary ELISA as described above.

2.3 Results

2.3.1 Construction of pRT-CTB-p277 plasmid and plant transformation

The expression vector pRT-CTB-p277, which contains an in-frame fusion of the *ctxB* gene and the p277 fragment of human heat shock protein 60, is shown in Fig. 2.1. Construction of pRT-CTB-p277 has been described in detail in Methods. The fusion gene was under the transcriptional control of a doubled CaMV 35S promoter.

Following the leaf disc transformation with *Agrobacterium* containing pRT-CTB-p277, more than 25 independent transgenic plants were produced. The presence of the fusion gene was confirmed by PCR analysis of transgenic plants using primers that spanned the entire fusion gene (Fig.2.2).

2.3.2 Detection of the CTB-p277 fusion gene transcripts in transgenic tobacco plants by RT-PCR

RT-PCR analysis was employed to detect the expression of the CTB-p277 hybrid mRNA in transgenic tobacco lines. When primers spanning the full length of CTB-p277 were used, an amplification product of expected size (480 bp) was detected following reverse transcription of total RNA extracted from leaf tissues of tobacco lines transformed with pRT-CTB-p277. Representative data are shown in Figure 2.3. No RT-PCR products were detected using the total RNA from the wild-type tobacco plants. To rule out the possibility of amplification of contaminant DNA in the samples, direct PCR amplification without reverse transcription was

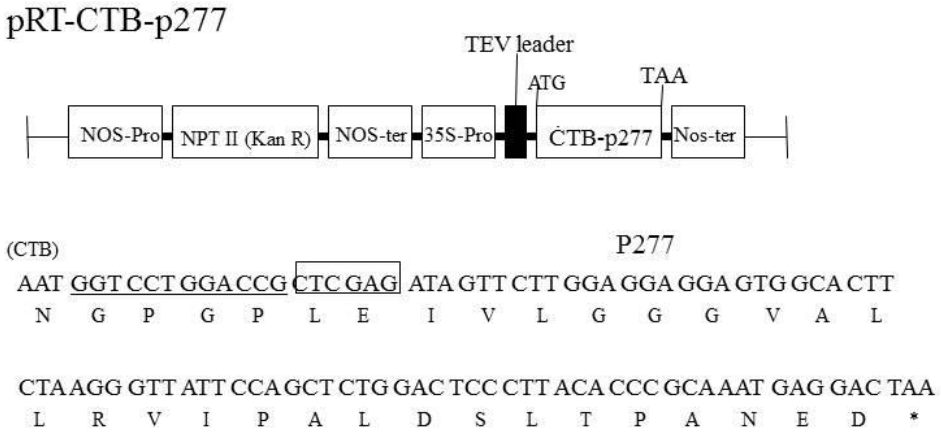

Figure 2.1 Schematic diagram of the plant expression vector pRT-CTB-p277. The T-DNA region inserted into the plant genome contains the nopaline synthase expression cassette (Kan^R), which confers kanamycin resistance on transformed cells, and the CTB-p277 fusion cassette consisting of the CaMV 35S promoter fused to a tobacco etch virus5'-untranslated region (TEV) and nopalin synthase terminater (Nos-ter). The nucleotide and amino acid sequences of p277 as well as the sequence surrounding the fusion site are shown at the bottom. The underlined sequences correspond to the flexible hinge region. The boxed sequences represent the joining site between the two fusion partners. The stop codon is indicated by an asterisk.

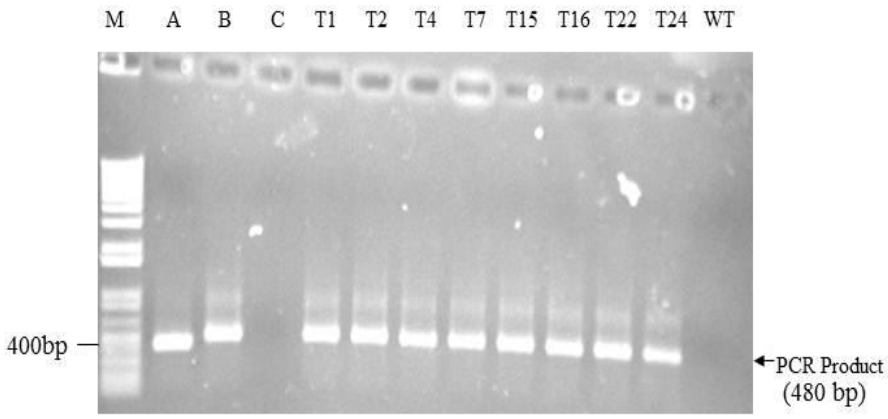

Figure 2.2 Detection of CTB-p277 integration into transgenic tobacco genomic DNA by PCR. The CTB-p277 fusion gene was amplified from transgenic plant DNA by PCR, and the PCR products were separated by agarose gel electrophoresis. M, DNA ladder; A, PCR product amplified from pRT-CTB-p277 plasmid DNA with primers specific for full-length CTB (positive control); B, PCR product amplified from pRT-CTB-p277 plasmid DNA with primers specific for full-length CTB-p277 (positive control); C, PCR amplification of pUC19-CTB with primers specific for full-length CTB-p277 (negative control). T1 to T24, independent transgenic lines amplified with primers specific for full-length CTB-p277. WT, wild-type tobacco amplified with primers specific for full-length CTB-p277.

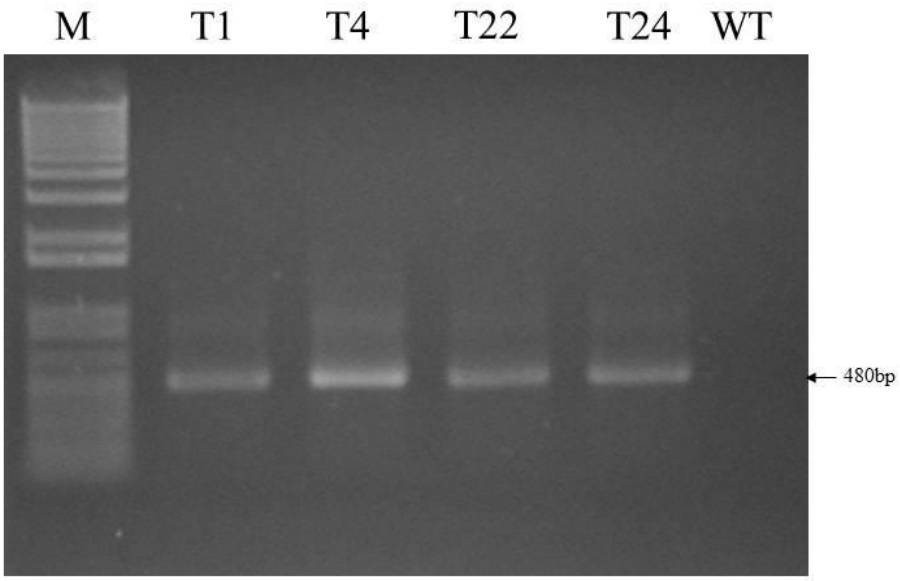

Figure 2.3 RT-PCR analysis of CTB-p277 transcripts in RNA from transgenic plants. Total RNA was extracted from leaf tissues of individual transgenic lines. RT-PCR reaction conditions were described in Methods. Primers used were the same as described above for PCR detection of full-length CTB-p277 from plant genomic DNA. M, molecular size marker; T1 to T4, independent transgenic lines; WT, wild-type tobacco.

performed on the RNA preparations. No amplified PCR products were seen under the same conditions (data not shown), confirming the specificity of the RT-PCR reaction. Sequencing of the RT-PCR products confirmed the correct full-length in-frame fusion of the CTB-p*277* fusion (Data not shown).

2.3.3 Accumulation of CTB-p277 fusion protein in transgenic tobacco plants

To examine the accumulation of CTB-p277 fusion protein in the transgenic plants, Western blot analysis was performed. Proteins extracted from leaves of the pRT-CTB-p277 tobacco plants were probed with anti-CTB polyclonal antibody (Fig. 2.4). The anti-CTB antibody detected a major band of 14 kDa, which is about 2 kDa larger than the control CTB in size, as well as two minor bands between 19 and 35 kDa when the samples were boiled prior to loading. The major band was consistent with the expected size of the monomeric form of CTB-p277, while the two minor bands may represent the dimeric and trimeric forms of CTB-p277, respectively. When the samples were loaded unboiled, a single 81-kDa band was seen in extracts from pRT-CTB-p277 tobacco plants but not from wild-type (Fig. 2.4). The 81-kDa band appears to correspond to pentameric CTB-p277. The presence of a single band suggests that the protein is stable and assembled as a biologically active CTB pentamer. The same bands could not be detected when the extracts were probed with anti-human hsp60 monoclonal antibody (data not shown). This may be due to the low affinity of the anti-human hsp60 antibody towards the peptide p277.

2.3.4 Quantification of CTB-p277 fusion protein in transgenic tobacco leaf tissues

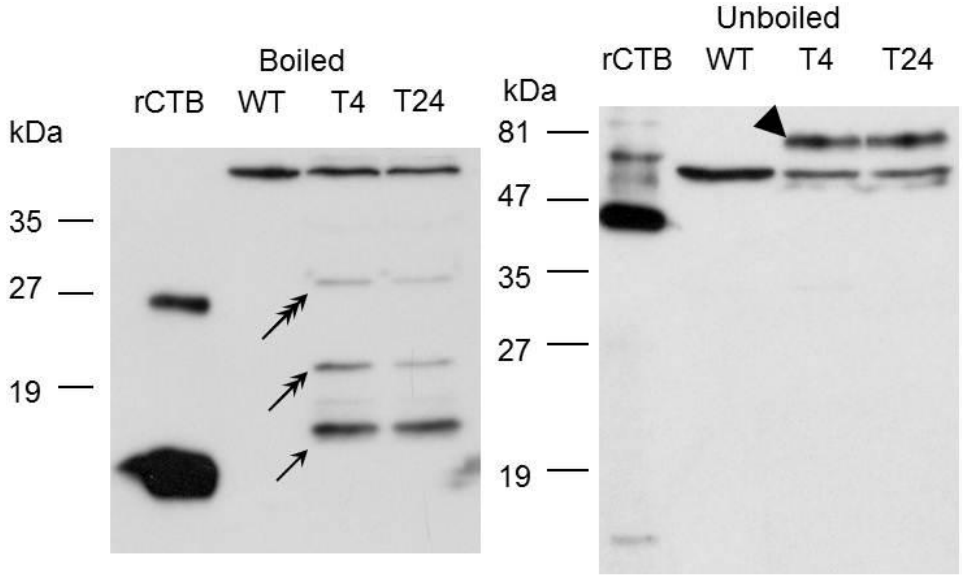

Figure 2.4 Western blot analysis of CTB-p277 fusion protein expression in transgenic plants. Total protein extracts (40 μg/lane) from leaf tissues of transgenic plants were fractionated by SDS-PAGE gel, blotted onto PVDF membrane, and probed with anti-CTB antibody. Both boiled and unboiled samples were analysed. T4 and T24 represent two individual transgenic lines; WT, wild-type tobacco; rCTB, recombinant bacterial CTB standard (Sigma). Bands for CTB-p277 monomer (single-headed arrow), dimer (double-headed arrow), trimer (triple-headed arrow) and pentamer (non-tailed arrow) are indicated. Numbers on the left indicate positions of protein size markers in kDa.

The levels of CTB-p277 fusion protein expression in transgenic leaf tissues were determined by ELISA. Using this method, the amount of plant CTB-p277 fusion protein was calculated by comparison of the optical density (OD) values obtained for transgenic extracts with the OD values for a known amount of bacterial CTB standard. The amount of the fusion protein was then expressed as a percentage of the total soluble plant protein (TSP). Representative data are shown in Fig. 2.5a. Transgenic line T4 showed the highest expression of CTB-p277 fusion protein, accounting for 0.1% of TSP.

2.3.5 GM1 receptor-binding assays of plant-derived CTB-p277 fusion protein

Biological functions of CTB, such as the ability to bind to GM1 ganglioside, depend on the formation of a pentameric structure composed of identical monomers (Hardy et al. 1988). To demonstrate that the plant-derived CTB-p277 fusion protein binds to GM1gangliosides, a GM1-ELISA was performed on the leaf extracts from transgenic line T4. The results show that both plant-derived fusion protein and commercial CTB bind gangliosides efficiently (Fig. 2.5b). No binding activity was detected when the plate was coated with irrelevant bovine serum albumin (BSA). These results further suggest that the CTB-p277 pentamer is required for biological activity. Thus, the addition of the p277 peptide to the C-terminus of CTB protein did not affect pentamerization or GM1 receptor binding of the protein.

2.4 Discussion

We report here the production of a fusion protein containing the anti-diabetic peptide p277 fused to the C-terminal of the B subunit of cholera toxin (CTB) in transgenic plants, a first step towards the development of a plant-based oral peptide vaccine for the treatment of Type 1 diabetes by inducing oral immune tolerance. The human hsp60 peptide p277 represents a functional important target in Type 1 diabetes, and injection of p277 has been shown to be very

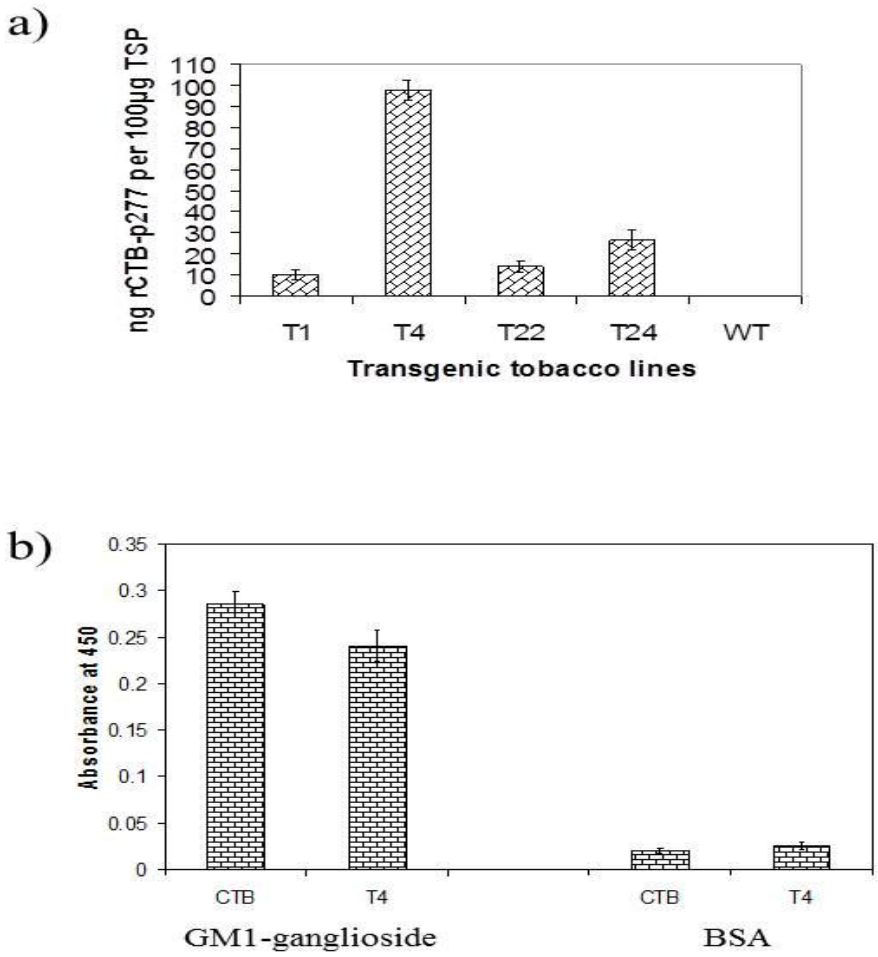

Figure 2.5 a) ELISA quantification of the CTB-p277 fusion protein. The amount of the CTB-p277 in total soluble protein (TSP) of transgenic tobacco leaf tissues was estimated by using ELISA as described in the Methods. The CTB-p277 fusion protein concentration was expressed as a percentage of TSP. Data shown here represent averages of three experiments. The numbers on the bottom of the figure represent the different tobacco lines. WT: wild-type tobacco. The error bar represents the standard deviation. b) GM1 binding analysis of plant-derived CTB-p277 fusion protein. The protein samples used in this assay were prepared from transgenic line T4. The amount of total protein added to the microplate well coated with GM1-ganglioside or BSA (control) was adjusted to obtain the concentration of CTB-p277 at proximately 8 ng/ml. The concentration of bacterial CTB used was 10 ng/ml. The absorbance of the GM1-ganglioside-CTB-p277 complex in each well was measured. The values represent the averages of three experiments. The error bar represents the standard deviation.

effective at treating Type 1 diabetes in both humans and NOD mice (Abulafia-Lapid et al. 1999). However, injection is not the preferred route of administration. Injections can cause discomfort and distress, and require trained people to administer it safely. This method also risks introducing harmful microbes into the patient's circulation system. Moreover, as most peptide drugs are chemically synthesized, the cost of their production is high and mass production would be difficult. Administration of p277 by the oral route offers practical advantages over parenteral administration: requiring neither sterile needles nor trained personnel, lower cost, increased quality of life, reduced side-effects, and greater patient acceptability and compliance. However, administration of therapeutic peptides by the oral route represents a major challenge. When administered orally, peptides are subjected to quick degradation by the enzymatic environments of the gastrointestinal tract and especially by the acidic environment of the stomach, thus requiring much higher amounts of peptide than subcutaneous injection. To address these limitations, we have therefore expressed p277 as a fusion with CTB. CTB is a highly stable protein, and has been shown to be an excellent oral delivery carrier and adjuvant for other peptide or protein antigens (Holmgren et al. 1993).

As the capacity of CTB as a mucosal adjuvant or carrier molecule for conjugated antigens is critically dependant on its ability to bind GM1 ganglioside in its pentameric form, it is essential to ensure that the CTB-p277 fusion protein retained the ability to form pentamers and bind the GM1 ganglioside receptor. Western blot analysis of unboiled transgenic leaf extracts with anti-CTB antibody showed that the fusion protein was apparently assembled into a pentameric structure (Fig. 2.4). Moreover, GM1-ELISA showed that plant-derived CTB-p277 binds to GM1-receptors as efficiently as the native CTB (Fig. 2.5B). These suggest that the p277 peptide carried by the fusion protein had no demonstrable adverse effect on assembly or GM1

binding of the parental CTB molecule. These results agree with our previous observations that genetic fusion of human insulin B-chain to the C-terminus of CTB had no detectable negative effect on pentamerization and GM1-binding capacity of CTB (Li et al. 2006a). Previous work by Liljeqvist et al. (1997) has suggested that pentamerization and GM1 binding capacity of CTB linked with proteins or peptides can be affected by the length of the partner protein or peptide, or by the conformational changes induced by the fusion partner. To minimize any possible negative perturbation effects of the fusion partner on the pentamer formation and ganglioside-binding capacity of CTB, the p277 was purposely fused to the C-terminus of CTB, as the GM1 binding properties of CTB does not critically depend on its C-terminal amino acid sequences (Zhang et al. 1995). Furthermore, a flexible hinge tetrapeptide (GPGP) was introduced between the CTB and p277 to reduce potential sterical hindrance and permit high intramolecular flexibility between the two partners of the fusion protein.

Analysis of boiled transgenic leaf extracts by Western blot using the same anti-CTB antibody revealed one specific band of about 14, and two bands between 19 to 35 kDa (Fig. 2.4). The 14-kDa band with the highest intensity was consistent with the expected size for the CTB-p277 monomer, whereas the two larger bands may correspond to the CTB-p277 dimer and trimer, respectively. The detection of both dimeric and trimeric forms of CTB-p277 in boiled samples suggests that the fusion protein is rather heat stable. The failure to detect the CTB-p277 fusion protein in the same transgenic leaf extracts by using a commercially available anti-human hsp60 monoclonal antibody (Clone 264233, R&D Systems) is not surprising. As this anti-human hsp60 antibody was raised against the whole hsp60 protein, it may not have a high binding affinity towards the peptide p277 that is only 24 amino acids in length. However, two lines of evidence support our conclusion that plant-derived fusion protein contains the amino acid

sequence of peptide p277. First, PCR amplification of the genomic DNA isolated from pRT-CTB-p277 transgenic tobacco lines resulted in an anticipated 480-bp DNA fragment, when primers specific for full-length CTB-p277 were used (Fig. 2.2, lanes T1 to T24). The same band was amplified when pRT-CTB-p277 plasmid DNA was a template using the same primer pair (positive control) (Fig. 2.2, lane B), whereas no PCR products were obtained when pCU19-CTB plasmid DNA was amplified with this primer pair (negative control, as this plasmid contains CTB without p277 sequence) (Fig. 2.2, lane C). On the other hand, an anticipated smaller DNA band (400 bp) was obtained when pRT-CTB-p277 plasmid DNA was amplified using primers specific for CTB but not for the fusion gene (Fig. 2.2, lane A), due to the presence of p277 sequence not being amplified. Secondly, RT-PCR amplification of total RNA isolated from these pRT-CTB-p277 transgenic tobacco lines also gave a 480-bp DNA product when the same CTB and p277 specific primer was used (Fig. 2.3), suggesting the expression of full-length CTB-p277 mRNA. As anticipated, RT-PCR amplification of total RNA isolated from wild type tobacco plants gave no product (Fig. 2.3). The in-frame fusion of CTB and p277 was further confirmed via cloning of the RT-PCR product and DNA sequencing (data not shown). Taken together, these results provide solid evidence that the fusion protein contains the amino acid sequence of p277.

The expression level of CTB-p277 fusion protein accounted approximately for 0.1% of total protein in tobacco leaf tissues (Fig. 2.4), which is higher than that reported for CTB or CTB fusion protein expressed in transgenic tobacco by other groups (Wang et al. 2001; Jani et al. 2004). This may be partly attributed to our use of a plant signal peptide to replace the native signal of CTB. We have previously shown that the use of a plant signal improves the expression of CTB in transgenic tobacco (Li et al. 2006a). It is anticipated that the current level of CTB-p277 expression can be further improved. One strategy is to express the fusion protein in the

chloroplast of tobacco plants. Daniell et al (2001) reported the expression of CTB in transgenic tobacco chloroplasts at levels up to 4.1 % of total soluble tobacco leaf protein. Another strategy is to express the CTB-p277 as an ubiquitin fusion. Recently, Mishra et al. (2006) demonstrated a significant increase in accumulation of CTB when it was expressed as an ubiquitin fusion in transgenic tobacco plants.

In summary, we have produced transgenic plants expressing CTB-p277 fusion protein. Functional analysis of the plant-derived fusion protein showed that it retains the biological and immunological characteristics of the native CTB including its ability to pentamerize and to bind to GM1 ganglioside receptor. Oral administration of transgenic plants expressing high levels of CTB-p277 may prove to be an effective method for inducing oral tolerance to treat Type 1 diabetes.

2.5 References

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Chapter 3

THE DEVELOPMENT OF A HIGH-YIELD RECOMBINANT PROTEIN BIOREACTOR THROUGH RNAi INDUCED KNOCKDOWN OF *ATP/ADP TRANSPORTER* IN *SOLANUM TUBEROSUM*

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3.1 Introduction

In recent years, there has been a marked increase in demand for recombinant protein therapeutics, including monoclonal antibodies and vaccines, largely due to a better understanding of disease pathogenesis and the identification of new molecular targets. On the other hand, it has become more apparent that the use of conventional cell culture-based expression systems, such as *E. coli and* mammalian cells, is difficult to meet the increasing need for therapeutic proteins, necessitating the development of alternative expression systems capable of satisfying this demand (Desai et al. 2010). Plants have emerged as a promising alternative to cell culture-based expression systems for recombinant protein production, offering simple, low-cost growth requirements, nearly unlimited scalability and a variety of different sub-cellular targeting options tailored for each protein's specific requirements, from protein storage vacuoles for cytotoxic compounds to high-yield non-glycoprotein production in chloroplasts (Tremblay et al. 2010b). Moreover, being eukaryotes, cells of plants can perform the complex post-translational modification and processing required by many mammalian therapeutic proteins for biological and/or immunological function.

Although many plants can be chosen as host species for the expression of foreign proteins, potato (*Solanum tuberosum*) offers a number of advantages as a bioreactor. First and foremost, potato tubers are natural long-term storage tissues, with a host of protease inhibitor complexes that have evolved to minimize degradation as the tubers lie dormant (Weeda et al. 2009). Indeed, it was shown that transgenic tubers synthesizing full-size IgGs or Fab fragments could be stored for up to 6 months without significant loss of antibody amount or activity (De Wilde et al. 2002). Potato also generates significant biomass compared to other plant production systems, with an average of over 30 tonnes per hectare according to Statistics Canada

(statcan.gc.ca). Potato is a vegetable crop with high nutritional value, making it attractive as a carrier for oral delivery of vaccines and possibly other biologics. There have been an increasing number of pharmaceutical proteins produced in potato, including cholera vaccines (Arakawa et al. 1997), human lactoferrin (Chong and Langridge 2000), interleukin-2 (Park and Cheong 2002), hepatitis B surface antigen (Richter et al. 2000), rotavirus capsid protein (Yu and Langridge 2003), foot and mouth disease antigen (Carrillo et al. 2001), and diabetic autoantigen (Ma et al. 1997). Although promising, relatively low-level foreign protein accumulation is a common problem in all plant systems, including potato. It is therefore essential to increase the efficiency of potato as a bioreactor.

Potato tubers develop at the terminal end of underground stems called stolons and are major carbon sinks in mature plants. Starch accounts for up to 70% of the dry weight of potato tubers (Oparka et al. 1990). Alteration of starch biosynthesis has been shown to affect other biosynthetic pathways in tuber development. For example, inhibition of starch synthesis through antisense inhibition of its one of the key enzymes, ADP-glucose pyrophosphorylase *(*AGPase), results in inhibition of patatin production and, in addition, affects the overall process of tuber formation, resulting in significantly more tubers per plant (Muller-Rober et al. 1992). Patatin is a plant storage glycoprotein accounting for up to 40% of the total soluble protein in potato tubers. Antisense inhibition of plastidic *ATP/ADP* transporter (*AATP1*), an adenylate transporter required for energy import in non-green plastids, results in a decrease in biomass and increased tuber number (Tjaden et al. 1998). More recently, however, Riewe and colleagues (2009) reported that altered ATP/ADP balance through the introduction of a plastidically targeted apyrase results in a 20 to 30% reduction in starch, but with a potential increase in biomass in lines with the lowest alteration in starch accumulation. Therefore, it is possible that a reduction

or inhibition of starch synthesis in tubers may provide a new effective strategy for enhancing the efficacy of potato plants as a bioreactor.

The goal of this research was to determine if potato tuber biomass and total protein levels could be increased through the disruption of starch synthesis, as we intend to develop new potato lines with improved efficiency for application as a molecular farming system. To this end, we utilized a hairpin RNA knockdown approach to disrupt the expression of platidic *AATP1* in order to generate a large number of putative transformants. We identified several *riAATP1* lines with increases in biomass compared to wild-type and selected a single line, designated riAATP1-10, for further study. The increase in biomass in riAATP1-10 was associated with both an increase in tuber number as well as in individual tuber size, and with less than a 15% reduction in starch compared to wild-type. Additionally, riAATP1-10 fresh tubers have an increased soluble protein concentration compared to wild-type tubers of similar size. We then tested the potential of riAATP1-10 for use as a new protein expression system. Here we show that when transformed with a construct containing the gene coding for a human scFv, riAATP1-10 line produced recombinant protein at expression levels over 0.5% of total soluble protein, a 2-fold increase over the highest accumulating line in a wild-type background. Together with increased biomass and increased levels in total protein content, foreign protein expression in riAATP1-10 line would translate, on the overall, into a nearly 4-fold increase in product yield. Our results indicate that riAATP1-10 line provides a superior system for recombinant production of foreign proteins, when potato is chosen as an expression system.

3.2 Materials and Methods

3.2.1 ATP/ADP Transporter RNAi construction

The theoretical gene structure of *S. tuberosum AATP1* was generated by aligning *aat1* mRNA (gi:4138582) with *Arabidopsis thaliana NUCLEOTIDE TRANSPORTER 1* (*NTT1*) gene (gi:844370). The RNAi construct was generated by cloning a portion of the predicted first exon of *AATP1*, from base pair 627 to 780 downstream of the predicted start codon, using the primers F1 F 5' TCTAGACCATGGTACAGCTTTTGCTGATAAGCTTCTC 3' with the restriction sites *XbaI*, *NcoI* and *HindIII* underlined, and F1 R 5' GAGCTCCTCGAGATTAGCAAATCCCCAAAAGAGTAC 3', with the restriction sites *SacI* and *XhoI* underlined. The intron from the Patatin gene was also cloned to use as an intron between the inverted repeat, using the primers PatF 5'GAATTCCCATGGCTCGAGGAATTGGGATCGTAAAAATATTTTG 3', with the restriction sites *EcoRI*, *NcoI* and *XhoI* underlined, and PatR 5' AAGCTTTCTAGAGAGCTCTTCGAACCCAATTTCCCAACTGACTCGAAAAGAAAAATA AGTCG 3', with the restriction sites *HindIII*, *XbaI* and *SacI* underlined. The restriction sites were added to each primer to facilitate the assembly of the inverted repeat. The gene fragments were cloned into pUC19, confirmed by sequence analysis, digested and ligated together and sequenced again to confirm the correct assembly of the inverted repeat. The entire cassette was then digest with *NcoI* and *XbaI* and ligated into pRTL2 in place of the GUS gene (Carrington and Freed 1990). The expression cassette was then digested with *HindIII* and ligated into pBI 101.1 to create pBI-F1IR. The pBI-F1IR was transferred to *Agrobacterium* via tri-parental mating (Ma et al., 2005).

3.2.2 scFv construct

The construct bearing the monoclonal antibody, pMB 5-61, was a gift from Klaus Döring (Axara Consulting, Frechen, Germany). The binary vector contained the monoclonal human scFv

L1G6 antibody with a C-terminal c-myc tag and a 6xHis tag and h*ygromycin B* as a selectable marker. The plasmid was provided in *Agrobacterium* and was used directly for plant transformation as described below.

3.2.3 Potato transformation and growth

Potato mini-tubers (var Désireé) were generated from sterile stocks as previously described (Bourque et al. 1987). Mini-tuber transformation was carried out using overnight cultures of *Agrobacterium* containing the pBI-F1IR plasmid or pMB 5-61 as previously described (Ma et al. 2005). Briefly, mini-tubers were peeled and cut into 5 mm discs. The discs where then submerged in MSO (4.4 g/L Murashige and Skoog basal media, 15 g/L Sucrose) in Petri plates. *Agrobacterium* cultures were centrifuged for 10 minutes at 1,000g in 1 mL aliquots, rinsed with MSO 4 times and then a single aliquot was added to the disc/MSO plates. After 30 minutes, discs were dried on sterile filter paper and placed on non-selective regeneration media (for 1 L add 4.4 g Murashige and Skoog basal media, 30 g Sucrose, 0.5 mg Nicotinic acid, 1 mg Thiamine HCl, 0.5 mg Pyridoxine, 8 g Phytagel, autoclave 20 minutes, add 2.5 µM Zeatin riboside, 4.8 µM IAA), covered in foil and incubated for 3 days in the growth chamber. Following incubation, discs were rinsed with MSO and transferred to regeneration media with the appropriate selective antibiotic (200 µg carbenicillin with either 50 µg/L kanamycin or 25 µg/L hygromcyin). Regenerating plantlets were transferred to Magenta boxes containing MS agar (4.4 g/L Murashige and Skoog basal media, 15 g/L Sucrose, 4 g/L Phytagel, pH 5.7) supplemented with 50 ug/L kanamycin for the pBI-F1IR selection, or 25 ug/L hygromycin for the scFv work. PCR was used to confirm successful transformation and positive plants were used to induce mini-tubers. Mini-tubers were screened via RT-PCR for the presence/absence of *aatp1* expression (described below) and those that lacked detectable levels were transplanted to soil

and transferred to the greenhouse along with wild-type desiree. Greenhouse potato plants were grown for ~3 months until the leaves began to die off, at which point tubers were harvested and either used immediately for experimentation or stored in the dark at room temperature for use as tuber seed stock. Each individual plant was harvested separately, with tuber number and biomass determined at the time of collection, as well as notation of changes in leaf scar number and photographs of any morphological differences compared to wild-type.

3.2.4 RT-PCR screening

Mini-tubers from independent transformants were screened for the expression of *aatp1* using RT-PCR. Total tuber RNA was extracted using the RNEasy Plant Mini Kit (Qiagen #74903) according to the manufacturer's instructions. RNA was converted into cDNA using Superscript II Reverse Transcriptase (Invitrogen #18064) according to the manufacturer's instructions. *aatp1* was amplified using primers designed to span the predicted first intron: F1 F (sequence above) and F2 R 5' CAACATCGATTGCTGCCTTCC 3'. The expression of *tubulin* was used as a control: TubF 5' AATGTGCAGAACAAGAACTCATCC 3' and TubR 5' CATAACAAGTTCACTTTGGCAG 3'. PCR conditions were 10 minutes at 94^{o}C, 30 or 37 cycles of 2 minutes at 94^{o}C, 30 seconds at 60^{o}C, 2 minutes at 72^{o}C, followed by 10 minutes at 72^{o}C.

3.2.5 Starch analysis

Starch quantification was carried using the Starch Assay Kit GO/P (Sigma, STA20-1KT) according to the manufacturer's instructions, with corn starch used as a control. Individual lines were compared against wild-type, with starch extracted from a transverse slice through peeled potatoes between 20 to 30 mm in length, with the exception of riAATP1-47 as there were no tubers over 15 mm, and each line was sampled in triplicate.

3.2.6 Protein extraction

Protein was extracted as previously described (Tremblay et al. 2010a). Briefly, potato leaf and tuber tissue was ground using a mortar and pestle with the addition of 3 times volume protein extraction buffer (200 mM Tris pH 8.0, 100 mM NaCl, 400 mM Sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 14 mM β-mercaptoethanol, 0.05% (v/v) Tween-20, 2 µg/mL leupeptin, 2 µg/mL aprotinin). The samples were incubated for 30 minutes on ice and spun at top speed in a micro-centrifuge for 10 minutes at 4^{o}C. The supernatant was collected and quantified for total soluble protein concentration using the Bradford method (Bradford 1976) in triplicate and subsequently used for protein analysis.

3.2.7 Protein analysis

Total soluble proteins were loaded onto a 12.5% (w/v) SDS-PAGE gel. Following separation, gels were either transferred to PVDF membrane for Western blotting or stained with Coomassie blue for protein profiling. For Western blot analysis of recombinant c-myc tagged scFv L1G6 expression, the blots were blocked with 5% milk (w/v) in TBS-T, washed 3 times in TBS-T for 5 minutes and then incubated with 1:1000 mouse anti c-myc (Santa Cruz Biotechnology Inc. #sc-40) in 1/3 block:2/3 wash buffer overnight at 4^{o}C. The blots were then washed for 3 times 10 minutes, and then treated with 1:5000 goat anti-mouse HRP for 1 hour at room temperature. The blots were then treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific #34080) and imaged using film.

3.2.8 scFv quantification

The amount of accumulated recombinant c-myc tagged human scFv L1G6 in *S tuberosum* tubers for both wild-type and riAATP1-10 was quantified by enzyme linked immunosorbent assay (ELISA). Briefly, commercially available c-myc protein standard (Abcam

#84132) and triplicate samples of untransformed wild-type (negative control), pMB 5-61 in wild-type or riAATP1-10 background were bound to 96 well plates by incubation in phosphate buffer overnight at 4^{o}C. The plates were washed with PBS-T and blocked with 3% BSA in PBS-T for 1 h at room temperature. The plates were then washed 3 times and incubated overnight at 4^{o}C in 1:2,000 mouse anti c-myc in ½ blocking:½ wash buffer. The plates were then washed 3 times and incubated for 1 h at room temperature with 1:5,000 goat anti-mouse-HRP in ½ blocking : ½ wash buffer and then rinsed 3 times. The plates were incubated with Substrate Reagent Pack (R&D Systems # DY999) according to the manufacturer's instructions. The color was developed for 20 min and then stopped with the addition of 2 M H_2SO_4. The plate was read using a Thermomax microplate reader (Molecular Devices, USA). Standard curves were calculated and used to determine scFv concentrations of the individual samples.

3.3 Results

3.3.1 Generation of knockdown AATP1 lines

A 150 bp sequence of the 3' end of the predicted first exon of *AATP1* gene, as determined through a comparison of the *aatp1* mRNA sequence with *Arabidopsis thaliana NTT1* genomic sequence, was selected and amplified by PCR (Figure 3.1a). A sense and antisense sequence of the cloned *AATP1* fragment were ligated with each other, separated by the first intron of the *patatin* gene from *S. tuberosum* and the expression was placed under the control of a constitutive 35S promoter (Figure 3.1b). The intron was included as this approach has been shown to be more efficient than either simple loop or directly linked inverted repeats (Wesley et al. 2001). Putative transformed plantlets were generated following tuber disc transformation via *Agrobacterium*, and transferred to rooting medium for shoot elongation and root development. Explants from *in vitro* grown plants were cultivated on microtuber inducing medium for the induction of minitubers. *In*

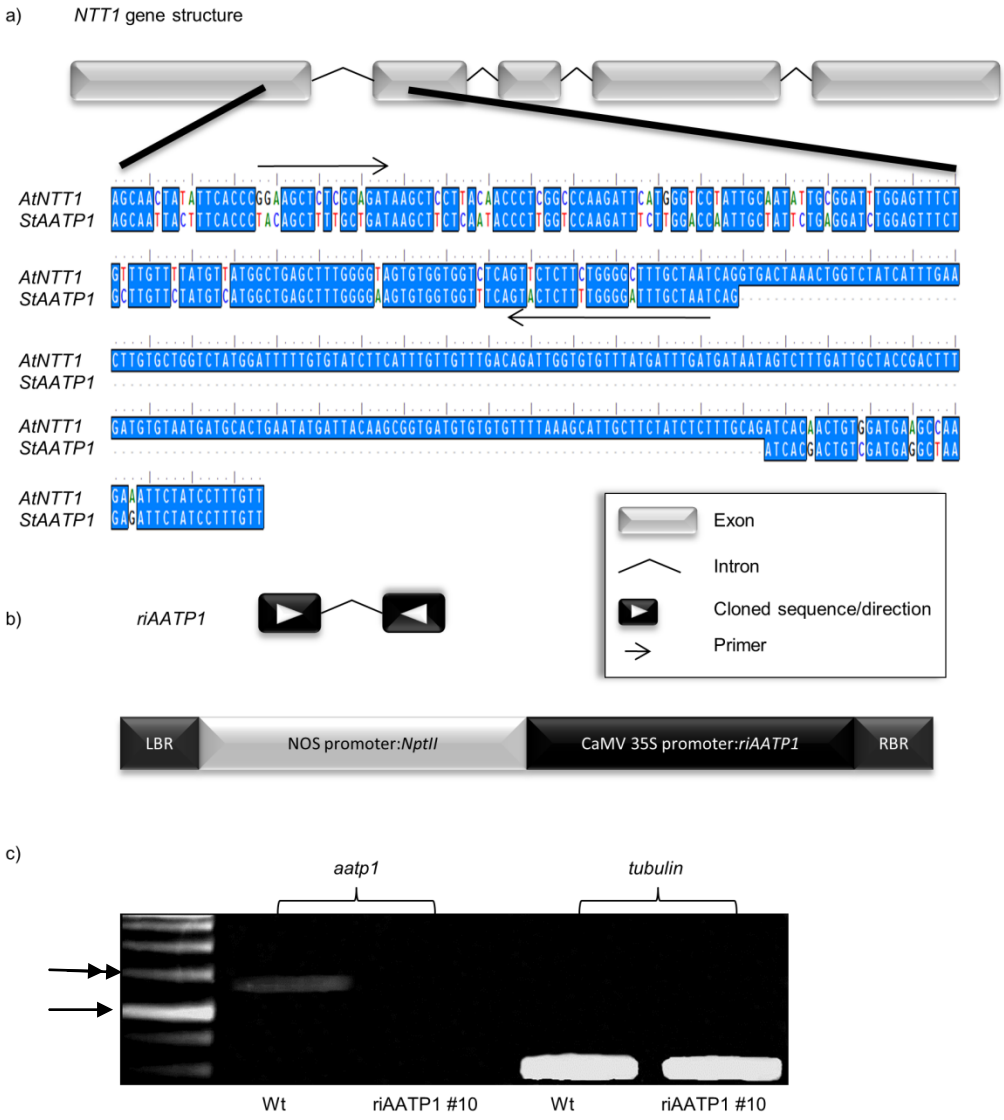

Figure 3.1: Construction of the riAATP1 knockdown lines. a) Alignment of *S. tuberosum aatp1* mRNA and the *A. thaliana NTT1* gene. Gene structure for *NTT1* is displayed at top. Arrows indicate position of primers used for amplification. b) The cloned *aatp1* sequence was ligated to the 1st intron from the *PATATIN* gene in both sense and antisense directions. The entire casette was cloned into the pBI expression vector, conferring kanamycin resistance in plants. c) RT-PCR to confirm *aatp1* knockdown. Expression of *tubulin* was used as a control for RNA quality and quantity. Single arrow and double arrow indicate positions of 500bp and 650bp marker bands, respectively.

vitro grown microtubers were screened via RT-PCR to confirm knockdown of *aatp1* expression (Figure 3.1c). Among over 60 independent transgenic lines screened, 47 lines showed an absence of *aatp1* expression following 30 PCR cycles, but in the majority of the samples screened, a faint product was detected following 37 cycles (data not shown). The 47 transgenic lines were then transferred to soil in the greenhouse and grown for 3 months to screen for changes in tuber biomass. There were no observable changes in above ground biomass accumulation or morphology.

3.3.2 riAATP1-10 has increased tuber biomass

Tubers were harvested following the senescence of above ground biomass, about 3 months post-transplantation. Each potato line was harvested independently and the fresh tuber weight was compared to highest accumulating wild-type control plant transferred to greenhouse at the same time (Figure 3.2a). Under identical growth conditions, 5 lines showed an increase in tuber biomass production compared to that of wild-type, with the transgenic line riAATP1-10 yielding the highest relative biomass. Tubers from riAATP1-10 were used as stock for a second 3 month growth cycle along with wild-type tubers. Each plant was harvested independently and the tubers weighed, with riAATP1-10 producing nearly 30% more biomass on average than wild-type, as well as a greater number of tubers (Figure 3.2b,c).

The increase in biomass was not strictly associated with increased tuber number, however, as when we examined the average biomass of the 4 largest tubers from both wild-type and riAATP1-10, we found significantly heavier tubers from riAATP1-10 plants (Figure 3.2d, $P<0.05$). This suggests that the increase in biomass is a combination of both increased tuber number as well as an overall increase in tuber size.

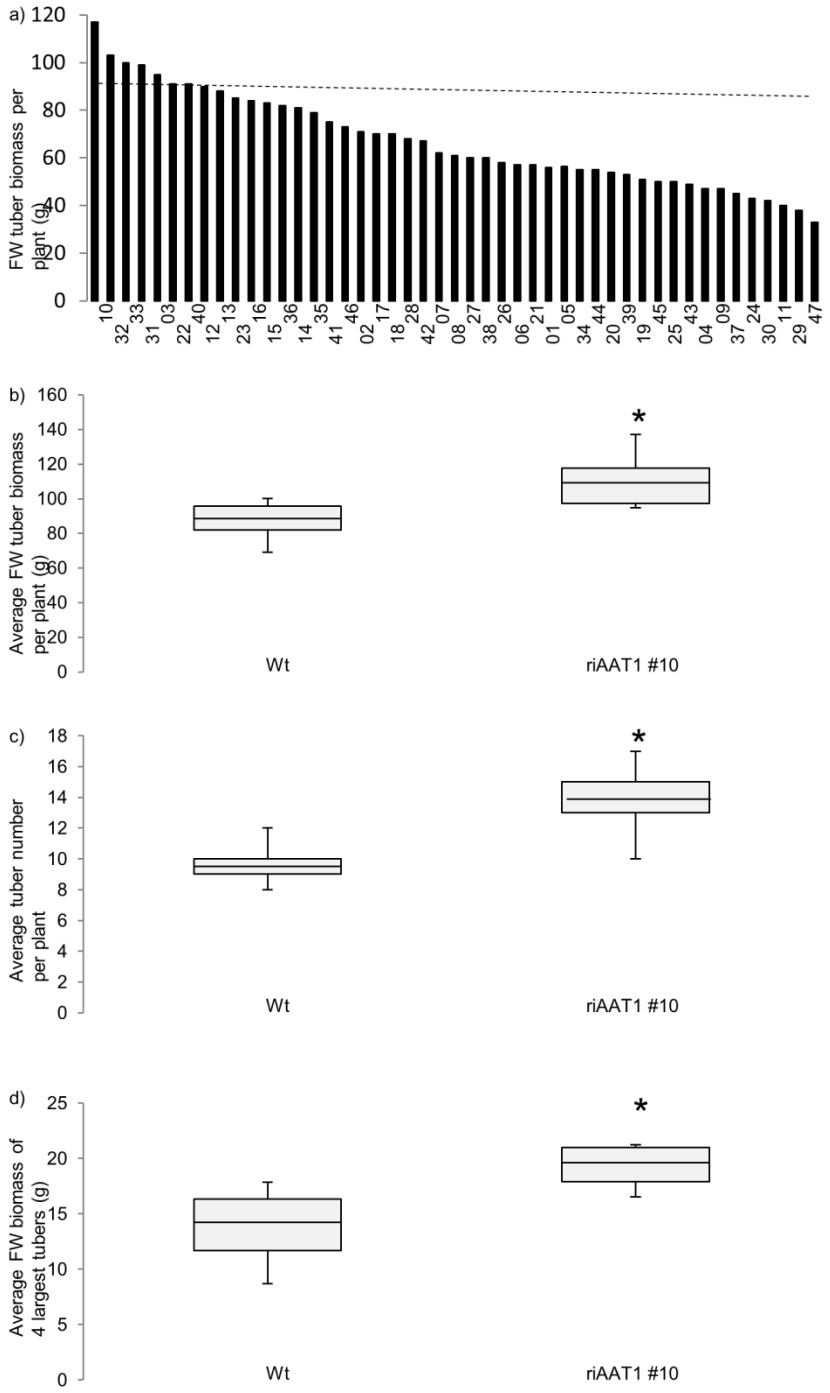

Figure 3.2: Biomass accumulation analysis. a) Tuber biomass accumulation of 47 independent riAATP1 lines. Dashed line represents the maximum accumulation of wild-type plants grown at the same time. b) Average tuber biomass accumulation of riAATP1-10 compared to wild-type (Wt). c) Average tuber number per plant for riAATP1-10 compared to wild-type. d) Average biomass for the 4 largest tubers from both riAATP1-10 and wild-type. * = significant difference ($p>0.05$), n = 6 (Wt), 5 (riAATP1-10) for b,c,d.

3.3.3 Morphological changes in riAATP1-10 tubers

Tubers were also markedly different in morphology compared to wild-type. As seen in figure 3.3a, the 4 largest riAATP1-10 tubers appear darker in colour, with a reddish hue compared to the largest 4 wild-type tubers. In addition, the tubers have an increased number of leaf scars, with nearly twice the number per tuber on average (Figure 3.3b). This trend holds true when tubers of identical length are examined (data not shown). As expected with an increase in biomass, there are differences in the tuber dimensions. The riAATP1-10 tubers are significantly longer than wild-type (Figure 3.3c) but have similar radial dimensions (data not shown). Indeed, regardless of the size of tuber examined, riAATP1-10 tubers are typically longer than wild-type tubers with similar radial dimensions. Conversely, tubers from the lowest biomass line, riAATP1-47, had a greater number of smaller tubers, with each tuber have multiple tuber nodules forming at within each leaf scar (data not shown).

3.3.4 riAATP1-10 has decreased starch

The plastidic *AATP1* is involved in starch production through the importat of ATP into heterotrophic plastids (Emes and Neuhaus 1997). Previous knockdown of *AATP1* using antisense approach resulted in a range of starch reduction, with a 20% reduction resulting in slightly lower biomass than wild-type and nearly 50% less starch yielding nearly 25% less biomass, although this study was limited to 4 individual transformants (Tjaden et al., 1998). As riAATP1-10 had an increase in biomass, we examined the quantity of starch in this line order to determine if starch level is disrupted, and to what degree. Starch extraction and quantification of riAATP1-10 tubers showed a 15% reduction in starch levels compared to wild-type tubers (Figure 3.4).

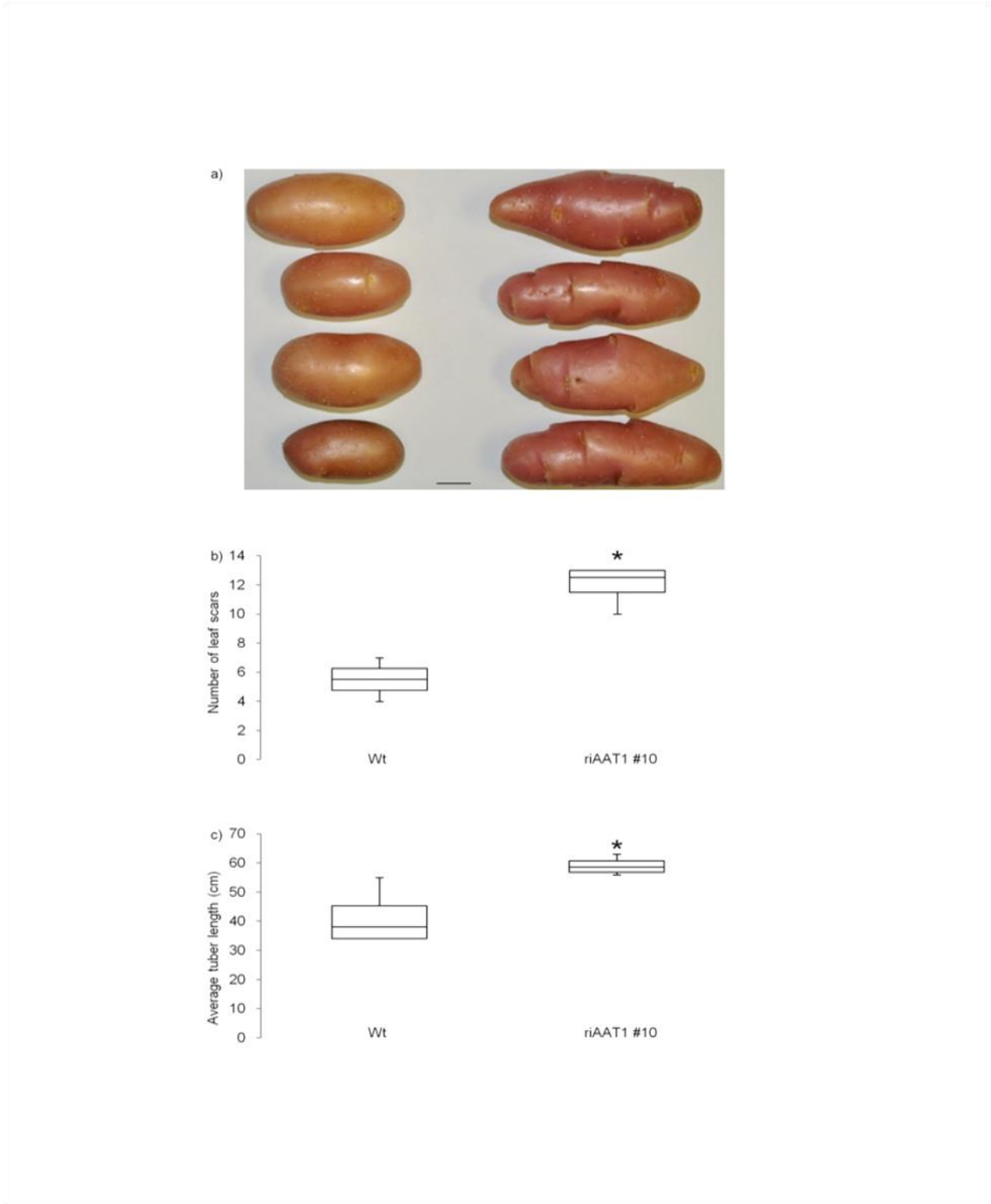

Figure 3.3: Morphological changes in riAATP1-10. a) Picture of the 4 largest wild-type tubers (left) and riAATP1-10 (right). Bar = 1 cm. b) Box plot of the number of leaf scars per tuber for the 4 largest wild-type (Wt) and riAATP1-10 line. c) Box plot of average tuber length for 4 largest tubers from wild-type and riAATP1-10 line. * = significant difference ($p>0.05$).

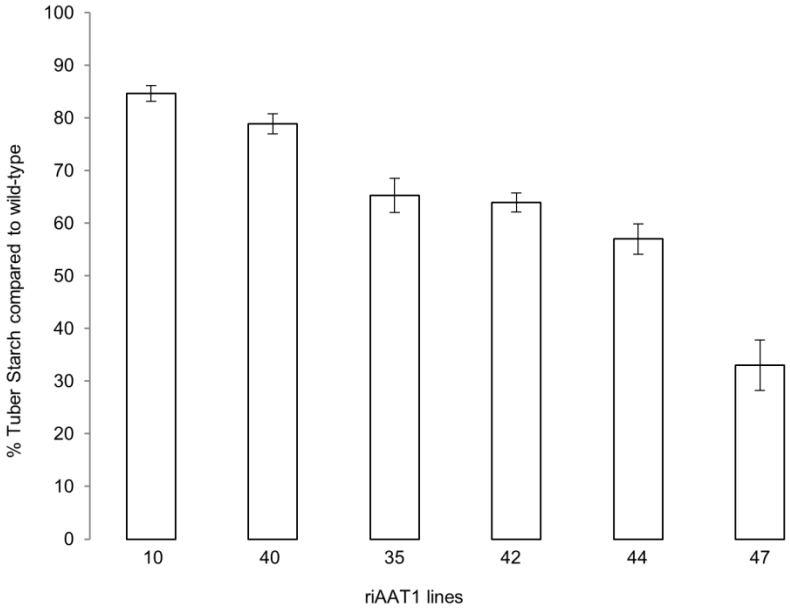

Figure 3.4: Starch accumulation of riAATP lines. Accumulation is present as percent of starch accumulation against the average accumulation of wild-type tubers from the same experiment (n=3, ± S.E.M.).

3.3.5 riAATP1-10 has an altered protein profile

We next examined the protein profile of tubers derived from riAATP1-10 potato line in comparison to wild-type. As shown in Figure 3.5a, notably altered are the bands that correspond to the expected size for patatin, which can accounts for up to 40% of the total soluble protein in potato tubers, or for PKPI, a 20 kDa potato Kunitz-type protease inhibitor (Weeda et al. 2009). There was also a protein band for an unknown protein(s) in riAATP1-10 lines that appears stronger than wild-type. We also noted similar alterations in the protein profile of the other riAATP1 knockdown lines (data not shown). Further differences were seen in between wild-type and riAATP1-10 line when we examined the amount of soluble protein per tuber. There was no statistical difference in protein concentration between either wild-type or riAATP1-10 in tubers between 5 and 10 mm in length (figure 3.5b). Soluble protein concentration remains similar for riAATP1-10 lines regardless of tubers size while the concentration in wild-type tubers decreases by an average of 34% as the tubers increase to over 30 mm in length ($p>0.05$). We also examined the effect of cold storage on the stability of the soluble proteins. riAATP1-10 tubers have nearly 50% less protein on average after 5 months of cold storage, while wild-type lost less than 10% on average over the same time period (Figure 3.5c).

3.3.6 riAATP1-10 accumulates more recombinant scFv than wild-type

We examined the potential uses of riAATP1-10 as a new potato line to improve recombinant protein expression due to its increased biomass accumulation and soluble protein concentration. We selected a human scFv for testing as monoclonal antibodies represent an increasingly important class of therapeutic agents that are in high demand. To this end, we transformed both wild-type and riAATP1-10 with human L1G6 scFv (Figure 3.6a). Initial screening was performed on the leaf tissue, but there was no difference in maximal accumulation

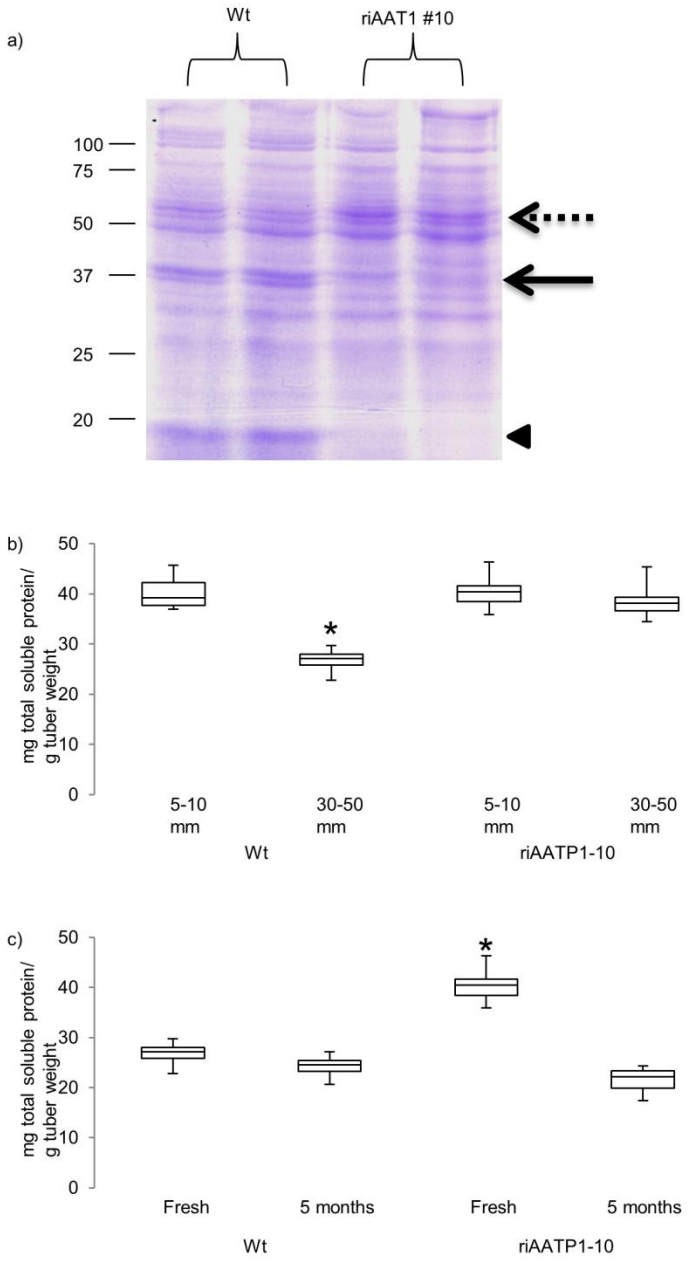

Figure 3.5: Protein accumulation in tuber lines. a) Protein profile of tubers from 3 wild-type and 3 riAATP1-10 plants. Numbers on left indicate protein standard sizes (kDa); arrow/arrowheads mark major protein band differences between wild-type and riAATP1-10. Arrow indicates position of the expected size for the patatin protein; arrowhead for the expected size for the PKPI protease inhibitor; dashed arrow indicates unknown protein. 20µg of TSP loaded per lane b) Comparison of concentration of total soluble protein from freshly harvest tubers with lengths between 5 and 10 mm or 30 to 50 mm (n=10 each). c) Comparison of concentration of total soluble protein from 30-50 mm tubers either post-harvest (fresh) and after 5 months cold storage (5 months) (n=10 each). * = significant difference ($p>0.05$).

between the wild-type and riAATP1-10 backgrounds (Figure 3.6b). Following the screening of 72 transformants in the wild-type background, we found a maximal accumulation of the antibody in the tubers of 0.25% of TSP (Figure 3.6c,d). We screened 20 individual transformants in the riAATP1-10 background and found that in addition to an increase in maximal accumulation, with one line yielding a 1-fold increase to over 0.5% of TSP (Figure 3.6c,d). In addition, the range of scFv accumulation in riAATP1-10 lines was found to be higher overall (Figure 3.6e).

3.4 Discussion

We have demonstrated that through the disruption of starch synthesis it is possible to increase the efficiency of recombinant protein production in potato tubers. The disruption of starch synthesis was achieved by using RNAi technology to knock down the activity of the plastidic ATP/ADP transporter AATP1. Using this technology, we were able to generate a much larger number of AATP1 knockdown transformants than previously possible when antisense methods were used, and the resulting screen yielded a variety of biomass accumulation. One such line, riAATP1-10, produces nearly 30% more biomass per plant with nearly 50% more soluble proteins in larger tubers, and accumulated just over 100% more recombinant scFV antibody as a percent of TSP compared to wild-type. Together with increased biomass yield and increased levels in total protein content, there is potential for a nearly 4-fold increase in recombinant protein production.

Perturbation of starch synthesis can have profound and drastic impacts on tuber development, as the degree of alteration of the carbon balance between starch and soluble sugars is important in maintaining tuber sink strength (Oparka et al. 1990). When starch synthesis is almost completely abolished, tubers have a drastic increase in soluble sugars and each plant produced many more tubers but had significantly lower overall tuber biomass per plant compared to wild-type (Muller-Rober et al. 1992). Previously reported knockdown of *AATP1*

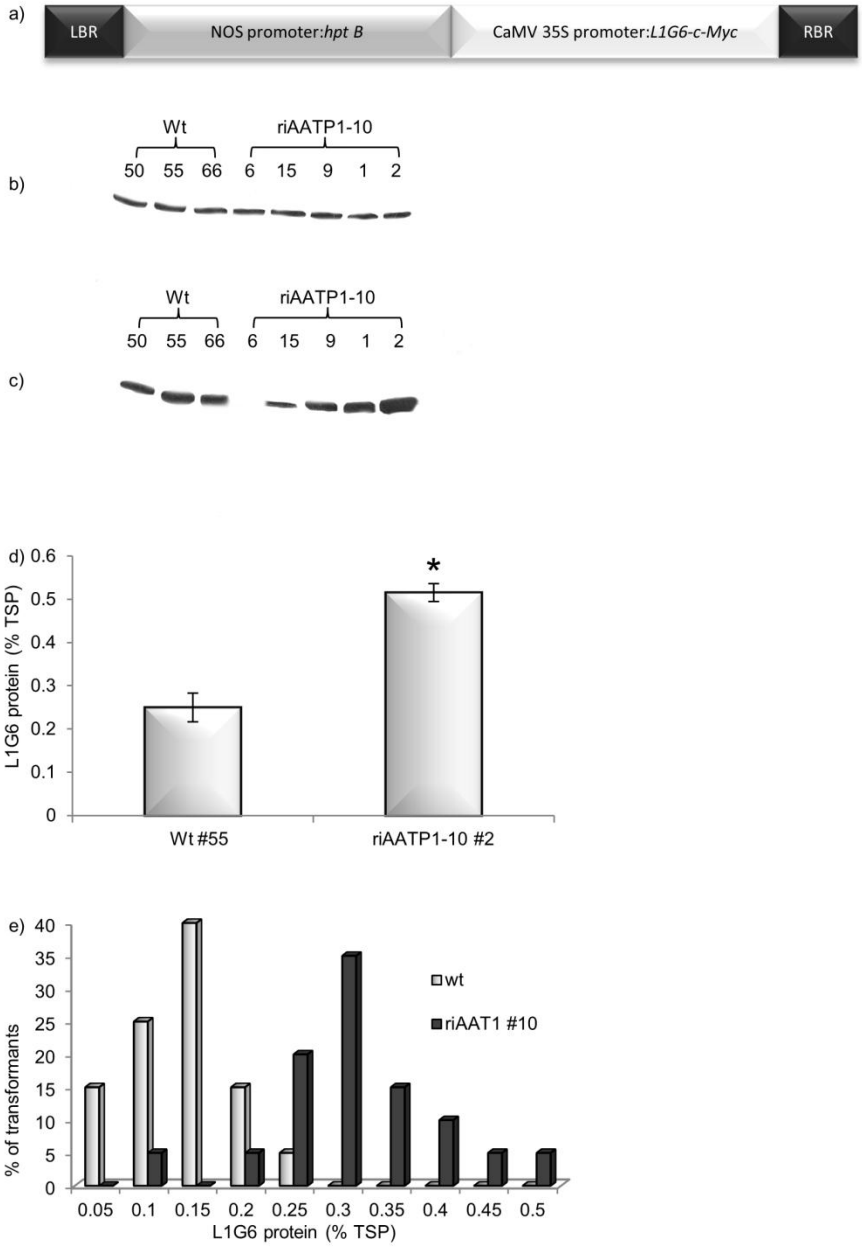

Figure 3.6: Comparison of scFv production in wild-type versus riAATP1-10. a) Expression cassette for scFv L1G6 under the control of a 35S promoter and with a linked c-Myc tag. The *hpt-B* gene was included to allow for hygromycin selection. b) Western blot of expression of L1G6 in leaves of top expressing wild-type backgrounds and a range of riAATP1-10 plants (20 µg TSP loaded). c) Western blot of expression of L1G6 in tubers of top expressing wild-type backgrounds and a range of riAATP1-10 lines (20 µg TSP loaded). d) L1G6 accumulation in top wild-type and riAATP1-10 lines (± S.E.M., * = significant difference ($p>0.05$)). e) Diagram of scFv accumulation levels in wild-type and riAATP1-10. The range of plant accumulations are divided into 0.05% of TSP intervals, with the y-axis representing the percentage of plants within that interval. n=72, 20 for wild-type and riAATP1-10, respectively.

using antisense methods resulted in a less drastic decrease in starch content, with up to a 40% reduction in amylose, but as with starch abolishment, researchers also noticed an increase in the number of tubers and decrease in overall weight compared to wild-type (Tjaden et al. 1998). However, relatively few lines were screened and with the primary focus on starch quantification. In our work, we focused instead on biomass and screened a large number of individual transformants. We likewise noted an increase in tuber number and typically lower biomass accumulation in about 80% of the lines. Several lines, however, demonstrated an increase in biomass during the initial screening, with riAATP1-10 having the greatest increase in biomass compared to wild-type. In riAATP1-10, we noted a decrease of nearly 15% in starch accumulation, while those lines with biomass lower than wild-type had significantly greater starch disruption. This suggests that there may be a threshold for perturbation of the balance between starch and soluble sugar accumulation, where small reductions in starch production are sufficient to maintain sink strength while at the same time allowing for higher biomass accumulation due to the increase in available energy in the form of soluble sugars.

The increase in tuber biomass for riAATP1-10 appears to be a combination of both an increase in tuber number and individual tuber biomass. When we examined the four largest tubers from wild-type and riAATP1-10, we found that the increase in tuber biomass appears linked with an increase in tuber length as opposed to radial width, as the largest tubers all had very similar radial dimensions, regardless of whether wild-type or riAATP1-10. When examining the protein expression profile of the transgenic tubers, the reduction in bands corresponding to patatin and to the protease inhibitor complex appears similar to previous knockdown of AGPase, although in that case the disruption of starch was significantly more

severe, as was the resulting protein profile disruption (Muller-Rober et al. 1992). Based on this profile, it is possible that the tubers are remaining in a post-initiation stage of development. This can be seen when comparing the protein profile we observed with previously published reports demonstrating the initiation of patatin and protease inhibitors produced during development (Weeda et al. 2009). The protein profile for riAATP1-10 is reminiscent of early stage tuber development, just prior to tuber bulking when wild-type tubers begin to accumulate higher percentages of patatin and various protease inhibitors.

Soluble protein production in wild-type tubers of *S. tuberosum* declines as tubers enter the bulking stage of development (Weeda et al. 2009). At the same time, proteolytic degradation declines as tubers increase in size, with large tubers having over 50% of soluble protein committed to a variety of protease inhibitors. This increase in protease inhibitors is one potential reason for the high stability of recombinant proteins produced in tubers, even months post-harvest (De Wilde et al. 2002). We found a similar decrease in soluble protein in our wild-type tubers, with the largest tubers having 34% lower protein concentration than the smallest tubers examined. In opposition to this, riAATP1-10 tubers retained the same protein concentration through tuber expansion, with no difference between small and large tubers. The lower quantities of the protease inhibitors found in large riAATP1-10 tubers do appear to have a negative impact on long-term storage of riAATP1-10 tubers. After 5 months of storage, we found nearly half the amount of soluble protein in large riAATP1-10 tubers, while wild-type lost a small fraction of this amount. The higher rate of protein turnover in riAATP1-10 may thus make it unsuitable for long-term storage, although this should not present a major problem for its use as a bioreactor, as typical post-harvest processing would take place with minimal delay, unlike post-harvest consumption of potato as a food staple.

The level of recombinant scFv produced in the riAATP1-10 line is significantly higher than that achieved in the wild-type background. While the level of expression, just over 0.5% of total soluble protein, is significantly less than that for production in high yield chloroplast-expression systems, this system offers a number of significant advantages. While reports have claimed accumulation of over 50% TSP for recombinant production, they are unable to produce glycoproteins (Cardi et al. 2010). While the requirements for glycosylation vary from glycoprotein to glycoprotein, they typically are important, at least in part, for protein stability and resistance to degradation. Genomic expression in eukaryotic potato tubers can achieve significant recombinant glycoprotein yields.

In summary, we have developed riAATP1-10 as a new potato line for use as a molecular farming system, offering combined advantages of increased biomass yield and increased total protein levels. We have demonstrated its efficacy as well as its potential value in expressing a human scFv antibody. It is expected that the riAATP1-10 line will also be highly valuable for producing many other therapeutic proteins.

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CHAPTER 4

HIGH-YIELD EXPRESSION OF RECOMBINANT SOYBEAN AGGLUTININ IN PLANTS USING TRANSIENT AND STABLE SYSTEMS

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A version of this chapter has been published in Transgenic Research. Permission has been granted for inclusion of the article "High-yield expression of recombinant soybean agglutinin in plants using transient and stable systems", published in Transgenic Research 20 (2):345-56 in this thesis (See Appendix 1).

4.4 Introduction

Soybean agglutinin (SBA) is a legume lectin glycoprotein that binds non-covalently to specific cell surface carbohydrates, provoking agglutination of the bound cells when in solution. SBA has been used to fractionate different cell types for use in clinical and biomedical applications. One such application is the separation of pluripotent stem cells from human bone marrow. Cells fractionated by SBA do not produce graft versus host disease (GVHD) and can be used in bone marrow transplantation across histocompatibility barriers (Yura et al. 2008). Another application is the enrichment and isolation of fetal cells from the blood of pregnant women as a means to detect fetal genetic and chromosomal abnormalities. It was shown that the number of fetal erythroblasts recovered by a soybean agglutinin galactose-specific lectin method was approximately eightfold higher than the number obtained by a standardized magnetic cell-sorting (MACS) protocol (Babochkina et al. 2005). In addition, SBA binds very effectively to certain tumour cells and has been used to detect and treat several cancers including breast cancer (Pusztai et al. 2008). SBA and other lectins, which are carbohydrate-binding proteins that bind sugar residues reversibly and specifically, have also been exploited as a ligand for targeted oral drug delivery as their glycoprotein and glycolipid targets are integral parts of the enterocyte membrane (Smart 2004). Protocols for high recovery and purity of SBA have been developed, with extraction of SBA from soybean flour resulting in high quality purification with over 90% yield (Percin et al. 2009), opening the door for the use of recombinant SBA as a potential novel affinity tag for purification of genetically fused proteins. The potential production of a variety of recombinant proteins genetically fused to SBA, however, necessitates the generation of an efficient recombinant production system.

Native SBA is produced in the developing seeds of *Glycine max*, with maximal production in seeds reaching over 2% TSP (Lindstrom et al. 1990). It is relatively simple to obtain large amounts of purified native SBA given that soybean seeds express high levels of native SBA together with the establishment of efficient purification methods. However, many potential applications of SBA in biotechnology and medicine could not be achieved or are difficult to achieve with the use of unmodified native SBA. For example, it is difficult to use native SBA as an affinity tag for protein purification, unless the target protein is genetically fused to SBA and produced as a recombinant fusion protein. SBA has previously been expressed in transgenic tobacco seeds in order to study the upstream and downstream cis-regulatory sequences mediating the expression of native SBA (Lindstrom et al. 1990). Although a protein band with a molecular mass expected for the monomer of SBA protein was seen on Western blot, no analysis or characterization of the recombinant protein itself was performed, merely using it as a marker for gene expression. Others have successfully expressed SBA in both *E. coli* and monkey BS-C-1 cells in an attempt to generate recombinant SBA in order to analyze the relationship between glycosylation and tetramer assembly (Adar et al. 1997). They demonstrated that the recombinant SBA retained its agglutination ability and specificity for N-acetylgalactosamine. The SBA generated in bacteria, however, lacks glycosylation that, while not required for agglutination or assembly, is important in the stability of the bioactive tetramer (Sinha and Surolia 2005). Production in BS-C-1 cells resulted in a similarly glycosylated protein to native SBA with an identical sugar binding profile but for some unknown reason had a lower agglutination ability. The minimal amount of BS-C-1 cell derived SBA required to induce hemagglutination is 10-20 µg/ml, which is 4-5 times larger than that of native SBA. In addition, for production of a therapeutic protein, animal cells carry an inherent risk of pathogen

contamination and have a high production cost due in large part to media costs. Therefore, both bacteria and mammalian cells are not ideal bioreactors for the SBA production for therapeutic uses.

Plants offer a suitable alternative expression system for recombinant SBA production. As bioreactors, plants permit unlimited scalability, elimination of product contamination by mammalian pathogens, as well as reduced production costs compared to microbial or animal cell-based systems (Ma et al. 2008; Boehm 2007; Tremblay et al. 2010b). Due to their eukaryotic nature, plants can perform the complex post-translational modification and processing required by many transgenic therapeutic proteins for biological and/or immunological function. Furthermore, plant bioreactors offer the short turn-around time to obtain gram quantities of a recombinant protein in a matter of weeks when expressed transiently. This is not only economically advantageous, but is also critical to meet challenges related to quick access to life-saving biotechnology drugs and therapies. In addition, stable edible transgenic plant tissue offers the possibility of allowing direct oral delivery of plant-derived therapeutic proteins and peptides, eliminating the need for expensive downstream protein purification and processing.

Our long-term goal is to generate recombinant SBA for use as a carrier molecule for oral delivery of protein and peptide drugs and as an affinity tag for simplified and high-yield, high purity isolation of recombinant proteins. As a first step toward these goals, we report here the transient production of recombinant SBA (rSBA) in *Nicotiana benthamiana* (Nb), a close relative of tobacco (*Nicotiana tabacum*), and stable production in *Solanum tuberosum* (St) under the control of a ubiquitous CaMV35S promoter. We demonstrate transient expression of SBA in Nb plants at levels as high as 4% of total soluble protein (TSP), whereas its stable expression in St tubers reaches 0.3% TSP. Furthermore, NbrSBA and StrSBA are similarly glycosylated

compared to native SBA, retain their ability to induce hemagglutination, bind specifically to N-acetylgalactosamine, are stable in simulated gastric fluid (SGF) containing pepsin at acidic pH and are rapidly isolated to a high degree of purity from total soluble protein.

4.2 Materials and Methods

4.2.1 Plasmid construction

The cDNA of soybean agglutinin (SBA) was cloned from *Glycine max* cDNA derived from 1-5mm developing seeds. In brief, total messenger RNA was extracted from the seeds using the RNeasy Plant Mini Kit (QIAGEN #74903) and converted to cDNA following the Superscript II protocol (Invitrogen #18064). The primers F 5' AATCCATGGCTACTTCAAAGTTGAAAACC 3' and R 5' TCTAGATTAATGATGATGATGATGATGGATGGCCTCATGCAACACAAAACTTG 3' with the addition of a 6XHis-tag to the C-terminus underlined, restriction sites bolded and a silent mutation to remove an internal HindIII site italicized, were constructed using the previously published sequence for SBA (Genbank Accession #K00821.1). The generated SBA cDNA was then inserted into pUC-19. After confirmation by sequence analysis, the SBA cDNA was inserted into pRTL-2 via digestion with *NcoI* and *XbaI*, replacing the GUS gene, providing a 35S promoter, 5' and 3' UTR (Carrington and Freed 1990). The resulting expression cassette was digested with *HindIII* and inserted into pBI-101 to create pBI-rSBA. Tri-parental mating was used to transfer pBI-rSBA to *Agrobacterium tumifaciens* strain LBA4404 and confirmed via PCR using specific primers.

4.2.2 Transient expression of SBA in *N. benthamiana*

Transient expression of pBI-rSBA was accomplished through infection of 6-8 week old leaves of *Nicotiana benthamiana* as described in (Sparkes et al. 2006). Briefly, overnight

cultures of *Agrobacterium* were grown until cell density reached 0.5-1.0 OD_{600}, at which time the cells were centrifuged at 800xg for 10 minutes, rinsed 4 times with infiltration media (0.5% w/v D-glucose, 50mM MES, 2mM Na3PO4, 0.0001M Acetosyringone) and then resuspended to the desired cell density, between 0.01 to 0.75 OD_{600}. The cells were then infiltrated into the abaxial side of the leaves using a 1mL syringe and infected tissue was harvested each morning at day 1 through 9 post-infection. For co-infiltration with a second *Agrobacterium* harboring the T-DNA vector encoding the p19 suppressor gene of tomato bushy stunt virus (Lakatos et al. 2004), the two agrobacterial cultures were mixed at equal concentration prior to infiltration.

4.2.3 Stable expression of SBA in *S. tuberosum*

Solanum tuberosum mini-tubers were generated as described previously (Bourque et al. 1987). Overnight cultures of *Agrobacterium* with pBI-rSBA were used to transform tubers as previously described (Ma et al. 2005). Regenerating plantlets were transferred to magenta boxes containing MS media supplemented with 50µg/L kanamycin. The presence of the transgene in transgenic plants was confirmed by PCR, and PCR-positive plants were then selected to produce mini tubers for protein expression analysis.

For mini tuber induction, a stem section (1 cm) with one resting auxiliary bud and one fully developed leaf was excised from a sterile magenta-grown transgenic plant. The leaf was removed and the stem was transferred to tuber inducing medium as described above and placed in darkness at 20°C. After 4 weeks, the formed tubers (3 mm in diameter) were harvested and used for protein analysis.

4.2.4 Accumulation of rSBA in *Nicotiana benthamiana* and potato plants

Total soluble protein was extracted from infected *N. benthamiana* leaf tissue or potato tuber and then quantified as described previously (Tremblay et al. 2008). Briefly, approximately

100mg of plant material, either *Solanum tuberosum* tuber or *Nicotiana benthamiana* leaf, was ground in 1.5mL tube with a plastic pestle and mixed with protein extraction buffer (200 mM Tris pH8.0, 100 mM NaCl, 400 mM Sucrose, 1 mM phenyl-methylsulfonyl fluoride, 10 mM EDTA, 14 mM ß-mercaptoethanol, 0.05% v/v Tween-20, 2 µg/ml leupeptin, 2 µg/ml aprotinin). The mixture was incubated on ice for 30 minutes and clarified by centrifugation at 10,000xg for 10 minutes at 4^{o}C. The supernatant was transferred to a new 1.5mL tube and used for all subsequent assays. Protein samples, boiled or unboiled, were loaded on to a 12.5% (w/v) SDS-PAGE gel and resolved. Gels were then either stained with Coomassie Blue to visualize protein profile or transferred to PVDF membrane as described previously (Tremblay et al. 2008). The blot was then blocked for 1 hour at room temperature with 5% w/v milk in TBS-T and washed 3 times 5 minutes with TBS-T. The blots were incubated overnight at 4^{o}C in 1:2000 rabbit anti-SBA (Cedarlane AL-1301-2) in 1/3 blocking buffer 2/3 wash buffer. The blots were washed 3 times for 10 minutes in wash buffer and incubated in 1:5000 goat anti-rabbit-HRP (G-7641, Sigma) for 1 hour at room temperature. The blots were washed 3 times for 10 minutes in wash buffer and then incubated with SuperSignal® West Pico Chemiluminescent Substrate (34080, Pierce, Rockford, IL). Blots were exposed and then developed using a film processor.

4.2.5 Quantification of plant-derived rSBA

The amount of accumulated rSBA in *Nicotiana benthamiana* leaves or *Solanum tuberosum* tubers was quantified by enzyme linked immunosorbent assay (ELISA). Briefly, commercial native SBA standard (Sigma L-1395) and triplicate NtrSBA or StrSBA samples were bound to 96 well plates by incubation in phosphate buffer overnight at 4^{0}C. The plates were then washed with PBS-T and blocked with 3% (w/v) BSA in PBS-T for 1 hour at room temperature. The plates were washed 3 times and then incubated overnight at 4^{o}C in 1:2000

rabbit anti-SBA in 1/2 blocking:1/2 wash buffer. The plates were washed 3 times and incubated for 1 hour at room temperature with 1:5000 goat anti-rabbit-HRP in 1/2 blocking:1/2 wash buffer and then rinsed 3 times. The plates were incubated with Substrate Reagent Pack (DY999, R&D Systems) according to the manufacturer's instructions. The color was developed for 20 minutes and then stopped with 1 times volume 2M H_2SO_4. The plate was then read using a Thermomax microplate reader (Molecular Devices, USA). Standard curves were calculated and used to determine protein concentrations of the individual samples. Negative control was protein extract prepared from wild-type plant tissue.

4.2.6 Purification of plant-derived rSBA

Purification of rSBA from *Nicotiana benthamiana* or potato tubers was carried out according to the protocol for GE Healthcare HisTrap HP column (#17-5248-01) or using an N-acetylgalactosamine-agarose column. For purification with the N-acetylgalactosamine-agarose column, agarose columns with pre-bound N-acetylgalactosamine (Sigma A2787-5mL) were washed and equilibrated with 0.1M NaCl. The TSP containing rSBA was then applied to the column and rinsed with excess 0.1M NaCl. Samples were taken throughout rinsing and total protein in rinse was determined by spectrophotometry at A_{280}. Once protein levels were negligible, rSBA was eluted with 0.5M galactose/0.1M NaCl. The purified protein was confirmed via Western blot and then desalted via dialysis against excess 0.5xPBS buffer.

4.2.7 Hemagglutination Assay

Hemagglutination assay was performed using 2% rabbit red blood cells (RBC) suspended in saline buffer. Commercial native SBA standard and purified NtrSBA and StrSBA were used for the assay. The proteins were diluted into saline at varying concentrations in order to determine the effective unit for each sample, with one unit defined as the amount of SBA

required to induce agglutination (Lin et al. 2008). All agglutination assays were repeated in triplicate. Fifty µl of 2% RBC were added to round bottom 96-well plates and then mixed with 50µl protein dilutions. The mixture was then allowed to settle for 1 hour and images were taken, with agglutination visually scored as positive or negative for agglutination in the well.

4.2.8 Competitive sugar binding assay

Sugar binding assay was carried out as described above for hemagglutination, with the following modifications. One unit of native SBA or NbrSBA or StrSBA was mixed with saline containing 40µM or 400µM concentrations of one of the following sugars: N-acetylgalactosamine, N-acetylglucosamine, arabinose, lactose or raffinose. Fifty µl of the mixture was then added to 50µl 2% RBC and incubated for one hour. The results were then photographed and each well was visually scored as positive or negative for hemagglutination.

4.2.9 Deglycosylation of plant-derived rSBA

Deglycosylation of NbrSBA and StrSBA was carried out with PNGase F according to the manufacturer's protocol (Sigma P-9120-1SET). The samples were then loaded onto an SDS-PAGE gel followed by Western blot analysis using anti-SBA antibody. N-linked glycan removal was confirmed via a band shift on the Western blot. To confirm the presence of mannose-type glycans on plant-derived rSBA, blots containing both PNGase F–treated and untreated samples were incubated with 20 µg/mL of Concanavalin A (C-2010, Sigma) at room temperature for 1 h. After several washes with 0.5% (v/v) Tween-20 in PBS (pH 7.5), the blot was incubated with HRP-conjugated anti-ConA antibodies (HAL-1104-1, E.Y. Laboratories Inc) at 1000-fold dilution for 1 h. After the same wash step, the blot was incubated with SuperSignal® West Pico Chemiluminescent Substrate. The signals were then developed using a film processor.

4.2.10 Digestion of rSBA in SGF and SIF

The analysis of *in vitro* digestion of NbrSBA was carried out using either simulated gastric fluid (SGF) or simulated intestinal fluid (SIF). For SGF (0.2g NaCl, 0.32g pepsin, 700 µl HCl, in 100mL H_2O, final pH 2.5) and SIF (0.68g monobasic KH_2PO_4, 7.7mL 0.2N NaOH, 1.0g pancreatin, in 100mL H_2O, final pH ~6.8), purified protein was incubated in a 37^oC water bath with samples taken and mixed in neutralization buffer (1.7g Na_2CO_3 in 100mL H_2O) at time 0, 15s, 30s, 1m, 5m, 15m and 30m. Samples were boiled for 10 min, separated on a 12.5% (w/v) SDS-PAGE gel and subjected to Western blot analysis as described above. Control native SBA and a non-glycoprotein, human GAD65 made in *E. coli* (Plantigen), were also tested.

4.3 Results

4.3.1 Isolation and cloning of cDNA encoding SBA

To obtain a cDNA clone encoding SBA, RNA was extracted from wild-type soybean seeds that were approximately 1-5mm in size, converted into cDNA that was then used as a template for the PCR cloning of the SBA coding sequence. The primers included the addition of a 5' *NcoI* and 3' *XbaI* restriction sites to facilitate sub-cloning of the PCR products, as well as the addition of the 6xhistidine tag to the C-terminus. The cloned SBA gene was confirmed by sequence analysis. For the convenience of sub-cloning, the internal *HindIII* site within the native SBA coding sequence was removed by converting a G to an A in 2nd codon position encoding serine, resulting in no change of the amino acid. The complete SBA expression cassette was released from the pRTL-rSBA via *HindIII* digestion and inserted into pBI101.1 to form pBI-rSBA (Figure 4.1). The plasmid pBI-rSBA was then transferred into *A. tumifaciens* strain LBA4404 for plant transformations.

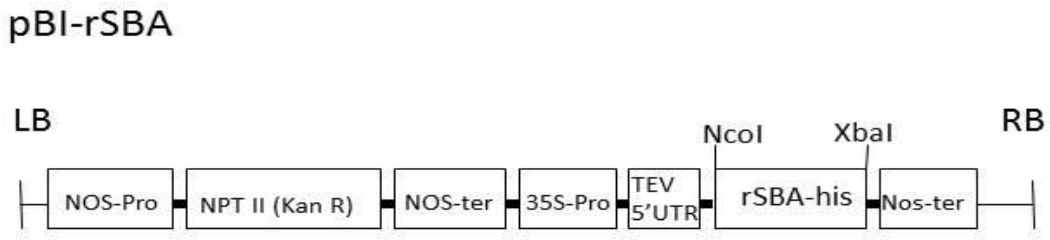

Figure 4.1: Construction of plasmid pBI-rSBA. Construction of pBI-rSBA was described in detail in Methods. The SBA gene contains an engineered 6xhistidine tag at its 3' end prior to the stop codon. rSBA is expressed under the control of a CaMV 35S promoter, the 5'UTR from tobacco etch virus (TEV) and the 3' terminator from nopaline synthase (Nos). The expression cassette contains resistance to kanamycin (NptII).

4.3.2 Transient production of rSBA in *Nicotiana benthamiana*

Six week old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* clone harboring pBI-rSBA or mixed with cultures of *A. tumefaciens* containing the p19 expression cassette, a viral suppressor of post-transcriptional gene silencing that has proven effective in increasing the yield of transiently expressed proteins (Lakatos et al. 2004) (Figure 4.2A). Leaf samples were collected from three independent plants on different days post infiltration with different concentrations of *A. tumefaciens* cultures, with an equal amount of p19 added for each assay, to determine the peak of rSBA expression in the agroinfiltrated *N. benthamiana* plants. The results of immunoblot analysis showed that the highest expression using the PTGS suppressor p19 was reached at day 5, with an optimal density of *Agrobacterium* of 0.25 OD_{600} (Figure 4.2B,C). As calculated by ELISA, the accumulation level of rSBA at day 5 reached up to 4% of TSP (Figure 4.2D). Examination of unreduced samples on SDS-PAGE gels followed by Western blotting showed the presence of two bands, one corresponding in size to the monomer and the other equivalent to the size of the tetramer of SBA (Figure 4.3), suggesting that *N. benthamiana* made rSBA was assembled into a tetrameric protein complex, essential for its biological activity.

4.3.3 Production of rSBA in stable *Solanum tuberosum*

Stably transformed potato plants were also tested for the expression of rSBA. Thirteen individual transgenic potato lines were generated following *Agrobacterium*-mediated transformation and transferred to mini-tuber inducing medium to generate consistently sized tubers for further analysis. Immunoblot analysis of rSBA expression in unboiled samples of tuber extract showed two major bands of 32 kDa and just over 100 kDa (Figure 4.3), expected for the

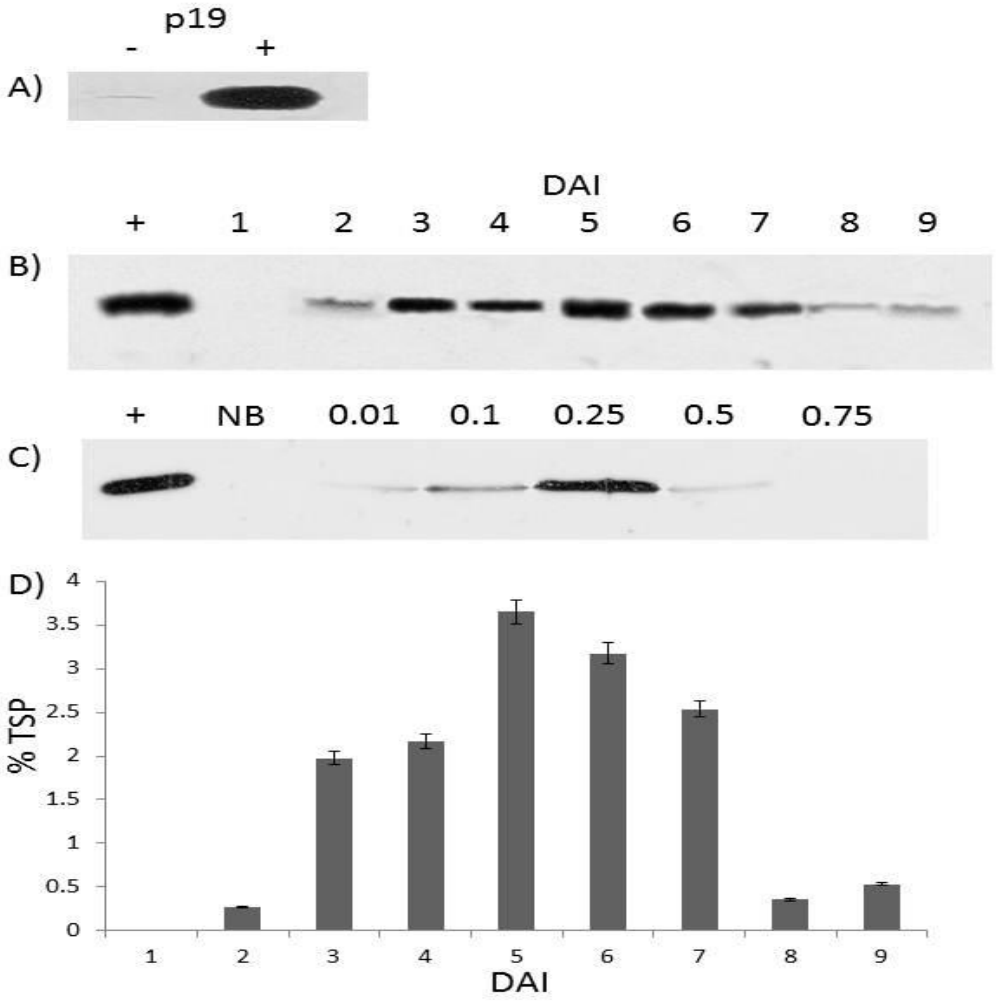

Figure 4.2: Western blot analysis of transient protein expression of rSBA in Nicotiana benthamiana. A) Western blot showing the accumulation of NbrSBA with (+) and without (-) p19, which was used in all subsequent transient assays. B) Accumulation of rSBA from 1 day after infiltration (DAI) to 9 DAI. + is native SBA. C) Accumulation of rSBA at day 5 with different OD_{600} of *Agrobacterium*. + is native SBA, NB is *Nicotiana benthamiana* wild-type TSP. D) ELISA data for accumulation at days 1-9 after infection at optimized concentration. Value expressed as percent total soluble protein (TSP), with each bar representing the mean value of the three collected samples repeated in triplicate from each day with standard error. All Western blots were loaded with approximately 30µg of TSP per lane, 150ng native SBA control.

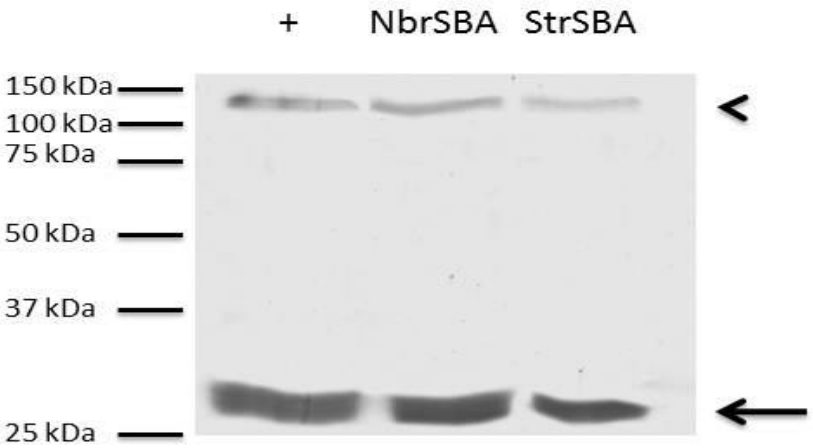

Figure 4.3: Detection of tetrameric structure of rSBA. Unboiled samples from *Nicotiana benthamiana* (Nb) and *Solanum tuberosum* (St) in were subjected to SDS-PAGE and Western blotting. Unboiled native SBA (+) sample was used as a positive control. The large arrowhead points to the tetramer, with an expected size of ~120 kDa. The solid arrow indicates the position of the monomer with expected size of 32 kDa. The amount of protein loaded per lane was 1 µg for native SBA, ~ 30µg TSP from N. *benthamiana* and 150µg TSP from mini tubers.

monomer and tetramer of SBA, respectively. Immunoblot analysis of boiled samples of tuber extract showed only the small band of 32 kDa (data not shown). The level of StrSBA accumulation in potato mini-tubers was variable among individual transgenic lines, ranging from no detectable signal to 0.31% TSP (Figure 4.4).

4.3.4 Purification of plant-derived rSBA

SBA specifically binds to N-acetylgalactosamine, allowing for the purification of this protein via an N-acetylgalactosamine-agarose column to a high degree of purity. The purification through this column also serves as confirmation for authentic SBA production. Figure 3.4A shows that sugar-specific purification of rSBA from *Nicotiana benthamiana* leaf extracts resulted in efficient protein retention during purification and elution. In addition, the sugar-purified sample was of high-quality when examined on SDS-PAGE gel and Coomassie blue staining (Figure 4.5B). Sugar-specific purification of rSBA from potato mini tubers also resulted in high-quality, purified protein (data not shown). As a control, the isolation of *Nicotiana benthamiana*-derived rSBA using a traditional HisTrap column was additionally performed (Figure 3.5B).

4.3.5 Hemagglutinating activity of plant-derived rSBA

Soybean agglutinin is known to agglutinate human and animal red blood cells (hemagglutination). rSBA was therefore assessed for its ability to agglutinate rabbit erythrocytes. Purified NbrSBA was found to induce the agglutination (clumping) of rabbit erythrocytes within 1 hour of treatment (Figure 4.6A). Similar results were obtained with StrSBA (data not shown). The minimum amount of native SBA required to induce agglutination in the assay was 2.5 µg, while NbrSBA and StrSBA required approximately 2 and 3.5 µg, respectively. As agglutination of erythrocytes by SBA is due to its ability to bind to specific sugar residues on the cell surface,

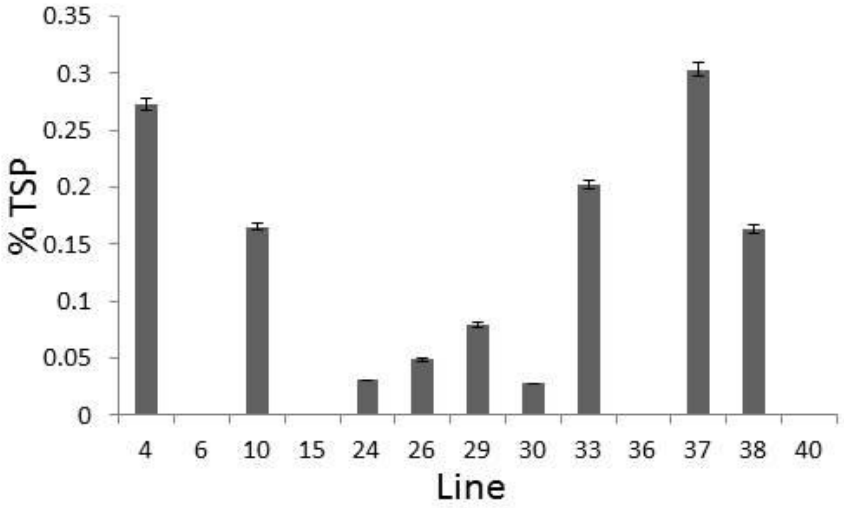

Figure 4.4: Accumulation of rSBA in potato tubers. Accumulation levels of rSBA in independent *Solanum tuberosum* transgenic lines were determined by ELISA. Value expressed as percent total soluble protein (TSP), with each bar representing the mean value of each sample repeated in triplicate from each day with standard error.

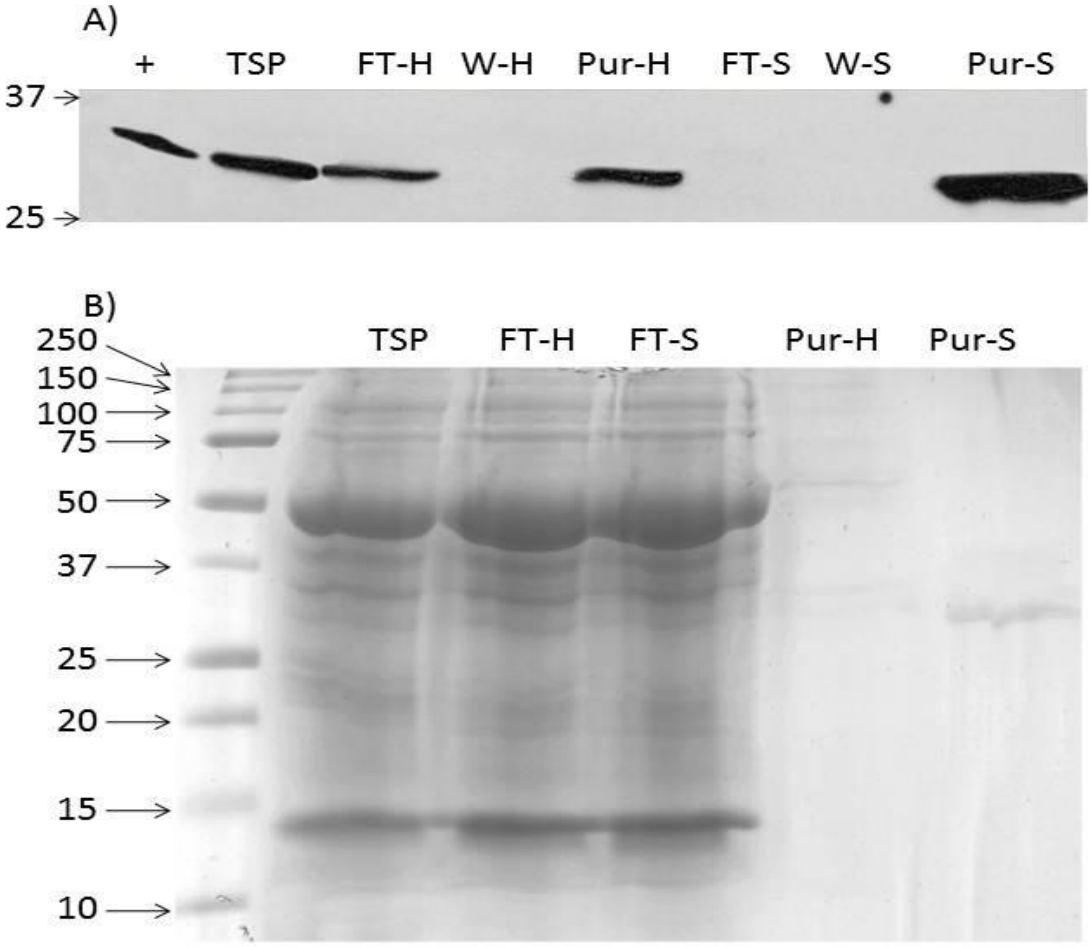

Figure 4.5: Purification of rSBA. A) Western blot showing the purification of NbrSBA using either His purification (H) or N-acetylgalactosamine (S). + is native SBA, TSP is total soluble protein prepared from *Nicotiana benthamiana*, FT is flow through, W is wash, Pur is purified. No signals were detected in FT-S. B) Coomassie stained SDS-PAGE gel. Note the single band in Pur-S at 32 kDa, equivalent to the expected monomer size. Numbers on the left of the blots represent the molecular mass (kDa) of the protein ladder.

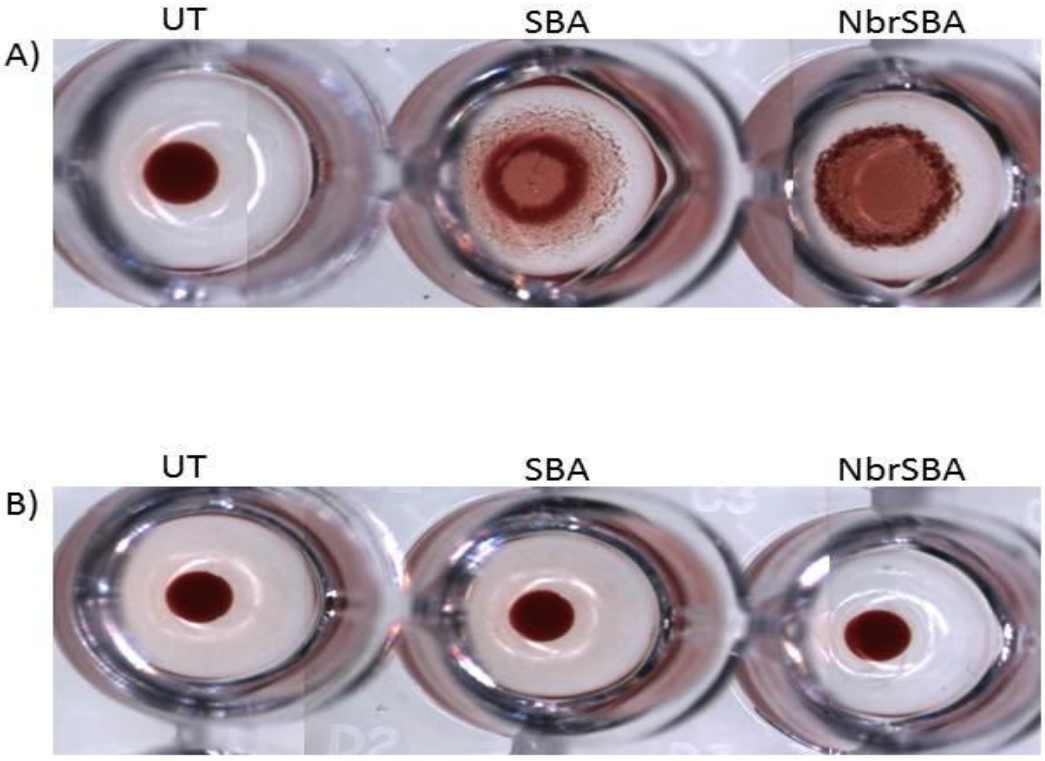

Figure 4.6: Hemagglutination/Sugar inhibition study. Rabbit red blood cells undergo agglutination when treated with SBA, due to the presence of N-acetylgalactosamine on membrane bound proteins. A) Agglutination of rabbit red blood cells (RBC) following treatment with native SBA control or *Nicotiana benthamiana-derived rSBA*, resulting in clumping around the bottom of the well. UT, untreated RBC, as characterized by RBC settling to the bottom of the well. B) N-acetylgalactosamine inhibition. Both native SBA and *Nicotiana benthamiana*-derived rSBA are inhibited by N-acetylgalactosamine, allowing RBCs to pellet.

it is therefore speculated that the agglutinating activity of SBA could be inhibited by addition of specific sugars. Indeed, addition of N-acetylgalactosamine even at low concentrations was able to prevent the agglutinating ability of NbrSBA, similarly to the native SBA control (Figure 4.6B). In contrast, the agglutinating ability of NbrSBA or native SBA was unaffected by high concentrations of non-specific sugars (Table 4.1 and also see Figure 4.6b).

4.3.6 Plant-derived rSBA is glycosylated

Native SBA is a glycosylated protein with a single high-mannose glycan (Adar et al. 1997). The presence of glycosylation, while not required for assembly or function, is important in maintaining the stability of SBA (Sinha and Surolia 2007). To determine the glycosylation status of plant-derived rSBA, NbrSBA, StrSBA and SBA standard were digested with PNGase F and analyzed by immunoblotting following separation by SDS-PAGE gels. Both SBA standard and plant-derived rSBA had a decrease in molecular size of approximately 2 to 3 kDa, which is an expected size for a single glycan (Figure 4.7A). To determine the type of glycans of plant-derived rSBA, blots containing both PNGase F–treated and untreated NbrSBA, StrSBA and SBA standard were incubated with ConA, which binds to N-linked mannose, and then with anti-ConA antibody. Figure 4.7B showed that binding of ConA occurs only in the untreated samples, but no binding to any of the PNGase F treated SBA proteins, suggesting that plant-derived rSBA contains high-mannose type glycans, similar to native SBA.

4.3.7 Plant-derived rSBA is stable in simulated gastric and intestinal fluids

Plant-derived rSBA was further investigated in simulated gastric and simulated intestinal fluid (SGF and SIF) to assess its stability in the digestive system. SGF (3.2 g/L pepsin, pH 2.5) has been used to mimic the acidic stomach environment in animals, while SIF (10 g/L pancreatin

Table 4.1: Sugar inhibition comparison of native SBA and rSBA

40/400 µM Sugar	Native SBA	NbrSBA
N-acetylgalactosamine	+/+	+/+
N-acetylglucosamine	-/-	-/-
Arabinose	-/-	-/-
Lactose	-/-	-/-
Raffinose	-/-	-/-

All sugars except N-acetlygalactosamine showed no effect on hemagglutination at 40 or 400 µm concentrations. + = hemagglutination, - = no hemagglutination

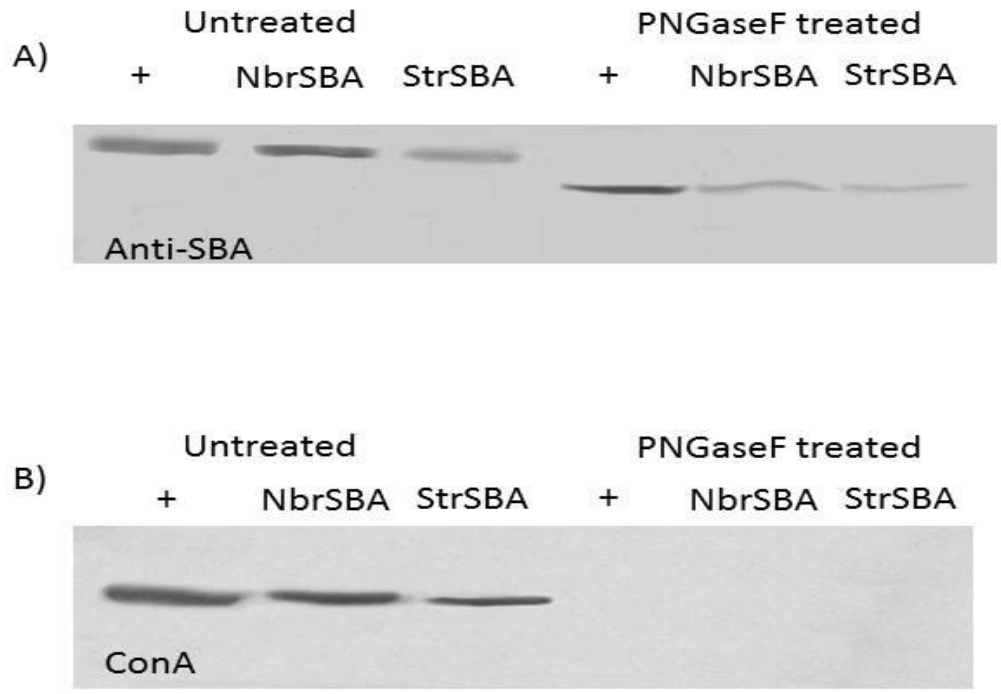

Figure 4.7: Determination of glycosylation of plant-derived rSBA. A) Deglycosylation of NbrSBA and StrSBA using PNGase F, which cleaves N-linked glycans. Native SBA (+), NbrSBA and StrSBA were treated by PNGase F, and analysed by SDS-PAGE and Western blotting. Untreated samples were used as controls. + is native SBA. B) Concanavalin A binding analysis. Plant-derived rSBA and native SBA, both untreated and treated with PNGase F, were treated with ConA, which binds to N-linked mannose. ConA treated samples were analysed by SDS-PAGE and Western blotting using mouse anti-ConA antibody. Binding to both untreated native and recombinant SBA, and no binding to PNGase F treated samples, suggests the presence of high mannose-type glycans for both recombinant and native SBA.

pH 6.8) is used for proximal small bowel conditions. To this end, purified NbrSBA and native SBA were used in simulated digestion experiments. The effect of SGF and SIF on the degradation of NbrSBA, as evaluated by immunoblot analysis, is shown in Figure 4.8A,B. As can be seen, NbrSBA remains relatively intact following digestion even for 30 minutes in either solution. Native SBA showed similar resistance to SGF and SIF digestion (data not shown). To ensure proper activity of the simulated fluids, *E. coli*-derived recombinant GAD65 (glutamic acid decarboxylase) previously produced in our laboratory was tested as a control. The protein lasts less than 1 minute in SGF and 5 minutes in SIF (Fig 4.8C,D).

4.4 Discussion

We have demonstrated the successful recombinant production of SBA through both stable and transient expression in *Solanum tuberosum* and *Nicotiana benthamiana*, respectively. SBA has served as an excellent system of choice to study the fundamentals of protein–carbohydrate interaction (Loris et al. 1998; Sharon and Lis 1990). With a vast increase in knowledge of SBA and other lectins, it has become apparent that SBA has many additional applications, especially in medical and biotechnology-focused industrial areas such as the utilization of SBA to selectively remove cancerous cells from whole blood without removing normal T cells, to prevent graft-versus-host disease following transplant, or to enrich hematopoietic stem cells (Bakalova and Ohba 2003; Yura et al. 2008; Kernan et al. 1987). Moreover, recombinant SBA has the potential to serve as a carrier system for oral drug delivery or to be used as an affinity tag for isolation of high-purity fusion proteins. To make these

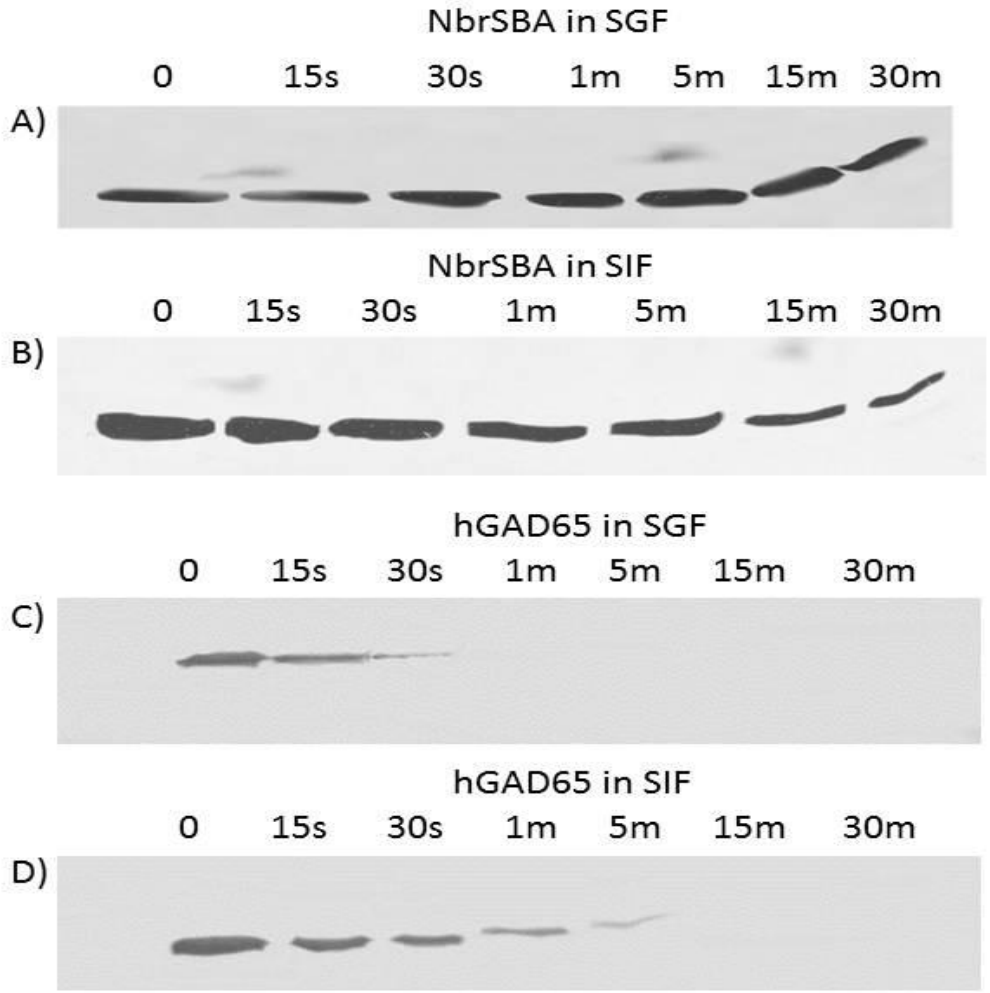

Figure 4.8: *In vitro* digestion of Nicotiana benthamiana-derived rSBA in SGF and SIF. A) NtrSBA is not degraded in simulated gastric fluid (SGF), even after 30 minutes (m) incubation, demonstrating resistance to both low pH and pepsin digestion. B) NtrSBA is stable during simulated intestinal fluid digestion (SIF). C) and D) *E. coli* made hGAD65 is rapidly degraded in SGF and SIF, respectively. Each lane contains the starting equivalent of ~500 ng of purified protein.

applications feasible, however, a more affordable source of high-quality and quantity rSBA is required. Previous recombinant production of SBA in tobacco was incidental, as it was simply used as a marker for studying the promoter activity and as such, there were no assays performed to confirm that the protein made retained authentic SBA activity (Lindstrom et al. 1990). Similarly, recombinant production in both bacterial and mammalian cell culture was for the purpose of elucidating the functional role of glycosylation and the produced recombinant SBA was either lacking glycosylation (*E. coli*-derived) or had reduced agglutinating ability (mammalian cell-derived) (Adar et al. 1997). Our goal was to develop a robust expression system that can generate large quantities of low-cost authentic rSBA, which retains its binding specificity to facilitate high-purity simplified protein isolation and maintains its stability in the gastrointestinal tract (GI) needed for future use as a carrier molecule for oral drug delivery.

Authenticity of both NbrSBA and StrSBA was confirmed by several results. The foremost is the binding and inhibition studies using rabbit red blood cells. SBA binds to different surface proteins bearing N-acetylgalactosamine, and in the case of red blood cells, results in hemagglutination (Gordon and Marquard 1974). Both of the plant-derived rSBAs were able to induce hemagglutination of rabbit red blood cells, as did commercial native SBA standard. In addition, a similar minimum amount of SBA, whether native or from *N. benthamiana* or *S. tuberosum*, were able to induce agglutination. This suggests that, unlike monkey cell made SBA, Nb/StrSBA is functionally equivalent to native SBA. Many lectins can induce hemagglutination, however, so it is important to confirm the specificity of rSBA for its ligand. For example, the Chinese black soybean variety is able to cause agglutination of rabbit red blood cells but is inhibited by a different sugar, in that case melibiose (Lin et al. 2008). The inhibition of hemagglutination through the addition of N-acetylgalactosamine but not other sugars confirms

the rSBA retains the authentic binding profile of native SBA. In addition to this, the ability to specifically purify high-quality rSBA via N-acetylgalactosamine bound to agarose further confirms the authenticity of the recombinant protein, as there was no detectable level of rSBA in either the flow-through or wash steps, only in the elution. The capture rate for this technique has been reported at over 90% efficient, which is supported by the lack of rSBA signal in the flow-through steps seen in figure 4.2b (Percin et al. 2009).

The ability to purify high quality rSBA also lends the potential to be used as an affinity tag. Through genetic fusion with a gene of interest (GOI), it may be possible to first purify the fusion protein on the sugar columns, followed by subsequent cleavage of the desired GOI via specific endoproteolytic cleavage, such as with tobacco etch virus (TEV) protease (Tubb et al. 2009). A second possibility would be in combination with a linker intein, an intervening protein sequence that can be induced to undergo n-terminal, c-terminal or both termini cleavage under specific conditions (Evans et al. 2005). This could allow for cleavage of the GOI from rSBA without any expensive proteases and result in high-purity protein. It should be mentioned that due to the requirement of the tetrameric formation for proper SBA activity, the addition of a fusion partner may affect the formation of a tetrameric SBA. However, several groups, including our own, have previously demonstrated that other multimer forming proteins, such as the non-toxic B subunit of cholera toxin, retain their ability to form pentamers and bind to their target ligand when fused with other proteins (Arakawa et al. 1998; Li et al. 2006a; Ruhlman et al. 2007; Tremblay et al. 2008).

The production of rSBA was accomplished using both transient and stable transformation of *Nicotiana benthamiana* and potato, respectively. Transient gene expression in plants offers a number of advantages over stable genomic transformation in plants or other recombinant

expression platforms. First and foremost is the rapidity with which new recombinant proteins can be generated in sufficient quantities for *in vitro* analysis as well as *in vivo* studies at a speed not possible with traditional bioreactors. This is best demonstrated in the generation of idiotpe specific scFvs to treat non-Hodgkins lymphoma patients. Researchers were able to generate patient-specific scFvs by first isolating the antibody fragments from the patient's biopsy, cloning and ligating it into a plant expression vector followed by transient expression in *N. benthamiana*, generating recombinant plant-made scFvs in less than 16 weeks, something unachievable with any other biological system (McCormick et al. 2008). Transient expression can also be used to generate and validate new vaccines against future pandemics such as H1N1 or SARS, where large quantities of vaccine are needed in a short-period of time in order to meet world-wide demand. An additional benefit of transient expression in plants is the level of accumulation. In our work, we achieved almost 4% accumulation of rSBA in less than a week. This is a 10-fold increase over the accumulation seen in our stably transformed potato lines. Based on our data, the transient system can generate an approximate yield of between 1.5-3g of rSBA per kg fresh weight in one week, meaning that a greenhouse setting capable of producing 1000kg of tissue per week could generate up to 3kg of purified rSBA every week.

The use of stable lines expressing rSBA can likewise prove advantageous. The production of a stable expression platform provides long-term low-cost therapeutic proteins, typically required where the therapeutic is commonly used in multiple individuals over a long period of time, such as in the potential prevention of type 1 diabetes using glutamic acid decarboxylase 65 (GAD65) (Ma et al. 2004). We were able to accumulate rSBA to over 0.3% of TSP in transgenic potato tubers. A major benefit of tuber accumulation is the stability of recombinant proteins during long periods of storage. In the production of monoclonal antibodies,

for example, the recombinant antibodies were stable for over 6 months when stored in the dark at room temperature, with little to no loss in either quantity or activity (De Wilde et al. 2002). This makes potatoes an excellent system for recombinant protein production, as one of the major disadvantages of field/greenhouse grown plants for recombinant production is losses in yield due to protein degradation during transit and processing. An additional benefit for the use of tubers is that they can be used to orally deliver the target protein with minimal processing, especially given that potato tubers form a staple part of many cultures diets and can be processed for consumption via freeze-drying that allows for ingestion without cooking and thus could help minimize protein denaturation/loss during food preparation.

In vitro digestion of *N. benthamiana*-derived rSBA under simulated gastric and intestinal conditions proved to be stable. There was no significant loss or degradation of the protein after incubation of NbrSBA in SGF for 30 minutes as seen in figure 4.5A,C. These results suggest that the plant-derived rSBA may hold great promise for using as a carrier system for oral delivery of protein and peptide drugs. The oral route for drug delivery is the most preferred route and offers considerable advantages: requiring neither sterile needles nor trained personnel, lower cost and increased quality of life, increased access to large population, reduced side-effects often seen with systemic delivery, and greater patient compliance and acceptability. However, administration of therapeutic peptide or protein drugs by the oral route represents a major challenge. Orally administered peptide or n protein drugs are readily degraded due to their exposure to the harsh environment of the GI (low pH and various proteinases and peptidases). Therefore, development of suitable delivery systems is crucial to the success of oral administration of protein drugs. Plant-derived rSBA may provide an ideal vehicle for oral drug

delivery. Moreover, SBA undergoes endocytosis through epithelial lining of the intestine, adding to its potential as an adjuvant for therapeutic protein delivery (Benjamin et al. 1997).

A potential concern with the use of rSBA as a carrier system for targeted drug delivery is its anti-nutritional property. Indeed, SBA is one of the predominant anti-nutritional factors found in the raw soybean and accounts for approximately 50% of the growth inhibition in rats fed unheated soybean (Liener 1996). However, its anti-nutritional activity is strictly dose-dependent. A negative effect of SBA on growth and immune function in rats was observed only when very high levels of this protein was given as diets (14 mg/per rat daily or equivalent to 0.2% of its body weight). In our previous work on oral tolerance induction in mice using plant-derived autoantigenic protein, we showed that only microgram quantities (or <0.005 % of the animal's body weight) of the plant-derived antigen is required to induce a response (Ma et al. 2004). This suggests that when used as a carrier system, the minimum effective dose of rSBA-antigen fusion protein required to be delivered is in the range of micrograms, not milligrams. Delivery of microgram quantities of SBA to animals is safe (Tang et al. 2006; Buttle et al. 2001). However, as with all therapeutics destined for human use, thorough studies would be required to determine the overall bio-safety.

In conclusion, our work has demonstrated the feasibility of high accumulation, high-purity recombinant production of SBA. This is the first molecular characterization of rSBA in plants. The availability of low-cost, high-quality rSBA may make many important applications of this protein possible, especially in medical areas.

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CHAPTER 5

THE DEVELOPMENT, CHARACTERIZATION, AND DEMONSTRATION OF A NOVEL STRATEGY FOR PURIFICATION OF RECOMBINANT PROTEINS EXPRESSED IN PLANTS

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5.1 Introduction

Plants have emerged as an increasing attractive expression system for production of recombinant therapeutic proteins, including antibodies and vaccines. As bioreactors, plants enable unlimited scalability, elimination of product contamination by mammalian pathogens, and reduced production costs compared with microbial or animal cell-based systems (Boehm 2007; Ma et al. 2008; Tremblay et al. 2010b). Plants can perform the complex post-translational modification and processing required by many transgenic therapeutic proteins for biological and/or immunological function. Furthermore, plant bioreactors have the short turn-around time needed to obtain gram quantities of a recombinant protein in a matter of weeks when expressed transiently. This is not only economically advantageous, but is also critical for rapid access to life-saving biotechnology drugs and therapies. Additionally, edible transgenic plant tissue may offer the possibility of allowing direct oral delivery of plant-derived therapeutic proteins and peptides, eliminating the need for expensive downstream protein purification and processing. However, compared to other expression systems, nuclear-transformed plants accumulate relatively low levels of foreign proteins, thus necessitating the development of an efficient and cost-effective system for high recovery of foreign proteins from plant tissues.

Soybean agglutinin (SBA), naturally occurring in soybean seeds, is a homotetrameric lectin glycoprotein that binds to *N*-acetyl-D-galactosamine and is able to induce the agglutination of cells with this glycan on their surface. SBA has shown great potential for medical applications, including screening and treatment of breast cancer, isolation of fetal cells from maternal blood for genetic screening, and the possibility as a carrier system for oral drug delivery (de Mejia et al. 2003; Tremblay et al. 2010a). Furthermore, because of its unique and highly specific binding affinity for *N*-acetyl-D-galactosamine, SBA could be potentially used to develop

novel affinity purification methods for low-cost, simplified and high-purity isolation of high-value foreign proteins when they were genetically fused to SBA. Previously it has been shown that high purity tetrameric SBA could be obtained from soybean flour with more than 90% yield following one-step purification on beads bearing *N*-acetyl-D-galactosamine (Percin et al. 2009). However, to use SBA as an affinity tag for purification of proteins, it is often necessary to produce the target protein as a SBA-tagged fusion protein. Consequently, a robust expression system enabling to produce large amounts of recombinant SBA fusion proteins needs to be established. Recently, we demonstrated the suitability and efficiency of transiently transformed *Nicotiana benthamiana* for high-level production of recombinant SBA (rSBA) (Tremblay et al. 2010a). As expected, plant-derived rSBA forms stable tetramers, bind specifically to *N*-acetyl-D-galactosamine, is rapidly isolated in high purity from total soluble protein and retains its ability to induce agglutination. This demonstration opens the door to the use of plants as an economic source of large quantities of recombinant SBA and potential SBA fusion proteins.

The goal of the present study is to determine whether plants are suitable for generating large quantities of recombinant SBA fusion proteins, as one of our long-term goals is to develop plant-derived SBA as a novel affinity tag for efficient and rapid purification of precious human therapeutic proteins by genetic fusion. As a first step toward this goal, we have expressed a SBA fusion protein containing a reporter protein, green fluorescent protein (GFP), in transiently transformed *N. benthamiana*. GFP has been widely used to monitor transgene expression and protein localization as it allows for non-invasive *in vivo* monitoring in the absence of any added cofactor or substrate. In addition, a fusion SBA-GFP protein may have a variety of diagnostic applications, including cancer diagnosis, reducing the costs associated with either immunolabelling or chemical conjugation of SBA with a fluorophore for visualization. We

demonstrate transient expression of SBA-GFP in *N. benthamiana* at levels higher than 2% of total soluble protein (TSP). Furthermore, the plant-derived fusion protein assembles to form tetramers essential for its stability, retains the capacity of SBA to induce cell agglutination and the ability of GFP to fluoresce. Importantly, the fusion protein can be quickly isolated to high purity via *N*-acetyl-D-galactosamine-column.

5.2 Materials and Methods

5.2.1 Generation of rSBA-GFP

GFP was cloned from pEarleyGate 103 (Earley et al. 2006) using the primers GFP F 5' GTCGACATGGTAGATCTGACTAGTAAAGG 3' and GFP R 5' TCTAGATCAGCTAGCTTTGTATAGTTCATCCATGCC 3', with restrictions SalI and XbaI, respectively, underlined. The rSBA to be used for the fusion was cloned from pBI-rSBA (Tremblay et al. 2010a) with the primers rSBA F 5' AATCCATGGCTACTTC AAAGTTGAAAACC 3' and rSBA-Fus R 5' CTCGAGGATGGCCTCATGCAACACAAAAC 3', with restriction sites *NcoI* and *XhoI*, respectively, underlined. PCR amplification for both products were as follows: 10 minutes at 94^{o}C, 35 cycles of 1 minute at 94^{o}C, 30 seconds at 60^{o}C, 2 minutes at 72^{o}C, followed by 10 minutes at 72^{o}C. Both genes were cloned into pUC19 and subsequently sequenced. The cloned genes were then ligated together and used to substitute the GUS gene in pRTL-2, providing a 35S promoter and 5' untranslated region from tobacco etch virus (TEV) (Carrington and Freed 1990) and 3' untranslated region from *Agrobacterium* nopaline synthase gene (NOS). The entire cassette was then ligated into the pBI 101 binary vector to generate pBI-rSBA-GFP. The newly generated plasmid was transferred to *Agrobacterium tumefaciens* strain LBA4404 via tri-parental mating (Ma et al. 2005), confirmed by PCR using the rSBA F and GFP R primers.

5.2.2 Transient expression in *N. benthamiana*

Transient expression was performed as described previously (Tremblay et al. 2010a). *Agrobacterium* carrying pBI-rSBA-GFP were mixed with *Agrobacterium* carrying p19 at a ratio of 1:1 according to cell density readings at OD_{600}, with concentrations of 0.1, 0.25 and 0.5 used for infection. Samples were collected at day 3, 4 and 5 to determine optimal collection date and concentration.

5.2.3 Accumulation of rSBA-GFP in *N. benthamiana*

Total soluble protein was extracted as previously described and quantified by the Bradford method (Tremblay et al. 2010a). Western blotting was performed as previously described, with either 5 or 20 µg of TSP loaded into each well. Following transfer onto PVDF membrane, blots were blocked with 5% fat-free powdered milk (w/v) in TBS-T for 1 hour and then incubated overnight at 4^{o}C in primary antibody. Rabbit anti-SBA (Cedarlane AL-1301-2) at 1:2000 dilution was used for SBA detection and Mouse anti-GFP (Sigma G-1546) at 1:1000 was used for GFP detection, both in 1/3 blocking buffer 2/3 TBS-T. Goat anti-rabbit HRP or goat anti-mouse HRP secondary antibody was diluted 1:5000 in 1/3 blocking buffer 2/3 TBS-T and incubated for 1 hour at room temperature. The blots were washed in TBS-T and then incubated with SuperSignal West Pico Chemiluminescent Substrate (34080, Pierce, Rockford, IL, USA). Blots were developed using film and a film processor. ELISA was performed as previously described to determine the quantity and expression level of rSBA and rSBA-GFP prior to purification.

5.2.4 GFP imaging

Stereoscopic images of leaves transiently expressing rSBA or rSBA-GFP were obtained using a Zeiss SteReo Lumar V12 microscope (Carl Zeiss International, Germany) with attached

fluorescence filters. All confocal imaging was achieved using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Germany). Plant material was either whole mounted (Stereoscopic images) or leaf punches suspended on water between cover slips (Confocal images). For confocal images of rSBA-GFP with red blood cells (RBC), agglutinated blood was smeared between two coverslips.

5.2.5 rSBA-GFP purification

Purification of rSBA-GFP was carried out using an *N*-acetly-D-galactosamine-agarose column as previously described (Tremblay et al. 2010a). Washes were monitored by spectrophotometry at A_{280} and once the readings reached zero, samples were eluted with 0.5 M galactose/0.1 M NaCl. Samples were confirmed by Western blot and dialyzed against excess 0.5 x PBS buffer to remove NaCl and galactose. Coomassie blue staining of SDS-PAGE gels was used to determine the relative purity of the purified rSBA and rSBA-GFP samples, and a Bradford method was performed to each dialyzed sample to determine the total amount of protein present following purification. Purification efficiency was determined by dividing the post-purification value by the pre-purification value.

5.2.6 Hemagglutination

Rabbit RBC agglutination was performed using a 2% RBC suspension in saline buffer. rSBA was used as a control. Purified rSBA or rSBA-GFP was mixed with the RBC and allowed to settle over the course of 1 hour at room temperature and then recorded. The agglutination assays were performed in triplicate. For the induction of agglutination, 1 unit is defined as the minimum amount of the protein required to induce agglutination (Lin et al. 2008). In this experiment, 1 unit of rSBA was 3 µg, rSBA-GFP was 4.5 µg. Inhibition of agglutination was performed by adding *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, arabinose, lactose or

raffinose to a concentration of 40 mM or 400 mM, allowing cells to settle for 1 hour at room temperature and recording for induction of agglutination.

5.3 Results

5.3.1 Plasmid construction

We previously cloned SBA cDNA and used it as a template for the construction of a SBA-GFP fusion gene (Tremblay et al. 2010a). To this end, a cloned GFP sequence was fused in frame to the 3' end of the SBA sequence and the resulting fusion construct was then confirmed by sequence analysis. Expression of the fusion gene was placed under the control of a 35S promoter (Figure 5.1a). The entire cassette was transferred into the pBI binary vector and confirmed via PCR (data not shown). *Agrobacterium* containing the plasmid was used to transiently express the recombinant SBA-GFP (rSBA-GFP) protein in *Nicotiana benthamiana*.

5.3.2 Transient expression of SBA-GFP

Western blotting was used to confirm the accumulation of rSBA-GFP fusion protein. Transient expression was achieved with co-cultivation with p19, a potent inhibitor of plant RNA silencing (Voinnet et al. 2003). Antibodies against either SBA or GFP identified a band corresponding to the expected size of the fusion protein, ~65 kDa (Figure 5.1b,c). We used rSBA alone or commercially available GFP as controls during Western blot analysis and identified the expected band shift for the fusion protein, approximately 30 kDa in both cases. Optimization experiments were carried out to determine the optimal initial concentration/date of collection for rBSA-GFP. Maximum accumulation occurred on day 5 post infection with an *Agrobacterium* cell density at OD_{600} of 0.5 (Figure 5.1c). On day 5, rSBA-GFP accumulated to just below 2.5%

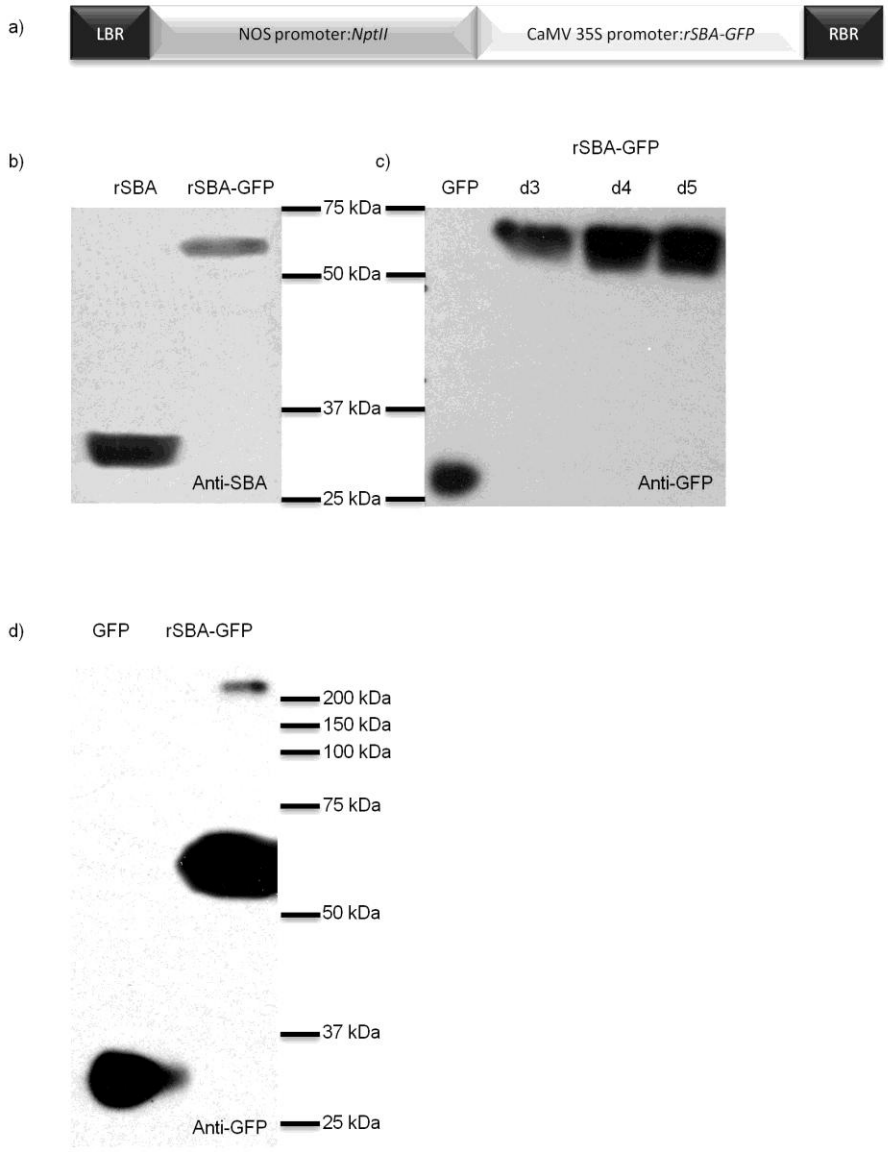

Figure 5.1: Construction and expression of rSBA-GFP. a) Expression cassette used for transient expression in *N. bethamiana*, with kanamycin resistance (nptII) and rSBA-GFP fusion. b) Western blot of rSBA-GFP using anti-SBA antibody. Control protein is tsp from rSBA-transformed plants. 20 µg of TSP loaded. c) Western blot of rSBA-GFP from day 3 to day 5 post infection using anti-GFP antibody. Control is commercial GFP. 5 µg of TSP loaded. d) Overexposed Western blot of rSBA-GFP using anti-GFP antibody. Samples were boiled for 10 minutes prior to loading. 20 µg of TSP loaded.

of total soluble protein. In addition, when using the anti-GFP antibody and a longer exposure, a high molecular weight band was detected in samples boiled for 10 minutes that was larger than the 200 kDa marker (Figure 5.1d). The predicted molecular mass for the rSBA-GFP tetramer is ~250 kDa.

5.3.3 rSBA-GFP retains the fluorescence characteristics of GFP

Fluorescence microscopy was used to confirm the correct folding of the GFP portion of the fusion protein. Fluorescence was first detected using a stereoscope, which showed localized regions of fluorescence that corresponded to the regions infiltrated with the rSBA-GFP-containing *Agrobacterium*, while no signal was detected in leaves infected with rSBA alone (Figure 5.2a,b). More detailed imaging was performed using scanning laser confocal microscopy, again with rSBA alone as a control (Figure 5.2c). Two-channel detection was used to separate the GFP signal from autofluorescence. The resulting images showed separate regions of intensity, with punctate GFP signal detected in leaves expressing rSBA-GFP, but no GFP channel signal in leaves expressing rSBA alone.

5.3.4 rSBA-GFP purification and recovery

Soybean agglutinin (SBA) specifically binds to *N*-acetyl-D-galactosamine, permitting one-step affinity purification of this protein via an *N*-acetyl-D-galactosamine–agarose column to a high degree of purity. Purification through ligand-based affinity chromatography also serves as confirmation of authentic SBA production. The major goal of this work was to demonstrate if SBA can be used as a tag for affinity purification of SBA-tagged fusion proteins expressed in plants, and to determine if foreign proteins fused to SBA could have an adverse effect on the structure and function of SBA. Using Coomassie brilliant blue stained SDS-PAGE gels, we

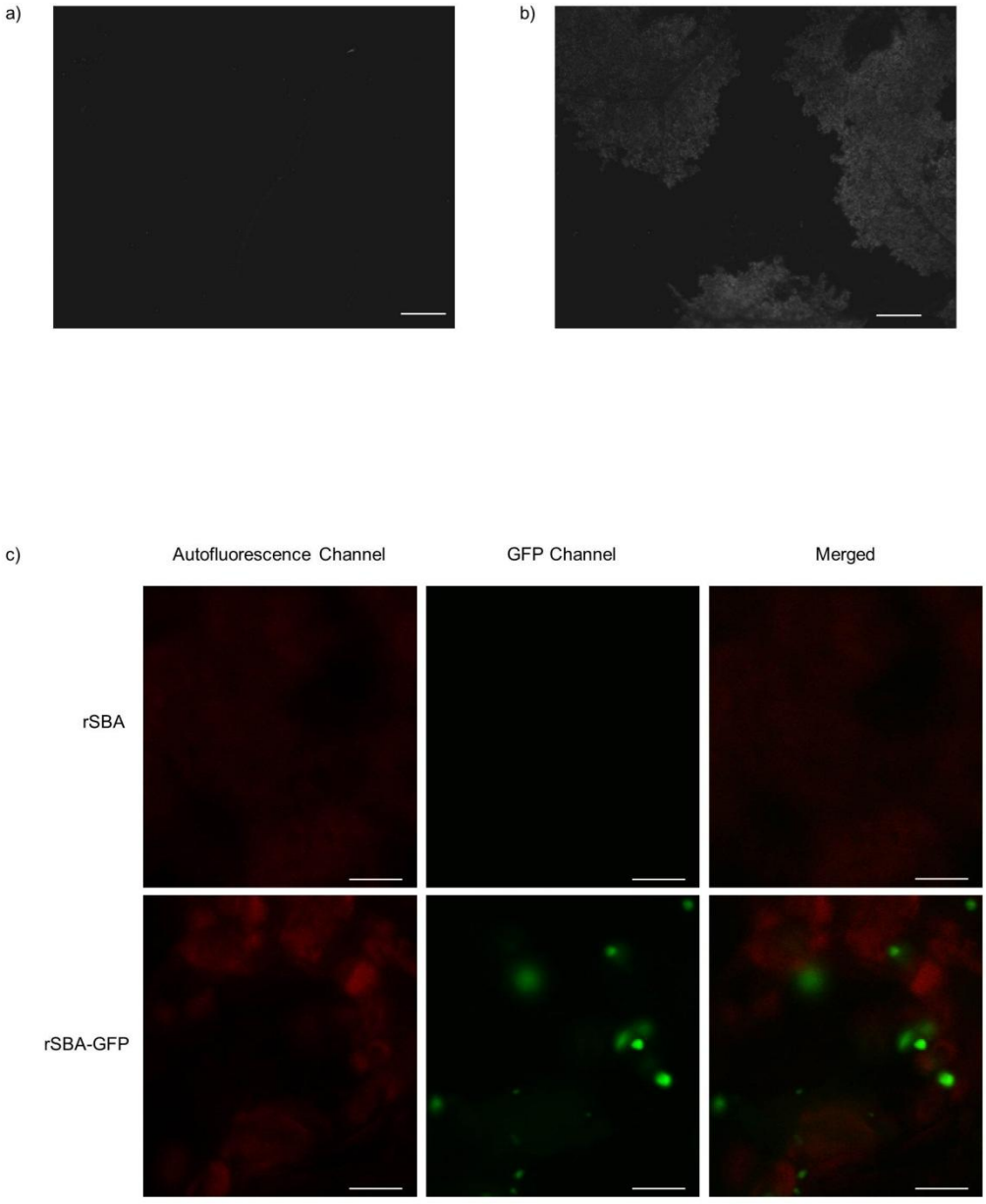

Figure 5.2: *In vivo* detection of GFP signal. a) Fluorescence sterioscopic image of transient expression of rSBA in *N. bethamiana*. b) Fluorescence sterioscopic image of transient expression of rSBA-GFP in *N. bethamiana*. c) Fluorescence confocal images for rSBA and rSBA-GFP. Autofluorescence Channel = over 600nm, GFP channel = between 505-535nm. Bar = 1cm (a,b), 10µm (c)

detect a single band in the elution fraction from the column, with the size expected for rSBA-GFP fusion protein (Figure 5.3). No other bands were detectable. Bradford quantification following purification demonstrated the recovery of ~ 92% and 91% of rSBA and rSBA-GFP proteins, respectively (Table 5.1). Our results suggest that plant-derived rSBA-GFP fusion protein was properly assembled into a tetrameric form, which is required for ligand binding and hence purification, and is readily purified with efficient recovery.

5.3.5 rSBA-GFP retains its ability to agglutinate red blood cells

Native SBA is able to induce the agglutination, or clumping, of red blood cells. We performed a hemagglutination assay in order to confirm that rSBA-GFP retains the ability to induce agglutination of rabbit erythrocytes following purification. As shown in Figure 5.4a, rSBA-GFP was able to induce agglutination within 1 hour of treating the cells, similar to native SBA. The induction of agglutination required 4 µg of rSBA-GFP, with native SBA requiring 3 µg. rSBA-GFP agglutination was inhibited in the presence of 40mM *N*-acetyl-D-galactosamine, but was unaffected by a variety of other sugars (Table 5.2).

5.3.6 rSBA-GFP fluorescence is detected on agglutinated cells

We next examined whether rSBA-GFP agglutinated red blood cells produced fluorescence signal due to the activity of GFP protein. Agglutinated blood cells were smeared between 2 coverslips and then examined under the confocal microscope. Agglutinated bloods cells can be seen as clumps of cells under white light (Figure 5.4b). When excited by 488 nm light, fluorescence was detected from around cells agglutinated with rSBA-GFP, while those cells agglutinated with rSBA alone showed no signal.

5.4 Discussion

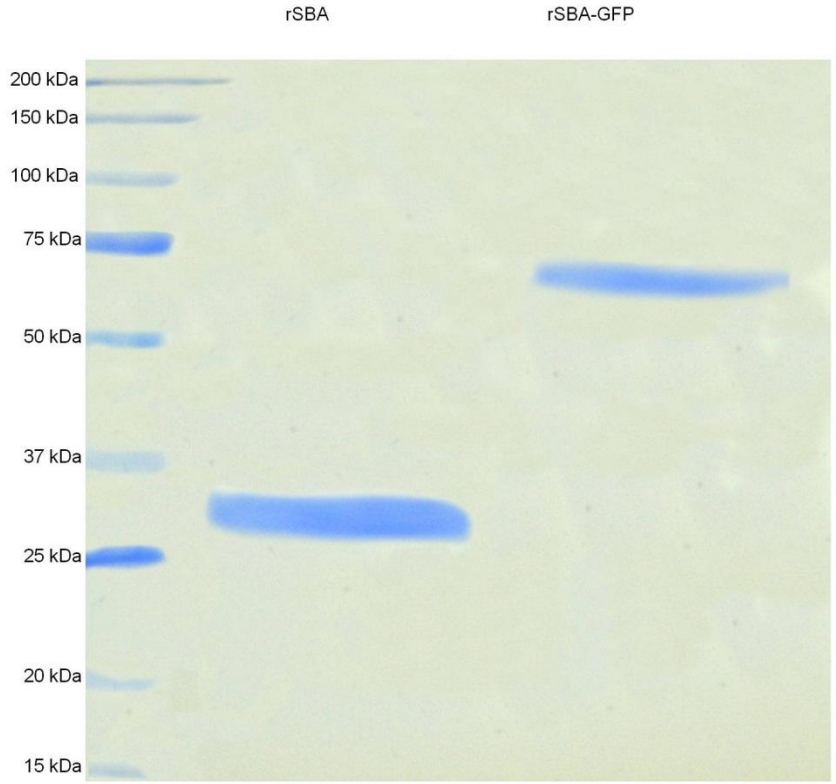

Figure 5.3: Coomassie Blue stained 12.5% (w/v) SDS-PAGE gel demonstrating purity of rSBA-GFP following purification on *N*-acetyl-D-galactosamine agar column. Purified rSBA from *N. benthamiana* was used as a control. The single band in each lane corresponds to the expected size for rSBA and rSBA-GFP, respectively. ~12ug and 9ug of purified rSBA and rSBA-GFP loaded, respectively.

Table 5.1: Purification efficiency

	Pre-purification				Post-purification			
	EV (mL)	TSP (µg/µl)	% TSP	Quantity (µg)	PV (mL)	TSP (µg/µl)	Quantity (µg)	Purification efficiency (%)
rSBA	25	3.038	3.733	2835.2	2.5	1.038	2595.8	91.6
rSBA -GFP	25	3.155	2.416	1905.6	2.0	0.868	1736.1	91.1

EV = Volume of extracted protein for purification. TSP = Total soluble protein. % TSP = Recombinant protein percentage of TSP. PV = Purified sample volume.

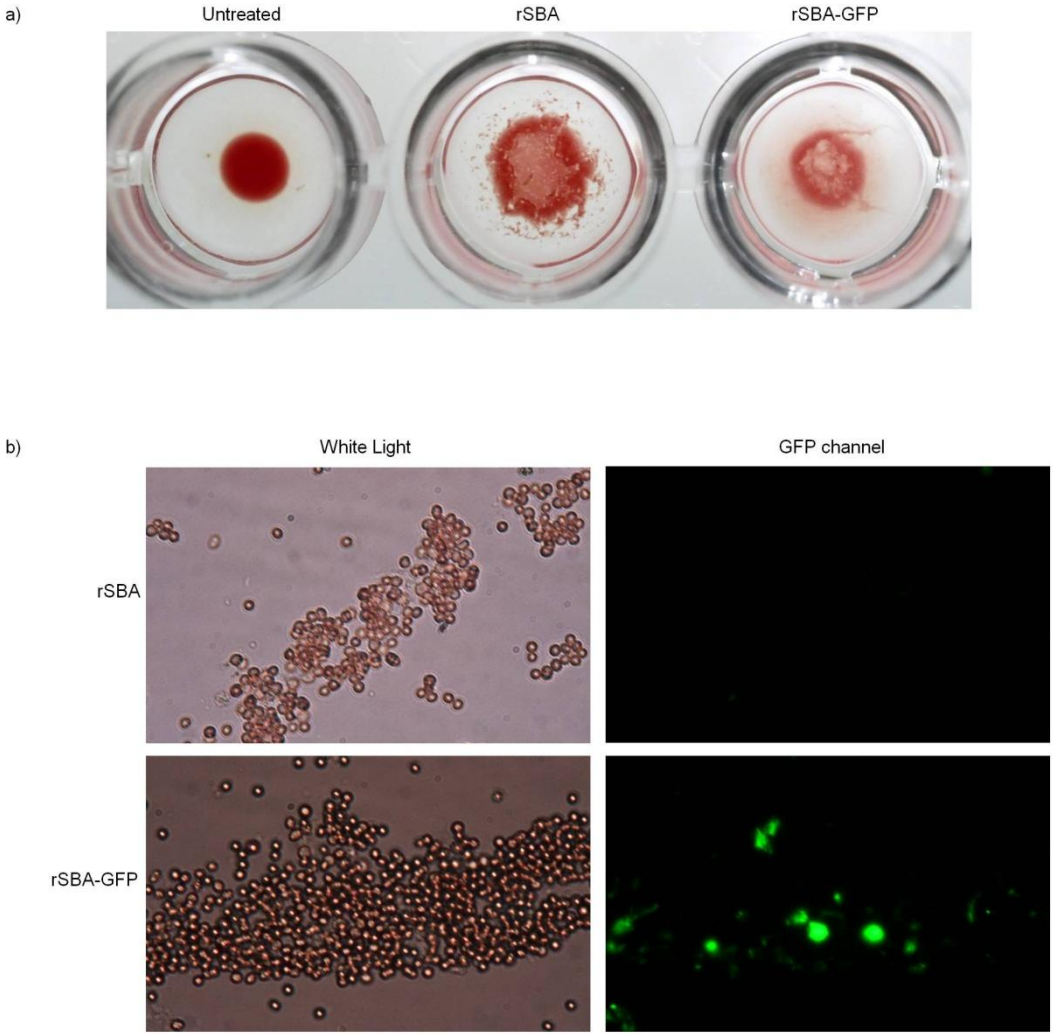

Figure 5.4: rSBA-GFP interacts with rabbit red blood cells. a) Agglutination of RBCs by rSBA-GFP. 3 µg of rSBA and 4.5 µg of rSBA-GFP induced agglutination. b) Fluorescence and white light confocal images of RBCs agglutinated by either rSBA or rSBA-GFP.

Table 5.2: Sugar inhibition comparison of rSBA and rSBA-GFP

40/400 uM Sugar	rSBA	rSBA-GFP
N-acetylgalactosamine	+/+	+/+
N-acetylglucosamine	-/-	-/-
Arabinose	-/-	-/-
Lactose	-/-	-/-
Raffinose	-/-	-/-

All sugars except N-acetlygalactosamine showed no affect on hemagglutination at 40 or 400um concentrations. + = hemagglutination, - = no hemagglutination

We have developed a novel strategy for purifying recombinant proteins expressed in plants using SBA as an affinity tag. This was demonstrated through the design, production, and characterization of a recombinant SBA-GFP fusion protein. We produced the SBA-GFP fusion protein in *N. benthamiana* with accumulation near 2.5% of TSP. The fusion protein retained both GFP and SBA activity, with detection of intra-cellular GFP in plants as well as on agglutinated RBC. Importantly, rSBA-GFP was successfully purified to a high degree on a ligand specific column and induced agglutination of RBC.

A number of assays confirmed the correct assembly and viability of both SBA and GFP. The first step was the confirmation of assembly of the fusion protein, with Western blotting using either anti-SBA or ant-GFP antibodies showing a band around the expected mass of the fusion, 62 kDa. This confirmed that the fusion protein was expressed as a single recombinant protein. In addition, with long exposure of the blots, a larger band over 200 kDa was detected using the anti-GFP antibody, demonstrating the presence of the expected tetramer. While previously we were only able to detect the tetramer form of rSBA without boiling the sample (Tremblay et al. 2010a), we found the antibody against GFP is far more sensitive than against SBA, allowing us to resolve tetramers even after extended boiling prior to loading. The ability to purify the recombinant rSBA-GFP protein on the *N*-acetyl-D-galactosamine-agar column and the ability to induce agglutination, both of which require SBA to assemble into a homotetramer, confirms that SBA retains its biological activity in the presence of a C-terminal fusion with a protein of interest, in this case GFP. GFP function was assayed through microscopic analysis using both a stereomicroscope and scanning laser confocal microscope. In the case of the stereoscopic analysis, regions immediately surrounding the sites of infection were obviously fluorescing, while intervening regions as well as rSBA alone samples showed little detectable

signal. The fluorescence confocal work demonstrates that the signal obtained is clearly GFP, as there is no overlap with chloroplast autofluorescence and no signal was detected in the rSBA controls. In addition, the signal detected was punctate, not dispersed, which may indicate that the protein is being stored in protein bodies as opposed to throughout the ER or the cytosol. Given that native SBA has been previously been shown to localize within protein bodies in Glycine max, it is probable that recombinant SBA is also able to form protein bodies, given that it retains the native SBA signal peptide, although further experiments would be required to confirm this (Horisberger and Vonlanthen 1980).

Transient expression of the rSBA-GFP fusion protein was selected due to our previous ability to achieve high level accumulation of rSBA alone (Tremblay et al. 2010a). Transient expression offers a number of advantages for recombinant protein production, including a rapid turn-around time from the conceptualization of the gene to be expressed to accumulation of sufficient quantities of the gene of interest for further analysis and validation. This is especially important with the design and implementation of new therapeutics to combat human pandemics and disease. For instance, the ability to isolate and generate sufficient quantities of an idio-type specific ScFvs for treatment of Non-Hogkin's lymphoma was achievable in under 16 weeks using transient expression in *N. benthamiana*, something unachievable with other bioreactor platforms (McCormick et al. 2008). We did note a decrease in the percent of TSP for rSBA-GFP compared to rSBA alone, although the accumulation still reached nearly 2.5% of TSP. There are a variety of possible reasons for lower accumulation compared to rSBA alone. It is possible that rSBA-GFP is less stable due to changes imparted by GFP on rSBA assembly. However, there seems little evidence to suggest this possibility, as the recombinant rSBA-GFP performed similarly to rSBA in purification, agglutination and was sufficiently stable to resist 10 minutes of

boiling prior to loading on the SDS-PAGE gels, as demonstrated by the detection of the tetramer during Western blotting. Another possibility is that the reduction in accumulation may be due to the increase in recombinant protein size, nearly double that of rSBA alone. Additionally, we may not have fully optimized the conditions for transient expression, as we performed minimal optimization based on those conditions which proved effective for rSBA alone.

We were able to achieve a greater than 90% recovery of rSBA-GFP fusion protein from total leaf extract in a simple, one-step process using *N*-acetyl-D-galactosamine-agarose columns (Table 4.1). When examined for purity of the purified fusion protein by SDS-PAGE gels followed by Coomassie blue staining, no other protein bands were detectable, suggesting very high levels of purity (Figure 4.3). Compared to isolation of plant-derived rSBA alone, there were no measurable differences found in terms of recovery rate and purity of the two recombinant proteins isolated by the same agar column method (Table 4.1 and Figure 4.3). Our results indicate that the binding affinity of SBA for specific carbohydrate residues is not affected when it is expressed as a SBA fusion protein in plants. This demonstration is important, as it suggests that SBA can be used not only as a fusion partner for the high-level expression of many heterologous proteins in plants, but can also be used for their one-step purification with high purity in a rapid and inexpensive way by the use of specific sugar ligands for SBA. It should be mentioned that although the recovery efficiency of rSBA and rSBA-GFP is similar to that previously described for native SBA extracted from soybean seeds (ie, over 90%) (Bajpai et al. 2005), the purified rSBA and rSBA-GFP from *N. benthamiana* leaf extract appears to have less contaminating proteins SBA purified from soybeans. Our previous attempts to purify native SBA from soybean seeds resulted in a product that still had minor contaminating protein bands following identical purification conditions (data not shown). The most likely cause for this is the

differences in the tissues used for extraction, with *N. benthamiana* leaves likely having less proteins that are able to interact with the *N*-acetyl-D-galactosamine that is bound to the agar or that are able to interact with SBA and thus be co-purified. Taken together, this supports the use of transient expression in *N. benthamiana* as a vehicle for recombinant SBA-fusion production.

The generation of rSBA-GFP serves more than just providing an example of proof of concept to demonstrate the feasibility and usefulness of SBA as a new affinity tag for simple, rapid, and efficient purification of tagged proteins from plants. A fluorescent rSBA-GFP fusion protein itself could have many potential applications, including detection of cell surface and intracellular glycoconjugates with *N*-acetyl-D-galactosamine molecules by microscopy and flow cytometry, localization of glycoproteins in gels, precipitation of glycoproteins in solution and agglutination of specific cell types. Moreover, it should be mentioned that fluorescent rSBA-GFP fusion protein may be particularly useful for the detection of cancer cells or monitoring tumour progression. Benbassat et al (1987) reported the use of fluorescein-isothiocyanate-conjugated SBA to assess the stage of lymphoid cell differentiation in human leukemic–lymphoma cell lines and showed that a gradual increase in SBA labelling was correlated with stages of the cell differentiation as reflected by increased fluorescence intensity. High affinity binding of fluorescent SBA conjugates to other types of cancer cells has also been shown, including mouse lymphoma T cells (Reese and Chow 1992) and human gastric carcinoma cells (Terashima et al. 1997). Fluorescent SBA conjugates are also reported to selectively bind to murine glial cells (Luth et al. 1992) and the zona pellucida of the mammalian egg (Aviles et al. 1997). Currently, a series of fluorescent SBA conjugates is commercially available but far too expensive for routine use, partly because they were produced by using expensive chemical conjugation methods. Our

demonstration that plants can express a functional recombinant SBA-GFP fusion protein at high levels may provide a higher quality, more reliable, lower cost fluorescent SBA product.

In summary, we have developed a novel strategy for purification of recombinant proteins expressed in plants, based on genetic fusion to SBA. We demonstrated the applicability of this strategy by producing a SBA-GFP fusion protein at high levels in transiently transformed N. benthamiana plants. The fusion protein was purified to a high degree in a one-step process on *N*-acetyl-D-galactosamine-agarose columns and retained both GFP and SBA activity. The detection of GFP fluorescence on SBA-GFP agglutinated red blood cells further demonstrate that plant-derived rSBA-GFP fusion protein itself may have many biological and medical applications such as bio-sensing, detection of cancer cells, and recognition and isolation of targeted cells.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSSIONS

6.1 Plants as bioreactors

The worldwide increase in demand for recombinant proteins are ever increasing as new applications are developed and therapeutics are approved for human use. Green plant bioreactors may be able to help meet these rising demands but are still hamstringed by a number of major hurdles. One such hurdle is the typically low levels of accumulation achievable in nuclear-transformed plants. It has been estimated that stable protein accumulation will need to reach over 1% of TSP to compete commercially with traditional cell culture-based bioreactors (Kusnadi et al. 1997). Chloroplast-based expression systems are currently the only green bioreactors able to routinely surpass this 1% barrier, but they are unable to perform eukaryotic post-translational modifications, such as glycosylation, drastically reducing the scope of potential applications (Grevich and Daniell 2005; Cardi et al. 2010). The second major hurdle for plant bioreactors is the cost associated with protein purification. Typically, purification accounts for nearly 80% of the final cost of a recombinant protein product requiring high purity, such as the production of protein therapeutics (Walsh 2002). The third major hurdle is in the production of short peptide fragments that are needed in a variety of therapies, including the prevention of T1DM.

6.2 Generating small peptides as fusions with CTB

It is extremely difficult to produce short peptide sequences in plants, due to instability and high turnover within the cells. One approach to combat this is through fusion with larger, more stable molecules that can facilitate their generation and/or isolation. I chose to use CTB as a fusion partner to help stabilize and express the p277 fragment of HSP60. CTB has been shown to be an effective adjuvant to increase the efficacy of its fused partner, drastically reducing delivery requirements (Arakawa et al. 1998). The fused protein was both stable and accumulated

to just over 0.1% of TSP, while no expression was detected when p277 was expressed on its own (data not shown).

Currently, phase III clinical trials for Diapep277 are underway, with results from the phase II trials showing promise. Other antigen-based therapies, such as using GAD65 or insulin, have not met with similar success. The generation of the CTB-p277 protein in transgenic plants may offer a less expensive alternative to Diapep277, which is chemically synthesized. Further pre-clinical feeding trials with NOD mice will help to validate CTB-p277 and its effectiveness in the animal model of T1DM. This would include determining its relative dose requirements, both in terms of quantity of plant material required as well as number of doses needed to induce tolerance.

6.3 Increasing recombinant protein yield

This thesis has demonstrated a variety of approaches to address each of the above hurdles. Through the disruption of starch in potato tubers by knocking down the expression of *AAT1*, as shown in chapter 2, I was able to increase tuber biomass accumulation, the concentration of soluble proteins in each tuber as well as increase the relative amount of a recombinant monoclonal antibody in said tubers. While on its own AATP1 knockdown lines did not achieve the theoretical 1% threshold, with the best line accumulating just over 0.5% of TSP, it did achieve a 4-fold increase in yield per plant, potentially resulting in commercial viability. There was a potential drawback in the newly generated potato lines, however. Tubers from the riAATP1-10 line have lower proteinase inhibitors than wild-type, and indeed, the protein appears less stable, with reduction of nearly 50% of TSP by 5 months post-harvest. This may reduce the utility of the line for some applications, such as for oral delivery of minimally processed tubers for the induction of oral tolerance in T1DM therapy. Further studies could focus on ways to

induce the expression of the proteinase inhibitor complexes and determine its impact of the tubers.

In addition, this work raised potential questions about the mechanisms involved in tuber development. The riAATP1-10 tubers had an increase in leaf scar number, as well as increased length to width ratios. Typically, tubers develop at the terminal end of a stolon and once the bulking stage occurs, all growth, both in length and width, is confined to the area already differentiated as a tuber. Hence, small tubers and large tubers typically have similar numbers of leaf scars, with the spacing between them increasing with size. In riAATP1-10, however, the largest tubers had significantly more leaf scars than either wild-type tubers (figure 2.3b) or smaller riAATP1-10 tubers (data not shown). This suggests that elongation of riAATP1-10 tubers may occur along the length of the stolon instead of being confined to the already differentiated portion. Further study into the impact of sink strength on delineating the axis of growth in tubers could prove interesting.

6.4 SBA is an effective affinity tag

The second major hurdle limiting the utility of plant bioreactors is that of purification. In order to surpass this hurdle, a novel affinity tag was developed and described in chapters 3 and 4. The use of the plant lectin SBA as an affinity tag offers the opportunity for high-efficiency one-step purification of a bound protein of interest, with GFP used as a first-step proof-of-concept in this thesis. Recombinant SBA was shown to retain native SBA features, even when genetically fused with GFP. These included the formation of stable tetramers *in vivo*, the ability to purify with high efficiency and purity using affinity chromatography with the ligand, *N*-acetyl-D-galactosamine, bound to agar, as well as the ability to induce the agglutination of rabbit RBC. Throughout the process, GFP retained its fluorescence ability, both *in vivo* during transient

expression in *N. bethamiana* and *in vitro* following purification and induction of agglutination of RBC. Follow-up experiments for this work should focus on expanding the range of proteins fused with SBA, as well as the introduction of protease cleavage sites, such as the tobacco etch virus protease recognition sequence, or the use of self-cleaving inteins (Carrington and Dougherty 1987; Evans et al. 2005).

GFP was selected as the fusion partner for these experiments for more than just its ability to be monitored at each step of the process. SBA has previously been shown to bind differentially to cell surfaces, a feature that has been led to its use in screening healthy tissues from those that are cancerous. Traditionally, this was achieved using either chemical conjugation of a fluorescent molecule, such as Alexa Fluor-488, or performing immunohistochemistry, where antibodies against SBA that are conjugated with a detectable signalling molecule, such as horseradish peroxidase. Both of these techniques are expensive, especially chemical conjugation with Alexa Fluor-488. The ability to generate relatively low-cost SBA-GFP fusion proteins may offer a less expensive alternative for distinguishing between cell types, although further research is needed to determine if the signal strength of GFP is sufficient for rapid identification.

6.5 Perspective

Plants really are several potential bioreactors all wrapped in one package. If what you need is large quantities of proteins that do not require post-translation modifications, chloroplasts can potentially yield over 50% of soluble protein (Lentz et al. 2010). If you encounter a new epidemic and need custom vaccines in a short period of time, transient expression can yield between 2 to 10% of soluble protein and can produce significant quantities of protein in under 2 months (McCormick et al. 1999). Plants also have the potential to deliver large quantities of low-cost glycoproteins over a long-period of time through stable nuclear expression.

There are, however, hurdles beyond simply making and purifying proteins from plants that must be addressed. Primarily, these are regulatory in nature, with grave concerns being raised about the safety of the food supply and possible risks of contaminating crops destined for consumption with transgenics. While a variety of options exist to limit this risk, such as transgene containment through male sterile lines and crop isolation within greenhouse spaces, they cannot eliminate the risk completely. This could limit the use of crop species, such as potato, in the production of recombinant proteins, despite the advantages that these crops may provide. A more realistic alternative is through recombinant protein production in non-food staple crops, such as tobacco and its relatives, in combination with greenhouse bio-containment.

The direction that plant green bioreactors will take will depend on the prevailing public sentiment. Dissemination of high quality, high impact results to the public sphere in a way that makes the data accessible, along with open discussions to address the fears and concerns over transgenics, may help to increase public awareness and opinion. An open dialogue with the public and government can ensure that regulations are implemented that protect the public interest while at the same time allowing the public to reap the benefits of low-cost recombinant proteins to help in their everyday lives, such as therapeutics and in the production of biofuels. As long as the sun keeps shining, green bioreactors should have a bright future.

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