PRODUCTION OF IMMUNOMODULATORY PROTEINS IN GLYCINE MAX FOR USE IN DIAGNOSTICS AND AS THERAPEUTICS

by

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ABSTRACT

REBECCA DIANE POWELL. Production of immunomodulatory proteins in *glycine max* for use in diagnostics and as therapeutics. (Under the direction of DR. KENNETH L. BOST)

Immunomodulatory proteins can be utilized in diagnostic assays, as tolerance therapies and in vaccine development. In the following studies, we describe the expression, characterization and use of two such proteins from the soybean expression platform: human thyroglobulin (hTG) and merozoite surface protein 1_{19} ($MSP1_{19}$).

We characterize the 1% TSP expression of human thyroglobulin, the primary auto antigen in Hashimoto's thyroid disease, in soybean. Our laboratory successfully purifies this vital diagnostic analyte and indicates its biological equivalency to human-derived thyroglobulin. Furthermore, we demonstrate the usefulness of soy-derived hTG in preventing and inhibiting the progression of Hashimoto's thyroid disease in the murine model.

While unable to express MSP 1_{19} at the same level in the soybean seed, we use the following studies to further characterize the expression constructs that generate the greatest success in producing a soy-derived protein and optimize the process that leads to the use of oral delivery in vaccine development.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AA	amino acid
APC	antigen presenting cell
DC	dendritic cells
hTG	thyroglobulin
kDa	kilodalton
MSP	merozoite surface protein
STD	standard
WT	wild type

CHAPTER 1: INTRODUCTION

1.1 MUCOSAL IMMUNOLOGY

In mammalian species, the majority of the immunological effort is focused at mucosal surfaces, with the greatest concentration of immunocytes being in the small intestine[1, 2]. This mucosal immune system serves to protect the body interior against invasive pathogens and accompanying antigens, as well as inhibit the invasion of commensal bacteria from the gut[3]. In addition, the mucosal immune system appears to be linked or common, indicating that an immune response at one mucosal surface will initiate responses at others [1, 3, 4]. Two potential outcomes, tolerance or a protective immune response, are possible, and regulating this response is the primary goal of oral immunomodulatory protein administration.

In a protective response, pathogenic bacteria and/or foreign antigens can be taken up by the mucosal system in several ways. First, antigen can gain access directly to the bloodstream from the gut, going on to interact with peripheral T-helper cells and creating a systemic response[2]. More directly, antigen can be taken up by so-called microfold (M) cells, specialized cells in the follicle-associated epithelium (FAE) of the gut[1, 2, 5]. Unlike professional antigen presenting cells (APCs), M cells do not express MHC II and cannot activate naïve B or T –cells[6]. Rather, M-cells act as an antigen transport

system across the intestinal epithelium, allowing the antigen to access the APCs found within the Peyer's patches[1, 2]. Alternatively, antigen can be sampled and taken up by myeloid Dendritic Cells (DCs). DCs use their dendrite properties to extend between the tight junctions of the epithelium and sample the gut antigens directly[7, 8]. Antigen-loaded DCs then drain via the lymph to the mesenteric lymph nodes(MLN)[2], where antigen is presented to naïve B-cells and $CD4^+$ T-cells[2, 8]. This induces T-cell differentiation to effector T-cells or T_{helper} cells, which produce immunoglobulin A (IgA)inducing cytokines[9]. In turn, naïve B-cells are induced to maturation and differentiation into high affinity, specific IgA producing plasma cells by the effects of TGF-β, IL-4 and Il-10[2, 3, 8]. Both B-cells and T-cells primed in the MLN migrate through the thoracic duct and peripheral bloodstream, finally accumulating in the mucosa[2, 10]. Targeting to mucosal surfaces in this manner is achieved by site-specific integrins on the cell surface, with addressins, or integrin receptors, expressed on vascular endothelial cells at mucosal surfaces (figure 1).

Alternatively, oral antigens can induce a tolerance response at the gut mucosa, in which a potentially harmful immune response is prevented or suppressed by a number of possible mechanisms which, in turn, elicit a non-harmful response (figure 2)[11]. The gut mucosa is sampled for antigens in the same manner as described above. However, subsets of dendritic cells, plasmacytoid DCs (pDCs) and CD8α (+) DCs, are implicated in inducing a tolerance response via T-regulatory cells (Tregs) or clonal deletion,

respectively[11]. It is likely the differentiation to such a response is, in part, due to antigen detection and uptake in the small intestinal mucosa[12, 13].

Upon antigen uptake, these DC subsets migrate to the MLN, subsequently activating antigen-specific T cells, which are then deleted or differentiate into Tregs. Newly differentiated Tregs migrate via the lamina propria through the thoracic duct and peripheral bloodstream to the gut mucosa, inhibit a Th1 inflammatory response in the gut[14]. The antigen presenting DCs then migrate to the portal circulation. Once in the liver, DCs activate natural killer T-cells and Kupffer cells. The activity of these three cells types, with the production of IL-10 and TGF-β, skew the liver's resident T cells to a Treg differentiation. Once these Tregs migrate through the portal vein to the body's systemic circulation, the desired systemic tolerance response is achieved[11, 14, 15]. This mechanism is thought to be driven by small amounts or doses of a specific oral antigen[15].

High dose antigen administration appears to be responsible for an anergic or clonal deletion response, in which T-cells are either unable to respond to the antigen or are activated and subsequently undergo apoptosis.

Anergy in T-cells is the result of the absence of one of the signals necessary to fully activate the cell. Typically, a signal from both the T-cell antigen receptor (TCR) and the CD28 costimulatory ligand is required for activation and response to a particular antigen[16]. In response to these stimuli, the cell produces Il-2, a T-cell growth factor which promotes CD8+ T cell memory and the production of inflammatory cytokines[17, 18], thereby augmenting a T-cell response. During an anergic response, however, T-cells fail to produce Il-2

in response to a stimulus, resulting in an abortive proliferative response and the deficient production of inflammatory cytokines[19]. While the mechanism of action is not fully elucidated, it is hypothesized that antigen presenting cells (APCs) that have failed to up-regulate costimulatory ligands (such as pDCs) are partially responsible for the lack of signal[17]. It is currently unclear why high doses of antigen initiate this pathway.

Alternatively, high dose antigen administration can lead to deletion of mucosal T-cells by apoptosis[15]. Apoptosis is induced in T-cells as a response to chronic TCR stimulation or cytokine deprivation[20, 21]. While a complete understanding of this mechanism has not yet been established, it is suggested that, upon T-cell activation, the survival cytokine Il-7 is down-regulated in the cell[22]. When presented with a weak, chronic stimulus, these cells are unable to respond and instead initiate apoptosis via the mitochondrial pathway[23]. Additionally, Fas/Fas ligand induced, Bim-dependent apoptosis induced by cytokine deprivation is thought to be a mechanism by which Tregs destroy autoreactive immune cells[24].

When these mechanisms, protective immunity and tolerance, fail; the body succumbs to infection and disease.

1.2 MALARIA

The obligate intracellular parasite, *Plasmodium*, is the causative agent of malaria disease and is responsible for more than 2 million deaths each year. The burden of disease is found predominantly in tropical regions of the developing world and is focused on the four major species that infect human hosts: *P. ovale, P. vivax,*

P. malariae and *P. falciparum*[33]. The parasite has an extremely complex lifecycle (figure 3), including both a vertebrate host and the *Anopheles* mosquito vector. Initial infection is generated via transmission of sporozoite parasites from the mosquito salivary glands to the bloodstream of the victim. Within minutes, these sporozoites migrate to the liver sinusoid, infecting hepatocytes and developing into mature asexual stage merozoites over the course of 5-10 days[34]. During this time, the victim displays no symptoms of disease pathology[35]. The liver schizont then ruptures, releasing up to 30,000 merozoite parasites into the bloodstream. This erythrocytic stage is responsible for disease pathology, including fever, anemia and blood vessel occlusion[36]. Additionally, merozoite proteins are exposed to the host immune system, while liver stage (pre-erythrocytic sporozoites) proteins are not. Therefore, it is the blood stage infection which has become the central focus for disease therapy and vaccine development.

Upon release from hepatocytes, merozoite parasites seek to invade erythrocytes. This invasion begins with initial contact between the merozoite surface and the erythrocyte membrane. The merozoite surface reorients to the erythrocyte membrane, such that the apical end of the parasite binds the membrane[33]. The contents of the apical organelles (micronemes, rhoptries and dense granules) are released, forming a tight junction between the cell and the parasite[37]. The formation of such a tight junction is irreversible, and the parasite is then committed to erythrocyte invasion. A series of proteolytic cleavages allows actual invasion of the erythrocyte by the merozoite and the

formation of the parasitophorous vacuole within the cell. The membrane of the vacuole fuses to the erythrocyte membrane, completing the process[33, 37]. Merozoite surface protein 1 (MSP 1) is a primary protein utilized during the invasion process, and its cleavage is required for successful erythrocyte infection. MSP 1 is, as implied, a surface protein found on the merozoite parasite. It ranges in size from 180-225kDa, depending upon the *Plasmodium* species, and is anchored to the surface via a C-terminal glycosyl-phosphatidyl-inositol (GPI) moiety[38]. The first proteolytic cleavage occurs upon liver schizont rupture, resulting in a protein complex that includes the abbreviated MSP 1 fragment, MSP 1-42. The second cleavage occurs during erythrocyte invasion. The protein complex is released, and the C-terminal MSP 1-42 fragment is cleaved, leaving the 11kDa MSP 1-19 fragment attached to the merozoite surface[38]. It is suggested that this cleavage is required for invasion due to the formation of a co-ligand between MSP 1-19 and MSP 9, another surface protein, which then binds the erythrocyte protein band, altering the erythrocyte membrane and allowing parasite entry[39].

MSP1-19 has become a primary vaccine target, unlike other fragments of MSP proteins that undergo cleavage, due to its rigid and highly conserved structure. MSP 1-19 has two epidermal growth factor-like (EGF-like) domains in tandem. These domains remain in contact and, together with invariant amino acid residues, generate an extremely rigid structure that resists polymorphisms. Additionally, this structure has been conserved across all *Plasmodium* species that infect the human host[40]. While no vaccine is currently available, a number

of studies involving both animal models and humans have demonstrated the efficacy and protection of anti-MSP1-19 antibodies, as well as their role in naturally acquired clinical immunity[41-43]. As such, it seems reasonable to suggest the successful malaria vaccine will contain this conserved element of MSP 1.

1.3 HASHIMOTO'S THYROIDITIS

While mammalian species, including humans, often succumb to disease related to pathogenic organisms, autoimmune disorders caused by the immune system's inappropriate protective response to self antigens are equally common and detrimental. These diseases can be a result of antibodies that react with host tissue or proteins, effector T cells which are specific for certain peptides and any number of supporting environmental and genetic factors[25]. Such autoimmune diseases are either systemic or organ specific. Systemic disease results in a widespread immune response to a ubiquitous molecule, while an organ specific disease is characterized by chronic T-cell or antibody attack of a certain organ[26]. Approximately 4-5% of the general population is affected by some form of autoimmune disease, most of which are more prevalent in women[26, 27]. Autoimmune thyroid disease represents over 30% of organ specific autoimmunity, and as such, is the most common archetype of disease, with Hashimoto's thyroiditis (HT) being the most prevalent form, affecting over 1% of the general population[26].

Patients with HT present with a variety of clinical symptoms, including dry skin, hair loss, weight gain, fatigue, temperature intolerance and mental

slowing[26, 27]. Immunologically, symptoms are the result of the body's failure to recognize the thyroid hormones, such as thyroglobulin, as self-antigens. In such individuals, autoantibodies are present in the sera, which bind to thyroglobulin, activating CD4+ T-lymphocytes to degrade it. This leads to decrease in thyroid function and, ultimately, complete thyroid destruction (figure 4)[28]. The reason for the breakdown in immune tolerance is unclear; however, multiple studies suggest a combination of factors. Some studies report the implications of both *MHC-HLA* and *CTLA-4* genes in development of susceptibility to autoimmune thyroiditis, but the mechanisms have not yet been elucidated. Furthermore, the evidence is tenuous, as sample sizes have been small and offer conflicting data[26]. Additionally, monozygotic twin studies further suggest the effects of environmental triggers (albeit unknown), as one twin is often afflicted and the other is not[29].

Currently, the only available treatment for this disease is the life long administration of the synthetic hormone replacement drug, levothyroxine sodium[30]. While this therapy can be effective, a narrow therapeutic dosing range must be determined for each individual and recalibrated as symptoms worsen[31, 32]. Ultimately, levothyroxine can control the symptoms of disease, but is unable to abrogate disease progression and thyroid destruction[27].

1.4 VACCINES AND ORAL TOLEROGENS

Vaccination is one of the most successful health interventions in human history. The first vaccination was performed over 200 years ago by Edward Jenner, when he inoculated a thirteen year-old boy with vaccinia virus (cowpox) to protect him

against variola virus (smallpox)[33]. Less than 100 years later, in 1885, Louis Pasteur used a similar method to produce the world's first widely used vaccine, against the virus that causes rabies[34, 35]. Since then, over 25 vaccines have been licensed for human use, including those against such scourges as poliomyelitis, diphtheria, pertussis, tetanus and influenzae[35]. While the greatest achievement in this area remains the eradication of smallpox, which was achieved in 1979, other vaccinations (including the ones previously listed) have led to the extension of life expectancy and the prevention of close to one million deaths each year[33, 35]. While such progress is impressive, the 22 new and reemerging diseases identified in the last 30 years underscore the necessity of improving vaccine development and seeking alternatives forms of vaccination[35].

Early vaccines were derived from live organisms. These organisms were either chemically attenuated or heat inactivated and administered live or killed prior to inoculation. This method of vaccine development is still seen today in such vaccines as the attenuated bacillus Calmette-Guerin (BCG) against tuberculosis, inactivated tetanus toxoid and the administrated of inactivated whole cell pertussis vaccine[34]. Live vaccines and inactivated toxoids, such as these, are highly immunogenic and, therefore, rarely require administration with an accompanying adjuvant to elicit a robust immune response[36]. Furthermore, the use of toxoids as carrier proteins allows conjugate vaccines to elicit an equally robust response. Conjugate vaccines are derived from poorly immunogenic molecules, such as polysaccharides and small peptides, covalently

linked to more highly immunogenic molecules, such as toxoids and other large proteins. Successful vaccines of this nature include *H. influenzae* type b and meningococcus[36].

More recently, vaccine manufacturing has shifted to produce alternative types of therapies. Subunit vaccines are ideal in that they are easily generated in large quantities and can be safely reproduced. They have no possibility of reversion and minimal cross-reactivity with human tissue, giving them an overall level of safety[34]. Subunit vaccines are produced via the identification of a highly immunogenic epitope or protein found in the infectious disease for which protection is desired. This protein is then produced in bulk, utilizing recombinant DNA technology, and administered parentally with an appropriate adjuvant formulation, such as aluminum salt emulsions.

Recombinant DNA technology can also be used in the production of genome-based vaccinations. Using live vectors, such as adenovirus or non-pathogenic bacteria, recombinant proteins can be expressed and presented to the host immune system. Such vectors can be further modified to express immunomodulatory cytokines, thereby skewing the immune response elicited by the host. Additionally, DNA technology can be used to encode a eukaryotic promoter plasmid with the gene responsible for the immunogenic protein. Following vaccination with the plasmid, the promoter is activated and the gene of interest expressed, producing the antigen *in situ*[36].

While such vaccine strategies are successful, many of these technologies are extremely costly and beyond the reach of all but the wealthiest countries.

Additionally, in order to safely administer these types of vaccines, it is necessary to ensure proper storage and handling (a cold chain), as well as appropriate 'sharps' training of qualified medical personnel[33, 37]. These requirements not only complicate the administration of such vaccines, but serve to further increase their cost.

To avoid obstacles such as cost and complicated administration, mucosal vaccination via the nasal and/or oral route can be utilized. Presenting antigens to mucosal surfaces generally results in the production of both mucosal and systemic antibodies, as well as the induction of a cell-mediated immune response. Together with the ease of administration available in oral or nasal routes, this makes mucosal immunization one of the leading vaccine technologies to date. A successful case study can be seen in the oral polio vaccine (OPV). While no longer used in the US due to polio eradication in North America, this only currently available oral vaccine is still used elsewhere to successfully combat poliomyelitis[3, 35]. Furthermore, the mucosal route can be utilized beyond the scope of traditional vaccines to administer tolerogens for the inhibition of allergies and autoimmune diseases[38].

The lack of mucosal therapies can be attributed, in part, to the difficulties presented in the gut mucosa once the antigen has been administered. There is considerable risk of antigen inactivation by mucosal enzymes, rapid elimination from the host and the potential lack of contact required for antigen uptake by M cells, which is necessary for eliciting a protective immune response or tolerance[3]. Moreover, while mucosal tolerance is a mechanism utilized by the

human host to inhibit unnecessary responses to harmless particles in the diet and can thus be readily exploited, the lack of approved adjuvants makes eliciting a protective response in this manner more difficult.

1.5 TRANSGENIC PLANT TECHNOLOGY

Regardless of the mechanism of administration, vaccinations against infectious diseases and treatments for allergies and autoimmune disorders have been largely beyond the scope of individuals in developing countries. Additionally, recent questions regarding the safety of avirulent and/or whole organism killed vaccines have led to an increase in subunit therapies, which utilize individual proteins known to elicit the desired immune response. Such subunit therapies and vaccines rely heavily upon recombinant expression systems such as *E. coli*, yeast and mammalian cell culture for production. New technologies in fermentation, purification, storage and sterile delivery have made the production of such vaccines and treatments more numerous but have also resulted in a 14-fold increase in their cost in the last ten years[39].

In response to these difficulties, a growing number of plant-based vaccine antigens and pharmaceuticals have been developed. Transgenic plant technology is desirable for a number of reasons. First, a plant expression system would have no risk of viral, bacterial or prion contamination, as no known human or animal pathogens are able to infect plants[40]. Secondly, a number of food crops are already utilized in the human and livestock diet, making production and processing of such crops for use as bioreactors extremely cost effective. Furthermore, transgenic plant pharmaceuticals would reduce the need for

maintaining a cold change during transport and storage, thus reducing the overall cost of production and delivery[41]. Most importantly, plant-based pharmaceuticals and vaccines could be administered orally, eliminating the need for peritoneal injection and specially trained personnel[40].

A variety of plants have been utilized in the production of vaccines and pharmaceuticals in recent years. Early studies used tobacco as the model organism and successfully produced a number of pharmaceutical proteins including human growth hormone and human serum albumin[42, 43]. Further studies demonstrated the ability of plants, including tobacco, *Arabidopsis thaliana* and Safflower, to produce functionally active serum and secretory antibodies, such as IgG1, IgM, SIgA and ICAM-1[44, 45].

More recently, edible plants have been the focus for production of vaccines and therapeutics. Tomatoes, potato tubers, lettuce and maize have recently been used to successfully express antigens against Hepatitis B virus, Rabies virus, Norwalk virus and an auto-antigen related to Diabetes[46-49]. While expression of these proteins has, in some instances, exceeded 7% of total soluble protein[45] (TSP), such expression models still present difficulties. Plants such as tomatoes, potatoes and lettuce have extremely high water content and, thus, a relatively low protein concentration. In order to produce commercial amounts of the desired antigen, the biomass of the plant would have to be increased dramatically. This reduces the cost-effectiveness of the plant-based model. In addition, proteins expressed in leaf or fruit tissue would require freeze-dried storage or purification for long term use. This further reduces the

economical value of the system. To restore the efficiency of the plant-based system, the expression platform should have a higher protein concentration, thereby making large-scale production of antigen much more accessible.

1.6 SOYBEAN

Like tobacco, maize and other crops used for protein expression, a *Glycine max* or soybean platform has little risk of contamination and is easily produced with processes already in use. Unlike other crops, however, soybean has the additional advantage of containing approximately 40% protein, dry weight[50]. This reduces the biomass of soybean necessary to produce commercial amounts of a desired protein. Furthermore, the soybean seed is a protein storage vacuole, which can be housed at ambient temperatures indefinitely. Therefore, any protein targeted to express in the seed could be stored for long term use without a cold chain or the need for flash frozen tissue materials[51]. Most importantly, proteins expressed at levels greater than 2% in soybean seeds can generate milligram amounts of protein in a single seed[52]. These seeds can then be further processed to produce small doses of soymilk for oral consumption and antigen delivery. These factors make soybean the leading platform in which transgenic plant technology should be developed.

In addition to the efficient and cost-effective advantages presented by the soybean platform, the use of *Agrobacterium tumefaciens* horizontal gene transfer transformation is a naturally occurring process that requires little additional technology (figure 5). The gene of interest in cloned into a binary vector, which can be transformed into both prokaryotic and eukaryotic hosts. The vector is

then transformed into the bacterium, and the bacterium is then allowed to infect the soybean plant. Using tissue culture methodology, the soybean tissue is grown up, and transgenic plants are identified via an herbicide resistance marker built into the original vector (figure 6).

We propose the utilization of the soybean platform for the production of the immunomodulatory proteins, human thyroglobulin (hTG) and merozoite surface protein (MSP) 1_{19} and 1_{42}. We hypothesize these proteins can be successfully expressed in soybean and used as diagnostic analytes and as oral therapies for tolerance and subunit vaccines.

Figure 1.1

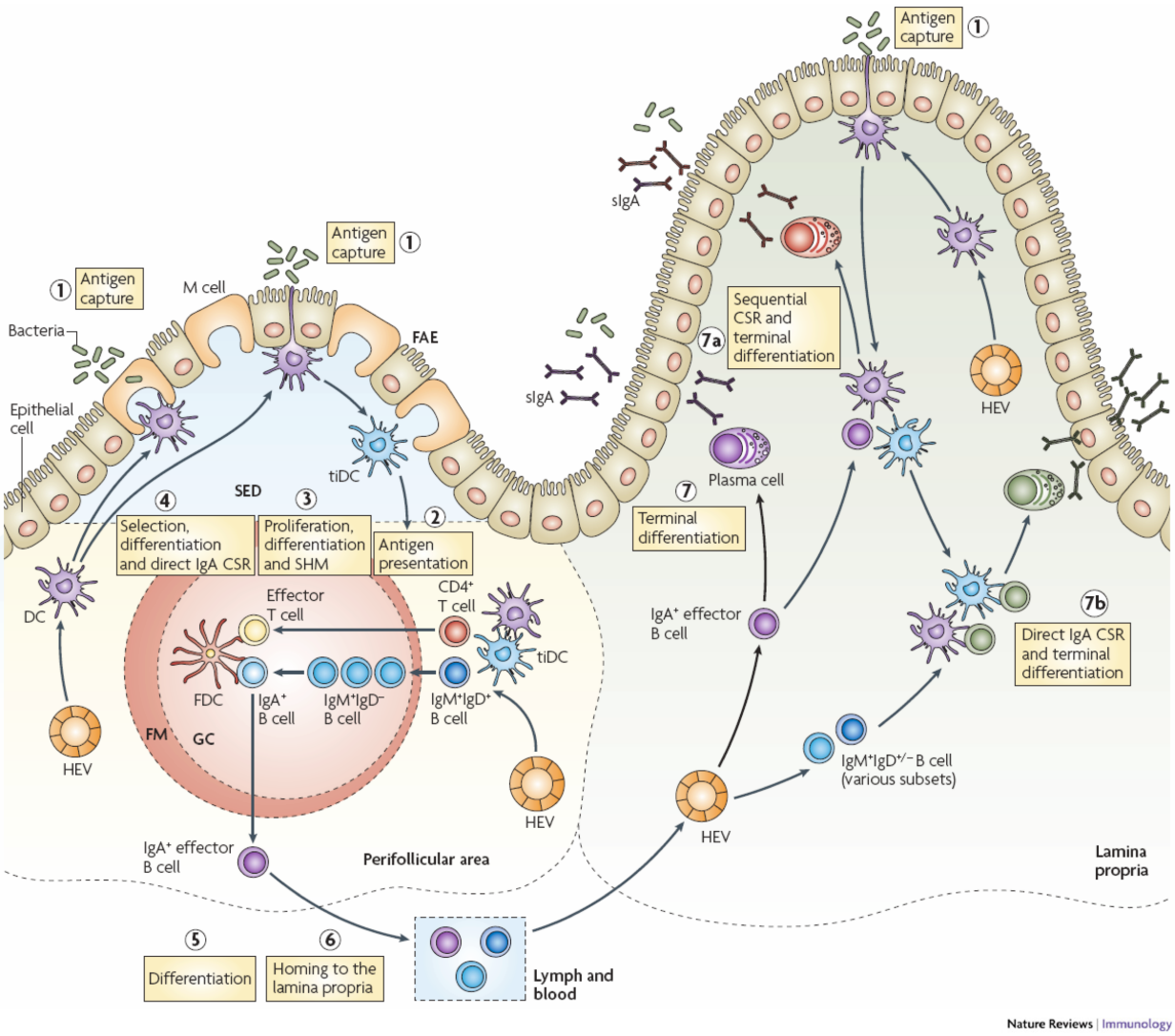

Figure 1.1: Mucosal Immunology: Dendritic cells (DC) can capture antigen from the gut lumen in one of two ways: 1) DC extend 'dendrites' through between the epithelial cells and sample the gut lumen directly or 1b) antigen from the gut passes through microfold cells (M-cells) to cross the epithelial barrier and DC sample in the perifollicular area. 2) Once the DC capture the antigen, they present it to CD4+ T cells (via the CD40 ligand) in the perifollicular space to activate them. 3) Upon activation, these T cells release TGF-beta, Il-4, 5,6, 10 and 13, which are IgA inducing cytokines leading to 5) hypermutation and antibody class switching in naïve B-cells, activating them to become IgA effector cells. 6) Activated B-cells then home to the lamina propria where they differentiate to high affinity plasma cells.

Figure 1.2

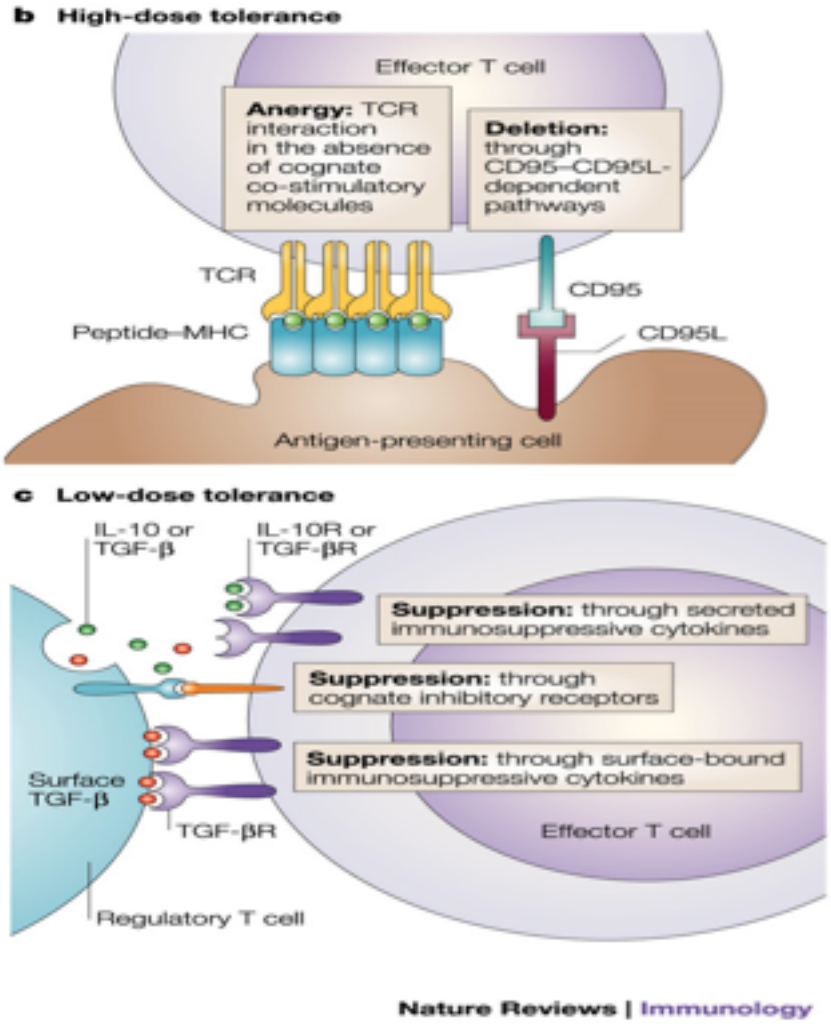

Figure 1.2: Mucosal Tolerance: Two tolerance mechanisms are currently elucidated. b) With high dose antigen administration, one sees the anergic response, the incomplete activation of the relevant T-cell. c) With lower doses of antigen, one observes the suppression of the response by T-regulatory cells via cytokine production and inhibitory receptors.

Figure 1.3

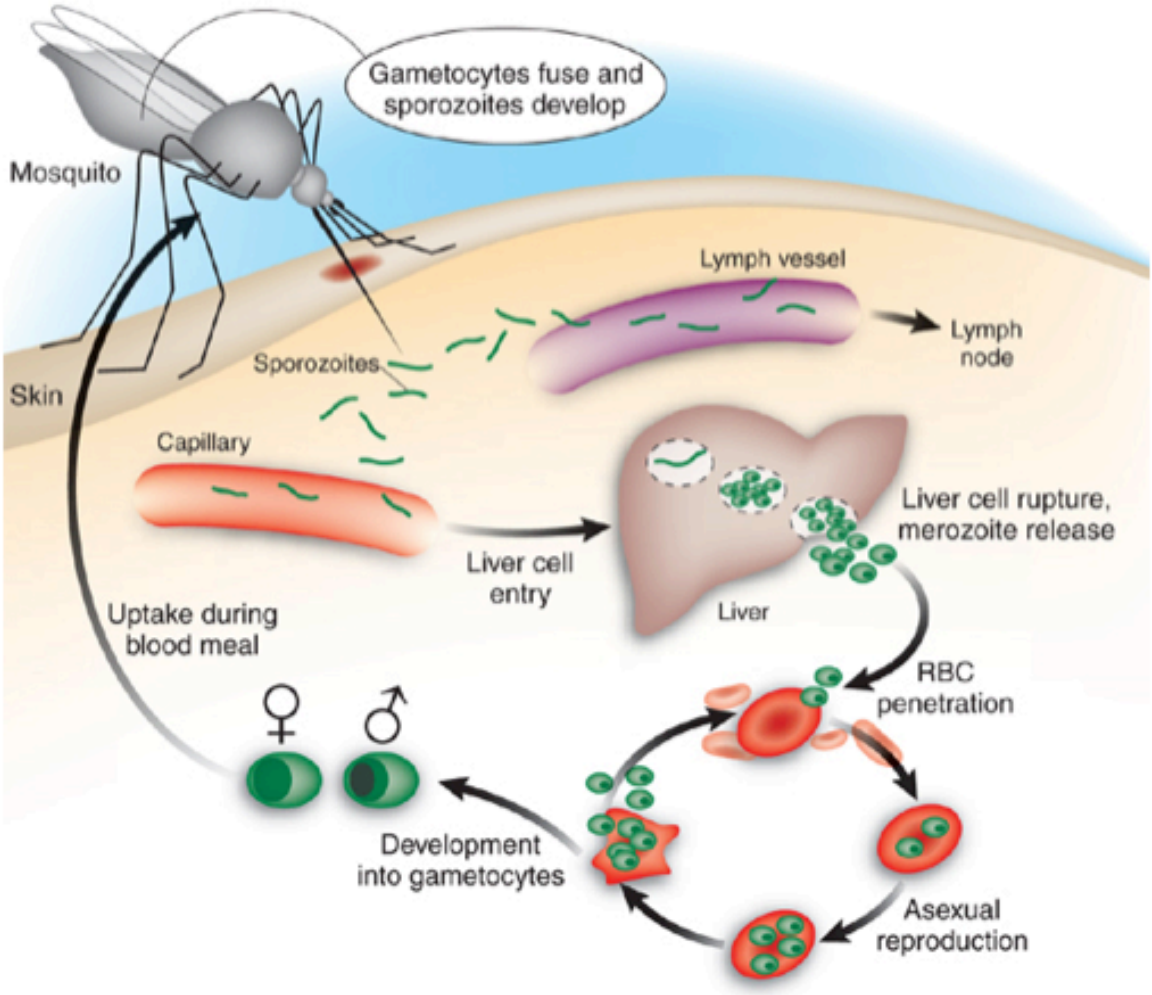

Figure 1.3: *Plasmodium* life cycle: As few as 10 plasmodium sporozoites are injected into the host by the bite of the female Anopheles mosquito. Within 30 minutes to an hour, these sporozoites make their way to the liver. Once there, they invade hepatocytes and mature into merozoites, a process of approximately two weeks. This stage of disease is asymptomatic. Once the merozoites mature, the liver cell ruptures, releasing thousands of merozoites into the bloodstream. Merozoites then invade erythrocytes. Inside the erythrocyte, they reproduce asexually for twelve hours, the erythrocyte bursts and the process begins again.

Figure 1.4

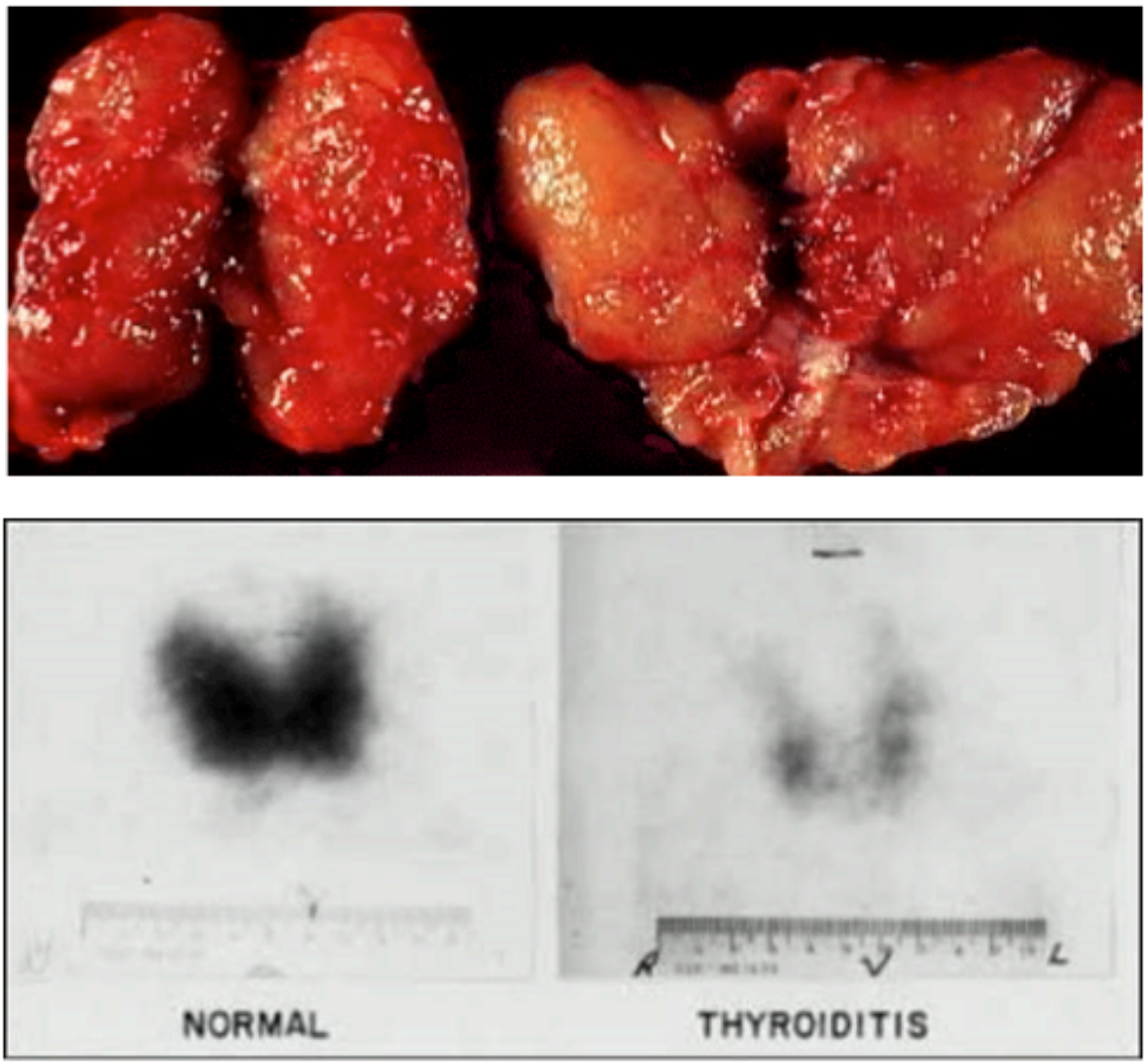

Figure 1.4: Hashimoto's thyroiditis glandular fibrosis: Thyroid disease accounts for more than 30% of all organ-specific autoimmunity, with Hashimoto's thyroiditis (HT) being the most prevalent, affecting over 3% of the global population, predominantly women. HT is the result of chronic thyrocyte depletion and, thus, a reduction in thyroid hormone production. Thyrocyte depletion is caused by immunocyte infiltration into the thyroid parenchyma and subsequent destruction of the thyrocytes, ultimately leading to gland fibrosis.

Figure 1.5

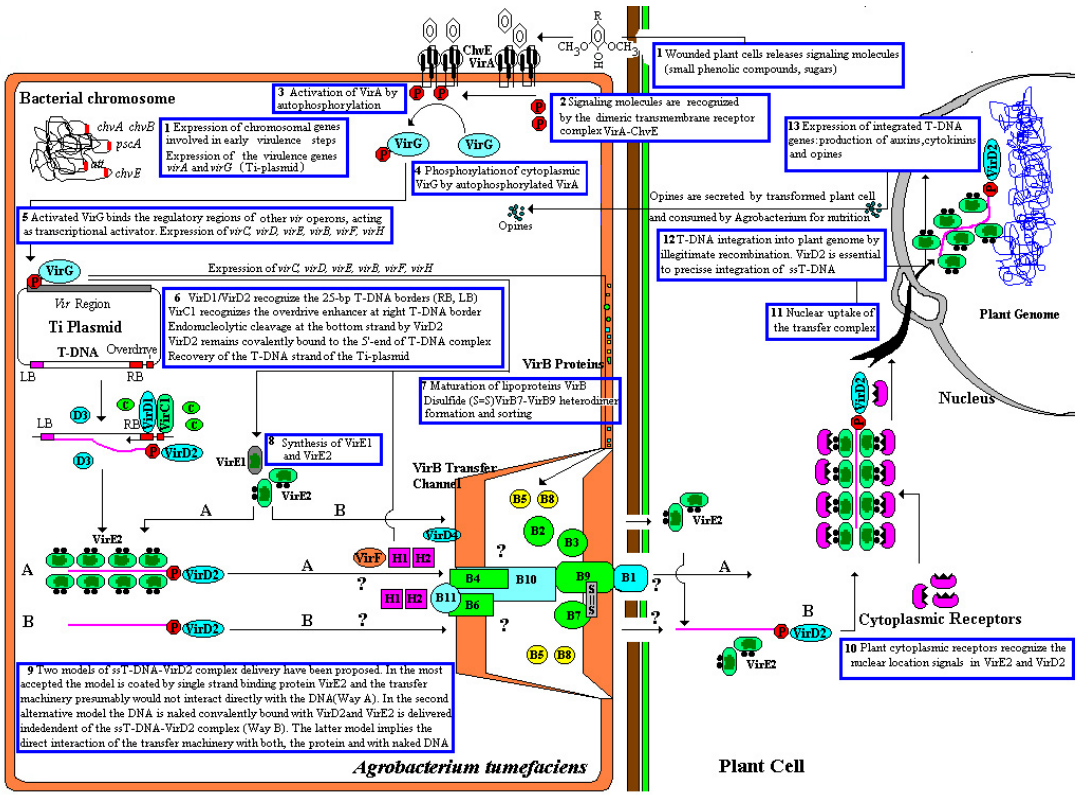

Figure 1.5: *Agrobacterium* transformation: Horizontal gene transfer is the integration to the plant genome of a specific fragment of DNA. These genes express proteins called *Opines*, a carbon source for the bacterium. This process is regulatedby a set of genes known as *Vir* genes, activated by wounded plant phenolic compounds (http://arabidopsis.info/students/agrobacterium/index.html).

Figure 1.6

Figure 1.6: Soybean Transformation: A binary vector, which can be transformed into both eukaryotic and prokaryotic hosts, is cloned with the gene of interest. The vector is transformed into *Agrobacterium*, and the bacteria are used to infect plants. It initiates horizontal gene transfer, permanently inserting pieces of its DNA into that of the plant. The resulting transgenic tissue is grown up in selective media, producing T1 transgenic seeds.

CHAPTER 2: UTILIZATION OF A SOYBEAN PLATFORM FOR THE PRODUCTION OF THE IMMUNOMODULATORY PROTEIN, THYROGLBULIN, FOR USE IN DIAGNOSTIC ASSAYS

2.1 INTRODUCTION

Hashimoto's thyroiditis (HT) is the primary cause of chronic autoimmune thyroiditis. Affecting approximately 3% of the general population, HT is characterized by self-antibodies to the thyroid hormone, thyroglobulin (hTG)[25]. Ultimately, these antibodies lead to the T-cell mediated destruction of the thyroid.

In addition to the thyroid destruction caused by circulating thyroglobulin autoantibodies, these antibodies have the potential to interfere with pharmacological assays which test for the recurrence of thyroid cancer. The U.S. National Cancer Institute reported that over 37,000 cases of thyroid cancer were diagnosed in 2008, with over 1500 of these cases resulting in death. A major obstacle in the early diagnosis of recurring thyroid cancer is due to the 30% subset of thyroid cancer patients who have circulating autoantibodies to thyroglobulin in their sera. These autoantibodies inhibit effectiveness of the only currently available assay used to test for thyroid cancer.

Previously, it has been demonstrated that the symptoms of HT can be abrogated via the oral feeding of human or porcine thyroglobulin[53, 54]. Such studies in combination with the ability of oral tolerance mechanisms to induce

systemic tolerance suggest that Hashimoto's thyroiditis may be clinically diminished or eliminated via oral tolerance dosing regimens.

One of the primary auto antigens in HT is human thyroglobulin (hTG), a 660kDa glycoprotein composed of two 330kDa monomers[55]. It is produced in the thyrocytes and functions as a hormone storage protein, later metabolized to produce thyroid hormones T3 and T4[56]. hTG is heavily glycosylated and iodinated in vivo, which contributes to its implication as an auto-antigen in hypothyroid disease[57, 58]. As previously indicated, hTG is post-translationally modified by both glycosylation and iodination. It has been demonstrated that glycosylation occurs in a step-wise fashion as the protein moves from the rough endoplasmic reticulum to the Golgi apparatus[59, 60]. This modification of the protein is necessary for proper folding and final tertiary structure. However, it has been shown that iodination is not required to maintain structural integrity of the protein and acts as an antigen enhancer in autoimmune disease[58, 61, 62]. Harvesting of thyroglobulin from human cadavers is the currently accepted method of obtaining the protein, although it presents a number of difficulties. This inefficient, costly source provides all the necessary thyroglobulin for medical assays related to thyroid cancer and autoimmune thyroid disease, as well as laboratory research. The inherent genetic differences in the sources present a significant problem in securing homogenous thyroglobulin. Glycosylation and iodination patterns vary from individual to individual depending on the maturity of the protein, which in turn inhibits cross talk capabilities between medical assays and detection methods[63]. This complication, along with the limited sourcing,

drastically increases the cost of testing for or obtaining human thyroglobulin. Despite the difficulties faced in the current means of harvesting thyroglobulin, due to the modifications and size of the protein, as well as the disulfide bonds required to maintain the dimer, it has not yet been expressed in any currently available recombinant protein expression system.

In response to these difficulties, we propose that production of thyroglobulin in *Glycine max* (soybean) efficiently and cost effectively addresses these issues. Soybean seeds are approximately 40% protein by dry weight, with the majority consisting of seed storage proteins[64]. Due to this increase in protein concentration, transgenic protein expression levels have been demonstrated to be much higher than those found in other plant systems[52, 65]. The increase in protein expression seen in the soybean system leads to a number of characteristics that emphasize the cost effectiveness of the platform. The amount of biomass required for commercial production of pharmaceuticals is greatly reduced in soybean, thereby reducing the cost of greenhouse production and cultivation, enabling the crop to be contained. Furthermore, production and harvesting processes are already in place, as soybean is an established food crop[66], thereby reducing the cost associated with establishing commercial harvesting processes and manufacturing facilities. In addition to high expression levels, soybean seeds are naturally stable at ambient temperatures. It has also been demonstrated that antigens expressed in the seed remain stable and retain antigenicity following long term storage at ambient temperatures[67].

Furthermore, early studies demonstrate the success of the soybean system, as shown by the expression of an approximately 200kDa monoclonal herpes antibody in Zeitlin et al[68]. Later studies go further, specifically targeting proteins to the seed[65] and even demonstrating retention in specific sub-cellular compartments within the seed storage parenchyma cells[52]. These most recent studies have achieved expression levels greater than 2% of total soluble protein in the seed, thereby demonstrating commercial production a viable option from this system. The success of these studies may be attributed to the favorable environment encountered by such proteins in the seed. The major seed storage protein, Glycinin, comprises approximately 35% of total seed protein[64]. Therefore, it is reasonable to suggest that the inherent storage capacity of the seed generates an environment in which proteins are easily expressed and retained as storage molecules. Therefore, we conclude a storage protein, such as human thyroglobulin, would be successfully expressed and retained in such an environment, potentially providing an alternative source of protein to that of the currently available cadaver-based system.

2.2 MATERIALS AND METHODS

Vector design and construction

The 8.3 kb human thyroglobulin gene containing a native signal peptide sequence, a histidine tag sequence, and 5’ and 3’ terminal restriction endonuclease sites was optimized for soybean codon usage and synthetically generated by GeneArt, Inc. (Burlingame, CA). Following digestion with NcoI and XbaI, the synthetic gene was isolated from an agarose gel and ligated with

pTN200 that had also been linearized with NcoI plus XbaI and gel-isolated. The resulting product, pTN-hTG, was verified for integrity and transformed into Agrobacterium.

Soybean transformation

Soybean transformation using the Agrobacterium-mediated half seed method was performed (by K. Wang in Iowa and T. Clemente in Nebraska) as described in Paz et al[69]. Briefly, half-seed explants (G. max cv Williams) were dissected and inoculated with Agrobacterium suspension culture (strain EHA101 carrying various binary vectors). The inoculated explants were placed adaxial side down on cocultivation medium at 24°C and under 18:6 photo period for 3-5 days. After cocultivation, explants were cultured for shoot induction and elongation under glufosinate selection (8 mg/L) for 8-12 weeks. Herbicide resistant shoots were harvested, elongated and rooted as described[69]. Acclimated plantlets were transferred to soil and grown to maturity in the greenhouse. Transformation resulted in a total of five independent transgenic events.

Preparation of genomic DNA and PCR

Genomic DNA was prepared from cotyledon tissue using the Maxwell 16 Instrument and Maxwell Tissue DNA Purification Kit (Promega, Madison, WI). Genomic DNA (100 ng), TG primers (forward: 5'-GCTCAACCACTTAGACCATGCGA-3'; reverse: 5'-TCAGCGCAGTGGCAATATCCTG-3'), vsp primers (forward: 5'-GCTTCCACACATGGGAGCAG-3'; reverse: 5'-CCTCTGTGGTCTCCAAGCAG-3'), and dNTPs were mixed with GoTaq Flexi DNA polymerase and buffer

(Promega Corp., Madison, WI) according to the manufacturer's directions. Following an initial denaturation cycle (5 minutes at 94°C) the reactions were subjected to 38 cycles of denaturation (45 seconds at 94°C), annealing (30 seconds at 58°C) and extension (60 seconds at 72°C). PCR products were visualized in 1.0% agarose gels stained with ethidium bromide.

Glufosinate screening

To monitor for expression of the herbicide selectable marker, plants were sprayed with glufosinate herbicide spray (80mg/l) a total of three times (days 1, 3, and 5). Plants with visible chlorosis similar to that observed in nontransgenic plants were scored as negative for resistance to the herbicide and discarded while positive plants were taken to maturity. Plants known to be positive for expression of Bar were included as a control for spray concentration and application.

Seed protein extraction

Seed slivers (chips) derived from cotyledon tissue (~10 mg) were resuspended in 150µl PBS and sonicated for 30 seconds using a Vibra-Cell ultrasonic processor (Newton, CT). Samples were clarified from soluble debris by centrifugation at 16.1 x 10^3g at 4ºC. Total soluble protein was quantified with the Bradford Reagent (Bio-Rad, Hercules, CA) using BSA as a standard.

Western blot analysis

Protein extracts from transgenic and wild type seeds were run in 5% native polyacrylamide gels for approximately 2 hours at 110V. Neither the gel nor the running buffer contained β-mercaptoethanol or SDS, and samples were not

boiled prior to loading onto the gel. Human TG protein and horse spleen apoferritin (Sigma, St. Louis, MO) were included for standards and a 400kDa molecular weight marker, respectively. Following electrophoresis, gels were equilibrated in 1X CAPS (recipe for 20X: 22.13g CAPS, Sigma, in 500ml dH2O, pH 11) buffer with 10% MeOH for 10 minutes and transferred to Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked overnight with 5% dry milk in PBS solution at 4ºC, incubated with rabbit anti-TG polyclonal antibody (Gene Tex Inc., Irvine, CA) for 3 hours at 23ºC, and washed three times (10 minutes each) with PBS containing 0.05% Tween. Membranes were then incubated with goat anti-rabbit HRP-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at 23ºC and washed as described above. Detection was carried out using the SuperSignal West Pico substrate (Thermo Scientific, Rockford, IL).

Confocal microscopy

Whole seed tissue was imbibed for 16 hours in 1X PBS and the seed coat was removed. Tissue was then fixed essentially as described previously by our laboratory (Oakes et al, 2009, also check the Piller Planta paper). Sections were permeabilized with 1X PBS containing 0.2% Tween for 10 minutes, and then nonspecific binding was blocked by incubation with 1X PBS supplemented with 3% BSA for 16 hours at 4°C. Tissue was then incubated with rabbit anti-hTG serum (1:200) for 2 hours at 23ºC, followed by incubation with an AlexaFlour488 goat anti-rabbit IgG-HRP conjugated secondary antibody (1:200) for 1 hour at 23ºC. Finally, tissue was incubated with DAPI stain (1:500) for 5 minutes. Cover

slips were added to the sections using Gel/Mount aqueous mounting media. Images were collected with a LSM 710 Spectral Confocor 3 Confocal Microscope (Carl Zeiss, Inc.) using a 20X objective and a 405nm laser to visualize DAPI stained nuclei, along with a 488nm laser to collect emitted fluorescence from the alexafluor. Stacks of images were collected in the Z plane of the specimens. These stacks were comprised of 14 optical sections that were ~80nm apart. Resultant stacks were projected to form a single image, and this image was exported to Adobe Photoshop 2.0. To improve clarity and reproduction quality, image colors were proportionally enhanced.

Quantification of recombinant protein in seed extracts

Samples of human thyroid purified thyroglobulin (20, 30, 40 and 50ng) were run on a 5% SDS-polyacrylamide gel. Samples were prepared in SDS-sample buffer without β-mercaptoethanol or boiling. 5ug of total protein extract from soy line 77-5 were also loaded. Western blots were performed as described above (see Western blot analysis). Using ImageJ software[70], we measured the Integrated Density of the resulting x-ray film to compare the optical density of the known standard amounts against that of the soy protein sample. The image was inverted and background subtracted to eliminate background pixel values. The Integrated Density of each lane was measured individually, and the quantification of hTG in the soy protein sample was determined by the resulting standard curve.

ELISA quantification was performed using the in-house sandwich ELISA as described below (see ELISA). Known amounts of human thyroid purified

thyroglobulin were plated (0.01ng-10ng) to generate a curve and identify the linear range of detection. 5ug soy protein samples were loaded using a number of dilutions to ensure results fell within the linear range (undiluted, 1:10, 1:100 and 1:1000). Absorbance values were compared to those of known values to determine the amount of hTG present in the soy protein samples. Considering dilutions, amounts were converted to percentage of hTG per sample.

Gel filtration chromatography

A Sephacryl S-300 HR gel filtration column (bed height 72 cm) was calibrated by determining the peak elution volumes (absorbance at 254 nm, BioLogic LP, BIO-RAD, Inc.) of a set of molecular weight protein standards (Sigma, Inc.). Crude, total soluble protein was then isolated from hTG-positive seeds, applied to the gel filtration column, and eluted fractions were collected. Similarly, human thyroid-purified thyroglobulin (Calbiochem, Inc.) was applied to the same column, and eluted fractions were also collected. Eluted fractions were then subjected to ELISA (Orgentec) to detect the presence of immunoreactive thyroglobulin in each fraction.

ELISA

Three different types of capture ELISAs were performed in this study. The first two utilized commercial kits that contained either polyclonal antibody pairs (Orgentec Immunometric Enzyme Immunoassay kit; Orgentec, Germany) or monoclonal antibody pairs (Kronus META-Tg Serum Thyroglobulin assay kit; Kronus, Boise, ID) for capture and detection of TG. ELISAs using both commercial kits were performed according to the specific manufacturer's

directions. A third sandwich ELISA was developed in house and utilized a monoclonal antibody for capture and a polyclonal antibody for detection. Briefly, 500 ng of capture antibody (GTX21984, GeneTex, Irvine, CA) was coated onto ELISA plates by incubation at 4°C for 16 hours. Unbound antibody was washed with phosphate buffered saline (PBS) and nonspecific binding sites were blocked by incubation with 1% BSA in PBS for 1 hour at 23°C. Soy protein samples and the bovine TG standard were then loaded onto plates and allowed to complex with the bound antibody for 2 hours at 23°C. Unbound products were washed and a rabbit polyclonal detection antibody (GTX73492, GeneTex, Irvine, CA) was allowed to bind to the antigen for 2 hours at 23°C. The secondary antibody was detected using a goat anti-rabbit IgG-HRP antibody (sc2004, Santa Cruz Biotechnology, Santa Cruz, CA) by incubation for 1 hour at 23°C. The antibody-antigen complexes were incubated with TMB Substrate (BioFX, Owings Mills, MD), and colorimetric reactions were stopped by the addition of 0.6 M sulfuric acid. Absorbance values were read at 450nm.

2.3 RESULTS

Transformation and Molecular Screening of T_1 events

Human thyroglobulin (hTG) is an extremely large protein (330kDa) that contains a signal peptide and is presumably translated by the secretory pathway. To maximize the likelihood that full length hTG would be stably expressed and accumulate within soybeans, a synthetic TG gene was designed based on the human amino acid sequence and optimized for high frequency codons used within Glycine max (Figure 2.1). The synthetic TG gene was constructed and

subcloned downstream of a strong seed-specific promoter, resulting in the binary vector pTN-hTG (Figure 2.2a) which contains a glufosinate resistance marker (Bar gene) and was used for Agrobacterium-mediated transformation of soybean. This transformation yielded five independent T_0 transgenic lines (77-3, 77-4, 77-5, 77-7 and 77-12) that were taken to maturity in the greenhouse and allowed to set seed. Parent T_0 plants, as well as T_1 and T_2 plants in subsequent generations, appeared phenotypically normal to nontransgenic plants with respect to leaf color, growth height, and seed yield (Figure 2.2b).

To screen for the presence of the hTG transgene, individual T_1 progeny from each of the five transgenic events were screened using a duplex PCR assay. Slivers of cotyledon material (~20 mg) were collected from T_1 seeds prior to germination. Genomic DNA was prepared from ~10 mg of the harvested material, and mixed with primer sets that should amplify a 325 bp fragment internal control (soybean vegetative storage protein gene) as well as a 650 bp fragment diagnostic of hTG in those progeny containing the transgene. Figure 3a shows that T_1 progeny from all five of the initial transformation lines acquired the hTG transgene. In one line (line 77-3) all progeny had acquired the hTG transgene while in the remaining four lines some of the progeny were PCR-negative, demonstrating that the locus or loci containing the T-DNA had segregated away. Despite the fact that only six progeny from each line were analyzed, the presence of both TG-positive and TG-negative individuals in some of the lines demonstrates that the transgene is segregating. Overall, the PCR results are consistent with a prior progeny screen experiment in which a different

subset of T_1 seeds were germinated and sprayed with glufosinate to demonstrate herbicide resistance consistent with expression of the bar gene (data not shown). Based on this PCR screen, T_1 progeny that were negative for the TG transgene were discarded while those positive for the gene were allowed to mature and set seed.

Since the presence of a transgene is not always correlated with transgene expression, western analysis was performed to visualize the profile of any transgenic protein that accumulated within T_1 progeny seeds. Total soluble protein was extracted in PBS from the remaining 10 mg of harvested cotyledon tissue, and 10µg of protein extract was separated in native polyacrylamide gels. Rabbit sera containing polyclonal anti-hTG antibodies were used to immunologically detect seed proteins with epitopes similar to those from hTG protein. To serve as positive and negative controls, purified hTG protein and soluble protein extract derived from nontransgenic seeds were also included on the gels. Figure 2.2b shows that progeny from lines 77-3, 77-5, 77-7 and 77-12 all accumulated immunoreactive protein detected by anti-TG antibodies. Control lanes containing nontransgenic seed extract or seed extract from isogenic samples shown to be PCR-negative for TG did not contain immunoreactive proteins, demonstrating the specificity of the antibodies for hTG epitopes. In contrast to the four lines containing immunoreactive protein, the six T_1 progeny derived from line 77-4 did not contain immunoreactive protein, suggesting that TG protein accumulation was either absent or below the level of detection in this assay. It should be noted that immunoreactive protein from several of the

individual progeny migrated at 330kDa and 660kDa, analogous to where monomers and dimers of the TG standard were detected. Thus, measurable amounts of proteins immunologically similar to hTG, and with a molecular weight consistent with that of purified hTG protein, accumulate within transgenic seeds. Time course studies show that the solubilized immunoreactive protein remains stable for at least 48 hours following extraction (data not shown).

Soybean-derived hTG remains intracellular

Although the synthetic hTG gene was designed to mimic the human gene, it was unclear whether the soybean-derived transgenic protein would be secreted into the apoplastic spaces or were retained within the cell. Immunohistochemistry was therefore carried out on cotyledon tissue using fluorescently-labeled hTG antibodies. The images in Figure 2.4 show immunoreactive protein was not secreted into apoplastic spaces, but instead remained intracellular and was localized to the cell membrane. Very little transgenic protein was found throughout the cytoplasm, and DAPI staining of nuclear material showed that transgenic protein was also excluded from the nucleoplasm. No fluorescence was observed in nontransgenic control tissues subjected to the same conditions.

Soybean-derived thyroglobulin protein is recognized by commercially available ELISAs

To further evaluate thyroglobulin protein expression by transgenic soybean seeds, two different commercially available ELISAs were used. Both of these ELISAs use pairs of antibodies in a capture/detection format and are frequently used in the diagnostic industry. Total soluble protein was isolated from T_1

cotyledon tissue derived from each of the five transgenic soybean lines. These T_1 soluble protein isolates were then assayed using a commercially available Orgentec ELISA kit to detect the presence of human thyroglobulin. Soluble extract from nontransgenic seeds and purified human TG served as negative and positive controls, respectively. Figure 2.5 demonstrates that four of the five transgenic lines tested exhibited seed-specific levels of immunoreactive thyroglobulin. Consistent with the western results shown in Figure 2.2, progeny derived from line 77-4 exhibited no detectable activity. The commercially available ELISA from Orgentec uses polyclonal anti-human thyroglobulin antibodies to capture and detect human thyroglobulin. Such polyclonal antibodies likely bind both linear and conformational epitopes along the length of the thyroglobulin molecule. Thus, the immunoreactivity observed in lines 77-3, 77-5, 77-7 and 77-12 suggests that soy-derived TG epitopes are intact and that the protein molecule is properly folded.

A more stringent test to evaluate the nature of soy-derived thyroglobulin was the use of an ELISA procedure that utilized monoclonal antibodies for capture and detection. The commercially available ELISA produced by Kronus, Inc. employs monoclonal antibodies that can simultaneously recognize two different conformational determinants on human thyroglobulin. We used this assay to indicate the levels of thyroglobulin present in selected soy protein samples that were identified as expressing this protein in Figure 2.4. Soluble extract from the highest expressing T_1 progeny identified in the Orgentec ELISA (figure 4) was chosen for analysis in the Kronus assay, and two dilutions (10^{-1}

and 10^{-2}) of each sample extract were tested to ensure absorbance readings remained within the linear range (0.1-1.0) of detection. The results from this Kronus ELISA experiment are shown in Figure 2.6. As predicted, representative soluble extracts from each of the four lines tested contained immunoreactive thyroglobulin. The fact that the Kronus ELISA uses two monoclonal antibodies to capture and detect thyroglobulin provides further support for the authenticity of soybean-derived thyroglobulin.

Soy-derived thyroglobulin is stable over generations

Purification of TG and potential use of this purified protein as a pharmaceutical or analyte is only practical if protein expression and accumulation is maintained over generations. To examine whether soy-derived TG also accumulates in T_2 seeds, polyacrylamide gel electrophoresis and western analyses were performed using 5µg of seed extract derived from ten individual T_2 seed slivers harvested prior to germination and T_2 growth in the greenhouse. Figure 2.7 shows that T_2 progeny from each of the four lead lines accumulated protein that reacted with polyclonal anti-hTG antibodies. Furthermore, the migration pattern of immmunoreactive protein in several of the T_2 progeny was consistent with the distinct 330kDa monomers and 660kDa dimers detected in the purified hTG standard. This was most notable in T_2 progeny derived from line 77-5 (e.g. T_2 progeny derived from 77-5 T_1-2, T_1-5 and T_1-6 parents). These western results therefore demonstrated that accumulation of soy-derived hTG is stable over at least two generations. Additionally, we identified several lead candidates with optimal expression that can be screened for zygosity and, if found to be

homozygous, can be bulked up for seed production, hTG protein purification and comprehensive protein analytical characterization.

Partial purification of soybean-derived hTG

To begin a physico-chemical characterization of soybean-derived thyroglobulin, gel filtration chromatography was used to partially purify soy-derived TG present in soluble protein extracts of ELISA-positive and Western-positive seeds. A Sephacryl S-300 HR gel filtration column was calibrated by determining the peak elution volumes of a commercial set of molecular weight protein standards ranging in size from 669,000 MW down to 29,000 MW. The elution volumes and molecular weights of the protein standards are indicated on the chromatogram plot in Figure 2.7a. It should be noted that the peak bovine TG standard (MW 669,000) eluted in fraction #20. After the gel filtration column was washed, soluble protein extract from lines 77-7 #6 and 77-3 #6 was applied to the column and eluted fractions were collected. Protein absorbance (280 nm) was recorded during elution and fraction collection and plotted as relative absorbance. The bulk of native soluble seed proteins eluted from the gel filtration column in fractions 16-19.

The eluted fractions were subjected to a sandwich ELISA that was previously developed in-house to detect the presence of immunoreactive TG. The sandwich ELISA utilized a monoclonal capture antibody and a polyclonal detection antibody. Figure 2.8b shows the immunoreactive profile for soybean-derived TG infractions 11-34, plotted as relative absorbance at 450 nm. Although fractions 16-24 all contained detectable levels of immunoreactive TG,

the majority of the immunoreactivity was detected in fractions 19-21. Notably, the bovine TG standard (669,000 MW) was previously shown to elute in fraction 20 (Figure 8a). Therefore, the ELISA results strongly suggests that soybean-derived TG folds in a manner similar to that of the commercial bovine TG standard and can exist as a ~660kDa dimer.

For a comparison, human thyroid-purified TG was also run over a Sephacryl column, and analogous fractions were collected and assayed for immunoreactivity using the ELISA described above. The immunoreactive profile for hTG was also plotted as relative absorbance and is shown in Figure 2.8a. In contrast to the tight peak of immunoreactivity observed in the soy-derived TG profile (fractions 19-21), the peak activity of thyroid-purified hTG was spread over fractions 16-27. This result suggested that commercial hTG may not be as homogenous as soybean-derived TG.

To analyze whether soy-derived TG dimers were present in eluted fractions, western analyses were performed. Equivalent volumes of fractionated sample from eluted fractions containing soy-derived TG and thyroid-purified TG were separated in native polyacrylamide gels, and immunoreactive proteins were detected using polyclonal anti-hTG antibodies. To serve as a visual marker, hTG was included as a standard in each experiment. Figure 8b shows that eluted fractions containing peak ELISA immunoreactivity also contained the greatest level of signal in the western experiments. Furthermore, the migration of immunoreactive protein in soy-derived TG eluted fractions was analogous to that of the 660kDa hTG dimers observed in the standard. These results further

demonstrate that native soy-derived TG is capable of forming functional dimers. Consistent with the profiles in Figure 2.8a, the greatest levels of soybean-derived TG signal were detected in fractions 19-21 while the greatest levels of thyroid-purified TG were spread over fractions 17-23. These results suggested a potential difference in the homogeneity of the human sample compared with that of the soy-derived sample.

2.4 DISCUSSION

To date, the sole source of purified hTG for use as a research and diagnostic reagent is derived from human cadaver and surgically removed thyroid tissue. In this study we have successfully expressed a recombinant form of hTG in soybean seeds. The expression of hTG in soybeans represents one of the largest recombinant proteins expressed in any plant host system to date and creates an alternate and novel source for this relevant protein. The authenticity of recombinant hTG was confirmed by ELISA using commercially-available kits developed specifically for the detection of hTG (Figures 2.5,2.6). Western analyses and gel filtration chromatography experiments demonstrated that soy-derived hTG and thyroid-purified hTG are biologically similar with respect to size, charge, mass and subunit interaction (Figures 2.3, 2.7, 2.8). The expression of hTG in transgenic seed extracts was stable over two generations, and represented ~0.8% of total soluble seed protein (Figure 2.7).

To our knowledge, no literature has reported the recombinant expression of hTG in any host system traditionally used for the production of recombinant proteins. It is not clear whether attempts to express recombinant forms of this

protein were unsuccessful or such attempts were never made. However, given the importance of hTG in the diagnostics industry, along with the absence of an accepted universal standard and the issues associated with purifying thyroglobulin from human tissue, it seems unlikely that attempts were never made using traditional systems such as yeast, insect cell culture and mammalian cell culture. We have demonstrated that soybeans represent a practical alternative to existing recombinant host systems for the expression of an extremely large and complex protein. We believe that recombinant expression of hTG in soybeans was possible due to the fact that soybean seeds contain a biochemically unique environment, serving as a “sink” for the accumulation and storage of large protein complexes. This is evident by the abundance of 7S and 11S protein complexes that comprise ~70% of total soybean seed protein [64, 71]. While the individual protein subunits that comprise the 7S and 11S complexes are not considered large, the mature complexes that subunit proteins form are indeed large in size and mass, very comparable to the size and mass of mature hTG. Thus, it was conceptually logical to assume that an environment that has evolved to store large protein complexes would therefore represent an ideal target site for the expression and accumulation of a recombinant protein which may otherwise be difficult or impossible to express in traditional host systems.

The accumulation of recombinant hTG in line 77-5 was shown to represent ~0.8% of total soluble protein as measured by western densitometry and ELISA. Although there have been relatively few published reports of

proteins targeted to soybean seeds, the observed accumulation level of seed-specific hTG is comparable to levels of recombinant proteins reported by other colleagues. For example, Ding et al [65] reported that recombinant basic fibroblast growth factor (bFGF) represented ~2.3% of total soluble soybean seed protein, while Moravec et al [52] reported that recombinant B subunit pentamers of E. coli heat labile toxin (LT-B) represented ~2.4% of total soluble soybean seed protein. Although the expression level of hTG is not as great as that of bFGF and LT-B in the soy system, it nonetheless represents a significant yield of recombinant protein. For example, with an expression level of 1.0%, a single transgenic soybean seed will contain approximately 0.6 mg of recombinant hTG (~180 mg seed weight x ~38% protein content x ~1.0% expression level), or about 150 mgs of transgenic protein per plant. It has been suggested that increased levels of transgenic protein can be obtained by targeting the protein to the ER by inclusion of an ER retention signal (H/KDEL). Given that optimal locations for subcellular expression are usually identified by trial and error, we have generated constructs to evaluate whether or not recombinant hTG targeted to the ER lumen can accumulate to levels >1% of total seed protein. However, given that hTG is a secreted protein, along with the fact that misfolded hTG protein complexes are degraded in vivo within the ER lumen of human thyrocytes [72], we chose not to include the ER retention signal in our original construct design.

During the course of these studies, we have found that recombinant hTG is stable in seed extracts for at least 1 week, and stable in seeds stored for > l

year at ambient temperatures in the laboratory. The stability of recombinant proteins in soybean seeds is not surprising given that the primary purpose of the seed is to store proteins for long periods of time under conditions that may not be optimal. Studies with other recombinant proteins targeted to soybeans have also demonstrated stability of transgenic proteins when seeds were stored for up to 4 years under ambient conditions [52, 67].

Stability, along with the production of a uniform product, will increase the means by which this alternative thyroglobulin source can be utilized. Commercially purified hTG is derived from human tissue and therefore represents a heterogeneous population comprised of immature and mature forms of hTG. Immature forms of hTG lack carbohydrates and iodine residues while mature forms contain up to 10% carbohydrate and 1% iodine [72]. Thus, different batches of commercially-purified hTG can be quite dissimilar with respect to purity and composition. This fact was borne out in Figure 2.7 where hTG purified from human thyroid tissue eluted as a relatively broad peak on molecular sizing chromatography columns. We speculate that soybean-derived hTG will be much more homogenous than thyroid-purified hTG. Since soybeans do not have enzymes for iodination, soy-derived hTG will not be iodinated. A non-iodinated soy-derived hTG standard represent a significant advantage to current thryroid-derived hTG standards since iodination increases the antigenicity of the protein, thereby introducing the possibility of false and/or inconsistent test results when used in ELISA-based screens[73, 74] Similarly, seed proteins are stored as “mature” forms of protein, thus it is likely that soy-derived hTG will be uniformly

glycosylated. While protein glycosylation in soybean differs from that in humans, the antibodies used in FDA-approved ELISAs utilizing hTG as a reference standard recognize the protein backbone, not the carbohydrate residues [75]. We are currently conducting protein analytical experiments to characterize the extent and composition of carbohydrate modification on soybean-derived hTG. We anticipate that the lack of iodination, along with uniform glycosylation of proteins within the seed will contribute significantly to overall hTG protein homogeneity.

While future studies will focus on purification and analytical characterization of soy-derived hTG, it will also be important to characterize the recognition of the soybean derived protein by antibodies found in human sera, particularly from individuals with autoimmune hypothyroid disease. The confirmation of soy-derived hTG as a bioequivalent to hTG purified from homogenates of human tissue will result in the first alternative sourcing of this protein. It is unclear what other uses may be derived from this never before available resource, but possibilities include the production of novel medical assays and devices to improve detection and/or treatment of thyroid cancer and disease. Furthermore, this resource could drastically improve the basic research relating to thyroid dysfunction and disease due to the increased supply and reduced cost of the recombinant molecule.

Figure 2.1

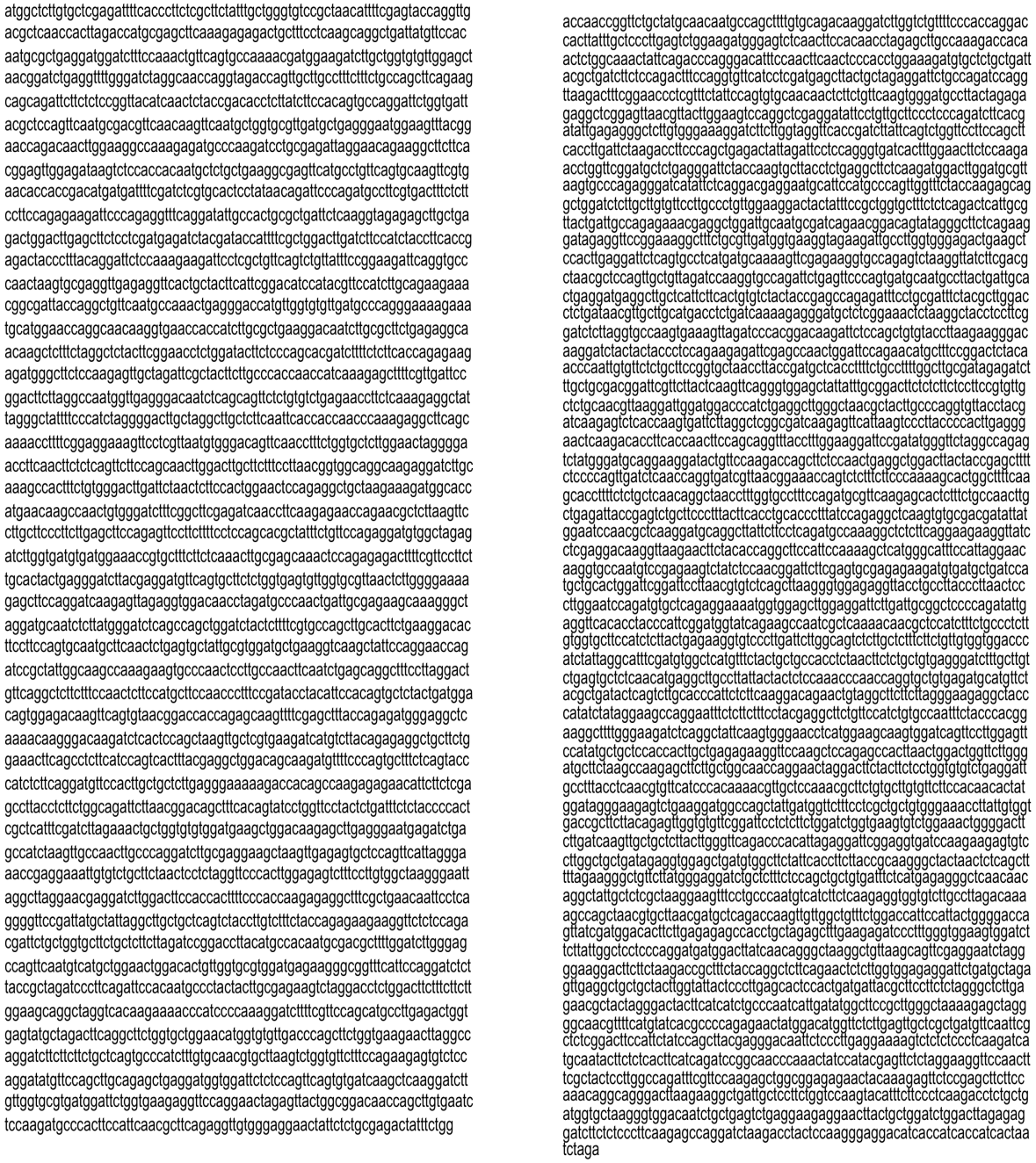

Figure 2.1: hTG gene sequence. hTG sequence optimized for soybean codon usage and cloned into the binary vector, pTN-200, for transformation into *Agrobacterium* and *Glycine max*.

Figure 2.2

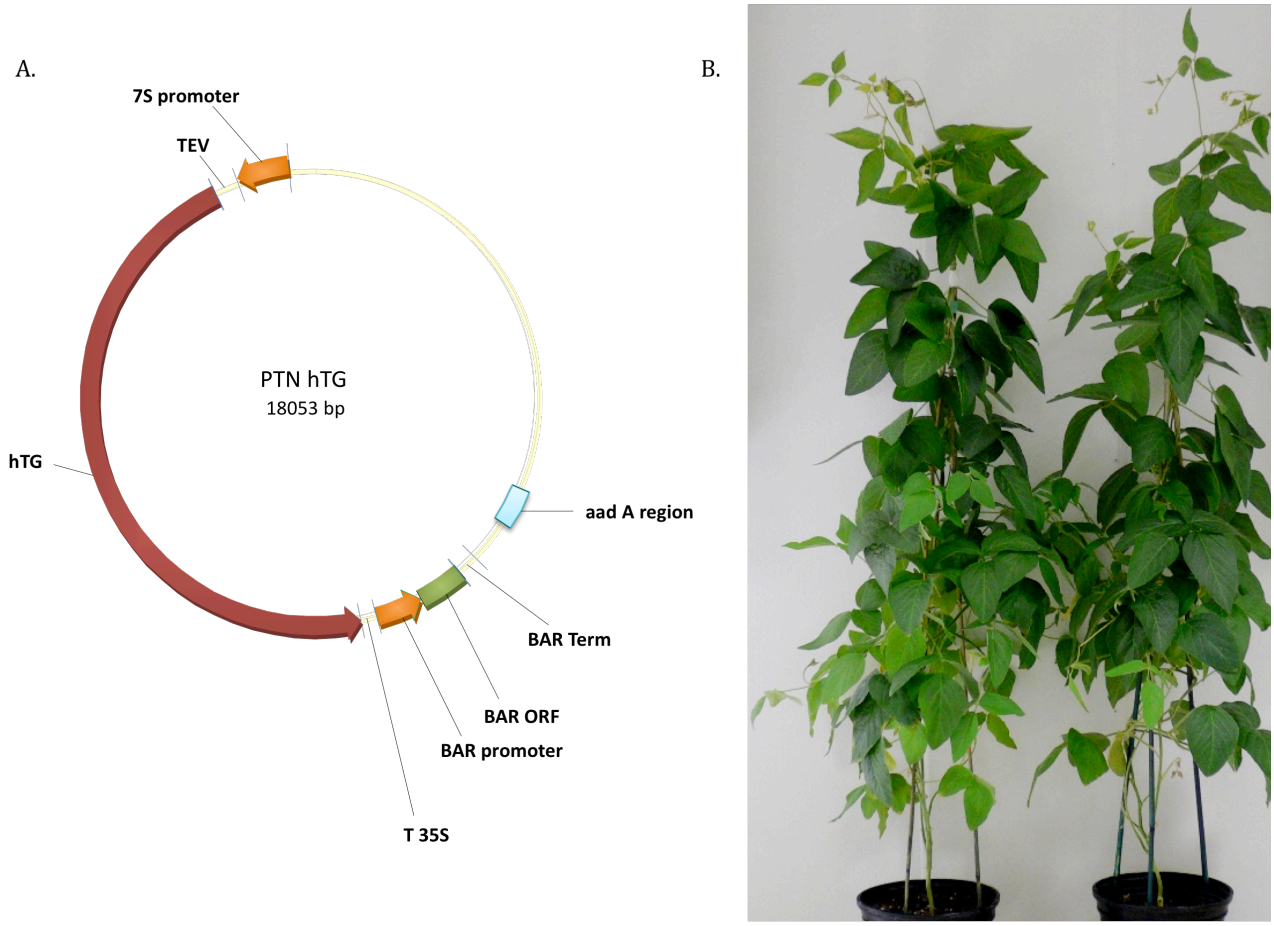

Figure 2.2: Gene design and soybean transformation. A. Binary vector pPTN-hTG used for *Agrobacterium*-mediated transformation. P-7S, soybean β-conglycinin promoter; TEV, tobacco etch virus translational enhancer element; hTG, synthetic human thyroglobulin gene; T-35S, cauliflower mosaic virus terminator element; P-nos, nopaline synthase promoter, Bar, phosphinothricin acetyltransferase gene for plant selection; T-nos, nopaline synthase terminator element; RB, right border sequence; LB, left border sequence; aad A region, streptomycin resistence gene for bacterial selection. B. 60-day old transgenic (line 77-5) and WT (control) plants.

Figure 2.3

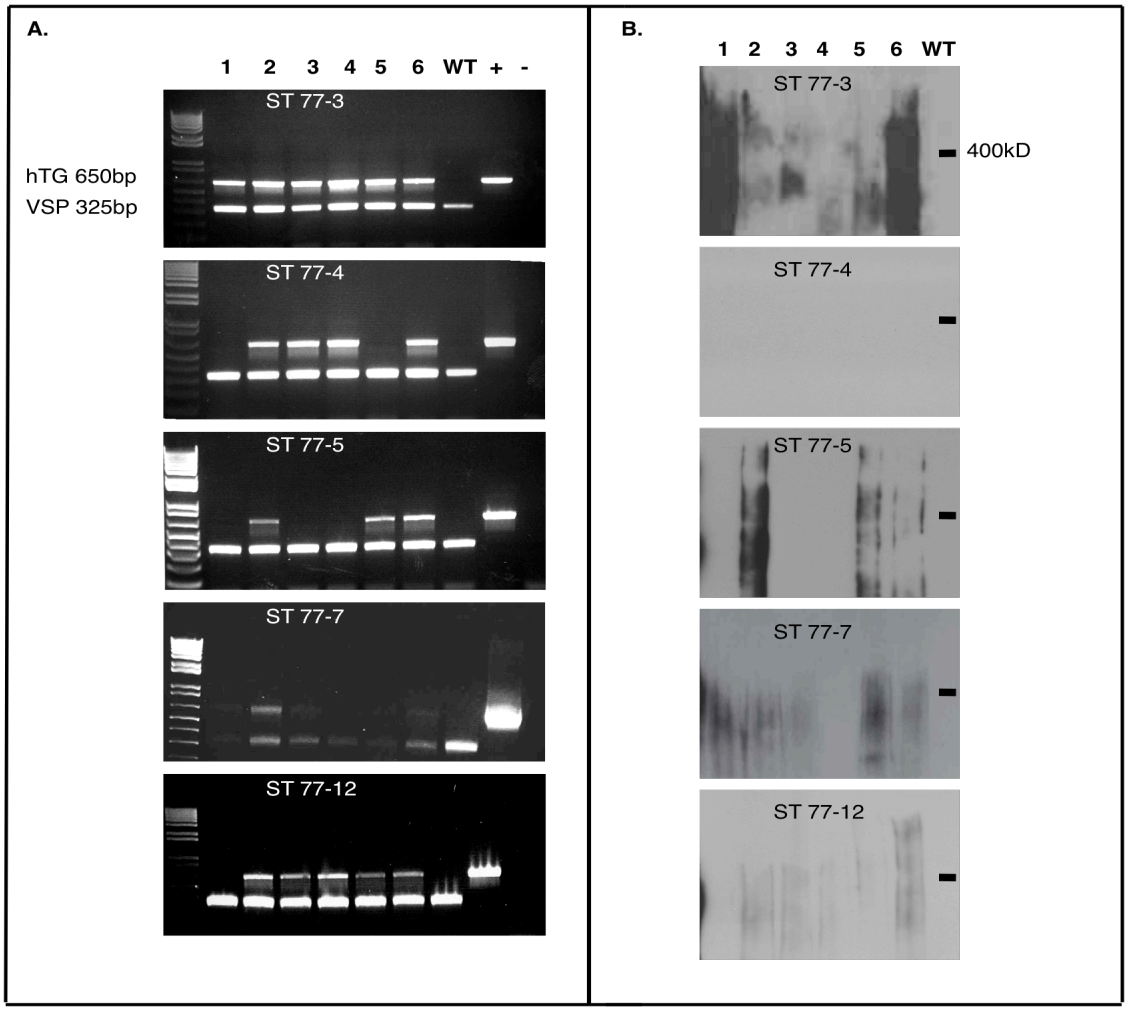

Figure 2.3: Molecular characterization of six individual T_1 progeny derived from each parent line. Top panels: Agarose gel (1%) showing results from duplex PCR used to detect vegetative storage protein (vsp, internal control) and hTG sequences in genomic DNA isolated from T_1 seed chips. Amplified PCR products diagnostic of vsp and hTG migrate at 325 bp and 659 bp, respectively. Genomic DNA from control reactions are designated "WT" (wild type, nontransgenic) and "+" (plasmid pPTN-hTG). The designations for molecular weight markers are shown in base pairs. Bottom panels: Western analysis of T_1 crude seed proteins separated in 5% native polyacrylamide gels. The migration of 250 kDa and 400 kDa proteins used as visual standards is shown. Arrows represent the locations of monomeric (M) and dimeric (D) forms of hTG as visualized in western blots by inclusion of an hTG standard (75 ng of commercially-purified hTG) run on each gel.

Figure 2.4

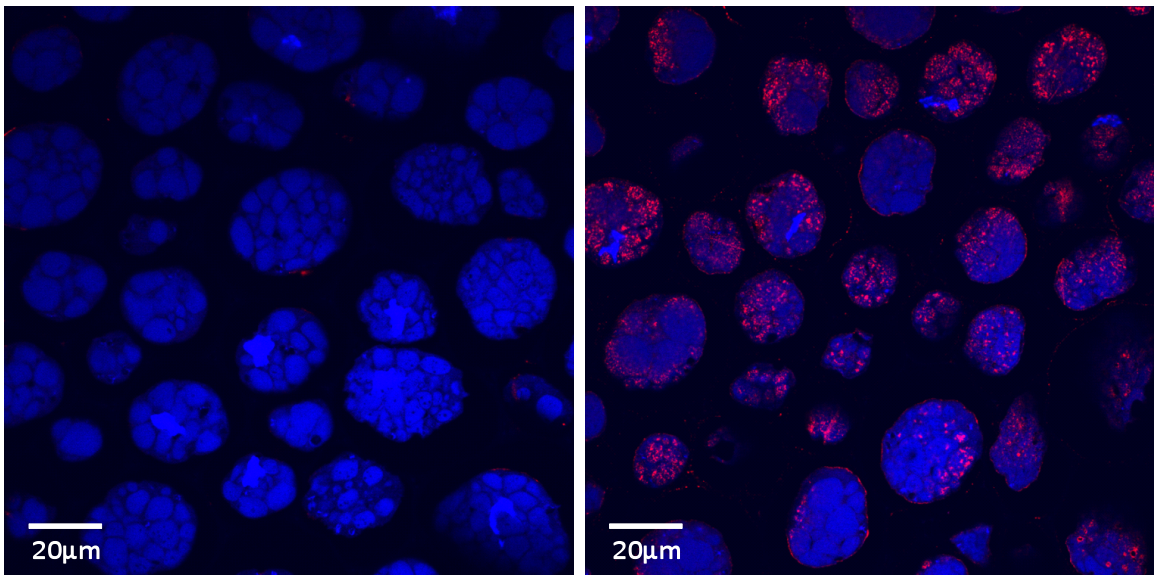

Figure 2.4: Representative confocal image demonstrating hTG accumulation in transgenic T_1 cotyledon tissue. The blue color represents DAPI staining while the red color represents fluorescence from the AlexaFluor antibody recognizing transgenic hTG.

Figure 2.5

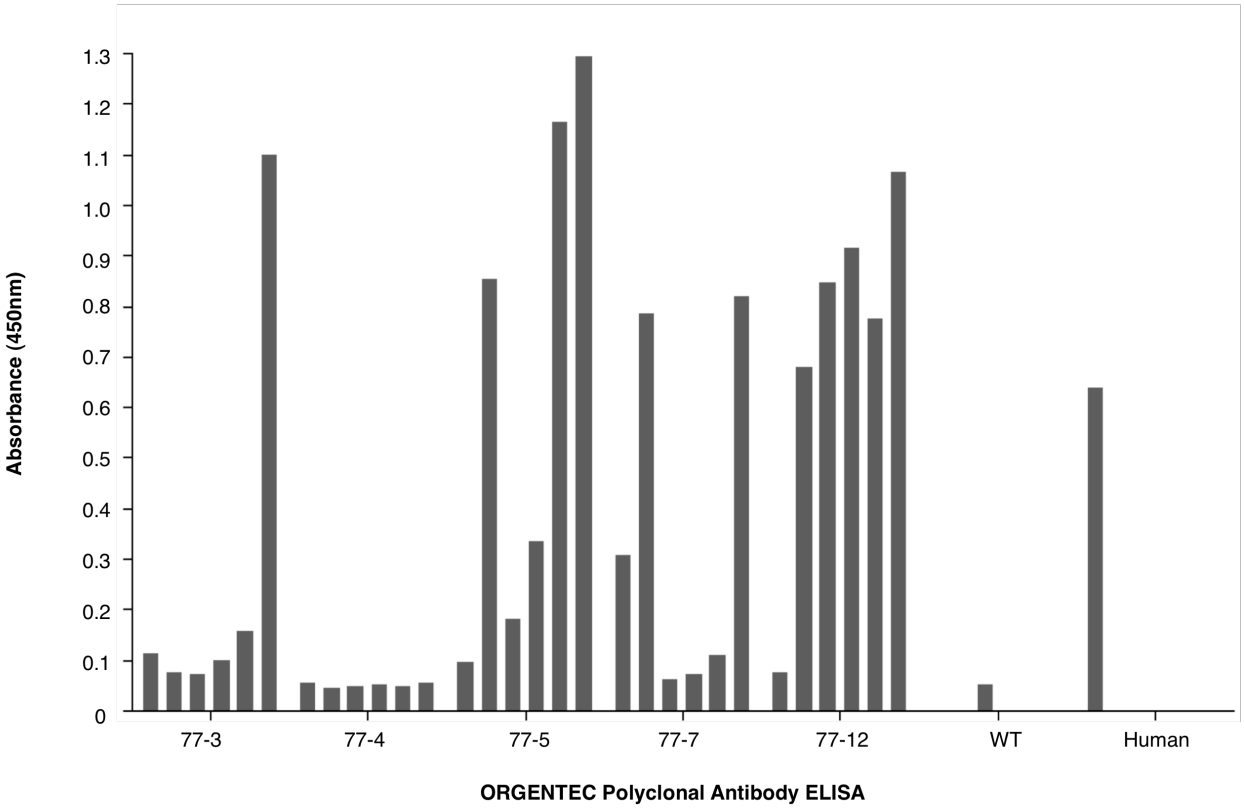

Figure 2.5: Orgentec ELISA used for detection of hTG in six T_1 progeny seed extracts derived from each T_0 transgenic parent. Soluble protein from nontransgenic seed (WT) and commercially-purified hTG (human) were used as negative and positive controls, respectively. Values shown are the absorbances (450 nm) from six individual T_1 progeny from each of the five transgenic lines, as well as controls. Samples denoted with an asterisk represent the individual sample chosen from each line for hTG detection using the Kronus assay (see Figure 5).

Figure 2.6

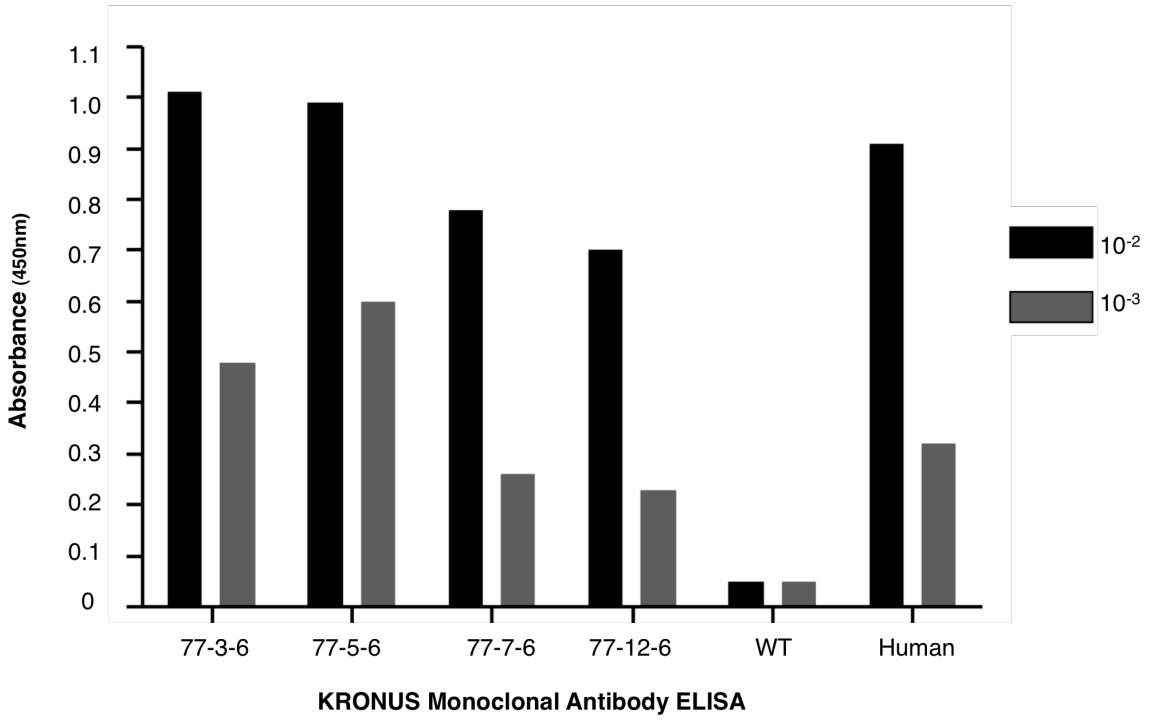

Figure 2.6: Kronus ELISA used for detection of hTG in select T_1 seed extracts, which had been normalized for TSP via Bradford assay. Crude seed extract from one representative T_1 progeny (indicated by an asterisk in Figure 4) was examined, along with soluble protein from a nontransgenic seed (WT) and commercially-purified hTG. Five different dilutions of each selected sample were tested in the ELISA and absorbance values for two of these dilutions (1:100 and 1:1000) are shown along with controls for comparison.

Figure 2.7

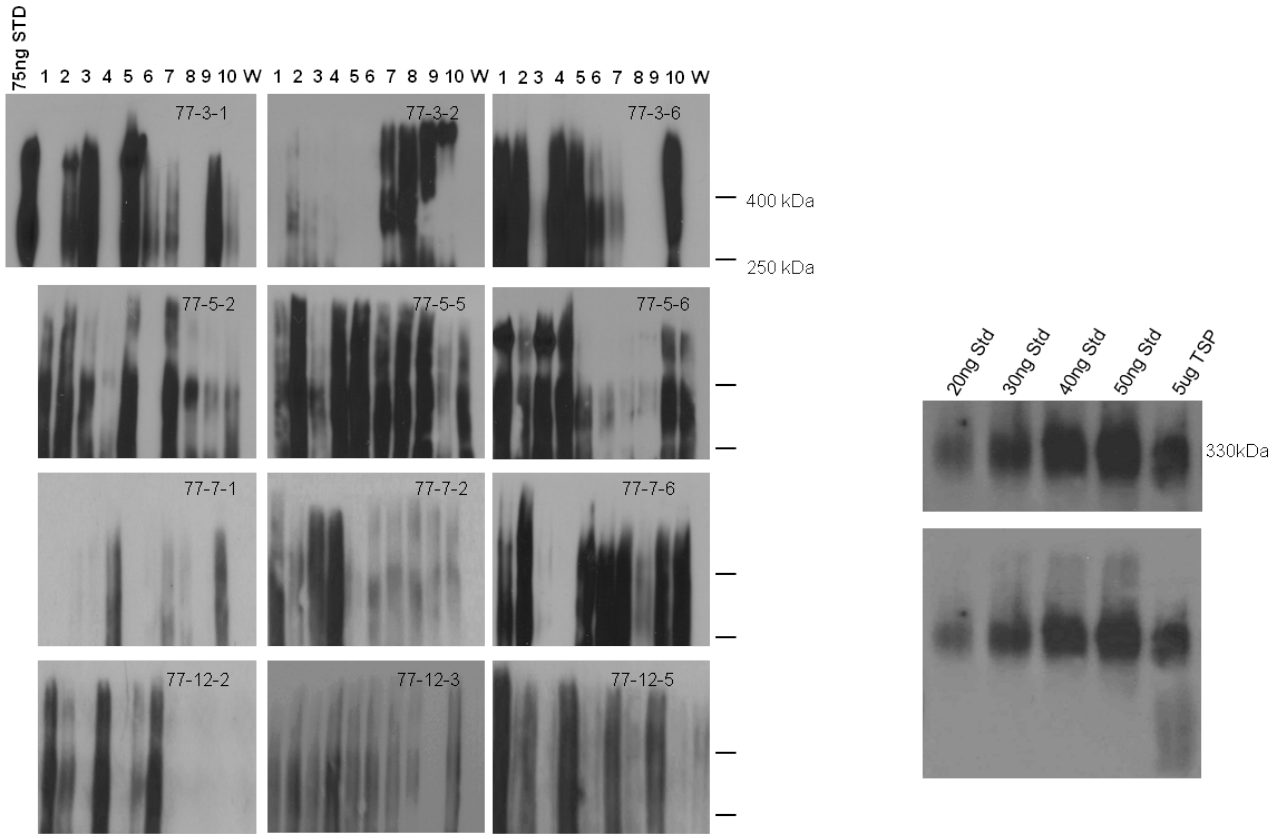

Figure 2.7: Western analysis of T_2 seed protein. A. Crude seed extracts (5 µg) from 10 random T_2 progeny were separated in 5% native polyacrylamide gels and screened by western blot analysis for the presence hTG. Thyroid-purified hTG (75 ng) served as a standard to visualize monomeric and dimeric forms of the protein. Nontransgenic seed protein (5 µg) served as a negative control. The locations of 400 kDa and 250 kDa molecular mass standards, as well as the monomeric (M) and dimeric (D) forms of hTG are indicated. B. Western quantification of recombinant hTG. The indicated amounts of commercially purified hTG protein and seed extract protein (line 77-5) were incubated in SDS-sample buffer (in the absence of β-mercaptoethanol and boiling) to relax dimeric protein and result in predominantly monomeric protein for visualization and quantification following western analysis. A densitometry curve was generated by scanning the gel image and plotting integrated density of each known standard using ImageJ software. Extrapolation from this curve revealed 27.97 ng of hTG protein in 5 ug of seed extract, representing ~0.8% of total soluble seed protein. (STD = Standard, TSP = total soluble protein)

Figure 2.8

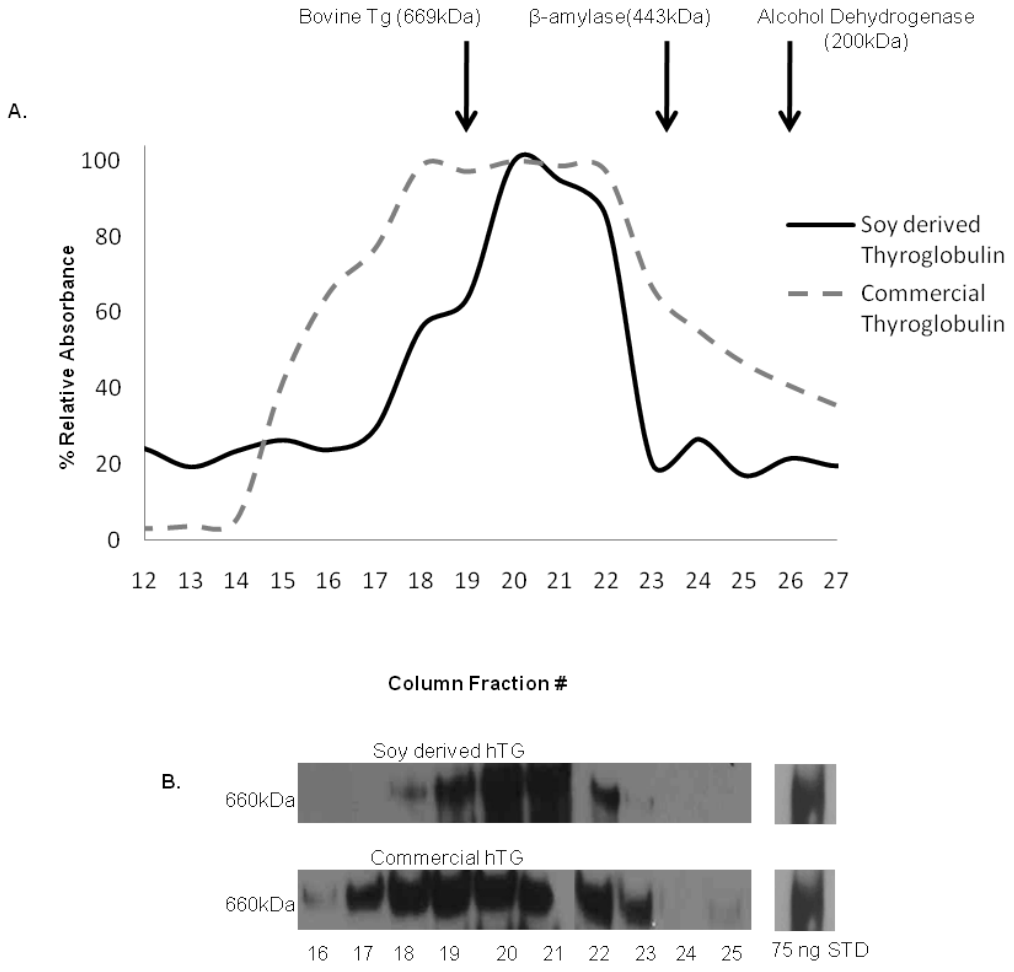

Figure 2.8: Fractionation and partial purification soy-derived hTG from crude seed extracts. A calibrated Sephacryl S-300 HR column was used to separate either a crude extract of seed protein or human thyroid purified thyroglobulin. A. Eluted fractions were subjected to a capture ELISA for detection of hTG. The ELISA results are shown as a percentage of the relative abosorbance (450 nm) for eluted fractions containing crude soybean seed protein (solid line) and human thyroid-purified protein (dashed line). Fractions 1-11 and 28-36 showed minimal absorbance and therefore are not included on the plot. The locations of molecular mass standards used for calibration are shown by arrows (669 kDa, bovine thyroglobulin; 443 kDa, β-amylase; 200 kDa, alcohol dehydrogenase). B. Western analysis of eluted fractions. Equal amounts of protein from the indicated fractions were separated in 5% native gels and subjected to western analysis. The location of the dimeric (D) form of hTG is indicated.

CHAPTER 3: ORAL ADMINISTRATION OF THE SOYBEAN-DERIVED IMMUNOMODULATORY PROTEIN hTG INDUCES TOLERANCE IN THE MURINE MODEL OF AUTOIMMUNE THYROIDITIS

3.1 INTRODUCTION:

Tolerance is the systemic and/or localized prevention of potentially harmful immune responses to a particular antigen. Most notably, this response is seen in the prevention of immune responses to self antigens, molecules necessary for various functions but able, if unchecked, to induce a robust and unnecessary immune response. Oral tolerance is generally described as the suppression of primarily the Th1 immune response to a specific antigen due to prior administration of that antigen via the oral route [15]. To date, two tolerance mechanisms of action have been described: active suppression via T-regulatory cells (Tregs) and induction of clonal anergy/deletion[14, 15, 38]. Studies have suggested that low doses of oral antigen favor cell-driven tolerance, while higher doses of antigen induce clonal deletion or anergy of antigen specific T-cells [38, 76, 77].

Tregs are a subpopulation of CD4+ T-cells and are required for the maintenance of self tolerance and the prevention of autoimmune disease [78]. Such cells secrete cytokines such as TGF-β, IL-10 and IL-4 to down-modulate or suppress the generation and activity of effector T-cells [14, 38]. In addition to cell contact upon recognition of a particular antigen, adaptive Tregs have the ability

to induce bystander suppression, a non-antigen specific form of tolerance. The cytokines produced by the antigen-specific Tregs can cross-prime CD4+ and CD8+ T cells in the area to induce the regulatory/suppressive phenotype in those cells[15]. The generation of antigen-specific Tregs seems to occur in the gut-associated lymphoid tissue (GALT), with migration to peripheral lymphoid tissue[79]. As with mucosal immunity, antigen is taken up from the intestinal milieu by microfold or M-cells and transported to the Peyer's Patches. Antigen is presented via gut-associated antigen presenting cells (APCs) to T-cells found in the GALT and the mesenteric lymph nodes (MLN). Retinoic acid from dendritic cells in both the GALT and MLN enhances the expression of the Foxp3 phenotype in T-cells, resulting in Treg generation. These Tregs can then migrate to the peripheral lymphoid tissues upon antigen recognition to induce systemic tolerance[14].

Clonal deletion functions via the apoptotic elimination of antigen-specific T-cells[35]. Studies suggest that feeding high doses of a particular antigen increases the susceptibility of antigen-specific T cells to undergo apoptosis following systemic challenge with the same antigen[80]. Gut-associated APCs then engulf the apoptotic cells and secrete the suppressive cytokine TGF-β, demonstrating that the mechanisms of tolerance most likely overlap[15].

Clonal anergy is the lack of function in antigen specific T cells when regulatory mechanisms are not present[15]. The role of anergic T-cells is best understood in peripheral tolerance to self-antigens[81]. However, as these cells have been shown to retain cytokine production capabilities and demonstrate

suppressive activity, it is clear they maintain a role in oral tolerance[14, 15, 38, 81].

Organ-specific autoimmunity (the failure of self tolerance) is characterized by chronic T-cell or antibody targeting of a particular organ. Thyroid disease accounts for more than 30% of all organ-specific autoimmunity, with Hashimoto's thyroiditis (HT) being the most prevalent, affecting over 3% of the global population, predominantly women[25]. HT is the result of chronic thyrocyte depletion and, thus, a reduction in thyroid hormone production. Thyrocyte depletion is caused by immunocyte infiltration into the thyroid parenchyma and subsequent destruction of the thyrocytes, ultimately leading to gland fibrosis[25, 65, 82]. While the presence of autoantibodies to thyroid hormones such as thyroglobulin and thyroid peroxidase is the diagnostic hallmark of disease and mechanisms including cytotoxic T-lymphocyte destruction and death receptor induced apoptosis have been proposed, it is much more likely that disease pathology is a result of the interplay between these factors[28].

As stated previously, T-regulatory cells suppress the activity of effector T-cells, thus inhibiting the pro-inflammatory Th1 immune response. The Treg population found in the thyroid is abundant, in both healthy individuals and those in the early stages of autoimmune thyroid disease (AITD)[83]. Both thyrocytes and resident Tregs are known to express death receptors and death receptor ligands, such as Fas and Fas-ligand. When induced, these molecules induce an apoptotic pathway, leading to the destruction of the cell[84]. Under normal physiological conditions, these receptors and ligands are not expressed at a

sufficient level in the thyroid to induce apoptosis of either the Tregs or the thyrocytes. However, T-lymphocytes infiltrating the thyroid under disease conditions demonstrate significantly increased expression of Fas and Fas-ligand. This upregulation of expression together with the pro-inflammatory cytokines produced by the infiltrating T-cells (Il-1β, IFN-γ, TNF-α) result in the induction of the apoptotic pathway in both thyrocytes and T-regulatory cells[84-86]. The decrease in resident Tregs in the thyroid leads to an increase in the Th1 immune response that Tregs typically suppress. This mechanism is particularly efficient as it exacerbates a positive feedback loop: as more pro-inflammatory cytokines are released in the Th1 response, T-regulatory cells will continue to diminish via apoptosis, prolonging the Th1 response, enhancing cytotoxic T-lymphocyte activity against thyrocytes and resulting in the chronic gland fibrosis characteristic of disease[28]. Additionally, the lack of Tregs decreases the expression of B-cell inhibitory markers, such as CD25, Foxp3 and TGF-β[87]. The increase in B-cell activity ultimately leads to production of autoantibodies against thyroid hormones and the activation of the complement cascade, culminating in the further reduction of thyroid hormones and glandular destruction.

Previously, we have demonstrated the successful expression of recombinant human thyroglobulin in the *Glycine max*, or soybean, platform. As thyroglobulin is one of the primary antigens in HT[73], it is reasonable to suggest that the induction of tolerance against this antigen could have inhibitory effects to early onset disease symptoms and progression. Based upon the limited success of previous studies[88] and the understanding of the mechanisms described

above, we anticipate tolerance to thyroglobulin can be achieved and HT inhibited prophylactically via the repeated oral administration of soybean-derived human thyroglobulin.

3.2 MATERIALS AND METHODS:

Preparation of soymilk extracts

A total of 40 seeds from either hTG line 77-5 or wild type plants were ground to a fine powder. For each gavage, 346mg of seed powder was resuspended in 3ml of phosphate buffered saline (PBS) and sonicated for 1 minute using a Vibra-Cell ultrasonic processor (Newton, CT). Samples were spun at 15,000 rpm for 5 minutes, and supernatant was transferred to a 1.5ml eppendorf tube. Tubes were spun for an additional 30 seconds at 16.1 x 10^3g for clarification.

Oral gavage of soymilk and I.P. immunization

Six to eight week old female Balb/c mice were gavaged every other day for 26 days as follows: using a 22 gauge feeding needle, 200ul of either hTG soymilk protein extract containing 280ug hTG or wild type soymilk protein extract was administered to each animal via oral gavage. On day 14, both groups were immunized intraperitoneally with 100ug of commercial human thyroglobulin (Calbiochem, UK) in aluminum hydroxide gel as an adjuvant (Sigma-Aldrich, St Louis, MO).

Sera collection and tissue culture

Following euthanasia on day 42, sera and splenocytes were isolated for quantification of antibody titers and T-cell restimulation assays. Spleens were ground through 30 mesh screens to isolate leukocytes. Resulting cells were cultured in RPMI-1640 with 20% FBS (BD Biosciences, Chicago, IL). Cells were

plated at 10^6 cells per well in 96-well flat bottom tissue culture plates, coated with 10ug commercial hTG or FBS and incubated for 72 hours.

T-cell restimulation and IFN-γ / IL-4 capture ELISA

Supernatants from cultured cells were taken after 72 hours. Supernatants were tested for IFN-γ and IL-4 via capture ELISA. Briefly, plates were coated with 5ug/ml capture antibody in 0.1M $NaHCO_3$ and incubated at 4ºC overnight. Plates were washed with PBS and blocked for 1 hour with 1% BSA-PBS. Plates were washed a second time, and 100ul of samples at varying dilutions were added to wells and incubated at room temperature for 2 hours. Following a third wash, 100ul of polyclonal rabbit antibody was added to each well. Plates were incubated for 2 hours. After washing with PBS, a polyclonal anti-rabbit-Ig-HRP conjugated antibody was added to each well and incubated for 45 minutes. Plates were washed and coated with TMB Substrate, with colorimetric reactions stopped by the addition of 0.6M sulfuric acid. Absorbance was read at 450nm.

ELISA determination of hTG antibody titers

ELISA plate was coated with 100ng of commercial hTG (Calbiochem) overnight at 4°C. Plate was then washed with PBS and blocked with 1% BSA-PBS for 1 hour. After a second wash, 100ul of sera samples of varying dilutions were loaded on to the plate and incubated at room temperature for 2 hours. Following a third PBS wash, 100ul of anti-mouse IgG-HRP antibody (Southern Biotech) at 1:500 dilution was added to each well and allowed to incubate for 1 hour. The antibody-antigen complexes were coated with TMB Substrate (BioFX, Owings

Mills, MD), and colorimetric reactions were stopped by the addition of 0.6 M sulfuric acid. Absorbance values were read at 450nm.

3.3 RESULTS:

Oral delivery of soybean-derived hTG results in lower antibody titers to thyroglobulin.

Sera collected from the wild type mouse group and the soy-derived hTG mouse group was used to determine antibody titers against commercial human thyroglobulin. To determine if there was a difference in antibody production between the groups, an ELISA was performed. Figure 3.1 demonstrates that, at three different dilutions, there is a difference in antibody titers between the wild type and the hTG group. While no statistically significant data was established, the individual data suggests 40% of the hTG group were tolerized. This suggests the mice that received the hTG soymilk formulation induced, at least partially, either a high or low-dose tolerance response to the antigen in the milk, while the wild type mice, having never received hTG prior to peritoneal immunization, were unable to do so at all.

T-cells from orally tolerized mice exhibit decreased response to thyroglobulin stimulation.

To further elucidate the type of tolerance response driven by the oral administration of soy-derived thyroglobulin, leukocytes isolated from spleens were subjected to restimulation with commercial hTG. The supernatants from these cell cultures were analyzed for IFN-γ and Il-4 production via ELISA. IFN-γ is a pro-inflammatory Th1 cytokine produced by activated T-cells. Figure 3.2

indicates the difference (*p=0.02)* in IFN-γ production between T-cells from the wild type group and those from the hTG group. The decreased production of IFN-γ indicates a shift to an anergic response by the T-cells to the stimulus. This is further supported by the high doses of tolerogen (280ug) administered in each gavage. Furthermore, figure 3.3 exhibits no difference in IL-4 production between the groups. IL-4 is a Th1 suppressive cytokine produced during a T-regulatory cell response. The lack of difference between the groups indicates that active suppression by Tregs does not play a significant role in the induction of tolerance against human thyroglobulin.

3.4 DISCUSSION:

Currently, the only available therapy for individuals with HT is lifelong treatment via levothyroxine, a synthetic hormone replacement. In these studies, we propose a potential one time prophylactic alternative. In the course of 28 days, we were successfully able to inhibit antibody production to thyroglobulin, a primary HT antigen, in mice, as indicated by figure 3.1. While the response was not completely abrogated, we maintain that continued therapy would further decrease the immune response, ultimately inhibiting it completely. This is supported by the data from the antibody titers, indicating that only mice who received hTG soybean formulations had decreased titers, while the control groups were entirely unaffected by the procedure. We propose such therapy would be most advantageous when used prior to disease diagnosis in the target population: briefly, women over 30 with a familial history of autoimmune disease. Furthermore, as HT is a progressive disease, such therapy could be utilized

during the early stages of symptom onset to inhibit further progression of disease pathology.

While the symptoms and diagnostic hallmarks, such as hTG antibody production, of HT are well characterized, the mechanism of disease is less so. Therefore, it is essential to determine the role of T-cells, as well as B-cell antibodies, in disease onset. Exploring the means by which tolerance induction inhibits CD4+ T-cell stimulation will shed greater understanding on the mechanistic failures in the immune response that serve to initiate this disease.

As indicated previously, there are two mechanisms of tolerance induction: T-regulatory cell activation and clonal anergy/deletion. During these experiments, we used a T-cell restimulation assay to determine the mechanism of tolerance being utilized in response to oral antigen delivery. A follow-up ELISA for IFN-γ (figure 3.2) demonstrates a pronounced difference in the cytokine production between the wild type and experimental groups. As IFN-γ is a Th1 cytokine and shows a decrease in production by experimental group (hTG) T-cells, it can be inferred that these cells are not responding enthusiastically to the thyroglobulin antigen and are, thus, exhibiting an anergic state. Alternatively, the high levels of IFN-γ produced by wild type T-cells indicate a robust immune response to thyroglobulin.

While IFN-γ is an indicator of T-cell activity, other cytokines, such as IL-4, can be used to determine the response of Tregs (figure 3.3). IL-4 is a suppressive cytokine secreted by Tregs upon activation. We demonstrate no difference between the two groups considering IL-4 production. Therefore, we

conclude that Tregs do not perform a major function in the suppression of CD4+ T-cell activity during tolerance induction to thyroglobulin. This cytokine data indicates a follow-up experiment that should be conducted to further support our conclusions. If IFN-γ is suppressed as our data indicates, then the corresponding antibody isotype, IgG1, should also be suppressed. In addition, as we saw no difference in the production of the Th2 cytokine, IL-4, the levels of the antibody isotype IgG2 should likewise be unchanged. A study of this nature will serve to further elucidate the mechanism of action generated in response to the high doses of antigen we utilized.

Although cytokine indicators point to clonal anergy as the mechanism for tolerance in HT disease pathology, it should be recalled that Tregs are eliminated by apoptosis during HT disease progression[84, 86]. Further studies should focus on the re-establishment of the Treg population in the thyroid following initial tolerance induction to determine the actual role of these cells. Additionally, studies should extend tolerance therapy to determine if complete abrogation of the immune response is, in fact, possible and if a pronounced difference in thyroid hormones T3 and T4 can be detected. Lastly, thyroid pathology analysis should be conducted with and without treatment in an attempt to visualize the degradation, or lack thereof, inflicted by disease progression. If these experiments prove successful, the cost-effectiveness of soybean-derived oral toleragens would radically alter the pharmaceutical approach to treating Hashimoto's thyroiditis.

Figure 3.1

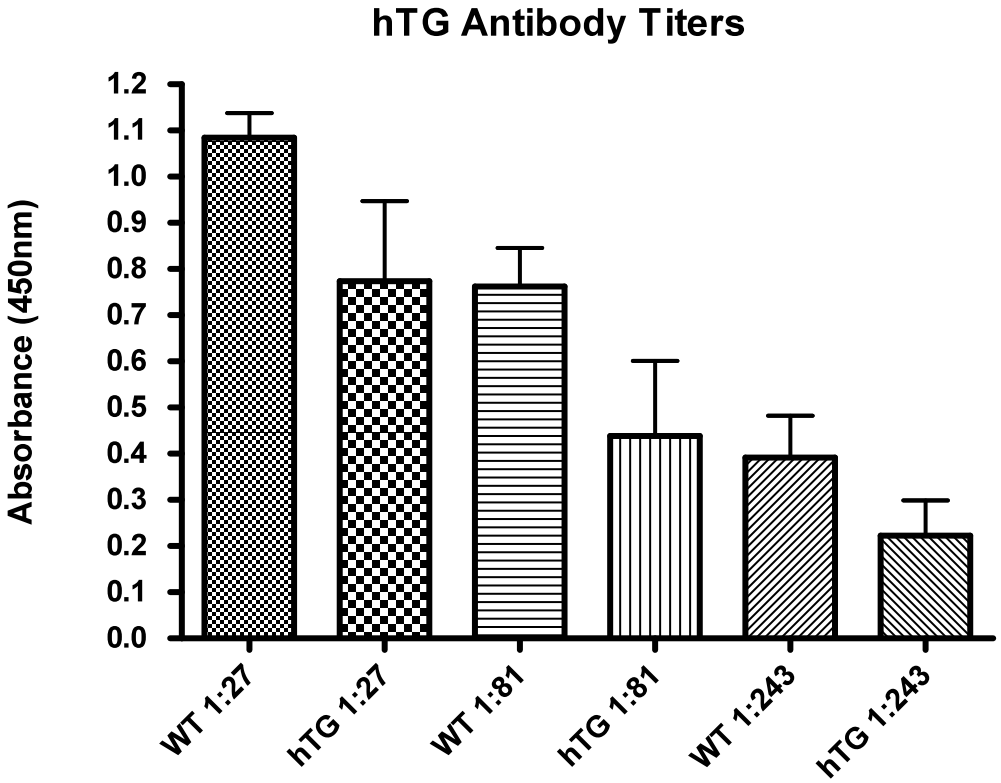

Figure 3.1: hTG ELISA of mouse sera to determine antibody titers. Dilutions of mouse sera from wild type (WT) and thyroglobulin (hTG) groups were analyzed. Eight serial dilutions of each sample were tested in the ELISA and absorbance values for three of these dilutions are shown. WT and hTG groups were both N=5. One way analysis of variance (ANOVA) indicated no statistical significance.

Figure 3.2

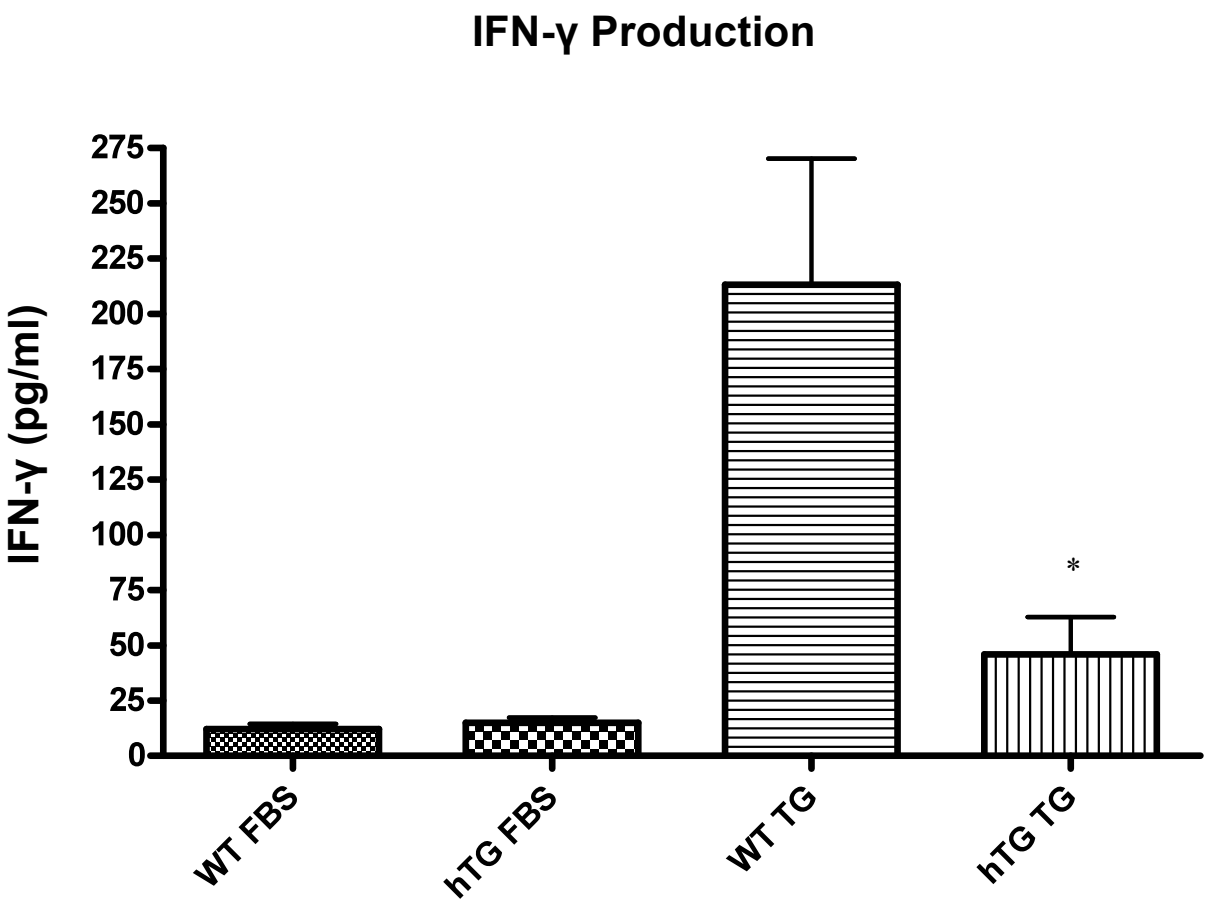

Figure 3.2: IFN-γ ELISA used to determine T-cell restimulation in the presence of commercial thyroglobulin. Splenocytes from wild type (WT) and thyroglobulin (hTG) groups were restimulated using commercial thyroglobulin (TG) and Fetal Bovine Serum (FBS) as a control. Supernatants were collected and analyzed via ELISA for the presence of IFN-γ. Results are shown in pg/ml. Both groups are N=5. One way analysis of variance (ANOVA) indicates a statistically significant difference between IFN-γ production in wild type splenocytes as compared to thyroglobulin group splenocytes (*p=0.01)*.

Figure 3.3

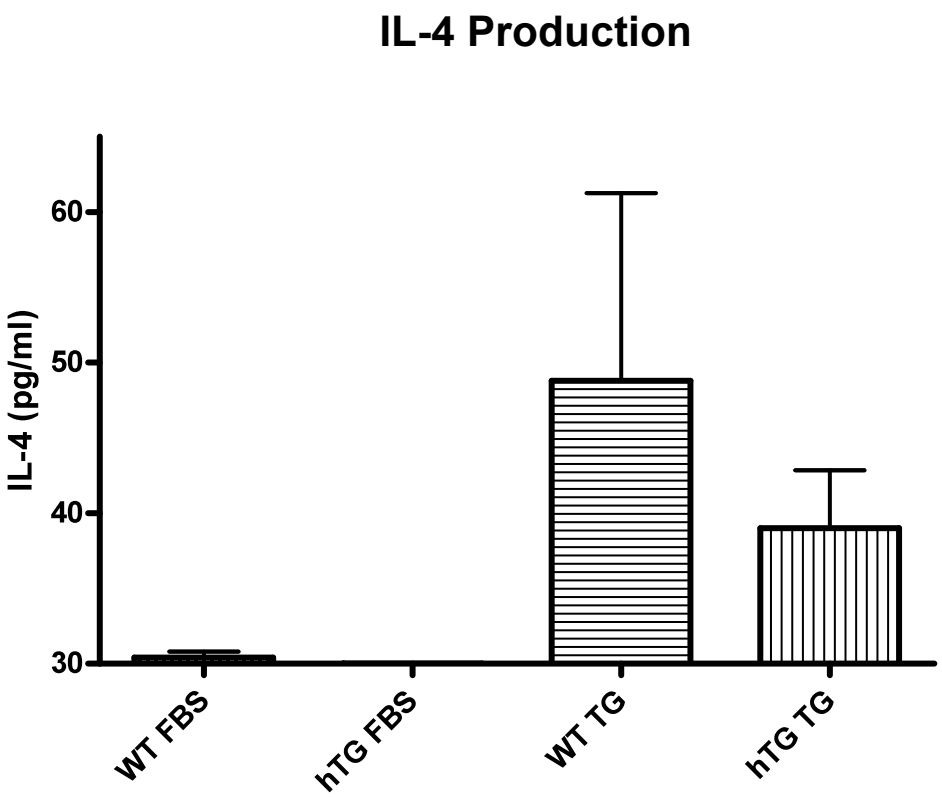

Figure 3.3: IL-4 ELISA used to determine T-cell restimulation in the presence of commercial thyroglobulin. Splenocytes from wild type (WT) and thyroglobulin (hTG) groups were restimulated using commercial thyroglobulin (TG) and Fetal Bovine Serum (FBS) as a control. Supernatants were collected and analyzed via ELISA for the presence of IL-4. Results are shown in pg/ml. Both groups are N=5. One way analysis of variance (ANOVA) indicates no significant difference between IL-4 production in wild type splenocytes as compared to thyroglobulin group splenocytes.

CHAPTER 4: PRODUCTION OF SOY-DERIVED MALARIAL ANTIGENS MSP119, MSP 4/5 AND MSP 142 FOR ORAL IMMUNIZATION IN THE MURINE MODEL

4.1 INTRODUCTION

As one of the world's major diseases, the *Plasmodium* species that cause malaria are responsible for the deaths of approximately 5 million people each year, with the majority of those being under 5 years of age. Largely endemic in sub-Saharan Africa and Asia, malaria is a disease of the vulnerable and impoverished populations found in developing countries. Transmitted via the bite of the female *Anopheles* mosquito, infection at some point during one's life span in these areas is all but guaranteed.

Following initial infection, there are two parts to the immune response. The first focuses around the pre-erythrocytic or live stage of infection, dependent upon CD8+ T-cells and a pro-inflammatory Th1 cytokine response. Upon 30 minutes of inoculation, as few as 10 malaria sporozoites make their way to the liver and invade host hepatocytes. The speed with which this happens renders an anti-sporozoite antibody response useless, as antibodies are generally not present in the circulation at high titers when infection occurs[89]. An attempt to clear liver stage infection is dependent upon T-cell activity, for which there are two potential mechanisms of action. The elimination of hepatocytes infected with *Plasmodium* could be achieved via the classical CD8+ cytotoxic T-lymphocyte

(CTL) activity: perforin/granzye-mediated apoptosis[90]. Additionally, CD4+ CTLs respond to infected hepatocytes in a similar manner, forming an immunological synapse upon contact and inducing apoptosis[91]. These cells are most efficient upon re-infection, activated in response to circumsporozoite protein (CSP), released by *Plasmodium* upon hepatocyte invasion[92].

Alternatively, non-cytotoxic CD8+ and CD4+ T-cells could eliminate sporozoites via their interaction with antigen presenting cells (APCs) in the liver, the production of IFN-γ and the induction of the nitric oxide (NO) pathway[89, 90, 93]. T-cells specific for CSP presented by infected hepatocytes via MHC class I in the liver produce large amounts of IFN-γ, which in turn induces the nitric oxide pathway in the hepatocytes, thereby killing the *Plasmodium* parasites within. As high concentrations of IFN-γ and NO are routinely found in individuals with naturally acquired immunity, it is likely this cytokine/NO pathway is the means by which malarial infection is arrested in the liver[93, 94].

The second part of the immune response focuses on blood stage infection, in which mature merozoites are released from the liver into the bloodstream to infect red blood cells (RBCs) and induce disease pathology. The hallmark of blood stage malaria is a severe fever with chills, cycling approximately every 12 hours. This stage of infection leads to RBC adherence to vascular walls, severe anemia and, ultimately, death. As thousands of merozoites are released into the bloodstream to infect RBCs, the immune response at this stage is generally over-enthusiastic, likening the infection to sepsis, in which the immune response does a large portion of the damage[93].

During the erythrocytic stage, the immune response is mediated largely by the humoral response: the activation of B-cells and the production of antibodies. The role of antibodies is multifaceted, as they serve to block RBC invasion, induce antibody-dependent cellular cytotoxicity (ADCC), activate T-cells and opsonize infected RBCs for the induction of the classical complement pathway[93, 94].

Plasmodium merozoites have surface proteins which are ideal for inducing B-cell antibody production. One such protein is merozoite surface protein 1 (MSP 1), a 200 kDa protein essential for RBC invasion by the merozoite. As this protein undergoes a number of proteolytic cleavages, only the highly conserved MSP 1_{19} 11kDa c-terminal fragment remains on the surface of the merozoite as it enters the RBC[95]. As an essential piece of erythrocytic infection that is conserved across all *Plasmodium* species, this protein is an ideal choice against which protective antibodies might be produced.

Based upon this understanding, a number of studies have demonstrated successful protection against blood stage infection in a number of mammalian models following immunization with a portion of MSP 1[96-99]. As malaria is a disease afflicting predominantly developing countries, it is essential to develop a vaccine that can be readily afforded by these populations. Plant-derived oral vaccination makes this possible. Antigens produced in plants for use as subunit vaccines can be delivered without special training, without the need for additional instruments such as needles and in the absence of a cold chain.

Previously, our laboratory has demonstrated the stability of such an antigen produced in soybean[51]. Soybean is the ideal organism for production of antigens of this nature. Soybean is approximately 40% protein by dry weight[52], is readily produced and processed as a food crop and easily stored for extended periods of time at ambient temperatures[51, 66]. As protective oral immunization has already been demonstrated in the murine model[97], it is reasonable to suggest that MSP 1 antigens produced in soybean could be administered via transgenic soymilk for the induction of protective antibody titers against *Plasmodium* species.

4.2 MATERIALS AND METHODS

Vector design and construction

The 350bp $MSP1_{19}$ gene containing a native signal peptide sequence, a histidine tag sequence, and 5' and 3' terminal restriction endonuclease sites was optimized for soybean codon usage and synthetically generated by GeneArt, Inc. (Burlingame, CA). Following digestion with NcoI and XbaI, the synthetic gene was isolated from an agarose gel and ligated with the binary vector pTN200 that had also been linearized with NcoI plus XbaI and gel-isolated. The resulting product, pTN-$MSP1_{19}$, was verified for integrity and transformed into Agrobacterium. The same gene was ligated into the binary vector pTF101, and the resulting pTF-$MSP1_{19}$ was verified and transformed.

A larger fragment of the MSP 1 protein, the MSP 1_{42} gene containing a chitinase leader peptide, 6x-histidine tag and KDEL endoplasmic reticulum retention signal was optimized and generated in the same manner as the $MSP1_{19}$ genes. The

synthetic gene was, again, digested with NcoI and XbaI, agarose gel purified and ligated into the pTN200 binary vector. The pTN-MSP1$_{42}$ plasmid was verified and transformed into *Agrobacterium*.

Soybean transformation

Soybean transformation using the Agrobacterium-mediated half seed method was performed as described in Paz et al[69]. Briefly, half-seed explants (G. max cv Williams) were dissected and inoculated with Agrobacterium suspension culture (strain EHA101 carrying various binary vectors). The inoculated explants were placed adaxial side down on cocultivation medium at 24°C and under 18:6 photo period for 3-5 days. After cocultivation, explants were cultured for shoot induction and elongation under glufosinate selection (8 mg/L) for 8-12 weeks. Herbicide resistant shoots were harvested, elongated and rooted as described[69]. Acclimated plantlets were transferred to soil and grown to maturity in the greenhouse.

Recombinant protein production

The MSP119 gene was PCR amplified with high fidelity Taq polymerase (Invitrogen) to replace the NcoI and XbaI restriction sites with XbaI and XhoI, respectively. The PCR product was agarose gel purified and ligated into the IPTG inducible expression vector, pET-303 (Invitrogen), which had been digested with XbaI and XhoI, then gel purified. The resulting pET-MSP119 was verified for integrity then transformed into the *E. coli* expression strain, BL21 DE3 (Invitrogen). Cells were coated onto luria broth (LB) plates with ampicillin (50ug/L) as a marker. Colonies were picked after 24 hours and incubated

overnight in 3 ml LB with ampicillin at 37°C. Overnight broths were added to 1 liter of LB with ampicillin and grown to OD_{280} = 0.6. 1M IPTG was added to media, and cultures were incubated for another 12 hours. Cultures were spun down at 8,000g x 45 minutes. Pellets were resuspended in 1x PBS with .25M NaCl and sonicated for 1 minute. Cultures were centrifuged for 30 minutes at 8,000g and pellets resuspended in 8M Urea. Following sonication, cultures were rocked at room temperature for 1 hour then spun down to clarify. Ni^{2+} column resin was added to the supernatant, and protein was purified via the manufacturer's instructions (Sigma-Aldrich).

Preparation of genomic DNA and PCR

Genomic DNA was prepared from cotyledon tissue using the Maxwell 16 Instrument and Maxwell Tissue DNA Purification Kit (Promega, Madison, WI). Genomic DNA (100 ng), BAR gene primers (forward: 5'-TACATCGAGACAAGCACG -3'; reverse: 5'-AGTTCCCGTGCTTGAAGC-3') and dNTPs were mixed with GoTaq Flexi DNA polymerase and buffer (Promega Corp., Madison, WI) according to the manufacturer's directions. Following an initial denaturation cycle (5 minutes at 94°C) the reactions were subjected to 35 cycles of denaturation (60 seconds at 94°C), annealing (60 seconds at 50°C) and extension (120 seconds at 72°C). 440 bp PCR products were visualized in 1.0% agarose gels stained with ethidium bromide.

Seed protein extraction

Seed slivers (chips) derived from cotyledon tissue (~10 mg) were resuspended in 200µl PBS and sonicated for ~30 seconds using a Vibra-Cell ultrasonic

processor (Newton, CT). Samples were clarified from soluble debris by centrifugation at 16.1 x 10^3g at 4°C. Total soluble protein was quantified with the Bradford Reagent (Bio-Rad, Hercules,CA) using BSA as a standard.

Western blot and Dot blot analysis

For western blots, protein extracts (10µg TSP) from transgenic and wild type seeds were run in 12% polyacrylamide gels for approximately 2 hours at 110V. Samples were boiled in SDS with β-mercaptoethanol prior to loading onto the gel. Recombinant $MSP1_{19}$ (Sigma, St. Louis, MO) was included for a standard. Following electrophoresis, gels were equilibrated in 1X CAPS (recipe for 20X: 22.13g CAPS, Sigma, in 500ml dH2O, pH 11) buffer with 10% MeOH for 10 minutes and transferred to Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked overnight with 5% dry milk in PBS solution at 4°C, incubated with mouse anti-MSP 1 monoclonal antibody (Abcam) for ~3 hours at 23°C, and washed three times (10 minutes each) with PBS containing 0.05% Tween. Membranes were then incubated with goat anti-mouse HRP-conjugated IgG (Southern Biotech) for 30 minutes at 23°C and washed as described above. Detection was carried out using the SuperSignal West Pico substrate (Thermo Scientific, Rockford, IL).

For dot blot analysis, Immobilon-P membranes (Millipore, Billerica, MA) were equilibrated in 1x CAPS buffer with 10% MeOH for 5 minutes. Membrane was secured to the Bio-Dot Apparatus (BioRad), per the manufacturer's instructions. 40µg TSP of each protein extraction sample and wild type sample were loaded onto the membrane via vacuum filtration. Following membrane

removal from the apparatus, protocol continued as described for a western blot (see above).

Preparation of soymilk extracts

A total of 12 seeds from line ST 95-25 or wild type plants were ground to a fine powder. For each gavage, 475mg of seed powder was resuspended in 3ml of phosphate buffered saline (PBS) and sonicated for 1 minute using a Vibra-Cell ultrasonic processor (Newton, CT). Samples were spun at 15,000 rpm for 5 minutes, and supernatant was transferred to a 1.5ml eppendorf tube. Tubes were spun for an additional 30 seconds at 16.1 x 10^3g for clarification.

Oral gavage and sera collection

Six to eight week old female Balb/c mice were orally immunized three times as follows: using a 22 gauge feeding needle, 200µl of either hTG soymilk protein extract containing ~8µg $MSP1_{19}$ or wild type soymilk protein extract was administered to each animal on day 0, 5 and 10. The first immunization was given in conjunction with 5µg cholera toxin (CT) as an adjuvant, and the other two were given with 10µg CT as an adjuvant. On days 17 and 23, mice were bled (25µl) via the saphenous vein. Blood was diluted 1:3 in 1x PBS and spun down at 8,000g for 10 minutes. Sera was collected and frozen at -20°C for future ELISA analysis.

ELISA analysis

ELISA plate was coated with 500ng of recombinant $MSP1_{19}$ overnight at 4°C. Plate was then washed with PBS and blocked with 1% BSA-PBS for 1 hour. After a second wash, 100µl of sera samples of varying dilutions were loaded on

to the plate and incubated at room temperature for 2 hours. Following a third PBS wash, 100µl of anti-mouse IgG-HRP antibody (Southern Biotech) at 1:500 dilution was added to each well and allowed to incubate for 1 hour. Following a fourth wash, the antibody-antigen complexes were coated with TMB Substrate (BioFX, Owings Mills, MD), and colorimetric reactions were stopped by the addition of 0.6 M sulfuric acid. Absorbance values were read at 450nm.

4.3 RESULTS

Transformation and Molecular Screening

MSP 1_{19} has a molecular mass of ~11kDa. A synthetic gene was designed to optimize codon usage as seen in *Glycine max*. The gene was cloned downstream of the soybean 7S-promotor and a transcription enhancing sequence from tobacco etch virus (TEV) to generate pRDP2 (Figure 4.1 and Figure 4.2a). *Agrobacterium* transformation resulted in two T_0 lines, ST 68-6 and ST 68-19. To screen for the presence of the transgene, four T_1 seeds from each line were subjected to PCR using genomic DNA isolated from seed chips (Figure 4.4a). The PCR primers utilized amplified a 440 bp region of the BAR herbicide resistance marker cloned alongside the gene of interest. While PCR evidence indicated the seeds were indeed transgenic, western blot analysis failed to demonstrate any detectable protein expression (data not shown). As such, the first generation constructs were not pursued further.

Using the same synthetic gene, a second construct was made. The MSP 1_{19} gene was subcloned downstream of the soybean β-conglycinin promotor and a *Staphylococcus* signal peptide (SEB), which targets the secretory pathway, to

generate pRDP4 (Figure 4.1 and Figure 4.2b). The transformation resulted in four transgenic lines: ST 95-2, 95-3, 95-18 and 95-25. Eight seeds from each line were subjected to PCR as described above to verify the presence of the transgene. It was suspected based on earlier constructs that protein expression levels might be extremely low. Therefore, a dot blot analysis was done in lieu of a western blot so as to be able to load additional protein. As indicated in Figure 4.5a, only ST 95-25 demonstrated detectable levels of MSP 1_{19}.

As MSP 1_{19} is a fragment of the larger 200kDa protein MSP 1, we also generated the 42 kDa fragment, MSP 1_{42}. As with the other two constructs, the gene was optimized for codon usage in *Glycine max*. MSP 1_{42} is a membrane bound protein secured by a glycosolphosphatidylinosital (GPI) anchor. As such, the synthetic gene was designed to contain a KDEL endoplasmic reticulum membrane retention signal in an attempt to optimize expression in a manner similar to the protein's native environment. The gene was subcloned downstream of the soybean derived 7S-promotor, TEV transcription enhancer and chitinase leader peptide[52]. Additionally, the GPI anchor was added to the gene to improve immunogenicity of the resulting protein[100] (Figure 4.3). The *Agrobacterium* transformation of pRDP5 (Figure 4.3) yielded extremely low numbers of T_1 seeds across three lines: ST 115-4, 115-12 and 115-29. Four seeds from each line with the exception of 115-4, which yielded only 2 seeds, were analyzed via PCR for the presence of the BAR herbicide resistance marker (Figure 4.4c).

Both a dot blot and western blot analysis were done on four seeds from each line, when available, as it was believed the changes in the construct would increase the expression levels from those seen in pRDP4 (Figure 4.2b). An anti-mouse antibody against MSP 1 (the entire protein) was used for detection. This served as a means of detecting the intact MSP 1_{42} protein and any degraded fragments that might exist. No detectable MSP 1_{42} protein or protein fragments were observed in either analysis (Figure 4.5b).

Oral delivery of soybean-derived MSP 1_{19} fails to generate protective antibody titers in the murine model.

ST 95-25 seeds were used to orally immunize Balb/c mice at 0, 5 and 10 days (Figure 4.6). Sera collected from the wild type mouse group and the soy-derived MSP 1_{19} mouse group was used to determine antibody titers against recombinant MSP 1_{19}. To determine if there was a difference in antibody production between the groups, an ELISA was performed. Figure 4.7 demonstrates that, over the course of the experiment, there is no significant difference in antibody titers between the wild type and the MSP 1_{19} group. This suggests the mice that received the MSP 1_{19} soymilk formulation did not receive an adequate amount of antigen to induce an immune response. This is unsurprising, considering the low expression levels of the transgenic soybeans used.

4.4 DISCUSSION

Over the course of this study, three different vector constructs were generated to produce optimum protein expression levels (Figures 4.1 and 4.2). The original construct, consisting of the 7S promotor and TEV enhancer (Figure 4.2), no

detectable levels of protein were generated. Using the modified construct with the β-conglycinin promoter and no TEV transcription enhancer, extremely low levels (~0.01%) of MSP 1_{19} expression were achieved. Based on previous studies that indicated protein retention in the ER improved protein expression[52] and studies done in our own lab that indicated the success of our first generation construct (unpublished data), a third construct was generated with the expectation of improved expression. While no protein was detactable from this third construct, there are a number of possible explanations. First, transgene expression is not entirely dependent upon construct design. Other factors include position effects, the position of transgene integration in relationship to surrounding chromatin, the structure of the locus, as well as copy number and the generation of truncated transgene copies. Together, these factors can inhibit protein expression and possibly silence it altogether [45]. Furthermore, the post-translational modification, particularly glycosylation, in plants varies in pattern from other mammals and, of course, does not occur in prokaryotic organisms such as *Plasmodium*. This difference in protein production can cause dramatic changes in protein longevity, inducing its degradation immediately upon translation[45, 101]. Additionally, it should be noted that the transformation events for the MSP 1_{42} construct produced very limited numbers of T_1 seeds. Therefore, it is reasonable to suggest that, based upon the poor transformation results, the construct and/or protein expressed is, in some manner, toxic to *Glycine max*.

Lastly, one should consider the limitation of detection antibodies when drawing conclusions of this nature. We have reported that only one construct of the three had produced detectable protein. This does not mean that the first and third generation constructs were unsuccessful, only that the currently available antibodies are not sensitive enough to detect the protein that is possibly being produced. While it is logical to address this problem with a replacement antibody, the type of antibody in question, against MSP 1, is only available in small quantities and from a limited number of commercial sources. The antibody utilized in our studies is the most sensitive of the currently available options. Therefore, future studies involving these constructs should consider the production of a more robust and sensitive antibody, perhaps generated within our laboratory.

As only pRDP4 (figure 4.2b) produced detectable levels of protein, these seeds were utilized in the murine oral immunization study. Other laboratories have demonstrated successful immunization with oral delivery of MSP 1 and other malarial antigens[97]. However, these studies were accomplished with the equivalent of 25μg of purified antigen in each dose. Given the low expression levels observed in the transgenic seeds, it was not possible to administer greater than 10μg of antigen per gavage. Furthermore, the additional step required to purify the antigen in question serves to render void the economic feasibility of the soybean platform and, therefore, was not done. For these reasons, we were unable to induce a protective titer (A_{450} > 2.0, [97]) in the mouse model. Despite this setback, future studies should progress to establish higher protein

expression in the soybean platform that would enable higher doses to be administered more frequently in order to establish the antibody titers necessary to test via parasite challenge.

Figure 4.1

pRDP2 (pTN-MSP119):

AAGCTTGCATGCTGCAGGATCCATGCCCTTCATTTGCCGCTTATTAATTAATTTGGTAACAGTCCGTACTAATCAGTTA
CTTATCCTTCCTCCATCATAATTAATCTTGGTAGTCTCGAATGCCACAACACTGACTAGTCTCTTGGATCATAAGAAAA
AGCCAAGGAACAAAAGAAGACAAAACACAATGAGAGTATCCTTTGCATAGCAATGTCTAAGTTCATAAAATTCAAACAA
AAACGCAATCACACACAGTGGACATCACTTATCCACTAGCTGATCAGGATCGCCGCGTCAAGAAAAAAAAACTGGAC
CCCAAAAGCCATGCACAACAACACGTACTCACAAAGGTGTCAATCGAGCAGCCCAAAACATTCACCAACTCAACCCA
TCATGAGCCCTCACATTTGTTGTTTCTAACCCAACCTCAAACTCGTATTCTCTTCCGCCACCTCATTTTTGTTTATTTCA
ACACCCGTCAAACTGCATGCCACCCCGTGGCCAAATGTCCATGCATGTTAACAAGACCTATGACTATAAATATCTGCA
ATCTCGGCCCAGGTTTTCATCATCAAGAACCAGTTCAATATCCTAGTACACCGTATTAAAGAATTTAAGATATACTAAC
AGCGGCCGCTGACACAATAGCCCTTCTTCCCTGTTTCCAGCTTTTCTCCTTCTCTCTCTCCATCTTCTTCTTCTT
TCACTCAGTCAGATCCAACTCCTCAGATAACACAAGACCAAACCCGCTTTTTCTGCATTGCAAGACTAGACGTTCTAC
CGGAGAAGCGACCTTAGAAATTCATTGCGTTCTCAACACAACATATACAAAACAAACGAATCTCAAGCAATCAAGCAT
TCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTCTGAAAATTTTCACCATTTACGAACGAT
AGACCATGGATGGAATGGACCTCCTTGGTGTGGACCCTAAACACGTGTGCGTTGATACCAGGGACATACCTAAGAAC
GCTGGATGTTTCCGCGACGATAATGGTACCGAAGAGTGGAGATGCCTTCTCGGTTATAAGAAAGGTGAAGGAAACAC
ATGCGTTGAAAACAATAACCCAACCTGCGACATTAACAACGGTGGATCGACTCCAACCGCATCTTGCCAAAACGCCG
AGAGCACTGAAAACAGCAAGAAGATTATCTGTACTTGTAAGGAGCCTACACCAAACGCCTACTACGAGGGAGTGTTC
TGTAGCAGTAGCAGCGGTGGACACCACCATCACCACCACTGATCTAGAGTCCGCAAAAATCACCAGTCTCTCTCTAC
AAATCTATCTCTCTCTATTTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTT
TCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATT
CCTAAAACCAAAATCCAGTGACCTGGTGGCATGCAAGCTT

pRDP4 (pTF-MSP119):

AAGCTTGCATGCCTGCAGGATCCTAGCCTAAGTACGTACTCAAAATGCCAACAAATAAAAAAAAAGTTGCTTTAATAAT
GCCAAAACAAATTAATAAAACACTTACAACACCGGATTTTTTTTAATTAAAATGTGCCATTTAGGATAAATAGTTAATAT
TTTTAATAATTATTTAAAAAGCCGTATCTACTAAAATGATTTTTATTTGGTTGAAAATATTAATATGTTTAAATCAACACAA
TCTATCAAAATTAAACTAAAAAAAAAAATAAGTGTACGTGGTTAACATTAGTACAGTAATATAAGAGGAAAATGAGAAATT
AAGAAATTGAAAGCGAGTCTAATTTTTAAATTATGAACCTGCATATATAAAAGGAAAGAAAGAATCCAGGAAGAAAAGA
AATGAAACCATGCATGGTCCCCTCGTCATCACGAGTTTCTGCCATTTGCAATAGAAACACTGAAACACCTTTCTCTTTG
TCACTTAATTGAGATGCCGAAGCCACCTCACACCATGAACTTCATGAGGTGTAGCACCCAAGGCTTCCATAGCCATG
CATACTGAAGAATGTCTCAAGCTCAGCACCCTACTTCTGTGACGTGTCCCTCATTCACCTTCCTCTCTTCCCTATAAAT
AACCACGCCTCAGGTTCTCCGCTTCACAACTCAAACATTCTCTCCATTGGTCCTTAAACACTCATCAGTCATCACCATG
ATGGCAAAGCTCGTGTTCTCTCTTTGCTTCCTTCTTTTCTCCGGATGCTGCTTCGCTTTCTACCATGGATGGAATGGA
CCTCCTTGGTGTGGACCCTAAACACGTGTGCGTTGATACCAGGGACATACCTAAGAACGCTGGATGTTTCCGCGACG
ATAATGGTACCGAAGAGTGGAGATGCCTTCTCGGTTATAAGAAAGGTGAAGGAAACACATGCGTTGAAAACAATAAC
CCAACCTGCGACATTAACAACGGTGGATGCGATCCAACCGCATCTTGCCAAAACGCCGAGAGCACTGAAAACAGCAA
GAAGATTATCTGTACTTGTAAGGAGCCTACACCAAACGCCTACTACGAGGGAGTGTTCTGTAGCAGTAGCAGCGGTG
GACACCACCATCACCACCACTGATCTAGAGTCCGCAAAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATTT
TTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCAT
ATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGT
GACCTGGTGGCATGCAAGCTT

pRDP5 (pTN-MSP142):

ATGGGACCCCAGAAAGAAAACCATAGGACCCTCAACAAGATGAAGACCAACCTTTTCCTCTTCCTCATTTTCTCCCTT
TTGCTTTCTTTGTCATCTGCTGAGCAGCATCATCATCACCATCACGCTATTTCCGTGACTATGGACAACATTCTTTCCG
GATTCGAGAACGAGTACGATGTGATCTACCTTAAGCCACTTGCTGGTGTGTATAGATCTCTCAAGAAGCAGATCGAGA
AGAACATCTTCACCTTCAACCTTAACCTCAACGACATTCTTAACTCCAGGCTCAAGAAGAGAAAGTACTTCCTTGATGT
GCTTGAGTCTGATCTCATGCAGTTCAAGCACATTTCTTCCAACGAGTACATCATCGAGGATTCCTTCAAGTTGCTTAAC
TCCGAGCAAAAGAACACCCTTCTCAAGTCCTACAAGTACATCAAAGAGTCCGTTGAGAACGACATAAGTTCGCTCAAG
AGGGAATTTTCTTACTACGAGAAGGTGCTCGCTAAGTACAAGGATGATCTCGAATCCATTAAGAAGGTGATCAAAGAG
GAAAAAGAGAAGTTCCCATCTTCTCCACCAACTACTCCACCATCTCCAGCTAAGACTGATGAGCAGAAGAAAGAGTCT
AAGTTCCTTCCATTCCTCACCAACATTGAAACCCTCTACAACAACCTCGTGAACAAGATTGATGACTACCTCATCAACC
TCAAGGCTAAGATTAACGATTGCAACGTGGAAAAGGATGAGGCTCATGTGAAGATTACCAAGCTCTCTGATCTCAAG
GCTATCGATGATAAGATCGACCTCTTCAAGAACCCATATGATTTCGAGGCTATCAAGAAGCTCATTAACGACGATACC
AAGAAAGATATGCTCGGAAAGTTGCTTTCTACTGGACTTGTTCAGAACTTCCCCAACACCATTATTTCCAAGCTCATCG
AGGGAAAGTTCCAGGATATGCTCAACATTTCTCAGCATCAGTGCGTTAAGAAGCAATGCCCAGAAAACTCTGGATGCT
TCAGGCATCTTGATGAGAGGGAAGAATGCAAGTGCCTTCTCAACTATAAGGGTGAGGGTGATAAGTGCGTTGAAAAC
CCAAACCCAACTTGCAACGAAAACAACGGTGGATGTGATGCTGATGCTACTTGCACTGAAGAGGATTCTGGATCTTC
CAGGAAGAAGATTACTTGCGAGTGCACTAAGCCAGATTCTTACCCACTCTTCGATGGAATTTTCTGCTCCTCTTCCAA
CTTCCTTGGAATTTCCTTCCTCCTCATCCTTATGCTTATCCTCTACTCCTTCATCAAGGACGAGCTGTGATAATCTAGA

Figure 4.1: *Plasmodium* construct sequences. pRDP2 is the DNA sequence cloned into the pTN vector backbone for the first generation malarial constructs (Figure 4.2) pRDP4 is the sequence for the second generation pTF vector construct and pRDP5 indicates the sequence changes made for the third and final generation construction in the pTN vector backbone.

Figure 4.2

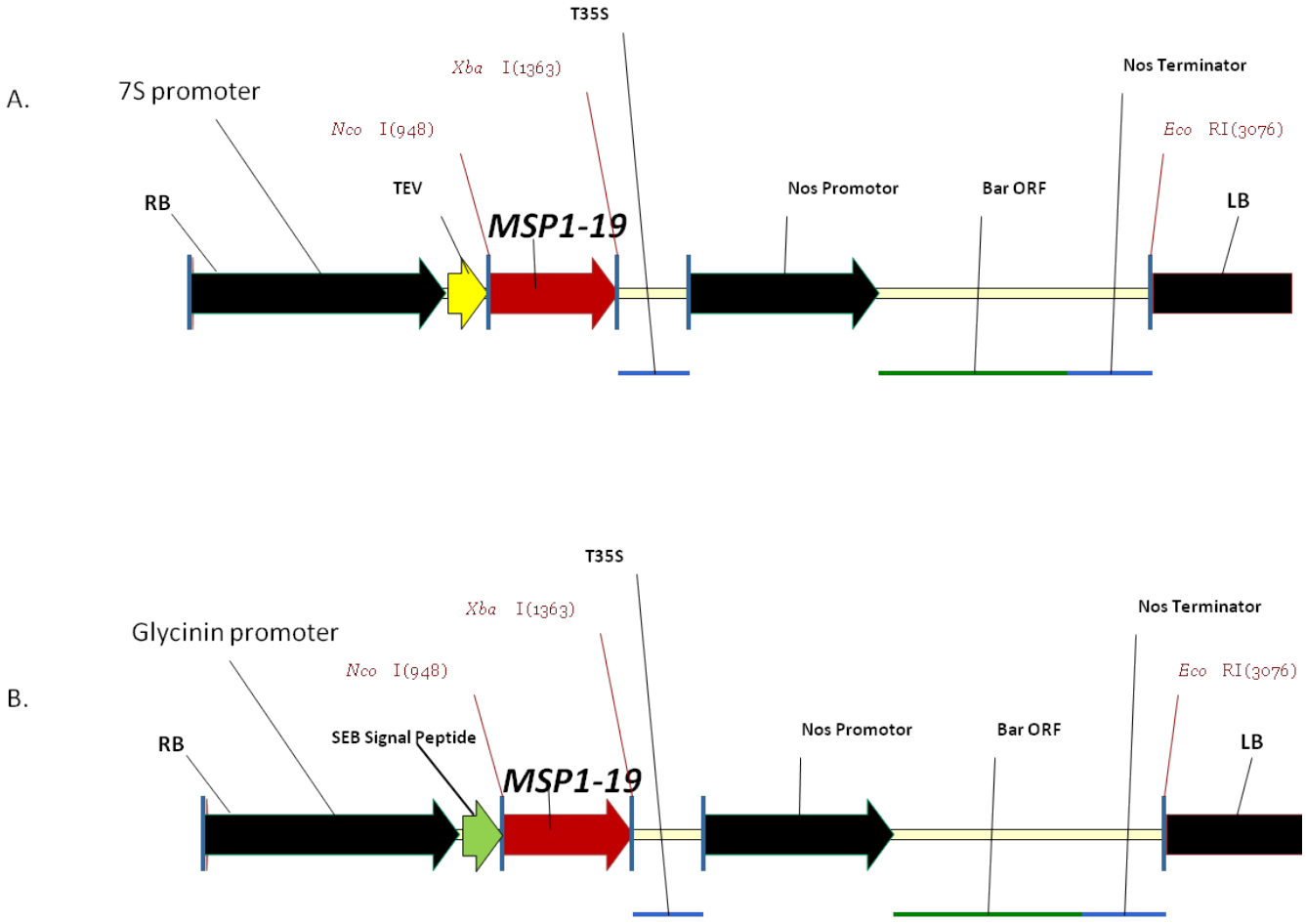

Figure 4.2: Gene design and soybean transformation. A. Binary vector pTN-MSP1$_{19}$ (pRDP2) used for *Agrobacterium* transformation with the following elements: 7S-promotor, tobacco etch virus (TEV) translational enhancer, MSP1$_{19}$ synthetic gene, cauliflower mosaic virus terminator (T-35S) nopaline synthase promotor (Nos-p), phosphinothricin acetyltransferase gene for selection (Bar ORF), Nos terminator element, right border (RB) and left border (LB). B. Binary vector pTF-MSP1$_{19}$ (pRDP4) used for *Agrobacterium* transformation, modified from the original pRDP2 with the following: soybean Glycinin promotor, SEB signal peptide targeting secretory pathway and removal of TEV element.

Figure 4.3

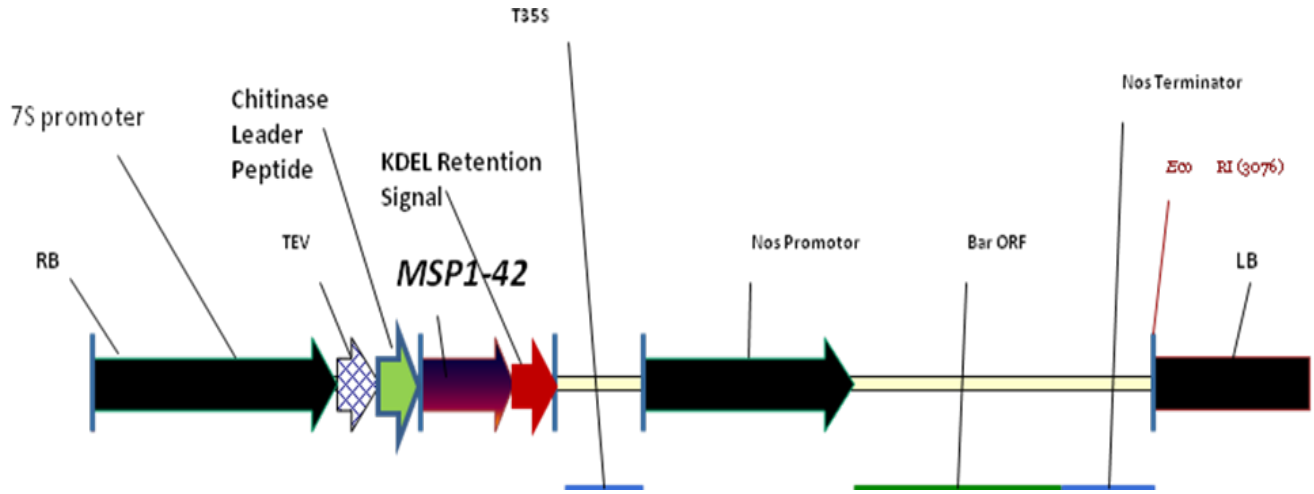

Figure 4.3: Second generation gene design and soybean transformation. Binary vector pTN-MSP1$_{42}$ (pRDP5) used for *Agrobacterium* transformation with the following elements: 7S-soybean β-conglycinin promotor, tobacco etch virus (TEV) translational enhancer, chitinase leader peptide for targeting to the endoplasmic reticulum, MSP1$_{42}$ synthetic gene, KDEL endoplasmic reticulum retention signal, cauliflower mosaic virus terminator (T-35S) nopaline synthase promotor (Nos-p), phosphinothricin acetyltransferase gene for selection (Bar ORF), Nos terminator element, right border (RB) and left border (LB).

Figure 4.4

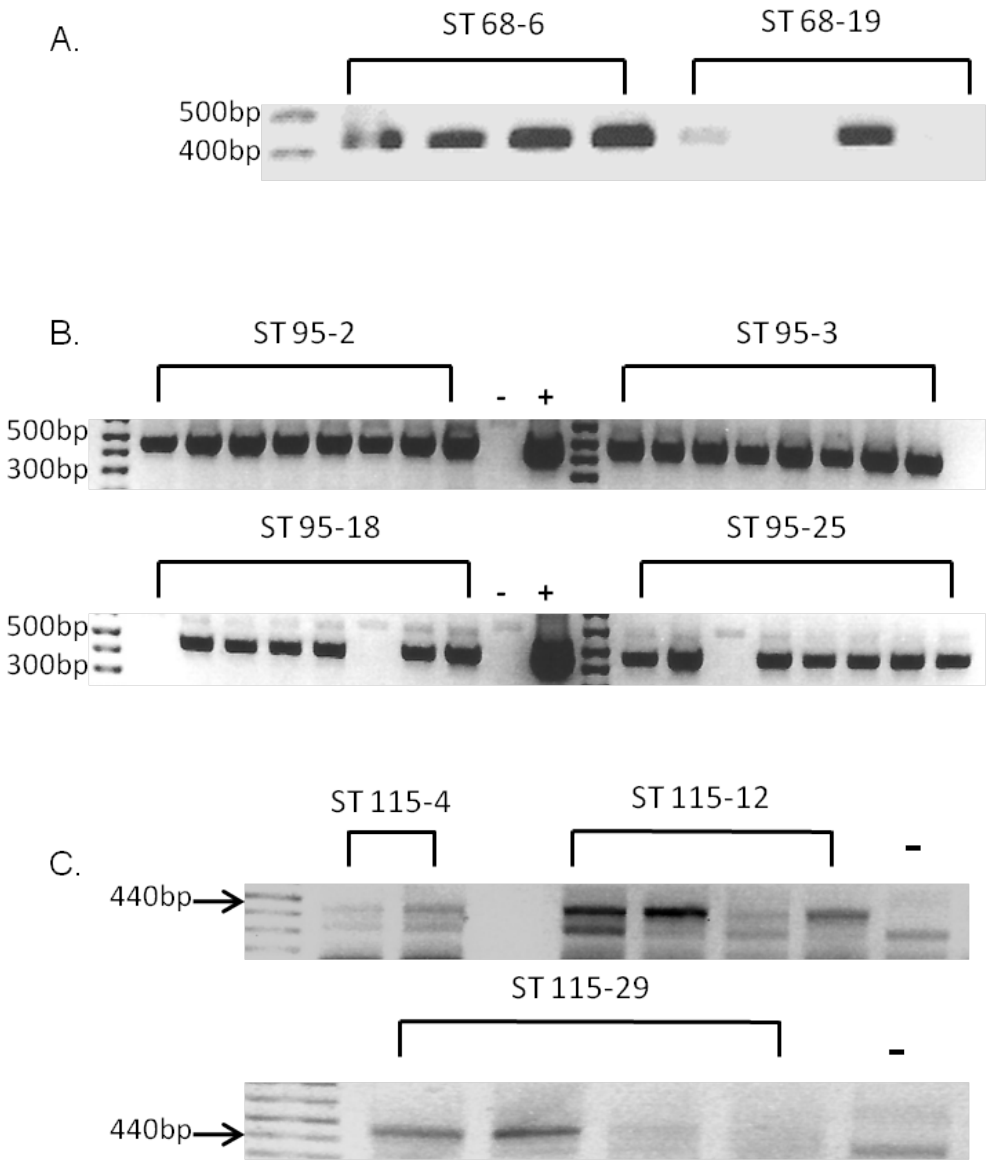

Figure 4.4: PCR analysis of transgenic soybean seeds to confirm transgenicity. A. pTN-MSP1$_{19}$ PCR analysis detecting Bar ORF in genomic DNA isolated from T_1 seed chips. Amplified PCR product migrates at 440 base pairs (bp). Genomic DNA from wild type plant is designated as the negative control (-), and the positive control (+) is pTN-MSP1$_{19}$ plasmid DNA. B. pTF-MSP1$_{19}$ PCR analysis detecting Bar ORF in genomic DNA isolated from T_1 seed chips. Amplified PCR product migrates at 440bp. Genomic DNA from wild type plant is designated as the negative control (-). C. pTN-MSP1$_{42}$ PCR analysis detecting Bar ORF in genomic DNA isolated from T_1 seed chips.

Figure 4.5

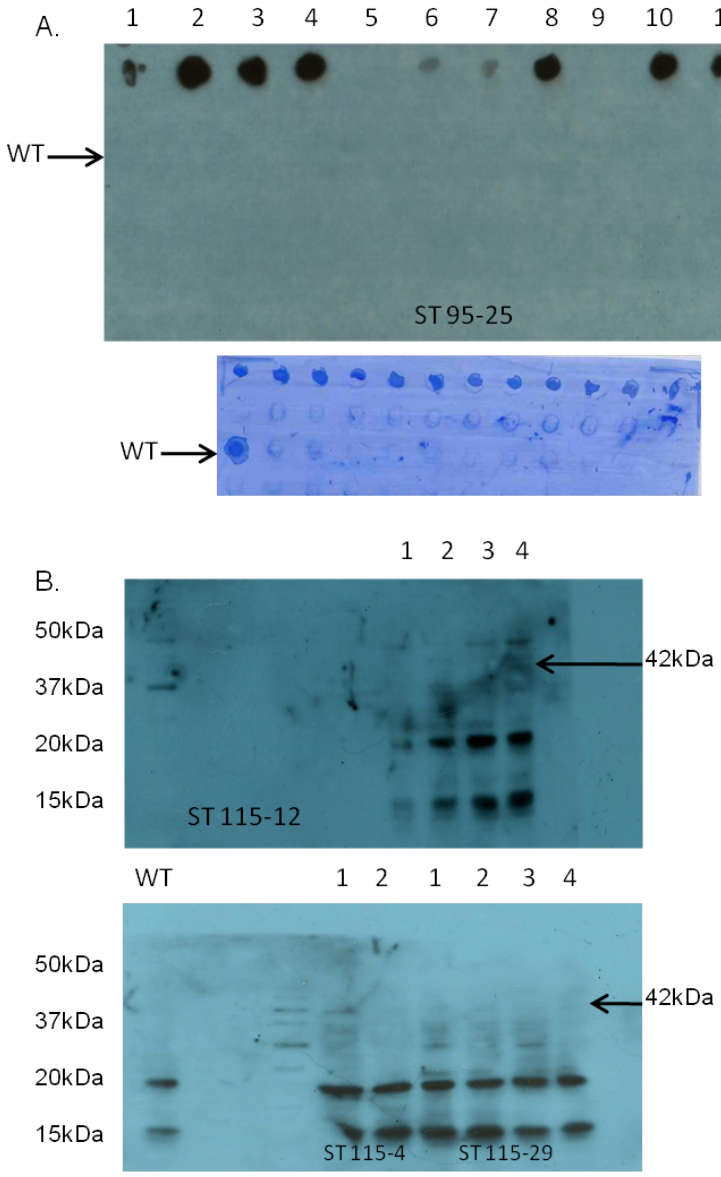

Figure 4.5: Dot blot and western blot soybean analysis. A. Dot blot analysis of pTF-MSP1$_{19}$ seeds. Top panel is western blot, indicating, via MSP 1 antibody, those seeds expressing protein at ~0.01% TSP. Bottom panel is the coomassie stain of the western membrane, demonstrating protein loading, specifically wild type (WT). B. Western blot analysis of pTN-MSP1$_{42}$ seeds with wild type (WT) control. Protein of interest should run at 42 kDa, as indicated by the arrows. No detectable protein is present. (TSP = total soluble protein)

Figure 4.6

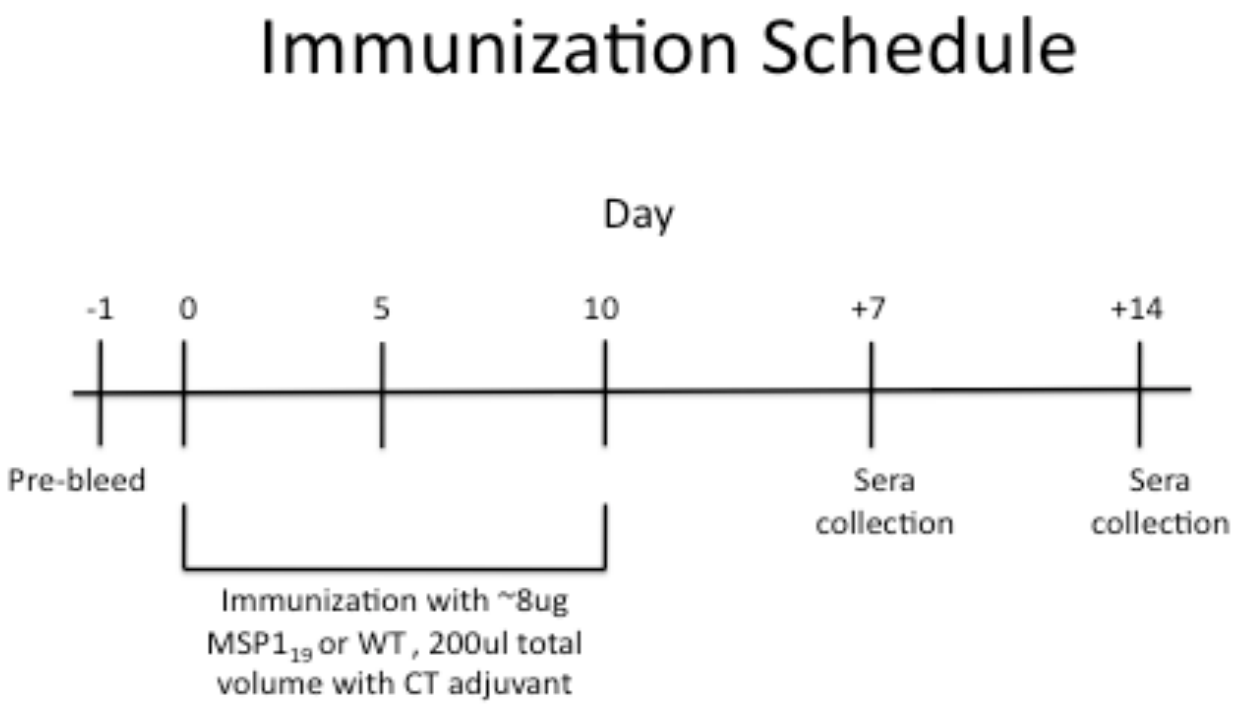

Figure 4.6: *Plasmodium* Oral Immunization Schedule: Six to eight week old balb/c mice were pre-bled one day prior to the beginning of the immunization regimen. On day 0, 5 and 10, mice in both the MSP and WT group were gavaged with 200ul soybean formulation. One and two weeks following the final immunization, mice were bled for sera antibody titer detection.

Figure 4.7

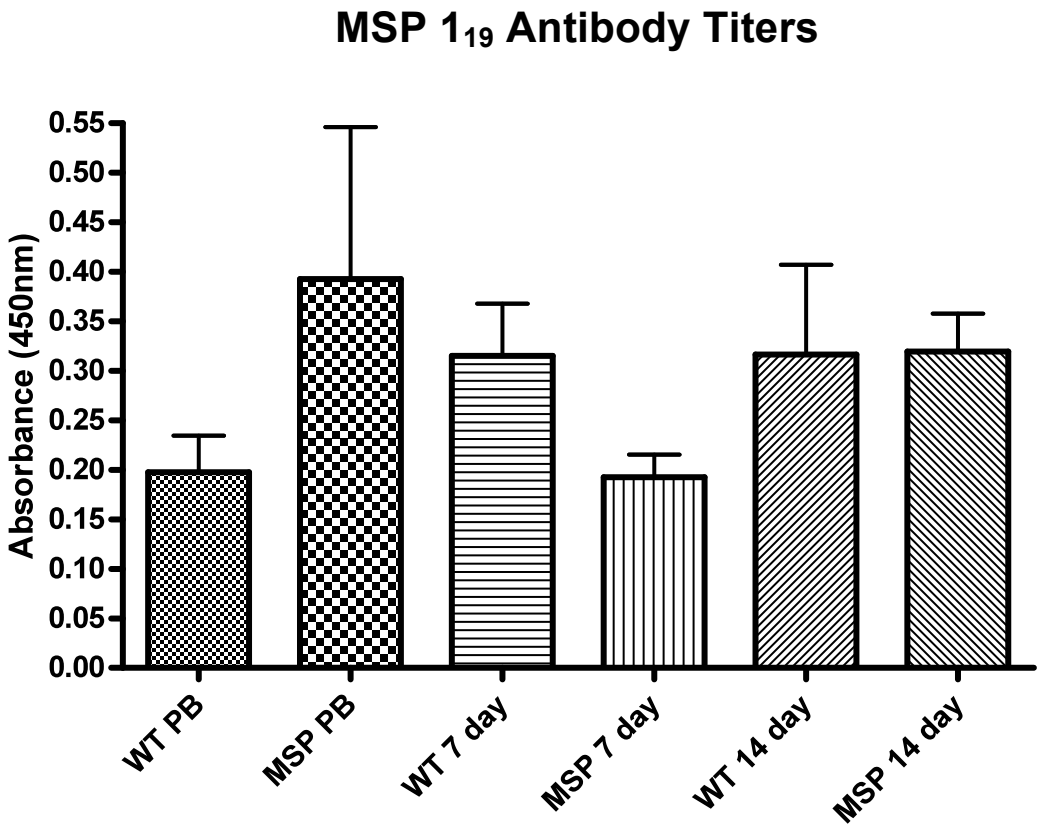

Figure 4.7: MSP1$_{19}$ ELISA to determine antibody titers. Sera from wild type and MSP mice was taken at day zero (PB), 7 and 14. Sera was analyzed via ELISA to determine if there was a difference between the groups in terms of MSP1$_{19}$ antibody titers. Absorbance was measured at 450nm. One way analysis of variance (ANOVA) indicated no statistical difference at any point in the experiment.

CHAPTER 5: SUMMARY AND CONCLUSIONS

The cost of production and safety concerns surrounding traditional vaccines has led the scientific community to search for new methods of vaccine administration. Mucosal immunology has generated an interest in oral antigen delivery. The majority of an individual's immune response is localized in the gut mucosa, and the response generated there leads not only to a common response at all mucosal surfaces but also to a systemic response. Furthermore, while vaccination is of interest, such a delivery mechanism could be used to initiate tolerance responses to particular antigens, abrogating difficulties with autoimmune disorders and allergies.

Our laboratory is currently exploring the production of a soybean-based delivery platform for subunit vaccines and tolerogens. Edible therapies are, in general, an ideal means of delivering oral therapeutics. However, many plants currently being explored for this process are deficient in protein expression, difficult to store long term and require special processes such as flash freezing to maintain protein stability during transportation. Soybean, however, is approximately 40% protein and, as antigens can be directed to express in the seed, can be stored indefinitely at ambient temperatures. Furthermore, the production of soymilk for consumption is well established, and the same

processes can be utilized to produce soymilk from transgenic seeds for therapeutic delivery.

In the studies outlined in previous chapters, our laboratory has examined antigens for both tolerance and immunization. Human thyroglobulin (hTG) is one of the primary auto-antigens in the establishment of Hashimoto's hypothyroiditis (HT). This protein is used in diagnostic assays to determine the presence of autoantibodies in patients suspected of having HT. The only currently available source of thyroglobulin is from cadaver tissue. The means of extracting thyroglobulin and testing it for contaminants and pathogens is extremely expensive and inefficient, driving up the cost of the assay for the thousands of individuals diagnosed with HT each year. Additionally, the only currently treatment for HT is lifelong hormone replacement therapy with levothyroxine, the synthetic form of thyroglobulin.

Our laboratory was able to express human thyroglobulin at ~ 1.0% TSP in soybeans. This success creates a second source for a very important pharmacological protein. More importantly, this source of protein is cost effective and easily reproducible. Since expression has been established, future studies should focus on further analytical verification of the soybean-derived protein. Although partial purification has been achieved, further analysis to purify the protein for use in diagnostics is required. In addition, focus should be placed on the establishment of a column device to remove autoantibodies that interfere with thyroid cancer assays and generate false negative results.

Despite the myriad applications for hTG in diagnostics, our laboratory has also placed an emphasis on initiating a tolerance response to the antigen in an attempt to inhibit HT disease induction and progression. In these studies, we demonstrate successful inhibition of hTG antibody production in the murine model. As this is the diagnostic hallmark of disease, it is reasonable to conclude that disease inhibition could be achieved via oral delivery of soybean-derived hTG. Future studies will focus on the time course and dose of therapy required to completely inhibit disease progression, examine the pathology of murine thyroids in relationship to disease inhibition or lack thereof and determine T3 and T4 hormone levels in mice that have been successfully tolerized as indicated by other markers. If successful, these studies could demonstrate an alternative to traditional hormone replacement therapy and offer a more cost-effective, permanent one time therapy for individuals at risk for HT or in the early stages of disease onset.

Given our success with tolerance induction using the soybean platform, our studies expanded to examine the production of malarial antigens in soybean for oral vaccination. We designed three constructs for transformation into soybean, producing only one that successfully expressed the MSP 1_{19} protein in the soybean platform. Our analysis indicated extremely low levels of expression (~0.01%). The potential reasons for this are varied and will require additional analysis of the transgenic soybeans, including southern blot analysis to test for position effects and multi-copy insertions.

Using the positively expressing seeds, a small murine study was conducted to determine if protective titers against MSP 1_{19} could be established. Given the low levels of antigen expression, it is unsurprising that this study was not successful. Second generation experiments should be conducted only when expression levels of 0.5-1.0% have been achieved, making it possible to administer greater doses of antigen via oral gavage.

Taken together, these experiments indicate the extreme versatility of the soybean platform. Numerous options are available for construct design, protein expression and commercial applications. Once optimized, the soybean platform will offer a cost effective, reproducible and readily obtainable alternative to diagnostic analytes and pharmacological therapeutics.

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