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Transgenic Plants as Novel Bioreactors to Produce Human Protein

Nadiyah Alqazlan
The University of Western Ontario

Supervisor
Dr. Shengwu Ma
The University of Western Ontario

Joint Supervisor
Dr. Jim Karagiannis
The University of Western Ontario

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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TRANSGENIC PLANTS AS NOVEL BIOREACTORS TO PRODUCE HUMAN INTERLEUKIN-37

(Thesis format: Monograph)

by

Nadiyah Alqazlan

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Interleukin-37 (IL-37) is a newly discovered cytokine, which is anti-inflammatory by its inhibition of pro-inflammatory cytokine production. An increasing number of studies suggest that IL-37 holds great promise for treating many inflammatory diseases. Currently, *E. coli*-derived recombinant human IL-37 is commercially available, but it is prohibitively expensive for use in clinical practice. This project aims to develop an alternative method for large-scale and cost-effective production of human IL-37 based on the use of plants as an expression system. Three binary plant expression vectors were designed and constructed to express human IL-37 as a recombinant native protein form, a His-tagged protein form (IL-37:6xHis) and a fusion protein form with soybean agglutinin (SBA:linker:TEV:IL-37), respectively. Soybean agglutinin (SBA) is a lectin glycoprotein that was previously shown to increase the accumulation levels of recombinant proteins and to facilitate downstream protein purification when used as a fusion partner. The constructed plant expression vectors were transferred to *Agrobacterium* prior to plant transformation. By using *Agrobacterium*-mediated plant transformation, both transient expression of IL-37 in *N. benthamiana* plants and stable expression of IL-37 in *Nicotiana tabacum* cv.81V9 were achieved. Immunoassays such as Western blot and ELISA (the enzyme-linked immunosorbent assay) showed high-level accumulation of IL-37 in plant cells. Functional testing showed that plant-derived IL-37 retained the ability to suppress the secretion of lipopolysaccharide (LPS)-induced tumor necrosis factor–α (TNF-α), a pro-inflammatory cytokine, from mouse kidney primary cells. These results suggest that plants hold a promising potential to be a green bioreactor for a new source of functional human IL-37 at high yield and low cost.
Key Words: *Agrobacterium*, interleukin-37, transient expression, transgenic tobacco, Soybean Agglutinin, pro-inflammatory cytokines, lipopolysaccharide, plant bioreactors, molecular farming.
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LIST OF ABBREVIATIONS

5’ Upstream of the sequence of interest
3’ Downstream of sequence of interest
% Per cent
°C Degree Celsius
bp Base pair
BSA Bovine serum albumin
CaMV Cauliflower mosaic virus
cDNA Complementary DNA
ConA concanavalin A
cv cultivar
CXCL2 Chemokine (C-X-C motif) ligand 2
DAI Days after infiltration
ddH₂O Double distilled water
DMEM Dulbecco’s modified Eagle medium
DNA Deoxyribonucleic acid
dNTPs Deoxyribonucleotide triphosphate
DTT Dithiothreitol
ECL Enhanced chemiluminescence
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GAD65</td>
<td>Glutamic acid decarboxylase 65</td>
</tr>
<tr>
<td>GAD67</td>
<td>Glutamic acid decarboxylase 67</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GTE</td>
<td>Glucose/Tris-HCL/EDTA</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-13</td>
<td>Interleukin-13</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin-18</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>L</td>
<td>Linker sequence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani media</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MSO</td>
<td>Murashige and Skoog salt</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>Ni–NTA</td>
<td>nickel-nitrilotriacetic acid chelate</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NOS</td>
<td>nopaline synthase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffer saline with Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl-methylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polvinylidene difluoride</td>
</tr>
<tr>
<td>rpm</td>
<td>Round per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SBA</td>
<td>Soybean agglutinin</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal broth with Catabolite repression (glucose)</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transfer DNA</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline with Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’, N’-tetramethylethylmenediamine</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factors</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>TSP</td>
<td>Total soluble protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>TYC</td>
<td>Tryptone/yeast extract/calcium chloride</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>USS$</td>
<td>United States dollar</td>
</tr>
<tr>
<td>UTL</td>
<td>Untranslated leader sequence</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction

1.1 Expression systems for production of recombinant proteins of pharmaceutical interest

To date, several expression hosts have been used for the production of recombinant proteins of pharmaceutical interest, including bacteria, yeast and mammalian cells (Manuell et al., 2007). Among them, bacteria such as *Escherichia coli* are the most widely used expression hosts for therapeutic protein production. The main advantages of bacteria as an expression system are its ability to achieve high levels of protein expression as well as the potential for large-scale production (Skerra, 1993). However, bacteria are prokaryotic organisms and as such, cannot perform adequate post-translational modification such as glycosylation that is required for proper protein folding of many therapeutic proteins of mammalian origin. Therefore, in most cases, bacteria are only suitable for use in the expression of those recombinant proteins that require no post-translational modifications. For the production of complex proteins such as antibodies, bacteria are not ideal hosts (Georgiou and Valax, 1996; Balbas, 2001).

Yeast is another host that is often used to produce proteins of therapeutic interest. Unlike bacteria, yeast is a eukaryotic organism that is able to carry out post-translational modifications such as glycosylation and is easy to handle, safe to use, and simple to
grow. However, yeast’s ability to successfully express, fold and post-translationally modify heterologous proteins is limited (Hamilton et al., 2003; Wildt and Gerngross, 2005). Moreover, the biological activity of yeast-derived proteins is often affected due to hyper-glycosylation (by the repeated addition of mannose at N-linked sites) (Kniskern et al., 1994).

On the other hand, mammalian cells have the capacity to produce recombinant proteins that are as close as possible to the native form with accurate post-translational modifications (O’Callaghan & James, 2008). However, mammalian cells bring with them the risk of pathogen contamination (Wang & Ma, 2012). Moreover, the economic and commercial value of recombinant therapeutic proteins is affected by the high production costs associated with mammalian cell culture media and subsequent purification methods. Compared to bacterial and yeast systems, the protein yields from mammalian cells are lower. Also, scaling up of the culture of mammalian cells can be both difficult and costly (O’Callaghan & James, 2008).

Over the last decade, the demand for protein therapeutic drugs, especially monoclonal antibodies, vaccines, growth factors and hormones, has been rising dramatically. The use of conventional cell culture-based production systems, such as bacteria, yeast or mammalian cells, can hardly meet the increasing demand. Therefore, new expression strategies allowing for low-cost and large-scale production of recombinant proteins must be developed.
1.1.1 Plants as an expression system for recombinant protein production

In the past two decades, advances in molecular biology and genetic engineering have created opportunities to expand the use of plants to produce biopharmaceuticals. Indeed, the use of plants as a green bioreactor to produce recombinant proteins of pharmaceutical importance, commonly known as molecular farming, has attracted ever-increasing attention. Plants as green bioreactors for protein production offer considerable advantages. One of the main advantages is the capacity of carrying out eukaryotic post-translational modifications such as glycosylation and disulfide bridging, which are often essential for the biological activity of many mammalian proteins (Ma et al., 2003; Tremblay et al., 2011a). Another advantage is the reduced risk of contamination with human pathogens (always a major concern if using mammalian cell culture expression system) (Tremblay et al., 2010). Additionally, there are advantages in terms of simplicity and the low cost of growing plants compared to traditional cell culture systems, which allows for the inexpensive scale-up of the production of recombinant proteins (Levi, 2000; Vianna et al., 2011; Wang & Ma, 2012). Moreover, plant systems are powerful and robust, which allow for the simplified purification of recombinant proteins and the ability to exploit plants as vehicle for the delivery of their expressed proteins with minimal processing (Ma et al., 2004).

Human growth hormone was the first pharmaceutical protein expressed in plants (Barta et al., 1986). Since then, a growing number of pharmaceutical proteins have been produced in plants, including monoclonal antibodies, vaccines, growth factors, cytokines and enzymes (for additional information, please see recent reviews by Karg et al., 2009; Melnik et al., 2013; Scotti et al., 2013). Moreover, many plant-made pharmaceutical
proteins have been advanced to preclinical animal studies or clinical human testing (Obembe et al., 2011). For example, production of interferon-alpha (IFN-α) in transgenic duckweed was reported, and plant-made IFN-α has already been assessed in Phase I clinical trials to treat hepatitis C virus (HCV) (Komarova et al., 2010). Aviezer and colleagues reported the production of human glucocerebrosidase in transgenic carrot cell cultures to treat Gaucher’s disease and it has progressed into a Phase III clinical trial (2009), and received approval by the Food and Drug Administration (FDA) to market in May 2012. Another example is the production of H5N1 pandemic influenza vaccine in *Nicotiana benthamiana*. It was shown that plant-made H5N1 forms virus-like particles. A Phase I clinical trial to test plant-made H5N1 influenza vaccine is underway (D’Aoust et al., 2010).

1.1.2 Methods for expressing recombinant proteins in plants

There are four main plant-based expression systems for the production of foreign proteins in plants. Each system displays distinct advantages and has its own characteristics. This offers the flexibility for researchers to choose the most efficient plant-based expression system for a particular target protein production.

Stable nuclear transgenic plants are genetically engineered plants created by introducing a foreign gene (transgene) into the host plant nuclear genome using recombinant DNA technology. One major advantage is that stable nuclear transgenic plants are able to meet the long-term demands for the production of recombinant proteins because the gene’s integration into the host plant nuclear genome allows it to be passed
on to the next generation. Moreover, stable nuclear transgenic plants can be used to scale up recombinant protein production, simply by planting more acres of the genetically engineered crops (Tremblay et al. 2010). Furthermore, foreign protein-encoding transgenes can be targeted to edible plant organs such as leaves, seeds or fruits for recombinant protein oral delivery without downstream processing. However, the production of recombinant proteins in stable nuclear transgenic plants is relatively time-consuming. There are also some concerns about the biosafety of using whole plants as bioreactors since the transgenes may escape to wild species through seed mixing or cross-pollination.

On the other hand, chloroplast transformation presents a valuable alternate stable expression system to nuclear transformation for the production of therapeutic proteins in plants. One important advantage of chloroplast-transformed plants is the ability to express foreign proteins at a very high level of accumulation with transgene containment. A typical tobacco leaf contains as many as 100 chloroplasts per cell with up to 100 genome per chloroplast which amounts to an extraordinarily high ploidy degree of up to 10,000 plastid genomes per cell (Chebolu and Daniell, 2009). Therefore, insertion of the transgene into the chloroplast genome usually leads to much higher amounts of recombinant protein expression. Furthermore, there is a low chance of transgene transmission through cross pollination to wild populations because chloroplast genomes are maternally inherited in most plant species. Additionally, chloroplast-transformed plants offer the precise integration of transgenes by homologous recombination, thus there are no position effects as normally experienced with random insertion of transgenes in nuclear transformation. Also, gene silencing does not occur in plastids and therefore
transgene expression is stable in progeny of transplastomic plants (Svab et al., 1990). However, a limitation to foreign protein expression in chloroplasts is that chloroplasts do not have the ability to carry out post-translational modifications to the expressed proteins (Gao et al., 2012; Tremblay et al., 2010).

Apart from stable nuclear and chloroplast-based expression, transient gene expression systems can also provide a plant-based platform for the production of recombinant pharmaceutical proteins in plants. Transient expression is done through the soil-borne plant pathogen *Agrobacterium tumefaciens*. *Agrobacterium*-mediated transformation is a well-known phenomenon that has been widely used for the transformation of plants such as tobacco (McCullen et al., 2006). The major advantage of transient gene expression system is its ability to produce a desired protein in a very short period of time (Tremblay et al., 2010). Target proteins can be generated in useful amounts within a matter of days or weeks. Furthermore, transient expression systems display a simplicity and ease of performance without the need for expensive supplies and equipment. Moreover, transient expression does not depend on the chromosomal integration of foreign genes, thus its expression is not affected by chromosomal position effects (Komarova et al., 2010). However, transient expression systems are limited in scale. Furthermore, expression is not permanent or heritable because the transgene does not integrate into the nuclear genome of plant cells.

Plant cell suspension culture offers another alternative plant-based expression system for the production of pharmaceutical proteins. This expression system combines the features of whole-plant systems and those of microbial and animal cell cultures (Xu et al. 2011). Plant cell suspension culture preserves the advantages of whole-plant systems,
such as post-translational modifications, product safety, easy scale-up, and the ability to synthesize correctly folded and assembled multimeric proteins. Additionally, plant cell suspension culture, like bacteria suspension culture, uses simple synthetic media. Furthermore, the use of plant cell suspension culture makes product recovery and purification simpler and cheaper than when using plant biomass. This is because of plant cells ability to secrete the expressed proteins into the culture medium (Xu et al. 2011). However, a main drawback of plant cell suspension culture is the relatively low protein yield (Hellwig et al. 2004).

1.1.3 Tobacco is a good choice of host species for protein production

For the establishment of a green bioreactor, there are many plant species to choose from. Depending on the type of application desired, each plant species has its own advantages and disadvantages. The use of tobacco for molecular farming offers unique advantages over other plant species. First, in the last 20 years, tobacco has become a primary tool to demonstrate the idea of recombinant protein production, and it is amenable to genetic modification. Second, tobacco has a high biomass yield as a leafy plant that yields up to 100 tons of leaf biomass per hectare. Besides, tobacco has high soluble protein levels, which is an attractive characteristic compared with other plant species. Third, because of the availability of low-nicotine, low-alkaloid tobacco species such as cultivar “81V9”, tobacco can even be used for the direct oral delivery of its expressed therapeutic proteins (Menassa et al., 2007). Fourth, there is low probability of the food and feed chains being contaminated with transgenic material because tobacco is
not a food or feed crop. Finally, tobacco offers numerous protein production strategies: for example, stable nuclear and chloroplast based expression, transient based expression and tobacco plant-cell culture-based expression.

To date, an increasing number of pharmaceutical proteins have been produced in tobacco plants, ranging from antibodies, vaccines and cytokines. Table 1 provides a brief summary of some tobacco-made biopharmaceutical proteins.

**Table 1: Examples of tobacco-made biopharmaceutical proteins**

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Type</th>
<th>Application</th>
<th>Progress</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiotype specific</td>
<td>Antibody</td>
<td>Treat cancer (B-cell lymphoma)</td>
<td>Human Phase I Clinical trial</td>
<td>(McCormick et al., 2008)</td>
</tr>
<tr>
<td>Anti-BoNT/A scFv</td>
<td>Antibody</td>
<td>Treat Botulism disease</td>
<td>Animal pre-clinical trial</td>
<td>(Almquist et al., 2006)</td>
</tr>
<tr>
<td>CaroRX™</td>
<td>Antibody</td>
<td>Treat S. mutans colonization</td>
<td>Approved for sale</td>
<td>(Ma et al., 1998)</td>
</tr>
<tr>
<td>VP1</td>
<td>Vaccine</td>
<td>Treat foot and mouth disease</td>
<td>Animal pre-clinical trial</td>
<td>(Wu et al., 2003)</td>
</tr>
<tr>
<td>GAD65 &amp; GAD67</td>
<td>Vaccines</td>
<td>Treat Type 1 diabetes</td>
<td>Animal pre-clinical trial</td>
<td>(Ma et al., 2004; Ma et al., 1997)</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Vaccine</td>
<td>Treat Type 2 diabetes</td>
<td>In vitro</td>
<td>(Brandsma et al., 2009)</td>
</tr>
<tr>
<td>IL-4</td>
<td>Cytokine</td>
<td>Treat cancer/chronic inflammation/autoimmune disease</td>
<td>In vitro</td>
<td>(Ma et al., 2004)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Cytokine</td>
<td>Treat chronic inflammation</td>
<td>In vitro</td>
<td>(Menassa et al., 2007)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Cytokine</td>
<td>Treat cancer/chronic inflammation/autoimmune disease</td>
<td>In vitro</td>
<td>(Wang et al., 2008)</td>
</tr>
</tbody>
</table>
1.2 Interleukin-37 and its therapeutic potential in disease treatment

Human interleukin-37 (IL-37), formerly known as IL-1F7, is an anti-inflammatory cytokine that was first identified in 2000 (Kumar et al., 2000; Dinarello et al., 2010). Of the 11 members of IL-1 family, IL-37 is the most recently characterized. It has 5 different splice variants (IL-37a to IL-37e); IL-37b is the largest isoform of IL-37 consisting of 218 amino acids (Boraschi et al., 2011; Dinarello et al., 2013). The anti-inflammatory activity of IL-37 was initially demonstrated by its ability to inhibit the secretion of pro-inflammatory cytokines using a mouse macrophage RAW cell line as described by Nold et al. (2010). The macrophage RAW cell line secretes a number of pro-inflammatory cytokines and chemokines, such as TNF, GM-CSF, IL-1β, IL-1α and CXCL2 (a chemokine also known as MIP-2), upon stimulation with lipopolysaccharide (LPS). When RAW cells were transfected with the IL-37 gene, they showed significant reduction of secretion of those pro-inflammatory cytokines mentioned above. IL-37 was subsequently tested as an anti-inflammatory agent to treat local and systemic inflammation in concanavalin (ConA)-induced hepatitis and lipopolysaccharide (LPS)-induced sepsis (Bulau et al., 2011) and inflammatory bowel disease (IBD) (McNamee et al., 2011), and to reduce liver inflammatory injuries in mouse models (Sakai et al., 2012). Imaeda and colleagues reported the enhanced expression of IL-37b by intestinal epithelial cells in the inflamed mucosa of IBD patients, suggesting an anti-inflammatory role in human IBD (Imaeda et al., 2013).

Currently, only an Escherichia coli-derived non-glycosylated form of recombinant human IL-37 (rhIL-37) is commercially available (Kumar et al., 2002); however, it has reduced stability and is prohibitively expensive (i.e. 2,200 US$ / mg,
NovoProtein Scientific). This makes the use of this form of hIL-37 as a novel clinical therapy unrealistic. Therefore, it is urgent to devise new technologies that enable the production of affordable and clinically useful quantities of rhIL-37.

1.3 Soybean Agglutinin as a high affinity tag

Soybean agglutinin (SBA) is a lectin glycoprotein found in soybean seeds. SBA binds to N-acetyl-D-galactosamine and has the ability to agglutinate cells on their surface with this glycan (Percin et al., 2009). SBA is naturally produced in soybean seeds to nearly 2% of the total soluble protein. It is typically extracted from soybean flour with more than 90% yield following one-step affinity purification on beads bearing N-acetyl-D-galactosamine (Percin et al. 2009; Lindstrom et al., 1990). Therefore, SBA offers great potential as a novel fusion tag to increase the expression of plant-made proteins. Moreover, the high affinity of SBA for its ligand permits the use of affinity chromatography with an N-acetyl-D-galactosamine-bound agarose column. In our laboratory for example, SBA has been expressed as a fusion protein with the reporter green fluorescent protein (GFP). This system achieved expression levels higher than 2% of TSP (Tremblay et al., 2011b).

1.4 Hypothesis and research objectives

Development of a plant-based platform for the production of human interleukin-37 (hIL-37) was the objective of this thesis. It was hypothesized that tobacco could be
used as a plant host to produce high quantities of recombinant hIL-37. Moreover, the expression of hIL-37 as a fusion protein with SBA was predicted to allow for the simple, rapid, one-step purification of the expressed protein from tobacco leaf tissue via affinity chromatography using $N$-acetyl-$D$-galactosamine. It was also hypothesized that plant-made hIL-37 and hIL-37-SBA fusion protein would be biologically active. The long-term goal of this project is to develop a novel, reliable and rapid method for producing low-cost, large-scale recombinant hIL-37 based on the use of plants as an expression system. The specific objectives of the project were:

1) **To construct plant transformation vectors for expressing hIL-37 in tobacco plants.** To date, recombinant hIL-37 protein has only been produced in *Escherichia coli* system, and no reports on the expression of hIL-37 in plants have been seen. Plant transformation vectors that express various forms of hIL-37 were constructed. These forms included: 1) the unmodified native form, 2) a His-tagged form and 3) a fusion form with soybean agglutinin (SBA). Inclusion of the 6xHis-tagged form allowed for the comparison of the efficiencies of different affinity tags (6xHis vs. SBA tag) with respect to the purification of recombinant IL-37. The *Agrobacterium*-mediated method was used to transform tobacco plants, and the expression of IL-37 in tobacco was achieved using both stable nuclear expression and transient expression systems.

2) **To demonstrate hIL-37 protein expression in tobacco and to quantify expression levels.** Total protein was extracted from *N. benthamiana* leaf tissues transiently transformed with *Agrobacterium* containing hIL-37 expression vector or from the leaves of transgenic tobacco plants. The presence of recombinant IL-37 was
demonstrated by Western blot. The level of IL-37 accumulation in tobacco plant leaf tissues was estimated specific ELISA.

3) **To purify recombinant IL-37 from tobacco leaf tissues.** His-tagged hIL-37 was purified from total tobacco leaf extract using a nickel-nitrilotriacetic (Ni-NTA) agarose chromatography purification method. The fusion protein SBA-IL-37 was purified by using an N-acetylgalactosamine–agarose column.

4) **To study the biological activities of plant-derived recombinant IL-37 via in vitro assays.** The functionalities of plant-derived His-tagged IL-37 protein and SBA-IL-37 fusion protein were determined by their ability to inhibit LPS-induced secretion of proinflammatory cytokines from mouse kidney primary cells.
Chapter 2

Methods and Materials

2.1 Bacterial strains

All cloning was carried out using *Escherichia coli* strain DH5α (Invitrogen, Burlington, Ontario). The cell lines were maintained on Luria Bertani (LB) media [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] containing 1.5% (w/v) agar and the appropriate selective antibiotics as specified. Liquid cultures of DH5α cells used for plasmid DNA extractions were grown in Terrific Broth (TB) [1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (w/v) glycerol, 17 mM KH$_2$PO$_4$, 72 mM K$_2$HPO$_4$]. Once expression cassettes were constructed, they were integrated into pBI101.1 binary vector, which allows for expression of the selectable marker neomycin phosphotransferase (NPTII) for kanamycin resistance in both *E. coli* and tobacco plants (Bevan, 1984). Due to the large size of the binary vector backbone, the final DNA constructs were transformed into DH5α electrocompetent cells because of their higher transformation efficiency relative to calcium competent DH5α cells.

The *Agrobacterium tumefaciens* strain LBA4404 was employed for transient expression in *N. benthamiana* and for stable tobacco leaf disc transformation. The LBA4404 strain was maintained on Tryptone/Yeast extract/Calcium chloride (TYC) medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.066% (w/v) CaCl$_2$] containing
1.5% (w/v) agar and rifampicin at 25 mg/ml. Liquid cultures of LBA4404 strain were grown in TYC broth with rifampicin.

The antibiotics concentrations used for selection of transformants were as follows: *E. coli* cells were selected for resistance to carbenicillin at 100 µg/ml or kanamycin at 50 µg/ml; *A. tumefaciens* cells were selected for resistance to kanamycin at 50 µg/ml and rifampicin at 25 µg/ml.

2.2 Rapid Alkaline Lysis Mini-prep of plasmid DNA

Plasmid DNA was collected from bacterial strains by the alkaline mini-prep procedure described by Birnboim and Doly (1979). Briefly, cells from a single colony were inoculated into 2 ml of TB containing the appropriate antibiotic. Cells were grown overnight at 37°C with shaking. A total of 1.5 ml overnight cell culture was then transferred into an Eppendorf tube and centrifuged for 30 s at 12,000xg. The pellet was resuspended in 100 µl GTE buffer [50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0] and incubated at room temperature for 5 min. After incubation, 200 µl of NaOH/SDS [0.2 M NaOH, 1% (w/v) SDS] was added into the solution, gently mixed by inversion and incubated on ice for 5 min. Total of 150 µl of potassium acetate [29.5% (w/v) glacial acetic acid, adjusted to pH 4.8 by KOH pellets] was then added into the solution, mixed for 2 s by vortexing and placed on ice for 5 min. After centrifugation at 12,000xg for 5 min, the supernatant was collected, mixed with 600 µl of isopropanol and then incubated at room temperature for 5 min. Plasmid DNA was precipitated by a 5-minute spin at 12,000xg and washed with 1 ml of 70% (v/v) ethanol. The resulting plasmid DNA pellet
was vacuum dried and resuspended in 50 µl of double distilled water (ddH₂O) or TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0] and 1 µl RNase (Fermentas, Glen Burine, Maryland).

2.3 Polymerase Chain Reaction (PCR)

PCR was used to facilitate DNA cloning and gene modification. All primers used are outlined in Table 2. Approximately 50-100 ng of template DNA was used for each 50-µl reaction. Each reaction mixture contained 1x PCR buffer, 2 M dNTPs, 1 µM of both forward and reverse primers, 2.5 mM MgCl₂ and 1 unit of DNA polymerase. High fidelity polymerase (pfu DNA polymerase from Fermentas) was used in all PCR reactions. PCR products were purified with the QIAquick gel extraction kit (Qiagen, Mississauga, ON) after size separation by electrophoresis on a 0.7% (w/v) agarose gel.

2.4 DNA Ligations

All DNA ligations were performed with the Rapid Ligation kit (Fermentas). A 20 µl ligation reaction contained 3 µg of insert DNA, 1 µg of plasmid vector DNA, 4 µl of 5x reaction buffer, 1 µl T4 DNA ligase and topped up with ddH₂O. After 10 to 15 min incubation at room temperature, 3-7 µl of the mixture was transformed into chemically competent DH5α cells (Invitrogen) by heat shock or into electrocompetent DH5α cells by electroporation. The preparation of electrocompetent cells was described previously by Miller and Nickoloff (1995).
2.5 *Escherichia coli* Transformation

Chemically competent DH5α cells were transformed with plasmid DNA by heat shock transformations. In brief, approximately 50 ng of ligated DNA was added to 40 µl DH5α cells, gently mixed and placed on ice for 35 min. The cells were then heat shocked in a 37°C water bath for 5 min and incubated on ice for 10 min before transferred to a tube containing 800 µl SOC medium [0.5 % (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose]. After 1 h incubation at 37°C with shaking at 200 rpm, 200 µl of the culture was spread onto LB agar plates with the appropriate antibiotics.

Electrocompetent DH5α cells were transformed with plasmid DNA by electroporation (Inoue et al., 1990; Miller and Nickoloff, 1995). Briefly, 1 or 2 µl of ligated DNA was mixed with 40 µl electrocompetent cells and incubated on ice for 1 min. The mixture was then transferred to a cold electroporation cuvette and placed in the chamber slide of MicroPuler™ electroporation apparatus (Bio-Rad Laboratories). The cells were pulsed once in the electroporation apparatus. A total of 1 ml of SOC medium was added to the cuvette after the pulse, and the cells were gently resuspended and transferred to a culture tube. The resulting cell suspension was incubated at 37°C for 1 h, shaking at 200 rpm. A total of 50 µl of the culture was spread onto LB agar plates with appropriate antibiotics.
2.6 Construction of Plant Expression Vectors

Standard molecular biology techniques were used to construct plant expression vectors. Details are described below.

2.6.1 Construction of native IL-37 plant expression vector: pBI101.1-IL-37

The plasmid containing IL-37 gene was provided by Dr. Ma’s laboratory. The IL-37 cDNA was amplified by PCR using the forward primer IL-37 F1 and the reverse primer IL-37 R2 (Table 2). The forward primer IL-37 F1 contained a PscI restriction site (underlined), which contains a translation start codon. The reverse primer IL-37 R2 contained an engineered XbaI restriction site immediately after the stop codon (underlined). PCR reaction was performed under following conditions: denature at 95°C for 30 s, anneal at 60°C for 1:30 min and elongate at 72°C for 1 min, for total of 35 cycles followed by a final elongation at 72°C for 10 min. The amplified IL-37 sequence was blunt-end ligated into SmaI site of pUC19 vector and subsequently sequenced to confirm the sequence integrity (Sequencing Facility: Robarts Research Institute, London, ON, Canada).

To create the expression cassette, PCR-amplified IL-37 sequence was released from pUC19 as a PscI/XbaI fragment, and then inserted into pRTL2 vector by replacing GUS gene through NcoI/XbaI restriction sites; PscI and NcoI restriction sites have compatible sticky ends. The IL-37 expression cassette consisted of an enhanced
constitutive cauliflower mosaic virus (CaMV) 35S promoter, a viral untranslated leader sequence (5’UTL), IL-37 coding sequence and the *A. tumefaciens* nopaline synthase terminator (NOS) 3’-polyadenylation signal. The expression cassette was excised from pRTL2-IL-37 as a single DNA fragment by *Hind*III digestion, and then cloned into the final plant binary transformation vector pBI101.1 to produce pBI101.1-IL-37 (Figure 1).

### 2.6.2 Construction of IL-37:6xHis plant expression vector: pBI- IL-37:6xHis

The IL-37 cDNA was amplified by PCR using the forward primer IL-37 F1 and the reverse primer IL-37 R1 (Table 2). The forward and reverse primers have the same restriction sites as in the previous construct, but the reverse primer IL-37 R1 contained a 6xHis tag (bold) just before the stop codon of IL-37 sequence. PCR reactions were performed under the same conditions as described in Section 2.6.1. The amplified IL-37 sequence was blunt-end ligated into *Sma*I site of pUC19 vector and subsequently sequenced to confirm the sequence integrity (Sequencing Facility: Robarts Research Institute, London, ON, Canada).

To create pBI101.1-IL-37:6xHis, the same procedures were used as described above for construction of plant expression vector pBI-IL-37. The difference of pBI101.1-IL-37:6xHis from pBI101.1-IL-37 is that pBI101.1-IL-37:6xHis contains an additional 6xHis tag at the C-terminal end of IL-37 coding sequence (Figure 2).
Table 2: PCR Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Nucleotide sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-37 F1</td>
<td>5’ ATTAA[CATG][TGGTCATACA][AAGA][TGGTTTTCTTT] 3’</td>
<td>Cloning</td>
</tr>
<tr>
<td>IL-37 R1</td>
<td>5’ TATAT[CTAGA][TCA][TGATGATGATGATGATGATGATCGGACACCTCT][TA][AGGAGA] 3’</td>
<td>Cloning</td>
</tr>
<tr>
<td>IL-37 F2</td>
<td>5’ ATTG[TCGAC][GTTTA][TACAA][AGGATTTTCTTT] 3’</td>
<td>Cloning</td>
</tr>
<tr>
<td>IL-37 R2</td>
<td>5’ TATAT[CTAGA][TCA][ATCGGACACCTCT][GA][AAGGAGA] 3’</td>
<td>Cloning</td>
</tr>
</tbody>
</table>

The IL-37 cDNA was modified by removal of its native signal peptide sequence via PCR techniques. The forward primer IL-37 F2 contained a SalI restriction site (underlined) for in-frame fusion with SBA, whereas the reverse primer IL-37 R2 contained an XbaI restriction site (underlined), see Table 2. PCR reaction was performed under following conditions: denature at 94°C for 30 s, anneal at 60°C for 30 s and elongate at 72°C for 33 s, for total of 33 cycles followed by a final elongation at 72°C for 5 min. The PCR product was blunt-end ligated into SmaI site of pUC19 vector, and sequenced to confirm the sequence integrity (Sequencing Facility: Robarts Research Institute, London, ON, Canada). SBA coding sequence was modified previously by Dr. Ma’s laboratory by incorporation of an NcoI restriction site at its 5’ end to serve as a translational start site, and by the removal of the stop codon at its 3’ end with addition of a flexible linker-coding sequence (L), a cleavage site for tobacco etch virus protease (TEV) and followed by a XhoI restriction site for genetic fusion with other genes.

To create pBI101.1-SBA:linker:TEV:IL-37, IL-37 sequence was released from pUC19 as a SalI/XbaI fragment, while SBA sequence was released from pUC19 as a NcoI/XhoI fragment. IL-37 and SBA fragments were then isolated, mixed and ligated into pRTL2 vector that had been digested with NcoI/XbaI through three-fragment ligation. The expression cassette consisting of an enhanced constitutive cauliflower mosaic virus (CaMV) 35S promoter, a viral untranslated leader sequence (5’UTL), SBA:linker:TEV:IL-37 coding sequence and the A. tumefaciens nopaline synthase terminator (NOS) 3’-polyadenylation signal was excised from pRTL2 as a single DNA
fragment by *Hind*III digestion, and then cloned into the final plant binary transformation vector pBI101.1 (Figure 3) to produce pBI101.1- SBA:linker:TEV:IL-37.
Figure 1: Construction scheme of expression vector pBI101.1- IL-37.

(A) The coding sequence of IL-37 was amplified by PCR using primers incorporating restriction sites at the 5’ end and the 3’ end. Human IL-37 amplified sequence was placed into pUC19. (B) Human IL-37 amplified sequence was then cleaved and ligated into the pRTL2 vector containing promoter (CaMV 35S), untranslated leader sequence (UTL) and terminator (T\textsubscript{NOS}) sequences. (C) The IL-37 expression cassette was isolated as a single HindIII fragment from pRTL2 vector and ligated into the multiple cloning site in the T-DNA region of plant binary vector pBI101.
Figure 2: Construction scheme of expression vector pBI101.1- IL-37:6xHis

Same steps were done as in the Figure 1, but PCR reverse primer incorporated a 6xHis tag at the C-terminus.
Figure 3: Construction scheme of expression vector pBI101.1- SBA:linker:TEV:IL-37.

(A) The cDNA coding sequence of SBA was provided by Dr. Ma’s lab, which contains a NcoI site at its 5’ end to serve as a translational start site and a flexible linker sequence (L), tobacco etch virus protease cleavage site (TEV) followed by an XhoI restriction site at its 3’ end. The coding sequence of human IL-37 was amplified by PCR using primers incorporated SalI at the 5’ end and XbaI at 3’ end. SBA sequence and the amplified human IL-37 sequence were placed into pUC19 vectors separately. (B) SBA sequence and human IL-37 sequence were then cleaved and ligated all together into the pRTL2 vector containing promoter (CaMV 35S), untranslated TMV leader sequence (UTL) and terminator (T_NOS) sequences. (C) The fused SBA-IL-37 expression cassette was isolated as a single HindIII fragment from pRTL2 vector and ligated into the multiple cloning site in the T-DNA region of plant binary vector pBI101.1. (D) Nucleotide sequence and the deduced amino acids of SBA:linker:TEV:IL-37. The linker sequence is indicated in italics; the TEV protease cleavage site is underlined.
2.7 Production of IL-37 in tobacco plants

The engineered plant expression vector was first introduced into *A. tumefaciens* strain LBA4404 by tri-parental mating using helper plasmid pRK4013. Stable nuclear transformation was performed in order to transform low-alkaloid *Nicotiana Tobacum* cv.81V9 by co-cultivating leaf discs with *A. tumefaciens* LBA4404 containing the plant expression vector according to the method of Horsch et al. (1985). Transformed leaf discs were selected on callus inducing MS104 medium containing 100 µg/ml carbenicillin and 50 µg/ml kanamycin. New shoots that developed from calli were transferred to magenta boxes containing Murashige and Skoog (MS) medium with 100 µg/ml carbenicillin and 50 µg/ml kanamycin and maintained. As the transgenic plants matured, they were transferred into the green house and maintained for further analysis.

Transient expression of *Nicotiana benthamiana* was performed according to the method of Sparkes et al. (2006). Briefly, *A. tumefaciens* LBA4404 containing the plant expression vector and *A. tumefaciens* containing p19, a viral protein from Cymbidium ringspot virus that suppresses post-transcriptional gene and is known to boost the yield of transiently expressed proteins (Lakatos et al. 2004), were inoculated and grown overnight at 28°C. Cells were harvested and resuspended in infiltration media [50 mM MES, 2mM Na$_3$PO$_4$.12H$_2$O, 0.0001 M acetosyringone]. The diluted cell culture (OD$_{600}$ =0.5) was injected into the leaves of 6-week-old *N. benthamiana* plants. The transformed leaves were harvested each day at 2-6 days after infiltration (dai) and proteins were extracted for analysis.
2.8 Accumulation of recombinant protein

To determine the recombinant protein accumulation level, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting was used. Protein samples were prepared as described by Brandsma et al. (2010). Tobacco leaf tissues were homogenized in liquid nitrogen and resuspended in cold protein extraction buffer [25 mM Tris-HCl (pH 7.0), 50 mM NaCl, 2 mM β-mercaptoethanol, 1 mM phenyl-methanesulfonyl fluoride (PMSF), 2 µg/ml aprotinin, 2 µg/ml pepstatin A and 2 µg/ml leupeptin]. Samples were centrifuged at 13,000xg for 10 min at 4°C and the supernatant was collected. Protein concentration was measured by the method of Bradford (1976) using the Bio-Rad protein dye reagent (Bio-Rad, Hercules, CA, USA), and bovine serum albumin (BSA) as standard (Sigma-Aldrich Canada). The expression of recombinant proteins was analyzed by Western blotting. First, protein extracts were boiled for 10 min in sample buffer [0.0063 M Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1 M DTT, 0.01% (w/v) bromophenol blue (pH 6.8)] and separated by 10% SDS-PAGE then subsequently blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, Massachusetts). The membrane was blocked for 1 h at room temperature in 5% (w/v) skim milk-TBS-T [20 mM Tris, 150 mM NaCl, 0.02% (v/v) Tween-20 (pH 10)], washed with TBS-T. The membrane was incubated overnight at 4°C with rabbit anti-IL-37 primary antibody (abcam Cat No. ab116282) diluted 1:3000 (v/v). The membrane was washed again by TBS-T and incubated at room temperature with horseradish peroxidase conjugated swine anti-rabbit secondary antibody (KPL Cat. No. 074-1506) diluted 1:5000 for 1 h, shaking. Detection was performed using the
enhanced chemiluminescence (ECL) detection system (Perkin Elmer Life Sciences, Rockford, IL) according to the manufacturer’s instructions.

2.9 Quantification of plant-derived recombinant proteins

The accumulation of recombinant proteins in crude tobacco leaf extract was quantified using an indirect enzyme linked immunosorbent assay (ELISA) (Brandsma et al., 2010). The accumulated recombinant protein was compared against known quantities of standard IL-37 protein or standard SBA protein. The serial dilutions of standard IL-37 or standard SBA as well as crude tobacco extracts, in triplicate, were resuspended in sodium bicarbonate buffer (pH 9.6) and coated onto a 96-well microtiter plate then incubated at 4°C for overnight. Wells were washed three times with PBS-T [1x phosphate buffered saline containing 0.05% (v/v) Tween-20] and blocked with 3% (w/v) BSA in PBS-T for 1-2 h at room temperature. After washing with PBS-T, rabbit anti-IL-37 polyclonal antibody (abcam Cat No. C-terminal ab153889) or rabbit anti-SBA (Cedarlane AL-1301-2) diluted to 1:3000 (v/v) was added to each well and incubated overnight at 4°C. Following five subsequent PBS-T washes, horseradish peroxidase conjugated swine anti-rabbit IgG antibody (KPL Cat. No. 074-1506) diluted to 1:5000 (v/v) was added to each well and incubated for 1 h at room temperature. The plate was washed five times with PBS-T, and then tetramethyl benzidine (TMB) substrate (R&D Systems) was added according to the manufacturer’s protocol and incubated in the dark for 30 min at room temperature. The substrate reaction was stopped by adding 50 µl/well stop solution (1 M H₂SO₄). The plate was read as an optical density (OD) value measured
at 450 nm in a microplate reader (Bio-Rad 3550). The quantitative values of IL-37 in tobacco leaf samples are interpolated from standard reference curves constructed from values for serially diluted commercial IL-37 standard or SBA standard.

2.10 Purification of His tagged IL-37 protein

Plant-derived IL-37 proteins were purified from leaf extracts of agroinfiltrated tobacco leaves with histidine affinity chromatography using HiTrap Chelating HP columns (GE Healthcare) according to the manufacturer’s instructions. In brief, the leaf samples of tobacco plants were grinded in liquid nitrogen to a fine powder and homogenized in protein extraction buffer [25 mM Tris-HCl (pH 7.0), 50 mM NaCl, 2 mM β-mercaptoethanol, 1 mM phenyl-methysulfonyl fluoride (PMSF), 2 µg/ml aprotinin, 2 µg/ml pepstatin A and 2 µg/ml leupeptin] in a 1:4 (w/v) ratio of sample to buffer. The homogenate was filtered through a paper filter and centrifuged at 14,000xg for 10 min at 4°C. The supernatant was collected and centrifuged again at 14,000xg for 10 min at 4°C. The final supernatant was filtered through a 0.80 µm membrane filter then a 0.45 µm membrane filter and loaded onto the prepared HiTrap Chelating HP column and washed with wash buffer [2 mM imidazole, 20 mM Na₂HPO₄, 500 mM NaCl] to remove nonspecifically bound tobacco leaf endogenous proteins. The bound IL-37 protein was eluted with elution buffer [500 mM imidazole, 20 mM Na₂HPO₄, 500 mM NaCl] into 1-ml fractions for further analysis. The eluted IL-37 was dialyzed extensively against 0.5x PBS (pH 7.5) and concentrated by evaporation using speed vacuum at 4°C. Western blot was employed to confirm the purified plant-made IL-37 protein presence in
each eluted fraction. Purified plant-made IL-37 protein was further confirmed by Coomassie blue-stained SDS-PAGE gel.

2.11 Purification of SBA:linker:TEV:IL-37 protein

Purification of SBA:linker:TEV:IL-37 was carried using an N-acetyl-D-galactosamine-agarose column as previously described (Tremblay et al. 2011b). Washes were monitored by spectrophotometry at $A_{280}$ and once the readings reached zero, samples were eluted with 0.5 M galactose/0.1 M NaCl. Eluted protein fractions were dialyzed against excess 0.5 x PBS buffer to remove NaCl and galactose. SDS-PAGE gel stained with Coomassie blue was used to confirm the identity and purity of the purified plant-made SBA:linker:TEV:IL-37 protein.

2.12 Development of a cell-based anti-inflammatory functional assay for plant-derived IL-37

The functionality of plant-derived IL-37:6xHis and SBA:linker:TEV:IL-37 proteins was determined by its ability to inhibit LPS-induced secretion of proinflammatory cytokines from mouse kidney primary cells. His-tagged IL-37 and SBA:linker:TEV:IL-37 fusion proteins were purified from plant extracts according to the methods described in sections 2.10 and 2.11 respectively. Work was done according to the method of Sakai et al. (2012) with minor modifications. Briefly, B7 mice were purchased from the Jackson Laboratory. Mice were sacrificed by asphyxiation using
carbon dioxide, and kidneys were harvested. Primary cells were isolated from mouse kidneys and cultured and maintained in K1 medium [50:50 DMEM and Ham’s F12 (Invitrogen) supplemented with 10% (v/v) FBS, hormone mix (5µg/ml insulin, 34 µg/ml triiodothyronine, 5 µl/ml transferrin, 1.73 ng/ml sodium selenite, 8 ng hydrocortisone and 25 ng/ml epidermal growth factor), 100 U/ml pencillin and 0.1 mg/ml streptomycin]. Cells were seeded in one 24-well tissue culture plate in complete K1 medium and grown for 7-9 days at 37°C in an atmosphere of humidified air (95%) and CO₂ (5%) to allow cell attachment to the plate. The culture medium was replaced with fresh medium every 2-3 days and cells were used for the experiments once they had reached 80% confluence. To conduct the assay, cells were then treated with different concentrations (500 ng/ml, 400 ng/ml, 200 ng/ml) of plant-derived IL-37 or SBA:linker:TEV:IL-37. For comparison purposes, cells treated with commercial recombinant IL-37b were included in the assay to examine the difference in function compared to plant-derived proteins. Cells without any treatment with IL-37 were included as a negative control. After 24 h incubation with plant-made or commercial IL-37, the culture medium was removed and cells were treated with 1 µg/ml LPS for 24 h. The culture medium was collected and analyzed via an ELISA kit (R & D) for TNF-α (Sakai et al., 2012).

2.13 Statistical analysis

A paired two-tailed Student’s 𝑡-test was used to determine whether or not differences between samples were significant. Values of p<0.05 were held to be significant.
3.1 Construction of plant expression vectors

The construction of plant expression vectors pBI101.1- IL-37, pBI101.1- IL-37:6xHis and pBI101.1- SBA:linker:TEV:IL-37 was described in detail in section 2.6. As shown in Figure 4, the expression of IL-37, IL-37:6xHis and SBA:linker:TEV:IL-37 was placed under the control of an enhanced constitutive cauliflower mosaic (CaMV) 35S promoter. Incorporation of a tobacco etch virus (TEV) RNA 5’untranslated leader (UTL) sequence was used to increase recombinant protein accumulation in plant cells (Brandsma et al., 2010; Wang et al., 2008). A 6xHis tag in pBI101.1- IL-37:6xHis was also included to facilitate recombinant protein purification. The inclusion of SBA coding sequences in the pBI101.1-SBA:linker:TEV:IL-37 vector was for the purpose of improving expression and to facilitate the downstream purification process. To verify that each of the constructed plant expression vectors contained a specific IL-37 expression cassette, the vector DNA was digested with HindIII, and then analyzed by agarose gel electrophoresis. As expected, digestion of pBI101.1-IL-37 and pBI101.1- IL-37:6xHis with HindIII released a DNA fragment of approximately 2 kb, whereas digestion of pBI101.1- SBA:linker:TEV:IL-37 released a DNA fragment of approximately 2.8 kb (Figure 5).
Figure 4: Diagrammatic representation of the T-DNA region of the plant expression vectors used in this study.

(A) pBI101.1-IL-37, (B) pBI101.1-IL-37:6xHis, and (C) pBI101.1-SBA:linker:TEV:IL-37. P\textsubscript{NOS}, nopaline synthase promoter; NPT-II, neomycin phosphotransferase gene; T\textsubscript{NOS}, nopaline synthase terminator; MCS, multiple cloning site; CaMV 35S, Cauliflower Mosaic Virus 35S promoter with double enhancer; UTL, tobacco etch virus 5’ untranslated leader sequence; LB, left border; RB, right border; The line with scales under the plasmid name indicates the size of each component within the T-DNA region.
Figure 5: Verification of the presence of the IL-37 expression cassettes in the plant expression vectors used in this study.

Vector DNA was prepared and digested with *Hind*III. The *Hind*III-digested DNA was run on a 0.7% agarose gel followed by staining with ethidium bromide solution. (1) Empty pBI101.1 without insert served as a negative control; (2) pBI101.1- IL-37; (3) pBI101.1- IL-37:6xHis and (4) pBI101.1- SBA:linker:TEV:IL-37. Numbers to the left indicate the sizes of the DNA markers in base pairs. Arrows indicate the released DNA fragments.
3.2 Expression and accumulation of recombinant native IL-37 in transiently transformed *N. benthamiana* plants

Next, the pBI101.1-IL-37 plant expression vector was introduced into *A. tumefaciens* strain LBA4404 by tri-parental mating using the helper plasmid, pRK4013. Leaves of six-week-old *N. benthamiana* plants were then infiltrated with *A. tumefaciens* containing pBI101.1-IL-37 together with cultures of *A. tumefaciens* containing the gene for p19, a viral protein from Cymbidium ringspot virus that suppresses post-transcriptional gene and is known to boost the yield of transiently expressed proteins (Lakatos et al. 2004). Leaf samples were collected from infiltrated plants on days 2 to 6 after infiltration (dai) as described in section 2.7.

To analyze the expression and accumulation of the IL-37 protein, total protein was extracted from agro-infiltrated leaves, separated by SDS-PAGE and analyzed by Western blotting with commercial rabbit anti-IL-37 polyclonal antibody (abcam Cat No. ab116282). Commercially available recombinant IL-37 (R&D Cat No. aa 46-218) was used as a positive control. As shown in Figure 6, the anti-IL-37 antibody recognized a predominant protein band of approximately 50 kDa, corresponding to the predicted size of the dimeric form of IL-37. A faint band also recognized by the anti-IL-37 antibody had a molecular weight of approximately 25 kDa, corresponding to the predicted size of the monomeric form of IL-37. No protein bands of similar sizes were detected from TSP prepared from non-infiltrated leaves of *N. benthamiana* plants under identical conditions (Figure 6). Based on the signal intensity on Western blots, it appears that the expression of IL-37 reached its peak at day 4 post agro-infiltration. Protein quantification by ELISA
showed that the expression level of IL-37 in leave samples of *N. benthamiana* harvested at day 4 post agro-infiltration reached approximately 0.11 % of TSP (Figure 7).
Figure 6: Detection of recombinant native IL-37 protein expression in transiently transformed *N. benthamiana* by Western blot analysis.

Total soluble protein was extracted from leaves of *N. benthamiana* infiltrated with *Agrobacterium* harbouring pBI101.1-IL-37. Protein samples (25 µg) were separated by 10% SDS-PAGE gel electrophoresis, transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with anti-hIL-37 antibody. +, commercial recombinant IL-37 protein (250 ng loaded); dai, days after infiltration; WT, protein samples from uninfiltreted control *N. benthamiana* leaves. Numbers to the left indicate the sizes of the protein markers in Kilodaltons. Double-headed arrow indicates dimeric form of IL-37; single-headed arrow indicates monomeric form of IL-37.
Figure 7: Quantification of recombinant native IL-37 protein expression in transiently transformed *N. benthamiana* by ELISA.

(A) The amount of recombinant native IL-37 protein present in total protein extract prepared from leave samples of *N. benthamiana* harvested at day 4 post agro-infiltration was determined by IL-37 specific ELISA as described in Section 2.9. The level of IL-37 is expressed as percentage of IL-37 in TSP.

(B) A representative standard curve for human IL-37. It was constructed from OD values obtained from a serial dilution of known quantities of commercial IL-37.
(A)

![Bar Graph](image)

Leaf sample collected at 4 dai

(B)

![Standard Curve](image)

Standard Curve for IL-37

Mean OD vs. Concentration (ng/ml)
3.3 Expression and accumulation of His tagged form of IL-37 (IL-37:6xHis) in stably transformed plants

Next, the expression of the His tagged form of IL-37 (IL-37:6xHis) was evaluated in stably transformed tobacco plants. To this end, low-nicotine and low-alkaloid *N. tabacum* cultivar 81V9 was transformed with *A. tumefaciens* strain LBA4404 harbouring pBI101.1- IL-37:6xHis using a standard leaf-disc co-cultivation procedure (described in section 2.7). More than 20 independent transgenic tobacco plant lines were generated. There were no obvious phenotypic differences between transgenic and untransformed 81V9 control plants.

The presence of IL-37:6xHis protein was demonstrated by Western blot analysis using rabbit anti-IL-37 polyclonal antibody. As shown in Figure 8A, anti-IL-37 antibody identified two major bands: one had a molecular weight of ~ 25 kDa corresponding to the monomeric form of IL-37, and the other had a molecular weight of ~50 kDa corresponding to the dimeric form of IL-37. As expected, no bands were observed when using TSP extracted from untransformed wild-type tobacco plants (81V9). The expression levels of IL-37:6xHis protein were variable among individual transgenic lines. Based on the signal intensity on Western blots, it appears that transgenic lines T0 and T1 are the best expressor of IL-37:6xHis. Protein quantification by ELISA showed that the levels of IL-37:6xHis protein expression in T0 and T1 lines reached 0.40 % and 0.45 % of TSP, respectively (Figure 8B).
Figure 8: Detection and quantification of IL-37:6xHis protein expression in transgenic tobacco plants.

(A) Detection of IL-37:6xHis protein expression by Western blot analysis. Total soluble protein was extracted from the leaves of individual transgenic plants transformed with pBI101.1- IL-37:6xHis. Proteins were separated by 10% SDS-PAGE then transferred onto a PVDF membrane and probed with rabbit anti-IL-37 primary antibody followed by swine anti-rabbit secondary antibody. Approximately 50 µg of TSP were loaded for each transgenic line. +, commercial recombinant IL-37 protein was used as a positive control (250 ng loaded); M, protein markers; WT, untransformed wild-type 81V9 tobacco plant was used as a negative control; T0 to T11, representatives of independent transgenic tobacco lines. Numbers to the left indicate the sizes of the protein markers in Kilodaltons. Double-headed arrow indicates dimeric form of IL-37; single-headed arrow indicates monomeric form of IL-37.

(B) Quantification of IL-37:6xHis protein expression by ELISA. The amount of IL-37:6xHis protein in transgenic lines T0 and T1 representing the two best IL-37 expressor based on Western blot results was quantified by IL-37 specific ELISA. The level of IL-37:6xHis is expressed as percentage of IL-37 in TSP.
3.4 Expression and accumulation of the His tagged form of IL-37 (IL-37:6xHis) in transiently transformed \textit{N. benthamiana}

The expression of IL-37:6xHis was additionally evaluated in transiently transformed \textit{N. benthamiana}. Leaves of six-week-old \textit{N. benthamiana} plants via infiltration with\textit{A. tumefaciens} containing pBI101.1-IL-37:6xHis together with cultures of \textit{A. tumefaciens} containing p19 as described in Section 3.2. Leaf samples were collected from infiltrated plants from 2-6 days after agro-infiltration.

The presence of IL-37:6xHis protein was verified by Western blot analysis with rabbit anti-IL-37 polyclonal antibody. As shown in Figure 9A, the anti-IL-37 antibody detected one protein band with a molecular weight of approximately 50 kDa, corresponding to the predicted size of the dimeric form of IL-37. No bands of similar sizes were detected from TSP prepared from non-infiltrated leaves of \textit{N. benthamiana} plants under identical conditions. It appears that like native IL-37 protein, the expression of IL-37:6xHis reached its peak at day 4 post agro-infiltration. Protein quantification by ELISA showed that the expression level of IL-37:6xHis in leave samples of \textit{N. benthamiana} harvested at day 4 post agro-infiltration reached approximately 0.13 \% of TSP (Figure 9B).
Figure 9: Detection and quantification of IL-37:6xHis protein expression in transiently transformed *N. benthamiana*.

(A) Detection of IL-37:6xHis protein expression by Western blot analysis. Protein samples (35 µg) were prepared from leaves of *N. benthamiana* infected with *Agrobacterium* harbouring pBI101.1-IL-37:6xHis, separated by 10% SDS-PAGE gel electrophoresis, then transferred onto a PVDF membrane and probed with anti-hIL-37 antibody. +, commercial recombinant IL-37 protein (250 ng loaded); dai, days after infiltration; WT, protein samples from uninfiltrated control *N. benthamiana* leaves. Numbers to the left indicate the sizes of the protein markers in Kilodaltons. Double-headed arrow indicates dimeric form of IL-37.

(B) Quantification of IL-37:6xHis protein expression by ELISA. The amount of recombinant IL-37:6xHis protein in total protein extract prepared from leave samples of *N. benthamiana* harvested at day 4 post agro-infiltration was assessed by IL-37 specific ELISA as described in Section 2.9. The level of IL-37:6xHis is expressed as percentage of IL-37 in TSP.
3.5 Expression and accumulation of SBA:linker:TEV:IL-37 fusion protein in stably transformed plants

To examine the production of SBA:linker:TEV:IL-37 fusion protein using a stable expression system, low-nicotine and low-alkaloid *N. tabacum* cultivar 81V9 was transformed with *A. tumefaciens* strain LBA4404 harbouring pBI101.1-SBA:linker:TEV:IL-37 using a standard leaf-disc co-cultivation procedure (described in Section 2.7). More than 20 kanamycin-resistant putative transgenic tobacco lines were generated. There were no observable changes in morphology when compared to untransformed 81V9 control plants.

The production of SBA:linker:TEV:IL-37 fusion protein was analyzed by Western blot analysis using rabbit anti-IL-37 polyclonal antibody. As shown in Figure 10, multiple peptides were shown to react with the rabbit anti-IL-37 antibody. The protein band with a molecular weight of approximately 50 kDa, corresponds to the size of monomeric form of SBA:linker:TEV:IL-37 fusion protein. The protein bands of larger than 50 kDa likely represent different multimeric forms of the fusion protein, given that SBA is a tetrameric protein. Under identical conditions, no protein bands of similar sizes were detected from TSP prepared from untransformed wild-type tobacco plants (81V9) by Western blotting (Figure 10). Two additional protein bands with molecular weights of smaller than 50 kDa that were also reactive to anti-IL-37 antibody are likely the degradation products of SBA:linker:TEV:IL-37 fusion protein that occurred during sample preparation. The accumulation level of the fusion protein in transgenic tobacco lines T1 and T2 was quantified by both IL-37-specific ELISA and anti-SBA ELISA. ELISA results were shown in Figure 11 A and B. There is a discrepancy between the two
ELISA results, and this may reflect the difference in binding affinity between the anti-IL-37 antibody and the anti-SBA antibody towards SBA-IL-37 fusion protein.
Figure 10: Detection of SBA:linker:TEV:IL-37 fusion protein expression in transgenic tobacco plants by Western blot analysis.

Total soluble protein was extracted from the leaves of transgenic plants transformed with pBI101.1- SBA-linker:TEV:IL-37. Proteins were separated by 10% SDS-PAGE then transferred onto a PVDF membrane and probed with rabbit anti-IL-37 primary antibody followed by swine anti-rabbit secondary antibody. Approximately 25 µg of TSP were loaded for each transgenic line. +, commercial recombinant IL-37 protein used as a positive control (250 ng loaded); M, marker; WT, untransformed wild type 81V9 tobacco plant used as a negative control; transgenic lines T1-T12, representative independent transgenic tobacco lines. Numbers to the left indicate the sizes of the protein markers in Kilodaltons. Double-headed arrow indicates different multimeric forms of SBA:linker:TEV:IL-37; single-headed arrow indicates monomeric form of SBA:linker:TEV:IL-37; star indicates putative degradation products of SBA:linker:TEV:IL-37.
Figure 11: Quantification of SBA:linker:TEV:IL-37 fusion protein expression in transgenic tobacco plants by ELISA

(A) Quantification of SBA:linker:TEV:IL-37 fusion protein expression by IL-37 specific ELISA. Total protein samples from transgenic tobacco lines T1 and T4 were measured by IL-37 specific ELISA to determine the level of expression of SBA:linker:TEV:IL-37 fusion protein. The level of IL-37:6xHis is expressed as percentage of IL-37 in TSP.

(B) Quantification of SBA:linker:TEV:IL-37 fusion protein expression by SBA specific ELISA. Total protein samples from transgenic tobacco lines T1 and T4 were measured by IL-37 specific ELISA to determine the level of expression of SBA:linker:TEV:IL-37 fusion protein. The level of IL-37:6xHis is expressed as percentage of IL-37 in TSP.

(C) A representative standard curve for SBA. It was constructed from OD values obtained from a serial dilution of known quantities of commercial SBA.
3.6 Purification of His-tagged IL-37 protein (IL-37:6xHis)

Plant-made His-tagged IL-37 (IL-37:6xHis) was partially purified by affinity chromatography using a HiTrap Chelating HP column (as described in Section 2.10). Infiltrated leaves of *N. benthamiana* collected at 4 dai were used for total protein extraction, as they showed higher levels of IL-37:6xHis accumulation (Figure 9). The bound IL-37:6xHis protein in the HiTrap column was eluted with elution buffer and collected in 1-ml fractions. The presence of purified IL-37:6xHis protein in eluted fractions was confirmed by Western blot using anti-IL-37 polyclonal antibody. As shown in Figure 12A, Western blot analysis revealed the presence of purified plant-made IL-37:6xHis protein in eluted fractions, with the first (E1) and second (E2) eluted fractions containing the highest concentration of IL-37:6xHis. The identity of purified IL-37:6xHis protein was further confirmed by Coomassie blue staining of SDS-PAGE gel (Figure 12B) followed by Western blotting (Figure 12C).

3.7 Purification of SBA:linker:TEV:IL-37 fusion protein

Plant-made SBA:linker:TEV:IL-37 fusion protein was purified by affinity chromatography using an *N*-acetyl-D-galactosamine-agarose column (as described in Section 2.11). The fusion protein was purified from leaves of stable transgenic plants. The bound SBA:linker:TEV:IL-37 in the *N*-acetyl-D-galactosamine-agarose column was eluted with elution buffer and collected in 1.5-ml fractions. The presence of purified SBA:linker:TEV:IL-37 fusion protein in eluted fractions was confirmed by Coomassie blue staining of SDS-PAGE gel (Figure 13).
**Figure 12: Confirmation of IL-37:6xHis protein purification**

(A) The presence of IL-37:6xHis protein in the eluted fractions was confirmed by Western blot using anti-IL-37 antibody. TSP, total soluble protein; F, the flowthrough; E1 to E3, different eluted fractions.

(B) The identity of purified IL-37:6xHis protein was further confirmed by Coomassie blue staining of SDS-PAGE gel. Different amounts of eluted protein were loaded into each well (1µg, 2µg, 4µg and 8µg).

(C) Western blotting analysis of purified IL-37:6xHis from the above Coomassie-stained SDS-PAGE gel. Proteins were transferred from the Coomassie-stained SDS-PAGE gel to a PVDF membrane and probed with anti-hIL-37 antibody. M, marker. Numerical values on the left refer to the size in Kilodaltons of the protein marker. Double-headed arrow indicates dimeric form; single-headed arrow indicates monomeric form.
Isolation of SBA:linker:TEV:IL-37 from SBA:linker:TEV:IL-37 containing extract using $N$-acetyl-D-galactosamine-agarose column was described in detail in Section 2.11. The presence of SBA:linker:TEV:IL-37 protein in the eluted fractions was confirmed by staining SDS-PAGE gel with Coomassie blue. TSP, total soluble protein; F, flow-through fraction; W, wash fraction; E1-E6, eluted fractions. Numerical values on the left refer to the size in Kilodaltons of the protein marker.
3.8 Plant-derived IL-37 and SBA-IL-37 are biologically active

IL-37 is a strong anti-inflammatory cytokine that is capable of inhibiting the secretion of many inflammatory cytokines such as TNF-α by various cell types (Nold et al. 2010). To determine the biological activity of plant-derived IL-37 and its derivatives, we developed a cell-based assay using mouse kidney primary cells (as described in detail in Section 2.12). To perform the assay, plant-derived IL-37:6xHis and SBA:linker:TEV:IL-37 proteins were purified as described in Sections 2.10 and 2.11, respectively. Purified IL-37:6xHis or SBA:linker:TEV:IL-37 was then added to the culture of mouse kidney primary cells. Commercial recombinant IL-37 was used as a positive control, while medium alone was used as a negative control. After 24 h of co-incubation and removal of the supernatant, cells were treated with LPS for 24 h. Supernatants were then collected and assayed for the concentration of TNF-α using a commercial TNF-α specific ELISA kit. As shown in Figure 14, LPS, a key inflammatory component of gram-negative bacteria, stimulated a significant amount of TNFα release from mouse primary kidney cells. Treatment with plant-made IL-37, SBA-IL-37 or commercial IL-37 significantly (P<0.05) reduced the production of TNFα in a dose-dependent manner. These results suggest that plant-derived IL-37 is biologically active. Compared to commercial IL-37, SBA-IL-37 fusion protein appears to be more effective at inhibiting LPS-induced TNFα production, probably due to increased stability of IL-37 when fused to SBA. Partially purified IL-37:6xHis showed some toxicity in mouse primary kidney cells probably due to contamination with plant-endogenous proteins.
Figure 14: Effect of plant-made IL-37 and SBAIL-37 fusion protein on inhibition of the production of the inflammatory cytokine TNFα from mouse primary kidney cells.

Primary kidney cells isolated from mouse kidney were grown in complete medium K1 for 7-9 days until they had 80% confluence. The cells were then treated with different concentrations of prIL-37:6xHis, prSBA-IL-37 or commercial IL-37 for 24 h. After removal of the supernatant, cells were treated with 1 μg/ml LPS for 24 h. Culture medium was then collected and analyzed for the levels of TNF-α via a TNF-α specific ELISA kit. Med, medium only; Re-IL-37, commercial recombinant IL-37; prIL-37-His, plant-derived IL-37:6xHis protein; prSBA-IL-37, plant-derived SBA:linker:TEV:IL-37 fusion protein.
Chapter 4

Discussion

IL-37 is a cytokine with a broad range of anti-inflammatory activities, making it an attractive drug candidate for the treatment of several inflammation diseases by inhibition of pro-inflammatory cytokines (Horan et al., 2013). Currently, only \textit{E. coli}-derived recombinant human IL-37 is commercially available, but it is prohibitively expensive for use in clinical practice. The major objective of this study was to develop an alternative method for large-scale, cost-effective production of human IL-37 based on the use of plants as an expression system. To this end, three plant transformation vectors were constructed, which express IL-37 alone, or in a fusion form with either the 6xHis or SBA tags. The expression of these IL-37 vectors was tested in tobacco plants using transient or and stable expression systems.

4.1 Major research findings

Three different forms of IL-37 were expressed in tobacco plants, including recombinant native IL-37, hIL-37 in a His-tagged form (IL-37:6xHis) and hIL-37 in a fusion form with soybean agglutinin (SBA:linker:TEV:IL-37).
Biological activity of the plant-derived IL-37:6xHis and SBA:linker:TEV:IL-37 was demonstrated by their ability to inhibit LPS-induced TNFα production from mouse kidney primary cells in vitro.

An analysis of the expression of IL-37 as a fusion protein with SBA (SBA:linker:TEV:IL-37) (Figure 3) was included in an attempt to increase the expression level of human IL-37, as well as to facilitate downstream protein purification. Soybean agglutinin (SBA) is a tetrameric lectin glycoprotein found in soybean seeds. SBA binds to N-acetyl-D-galactosamine and is able to induce the agglutination of cells with this glycan on their surface. SBA accumulates to nearly 2% of the soluble protein in soybean seeds and can be isolated from soybean flour with more than 90% yield following one-step affinity purification on beads bearing N-acetyl-D-galactosamine (Percin et al. 2009). Therefore, SBA has great potential for exploration as a novel fusion tag not only to increase the expression of plant-made proteins but also to allow their one-step affinity purification.

An analysis of the expression of a His-tagged form of IL-37 (IL-37:6xHis) was also included in an attempt to facilitate recombinant protein purification. The purification of His-tagged proteins is based on the use of a chelated metal ion as an affinity ligand. One commonly used ion is the immobilized nickel-nitrilotriacetic acid chelate [Ni–NTA], which is bound by the imidazole side chain of histidine.
4.2 Analysis of IL-37 expression in plants

The expression of IL-37 protein in plants was determined by immunoassays such as Western blot and ELISA. Western blot analysis of total extract from infiltrated leaves of *N. benthamiana* with *Agrobacterium* containing pBI101-IL-37 or pBI101-IL-37:6xHis demonstrated that plant-derived recombinant native IL-37 or IL-37:6xHis is present mainly in a dimeric form (Figures 6 and 9), although IL-37:6xHis expressed in stable transgenic tobacco plants was found to be the presence of both monomeric and dimeric forms (Figure 8). While IL-37 is a single polypeptide, it can form homodimers in solution (Kumar et al., 2002) and this also occurs under physiological conditions in LPS stimulated peripheral blood mononuclear cells (PBMC) (Nold et al., 2010).

Western blot analysis of extracts from leaves of transgenic tobacco plants transformed with *Agrobacterium* containing pBI-SBA:linker:TEV:IL-37 (Figure 10) revealed the presence of multiple bands specifically reactive to anti-IL-37 antibody. This is not surprising because structurally SBA is formed as a tetrameric protein. The band with a molecular weight of approximately 50 kDa may represent the monomeric form of SBA:linker:TEV:IL-37 fusion protein, while other bands with molecular weights between 75 kDa and 250 kDa may represent different multimeric forms of SBA:linker:TEV:IL-37 fusion protein, such as dimer, trimer and tetramer.
4.3 Factors contributing to relative high-level expression of IL-37 in plants

Data gained from ELISA analysis indicated that IL-37:6xHis protein accumulated up to 0.45% of TSP in stable transgenic plants, although lower expression levels of IL-37:6xHis were achieved in *N. benthamiana* when used as a transient expression system. Moreover, when expressed as a fusion protein with SBA in stable transgenic plants, the expression level of IL-37 was found to be substantially increased, accounting for more than 2.5% of TSP as measured by SBA specific ELISA (Figure 11). These expression levels are very encouraging, suggesting a great potential for the use of plants as an alternative system for production of human IL-37. Cytokines are generally unstable proteins, and it is usually difficult to achieve high levels of expression when a recombinant form of a cytokine is expressed. For example, Ma et al. (2005) expressed human IL-4 in both tobacco and potato plants and showed that the expression level of IL-4 was lower than 100 µg per g of fresh weight in transgenic tobacco leaves. Menassa et al. (2001) showed the expression of human IL-10 in transgenic tobacco plant, but with a low-level of protein accumulation. The relatively high level of accumulation of rhIL-37 achieved in stable transgenic tobacco plants in this study may result from several factors acting cooperatively; one factor could be the use of a robust CaMV 35S promoter coupled with a viral untranslated leader sequence (5’UTL). The 35S promoter is one of the most widely used, general-purpose constitutive promoters and is a very strong promoter, which regulates the expression of a transgene causing high levels of gene expression in dicot plants. Equally important, the viral untranslated leader sequence (5’UTL) enhances transgene’s translation efficiency (Carrington & Freed, 1990). The other factor that may contribute to this relatively high level of IL-37 expression in
transgenic tobacco plant is that IL-37 is a relatively stable protein compared to other cytokines such as IL-4 and IL-10 because it shares a similar β-barrel structure with other members of the IL-1 family (Botaschi et al., 2011). β-barrels are known to form very stable secondary or tertiary structures (or fold) of a protein (Wimley, 2003). Results from the expression and purification of IL-37 as a fusion with SBA suggested that SBA has promising potential for use as a new fusion partner not only to increase foreign protein expression in plant cells but also to facilitate downstream processing of proteins.

There is a discrepancy regarding the expression level of SBA:linker:TEV:IL-37 when measured by both SBA-specific ELISA and IL-37-specific ELISA. While results from SBA-specific ELISA showed that the levels of SBA:linker:TEV:IL-37 expression in transgenic lines T1 and T4 reached as high as 2.7 % and 1.5% of TSP respectively, the result from IL-37 specific ELISA showed that the fusion protein expression levels in the same T1 and T4 transgenic lines accounted for 0.5 and 0.2% TSP (Figure 11). This difference may reflect a reduction in binding affinity of the anti-IL-37 antibody to the IL-37 moiety when it is present as a fusion protein with SBA compared to its binding affinity when IL-37 is in its native form. The difference in binding affinity may be a result of the structural differences between the native IL-37 protein and the fusion protein.
4.4 Biological activity of plant-derived IL-37

IL-37 is a recently discovered and potent anti-inflammatory cytokine with broad therapeutic and clinical potential. Over-expressed human IL-37 has been shown to suppress the TLR (toll-like receptor)-induced pro-inflammatory cytokines such as IL-1β, TNF-α, IFN-γ and IL-18 in a mouse macrophage RAW cell line, in human monocyte cell line THP-1 and in alveolar epithelial A549 cells (Akdis et al., 2011; Nold et al., 2010). Biological activity of plant-derived IL-37 protein was demonstrated by its ability to inhibit the secretion of pro-inflammatory cytokines from isolated mouse kidney primary cells in vitro. A previous study by Wang et al. (2004) indicated that kidney tubule cells can produce a variety of proinflammatory cytokines such as TNF-α when stimulated with LPS. We therefore determined the biological activity of plant-derived IL-37 by testing whether or not it can inhibit LPS-induced proinflammatory cytokine production in primary mouse renal cells. IL-37:6xHis and SBA:linker:TEV:IL-37 protein used for testing were purified. ELISA data showed that mouse renal cells treated with plant-derived IL-37:6xHis or SBA:linker:TEV:IL-37 had a reduction in the production of TNF-α upon LPS stimulation compared to non-treated control cells (Figure 14). As the inhibitory effect of plant-derived rhIL-37:6xHis is comparable to that of recombinant standard IL-37, the result suggests that the His tag added to the C-terminus of plant-derived IL-37 did not exert a negative effect on the biological function of plant-derived rhIL-37. On the other hand, when compared to standard IL-37 or plant-derived rhIL-37:6xHis, SBA-IL-37 fusion protein appears to be more effective at inhibiting LPS-induced TNFα production. This may be probably due to increased stability of IL-37 when fused to SBA.
4.5 Future directions

The use of tobacco as a production platform for IL-37 was assessed and the results are very promising. Nevertheless, there is still room to further increase the expression levels of IL-37 in plants. One potential strategy for improving IL-37 yield in plants is through chloroplast transformation. Recent data suggests that use of transgenic chloroplasts as bioreactors offers certain advantages over nuclear transformed plants. These include high-level protein accumulation due to increased foreign gene content in chloroplasts (up to 10,000 copies/leaf cell in tobacco), expression of multiple genes through a single transformation event and increased transgene containment because of maternal plastid inheritance, as well as a lack of position effects on foreign genes (Daniell, 2006).

Expression of IL-37 as a fusion protein with human transferrin in plants for oral delivery of IL-37 should be considered as another further research direction. The oral route for drug delivery is the most preferred route and has considerable advantages: requiring neither sterile needles nor trained personnel, lower cost, increased access to a large population and greater patient compliance and acceptability (Ma et al., 2008). However, administration of therapeutic peptide or protein drugs by the oral route is a major challenge. Orally administered peptide or protein drugs are readily degraded because of their exposure to the harsh environment of the human gastrointestinal tract (GI tract) (low pH and various proteinases and peptidases). Transferrin (Tf) is an abundant, naturally occurring serum protein with the capacity to bind and transport iron to cells through Tf receptor-mediated endocytosis. Tf receptor (TfR) is present on the surface of most proliferating higher eukaryotic cells. TfRs are also highly expressed in human GI
epithelial cells. Furthermore, Tf is stable and resistant to proteolytic enzymes, which leads to a tremendously long plasma half-life (14–17 days) (Brandsma et al., 2010; Li and Qian, 2002; Melanie, 2005). These properties of human Tf make it a very valuable tool in developing Tf-based novel fusion protein technology to enhance protein expression, extend the serum half-lives of protein/peptide drugs and to achieve active targeted (smart) drug delivery. Being an endogenous protein, Tf as a drug delivery system is non-immunogenic and nontoxic in humans (Jiang et al., 2007). Choi et al. (2013) have recently demonstrated that plant-derived exendin-4, an anti-diabetic peptide, fused to Tf can be effectively delivered orally for the treatment of type 2 diabetes in animal models, while oral delivery of exendin-4 alone had no effect.
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CURRICULUM VITAE

NAME  Nadiyah Alqazlan

POST-SECONDARY EDUCATION

- 2012-2014  Master of Science in Biology
  University of Western Ontario, London, Ontario, Canada
- 2010-2011  Certificate of English Proficiency
  University of Guelph, Guelph, Ontario, Canada
- 2007-2009  Diploma in Computer Science
  Cultural Center for Women, Riyadh, Saudi Arabia
- 2001-2005  Honors Bachelor of Science
  Princess Nora University, Riyadh, Saudi Arabia

AWARDS

- 2010-2014  King Abdullah Scholarships Program
  Ministry of Higher Education, Riyadh, Saudi Arabia

RELATED EXPERIENCE

- 2012  Teaching Assistant
  University of Western Ontario