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TRANSGENIC PLANTS AS A NOVEL BIOREACTOR TO PRODUCE ANTIDIABETIC PROTEINS FOR THE TREATMENT OF TYPE 2 DIABETES

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TRANSGENIC PLANTS AS A NOVEL BIOREACTOR TO PRODUCE ANTIDIABETIC PROTEINS FOR THE TREATMENT OF TYPE 2 DIABETES

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) and exendin-4 (Ex-4) are small peptides with potent insulin secretory effect which makes them attractive drug candidates for the treatment of Type 2 diabetes mellitus (T2DM). However, the synthesis of these small peptides is difficult due to recombinant protein degradation and instability. Human serum transferrin (hTf) is an iron-transport protein that has great potential as an efficient carrier system for protein-based drugs. The use of hTf as a fusion partner will provide a new strategy to enhance the therapeutic potential of GLP-1 and Ex-4. This project investigates the development of transgenic tobacco plants as bioreactors for generating recombinant fusion proteins, GLP-1 – hTf and Ex-4 – hTf. Here, transgenic tobacco plants have successfully accumulated recombinant fusion proteins. Plant-derived proteins showed stability in simulated gastrointestinal environment and confirmed their ability to stimulate insulin secretion from a pancreatic β-cell line *in vitro*. Lastly, hTf-fused proteins were shown to internalize into human intestinal epithelial cells *in vitro*. Collectively, the results suggest that transgenic plants are an effective expression and delivery system of recombinant anti-diabetic proteins.

Key Words: transgenic tobacco, glucagon-like peptide-1, exendin-4, oral administration, recombinant therapeutic proteins, fusion proteins, *Agrobacterium*-mediated transformation, Type 2 diabetes mellitus

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LIST OF ABBREVIATIONS

5'	Upstream of the sequence of interest
3'	Downstream of sequence of interest
%	Percent
°C	Degree Celsius
BA	Benzylaminopurine
BHK	Baby hamster kidney
bp	Base pair
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
cm	Centimetre
ddH_2O	Double distilled water
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetate
ELISA	Enayme-Linked Immunosorbent Assay
Ex-4	Exendin-4
g	Gram
G	Gavitational constant (6.67×10^{-11} $m^3/kg/s^2$)
gDNA	Genomic DNA
GI	Gastrointestinal
GLP-1	Glucagon-like peptide 1
GTE	Glucose/Tris-HCL/EDTA
h	Hour
hTf	Human transferrin
IgG	Immunoglobulin G

IMAC	Immobilized metal affinity chromatography
kb	Kilobase
kDa	KiloDalton
kg	Kilogram
kV	Kilovolt
L	Litre
LB	Luria-Bertani media
m	Metre
µg	Microgram
µl	Microlitre
µM	Micromolar
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
M	Molar
MSO	Murashige and Skoog salt
ng	Nanogram
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with Tween-20
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PMSF	Phenyl-methylsulfonyl fluoride
PNGaseF	Peptide N-glycosidase F
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
rmp	Revolutions per minute
RTP	Recombinant therapeutic protein

RT-PCR	Reverse transcriptase polymerase chain reaction
s	Seconds
SDS	Sodium dodecyl sulphate
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
siRNA	Small interfering RNA
TB	Terrific broth
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TEMED	N,N,N', N'-tetramethylethylmenediamine
TEV	Tobacco etch virus
Tris	2-amino-2-hydroxylmethyl-1, 3-propandiol
TSP	Total soluble protein
TYC	Tryptone/yeast extract/calcium chloride
U	Unit
UTL	Untranslated leader sequence
UTR	Untranslated region
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

Chapter 1

General Introduction

1.1 Expression systems for production of recombinant proteins of pharmaceutical interest

To date, a number of biological expression systems such as bacteria, yeast, and mammalian cells have been widely available for the production of recombinant pharmaceutical proteins. Recombinant proteins are generally synthesized by large-scale cultivation of genetically engineered "host" cells, which harbour artificially transfected genes encoding proteins of interest (Manuel et al., 2007). For protein therapeutics to be effective, they must be synthesized in a biologically active form, which requires proper folding and appropriate post-translational modifications (Sethuraman and Stadheim, 2006). Conventional systems, including bacteria, yeast, and mammalian cell cultures, have advantages and disadvantages for production of recombinant proteins.

Bacteria such as *Escherichia coli* have been the most common host for therapeutic protein production. Bacterial systems provide relatively high expression level of recombinant protein as well as the potential for large-scale production (Skerra, 1993). However, all bacteria are prokaryotic organisms and cannot perform post-translational modifications that regulate protein folding and biological activity

of eukaryotic proteins, which makes bacteria not suitable for expressing complex proteins such as antibodies and therapeutics (Georgiou and Valax, 1996; Balbas, 2001).

Unlike the bacterial systems, yeast, a single-cell eukaryotic organism, harbours the machinery able to perform some of the post-translational modifications and provides easy manipulation and cultivation. However, the ability of yeast to perform post-translational modification of heterologous proteins is limited (Hamilton et al., 2003; Wildt and Gerngross, 2005). Moreover, yeast-derived proteins are often hyper-glycosylated due to the repeated addition of mannose at N-linked sites which can hinder biological activity of proteins (MacKays, 1987; Kniskern et al., 1994).

Mammalian cell cultures offer a better choice as they can produce recombinant proteins with accurate post-translational modifications. Despite this, mammalian cell cultures are limited by their production cost due to expensive media and subsequent purification methods, thereby impeding the economic and commercial value of recombinant proteins for therapeutic use. The scale-up is low and often not feasible, and protein yields are lower than in any other systems. In addition, there is a possibility of viral contamination, and if the host is human cell-derived or the virus is capable of cross-species infection, serious complications can arise (Kojima et al., 1996; O'Callaghan and James, 2008)

Recently, there has been a significant increase in the demand of protein-based therapeutics such as antibodies, growth factors and insulin. Novel production strategies must be implemented in order to meet the ever increasing demand for recombinant therapeutic market, while allowing cost-effective large-scale production.

1.1.1 Plant expression system for recombinant proteins

Major advances have been made over the last two decades to improve the expression and accumulation of recombinant proteins in plant systems. The recombinant production of pharmaceuticals, functional proteins, industrial enzymes and functional secondary metabolites in plants is referred to as "plant molecular farming" (PMF) (Ma et al., 2005). The first reported pharmaceutical protein made in plants was tobacco callus expressing human growth hormone (Barta et al., 1986). To date, several proteins of medical interest have been expressed successfully in plants, including a variety of antibodies, vaccine antigens, protein allergens, enzymes, cytokines and hormones (Hiatt et al., 1989; Mason et al., 1992; Ma et al., 1995, 2003, 2005; Kirk and Webb, 2005; Floss et al., 2007). As our understanding of the factors that impact transgene expression in plants improves, we will see enhancement in recombinant protein production and greater overall exploitation of plant-based expression systems.

1.1.2 Advantages of plant expression systems

Plants have advantages compared with traditional systems for molecular farming of pharmaceutical proteins. These include: 1) Low cost. The production of recombinant proteins in plants does not require the costly equipment or media that bacteria or mammalian cell cultures demand. Essentially, plants can be grown in the field requiring only water and sunlight. Furthermore, protein isolation and purification may not even be necessary if the recombinant proteins are produced in edible plant tissues and intended to be used for oral consumption (Barta et al., 1986;

de Smit et al., 1995; Mason et al., 1998; Tacket et al., 1998). 2) Easy scale-up. Transgenic plants have a high production capacity with nearly unlimited scalability. Industrial level production can be achieved by increasing acreage. 3) Ability to perform post-translational modifications required to assemble functional recombinant proteins, in a similar fashion to a mammalian cell (Gomord and Faye, 2004). 4) Safer products. Recombinant proteins produced in transgenic plants are less likely to be contaminated with human pathogenic microorganisms, such as viruses and prions, than those derived from mammalian cell, since plants do not act as hosts for human infectious agents (Giddings et al., 2000).

1.1.3 Strategies for plant transformation

There are two basic methods for expressing recombinant foreign proteins in plants. One involves the generation of transgenic plants by stable genomic integration of a transgene. The other involves the expression of transgenes in transiently transformed plants or tissues. Each approach has its advantages and disadvantages (Fisher et al., 2000). Both strategies involve a soil-borne plant pathogen known as *Agrobacterium tumefaciens*, which has been widely used for the transformation of plants such as tobacco (Horsch et al., 1985). The molecular mechanisms of *Agrobacterium*-mediated transformation are now well understood (McCullen et al., 2006; Wenck et al., 1997).

Compared to stable plant transformation, transient gene expression is rapid and relatively simple to handle, and the experiment is usually completed within a week. Furthermore, the absence of chromosomal position effects allows for better

reproducibility and reliable levels of gene expression in transient assays (Janssen et al., 1990). Transient expression allows higher protein accumulation when recombinant gene is co-expressed with p19. This viral p19 protein suppresses the post-transcriptional gene silencing in plant systems (Voinnet et al., 2003). Transient gene expression, however, is limited in scale and is neither permanent nor heritable. In contrast, stably transformed plants allow for the permanent and heritable expression of a particular gene, although generating stably transformed plants requires a longer scale-up time. With these advances in plant expression systems, the development of protein drugs for human disease, such as diabetes, is gaining an increased interest.

1.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a progressive and complex disorder that is characterized by insulin deficiency and insulin resistance (Bethel and Feinglos, 2005). Most conventional anti-diabetic agents, including sulfonylureas, thiazolidinediones, and insulin, improve glycaemic control, but these are associated with pancreatic β-cell reduction, cardiovascular problems and undesired weight gain (Holst, 1997). New treatment options are needed for patients to gain glycaemic control and to reduce the risk of serious health complications.

1.3 Glucagon-like peptide-1 and Exendin-4 for T2DM treatment

Glucagon-like peptide-1 (GLP-1) is a major incretin hormone, a potent blood glucose-lowering hormone, secreted by intestinal L cells in the small intestine and

colon (Holst, 2007). GLP-1 is a small peptide (31 amino acids) and is released in response to food ingestions, particularly to intake of carbohydrates and fats (Holst, 2007). Secreted GLP-1 promotes insulin production from pancreatic β-cells in a glucose-dependent manner, which reduces the risk of hypoglycaemia (Holst, 2007). Besides its insulinotropic activities, GLP-1 has been shown to inhibit glucose-dependent glucagon secretion, delay gastric emptying and decrease food intake, resulting in weight loss (Turton et al., 1996; Flint et al., 1998; Drucker, 2003). Furthermore, GLP-1 improves β-cell function by promoting β-cell proliferation and inhibiting apoptosis (Gromada et al., 2004; McIntosh et al., 2005). However, GLP-1 released into the blood circulation is rapidly cleaved by dipeptidyl-peptidase-IV (DDP-IV) into smaller peptides causing its inactivation (Orskov et al., 1993). The rapid degradation of GLP-1 shortens its biological half-life to less than 2 minutes (Schepp et al., 1994), prompting the search for analogs with longer durations of action.

Exendin-4 (Ex-4) is a 39 amino acid peptide isolated from the salivary glands of Gila monster (*Heloderma suspectum*) (Eng et al., 1992). It is a functional homolog of GLP-1 (53% amino acid identity) and it is reported to show agonistic activity to GLP-1 receptors (GLP-1R) (Fehmann et al., 1994; Deacon et al., 1995; Kieffer et al., 1995; Nielsen et al., 2004). It has all the same therapeutic benefits as GLP-1 and more importantly, it is resistant to DPP-IV cleavage that allows for a longer biological half-life than GLP-1 (Deacon et al., 1995; Kieffer et al., 1995). Exendin-4 showed a glucose-lowering action that endured for several hours in a diabetic mouse model (Martin et al., 2009). In addition, Exenatide (a synthetic form

of exendin-4) improved glycemic control, reduced cardiovascular risk factors, and allowed weight loss in T2DM patients in a 3-year clinical study (Nielsen et al., 2004). The therapeutic advantages of exendin-4 and GLP-1 offer a great potential for the treatment of T2DM patients.

1.4 Human Transferrin as a molecular carrier

Much research has focused on identifying and characterizing molecular carriers that have the potential to transport therapeutic agents to specific target cells. Human transferrin (hTf) is a 80kDa monomeric glycoprotein consisting of approximately 679 amino acids (Gomme and McCan, 2005). The molecule contains functionally related N-terminal and C-terminal domains that serve as binding sites for iron (Fe^{3+}) (Qian et al., 2002). Human Tf is synthesized predominantly in the liver by hepatocytes. It is readily found in various body fluids including plasma, bile, amniotic, cerebrospinal and lymph fluids (Li and Qian, 2002). As a major iron transport protein, hTf plays a vital role in human physiology. It is involved in iron uptake and regulation, cellular growth and differentiation, and is also involved in cytokine production and antimicrobial activity (Gomme and McCann, 2005; Park et al., 2009). It regulates iron concentration in many metabolic processes and prevents free iron from reaching toxic levels in the body which can lead to free radical formation and lipid peroxidation (Li and Qian, 2002). Studies have reported hTf's efficiency in iron transportation to different cell types possessing surface transferrin receptors (TfR) (Li and Qian, 2002). Human Tf receptors are found on the surfaces of erythrocytes, thyroid cells, hepatocytes, intestinal cells and brain cells (Padron et

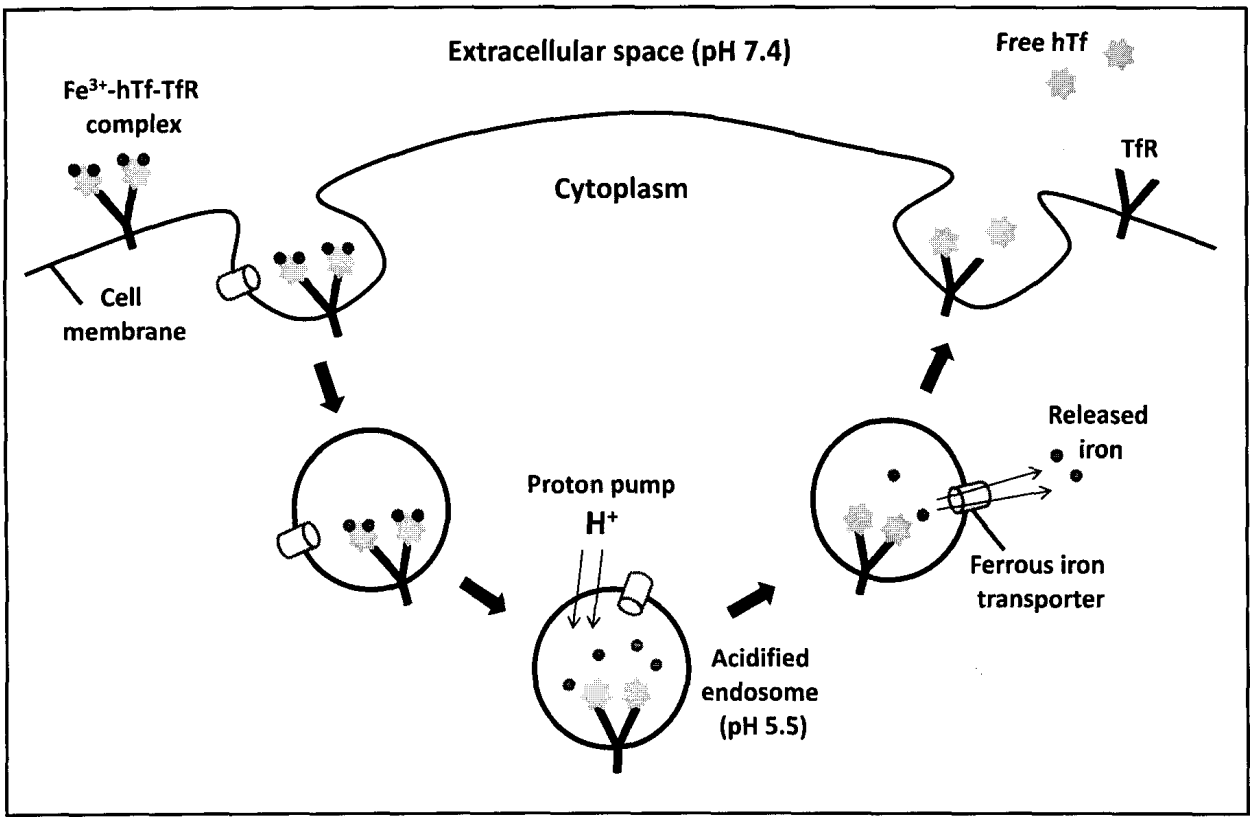
al., 2006). The cellular uptake of iron is carried out by ligand-receptor-mediated endocytosis (Figure 1; Li and Qian, 2002). The process is initiated by high-affinity binding of the hTf-Fe^{3+} complex to TfR (Morgan and Appleton, 1969). The Fe^{3+}-hTf-TfR complex enters the cell through endosome formation (Qian et al., 2002). The pH inside the matured endosome drops to about 5.5, at which point the irons are released from hTf (Klausner et al., 1983). The released irons are sent to the cytosol via a ferrous iron transporter to be used in cellular processes (Qian et al., 2002). This mechanism of iron delivery has attracted increased interest from the field of therapeutic drug delivery because it shows potential for engineering hTf to introduce drugs or therapeutic proteins to specific target cells.

Besides its efficient transport mechanism, hTf presents many advantages as a molecular carrier. Aside from iron, hTf can also bind to other metal ions such as bismuth (Bi) and gallium (Ga) which are used in therapeutic and diagnostic procedures, respectively (Li and Qian, 2002). Furthermore, because hTf is a naturally-occurring protein in humans, it does not induce immunogenic responses and is virtually nontoxic (Jiang et al., 2007). All these features make hTf a very suitable carrier and have led to the development of hTf conjugates as a drug delivery system in anticancer therapies (Kratz et al., 1998; Jiang et al., 2007).

With the aim of producing an efficient drug delivery system, hTf conjugates have been created by attaching chemical linkers (Qian et al., 2002). For example, doxorubicin, an antineoplasmic agent, was conjugated to hTf by forming a stable amide linkage (Kratz et al., 1998). Likewise, tumour necrosis factor alpha (TNF-α), an antitumor cytokine, was linked to hTf by a polyethylene glycol (PEG) linker

Figure 1.

Receptor-mediated endocytosis of hTf. The cellular uptake of iron is carried out by hTf-TfR -mediated endocytosis. Iron-bound hsTf binds to TfR on the cell surface. The resulting complex enters the cell via endosome formation. Acidification caused by proton pump activity triggers iron release from hTf. Released iron enters the cytoplasm through a ferrous iron transporter (DMT1). Free hTf (Apo-hTf) are then released back to extracellular space to repeat the cycle (Li and Qian, 2002).


Extracellular space (pH 7.4)
Free hTf
Fe3+-hTf-TfR complex
TfR
Cytoplasm
Cell membrane
Released iron
Proton pump
H+
Ferrous iron transporter
Acidified endosome (pH 5.5)

method, where one side of the linker molecule (maleimide-PEG) is attached to TNF-α via amide bond and the other side is joined to hTf by forming a thioether bridge (Jiang et al., 2007). Results of these hTf conjugates show increased potential for specific targeting and inhibition of malignant cancer cells without causing any toxicity (Kratz et al., 1998; Jiang et al., 2007). The targeting is caused by an increased cellular uptake of conjugated drugs in response to greater-than-normal TfR expression on malignant cells, which is required for rapid cell proliferation (Kratz et al., 1998; Jiang et al., 2007). Furthermore, hTf has attracted a great deal of attention as a transport chaperone for fused therapeutics, intended for enhanced transport across blood-brain barrier (Friden and Walus, 1993; Osborn et al., 2008) or to enhance the therapeutic bioavailability upon oral delivery of protein and peptide drugs (Bai et al., 2005; Widera et al., 2004). Human Tf seems to be an ideal candidate for further development of delivering various therapeutic agents including anti-diabetic agents.

1.5 Progresses in the production of GLP-1, Ex-4 and hTf

For generation of small peptides such as GLP-1, the most commonly used method is chemical synthesis which is often expensive and results in a low yield of active peptide product (Kimmerlin and Seebach, 2005). Recombinant DNA technology has provided a wide venue of expression systems for the recombinant protein production. Recombinant GLP-1 has been produced in both yeast and *E. coli* as a multimer (fused tandem repeat of itself) with enhanced stability (Hou et al., 2007a, b). However, expensive and sophisticated fermentation facilities are required

to scale-up the production. Yasuda and colleagues (2006) reported a synthesis of rice-derived GLP-1 pentamer, but it failed to retain the original protein function. In another study, recombinant GLP-1 fused to globulin protein was generated in rice-seed (Sugita et al., 2005). However, the activity of GLP-1 was detected only when the fusion partner was cleaved by enzymatic digestion.

Synthesis of hTf has been investigated in bacteria, yeast, insect cells and mammalian cell cultures (Funk et al., 1990; Steinlein and Ikeda, 1993; de Smit et al., 1995; Ali et al., 1996; Mason et al., 2001; Sargent et al., 2006). As described in section 1.1, these systems are not suitable for large-scale production of proteins at low cost.

Previously, our lab has successfully produced recombinant GLP-1 decamer and recombinant hTf in transgenic tobacco plants (Brandsma et al., 2009, 2010). Plant-derived GLP-1 decamer was stably accumulated in tobacco and maintained its insulinotropic property, although with reduced efficacy. Recombinant hTf showed stable accumulation level in transgenic tobacco and retained many of the biological functions of native hTf.

Most recently, generation of hTf fusion proteins were explored. Recombinant GLP-1 – hTf and Ex-4 – hTf were produced in yeast (Kim et al., 2010). These fusion proteins retained the ability of GLP-1 and Ex-4 to stimulate insulin secretion both in vivo and by pancreatic islet cells in vitro. Nevertheless, the limitations of conventional bioreactors, i.e. yeast, prompt the search for alternative production source for hTf-fusion proteins.

1.6 Hypothesis and research objectives

The purpose of this thesis was to investigate a novel plant-based production strategy for the synthesis of recombinant fusion proteins, GLP-1 – hTf and Ex-4 – hTf. It is hypothesized that transgenic tobacco will be capable of independently accumulating GLP-1 – hTf and Ex-4 – hTf following subsequent transformation with plant expression vectors. Furthermore, plant-derived GLP-1 – hTf and Ex-4 – hTf are hypothesized to retain their native biological functions. The long-term goal of this project is to develop a robust plant-based system for complex anti-diabetic therapeutics which can be orally administered and effectively delivered to target cells in T2DM patients. The specific objectives of the project were:

(1) **To test the feasibility of producing GLP-1 – hTf and Ex-4 – hTf fusion proteins in tobacco plants.** To date, GLP-1 – hTf and Ex-4 – hTf fusion proteins have been expressed in yeast system but never been demonstrated to synthesize in any transgenic plant systems. With this purpose in mind, fusion gene constructs were designed for their constitutive expression. *Agrobacterium*-mediated method was used to transform tobacco plants. The resulting transgenic plants were analyzed for the presence of recombinant fusion proteins. It was expected that GLP-1 – hTf and Ex-4 – hTf could accumulate at a detectable levels in transgenic tobacco lines.

(2) **To examine the biological activities of plant-derived recombinant GLP-1 – hTf and Ex-4 – hTf fusion proteins via *in vitro* assays.** The functionalities of plant-derived GLP-1 – hTf and Ex-4 – hTf were determined by the induction of insulin secretion to test GLP-1 and Ex-4

and by cellular internalization to test hTf portion of the fusion proteins. It was expected the plant-derived proteins would retain their original biological activities.

(3) **To determine the stability of GLP-1 – hTf and Ex-4 – hTf fusion proteins in GI environment *in vitro*.** The survival of recombinant proteins is important when the proteins are meant for oral administration. Recombinant fusion proteins were subjected to simulated gastrointestinal fluids which mimic the acidic conditions of the digestive system. It was expected that the fusion proteins would remain intact after incubation with SGF (used to mimic the acidic stomach environment) and SIF (used to mimic the intestine condition).

Chapter 2

Methods and Materials

2.1 Bacterial Strains

All manipulations and propagations of plasmid DNA to build the expression cassettes were carried out using the *E. 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 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_2PO_4, 72 mM K_2HPO_4). Once expression cassettes were constructed, they were integrated into pBI101.1 binary vector, which allows for expression of the selectable marker (NPTII, Kanamycin resistance) in both *E. coli* and tobacco plants. 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 assays and 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) CaCl2) containing 1.5% (w/v) agar and rifampicin at 25 mg/ml. Liquid cultures of LAB4404 strain were grown in TYC broth with rifampicin.

The antibiotics concentrations used for selection were as follows: *E. coli* cells were selected for by resistance to carbenicillin at 100 μg/ml or kanamycin at 50 μg/ml; *A. tumefaciens* cells were selected for by 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 while shaking. The following centrifugations were done using a Sigma I-15 centrifuge. A total of 1.5 ml cell culture was transferred into an Eppendorf tube and centrifuged for 30 seconds at 12,000×g. The pellet was resuspended in 100 μl GTE buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) and incubated on ice for 5 minutes. After the 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 minutes. 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 seconds by vortexing and placed on ice for 5 minutes.

After centrifugation at 12,000×g for 5 minutes, the supernatant was collected, mixed with 600 µl isopropanol and then incubated at room temperature for 5 minutes. Plasmid DNA was precipitated by a 5 minute spin at 12,000×g 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 (ddH_2O) or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1 µl RNase (Fermentas, Glen Burnie, Maryland)

2.3 Polymerase Chain Reaction (PCR)

PCR was used to facilitate cloning of all cDNA into plasmids by incorporating the specific restriction enzyme recognition sites in the primers. This technique was also used for screening the large populations of possible tobacco transformants. All primers are outlined in Table 2. Approximately 50-100 ng of template DNA was used for each 50 µl reaction. Each reaction mixture included 10× PCR buffer, 2 M dNTP, 1 M of both forward and reverse promers, 2.5 mM $MgCl_2$ and 1 unit of DNA polymerase. High fidelity polymerase (*pfu* polymerase from Fermentas) was used in all PCR reaction except for large screening, in which Taq DNA polymerase (Fermentas) was applied in place of *pfu*. For screening purposes, controls included untransformed plant gDNA in the place of transgenic plant gDNA, or contained no template DNA or no primers to ensure that the primers or gDNA was not contaminated, respectively.

2.4 DNA Ligations

All DNA ligations were performed with the Rapid Ligation kit (Fermentas). A total of 20 µl ligation mixture contained 3 µg of insert DNA, 1 µg of backbone plasmid DNA, 4 µl of 5× reaction buffer, 1 µl T4 DNA ligase and topped up with ddH_2O. After 10 to 15 min incubation at room temperature, 3-7 µl of the mixture was transformed into chemically competent DH5α cells (Invirtogen) by heat shock or into electrocompetent DH5α cells by electroporation. The preparation of electrocompetent cells were described previously by Miller and Nickoloff (1995).

2.5 *E. coli* Transformation

Chemically competent DH5α cells were transformed with plasmid DNA by heat shock transformations. In brief, approximately 50 ng of plasmid 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 media (0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). After 1 hour incubation at 37°C with shaking at 200 rpm, 200 µl of the culture was spread onto LB agar plates with appropriate antibiotics.

Electrocompetent DH5α cells were transformed with plasmid DNA by electroporation (Miller and Nickoloff, 1995). Briefly, 1 or 2 µl ligation mixture 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 MicroPulser™ 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. The resulting cell suspension was incubated at 37°C for 1 hour, 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

PCR was used to facilitate cloning of all cDNA into plasmids by incorporating the desired restriction enzyme recognition sites in the primers. The primers used for the vector construction are listed in Table 2. The construction scheme is illustrated in Figure 2. All PCR products were subcloned into appropriate vectors and transformed into DH5α cells according to *E. coli* transformation methods described in Section 2.5.

2.6.1 Construction of GLP-1 – hTf plant expression vector

The plasmid containing *GLP-1* gene was provided by Dr. Ma's laboratory. The GLP-1 cDNA was amplified by PCR using the forward primer GLP-1-F and the reverse primer GLP-1-R (Table 1). The forward primer GLP-1-f contained *Nco*I restriction site (underline) at the start codon and it incorporated barley α-amylase signal peptide (bold) immediately upstream of the *GLP-1* sequence. The reverse primer GLP-1-R contained *Sca*I restriction site (underlined) for creating a fusion site,

Table 1. PCR Primers

Primer Name	Nucleotide sequence (5' →3')	Purpose
GLP-1-F	A**CCATGG**GGAAGAACGGCAGCCTGTGCTGCTTCTCTCTGCTGCTGCTG CTGCTTCTCGCCGGGTTGGCGTCCGGGCATTCTGAGGGAACCTTCACC	Cloning
GLP-1-R	GAC**AGTACT**TCCGCCGCCACCCGACCCACCTCCGCCCGAGCCACCGCC ACC*CCTTCCCTTCACCAACCAAGC*	Cloning
Ex-4-F	A**CCATGG**GGAAGAACGGCAGCCTGTGCTGCTTCTCTCTGCTGCTGCTG CTGCTTCTCGCCGGGTTGGCGTCCGGGCATGGTGAGGGTACCTTCACC	Cloning/Screening
Ex-4-R	AATTTCTAGA**TTA***ATGATGATGATGATGATGA*GAAGGTGGAGGA GC	Cloning
hTf-F1	*GGAGCTCCTCCACCT***GGTGGCGGTGGCTCGGGCGGAGGTGGGTCGGGT GGCGGCGGATCA**GTCCCTGATAAAACTGTGAGATGG	Cloning
hTf-F2	**GGCGGAAGTACT**GTCCCTGATAAAACTGTGAGATGG	Cloning
hTf-R	AATCTAGA**TTA***AAGCTCATCCTT*ATGATGATGATGATGATGAGGTCTACGGA AAGTGCAGGC	Cloning/Screening

flexible linker sequence (bolded) and last 21 nucleotide of *GLP-1* sequence. PCR reaction was performed under following conditions: denature at 95°C for 55 s, anneal at 65°C for 47 s and elongate at 72°C for 1 min, for a total of 35 cycles followed by a final elongation at 72°C for 10min.

The *hTf* cDNA clone was obtained from OriGene (Rockville, MD, USA). The coding region, excluding the native *hTf* signal peptide sequence, was amplified using forward primer hTf-F2 and reverse primer hTf-R. Forward primer hTf-F2 contained the last 12 nucleotides from 3' end of flexible linker sequence which has *Sca*I site (underline) for fusion with *hTf* sequence. Reverse primer hTf-R contained 3' His×6 tag (double underline), ER-retention signal KDEL (italics), stop codon (bold) and *Xba*I restriction site (underline). PCR reaction was performed under following conditions: denature at 94°C for 45 s, anneal at 55°C for 45 s and elongate at 72°C for 2 min, for a total of 35 cycles followed by a final elongation at 72°C for 10min. The amplified *GLP-1* and *hTf* sequences were blunt-end ligated into the *Sma*I site of pUC19 vector and subsequently sequenced to confirm the sequence integrity (Sequencing Facility; Robarts Research Institute, London, ON, Canada).

To create the fusion expression cassette, *GLP-1* sequence was released from pUC19 as *Nco*I/*Sca*I fragment and *hTf* sequence was released as *Sca*I/*Xba*I fragment then inserted into pRTL-2 vector using three-fragment ligation (Figure 2). The resulting expression cassette contained the enhanced constitutive cauliflower mosaic virus (CamV) 35S promoter, tobacco etch virus (TEV) 5'UTL, barley α-amylase signal peptide, GLP-1 – linker – hTf fusion sequence, a His×6 tag for protein

Figure 2.

Construction scheme of expression vector *pBI 101.1 – GLP-1 – hTf* and *pBI 101.1 – Ex-4 – hTf.* (A) The coding sequences of *GLP-1*or *Ex-4* were amplified by PCR using primers that incorporated barley α-amylase signal peptide (SP) sequence at the 5' end. The coding sequence of *hTf* was amplified by PCR using primers that incorporated linker (L) sequence. (B) Each amplified sequences were placed into pUC19 which were then cleaved and ligated into the pRTL2 vector containing promoter and terminator sequences. (C) The internal *Xba* I site was destroyed by Klenow treatment then the expression cassette was place into the intermediate pBluescript to allow proper integration of the entire cassette into final binary vector. (D) The expression cassette was ligated into the multiple cloning site in the T-DNA region of plant binary vector pBI101.1.

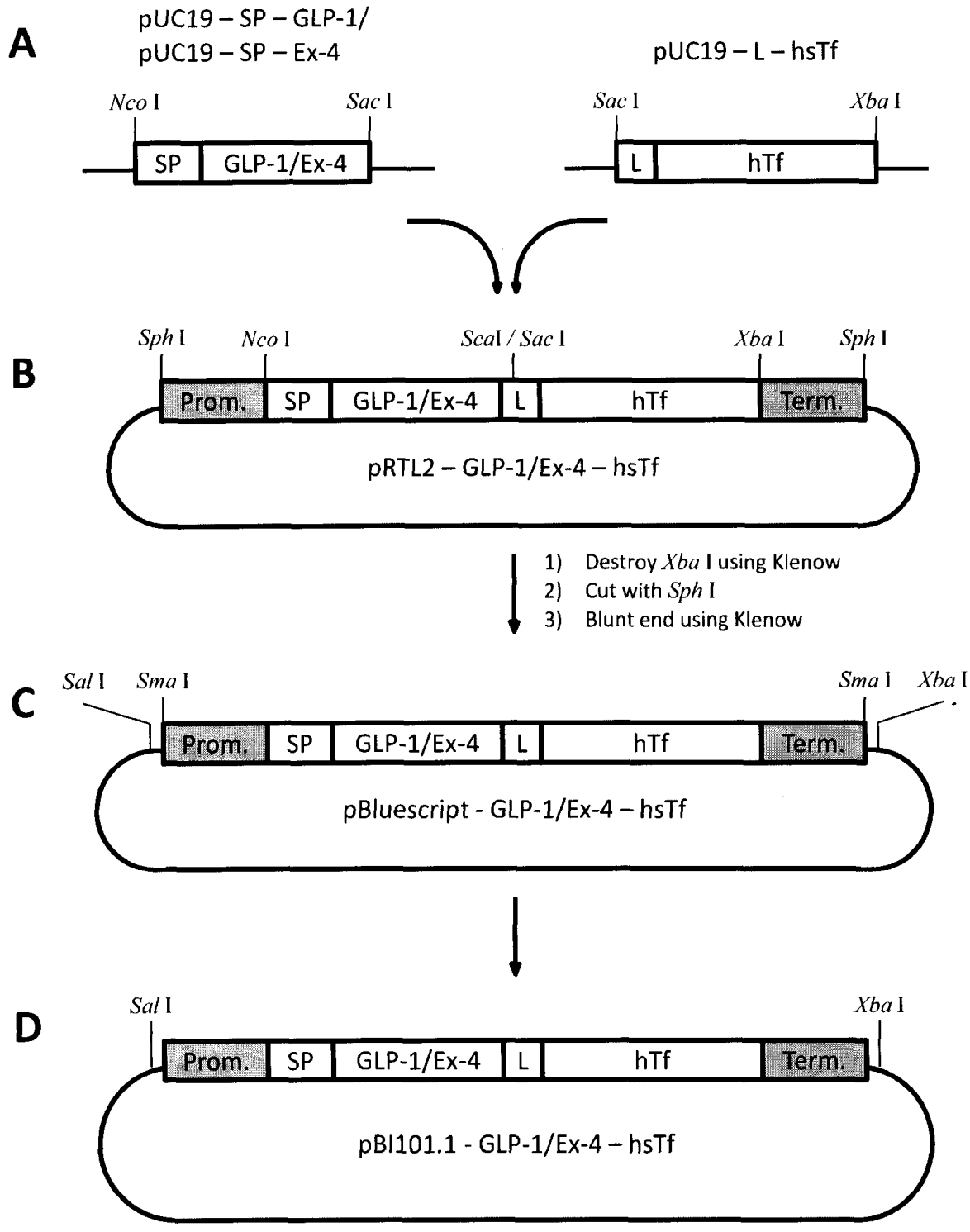
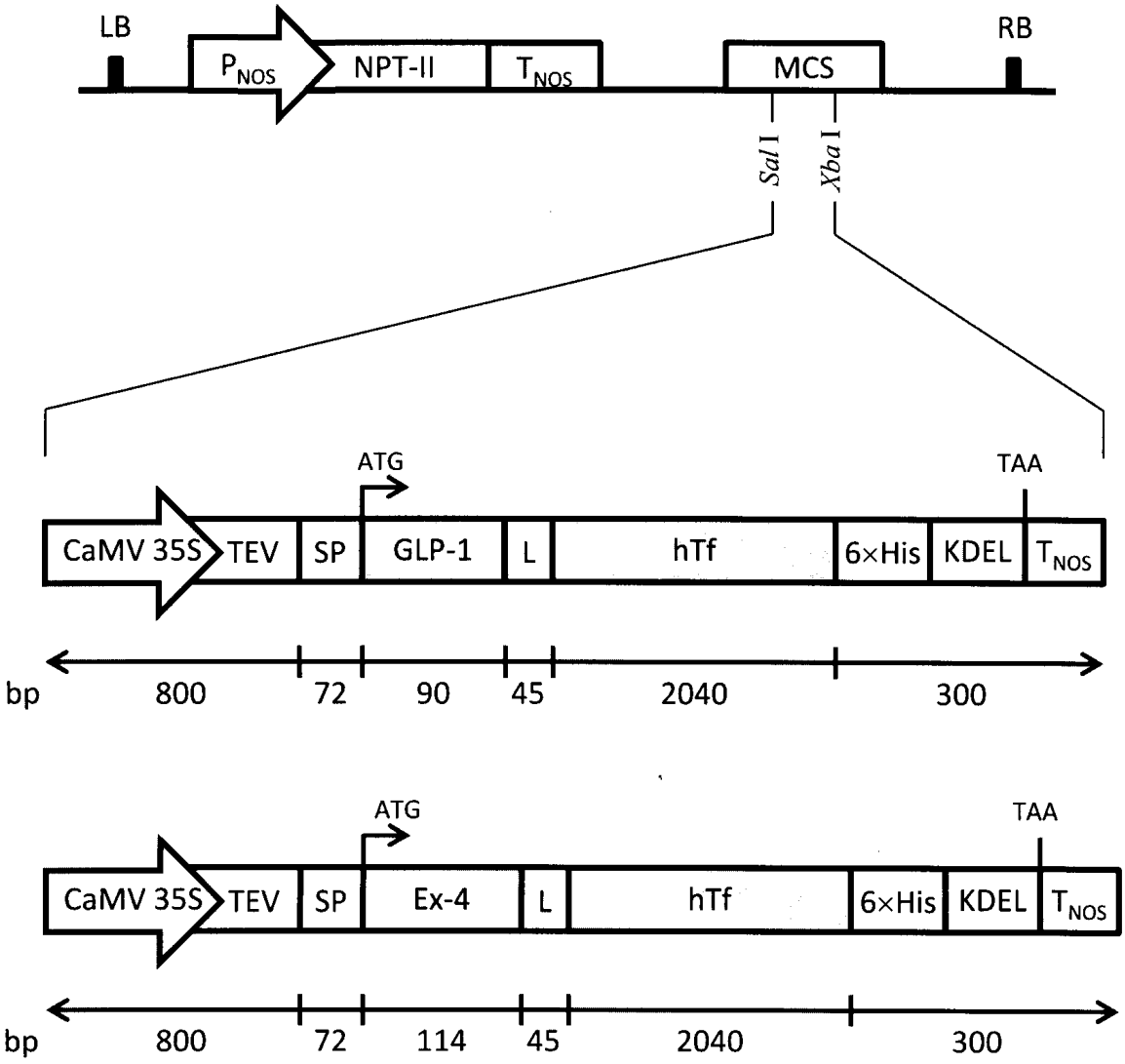

A
pUC19 – SP – GLP-1/
pUC19 – SP – Ex-4
pUC19 – L – hsTf
Nco I
Sac I
SP
GLP-1/Ex-4
Sac I
Xba I
L
hTf
B
Sph I
Nco I
ScaI / Sac I
Xba I
Sph I
Prom.
SP
GLP-1/Ex-4
L
hTf
Term.
pRTL2 – GLP-1/Ex-4 – hsTf
1) Destroy Xba I using Klenow
2) Cut with Sph I
3) Blunt end using Klenow
C
Sal I
Sma I
Sma I
Xba I
Prom.
SP
GLP-1/Ex-4
L
hTf
Term.
pBluescript - GLP-1/Ex-4 – hsTf
D
Sal I
Xba I
Prom.
SP
GLP-1/Ex-4
L
hTf
Term.
pBI101.1 - GLP-1/Ex-4 – hsTf

Figure 3.

Schematic diagram of plant expression vector. The T-DNA region inserted into the plant genome contains the nopaline synthase (NPT-II) expression cassette, which confers kanamycin resistance on transformed cells. The vector contains NPT-II promoter (P_{NOS}), NPT-II terminator (T_{NOS}), left border (LB) and right border (RB). The transgene was driven by Cauliflower Mosaic Virus 35S promoter (CaMV 35S) with a double enhancer sequence. The expression cassette also includes tobacco etch virus leader sequence (TEV), barley α-amylase signal peptide (SP), linker sequence (L), 6×His tag and ER retention signal (KDEL).


LB
P NOS
NPT-II
T NOS
MCS
RB
Sal I
Xba I
ATG
TAA
CaMV 35S
TEV
SP
GLP-1
L
hTf
6×His
KDEL
T NOS
bp
800
72
90
45
2040
300
ATG
TAA
CaMV 35S
TEV
SP
Ex-4
L
hTf
6×His
KDEL
T NOS
bp
800
72
114
45
2040
300

purification, KDEL signal for ER retention and the nopaline synthase (NOS) terminator (Figure 3). The *Xba*I site in pRTL-2 – GLP-1 – hTf was destroyed by Klenow treatment for fusion purpose. This vector was digested with *Sph*I then treated with Klenow to produce a blunt-ended fragment, which was ligated into pBluescript at *Sma*I site. The entire expression cassette was released from pBluscript by using *Sal*I and *Xba*I digestion then cloned into the final plant binary vector pBI101.1 to produce pBI101.1 – GLP-1 – hTf (Figure 2).

2.6.2 Construction of Ex-4 – hTf plant expression vector

The cDNA of *Ex-4* was provided by Dr. Ma's laboratory and amplified using designed forward primer Ex-4-F and reverse primer Ex-4-R (Table 1). Forward primer Ex-4-F contained *Nco*I restriction site (underline) at the start codon and it incorporated barley α-amylase signal peptide (bold) immediately upstream of the *Ex-4* sequence which replaced the native *Ex-4* signal peptide sequence. Reverse primer Ex-4-R contained 3' His×6 tag (italics), stop codon (bold) and *Xba*I restriction site (underline). PCR reaction was performed under following conditions: denature at 94°C for 45 s, anneal at 55°C for 45 s and elongate at 72°C for 45 s, for a total of 35 cycles followed by a final elongation at 72°C for 10min.

The *hTf* cDNA clone was obtained from OriGene (Rockville, MD, USA). The coding region, excluding the native *hTf* signal peptide sequence, was amplified using forward primer hTf-F1 and reverse primer hTf-R. Forward primer hTf-F1 contained the last 14 nucleotides from 3' end of *Ex-4* sequence (italics) which has

*Sac*I site (underline) for fusion and incorporated a linker sequence (bold) upstream of *hTf* sequence. Reverse primer hTf-R contained 3' His×6 tag (double underline), ER-retention signal KDEL (italics), stop codon (bold) and *Xba*I restriction site (underline). PCR reaction was performed under following conditions: denature at 94°C for 45 s, anneal at 55°C for 45 s and elongate at 72°C for 2 min, for a total of 35 cycles followed by a final elongation at 72°C for 10min. The amplified *Ex-4* and *hTf* sequences were blunt-end ligated into the *Sma*I site of pUC19 vector and subsequently sequenced to confirm the sequence integrity (Sequencing Facility; Robarts Research Institute, London, ON, Canada).

To create the fusion expression cassette, *Ex-4* sequence was released from pUC19 as *Nco*I/*Sac*I fragment and *hTf* sequence was released as *Sac*I/*Xba*I fragment then inserted into pRTL-2 vector using three-fragment ligation. The resulting expression cassette contained the enhanced constitutive cauliflower mosaic virus (CamV) 35S promoter, tobacco etch virus (TEV) 5'UTL, barley α-amylase signal peptide, Ex-4 – linker – hTf fusion, a His×6 tag for protein purification, KDEL signal for ER retention and the nopaline synthase (NOS) terminator (Figure 3). The *Xba*I site in pRTL-2 – Ex-4 – hTf was destroyed by Klenow treatment for fusion purpose (Figure 2). This vector was digested with *Sph*I then treated with Klenow to produce a blunt-ended fragment, which was ligated into pBluescript at *Sma*I site. The entire expression cassette was released from pBluscript by using *Sal*I and *Xba*I digestion then cloned Pinto the final plant binary vector pBI101.1 to produce pBI101.1 – Ex-4 – hTf (Figure 3).

2.7 Generation of transgenic plants

The engineered plant expression vector pBI101.1-Ex-4-hTf 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). Transgenic plants were selected on Murashige and Skoog (MS) medium containing 100 mg/L kanamycin. As the transgenic plants matured, they were transferred into 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 were inoculated and grown overnight at 28°C. Cells were harvested and resuspended in infiltration media (50 mM MES, 2 mM $Na_3PO_4 \cdot 12H_2O$, 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 for seven days and proteins were extracted for analysis.

2.8 Screening of putative transgenic plants using PCR

Total genomic DNA was extracted from individual transgenic tobacco lines using a basic phenol/chloroform extraction (Ma et al. 2005). In brief, tobacco leaf tissue was homogenized in DNA extraction buffer [0.2 M Tris-HCl (pH 7.5), 0.25 M

NaCl, 25 mM EDTA, 0.5% (w/v) SDS] and incubated at room temperature for 1 hour. The sample was centrifuged for 10 min at 12,000×g and supernatant was collected. Sodium acetate (3 M CH3COONa, pH 4.8) and phenol/chloroform (1:1) was added to the supernatant then the mixture was centrifuged for 5 min at 12,000×g. The aqueous phase was extracted once with 100% isopropanol and the resulting DNA pellet was washed with 70% ethanol. Extracted DNA was used as template for PCR analysis to confirm the integration of *Ex-4 – hTf* fusion. The primer pair EX-4-F and hTf-R was used under same PCR conditions as describe above. All PCR products were analyzed by electrophoresis on a 7.5% (w/v) agarose gel.

2.9 Tobacco RNA extraction and RT-PCR analysis

Total RNA was prepared from transgenic tobacco leaf tissue by using a Trisol RNA extraction kit (Invitrogen) according to the manufacturer's recommendations. Extracted RNA was transcribed into cDNA with SuperScript II Reverse Transcriptase following the manufacturer's protocol (Invitrogen). Briefly, 5 μg of total RNA extracted from individual plants, 1 μl Oligo(dT) (500 μg/μl), 1 μl dNTP mix (10 mM each) and 5 μl sterile distilled water were mixed and incubated at 65 °C for 5 min. Following the addition of 4 μl First-Strand Buffer and 2 μl 0.1 M Dithiothreitol (DTT), the reaction mixture was further incubated for 2 min at 42 °C. After incubation, 1 μl of SuperScript II Reverse Transcriptase was added and incubated at 42 °C for 50 min to produce cDNA. PCR analysis was performed using

primer pair EX-4-F and hTf-R under same PCR conditions as describe above. All PCR products were analyzed by electrophoresis on a 7.5% (w/v) agarose gel.

2.10 Accumulation of GLP-1 –hTf and Ex-4 – hTf in transgenic plants

Positive transgenic plants confirmed by PCR analysis were analyzed to determine the recombinant protein accumulation level using SDS-PAGE followed by Western blotting. In brief, tobacco leaf tissues were homogenized in protein extraction buffer [25 mM Tris-HCl (pH 7.0), 50 mM NaCl, 2mM β-mercaptoethanol, 1mM phenyl-methysulfonyl fluoride (PMSF), 2 μg/mL aprotinin, 2 μg/mL pepstain A and 2 μg/mL leupeptin]. Samples were centrifuged at 13,000×g for 10 min at 4°C and 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 GLP-1 – hTf and Ex-4 – hTf fusion proteins were 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 hour 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 over night at 4°C with goat anti-hTf primary antibody (Sigma-Aldrich Canada, Oakville,

Ontario) diluted 1:500 (v/v). The membrane was washed again by TBS-T and incubated with horseradish peroxidise conjugated swine anti-goat secondary antibody (Sigma-Aldrich Canada, Oakville, Ontario) for 1 hour. Immunodetection was performed using the enhanced chemiluminescence (ECL) detection system (Perkin Elmer Life Sciences, Rockford, IL) according to the manufacturer's protocol.

2.11 Quantification of plant-derived GLP-1 – hTf and Ex-4 – hTf

The accumulation of GLP-1 – hTf and Ex-4 – hTf fusion protein in crude tobacco leaf extract was quantified using an enzyme linked immunosorbent assay (ELISA). The accumulated recombinant protein was compared against known quantities of hTf reference protein (Sigma-Aldrich) as a standard. The serial dilutions of standard hTf as well as crude tobacco extracts, in triplicates, were resuspended in sodium bicarbonate buffer (pH9.6) and coated onto a 96-well microtiter plate then incubated at 4°C for overnight. Wells were washed three times with PBS-T [1× phosphate buffered saline containing 0.05% (v/v) Tween-20] and blocked with 3% (w/v) BSA in PBS-T for 3 hours at room temperature. After washing with PBS-T, goat anti-hTf monoclonal antibody (Sigma-Aldrich) diluted to 1:2000 (v/v) was added to each well and incubated overnight at 4°C. Following five subsequent PBS-T washes, horseradish peroxidise-conjugated swine anti goat IgG antibody diluted to 1:2000 (v/v) was added to each well and incubated for 1 hour at room temperature. The plate was washed eight 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 H_2SO_4). The plate was read as an optical density (OD) value measured at 450 nm in a microplate reader (Bio-Rad 3550). The OD measurement was converted as a percentage of total soluble protein by reference to an ELISA standard curve constructed with the hTf reference protein.

2.12 Purification of His-tagged GLP-1– hTf and Ex-4 – hTf

Plant-derived GLP-1– hTf and Ex-4 – hTf fusion proteins were purified from leaf extracts of transgenic tobacco plants 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, 2mM β-mercaptoethanol, 1mM phenyl-methysulfonyl fluoride (PMSF), 2 μg/mL aprotinin, 2 μg/mL pepstain A and 2 μg/mL leupeptin] in a 1:4 (w/v) ratio of sample to buffer. The homogenate was filtered through a filter paper and centrifuged at 14,000 g for 10 min at 4°C. Supernatant was collected and centrifuged again at 14,000 g for 10 min at 4°C. The final supernatant was filtered through a 0.45 μm membrane filter and loaded into the prepared HiTrap Chelating HP column and washed with wash buffer (2 mM imidazole, 20 mM Na_2HPO_4, 500 mM NaCl) to remove nonspecifically bound tobacco leaf endogenous proteins. The bound GLP-1– hTf and Ex-4 – hTf fusion

protein was eluted with elution buffer [500 mM imidazole, 20 mM Na_2HPO_4, 500 mM NaCl] with 1-mL fractions collected for further analysis. The eluted GLP-1– hTf and Ex-4 – hTf fractions were dialyzed extensively against 1× PBS (pH 7.5) and concentrated using speed vacuum at 4°C. ELISA was carried out to quantify the amount of fusion protein using hTf reference protein as a standard.

2.13 Insulin induction assay

The functionality of plant-derived GLP-1 – hTf and Ex-4 – hTf fusion proteins was determined by its ability to stimulate insulin secretion from a mouse pancreatic beta-cell line, MIN6 cells, according to the method of Miyazaki et al. (1990) with minor modifications. Briefly, MIN6 cells were cultured and maintained in 25 cm^2 tissue culture flasks using high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% (v/v) fetal calf serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%). The culture medium was replaced with fresh medium every 24 hours and cells were used for the experiments once they had reached 80% confluence. To determine the insulin-induction effect of plant-derived GLP-1– hTf and Ex-4 – hTf fusion proteins on MIN6 cells, the cells were washed with PBS and harvested using Trypsin-EDTA solution (0.125 M trypsin, 0.05 mg/ml EDTA) for 3 to 5 min. Harvesting was stopped with the addition of DMEM and subsequent centrifugation (1,500 rpm for 5 min). Cell pellets were resuspended in 10% (v/v) DMEM containing 10 mM glucose and counted using

hemacytometer. Cells were then added to each 1.5 mL culture tube (~1×10^6 cells/tube) containing 1.2 mL 10% (v/v) DMEM medium in the presence of 10 mM glucose. The insulin-induction effect of plant-derived GLP-1– hTf and Ex-4 – hTf fusion proteins were compared to commercial GLP-1 standard (Sigma) and DMEM only, and 200 ng/ml of each protein were added to the prepared MIN6 cell plates. Various concentrations of glucose (0, 5, 10, 25 mM) were added to each plate. MIN6 cells were incubated for 135 min at 37°C, with culture supernatants subsequently collected and assayed for insulin concentration using an insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA) according to the manufacturer's protocols.

2.14 Detection of internalized plant derived GLP-1– hTf and Ex-4 – hTf

Human intestinal epithelial cell line, Caco-2 cells, was used to determine the internalization of plant-derived GLP-1 – hTf and Ex-4 – hTf fusion proteins *in vitro*. Caco-2 cells were grown to the density of 0.5×10^6 cells/flask then maintained in fresh 10% (v/v) FBS-RPMI 1640 medium (Invitrogen). The cells were inoculated into 60×15 mm petri dishes as triplicates and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 24 hours. After the incubation, the medium was removed and the cells were washed twice the serum-free F12 medium. Following the wash, the cells were incubated in serum-free F12 medium at 37°C in an atmosphere of 5% CO_2 and 95% air for 1 hour to starve the cells. Two different concentration (0.2 and 1 μg/mL) of commercial hTf standard (Sigma), plant-derived hTf, GLP-1– hTf and and Ex-4 – hTf proteins were added to Caco-2 cells and incubated for another 1 hour with

the same condition. To stop the reaction, the plates were placed onto ice and washed with cold Dulbecco's-PBS to remove excess and unbound proteins. The cell membrane-bound proteins were removed using acid buffer [200 mM acetic acid, 500 mM NaCl, pH 4.5] by incubating for 5 min on ice then washed twice with cold Dulbecco's-PBS to neutralize the pH. The cells were removed from the dish using plastic scraper and place into 15 mL conical tubes. The cell pellet was collected by centrifugation for 5 min at 14,000 g, then cells were lysed with lysis buffer [1% (v/v) Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 1 mM vanadate, 1:100 (v/v) phosphatise inhibitor cocktail, pH7.4] (Sigma-Aldrich) by 10 min incubation on ice. The cell lysate was centrifuged at 10,000 g at 4°C for 10 min to remove cellular debris. The supernatant was collected and its protein concentration determined by ELISA followed by Western blotting using anti-hTf antibody

2.15 Digestion of GLP-1– hTf and Ex-4 – hTF in SGF and SIF

The stability of plant-derived GLP-1– hTf and Ex-4 – hTf fusion protein was determined by SGF and SIF digestion testing according to Tremblay et al. (2011). Briefly, His-purified protein sample was incubated in SGF (0.2 g NaCl, 0.32g pepsin, 700 µL HCl, in 100 mL dH_2O, pH 2.5) and SIF (0.68 g monobasic KH2PO4, 7.7 mL 0.2 M NaOH, 1.0 g pancreatin , in 100 mL dH_2O, pH ~6.8) at 37°C. The digestion was stopped by a neutralization buffer (3.4 g Na_2CO_3 in 100 mL dH_2O) at time 0, 15

s, 30 s, 1 min, 5 min, 15 min, and 30 min. The neutralized samples were boiled for 10 min and analysed using immunoblot analysis.

2.16 Immunoblot analysis of SGF and SIF

The proteins were boiled for 10 min in 5x 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 hour 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 over night at 4°C with goat anti-hTf primary antibody (Sigma-Aldrich Canada, Oakville, Ontario) diluted 1:500 (v/v). The membrane was washed again by TBS-T and incubated with horseradish peroxidise conjugated swine anti-goat secondary antibody (Sigma-Aldrich Canada, Oakville, Ontario) for 1 hour. Immunodetection was performed using the enhanced chemiluminescence (ECL) detection system (Perkin Elmer Life Sciences, Rockford, IL) according to the manufacturer's protocol.

2.17 Statistical analysis

All statistical analysis was completed independently in triplicate. 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.

Chapter 3

Results

3.1 Construction of plant expression vectors pBI101.1 – GLP-1 – hTf and pBI101.1 – Ex-4 –hTf.

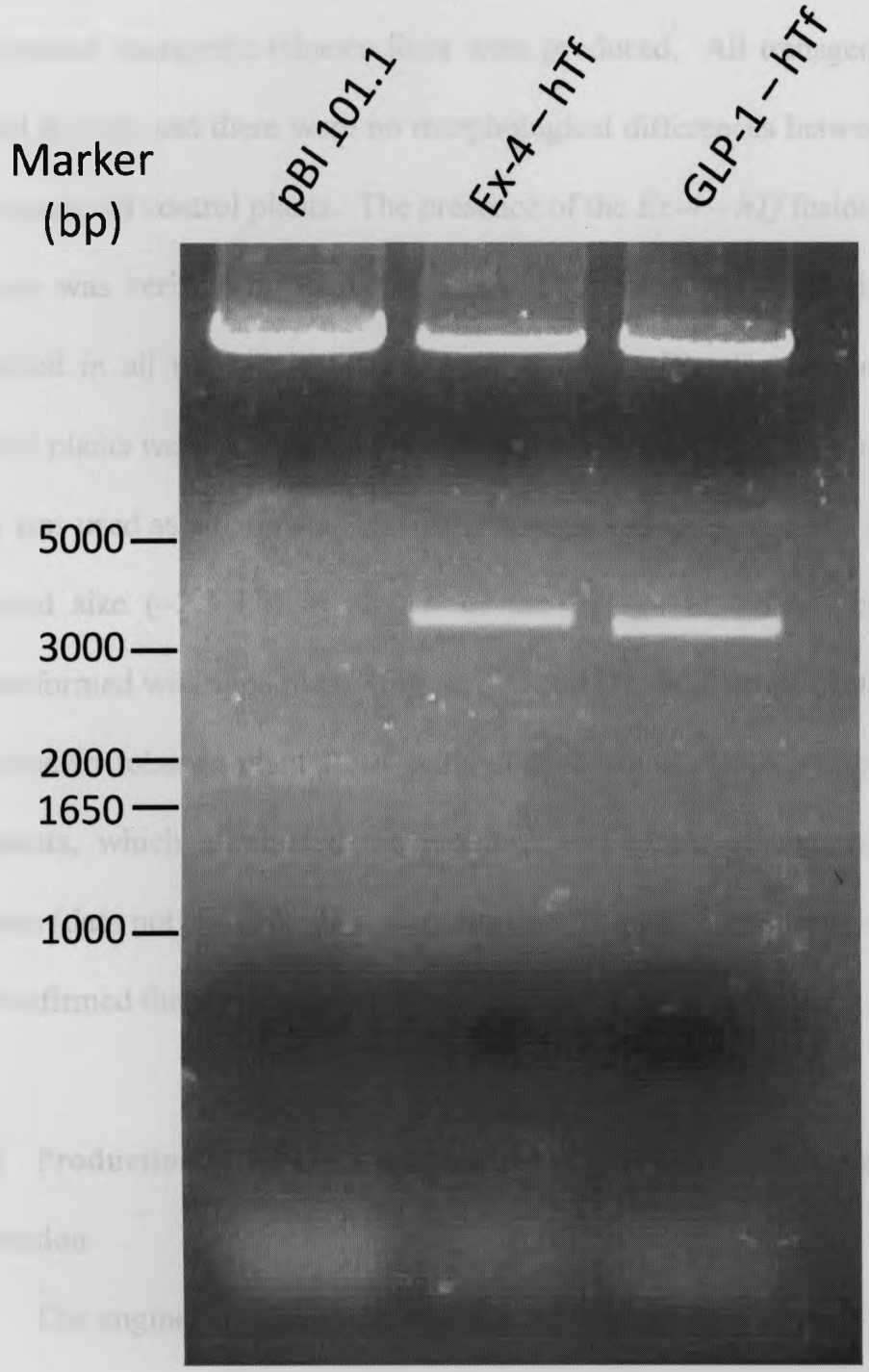
The construction of plant expression vectors *pBI101.1 – GLP-1 – hTf* and *pBI101.1 – Ex-4 – hTf* is explained in section 2.6 and shown in Figure 2. The resulting plant binary vectors were digested with *Sal*I and *Xba*I to confirm their proper integration. The digestion of *pBI101.1 – GLP-1 – hTf* produced 3.35 kb fragment and the digestion of *pBI101.1 – Ex-4 – hTf* produced 3.37 kb fragment which correspond to the correct size of each expression cassette (Figure 4). Both expression cassettes were under the control of enhanced constitutive cauliflower mosaic virus (CaMV) 35S promoter and contained tobacco etch virus (TEV) 5'UTL, barley α-amylase signal peptide, *GLP-1/Ex-4 – linker – hTf* fusion sequence, a His×6 tag for protein purification, KDEL signal for ER retention and the nopaline synthase (NOS) terminator (Figure 3).

3. 2 Production of transgenic tobacco plants

3.2.1 Production of Ex-4 – hTf in transgenic tobacco plants by stable nuclear transformation

Figure 4.

Confirmation of plant binary vector containing expression cassettes. The final binary vector was confirmed by restriction enzyme digestion. Both binary vectors, *pBI101.1 – GLP-1 – hTf* and *pBI101.1 Ex-4 – hTf*, were digested with *Sal* I and *Xba* I to test for proper integration of the expression cassettes. Empty *pBI101.1* was subjected to same digestion as a negative control.


Marker
(bp)
pBI 101.1
Ex-4 – hTf
GLP-1 – hTf
5000
3000
2000
1650
1000

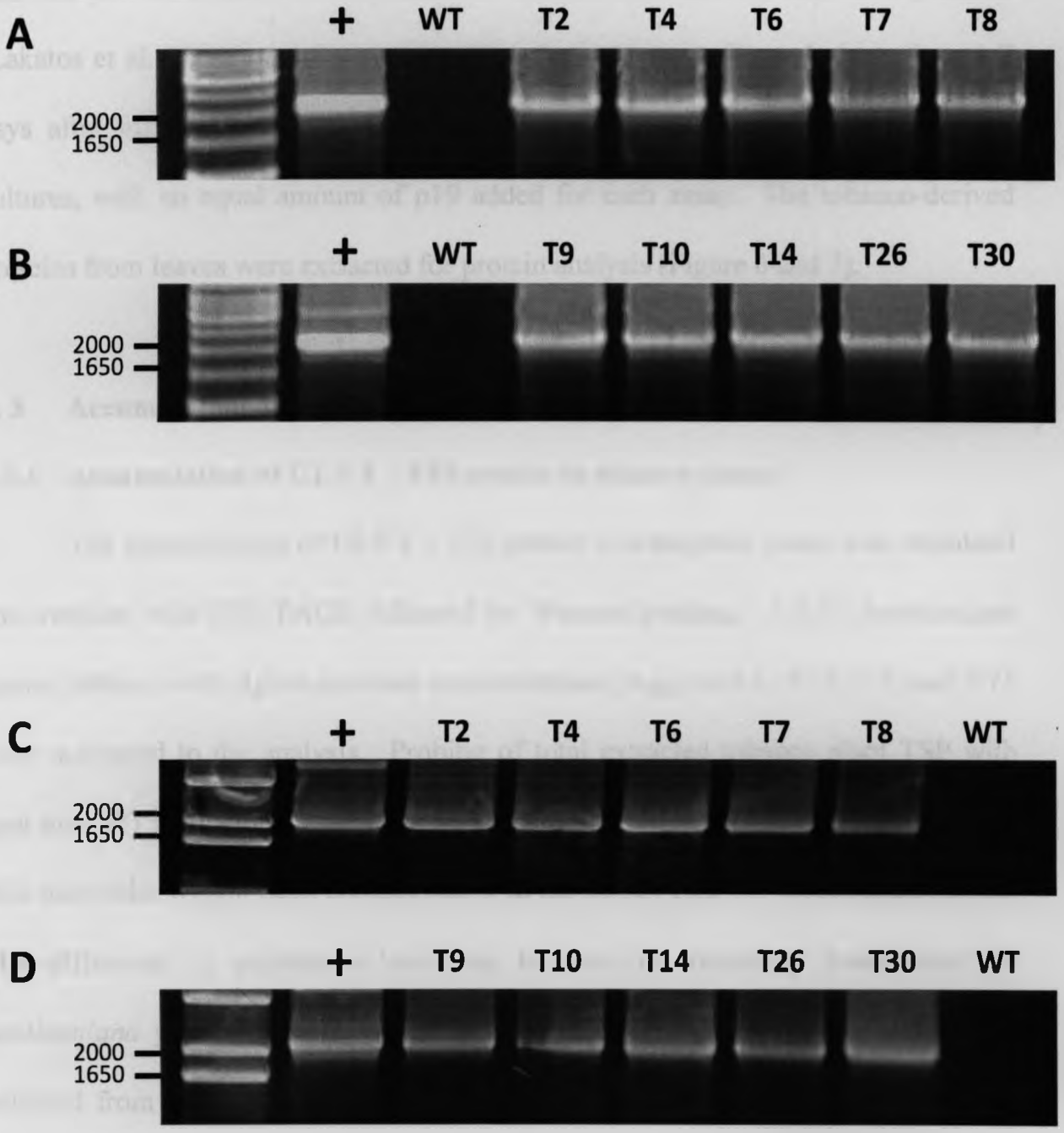
Low-nicotine and low alkaloid *N. tabacum* cultivar 81V9 was transformed with *A. tumefaciens* strain LBA4404 containing plasmid *pBI101.1 – Ex-4 – hTf* by tobacco leaf-disc transformation described by Horsch et al. (1985). Total of 10 independent transgenic tobacco lines were produced. All transgenic plants showed normal growth and there were no morphological differences between transgenic and untransformed control plants. The presence of the *Ex-4 – hTf* fusion gene in the plant genome was verified by PCR. A DNA fragment of expected size (~2.3 kb) was amplified in all transgenic lines (Figure 5 A and B). Furthermore, PCR-positive tobacco plants were analyzed at transcript level using RT-PCR. Total extracted plant RNA was used as a template. The result revealed the presence of a DNA fragment of expected size (~2.3 kb) in all ten of the transgenic tobacco plants, but not in untransformed wild type plant (Figure 5 C and D). PCR amplification of all samples of extracted tobacco plant RNA without first-strand cDNA synthesis produced no fragments, which eliminated the possibility of DNA contamination of the RNA samples (data not shown). This suggests that the plant transformation was successful and confirmed the integration of *Ex-4 – hTf* fusion gene in the plant genome.

3.2.2 Production of GLP-1 – hTf and Ex-4 – hTf in tobacco plants by transient expression

The engineered plant expression vector *pBI101.1 – GLP-1 – hTf* or *pBI101.1 – Ex-4 – hTf* was introduced into *A. tumefaciens* strain LBA4404 by tri-parental mating using helper plasmid pRK4013. Six-week-old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* clone containing *pBI101.1 – GLP-1 – hTf* or

Figure 5.

Detection of *Ex-4 – hTf* in transgenic tobacco plants by PCR and RT-PCR analysis. Total genomic DNA and RNA was extracted from the leaves of ten transgenic tobacco lines transformed with *pBI101.1 – Ex-4 – hTf.* (A and B) The genomic DNA was then used for PCR amplification in the presence of transgene specific primers. The resulting PCR products were analyzed by agarose gel electrophoresis. Total of 45 plants were examined and ten independent transgenic lines showed the successful integration of transgene. (C and D) The isolated RNA was reverse transcribed to synthesize cDNA templates. Primers specific for *Ex-4 – hTf* sequence were used for amplification of cDNA templates. As a positive control (+), *pRTL2 – Ex-4 – hTf* vector was used as a template and genomic DNA from untransformed wild type 81V9 tobacco plant was used as a negative control (WT).


A
+
WT
T2
T4
T6
T7
T8
2000
1650
B
+
WT
T9
T10
T14
T26
T30
2000
1650
C
+
T2
T4
T6
T7
T8
WT
2000
1650
D
+
T9
T10
T14
T26
T30
WT
2000
1650

pBI101.1 – Ex-4 – hTf together with cultures of *A. tumefaciens* containing the p19 expression cassette, a viral inhibitor of post-transcriptional gene silencing in plants that has proven effective in increasing the yield of transiently expressed proteins (Lakatos et al. 2004). Leaf samples were collected from infiltrated plants from 1-7 days after infiltration (DAI) with four different concentrations of *A. tumefaciens* cultures, with an equal amount of p19 added for each assay. The tobacco-derived proteins from leaves were extracted for protein analysis (Figure 6 and 7).

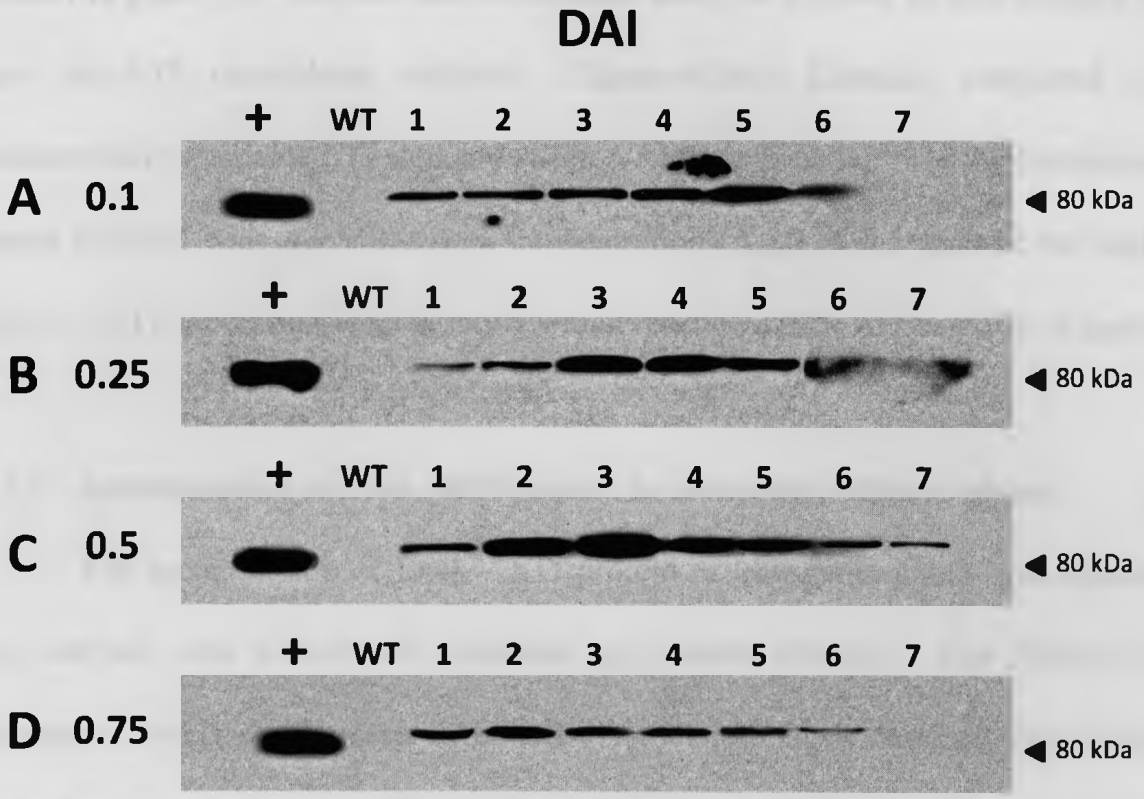
3. 3 Accumulation of recombinant proteins in transgenic plants

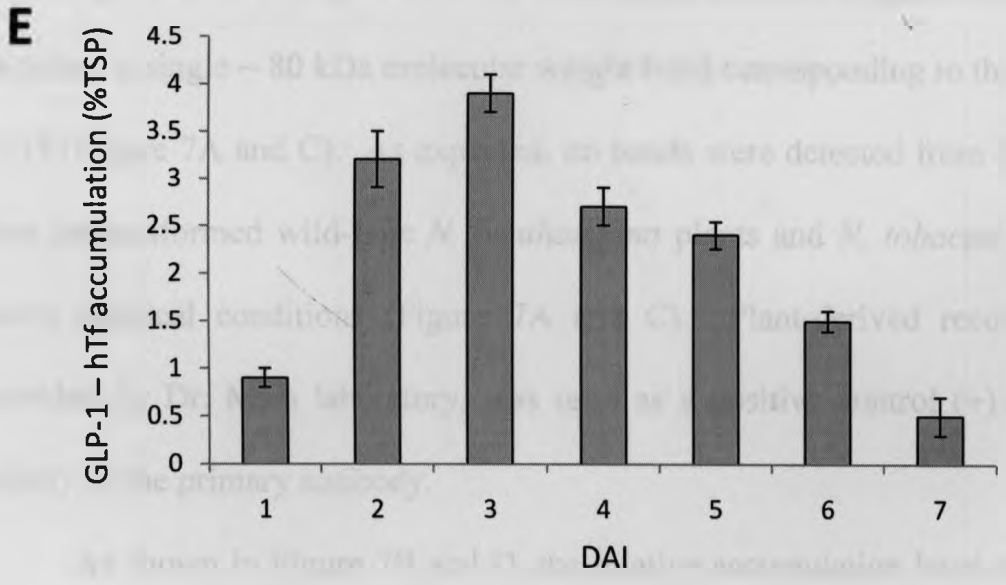
3.3.1 Accumulation of GLP-1 – hTf protein in tobacco plants

The accumulation of GLP-1 – hTf protein in transgenic plants was examined and verified with SDS-PAGE followed by Western blotting. All *N. benthamiana* plants infected with *Agrobacterium* concentrations (A_{600}) of 0.1, 0.25, 0.5, and 0.75 were subjected to the analysis. Probing of total extracted tobacco plant TSP with goat anti-hTf monoclonal antibody (Sigma-Aldrich Canada) identified a single ~ 80 kDa molecular weight band corresponding to the size of GLP-1 – hTf (Figure 6A-D). The difference in expression level can be seen in transiently transformed *N. benthamiana* plants at each day after infiltration. As expected, no bands were detected from TSP extracted from untransformed wild-type *N. benthamiana* plants under identical conditions (Figure 6A-D). Plant-derived recombinant hTf, provided by Dr. Ma's laboratory, was used as a positive control (+) to assess the affinity of the primary antibody.

Figure 6.

Western blot analysis and quantification of GLP-1 – hTf protein in transiently transformed *N. benthamiana*. The plants were infiltrated with *Agrobacterium* containing *pBI101.1 – GLP-1 – hTf* vector then leaves were harvested for 1 -7 days after infection (DAI). Total soluble protein was extracted from the leaves of agroinfiltrated *N. benthamiana* plants. Proteins were separated by 10% SDS-PAGE, transferred onto a PVDF membrane and probed with goat anti-hTf primary antibody followed by swine anti-goat secondary antibody. (A-D) Accumulation of GLP-1 – hTf in plants infected with *Agrobacterium* concentrations (A_{600}) of 0.1, 0.25, 0.5, and 0.75. Plant-derived recombinant hTf was used as a positive control (+) and untransformed wild type 81V9 tobacco plant was used as a negative control (WT). Approximately 30 μg of TSP were loaded for each western blot experiments. (E) ELISA data for GLP-1 – hTf accumulation in *N. benthamiana* plant at optimal *Agrobacterium* concentration (A_{600} = 0.5). Concentration levels are expressed as a percentage of total soluble protein (% TSP). Data shown represents average value of three independent experiments with error bars correspond to standard deviation.


DAI
+ WT 1 2 3 4 5 6 7
A 0.1
B 0.25
C 0.5
D 0.75
80 kDa


E
GLP-1 – hTf accumulation (%TSP)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7
DAI

As shown in Figure 6E, the relative accumulation level of GLP-1 – hTf protein in plant TSP extracts was determined using an indirect ELISA method with goat anti-hTf monoclonal antibody (Sigma-Aldrich Canada), compared to a commercially available hTf standard (Sigma-Aldrich Canada). The *N. benthamiana* plants infected with *Agrobacterium* concentration (A_{600}) of 0.5 showed the highest GLP-1 – hTf expression level at day 3 which reached 3.92% of plant TSP (Figure 6E)

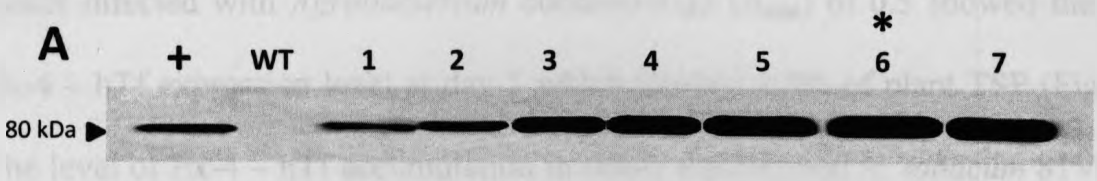
3.3.2 Accumulation of Ex-4 – hTf protein in transgenic tobacco plants

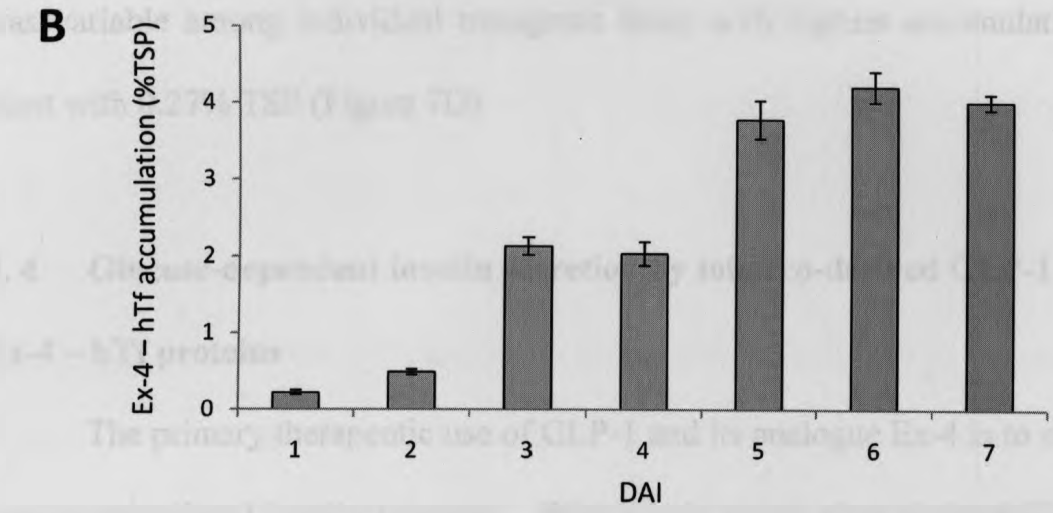
The accumulation of Ex-4 – hTf protein in transgenic plants was examined and verified with SDS-PAGE followed by Western blotting. The difference in expression levels can be seen in transiently transformed *N. benthamiana* plants at each day after infiltration (Figure 7A). Total of ten transgenic tobacco lines successfully expressed Ex-4 – hTf protein (Figure 7C). Probing of total extracted tobacco plant TSP with goat anti-hTf monoclonal antibody (Sigma-Aldrich Canada) identified a single ~ 80 kDa molecular weight band corresponding to the size of Ex-4 – hTf (Figure 7A and C). As expected, no bands were detected from TSP extracted from untransformed wild-type *N. benthamiana* plants and *N. tobacum* 81V9 plants under identical conditions (Figure 7A and C). Plant-derived recombinant hTf, provided by Dr. Ma's laboratory, was used as a positive control (+) to assess the affinity of the primary antibody.

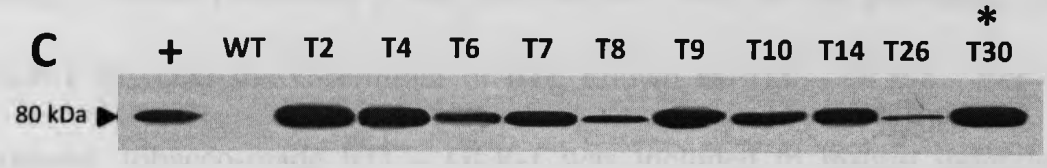
As shown in Figure 7B and D, the relative accumulation level of Ex-4 – hTf protein in plant TSP extracts was determined with an indirect ELISA method using goat anti-hTf monoclonal antibody (Sigma-Aldrich Canada), compared to a

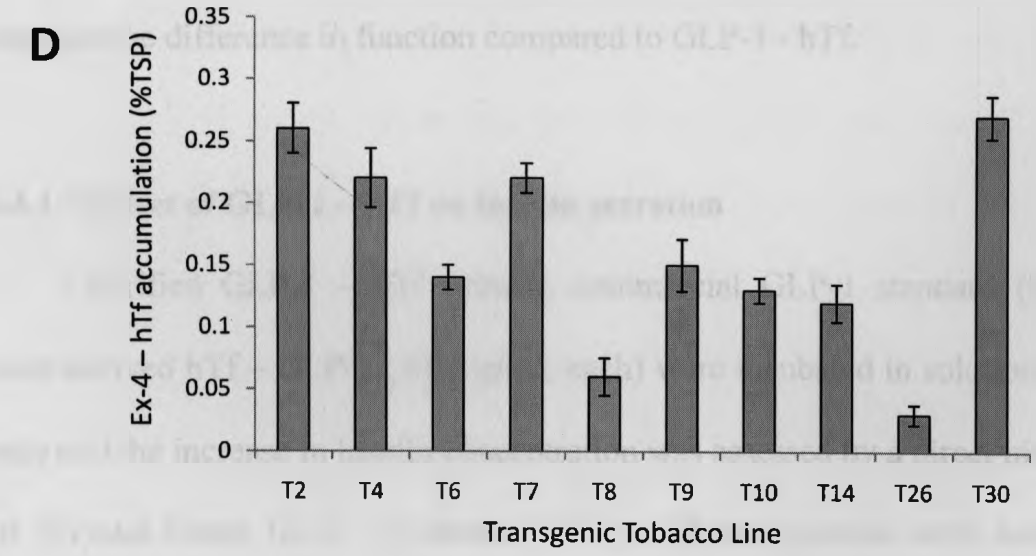
Figure 7.

Western blot analysis and quantification of Ex-4 – hTf protein in tobacco plants. (A) Total soluble protein was extracted from the leaves of agroinfiltrated *N. benthamiana* plants. Highest protein concentration reached at day 6 in *N. benthamiana* plant infected with A_{600} = 0.5 *Agrobacterium* concentration (*). (B) ELISA data for Ex-4 – hTf accumulation in *N. benthamiana* plant at optimal *Agrobacterium* concentration (A_{600}) of 0.5. (C) Total soluble protein was extracted from the leaves of transgenic plants transformed with pBI101.1 – Ex-4 – hTf. Proteins were separated by 10% SDS-PAGE, transferred onto a PVDF membrane and probed with goat anti-hTf primary antibody followed by swine anti-goat secondary antibody. Approximately 55 μg of TSP were loaded for each transgenic line. (B) Protein samples from each transgenic plant were used to determine the accumulation level of Ex-4 – hTf using ELISA. Data shown represent average value of three independent experiments with error bars correspond to standard deviation. For Western blot analysis, plant-derived recombinant hTf – GLP1 protein was used as a positive control (+) and untransformed wild type 81V9 tobacco plant was used as a negative control (WT). For ELISA result, concentration levels are expressed as a percentage of total soluble protein (% TSP).


A
+
WT
1
2
3
4
5
6
7
*
80 kDa


B
Ex-4 – hTf accumulation (%TSP)
0
1
2
3
4
5
1
2
3
4
5
6
7
DAI


C
+
WT
T2
T4
T6
T7
T8
T9
T10
T14
T26
T30
*
80 kDa


D
Ex-4 – hTf accumulation (%TSP)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
T2
T4
T6
T7
T8
T9
T10
T14
T26
T30
Transgenic Tobacco Line

commercially available hTf standard (Sigma-Aldrich Canada). The *N. benthamiana* plants infected with *Agrobacterium* concentration (A_{600}) of 0.5 showed the highest Ex-4 – hTf expression level at day 5 which reached 4.2% of plant TSP (Figure 7B) The level of Ex-4 – hTf accumulation in stably transformed *N. tobacum* 81V9 plants was variable among individual transgenic lines, with highest accumulation in T30 plant with 0.27% TSP (Figure 7D).

3. 4 Glucose-dependent insulin secretion by tobacco-derived GLP-1 – hTf and Ex-4 – hTf proteins

The primary therapeutic use of GLP-1 and its analogue Ex-4 is to enhance the glucose-stimulated insulin secretion. With this in mind, plant-derived GLP-1 – hTf and Ex-4 – hTf proteins were analyzed for their ability to stimulate insulin secretion using a mouse pancreatic β cell line, MIN6. Our lab has previously developed a GLP-1 fused at the C-terminal of hTf, known as hTf – GLP-1. For comparison purpose, tobacco-made hTf – GLP-1 was included in insulin induction assay to examine the difference in function compared to GLP-1 - hTf.

3.4.1 Effect of GLP-1 – hTf on insulin secretion

Purified GLP-1 – hTf protein, commercial GLP-1 standard (Sigma), and plant-derived hTf – GLP-1 (200 ng/mL each) were incubated in solution with MIN6 cells and the increase in insulin concentration was assessed by a direct insulin ELISA kit (Crystal Chem Inc.). As shown in Figure 8, the proteins were incubated with MIN6 cells in the presence of various glucose levels (0 – 25 mM). Tobacco-derived

hTf – GLP-1 protein was produced previously from our lab and was included in this study as a comparison with recombinant GLP-1 – hTf protein. A significant ($p<0.05$) increase in insulin concentration was observed in MIN6 cell-supernatant collected after incubation with tobacco-derived GLP-1 – hTf , as compared to MIN6 cells incubated with medium only (Figure 8A). Tobacco-derived GLP-1 – hTf increased the insulin level up to 1.42 μg/ml which is almost 5-fold higher than that of media only treatment. Both GLP-1 standard and recombinant GLP-1 – hTf protein showed a similar glucose concentration-dependent increase in insulin release form MIN6 cells with no significant differences. Tobacco-derived hTf – GLP-1 protein showed no significant increase in insulin production (Figure 8A)

3.4.2 Effect of Ex-4 – hTf on insulin secretion

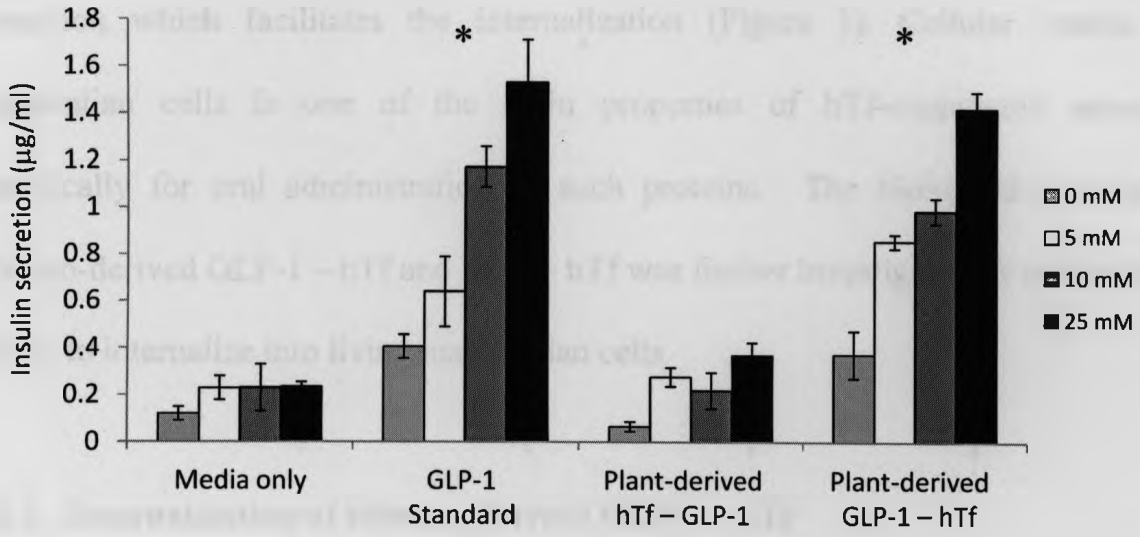
Purified Ex-4 – hTf protein and commercial GLP-1 standard (Sigma) (200 ng/mL each) were incubated in solution with MIN6 cells and the increase in insulin concentration was assessed by a direct insulin ELISA kit (Crystal Chem Inc.) as described above (Section 3.4.1). Tobacco-derived Ex-4 – hTf increased the insulin level up to 1.57 μg/ml which is almost 6-fold higher than that of media only treatment, and very close to GLP-1 standard. Both GLP-1 standard and recombinant Ex-4 – hTf protein showed a very similar increase in insulin release form MIN6 cells with no significant differences (Figure 8B).

Figure 8.

Effect of tobacco-derived GLP-1 – hTf and Ex-4 – hTf proteins on insulin secretion using MIN6 cell lines. (A) The mouse pancreatic beta-cell line (MIN6) was incubated with glucose in DMEM in the presence of GLP-1 standard, His-purified tobacco-derived hTF – GLP-1, His-purified tobacco-derived GLP-1 –hTf or DMEM media only (5 ng each). (B) MIN6 cells were incubated with GLP-1 standard, His-purified tobacco-derived GLP-1 –hTf or DMEM media only (5 ng each). Four different concentrations (0, 5, 10, 25 mM) of glucose were applied for the analysis. After incubation cell culture-supernatants were collected and insulin concentration was determined using an insulin ELISA kit (Crustal Chem Inc.) followed by reading of OD at 450 nm. Data represents the average insulin concentration in triplicate with error bars corresponding to standard deviation. The * indicates significantly higher insulin concentration determined by paired two-tailed Student's *t*-test.

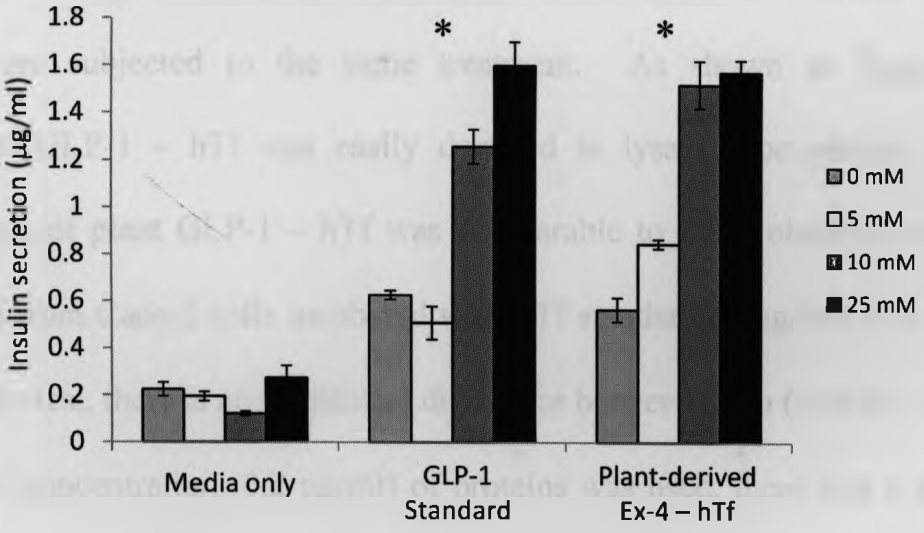
A

Glucose-dependent insulin stimulation by plant-derived GLP-1 – hTf



B

Glucose-dependent insulin stimulation by plant-derived Ex-4 – hTf



3. 5 Cellular internalization of tobacco-derived GLP-1 – hTf and Ex-4 – hTf proteins

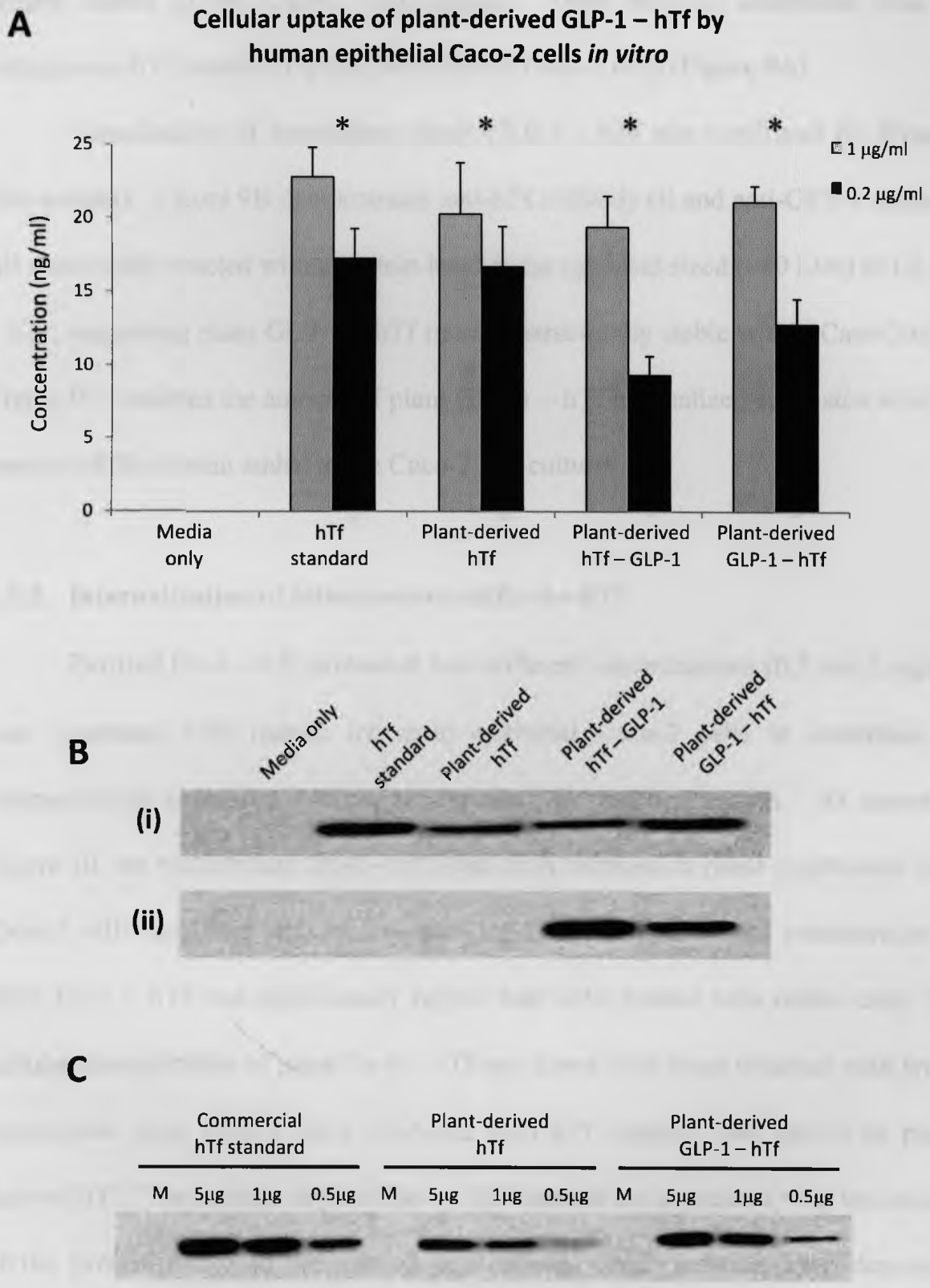
Internalization of hTf into mammalian cells involves a two-step process comprised of the binding to hTf receptor on the cell surface followed by endosome formation which facilitates the internalization (Figure 1). Cellular uptake by mammalian cells is one of the main properties of hTf-conjugated proteins, specifically for oral administration of such proteins. The biological activity of tobacco-derived GLP-1 – hTf and Ex-4 – hTf was further investigated by testing their ability to internalize into living mammalian cells.

3.5.1 Internalization of tobacco-derived GLP-1 – hTf

Purified GLP-1 – hTf protein at two different concentrations (0.2 and 1 μg/ml) was incubated with human intestinal epithelial Caco-2 cells to determine its internalization capability *in vitro*. For comparison purposes, the same concentrations of commercial hTf standard, plant-derived hTf and plant-derived hTf – GLP-1 proteins were subjected to the same treatment. As shown in Figure 9, the recombinant GLP-1 – hTf was easily detected in lysate supernatants. Cellular concentration of plant GLP-1 – hTf was comparable to those obtained with lysate supernatant from Caco-2 cells incubated with hTf standard (1 μg/ml) and according to student's t-test, there is no significant difference between them ($p<0.05$). However, when lower concentration (0.2 μg/ml) of proteins was used, there was a significant difference between plant-derived GLP-1 – hTf and the standard hTf. Furthermore,

Figure 9.

Determination of tobacco-derived GLP-1 – hTf internalization by Caco-2 cells *in vitro*. Human intestinal epithelial Caco-2 cells were grown in serum-free SFF12 medium then incubated with plant-derived recombinant proteins to analyze its cellular uptake level. Cells were incubated with SFF12 media only, hTf standard, plant-derived hTf, plant-derived hTf – GLP-1 or plant-derived GLP-1 – hTf at two different concentrations (0.2 and 1 μg/ml). (A) Each cell lysate was quantified by ELISA using goat anti-hTf antibody. The ELISA assay was performed in triplicate wells and repeated twice. Data represents the average concentration of internalized proteins in triplicate with error bars corresponding to standard deviation. The * indicates significant increase in internalization concentration compared to media only which was determined by paired two-tailed Student's *t*-test. (B) Caco-2 cells were incubated with SFF12 media only, hTf standard, plant-derived hTf, plant-derived hTf – GLP-1 or plant-derived GLP-1 – hTf. Following incubation, surface-bound proteins were removed by acetic acid treatment. Collected cells were lysed to release internalized proteins and confirmed by Western blot analysis using (i) goat anti-hTf antibody and (ii) mouse anti-GLP-1 antibody. (C) Different concentrations (0.5 – 5 μg/ml) of hTf standard, plant-derived hTf, or plant-derived GLP-1 – hTf were incubated with Caco-2 cells to determine the effect of protein internalization. M refers to SFF12 media without protein presence.


A
Cellular uptake of plant-derived GLP-1 – hTf by human epithelial Caco-2 cells *in vitro*
Concentration (ng/ml)
25
20
15
10
5
0
*
*
*
*
1 µg/ml
0.2 µg/ml
Media only
hTf standard
Plant-derived hTf
Plant-derived hTf – GLP-1
Plant-derived GLP-1 – hTf
B
Media only
hTf standard
Plant-derived hTf
Plant-derived hTf – GLP-1
Plant-derived GLP-1 – hTf
(i)
(ii)
C
Commercial hTf standard
Plant-derived hTf
Plant-derived GLP-1 – hTf
M
5µg
1µg
0.5µg
M
5µg
1µg
0.5µg
M
5µg
1µg
0.5µg

the amount of plant GLP-1 – hTf internalized correlated with the amount of the protein added to the Caco-2 cell culture. There was no detectable level of endogenous hTf protein in lysate from control Caco-2 cells (Figure 9A).

Visualization of internalized plant GLP-1 – hTf was confirmed by Western blot analysis. Figure 9B demonstrates anti-hTf antibody (i) and anti-GLP-1 antibody (ii) specifically reacted with a protein band at the expected sized (~80 kDa) of GLP-1 – hTf, suggesting plant GLP-1 – hTf remains structurally stable within Caco-2 cells. Figure 9C confirms the amount of plant GLP-1 – hTf internalized correlated with the amount of the protein added to the Caco-2 cell culture.

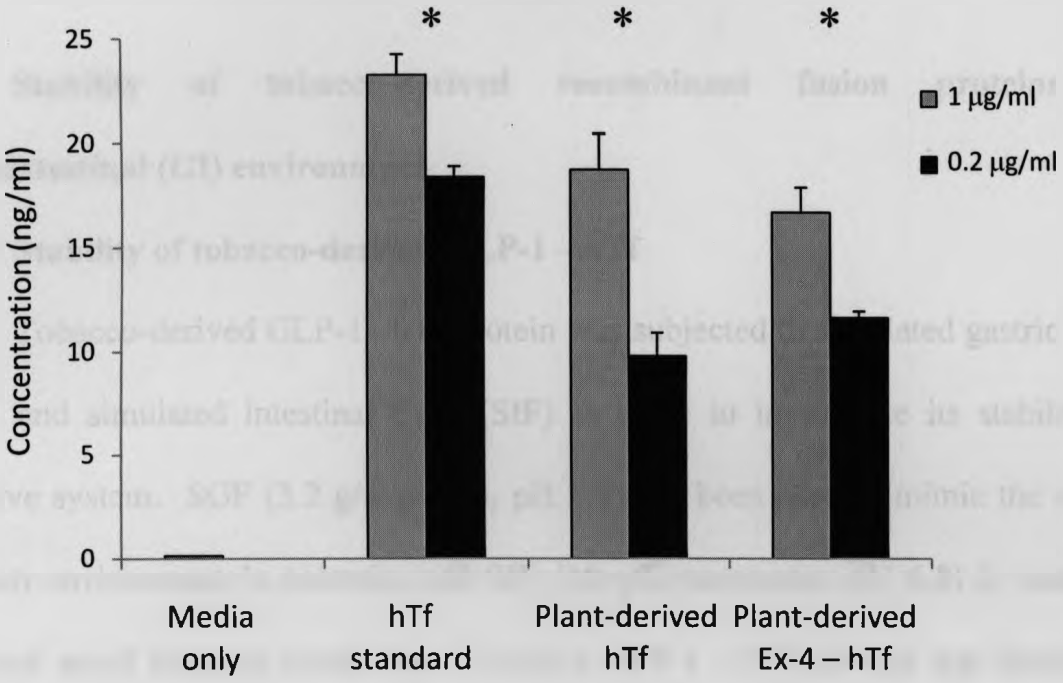
3.5.2 Internalization of tobacco-derived Ex-4 – hTf

Purified Ex-4 – hTf protein at two different concentrations (0.2 and 1 μg/ml) was incubated with human intestinal epithelial Caco-2 cells to determine its internalization capability *in vitro* as described in previous section. As shown in Figure 10, the recombinant Ex-4 – hTf was easily detected in lysate supernatant from Caco-2 cells incubated with recombinant Ex-4 – hTf. Internalized concentration of plant Ex-4 – hTf was significantly higher than cells treated with media only. The cellular concentration of plant Ex-4 – hTf was lower than those obtained with lysate supernatant from Caco-2 cells incubated with hTf standard, but similar to plant-derived hTf. The amount of plant Ex-4 – hTf internalized correlated with the amount of the protein added to the Caco-2 cell culture, which indicates dose-dependent uptake. There was no detectable level of endogenous hTf protein in lysate from control Caco-2 cells (Figure 10A).

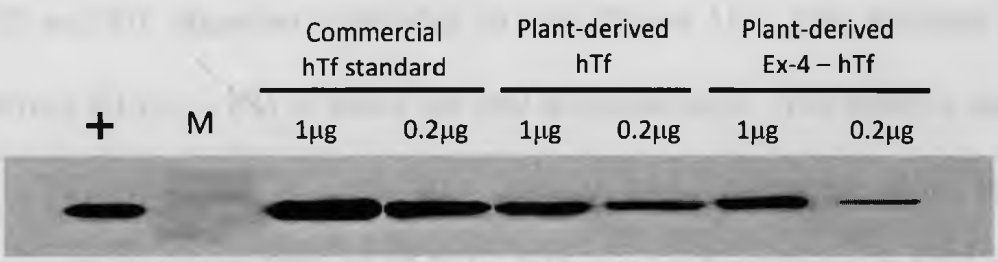
Figure 10.

Determination of tobacco-derived Ex-4 – hTf internalization by Caco-2 cells *in vitro*. Human intestinal epithelial Caco-2 cells were grown in serum-free SFF12 medium then incubated with plant-derived recombinant proteins to analyze its cellular internalization. (A) Each cell lysate was quantified by ELISA using goat anti-hTf antibody. The ELISA assay was performed in triplicate wells and repeated twice. Data represents the average concentration of internalized proteins in triplicate with error bars corresponding to standard deviation. The * indicates significant increase in internalization concentration compared to media only which was determined by paired two-tailed Student's *t*-test. (B) Immunoblot analysis of different concentrations (0.2 and 1 μg/ml) of hTf standard, plant-derived hTf, or plant-derived GLP-1 – hTf incubated with Caco-2 cells. M refers to SFF12 media without protein presence.

A **Cellular uptake of plant-derived Ex-4 – hTf by human epithelial Caco-2 cells *in vitro***



B



Visualization of internalized plant Ex-4 – hTf was confirmed by Western blot analysis of the same Caco-2 cell lysate (Figure 10B). In addition, Figure 10B shows the relative comparison of protein levels before and after acetic acid solution treatment.

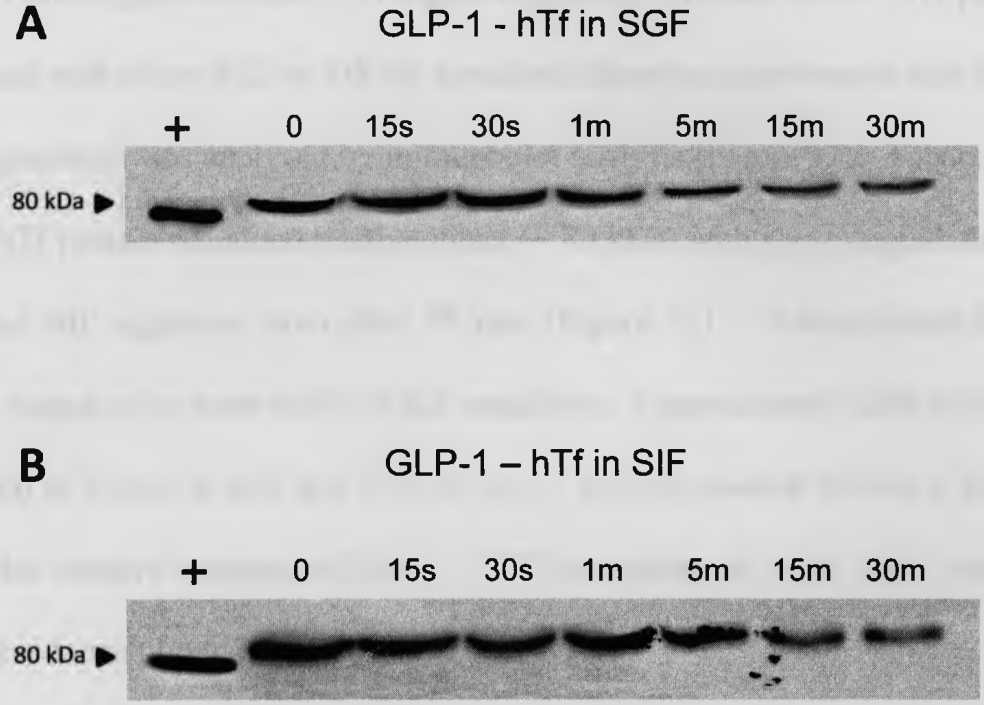
3. 6 Stability of tobacco-derived recombinant fusion proteins in gastrointestinal (GI) environment

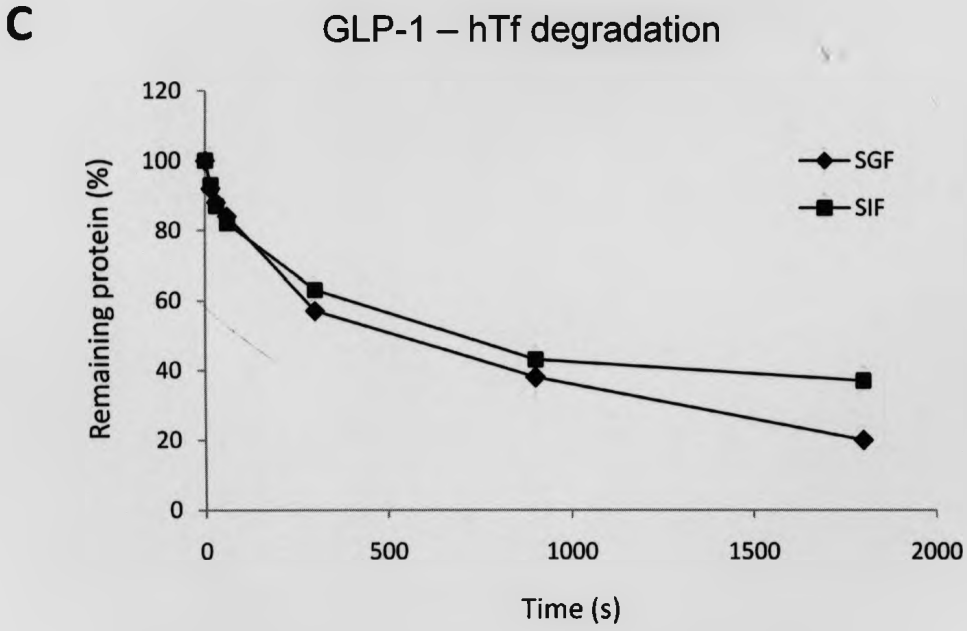
3.6.1 Stability of tobacco-derived GLP-1 – hTf

Tobacco-derived GLP-1 –hTf protein was subjected to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) in order to investigate its stability in digestive system. SGF (3.2 g/L pepsin, pH 2.5) has been used to mimic the acidic stomach environment in animals, and SIF (10 g/L pancreatin, pH 6.8) is used for proximal small intestine conditions. Purified GLP-1 – hTf protein was incubated with either SGF or SIF for simulated digestion experiments then the degradation of GLP-1 – hTf was analyzed by immunoblot analysis (Figure 11). Tobacco-derived GLP-1 –hTf protein remained relative intact (~ 80 kDa) with some degradation in both SGF and SIF digestion even after 30 min (Figure 11). This indicates that the plant-derived GLP-1 – hTf is stable for oral administration. The relative amount of GLP-1 – hTf remaining at each time interval was determined using ImageJ™ program. Approximately 20% of GLP-1 – hTf remained at 30 min in SGF and 37% of GLP-1 – hTf remained at 30 min in SIF (Figure 11C).

Figure 11.

In-vitro digestion of plant-derived GLP-1 – hTf protein in SGF and SIF. The recombinant GLP-1 – hTf protein was digested in SGF and SIF to test their stability in gastric intestinal environment. (A) The Western blot result of GLP-1 – hTf degradation in SGF from zero to 30 min demonstrates the stability of GLP-1 – hTf in low pH and pepsin digestion (SGF). (B) The Western blot result of GLP-1 – hTf degradation in SIF from zero to 30 min demonstrates the stability of GLP-1 – hTf in intestinal environment (SGF). As a positive control (+), non-digested plant-derived recombinant hTf – GLP-1protein was used. (C) Rate of degradation plotted using Image J program.


A
GLP-1 - hTf in SGF
+
0
15s
30s
1m
5m
15m
30m
80 kDa
B
GLP-1 – hTf in SIF
+
0
15s
30s
1m
5m
15m
30m
80 kDa

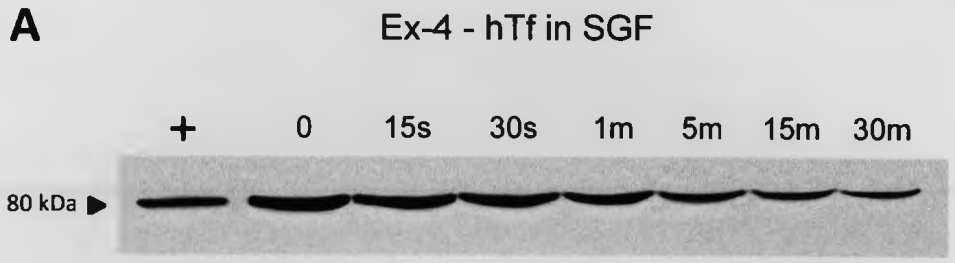

C
GLP-1 – hTf degradation
Remaining protein (%)
120
100
80
60
40
20
0
0
500
1000
1500
2000
Time (s)
SGF
SIF

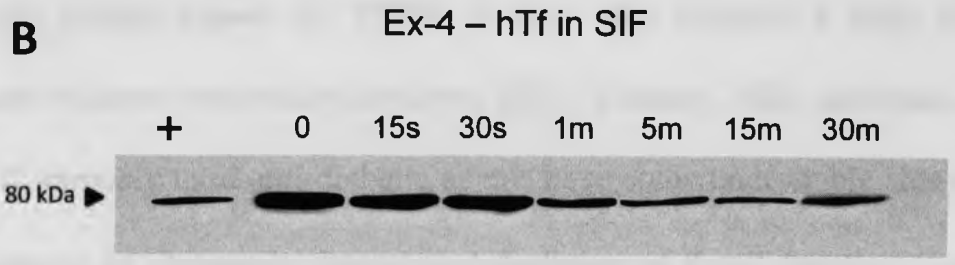
3.6.2 Stability of tobacco-derived Ex-4 – hTf

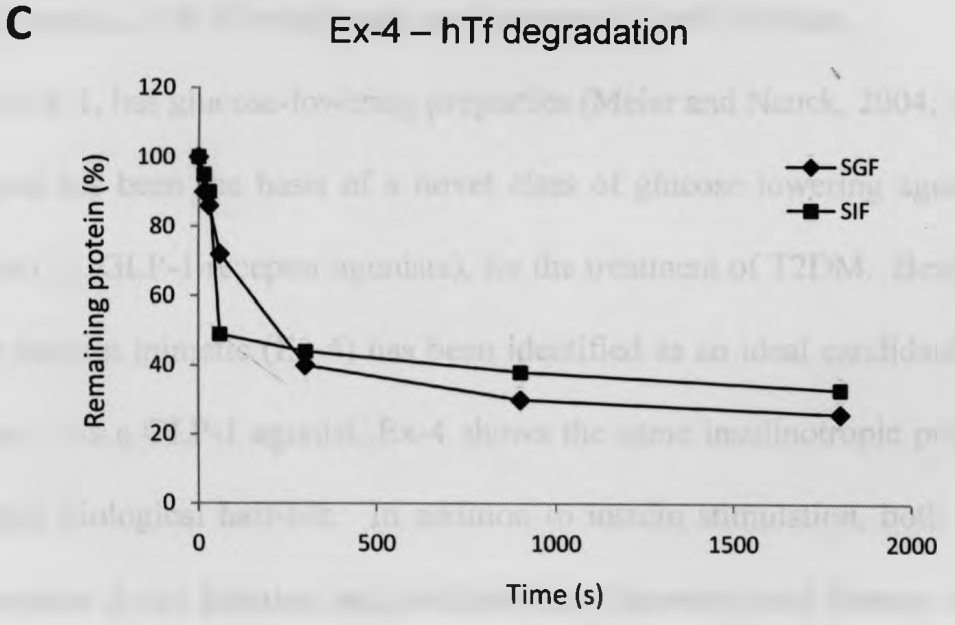
Tobacco-derived Ex-4 –hTf protein was also subjected to SGF and SIF in order to investigate its stability in digestive system. Purified Ex-4 – hTf protein was incubated with either SGF or SIF for simulated digestion experiments then the Ex-4 – hTf degradation was analyzed by immunoblot analysis (Figure 12). Tobacco-derived Ex-4 –hTf protein remained relative intact (~ 80 kDa) with some degradation in both SGF and SIF digestion even after 30 min (Figure 12). Recombinant Ex-4 –hTf protein seems to be more stable in SIF conditions. Approximately 26% of Ex-4 – hTf remained at 30 min in SGF and 33% of Ex-4 – hTf remained at 30 min in SIF (Figure 12C).The relative amount of Ex-4 – hTf remaining at each time interval was determined using ImageJ^{TM} program.

Figure 12.

In-vitro digestion of plant-derived Ex-4 – hTf protein in SGF and SIF. The recombinant Ex-4 – hTf protein was digested in SGF and SIF to test their stability in gastric intestinal environment. (A) The Western blot result of Ex-4 – hTf degradation in SGF from zero to 30 min demonstrates the stability of Ex-4 – hTf in low pH and pepsin digestion (SGF). (B) The Western blot result of Ex-4 – hTf degradation in SIF from zero to 30 min demonstrates the stability of Ex-4 – hTf in intestinal environment (SIF). As a positive control (+), non-digested plant-derived recombinant hTf – GLP-1protein was used. (C) Rate of degradation plotted using Image J program.


A
Ex-4 - hTf in SGF
+
0
15s
30s
1m
5m
15m
30m
80 kDa


B
Ex-4 – hTf in SIF
+
0
15s
30s
1m
5m
15m
30m
80 kDa


C
Ex-4 – hTf degradation
Remaining protein (%)
120
100
80
60
40
20
0
0
500
1000
1500
2000
Time (s)
SGF
SIF

Chapter 4

Discussion

Type 2 diabetes mellitus is a major global health problem with an expected prevalence in 380 million patients by 2025 (Jacobson, 2004). The health, social and economic burden caused by T2DM is great and presents a huge challenge to healthcare systems worldwide (de Groot, 2001; Wanless, 2002; Jacobson, 2004).

Commonly used anti-diabetic agents have some undesirable side effects such as worsening of glycaemic control and declining of β-cell function (Karaca et al., 2009). There is a need for newer therapies which can provide low risk of hyperglycaemia, lack of weight gain and improved β-cell function.

GLP-1, has glucose-lowering properties (Meier and Nauck, 2004; Nauck et al., 2003) and has been the basis of a novel class of glucose lowering agents, incretin mimetics (i.e. GLP-1 receptor agonists), for the treatment of T2DM. Besides GLP-1, another incretin mimetic (Ex-4) has been identified as an ideal candidate for T2DM treatment. As a GLP-1 agonist, Ex-4 shows the same insulinotropic properties and has longer biological half-life. In addition to insulin stimulation, both GLP-1 and Ex-4 improve β-cell function and proliferation. Incretin-based therapy has gained a huge interest following the observation that oral glucose produced a greater insulin

response compared to equivalent intravenous glucose (Elrick et al., 1964). However, in T2DM patients, the level of naturally-occurring incretin is greatly reduced which results in a lower insulin secretion (Nauck et al., 1986b). This prompts for the development of recombinant GLP-1 and Ex-4 production to meet the increasing demand of incretin therapy.

In previous studies, hTf-fusion technology has provided an effective method for the delivery of pharmaceutical agents (Jiang et al., 2007; Qian et al., 2002). The cellular internalization and the abundance of hTf receptors in intestinal lining make hTf an ideal candidate for transport of anti-diabetic agents for oral administration.

In this study, transgenic tobacco plants were used to synthesize GLP-1 – hTf and Ex-4 – hTf fusion proteins. The results demonstrated the transgenic plants stably accumulated the recombinant proteins which retained the original biological activities. Furthermore, tobacco-derived GLP-1 – hTf and Ex-4 – hTf proteins appear to be stable in GI environment which allows for oral administration.

4.1 Research findings

The main purpose of this research was to investigate a novel strategy to produce therapeutic GLP-1 – hTf and Ex-4 – hTf fusion proteins. To serve this purpose, the major objective of the study was to express and accumulate biologically active synthetic GLP-1 – hTf and Ex-4 – hTf fusion in transgenic tobacco plants intended as a novel source of therapeutic production.

Biological activity of the tobacco-derived GLP-1 – hTf and Ex-4 – hTf fusion proteins were confirmed by their ability to stimulate insulin secretion in vitro from a mouse pancreatic MIN6 cell line. Furthermore, cellular uptake of hTf fusion proteins was achieved by human intestinal epithelial Caco-2 cell line. This thesis is the first study to demonstrate the viable accumulation of recombinant GLP-1 – hTf and Ex-4 – hTf fusion proteins in transgenic tobacco.

Both GLP-1 and Ex-4 are small peptides with molecular mass of about 3 kDa. The GLP-1 – hTf and Ex-4 – hTf fusion strategy was intended to enhance recombinant protein stability, accumulation and allow for oral administration. A binary plant expression vector was generated for constitutive expression of synthetic GLP-1 – hTf or Ex-4 – hTf. Tobacco plant was either transiently or stably transformed by *Agrobacterium*-mediated gene transfer. Immunoblot analysis using anti-hTf antibody detected band of appropriate molecular mass (~80 kDa) corresponding to the size of GLP-1 – hTf and Ex-4 – hTf fusion proteins. No bands were detected with immunoblotting under identical conditions in untransformed wild-type tobacco control. Stability of fusion protein in GI environment was tested with SGF and SIF incubation. Both proteins remain intact after 30 min of incubation in both SGF and SIF, which indicated their stability in simulated gut environment.

4.2 Novel expression platform for recombinant anti-diabetic agents

The use of transgenic plant tissue for recombinant therapeutics has considerable clinical appeal not only for efficiency but also the simplicity of

production. Further advantages of transgenic plants include low cost, absence of risk from human pathogens, and perhaps if used in edible plants, it allows increase patient acceptance.

The GLP-1, Ex-4 and hTf proteins have been well characterized, with many associated therapeutic applications. However, if these proteins are to have significant future therapeutic use, an efficient and economical production system must be achieved. Bacterial and yeast systems have been used to produce these proteins as monomers or as fusion proteins (Kim et al., 2010; Chen et al., 2007; Hou et al., 2007a, b). However, these attempts were proven unsuccessful, generated non-functional proteins or have limitations with large scale production (Chen et al., 2007; Hou et al., 2007a, b). In the present study, transgenic tobacco plants were assessed as an alternative system for the production of GLP-1 – hTf and Ex-4 – hTf fusion proteins. The main goal of this research was to establish a novel bioreactor for fusion protein production that could overcome the limitations of traditional expression systems. Immunoblot analyses have confirmed the accumulation of GLP-1 – hTf and Ex-4 – hTf fusion proteins in transgenic tobacco lines (Figure 6 and 7). The result showed a band size corresponding to the molecular size of the fusion proteins (~ 80 kDa). Furthermore, data gained from ELISA results indicate that tobacco-derived GLP-1 – hTf accumulated up to 3.93% of plant TSP using transient transformation (Figure 6). Tobacco-derived Ex-4 – hTf accumulated 0.27% and 4.2% TSP using stable nuclear transformation and transient transformation respectively (Figure 7). Transient expression of both fusion proteins achieved high accumulation level. This

result suggests that large amount of therapeutics can be generated by plant bioreactor in a short period of less than a week.

The accumulation level of stably transformed Ex-4 – hTf is comparable to that of tobacco-derived GLP-1 decamer with 0.15% of TSP and tobacco-derived hTf with 0.25% of TSP (Brandsma et al., 2009). This is an encouraging accumulation level, suggesting that the expression and stability of plant-derived proteins are enhanced by fusion technology. In general, the expression level of stably transformed plants does not exceed 0.4% of TSP (Ma et al., 2008). However, the production of a stable expression platform provides long-term, low cost therapeutic proteins, typically required where the therapeutic agent is commonly used in multiple individuals over a long period of time.

4.3 Insulinotropic property of plant-derived GLP-1 – hTf and Ex-4 – hTf

GLP-1 and Ex-4 are potent insulinotropic peptide hormones, making them attractive drug candidate for T2DM treatment. The insulinotropic activity of the plant-derived GLP-1 – hTf and Ex-4 – hTf fusions was determined by their ability to stimulate insulin secretion from a mouse pancreatic β-cell line (MIN6) *in vitro*. The MIN6 cell line exhibits characteristics of glucose metabolism and glucose-dependant insulin secretion similar to those of normal islets (Miyazaki et al., 1990). Compared to the negative control, the concentration of insulin was significantly ($p<0.05$) higher in MIN6 cells incubated with plant-derived GLP-1 – hTf or Ex-4 – hTf (Figure 8). The results of the study also indicated the insulin concentrations of MIN6 cell-

supernatant collected after incubation with either plant-derived GLP-1 – hTf or Ex-4 – hTf are comparable to that of cell-supernatant collected after incubation with commercial GLP-1 standard (Figure 8). Furthermore, plant-derived GLP-1 – hTf and Ex-4 – hTf stimulated insulin secretion in a dose-dependent manner that is similar to GLP-1 standard. The insulinotropic activity of plant-derived hTf – GLP-1 was greatly reduced compared to plant-derived GLP-1 – hTf. This could be attributed to the fusion strategy. The presence of histidine at position 7 (His^7) in GLP-1 and Ex-4, as a free N-terminal amino acid, is critical for protein function (Xiao et al., 2001; Chang et al., 2002). The placement of hTf at the N-terminus of GLP-1 blocks His^7 and may have negative effect on the bioactivity of the transgenic protein. Plant-derived GLP-1 – hTf and Ex-4 – hTf were designed with barley alpha-amylase signal peptide at its N-terminal end and this signal peptide is removed by a protease to expose His^7 in GLP-1 for proper function of the protein.

4.4 Cellular delivery of plant-derived GLP-1 – hTf and Ex-4 – hTf

Plant-derived hTf fusion proteins may provide an ideal vehicle for oral drug delivery. Moreover, hTf undergoes endocytosis through the epithelial lining of the intestine, adding to its potential as a carrier for therapeutic protein delivery. Recombinant hTf-fusion proteins have shown the ability of hTf to transport the whole fusion through the GI tract and into systemic circulation following oral administration (Li and Qian, 2002; Shah and Shen, 1996; Widera et al., 2004). In this study, human epithelial cell line (Caco-2) was used to determine the

internalization of plant-derived GLP-1 – hTf and Ex-4 – hTf fusion proteins. The ELISA data indicate that internalization efficiency of plant-derived GLP-1 – hTf and Ex-4 – hTf was significantly higher than that of the negative control and was comparable to that of commercial hTf standard (Figure 9 and 10).

Cellular uptake of plant-derived GLP-1 – hTf and Ex-4 – hTf was confirmed by immunoblot analysis (Figure 9 and 10). The result showed a protein band at the expected sized (~80 kDa) of GLP-1 – hTf and Ex-4 – hTf, suggesting plant GLP-1 – hTf and Ex-4 – hTf were internalized and remained structurally stable within Caco-2 cells. Both ELISA and immunoblot results indicate that internalization of plant-derived GLP-1 – hTf and Ex-4 – hTf into Caco-2 cells occurs in a dose-dependent manner similar to plant-derived hTf protein. This shows that the fusion protein does not affect the internalization ability of hTf. This is important to the successful application of hTf as a carrier for targeted delivery of peptide and protein drugs.

4.5 Production of stable recombinant proteins for oral consumption

In-vitro digestion of tobacco-derived GLP-1 – hTf and Ex-4 – hTf under simulated gastric and intestinal conditions proved it to be stable. There was some degradation of the protein after 30 min incubation, however more than 20% of the proteins remained intact after both treatments (Figure 11-13). These results suggest that the plant-derived GLP-1 – hTf and Ex-4 – hTf hold great promise for oral delivery of protein and peptide drugs. 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. 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 GI (low pH and various proteinases and peptidases). Therefore, this result shows that hTf fusion strategy offers a promising delivery system for oral administration of protein drugs.

4.6 Future directions

The use of transgenic tobacco as a production platform for anti-diabetic agents was assessed and the results are very promising. Nevertheless, there are still questions that require further investigation regarding the activity of recombinant GLP-1 – hTf and Ex-4 – hTf.

The advantage of using GLP-1 and Ex-4 compared to traditionally available anti-diabetic agents is their ability to improve pancreatic β-cell function and to stimulate β-cell proliferation (Holst et al., 2009). Preclinical studies with GLP-1 and Ex-4 have demonstrated that, in addition to stimulating insulin secretion, they are capable of improving islet glucose responsiveness and stimulating insulin biosynthesis (Drucker, 2006). The effect of fusion proteins on the other function of the β-cell must be examined in order to verify the full capacity of plant-derived GLP-1 – hTf and Ex-4 – hTf proteins.

In the present study, the biological activities of plant-derived GLP-1 – hTf and Ex-4 – hTf proteins were analyzed prior to SGF and SIF digestion. Since plant-

derived fusion proteins were generated for the oral administration of recombinant proteins, the biological assay could be performed after SGF and SIF treatments. Although there were intact proteins remained after 30 min of digestion, the functionality of the proteins might have been hindered by acidic conditions. Therefore, *in vitro* assay could be repeated with SGF/SIF-digested proteins to fully validate the stability and functionality of these proteins.

Functionally active recombinant GLP-1 – hTf and Ex-4 – hTf have been synthesized in yeast and resulting proteins were tested on diabetic (*db/db*) mice (Kim et al., 2010). To provide further insight on T2DM, the comparison of therapeutic potency of plant-derived GLP-1 – hTf and Ex-4 – hTf need to be done using *in vivo* animal model. A long-term study is needed to establish the efficiency of plant-derived fusion proteins for T2DM therapy.

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