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## A Plant-Made Vaccine Candidate To Protect Ruminants Against Shiga Toxin-Producing Escherichia Coli

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Graduate Program in Biology

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

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**A PLANT-MADE VACCINE CANDIDATE TO PROTECT RUMINANTS  
AGAINST SHIGA TOXIN-  
PRODUCING *ESCHERICHIA COLI***

(Thesis format: Monograph)

by

Sean, Miletic

Graduate Program in Biology

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

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

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## **ABSTRACT**

Shiga toxin-producing *Escherichia coli* (STEC) are enteropathogens colonizing the digestive tracts of humans and animals worldwide. STEC are shed in the manure of cattle and frequently contaminate beef products, vegetables, and drinking water. Immunizing cattle herds against STEC is a promising strategy to reduce STEC colonization in cattle and therefore decrease contamination in the food supply. The goal of this project is to produce a plant-made vaccine to protect ruminants against STEC. Several recombinant proteins were designed as vaccine candidates and expressed transiently in *Nicotiana benthamiana* and transplastomically in *Nicotiana tabacum*. One vaccine candidate, an *E. coli* secreted protein A (EspA) fusion, accumulated in transplastomic lines up to 220.7 mg/kg of fresh leaf weight. Leaf tissue containing this antigen was lyophilized and will be orally administered to a sheep ruminant model to test vaccine efficacy.

**Key words:** Plant biotechnology, agroinfiltration, chloroplast transformation, tobacco, Shiga toxin-producing *Escherichia coli*, vaccine

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## LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bp	Base pair
BSA	Bovine serum albumin
BY-2	Tobacco bright yellow-2 cell line
CaMV	Cauliflower mosaic virus
CBD	Cellulose binding domain
CEC4	Chloroplast expression cassette 4
CEC5	Chloroplast expression cassette 5
DAEC	Diffusely Adherent <i>E. coli</i>
EAEC	Enteroadgregative <i>E. coli</i>
EDTA	Ethylenediaminetetracetic acid
EHEC	Enterohemorrhagic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
ELP	Elastin-like polypeptide
Esp	<i>E. coli</i> secreted protein
Esps	<i>E. coli</i> secreted proteins
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GOI	Gene of interest
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HFBI	Hydrophobin-I
HFBs	Hydrophobins
HRP	Horseradish peroxidase
HUS	Hemorrhagic uremic syndrome
IEE	Intercistronic expression element
IgG	Immunoglobulin G
IgA	Immunoglobulin A
ITC	Inverse transition cycling
kb	Kilobase
KDEL	ER retrieval signal
L	Linker
LB	Lysogeny broth
LEE	Locus of enterocyte effacement
MES	2-( <i>N</i> -morpholino)ethanesulfonic acid
NleA	Non-LEE encoded factor A
nos	nopaline synthase
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonylfluoride
Pr1b	Pathogenesis-related protein 1b of tobacco
psbA	Photosystem II protein D1
PTGS	Post-translational gene silencing

PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpolypyrrolidone
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase large chain
rbcS	Ribulose-1,5-bisphosphate carboxylase/oxygenase small chain
RT	Reverse transcription
SD	Shine Dalgarno
STEC	Shiga toxin-producing <i>E. coli</i>
Stx	Shiga toxin
Stx2b	Shiga toxin 2 B subunit
TBS	Tris-buffered saline
T3SS	Type three secretion system
tCUP	Tobacco cryptic upstream promoter
T-DNA	Transfer DNA
TEV	Tobacco etch virus
Ti	Tumor inducing
Tir	Translocated Intimin receptor
TMV	Tobacco mosaic virus
TSP	Total soluble protein
trnA	Transfer RNA alanine
trnI	Transfer RNA isoleucine
UTR	Untranslated region
VTEC	Verocytotoxin-producing <i>E. coli</i>

## 1. INTRODUCTION

### 1.1 Shiga toxin-producing *Escherichia coli*

Within hours after birth, bacteria colonize the gastrointestinal tract of infants and other mammals, beginning a lifelong symbiotic relationship where the host benefits from proper digestion and the bacteria are provided with an optimal, growth-supporting environment (Guarner and Malagelada, 2003). However, this mutualism can be short-lived. Consumption of contaminated food and water can introduce pathogenic bacteria to the gastrointestinal tract, causing a wide range of pathologies in animals and in humans.

Enteric *Escherichia coli* are part of the normal intestinal flora in humans however certain pathotypes have acquired toxic traits during their evolution. These traits are encoded on genetic elements that are either fixed in their genomes or are mobile and can move between strains, creating new combinations of virulence factors (Kaper et al., 2004). Strains acquiring highly virulent combinations have led to the evolution of six prevalent pathotypes characterized by their clinical outcomes in humans: Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), and Diffusely Adherent *E. coli* (DAEC) (Clements et al., 2012; Kaper et al., 2004). All EHEC produce Shiga toxins, originally named verocytotoxins, and are referred to as verocytotoxin-producing *E. coli* (VTEC) or more commonly, Shiga toxin-producing *Escherichia coli* (STEC). The latter designation will be used for the remainder of this thesis.

STEC is a major foodborne pathogen causing a variety of digestive complications in humans worldwide. STEC infection occurs predominantly in children and causes watery diarrhea which may be followed by bloody stool, severe abdominal cramps, and vomiting, clinically referred to as hemorrhagic colitis (Cleary, 2004). Around 5-8% of children with hemorrhagic colitis will develop hemorrhagic uremic syndrome (HUS) a condition characterized by acute kidney failure which can lead to permanent renal impairment or even death (Cleary, 2004; Mele et al., 2014).

STEC can be further classified into different serotypes depending on their O (lipopolysaccharide) and H (flagellar) antigens. Hundreds of STEC serotypes have been identified that cause disease in humans. *E. coli* O157:H7 is the most frequently isolated serotype in North America, but there are six other prevalent serogroups responsible for foodborne illness which are found across the continent: O26, O45, O103, O111, O121, and O145 (Brooks et al., 2005). There are an estimated 231,157 cases of STEC human infection in the United States each year with 93,094 cases caused by O157:H7 (Hale et al., 2012). Worldwide, it is estimated that STEC causes 2.8 million acute illnesses annually with 3,890 cases of HUS and 230 of these cases are estimated to cause death (Majowicz et al., 2014). STEC infection causes a considerable economic burden through medical care, loss of productivity, a decrease in quality of life, and death (Smith et al., 2014). Each O157:H7 human infection is estimated to cost \$10,048 while non-O157 STEC infections are estimated to cost \$1,334 per case in the United States with a total economic burden of \$635 and \$154 million per year, respectively (Scharff, 2012). The financial burden of non-O157 infections is lower as there is no estimate of the number of deaths, which accounts for the majority of the cost.

Cattle along with other ruminants are considered the primary reservoir of STEC (Beutin et al., 1993; Montenegro et al., 1990). The bacteria colonize cattle throughout the gastrointestinal tract (Brown et al., 1997) and are shed on the surface of cattle feces (Naylor et al., 2003). *E. coli* O157 shedding is highly variable and usually transient-lasting roughly a month (Besser et al., 1997). However, certain animals have been identified as “super shedders” and can shed *E. coli* O157:H7 at higher numbers, for longer periods, and are responsible for greater than 96% of all *E. coli* O157:H7 shed even though these animals consist of only 9% of the herd (Omisakin et al., 2003). Reasons as to why these individual animals shed STEC at such higher levels remain largely unknown. There appears to be no distinct genotype of *E. coli* O157:H7 responsible for super shedding (Arthur et al., 2013) and this phenomenon is probably caused by differences between cattle (Jeon et al., 2013). For instance, where the bacteria colonize in the gastrointestinal tract may impact the duration or amount of STEC shedding. Several studies have shown a link between colonization of the rectal-anal junction (RAJ) near the end of the cattle digestive system and high-level shedding (Cobbold et al., 2007; Naylor et al., 2003). However, it is worth mentioning that *E. coli* O157:H7 has been found in several sites throughout the intestinal tract of persistently shedding cattle (Baines et al., 2008b).

Interestingly, cattle show variation in the severity of symptoms depending on their age. Other than neonatal calves, which display symptoms ranging from diarrhea to enterocolitis (Dean-Nystrom et al., 1997), weaned calves are not susceptible to *E. coli* O157:H7 pathogenesis (Brown et al., 1997) and adult cattle are asymptomatic carriers when infected (Cray and Moon, 1995). However, some intestinal pathology has been

observed in adult animals infected with O157:H7 (Baines et al., 2008a). The mechanism behind the asymptomatic nature of STEC infection in adult cattle needs further elucidation and could explain why these animals are so frequently colonized.

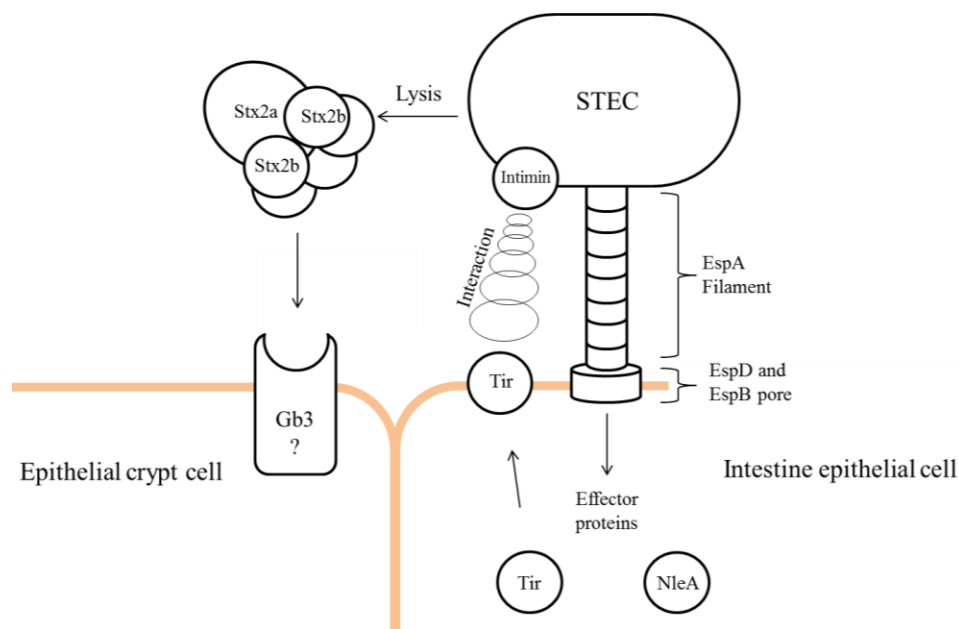
Shiga toxin-producing *E. coli* are transmitted to humans by direct or indirect contact with cattle feces. Humans primarily become infected with STEC through consumption of beef or dairy products (Armstrong et al., 1996). Consumption of undercooked ground beef is the main source of infection as the meat can be easily contaminated with cattle feces during butchering (Elder et al., 2000; Griffin and Tauxe, 1991). STEC outbreaks have also been linked to contaminated vegetables, drinking water, and direct contact with infected animals (Hilborn et al., 1999; Salvadori et al., 2009; Stirling et al., 2008). STEC have a low infectious dose with fewer than 700 bacteria present in raw ground beef being sufficient to cause infection (Tuttle et al., 1999). To emphasize this point, even swimming in water contaminated with STEC can cause infection (Keene et al., 1994).

STEC infection stems from its ability to colonize the gastrointestinal tract of ruminants, humans, and many other species. Colonization is very complex and 59 STEC genes are reported to be involved in the process (Dziva et al., 2004). The hallmark of STEC colonization is the formation of attaching and effacing (A/E) lesions on host intestinal epithelial cells. The A/E lesion consists of brush border microvilli destruction and the attachment of the bacterium to the host through a pedestal-like structure composed of actin (Donnenberg et al., 1997). The genes coding for this histopathology are encoded on a 43 kb pathogenicity island called the locus of enterocyte effacement (LEE) acquired through horizontal transfer during STEC evolution (Perna et al., 1998).

The LEE contains 51 open reading frames (ORFs) coding for gene regulators, chaperones, the virulence factor Intimin, the type three secretion system (T3SS), and many secreted effector proteins (Wong et al., 2011).

The T3SS is a needle-like structure used by several pathogens to inject virulence factors into the host cell cytosol (Hueck, 1998). STEC uses the T3SS to secrete several effector proteins into intestinal epithelial cells to form the A/E lesions. The T3SS consists of a filamentous structure made up of multiple *E. coli* secreted protein A (EspA) subunits (Delahay et al., 1999; Knutton et al., 1998) (Summarized in Figure 1). These subunits polymerize to form a hollow tube-like structure to translocate effector proteins through a pore, composed of EspB (Kenny and Finlay, 1995) and EspD (Lai et al., 1997) proteins, formed on the surface of the intestinal epithelial cells (Fivaz and van der Goot, 1999; Warawa et al., 1999). Once assembled, at least 39 effector proteins are secreted into the host cell (Tobe et al., 2006) which alter several host cell processes, enabling the bacteria to colonize and ultimately cause disease (Wong et al., 2011). The non-LEE-encoded effector A (NleA) (Gruenheid et al., 2004) is secreted through the T3SS and is believed to disrupt intestinal tight junctions and inhibit protein trafficking in the host cells (Kim et al., 2007; Thanabalasuriar et al., 2010). One of the most important effector proteins, the translocated Intimin receptor (Tir) is also secreted and integrates into the host plasma membrane where it serves as a receptor for the bacterial outer membrane protein, Intimin (Kenny et al., 1997). This binding results in the intimate attachment of the bacterium to the host cell and, with the help of other effector proteins, causes the polymerization of actin underneath the adherent bacteria leading to pedestal formation characteristic of A/E lesions (Garmendia et al., 2004).





**Figure 1. Schematic outlining the T3SS-mediated colonization of intestinal epithelial cells by STEC.** STEC use the T3SS consisting of a filament composed of EspA, and an EspD/EspB pore to inject several effector proteins including Tir and NleA into host cells. Tir embeds in the host cell membrane to create a receptor for Intimin which binds and allows STEC to adhere to the intestinal epithelia. Shiga toxins are released through phage-mediated lysis and bind to Gb3 receptors reportedly found on cattle intestinal crypt cells. Stx2 is believed to also contribute to STEC colonization.

Although Shiga toxin-producing *E. coli* carry several virulence factors including toxins, adhesins, proteases, iron acquisition systems, liposaccharides, and flagellin (Gyles, 2007), the principal virulence factor responsible for HUS is the Shiga toxin. There are two toxins, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) and some serotypes produce one or the other, while others produce both (Strockbine et al., 1986). Stxs were

originally called “Shiga-like toxins” as Stx1 only differs from the Shiga toxin of *Shigella dysentererae* by one amino acid (Takao et al., 1988) and Stx2 shares a 60% sequence homology to Stx1 (Smith et al., 2014). Stx2 is 1000 times more toxic to human endothelial cells than Stx1 (Louise and Obrig, 1995) and is more likely to cause HUS (Siegler et al., 2003)

Shiga toxins are encoded on the genomes of lysogenic lambdoid prophages and are produced as a result of phage induction in response to UV irradiation or antibiotics (Fuchs et al., 1999). Therefore, antibiotics used to treat STEC infection tend to worsen the symptoms by promoting the release of Stxs (Wong et al., 2000; Zhang et al., 2000). Stxs are AB<sub>5</sub>-type toxins composed of one A subunit and five smaller B subunits. The B pentamer specifically binds to globotriaosylceramide (Gb3) receptors on host endothelial surfaces, allowing the toxin to be endocytosed. The A subunit contains two smaller subunits A1 and A2 which are cleaved and the A1 subunit targets 28S RNA of the 60S ribosomal subunit inhibiting protein synthesis and causing cell apoptosis (Ivarsson et al., 2012). Cell surface expression of Gb3 is required for Stx toxicity (Jacewicz et al., 1994) and in humans Gb3 is restricted to the microvasculature of the kidneys (Obrig et al., 1993), endothelial cells in the lamina propria and paneth cells (Schuller et al., 2007), platelets (Cooling et al., 1998), and to B lymphocytes (Murray et al., 1985). Cattle appear to lack the Gb3 receptor in their GI tracts (Pruimboom-Brees et al., 2000) or they are at least confined to epithelial crypt cells (Hoey et al., 2002) which may explain their lack of symptoms from STEC infection. However, Stxs are reported to still be toxic to cattle enterocytes and Stx2 has been shown to contribute to STEC colonization (Baines et al., 2008a).

Limiting bacterial colonization in livestock through cattle immunization is an effective strategy to reduce STEC infection in humans. A vaccine causing a 50% reduction of STEC shedding from cattle can reduce human infection by nearly 85% (Matthews et al., 2013). There have been a multitude of attempts to create an effective vaccine to reduce STEC infection in cattle. Most efforts have been focused on priming the animal's mucosal immune system against secreted proteins involved in colonization. Vaccination with purified EspA and other secreted proteins significantly reduced *E. coli* O157:H7 shedding in cattle (Potter et al., 2004). Purified antigens Intimin, Tir, and EspA significantly reduced colonization in calves (McNeilly et al., 2010) and immunization with EspA, EspB, Intimin, and inactivated Stx2 produced antibodies in pregnant cattle which were transferable to calves (Rabinovitz et al., 2012). A recombinant vaccine containing EspA, Intimin, and Stx2 protected mice against *E. coli* O157:H7 (Gu et al., 2009). An EspA-Stx2a1 subunit vaccine was shown to protect mice from Stx2 (Cheng et al., 2009) yet vaccinating calves with EspA induced Immunoglobulin G (IgG) and Immunoglobulin A (IgA)-specific responses but failed to protect cattle against STEC colonization (Dziva et al., 2004). Similarly, vaccination with Intimin alone failed to reduce the duration of *E. coli* O157:H7 secretion (van Diemen et al., 2007). Based on these studies, it appears that the most promising vaccines contain multiple effector proteins and components of the T3SS.

Unfortunately, vaccination with T3SS proteins induces the production of serotype-specific antibodies (Asper et al., 2007) as T3SS proteins from different serotypes are antigenically distinct. To the best of my knowledge there is no currently available vaccine offering protection against multiple serotypes of STEC. Thus, future vaccines should

contain a variety of recombinant proteins from different STEC serotypes to generate a multi-serotype immune response and provide better protection against STEC.

## 1.2 Using plants to produce recombinant proteins

Plants are invaluable bio-production systems supplying humans with food, fiber, wood, and therapeutics. Early on, our ancestors began genetically manipulating plants through selective breeding to create new varieties with enhanced nutritional value, vigorous growth rates, and increased stress tolerance. The advent of molecular biology made it possible for scientists to precisely engineer several plant species and in 1983, research groups produced the first genetically modified (GM) plants, transgenic tobacco and sunflower (De Block et al., 1984; Horsch et al., 1984; Murai et al., 1983). Since then, several crop species have been genetically engineered to boost agricultural yield leading to the development of many GM crops planted today on over 175 million hectares globally (James, 2013).

Because plants share similar transcriptional, translational, and post-translational machineries with other eukaryotic and prokaryotic organisms, it was postulated that plants could be engineered into living bioreactors capable of producing valuable biologicals for human use. Hence, human growth hormone was the first pharmaceutical made in plants (Barta et al., 1986) and three years later the first recombinant protein, a functional mouse IgG antibody, was produced in *Nicotiana tabacum* or tobacco (Hiatt et al., 1989). These initial results enticed many scientists to further develop plants as production platforms to produce a variety of recombinant proteins for clinical, veterinary, and industrial uses. This seeded the growing field of *molecular pharming* where scientists “pharm” plants for pharmaceuticals and biologicals (Xu et al., 2012).

The major advantage of using plant bioreactors is the low capital and running costs required for large-scale production compared to existing bacterial, insect, or mammalian cell bioreactors (Twyman et al., 2003). Plants can be cheaply grown using current farming techniques and in greenhouses or in the field as long as measures are taken to ensure transgene containment. Depending on the application, recombinant proteins can be stored and administered in edible, unprocessed plant material removing costs associated with protein purification, vaccine formulation, and cold storage (Fischer et al., 2004). Plants can also correctly fold and modify complex proteins which bacterial-based expression platforms cannot, and do not carry any human or animal pathogens negating some of the risks associated with mammalian cell cultures (Menassa et al., 2012; Twyman et al., 2003).

The earliest example of genetically transforming plants utilized the gram-negative bacterium, *Agrobacterium tumefaciens*. This bacterial species causes crown gall disease in plants by naturally transferring DNA into plant cells where it integrates into the host genome leading to the formation of tumours or galls (Chilton et al., 1977). *A. tumefaciens* carries a large tumour inducing (Ti) plasmid carrying a region of DNA called transfer DNA or T-DNA which is marked by flanking left and right border sequences. Through the use of several virulence proteins, this region is excised from the plasmid and is transferred to the host cell where it inserts into the plant genome (Gelvin, 2003). After this discovery, plant biologists began adapting *A. tumefaciens* to use as a tool to genetically engineer plants. Through the development of a binary vector system, virtually any DNA sequence could be cloned in between the left and right border sequences of the T-DNA region and integrated into the plant genome by infecting the plant with *A.*

*tumefaciens* (Krenek et al., 2015).

It was discovered that T-DNA gene expression transiently peaks two to four days in plant tissues after infection with *A. tumefaciens* and if selection is applied to the tissue, gene expression increases dramatically after 14 days (Janssen and Gardner, 1990). This transient peak results from the expression of free T-DNA copies in the cell nucleus that have not integrated into the genome. Most of these free T-DNA species are degraded within a few days causing an initial decrease in transgene expression. The following increase in expression several days later can be explained by the expression of select T-DNA copies which have stably integrated into the plant genome (Janssen and Gardner, 1990; Krenek et al., 2015). This stable transformation event is heritable and can be selected for, with antibiotics, to generate fully transgenic plants where every plant cell contains an integrated T-DNA copy in the nuclear genome (Krenek et al., 2015).

However, the ability of free T-DNA to be transiently expressed while in the nucleus has gained much attention from plant biotechnologists and biotech companies. *A. tumefaciens*-mediated transient transformation allows for the quick production of recombinant protein within a matter of days by infiltrating *A. tumefaciens* or “agroinfiltrating” cultures carrying a gene of interest into plant leaves using a syringe or a vacuum (Menassa et al., 2012). Since this technique does not rely on stable integration into the nuclear genome, transient transformation is fast, does not produce GM plant material, and can be easily adopted by countries with strict GM crop regulations. *Nicotiana benthamiana* is the plant species of choice for transient expression due to its amenability to agroinfiltration (Menassa et al., 2012). This, along with its small size and short life-cycle, also makes this species an efficient platform for the transient production

of proteins (Conley et al., 2011b). To date, many recombinant proteins have been produced using agroinfiltration and several biotech companies have scaled-up agroinfiltration using enormous, automated infiltration chambers with large volumes of *A. tumefaciens*, and are actively producing biologicals at an industrial scale.

An alternative strategy to nuclear transgenic or transient protein production is to use intracellular organelles such as the chloroplasts for recombinant protein production. Chloroplasts contain a circular genome, of roughly 130 genes in seed plants, which resembles the genome of an early ancestral cyanobacterial endosymbiont. This genome can be engineered for recombinant protein production which is alluring to plant biotechnologists for several reasons. Foremost, there are many copies of the chloroplast genome per chloroplast and several chloroplasts per plant cell, equating to a very high copy number, creating the possibility to express foreign proteins at much higher levels than nuclear-transformed plants. Also, gene integration occurs by homologous recombination which can be used to precisely insert transgenes into any locus. Due to their cyanobacterial nature, chloroplasts do not have any gene silencing mechanisms and many genes are arranged as operons. This allows for the stacking of multiple transgenes in a single reading-frame which can be highly useful for metabolic pathway engineering (Bock, 2014c).

Chloroplast engineering would have remained impractical for large-scale protein production without the invention of the gene gun, a tool which bombards living tissue with microparticles coated in DNA. This allows for the biolistic (biological + ballistic) transformation of DNA into nuclear or organelle genomes of plants, animal cells, yeast, and bacteria (Sandford, 2000). The chloroplast genome was first transformed using

biolistic transformation in the unicellular alga, *Chlamydomonas reinhardtii* (Boynton et al., 1988). The plastid genome of tobacco was transformed soon after (Svab et al., 1990a) and the plastids of a few other plant species have been transformed since. Tobacco has remained the model organism of choice for transplastomic research and is one of the most established plant bioreactors in the field due to its high biomass and the fact that it is a non-food and non-feed crop, removing any potential risk of contamination in the food supply (Twyman et al., 2003). A plethora of research has been published documenting a wide variety of recombinant proteins produced using the transplastomic (transgenic + plastome) method. Despite these successes, industry has been slow to adopt this technology and no transplastomic crops are currently grown commercially.

Both transient and transplastomic technologies can be used to produce a subunit vaccine against STEC colonization in ruminants. A dual approach will allow for the quick screening of recombinant protein production in plants by transient transformation and for the generation of transplastomic bioreactors for high-level protein production. Once generated, transplastomic plants producing this vaccine could be grown in farm fields at a low cost and at a very large scale. Accumulating protein can be stored in lyophilized plant tissue at room temperature and can be directly fed to animals as an oral immunization. This will reduce the cost of vaccine formulation, ease administration to large cattle herds, and increase the likelihood of vaccine uptake by the beef cattle industry. Such a vaccine would be highly valuable for Canadians through revenue generated from this novel product and through a reduction of the number of STEC outbreaks and subsequent cases of human foodborne illness.



Since plant bioreactors have been proven to produce a variety of recombinant proteins, this Master's thesis is goal-oriented instead of hypothesis driven. The primary goal of this project is to produce a recombinant, multi-subunit vaccine using plant bioreactors to reduce STEC colonization in cattle. The objectives of this study are as follows:

### **1.3 Objectives**

1. Design gene constructs for the subunit vaccine candidates for transient expression in *N. benthamiana* and stable plastid expression in *N. tabacum*.
2. Transiently express constructs in *N. benthamiana* and quantify protein accumulation to determine the highest accumulating recombinant proteins and the best subcellular compartment for protein targeting.
3. Stably transform the gene constructs into the *N. tabacum* plastid genome and quantify protein accumulation.
4. Prepare lyophilized leaf tissue for immunological assays and STEC challenge experiments in sheep which will be completed by Dr. Tim McAllister and colleagues from Agriculture and Agri-Food Canada in Lethbridge, Alberta.

## **2 MATERIALS AND METHODS**

### **2.1 Gene Synthesis**

Peptide sequences for the gene candidates were obtained from the National Center for Biotechnology Information (NCBI) database and the accession numbers used for gene synthesis were: EspA O157:H7: AAG58820.1, EspA O26:H11: EHW67626.1, NleA O157:H7: ACT71538.1, Tir O157:H7: AAG58825, Stx2b: AAG55588.1, EspD O157:H7: AAG58819.1, and Colicin E1: AAA87379.1. Peptide sequences were

submitted to BioBasic Inc. (Markham, Ontario) and gene sequences were created and optimized for nuclear expression in *N. benthamiana* for the transient assays or for plastid expression in *N. tabacum* for the transplastomic assays. All constructs were fused to a *StrepII* tag (Schmidt et al., 1996) for protein purification and the transplastomic constructs were also fused to a *c-Myc* tag for protein detection via Western blot. Gene sequences were then flanked with *attL* sites for Gateway® recombination into the transient expression cassettes or restriction enzyme sites for cloning into the chloroplast transformation vectors.

## **2.2. Transient Assays**

### **2.2.1 Gateway® cloning**

For transient expression of the proteins, gene constructs were recombined into previously constructed destination vectors using Gateway® cloning technology (Invitrogen, Thermo Fischer Scientific, Waltham, U.S.A.). Gene constructs were synthesized with flanking *attL* sites to allow the direct recombination into destination vectors using the LR reaction while bypassing the BP reaction. Briefly, 100 ng of pUC57 vector, carrying the different genes of interest (GOI), was recombined with 150 ng of destination vector using 0.5 µl of LR Clonase II Enzyme Mix (Invitrogen) overnight at room temperature. Proteinase K solution was added to each sample to terminate the reactions which were then incubated at 37°C for 10 minutes.

### **2.2.2. *E. coli* transformations**

pCAMgate vectors with the GOIs were transformed in *E. coli* XL1-Blue using the Gene Pulser II system (Bio-Rad Laboratories Inc., Hercules, U.S.A.). Briefly, 1.5 µl of LR reaction was added to 40 µl *E. coli* which was then electroporated. Cells were diluted

with 960 µl of lysogeny broth (LB), incubated for 1 hour at 37°C, 250 rpm (Innova® 42 Incubator, Eppendorf, Hamburg, Germany), and 20 and 200 µl were plated on LB plates containing 50 µg/µl of kanamycin. LB plates were incubated overnight at 37°C.

PCR screening using gene-specific primers was conducted to screen for positive transformants containing the correct insert. Positive colonies were used to inoculate 5 ml of LB medium with kanamycin (50 µg/µl) and grown at 37°C, 250 rpm overnight. Plasmid DNA was extracted using the QIAprep® Spin Miniprep Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocols. A double restriction enzyme digestion using *Kpn* I and *Xho* I was done on the purified plasmids and digested fragments were visualized on a 1% (w/v) agarose gel to ensure the insertion of the correct transgene.

### 2.2.3 *A. tumefaciens* transformation

Electro-competent *A. tumefaciens* EHA 105 cells were transformed as mentioned for *E. coli* except for the following modifications. Electroporated *A. tumefaciens* cells were incubated at 28°C at 250 rpm for one hour and afterwards, spread on yeast extract broth (YEB) plates containing 50 µg/ml kanamycin and 10 µg/ml rifampicin and incubated for two days at 28°C.

### 2.2.4 Transient expression in *N. benthamiana* plants

Transformed colonies of *A. tumefaciens* with the GOIs or *A. tumefaciens* carrying the gene silencing suppressor p19, were used to inoculate 3 ml of YEB with 50 µg/ml kanamycin and 10 µg/ml rifampicin and cultures were incubated overnight at 28°C at 250 rpm. These were then used to inoculate 10 ml of YEB infiltration culture containing 50

$\mu\text{g/ml}$  kanamycin and  $10 \mu\text{g/ml}$  rifampicin,  $10 \text{ mM}$  2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 5.6 and  $100 \mu\text{M}$  acetosyringone, at a 1/1000 dilution for the GOIs and 1/850 dilution for p19. Cultures were incubated overnight at  $28^\circ\text{C}$ , 250 rpm until an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.5- 1.0 was reached using a Thermo Scientific Nanodrop 2000c spectrophotometer. Cultures were then centrifuged at  $6000 \times g$  for 30 minutes and were resuspended to an  $\text{OD}_{600}$  of 1.0 in Gamborg's solution containing 3.2 g/l Gamborg's B5 with vitamins, 20 g/l sucrose,  $10 \text{ mM}$  MES (pH 5.6) and  $200 \mu\text{M}$  acetosyringone. Cultures were then incubated at room temperature with gentle agitation for one hour. Equal volumes of *A. tumefaciens* cultures containing one of the GOIs were combined with *A. tumefaciens* culture carrying p19 and Gamborg's solution to give a total *A. tumefaciens*  $\text{OD}_{600}$  of 0.67. These suspensions were used to infiltrate 7-8 week old *N. benthamiana* plants grown in a growth room under 16 hour light/8 hour dark conditions at  $21\text{-}22^\circ\text{C}$ , 55% humidity, and receiving roughly  $100 \mu\text{mol/photons m}^{-2}\text{s}^{-1}$  of light. A needle was used to poke a hole in the underside of the leaves and then a 3 ml syringe was used to infiltrate the *A. tumefaciens* suspensions. Gene constructs were infiltrated on three middle leaves per plant and five replicate plants were used. After infiltration, plants were returned to the growth chamber for four days.

### 2.2.5 Tissue collection and protein extraction

A 7 mm diameter cork borer was used to sample tissue from leaves infiltrated as described above. Two leaf disks were collected from each infiltrated leaf of one plant. These six leaf discs were pooled to create one biological replicate. This was repeated for each of the five plants. One leaf disc was collected from all the leaves from all the plants and pooled to create the pooled samples. Tissue was collected in 2 ml tubes containing

three, 2.3 mm ceramic beads. Tissue weight was determined using a Sartorius (Göttingen, Germany) B 120S fine balance, and tissue was flash frozen in liquid nitrogen and stored at -80°C until use. For protein extraction, tissue samples were placed in homogenization blocks precooled to -80°C and were homogenized twice in 30 second pulses using a TissueLyser (Qiagen). Blocks were centrifuged at 1,000 x g for one minute and 200 µl of plant extraction buffer (PEB) containing 1X PBS, 0.1% (v/v) Tween-20, 2% (w/v) polyvinylpyrrolidone (PVPP), 100 mM ascorbic acid, 1 mM ethylenediaminetetracetic acid (EDTA), 1mM of phenylmethanesulfonylfluoride (PMSF) and 1 µg/ml leupeptin, was added to each sample. Tubes were vortexed for five seconds on both the top and bottom and were centrifuged at 4°C for 10 minutes at 20,817 x g. The supernatants were collected and centrifuged again in a 1.5 ml tube. Total soluble protein (TSP) concentrations for each sample were determined using a Bradford assay (Bradford, 1976). Briefly, a bovine serum albumin (BSA) protein standard curve ranging from 1 to 4 µg was used to quantify the amount of TSP present in each extract using a Bio-Rad iMark™ Microplate Reader. Eighty microliters of each sample extract was combined with 20 µl of sample buffer containing 0.3 M Tris-HCl pH 8.0, 5% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 100mM Dithiothreitol (DTT), and 0.05% (w/v) Phenol Red, and frozen at -80°C.

#### 2.2.6 Protein quantification

Sample extracts were immunoblotted to accurately quantify protein accumulation levels against a standard curve of known protein concentrations. Briefly, samples were boiled for ten minutes and loaded onto a Bio-Rad Mini-Protean® TGX™ Precast 4-20% (w/v) polyacrylamide gradient gel. Known amounts of a cellulose binding domain (CBD)

synthetic protein standard were loaded for densitometry analysis. Gels were run at 100 volts until the dye front reached the bottom of the gel. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a Bio-Rad Trans-Blot® SD Semi-Dry Transfer Cell apparatus at 25 volts and blocked overnight in a 5% (w/v) skim milk powder in TBS-T (Tris-buffered saline-Tween 20) blocking solution. Membranes were hybridized for one hour with a 1:5000 dilution of mouse anti-c-Myc antibody in 0.5% (w/v) milk blocking solution, under gentle agitation. Membranes were washed for 3 x 10 minutes in TBS-T and hybridized with a 1:5000 dilution of goat anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP), in a 0.5% (w/v) milk blocking solution. After another three washes, GE Healthcare Life Sciences (Little Chalfont, UK) ECL Prime Western Blotting Detection Reagent was incubated with the membranes for one minute which were then visualized on X-ray film by autoradiography.

Recombinant protein was quantified using Totallab TL100 software (Nonlinear Dynamics, Durham, USA). Autoradiograms were scanned and band intensities were compared to a calculated CBD standard curve. The percentage of recombinant protein of the TSP extracted was calculated. The amount of protein in mg per kg of fresh leaf weight was also calculated using the tissue weight in each sample. Differences between the mean accumulation levels were analyzed using a one-way, Welch's analysis of variance (ANOVA) followed by a Games-Howell Pairwise Comparison using Minitab statistical software (State College, Pennsylvania, USA).

## **2.3. Generation of transplastomic plants**

### **2.3.1 Gene cloning**

Transplastomic genes were digested with restriction enzymes and ligated into a previously constructed chloroplast expression cassette 5 (CEC5) transformation vector (Kolotilin et al., 2013). Briefly, 1 µg of the constructs in the pUC57 cloning vector and 1 µg of CEC5 transformation vector were digested with *Nhe* I and *Not* I restriction enzymes overnight at 37°C. Digestions were terminated at 65°C for 20 minutes and the fragments were separated on a 1% (w/v) agarose gel. Visualized bands corresponding to digested transformation vector and the digested GOIs were cut out and gel purified using the QIAquick Gel Extraction Kit (Qiagen). Digested GOIs were ligated into 50 ng of the transformation vector in a 3:1 ratio using 1 µl of T4 DNA ligase (Invitrogen).

Ligation reactions were transformed into XL1-Blue *E. coli* as described earlier but were plated on LB plates containing 100 µg/ml ampicillin. Colonies were PCR screened for correctly ligated insertions using gene specific primers and positive colonies were used to inoculate 20 ml of LB containing 100 µg/ml ampicillin. Cultures were grown overnight at 37°C at 250 rpm and plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen). DNA concentration was determined using a Nanodrop 2000c spectrophotometer. Constructs were confirmed by restriction enzyme digestion and DNA sequencing using an Applied Biosystems (Thermo Fisher Scientific) 3130xl genetic analyzer.

All constructs were additionally cloned into the same transformation vector but with the addition of a *T7g10* translation enhancer on the 5' end of the gene construct (Kolotilin et al., 2013). To achieve this, GOIs were PCR amplified out of the transformation cassette using gene specific primers which add *Sap* I site to the 5' end of

the PCR amplicon. The fragment was then digested with *Sap* I and *Not* I and ligated as described previously into the *T7g10* transformation vector.

### 2.3.2 *N. tabacum* seed sterilization and growth conditions

Male-sterile 81V9 *N. tabacum* seeds were sterilized by soaking in 70% (v/v) ethanol for 30 seconds followed by two 30 second washes in sterile MilliQ water. Sterilized seeds were grown on media (pH 5.8) containing 4.43 g of Murashige & Skoog Basal Medium with vitamins and 40 g of sucrose, in sterile Magenta boxes. Plants were grown for 8 weeks in a growth cabinet at 22°C under 16 hour light/8 hour dark conditions in a Conviron® (Winnipeg, Canada) TC16 growth cabinet. Potted, transplastomic plants were grown in a greenhouse receiving 16 hours of light, 8 hours of dark, supplemented with lamps to receive 25 mol/photons m<sup>-2</sup>s<sup>-1</sup> of light each day. Greenhouse temperatures ranged from 18°C at night to 26°C during the day.

### 2.3.3 Biolistic transformation and generation of transplastomic plants

Transplastomic plants were obtained using the biolistic method as previously described (Verma et al., 2008). Succinctly, 5 µg of transformation vector was coated to 50 µl of 0.6 µm gold particles (50 mg/ml) using 50 µl of 2.5 M CaCl<sub>2</sub> and 20 µl of 0.1 M spermidine. Gold particles were washed in 70% (v/v) ethanol followed by a wash in 100% ethanol and were resuspended in 50 µl of 100% (v/v) ethanol. Ten microliters of gold particles were coated on to each of the macrocarriers making five shots for bombardment. Leaves of 8-10 week old male-sterile, 81V9 *N. tabacum* plants were bombarded abaxial side up using the Bio-Rad PDS-1000 / He™ Biolistic Particle Delivery System following manufacturer's protocols and under sterile conditions. Ten



leaves were bombarded per construct at a time. The same transformation vector but with the green fluorescence protein (GFP) was used as a positive control.

Bombarded tissue was incubated in the dark at 22°C for two days on RMOP plates (pH 5.8) containing 4.43 g/l of Murashige & Skoog Basal Medium with Vitamins, 7 g of agar, 30 g/l of sucrose, 1 mg/l 6-benzylaminopurine, 0.1 mg/l 1-naphthaleneacetic acid, 1 mg/l thiamine hydrochloride, and 0.1 g/l myo-inositol. After the two days, leaves were cut into small pieces and placed abaxial side down on RMOP media containing 500 mg/l of spectinomycin. Plates were incubated in growth cabinets under 16 hour light/8 hour dark conditions at 22°C for 4 to 12 weeks. Positive transformants were identified using PCR with gene specific primers. Once plantlets were generated after 4-8 weeks, leaf tissue was cut and placed on RMOP media containing 500 mg/l of spectinomycin for the second regeneration. After 4-8 weeks of regeneration, meristems were cut and placed on rooting media containing 4.43 g/l of Murashige & Skoog Basal Medium with Vitamins, 7 g of agar, 30 g/l of sucrose, and 500 mg/l of spectinomycin for the third regeneration. Four weeks later, plantlets were transferred to soil and grown in the greenhouse until flowering.

#### **2.4 Southern blot analysis**

A Southern blot was conducted to investigate if all chloroplasts were transformed with the transformation cassette. Two plants from EspA, EspD, and Tir transplastomic lines as well as two wild type plants were used for the analysis. Six leaf discs were pooled to create one biological replicate from each plant. Tissue was collected in 2 ml tubes containing three 2.3 mm ceramic beads, flash frozen in liquid nitrogen, and stored at -80°C until use. Tissue samples were placed in homogenization blocks precooled to

-80°C and were homogenized two times in 30 second pulses using a TissueLyser (Qiagen). Blocks were centrifuged at 1000 x g for one minute and total DNA was extracted using the DNeasy Plant Mini Kit from Qiagen following the manufacturer's protocol. After quantification with a Nanodrop 2000c spectrophotometer, 3 µg of DNA was digested with *Rsr* II overnight at 37°C. The next day, samples were digested with an additional 0.5 µl *Rsr* II for 30 minutes to ensure complete digestion. Samples were run at 4°C on a large 0.8% (w/v) agarose gel at 70 volts for three hours and then 120 volts until the dye front reached the bottom of the gel. A CEC5 transformation vector containing *EspA* was linearized with *Ahd* I to be used as a positive control as well as a DIG-labeled molecular weight marker (Roche) was loaded. After electrophoresis, the gel was then depurinated in 500 ml of 0.2 M HCl in a gently shaking Pyrex dish at room temperature for 30 minutes and was denatured in 0.5 M NaOH, 1.5 M NaCl for 2X 30 minutes. The gel was washed twice for 30 minutes each time in 1 M Tris-Cl, 1.5 M NaCl, pH 8.0 and washed once with 10X SSC (1.5 M NaCl, 0.15 M sodium citrate) for 15 minutes.

DNA was then blotted onto a Hybond N+ Nylon Membrane using capillary transfer, overnight at room temperature as described (Brown, 1998). Afterwards, the membrane was crosslinked using a Thermo Fisher Scientific FB UVXL-1000 UV crosslinker, and incubated with dissolved DIG Easy Hyb Granules from the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Basel, Switzerland) for one hour at 50°C in an Amersham Pharmacia Biotech (GE Healthcare) hybridization oven at 8 revolutions per minute. The membrane was then hybridized with the DIG-labeled probe overnight at 50°C, at 8 revolutions per minute. The probe was synthesized previously (Kolotilin et al., 2012) using primers Probe-F 5'-

CACCACGGCTCCTCTCTTCTCG-3' and Probe-R 5'-

TTCCTACGGGGTGGAGATGATGG-3' and the PCR DIG Probe Synthesis kit (Roche Diagnostics). The membrane was washed twice in 2X SSC with 0.1% (w/v) SDS at room temperature for 15 minutes followed by three washes for 1.5 hours each in 0.5X SSC with 0.1% (w/v) SDS at 68°C and 10 revolutions per minute. The membrane was then washed in Maleic Acid Buffer with 0.3% (v/v) Tween20 for 5 minutes at 48°C and 8 revolutions per minute. Afterwards, the membrane was blocked in blocking buffer (1.25 g of Roche Blocking Reagent in 125 ml of Maleic Acid Buffer) for 30 minutes at 42°C at 8 revolutions per minute. The membrane was then incubated with anti-DIG antibody solution for one hour at room temperature at 8 revolutions per minute. Following two washes with Maleic acid buffer with 0.3% (v/v) Tween20, the blot was placed in a plastic envelope and incubated with CSPD chemiluminescent substrate for 5 minutes. The membrane was then incubated at 37°C for 10 minutes and imaged using a DNR Bio-Imaging Systems (Jerusalem, Israel) MicroChemi 4.2 imager.

## **2.5 Recombinant protein extraction and quantification**

Total soluble protein was extracted from transplastomic plants using PEB and recombinant protein was quantified by Western blot as described for the transient assays. Leaf discs were taken from random young and old leaves distributed throughout the plant and pooled to create one sample. Seven EspA O157:H7-O26:H11 and two EspA O111:H8-O121:H19 flowering plants (roughly 12 weeks of age) were sampled. To determine the spatial accumulation of EspA, leaves from one plant were sampled from top (young) to bottom (old) leaves and recombinant protein was quantified by Western blot.

To determine if different extraction techniques can influence the amount of recombinant protein extracted, TSP was extracted from fresh leaf tissue or 0.01 g of ground, pooled lyophilized tissue from EspA transplastomic plants as previously described, or with an additional sonication step. After buffer addition, samples were vortexed and then sonicated using a Fisher Scientific Sonic Dismembrator Model 100 for 30 short bursts, on ice. Additionally, total protein was extracted from the samples using a 2% (w/v) SDS, 10 mM Tris pH 8 extraction buffer and samples were then subjected to the additional sonication step. TSP was not determined for these samples by the Bradford assay as total protein was extracted. Recombinant protein was quantified by Western blot as described for the transient assays.

## 2.6 Gene expression analysis

A quantitative (q) reverse-transcription (RT)-PCR was conducted to determine differences in gene expression between the transplastomic constructs. Primers TrbcL3UTR-F 5'-GCCGCTCCACTAATTAATGTTC-3' and TrbcL3UTR-R2 5'-GAAAGGTTTGATTTCTTTGAGTCTT-3' were designed to amplify a 199 bp amplicon in the 3'UTR of all STEC constructs. These primers bind to the UTR of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) large chain (*rbcL*) from the *Populus alba* or white poplar plastome (XM\_011020386) and a NCBI BLAST using the primers returned no significant hits in the tobacco genome. Primers were designed to amplify cDNA from four reference genes: Aminoglycoside-3"-adenyltransferase (*aadA*; AIC32303), RuBisCo small chain (*rbcS*; AY220079), Photosystem II protein D1 (*PsbA*; JN559760), and the 16S ribosomal RNA (*16S RNA*; V00165). The primer sequences for each pair are as follows: *aadA* (5'-TTATCCAGCTAAGCGCGAAC-3' and 5'-

TCAGGAACCGGATCAAAGAG-3'), *rbcS* (5'-AATGGATGGGTTCCTTGTTT-3' and 5'-GTATGCCTTCTTCGCCTCTC-3'), *PsbA* (5'-TACCCAATCGGTCAAGGAAG-3' and 5'-TTGTGCTCAGCCTGGAATAC-3'), and *16S RNA* (5'-GGGAAGTGGTGTTCAGTG-3' and 5'-TTCCTCCTTTTGCTCCTCAG-3'). These primers amplified fragments of the following lengths, respectively: 177 bp, 177 bp, 92 bp, 119 bp.

To collect tissue, six leaf discs from random leaves of a single, flowering plant were pooled in a 2 ml tube with 2.3 mm ceramic beads and immediately frozen in liquid nitrogen. Three plants were sampled for each transplastomic construct: *EspA*, *EspD*, *Tir*, and wild type as a negative control. Tissue samples were placed in homogenization blocks precooled to -80°C and were homogenized two times in 30 second pulses using a TissueLyser (Qiagen). Blocks were centrifuged at 1000 x *g* for one minute and total RNA was extracted and cleaned using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocols with the following exceptions: a 30 minute DNA digestion using the RNase-free DNase Digestion set (Qiagen) and RNA was eluted in 30 µl of RNase-free water and mixed equally with EB buffer from the Qiagen QIAprep® Spin Miniprep Kit to accurately determine RNA concentration using a Nanodrop 2000c spectrophotometer. Five microliters of this dilution was run on a 1% agarose gel to visualize RNA integrity.

cDNA was synthesized using random hexamers and the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). One microgram of RNA was reverse-transcribed in a 20 µl reaction with 1 µl of SuperScript II reverse transcription (RT) enzyme under the following conditions: 25°C for 10 minutes, 42°C for 50 minutes, and

70°C for 15 minutes. 1 µl of RNaseH was added and reactions were incubated at 37°C for 20 minutes.

cDNA was diluted 1:5 prior to use. Each reaction contained 5 µl of SsoFast EvaGreen Supermix (Bio-Rad), 3.8 µl of water, 0.4 µl of 10 µM forward primer, 0.4 µl of 10 µM reverse primer, and 0.4 µl of diluted cDNA. Reactions were amplified using a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. A gradient PCR with annealing temperatures ranging from 53°C - 63°C was used to determine the optimum annealing temperature of the primers. A melt-curve analysis was done to determine the specificity of the primers which was further confirmed by visualizing the products on a 1% agarose gel. A dilution series of the cDNA was performed to determine the efficiency of each primer. PCR efficiencies were calculated for the *TrbcL*, *aada*, *rbcS*, *PsbA*, and the *16S RNA* primer pairs, respectively: 104.3%, 103.8%, 94.0%, 109%, and 98.2%.

For the qPCR assay, the PCR amplification cycle consisted of 95°C for 30 seconds, 95°C for 5 seconds, and 58°C for 30 seconds plus a plate read, repeated 40 times. This was followed by a melt curve from 65°C to 95°C in 0.5°C increments plus a plate read. Three technical replicates were used per sample. Two technical replicates were used for pooled no-template (NTC) and no-RT (NRT) controls. Quantification cycle (Cq) values were determined by the single threshold mode using the Bio-Rad CFX Manager 3.1 software. A one-way, Welch's ANOVA followed by Games-Howell Pairwise Comparison was used to determine significant differences between mean expression values using Minitab statistical software.

## **2.7 Transplastomic tissue collection and lyophilization for the vaccine trial**

Multiple transplastomic EspA plants were grown under greenhouse conditions until flowering (roughly 12 weeks) and all the leaves from these plants were collected, weighed, and frozen at -80°C for 24 hours. Frozen tissue was then lyophilized in a FreeZone 18 Liter Console Freeze Dry System (Labconco, Kanas City, USA) and stored at room temperature until use.

## **3 RESULTS**

### **3.1 STEC vaccine design**

Several Shiga toxin-producing *E. coli* genes are involved in cattle colonization (Clements et al., 2012) and represent potential targets for vaccine design. STEC use the T3SS to inject an array of effector proteins into intestinal epithelial cells and these proteins are targeted by the host immune system following infection (Potter et al., 2004; Puhar and Sansonetti, 2014). EspA, Tir, EspD, and NleA were reported to react with sera from experimentally infected cattle out of sixty-six tested *E. coli* O157:H7 proteins (Asper et al., 2011). The non-toxic B subunit of the Shiga toxin is also a potential vaccine antigen as Stx2 promotes STEC colonization in cattle intestine (Baines et al., 2008a). Collectively, these antigens were chosen for plant-based expression to produce a vaccine against STEC. In addition to these antigens, the Colicin was chosen as it is a known antimicrobial peptide against STEC (Callaway et al., 2004). This recombinant protein could serve as an antimicrobial feed-additive for an alternative strategy to reduce STEC colonization before slaughter.

STEC O157:H7 peptides used for the vaccine are from the EDL 933 strain isolated in the United States in 1982 (Wells et al., 1983), NleA O157:H7 is from strain

TW1439 isolated in 2006 (Kulasekara et al., 2009), EspA O26:H11 is from the DEC10C strain (Durso et al., 2005), and the Colicin is from the Colicin E1 plasmid (Yamada et al., 1982). EspA from the O157:H7 serotype was fused to EspA from the O26:H11 serotype using a flexible GSGGSG linker (Gu et al., 2009) to create a fusion protein to provide protection against both O157:H7 and O26:H11 serotypes (Figure 2A).

### 3.2 Transient production of the STEC antigens

The second objective of this study is to produce these vaccine antigens using *A. tumefaciens*-mediated transient transformation of *N. benthamiana*. The *EspA* fusion, *EspD*, *Tir*, *NleA* and the *Colicin* genes were optimized for expression in the nuclear genome of *N. benthamiana* and recombined using Gateway® cloning into expression vectors targeting different intracellular organelles and with different peptide fusions (Pereira et al., 2014). These cassettes target the endoplasmic reticulum (ER), the chloroplasts, or the cytoplasm (Figure 2B). In two cassettes, constructs targeting the ER and were also fused to either an elastin-like polypeptide (*ELP*) or a hydrophobin (*HFBI*) tag to potentially increase protein accumulation (Conley et al., 2011a). All constructs are driven by a double-enhanced cauliflower mosaic virus promoter (2X35S) (Covey et al., 1981), have a terminator from the nopaline synthase gene (*nosT*), a tCUP translation enhancer (Wu et al., 2001), and an N-terminal *Xpress* and a C-terminal *c-Myc* tag for immunodetection. Cassettes targeting the ER contain a signal peptide from the pathogenesis-related-1b protein (*PR1b*) (Huub and Van Loon, 1991) and an ER retrieval tag (*KDEL*) (Munro and Pelham, 1987). The chloroplast targeting cassette contains a RuBisCo small chain transit peptide (*rbcS*). All of the vectors contain the selectable marker *nptII*, coding for neomycin phosphotransferase which confers resistance to

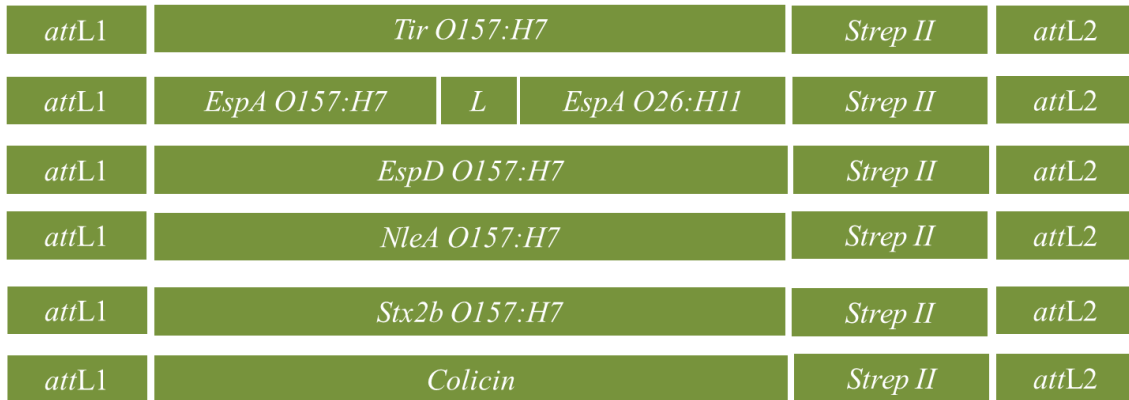


kanamycin and is located upstream of the gene of interest (Figure 2C). Constructs are flanked by left and right T-DNA border sequences for *A. tumefaciens*-mediated transformation.

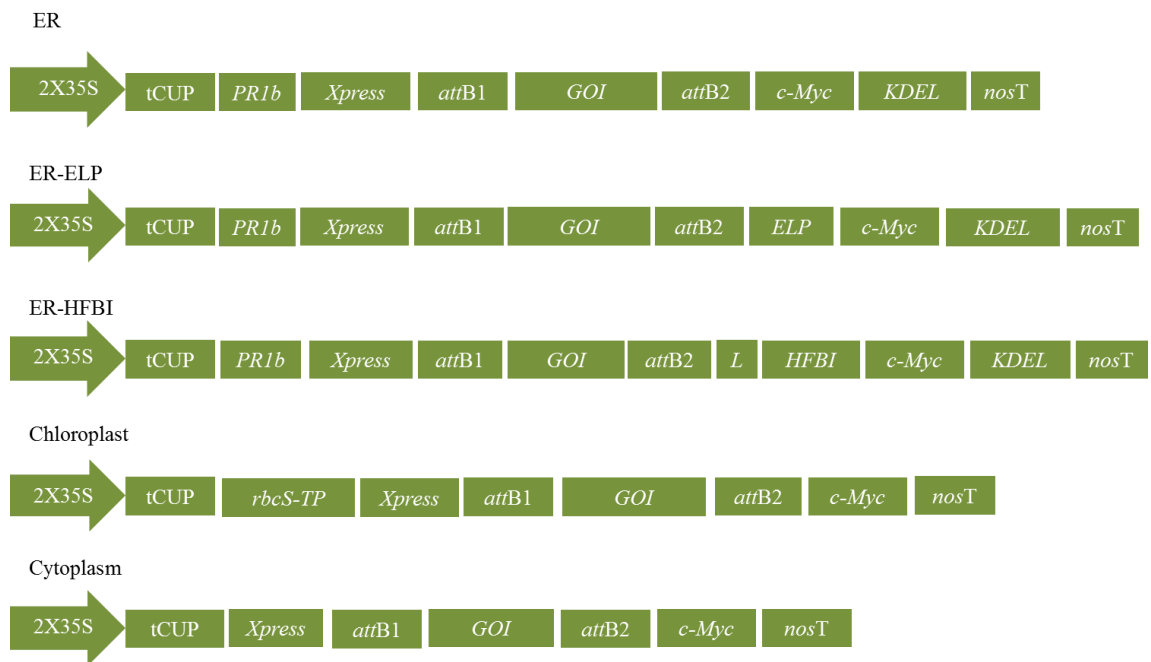
Post-transcriptional gene silencing (PTGS) is known to cause degradation of foreign transcripts (Voinnet et al., 2003). To avoid PTGS, cultures of *A. tumefaciens* containing the STEC constructs in each expression cassette were co-infiltrated along with *A. tumefaciens* carrying the gene silencing suppressor p19 from the cymbidium ringspot tomosvirus (Silhavy et al., 2002), into *N. benthamiana* plants.

Plant leaves had a slight yellow colour after infiltration. Leaf tissue from pooled and individual samples was collected four days after infiltration and protein extracts were analyzed by Western blot to detect and quantify recombinant protein production.

A)



B)



C)



**Figure 2. Designed gene constructs and expression cassettes for vaccine candidates.**

A) A schematic representing the gene constructs chosen for transient expression in *N. benthamiana*. All genes were fused to a *StrepII* tag for protein purification and have flanking *attL* sites for recombination into Gateway®-compatible expression vectors. Genes were codon-optimized for expression in the nuclear genome of *N. benthamiana*. The *EspA* construct consists of full-length *EspA* genes from the O157:H7 and the O26:H11 serotypes fused together with a flexible linker (*L*). B) Genes of interest (*GOI*) were recombined into vectors containing expression cassettes using Gateway® recombination. The cassettes target the endoplasmic reticulum (ER), the chloroplasts, and the cytoplasm. Two of the cassettes targeting the ER also contain sequences coding for an elastin-like polypeptide (*ELP*) or a hydrophobin (*HFBI*) tag with a linker (*L*). Expression and targeting elements incorporated into the expression vectors are: the double-enhanced 35S cauliflower mosaic virus promoter (2X35S); tCUP translational enhancer from tobacco; Tobacco Pathogenesis-Related-1b (*PR1b*) and the *KDEL* ER retrieval tetrapeptide, respectively; nopaline synthase terminator (*nosT*); and *Xpress* and *c-Myc* tags for detection. A sequence coding for the RuBisCo small chain transit peptide (*rbcS*) was used for targeting the chloroplasts. C) A zoomed-out schematic of the constructs in the T-DNA strand. LB and RB are the left and right borders of the T-DNA sequence. *nptII* codes for neomycin phosphotransferase conferring resistance to kanamycin, driven by a nopaline synthase promoter (*nosP*) and terminator (*nosT*). Schematic represents the same constructs in A) with different N-terminal fusions (*NTFs*) and C-terminal fusions (*CTFs*). Schematics are not to scale.

### 3.3 Quantification of transiently-produced recombinant protein

Western blots on pooled samples revealed that *EspA*, *NleA*, and *Stx2b* gene constructs accumulate recombinant protein of the expected sizes when transiently expressed in *N. benthamiana* (Table 1; Figure 3). Higher-migrating, more saturated bands were seen when these recombinant proteins were fused to ELP or hydrophobin tags indicating an increase in protein accumulation. Very faint banding was visualized for *Stx2b* when targeted to the chloroplast and cytoplasm and for the Colicin when targeted to the cytoplasm. Faint banding running around 60 kDa was also observed for the Tir construct when fused to an ELP tag, but it is of an incorrect size (Figure 3, Table 1). *EspD* and the Colicin do not accumulate when targeted to any of the different intracellular compartments as no visible bands can be seen (Figure 3). Individual biological replicates from accumulating *EspA*, *NleA*, and *Stx2b* constructs were then immunoblotted to accurately quantify protein accumulation levels against a standard curve of known amounts of the CBD protein standard.

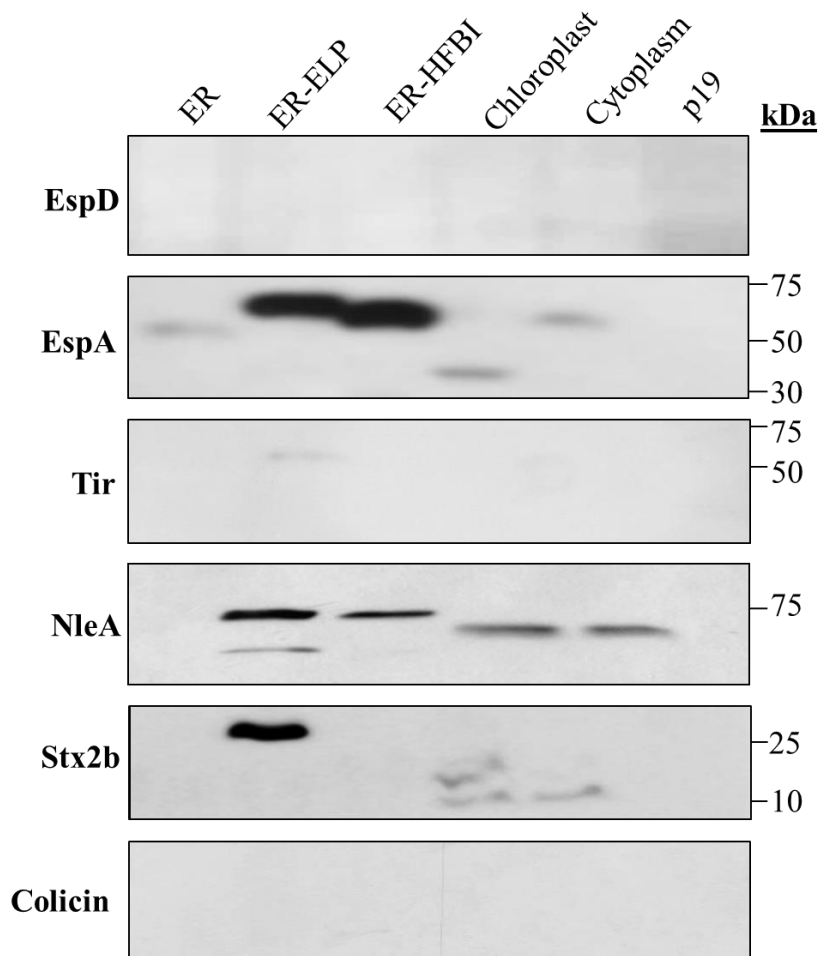
*EspA* accumulates the highest in *N. benthamiana* of all the transiently-produced STEC constructs. An ANOVA revealed that the mean *EspA* accumulation levels for each intracellular compartment are significantly different and a Games-Howell pairwise comparison demonstrated that the ER-ELP and ER-HFBI constructs accumulate higher than the ER or chloroplast targeted constructs as a percentage of TSP [ $F(3,8.05)= 11.75$ ,  $p=0.003$ ] and in mg/kg [ $F(3, 8.23)= 11.4$ ,  $p= 0.003$ ]. *EspA* accumulated the highest when fused to an ELP tag, accumulating to  $0.8 \pm 0.11\%$  of TSP or  $87.8 \pm 13.2$  mg/kg of fresh leaf weight (Table 1, Figure 4). This corresponds to an 8-fold increase in protein accumulation with the ELP tag as compared to the non-fused, ER-targeted construct.

Fusion to the hydrophobin tag resulted in a five-fold increase in protein accumulation compared to the non-fused, ER-targeted construct. When targeted to the chloroplasts, EspA accumulates to a similar level to the non-fused, ER-targeted construct and no protein was detected when targeted to the cytoplasm, though faint banding was seen in the pooled samples (Figure 3; Figure 4). Interestingly, additional banding of a lower molecular weight was seen for the ER-ELP construct which could represent protein degradation.

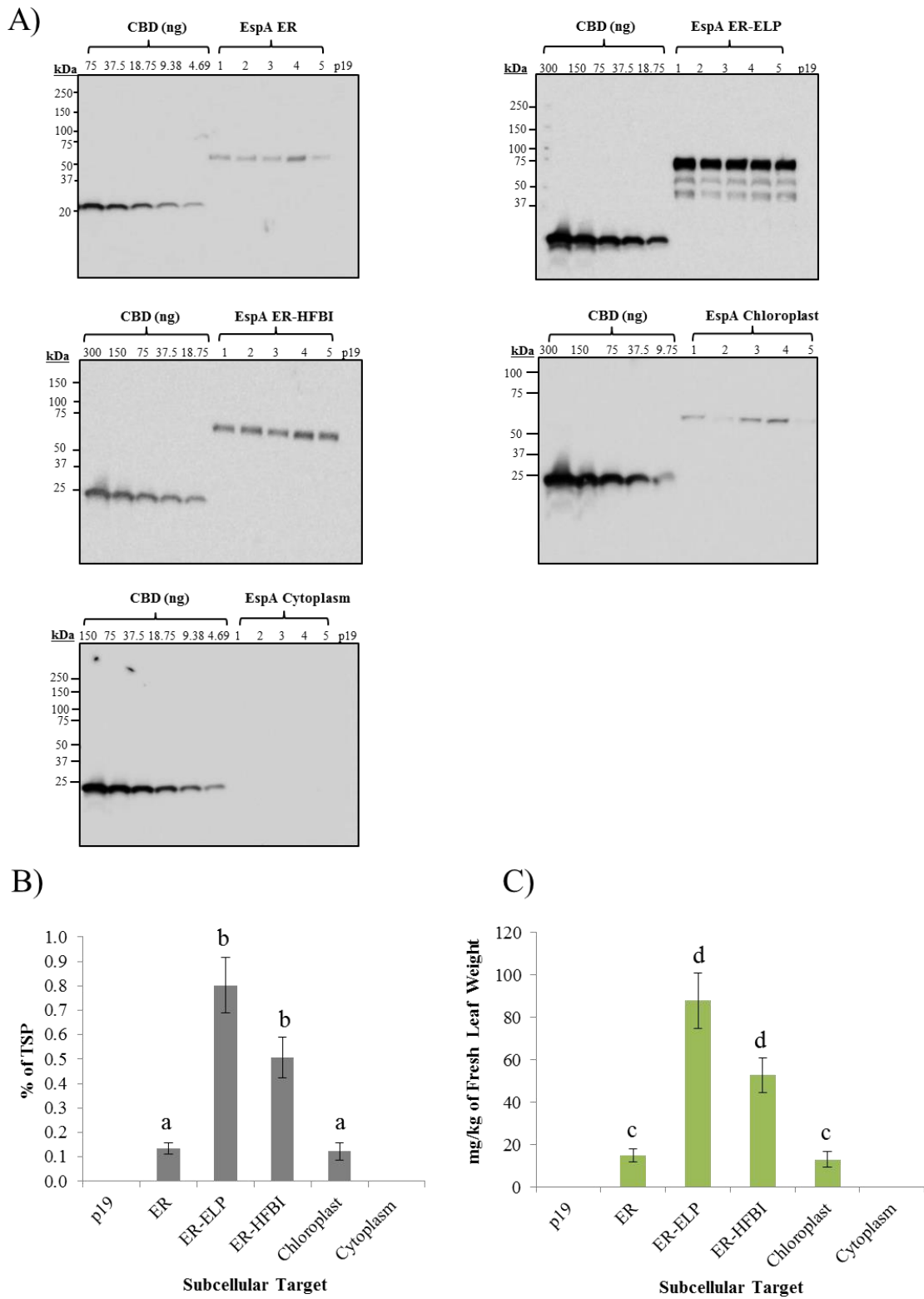
**Table 1. Predicted sizes and quantified accumulation levels of transiently produced recombinant protein in *N. benthamiana*.**

Recombinant protein	Target	Predicted size (kDa)	Accumulation as a percentage of total soluble protein <sup>a</sup>	Accumulation in mg/kg of fresh weight leaf tissue <sup>a</sup>
<b>EspA</b>	ER	51.5	0.1 ± 0.02	14.8 ± 3
	ER-ELP	62.9	0.8 ± 0.11	87.8 ± 13.2
	ER-HFBI	59.7	0.5 ± 0.08	52.7 ± 8.1
	Chloroplast	53.3	0.1 ± 0.03	13 ± 3.9
	Cytoplasm	48	0	0
<b>EspD</b>	ER	49	0	0
	ER-ELP	60.5	0	0
	ER-HFBI	57.3	0	0
	Chloroplast	50.9	0	0
	Cytoplasm	45.6	0	0
<b>Tir</b>	ER	67.9	0	0
	ER-ELP	79.4	0	0
	ER-HFBI	76.2	0	0
	Chloroplast	69.8	0	0
	Cytoplasm	64.5	0	0
<b>NleA</b>	ER	57.5	0	0
	ER-ELP	69	0.01 ± 0.007	1.3 ± 0.7
	ER-HFBI	65.8	0.01 ± 0.004	0.7 ± 0.4
	Chloroplast	59.4	0.004 ± 0.003	0.45 ± 0.3
	Cytoplasm	54.1	0.01 ± 0.004	0.6 ± 0.3
<b>Stx2b</b>	ER	20.2	0	0
	ER-ELP	31.7	0.3 ± 0.04	28 ± 4.9
	ER-HFBI	28.5	0	0
	Chloroplast	22.1	0	0
	Cytoplasm	16.8	0	0
<b>Colicin E1</b>	ER	67.2	0	0
	ER-ELP	78.7	0	0
	ER-HFBI	75.5	0	0
	Chloroplast	69.1	0	0
	Cytoplasm	63.8	0	0

<sup>a</sup>, ± standard error of the mean value of the biological replicates. The quantity of each recombinant protein was calculated as a mean of five biological replicates. NleA was calculated as a mean of two biological replicates.



**Figure 3. Immunoblots of total soluble protein extracted from pooled plant tissue transiently transformed with STEC constructs.** Constructs were agroinfiltrated into *N. benthamiana* and proteins were targeted to the ER, chloroplasts, cytoplasm, or were targeted to the ER and fused to ELP or HFBI tags. Leaf tissue was pooled from five biological replicates for each infiltrated construct. Total soluble protein was extracted from pooled samples and analyzed by Western blot. p19 is a negative control.





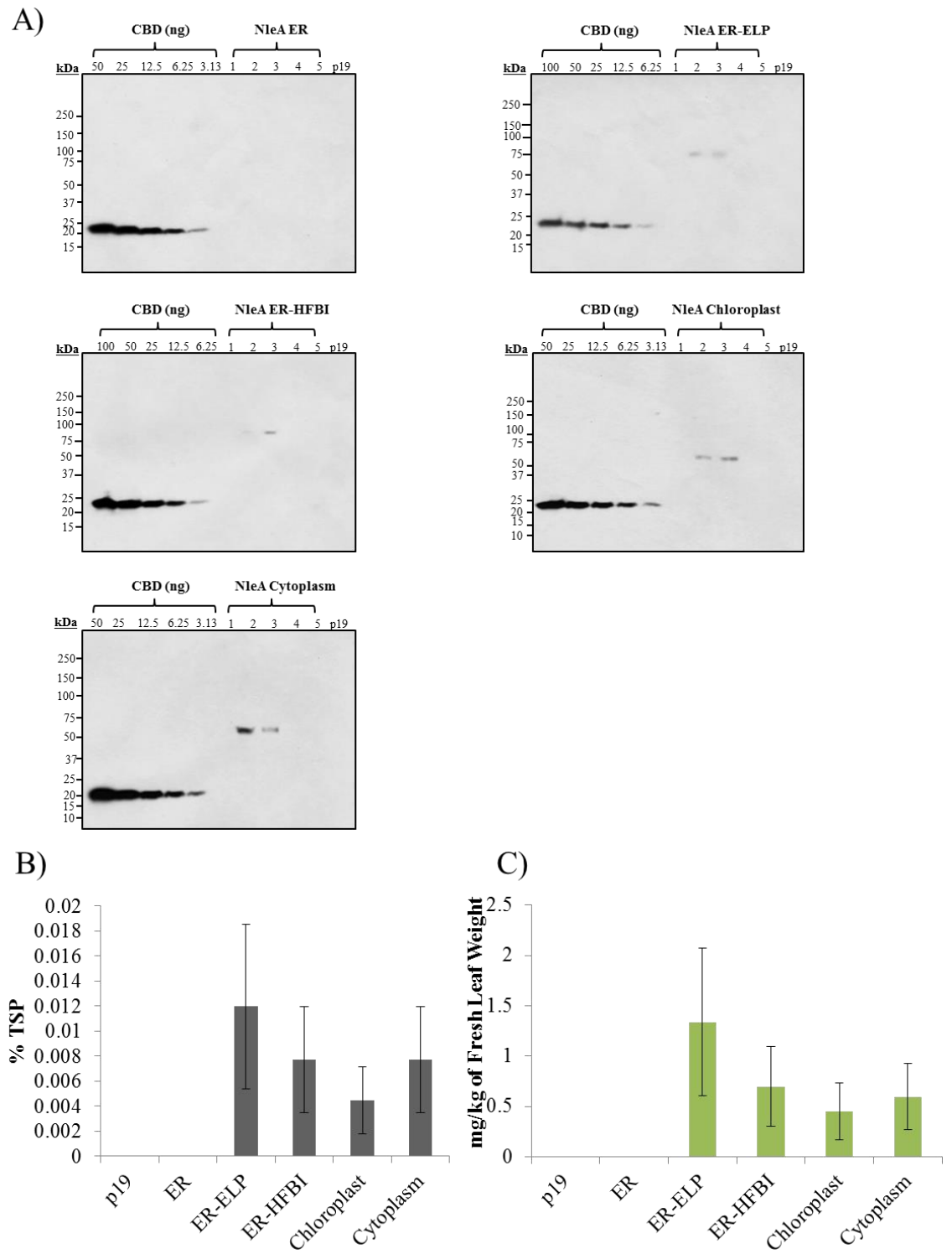
**Figure 4. Quantification of transiently produced EspA.** A) Immunoblots of total soluble protein (TSP) extracted from plants transiently transformed with the EspA fusion targeted to different intracellular compartments or fused with ELP or hydrophobin (HFBI) tags. Five biological replicates were used for each construct. p19 is a negative control and CBD is a protein standard for quantification. B) and C) Quantification of results shown in A) by densitometry against the CBD protein standard. B) Protein accumulation as a percentage of TSP extracted and C) in milligrams per kilogram of fresh leaf weight. Each column represents the mean value of five biological replicates and error bars indicate the standard error of the mean. Means that do not share a letter are significantly different as a percentage of TSP [ $F(3,8.05)= 11.75, p=0.003$ ] and in mg/kg [ $F(3, 8.23)= 11.4, p= 0.003$ ].

NleA accumulates in all intracellular compartments tested but to a lower level compared to EspA. Accumulation varied dramatically between biological replicates and only two of the plants accumulated protein to a quantifiable level (Figure 5). Consequently, the standard error of each mean value is quite large. As with EspA, NleA accumulates the highest when targeted to the ER and fused to an ELP tag, accumulating to  $0.1 \pm 0.02\%$  of TSP or  $1.3 \pm 0.7$  mg/kg of fresh leaf weight (Figure 5 B,C; Table 1). However, an ANOVA revealed that the mean NleA accumulation levels for each intracellular compartment are not significantly different in terms of TSP [ $F(3,8.50)=0.33$ ,  $p=0.804$ ] or in mg/kg [ $F(3,8.62)=0.31$ ,  $p=0.814$ ]. Extraneous, lower bands were visualized on over-exposed X-ray film for the ER-ELP construct representing potential protein degradation. No non-fused protein was detected when targeted to the ER, however, faint banding was visible at increased exposure times corresponding to NleA accumulating at a very low, non-quantifiable level.

Unlike EspA and NleA, the B subunit of the Shiga toxin only accumulates to a quantifiable level when expressed in one of the intracellular-targeting cassettes. Despite this, Stx2b accumulates to a higher level than NleA. ER targeted, ELP fused Stx2b accumulates to  $0.3 \pm 0.04\%$  of TSP or  $28 \pm 4.9$  mg/kg of fresh leaf weight (Figure 6 B,C; Table 1). Faint banding was observed in the chloroplast and cytoplasm targeted constructs but the intensity was too low to be accurately quantified (Figure 6A).

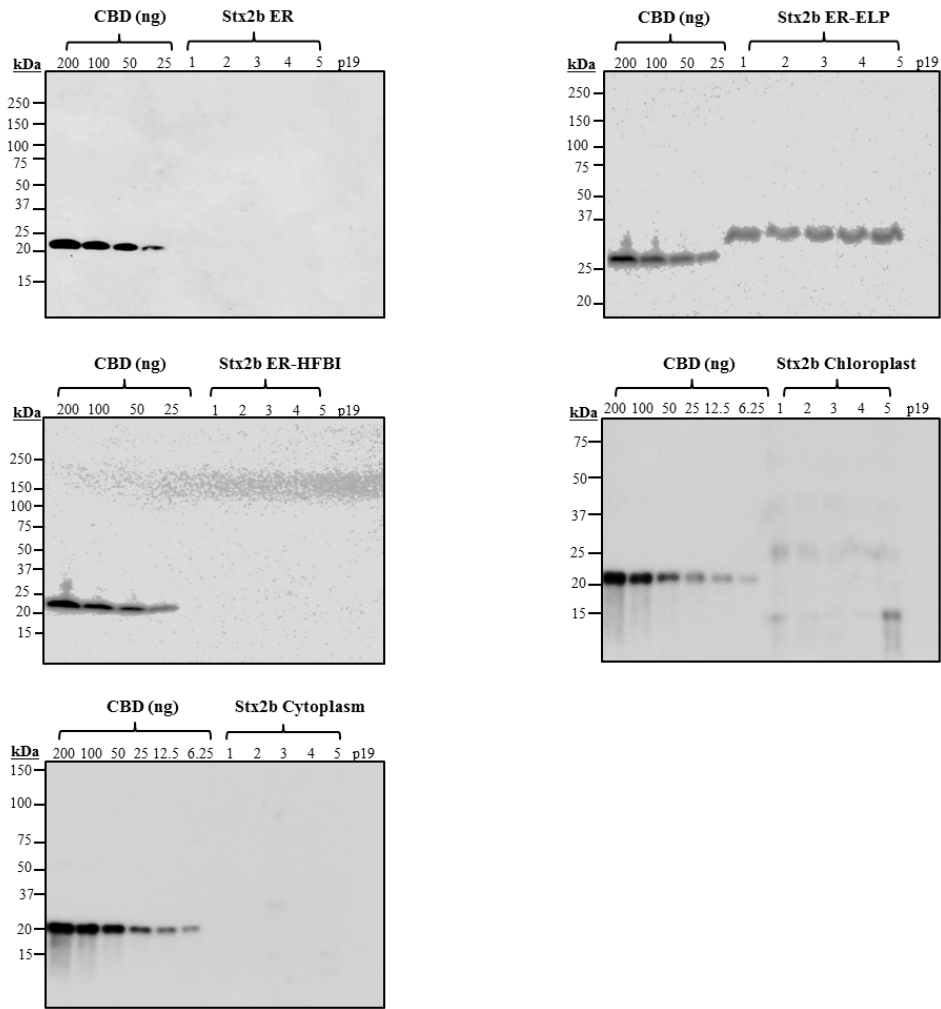
These results suggest that targeting the STEC proteins to the ER with an ELP fusion tag causes the largest increase in protein accumulation and of all the transgenes tested, only EspA, NleA, and Stx2b accumulate to a quantifiable level. EspA and NleA

were the only proteins to accumulate when targeted to the chloroplasts suggesting that these proteins are prime candidates for transplastomic production.

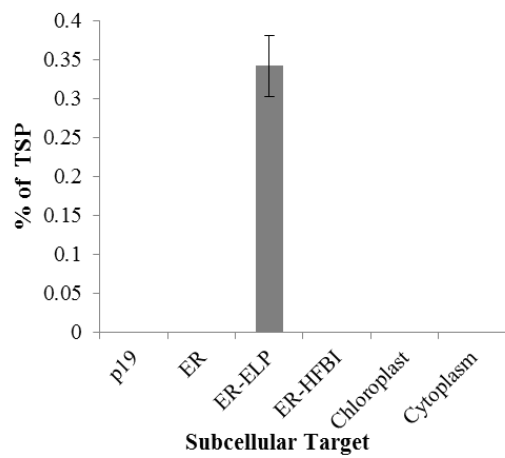


**Figure 5. Quantification of transiently produced NleA.** A) Immunoblots of total soluble protein (TSP) extracted from plants transiently transformed with NleA targeted to different intracellular compartments or fused with ELP or hydrophobin (HFBI) tags. Five biological replicates were used for each construct. p19 is a negative control and CBD is a protein standard for quantification. B) and C) Quantification of results shown in A) by densitometry against the CBD protein standard. B) Protein accumulation as a percentage of TSP extracted and C) in milligrams per kilogram of fresh leaf weight. Each column represents the mean value of two biological replicates and error bars indicate the standard error of the mean. NleA accumulation levels for each intracellular compartment are not significantly different in terms of TSP [ $F(3,8.50)=0.33$ ,  $p=0.804$ ] or in mg/kg [ $F(3,8.62)=0.31$ ,  $p=0.814$ ].

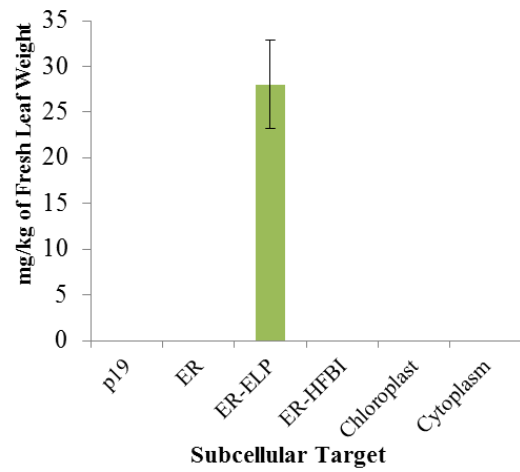
A)



B)



C)



**Figure 6. Quantification of transiently produced Stx2b.** A) Immunoblots of total soluble protein (TSP) extracted from plants transiently transformed with Stx2b O157:H7 targeted to different intracellular compartments or fused with ELP or hydrophobin (HFBI) tags. Five biological replicates were used for each construct. p19 is a negative control and CBD is a protein standard for quantification. B) and C) Quantification of results shown in A) by densitometry against the CBD protein standard. B) Protein accumulation as a percentage of TSP extracted and C) in milligrams per kilogram of fresh leaf weight. The column represents the mean value of five biological replicates and error bars indicate the standard error of the mean.

### 3.4 Generation of transplastomic *N. tabacum* for STEC antigen expression

The third objective of this study is to produce stable, transplastomic *N. tabacum* lines for high-yield production of the STEC antigens. Engineering the chloroplast genome for STEC gene expression is a better strategy for vaccine production as typically higher accumulation levels are possible with transplastomic protein production and no glycosylation machinery is present in the chloroplasts which could alter the antigenicity of the STEC proteins (Kolotilin et al., 2014). Although several STEC genes did not accumulate at all and only EspA and NleA accumulated when targeted to the chloroplasts, all the designed STEC genes, with the exception of the *Colicin*, were used for plastid transformation. This was done to determine if transient protein accumulation is a potential indicator for transplastomic protein accumulation which would contribute to the conclusions of this thesis.

To maximize transplastomic protein production, STEC genes were codon-optimized for expression from the chloroplast genome of *N. tabacum*. Genes were fused to a *c-Myc* tag for detection and a *StrepII* tag for protein purification (Figure 7) and were synthesized and ligated into a previously constructed chloroplast transformation cassette (Kolotilin et al., 2013). This cassette contains flanking transfer RNA isoleucine (*trnI*) and transfer RNA alanine (*trnA*) sequences to integrate in between the *trnI* and *trnA* genes of the ribosomal RNA (*rrn*) operon in the inverted repeat region of the *N. tabacum* chloroplast genome by homologous recombination (Figure 8). The cassette integrates two genes into the genome, the transgene of interest and the aminoglycoside-3'-adenyltransferase (*aadA*) gene coding for resistance to spectinomycin (Svab et al., 1990b). Expression of the *aadA* gene relies on read-through transcription from the *Prrn*

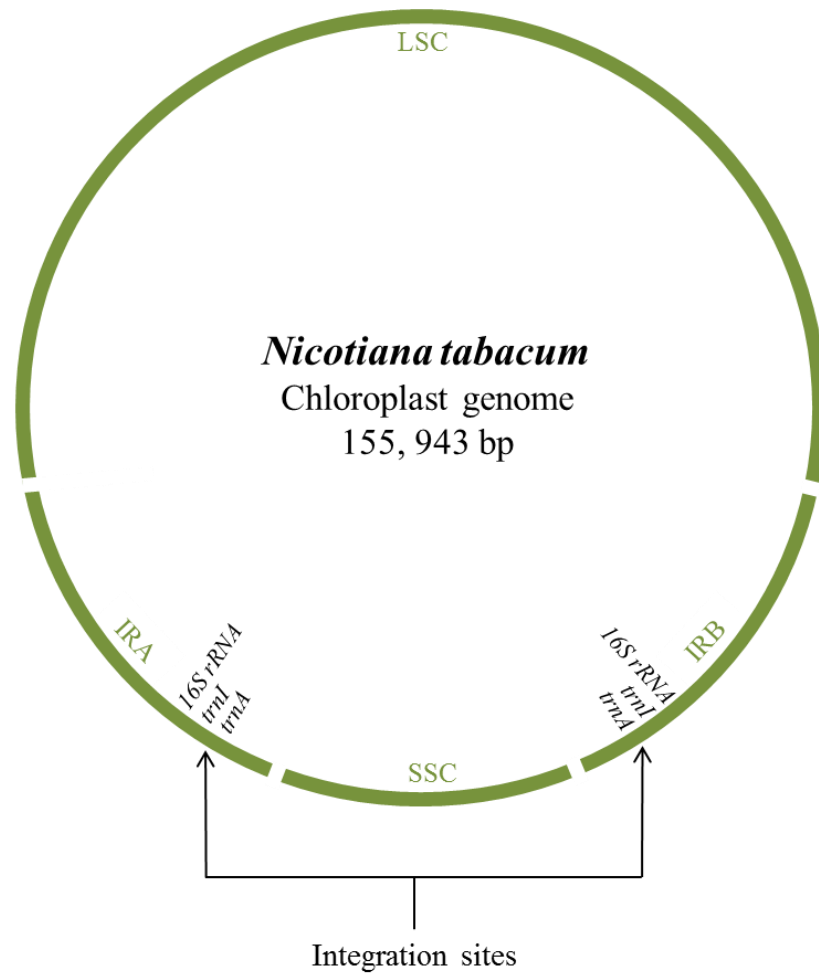


promoter of the plastid rRNA (*rrn*) operon (Verma and Daniell, 2007) and the 3'UTR from the *psbC* gene coding for the photosystem II chlorophyll protein (CP) 43. Transgene expression is driven by the plastid promoter and 5'UTR from photosystem II protein D1, *PpsbA*, (Verma and Daniell, 2007) and has the 3'UTR from the white poplar chloroplast RuBisCo Large chain (*TrbcL*) to stabilize the mRNA (Kolotilin et al., 2013; Shinozaki and Sugiura, 1982). The intercistronic expression element (IEE) which mediates cleavage of polycistronic transcripts into monocistronic transcripts (Zhou et al., 2007) and the Shine-Dalgarno (SD) ribosome binding site (Drechsel and Bock, 2011) reside upstream of the *aadA* gene to improve translation efficiency. STEC genes were also cloned into a second transformation cassette (Figure 8B) containing the 5' UTR and downstream box of the *E. coli* phage 7 gene 10 (*T7g10*) which enhances mRNA stability, translation, and has been shown to increase protein accumulation (Kuroda and Maliga, 2001) (Figure 8C).



**Figure 7. STEC constructs used for transplastomic production in *N. tabacum*.** STEC genes were fused to *c-Myc* and *StrepII* sequences for detection and purification respectively. Genes were optimized for expression in the chloroplast genome of *N. tabacum*. The EspA construct consists of sequences coding for two proteins, *EspA* from the O157:H7 and *EspA* from the O26:H11 serotype, fused together with a flexible linker sequence (*L*). Schematic is not to scale.

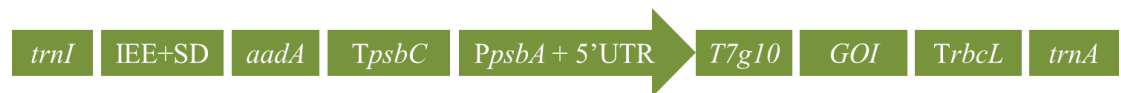
A)



B)



C)

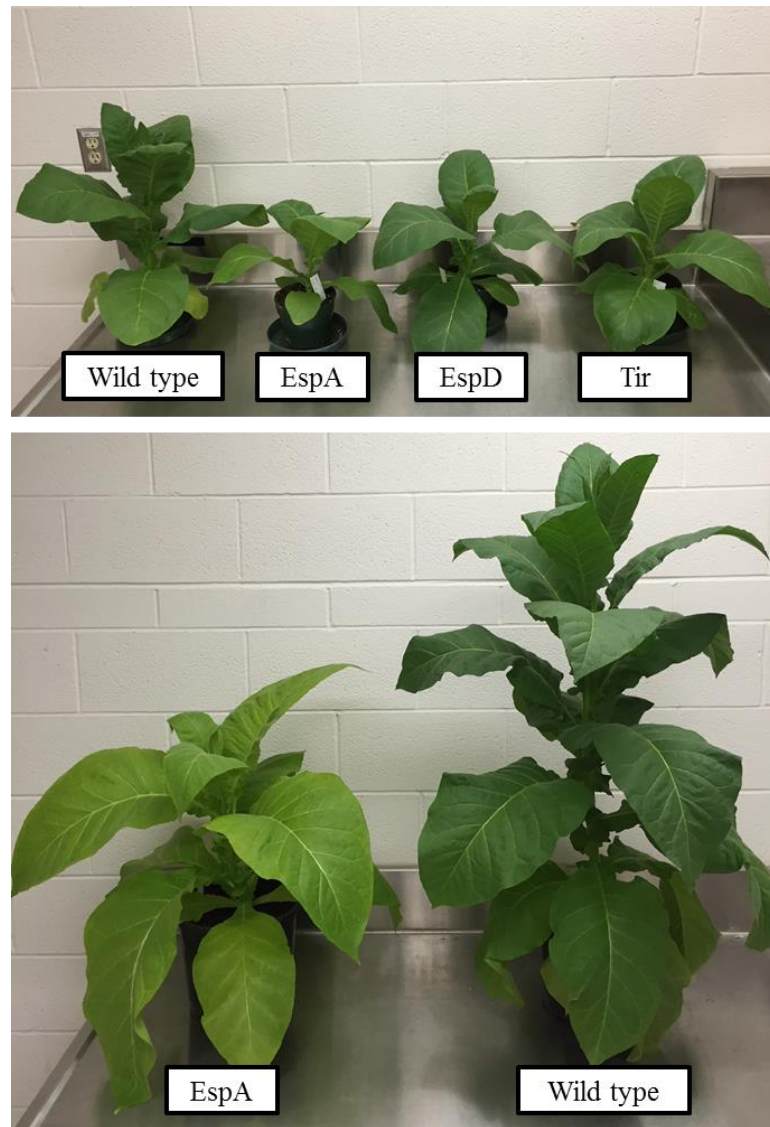


**Figure 8. Transformation of STEC constructs into chloroplast genome of *N.***

*tabacum*. A) A Schematic of the *N. tabacum* chloroplast genome showing where the expression cassettes recombine/integrate into the inverted repeat regions A and B (IRA, IRB). LSC: large single copy region, SSC: small single copy region. B) Genes of interest (GOI) were cloned into this cassette for biolistic transformation of *N. tabacum* tissue. GOI expression was driven by the promoter and 5'UTR from the PSII D1 protein of tobacco (*PpsbA* + 5'UTR) and terminated by the 3'UTR from RuBisCo (*TrbcL*) of white poplar. The selectable marker, *aadA*, relies on read-through transcription and is terminated by the 3'UTR from the *psbC* gene (*TpsbC*) of white poplar. The intercistronic expression element and Shine-Dalgarno sequence (IEE+SD) are upstream to mediate correct RNA cleavage and ribosome binding. The cassette is flanked by *trnI* and *trnA* gene sequences for recombination into the plastid genome. C) The same cassette with the addition of a *T7g10* 5'UTR and downstream box.

*N. tabacum* leaf tissue from the male-sterile, low alkaloid, 81V9 cultivar was bombarded with the STEC constructs and placed on antibiotic-containing media to select for positive transformation events. This cultivar was used to prevent transgene transfer through pollen and to reduce alkaloid levels in the oral vaccine (Menassa et al., 2001). Due to the high degree of polyploidy in the plastid genome, a chloroplast transformation event produces a heteroplasmic mixture of wild type and transformed copies. To prevent transgene loss by genome segregation, transformed plastid genomes must be selected for to remove residual wild type genomes and create a homoplasmic state where all the genome copies contain the transgene of interest (Bock, 2014b). To achieve homoplasmy, bombarded tissue was regenerated for three rounds on media containing spectinomycin. Transgenic plants were then potted in soil and grown until flowering under greenhouse conditions. Transgenic lines were generated for *EspA*, *Tir*, and *EspD* constructs. Transplastomic *EspA* lines grew slower and had a yellow-green colour compared to wild type, male-sterile 81V9 tobacco plants (Figure 9). Transplastomic *EspD* and *Tir* lines grew slower but were otherwise identical in appearance to wild type plants. No transplastomic lines for *NleA* or *Stx2b* could be successfully generated.

Flowering *EspA*, *EspD*, and *Tir* transplastomic plants were pollinated with pollen from wild type 81V9 tobacco plants to generate seed. All transplastomic seed germinated on spectinomycin-containing media and plants were transferred to soil and grown under greenhouse conditions to scale-up recombinant protein production.



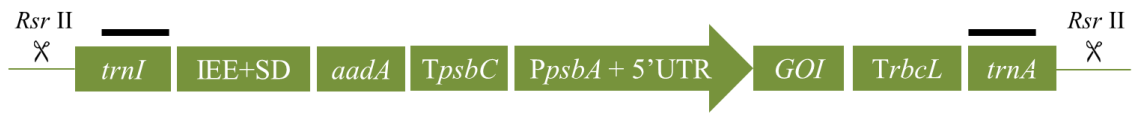
**Figure 9. Phenotype of transplastomic and wild type *N. tabacum* plants.** Top: Transplastomic EspA, EspD, Tir and wild type plants at 8 weeks of age. EspA plants grew slower and a pale green phenotype compared to wild type tobacco plants. Bottom: The pale green, slow growth phenotype of EspA plants (left) is more pronounced in older plants (10 weeks of age). EspD and Tir plants were identical to wild type tobacco plants (right) at this growth stage.

### 3.5 Determination of homoplasmy in transplastomic lines

Ensuring that the transplastomic lines are homoplasmic is essential to maximize transgene copy number, maximize transgene expression level, and to prevent transgene loss through plastid genome segregation. Therefore, EspA, Tir, and EspD lines were tested for homoplasmy using a Southern blot to probe for any wild type plastid genomes in the transformed lines. The probe used to hybridize DNA binds to regions of the plastid genome flanking the expression cassette and therefore can be used for all the transplastomic constructs (Figure 10A). This probe hybridized to the expected 1,054 bp *Rsr* II-digested fragment from the wild type plastid genome in the wild type samples. In the transplastomic samples, the probe hybridized to the expected 3,774 bp, 3,726 bp, 4,278 bp fragments from the EspA, EspD, and Tir lines, respectively. The increase in band size represents expression cassette insertion into the plastid genome. Since the probe did not hybridize to any DNA fragments corresponding to the wild type genome in both clones from the EspA and EspD lines and clone 1 from the Tir O157:H7 line, these clones are considered homoplasmic for the transformed plastid genome. Clone 2 from the transplastomic Tir line has two bands corresponding to both wild type and transformed genomes and is considered heteroplasmic (Figure 10). This clone was omitted from further experiments.

A)

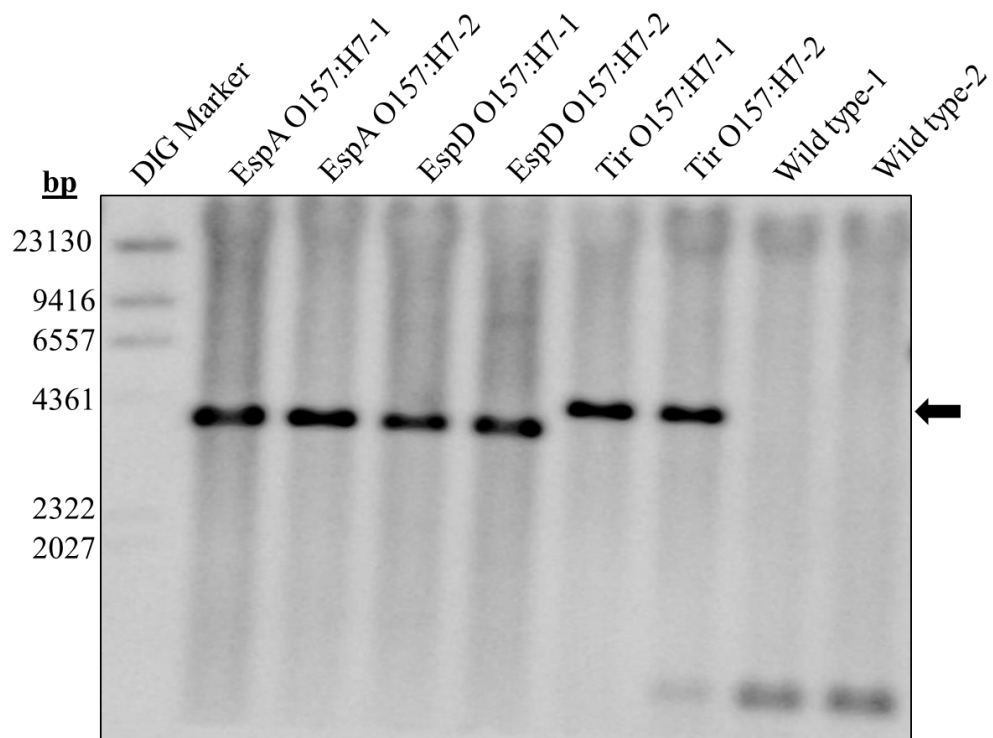
Transformed genome



Wild type genome



B)





**Figure 10. Southern blot of DNA from transplastomic lines to determine**

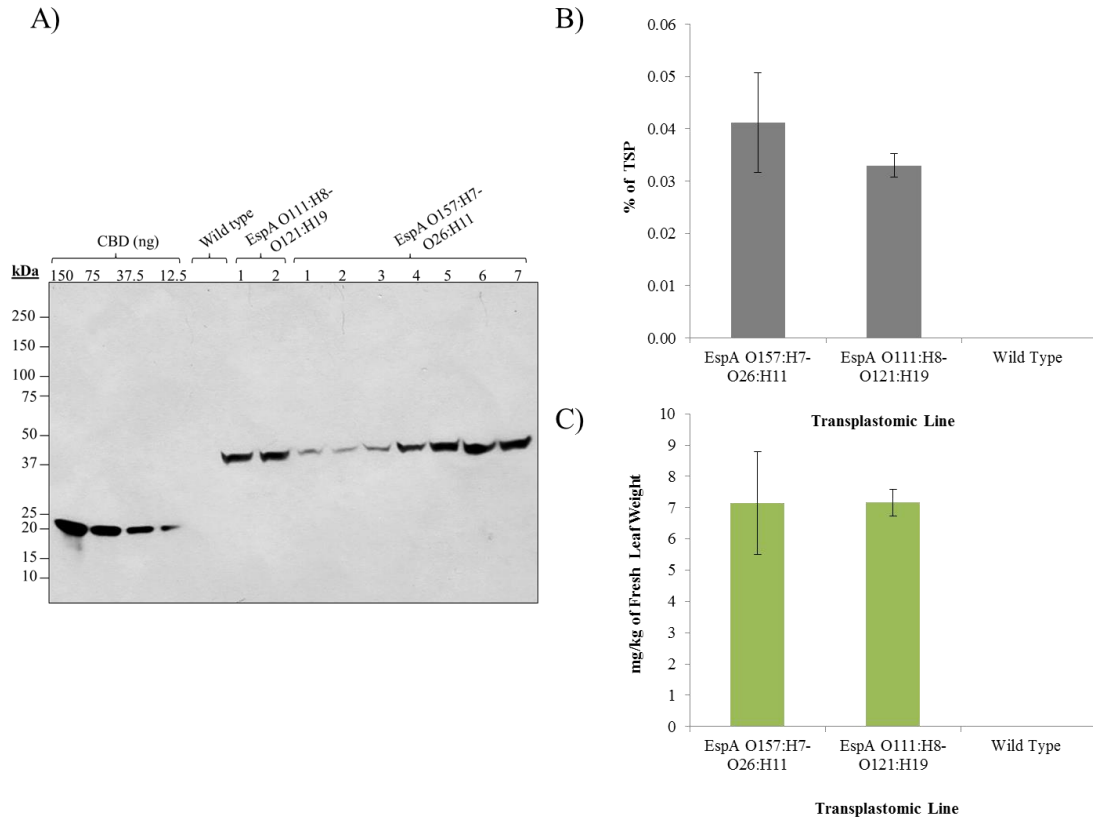
**homoplasmy.** A) A schematic showing the location of the probe (black bars) hybridizing to transformed and wild type plastid genomes. The probe binds to flanking regions of the transformation cassette and therefore was used for all STEC genes. Scissors mark *Rsr* II cut sites. B) A Southern blot of digested DNA using the probe described in A). Three micrograms of DNA from two transplastomic clones of each GOI and from two wild type plants was digested with the endonuclease *Rsr* II and electrophoresed on an agarose gel. In the wild type samples, a one kb fragment is visible corresponding to the probe binding to the non-transformed plastid genome. Transformed plastomes yield larger fragments of 3,774 bp, 3,726 bp, and 4,278 bp containing the inserted expression cassettes for EspA, EspD, and Tir, respectively (black arrow).

### 3.6 Quantification of EspA protein accumulation

To determine the amount of recombinant protein produced in the transplastomic plants, accumulation levels of EspA, EspD, and Tir were quantified. TSP was extracted from tissue samples pooled from six random leaves from each plant in each transplastomic line. Recombinant protein was detected by Western blot. No recombinant protein was detected for the Tir and EspD transplastomic plants. Only EspA was detected by Western blot and migrates on a SDS gel to the expected molecular weight (Figure 11A, Table 2).

Densitometry analysis to quantify the recombinant protein revealed that the EspA fusion accumulates on average to  $0.04 \pm 0.01\%$  of TSP or  $7 \pm 2$  mg/kg of fresh leaf weight (Figure 11, Table 2). Accumulation levels were compared to two previously-generated transplastomic plants expressing a fusion of EspA proteins from serotypes O111:H8 and O26:H11. Both EspA fusions run similar on a SDS gel (Figure 11A) and accumulate to similar levels. EspA O111:H8-O26:H11 accumulates to  $0.03 \pm 0.002\%$  of TSP or  $7 \pm 0.4$  mg/kg of leaf tissue (Figure 11 B,C; Table 2). This is expected as both proteins have similar peptide sequences. Interestingly, plants 1-3 were several weeks older than plants 4-7 and accumulated less EspA (Figure 11A). This provides evidence for EspA being unstable and consequently being degraded in older tissues.

STEC genes cloned into cassettes containing the *T7g10* translational enhancer sequence were bombarded into *N. tabacum* to try to increase recombinant protein production. As of writing, only EspD transgenic lines have been generated. No recombinant protein was detected in these lines (data not shown) and therefore, the *T7g10* enhancer appears to not directly influence EspD accumulation.



**Figure 11. Accumulation of EspA protein in transplastomic *N. tabacum* lines.** A) Immunoblot of total soluble protein extracted (TSP) from wild type or transplastomic plants transformed with the EspA fusion (O157:H11-O26:H11) or another EspA fusion of different STEC serotypes (O111:H8-O121:H19). B) Mean protein accumulation of EspA O157:H7-O26:H11 and EspA O111:H8-O121:H19 as a percentage of total soluble protein (TSP) extracted and C) in milligrams per kilogram of fresh leaf weight. Each column represents the mean value of seven plants for EspA O157:H7-O26:H11 and two plants for EspA O111:H8-O121:H19. Error bars indicate the standard error of the mean.

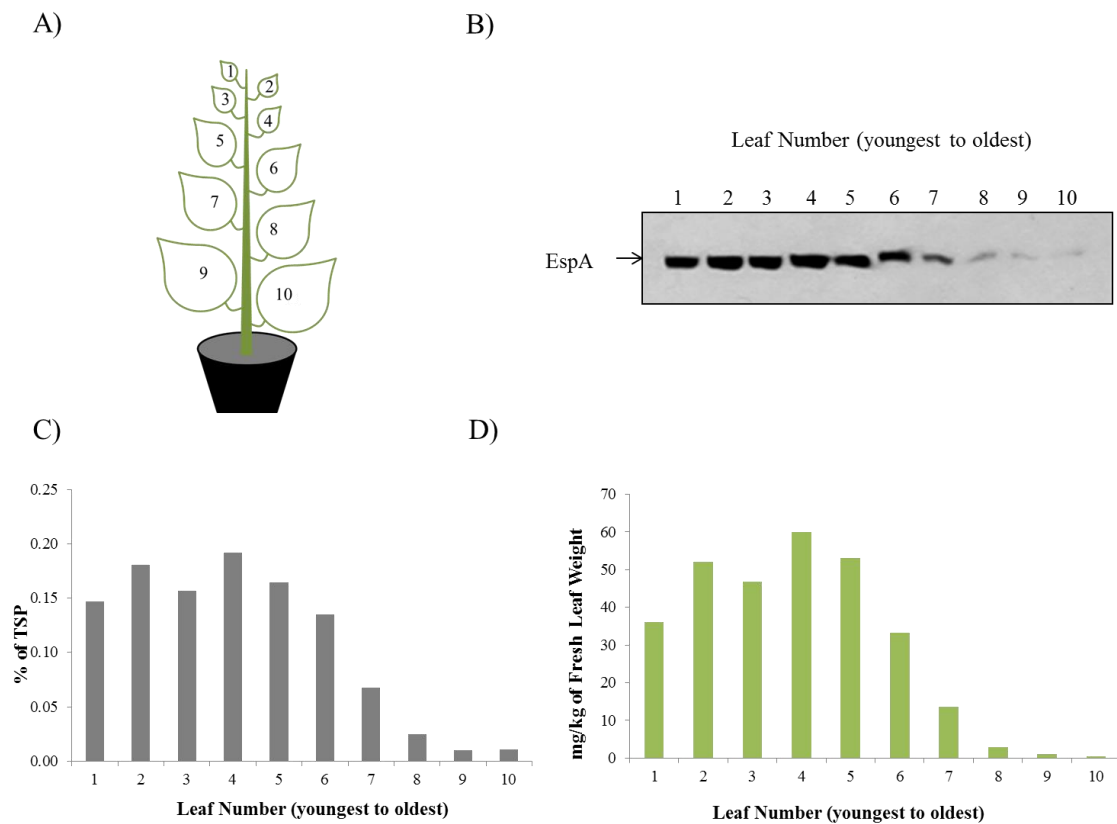
**Table 2. Predicted sizes and quantified accumulation levels of recombinant protein in *N. tabacum* transplastomic lines.**

Recombinant Protein	Predicted Size (kDa)	Accumulation as a percentage of total soluble protein <sup>a</sup>	Accumulation in mg/kg of fresh weight leaf tissue <sup>a</sup>
EspA O157:H7-O26:H11	43.8	0.04 ± 0.01	7 ± 2
EspA O111:H8-O121:H19	43.8	0.03 ± 0.002	7 ± 0.4
EspD O157:H7	41.4	0	0
Tir O157:H7	60.4	0	0
NleA O157:H7	50	N/A	N/A
Stx2b O157:H7	12.1	N/A	N/A

<sup>a</sup>, ± standard error of the mean value of the biological replicates. The quantity of recombinant protein was calculated as a mean of seven biological replicates for EspA O157:H7-O26:H11 and two biological replicates for EspA O111:H8-O121:H19. N/A, data is not available. Transplastomic plants could not be generated for these constructs.

### **3.7 Developmental dependence of EspA protein accumulation**

To gain insight into the stability of EspA, the spatial accumulation pattern of EspA in young to old leaf tissue was investigated. Tissue was collected from 10 leaves ranging from the highest, uppermost leaf to the bottom leaves of one transplastomic plant and analyzed for recombinant protein accumulation (Figure 12A). An immunoblot of total soluble protein extracts revealed that EspA accumulates to a higher level in younger, upper-middle leaves (Figure 12B). When quantified, EspA accumulates the highest in the middle leaves reaching 0.2% of TSP or 60 mg/kg of fresh leaf weight (Figure 12 C, D). There is an evident drop in protein accumulation in older tissue (leaves 6 to 10). EspA accumulates to the lowest level in leaf 10, reaching only 0.01% of TSP or 0.4 mg/kg of fresh leaf weight. Only one plant was used for this analysis and more plants will need to be analyzed to confidently conclude that accumulation decreases in older leaves. However, based on these results, EspA appears to be unstable in older tissue and therefore this indicates that tissue from younger leaves or plants should be harvested to maximize EspA production.



**Figure 12. Developmental dependence of EspA protein accumulation in transplastomic *N. tabacum* plants** A) Schematic of leaves used for analysis. Leaves sampled from younger (1) to older (10) leaves from one flowering plant. Image adapted from Kolotilin et al., 2012. B) Immunoblot of protein extracts from younger to older leaves from an EspA O157:H7-O26:H11 transplastomic plant. C-D) Spatial accumulation of EspA as a percentage of TSP and in milligrams per kilogram of fresh leaf weight. Each column represents the value from one plant.

### 3.8 STEC RNA expression in transplastomic lines

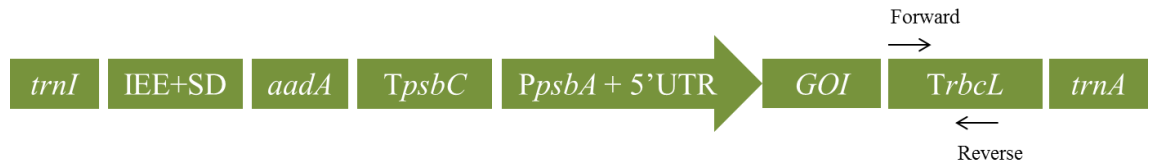
It is possible that differences in gene expression or mRNA stability could account for the striking differences in protein accumulation between *EspA*, *EspD*, and *Tir*. To determine if there are differences in transcript levels between constructs, mRNA levels from *EspA*, *EspD*, and *Tir* transgenic lines were compared using quantitative, reverse transcription (RT)-PCR. Leaf tissue from young and old leaves distributed throughout the plant was pooled to create one biological replicate. RNA was extracted from three plants from each transplastomic line, reverse-transcribed, and PCR amplified using primers specific to the 3' UTR of the transformation cassette. Primers were designed to anneal to the 3'UTR of the *rbcL* gene in each construct to amplify a 199 bp product and could be used for all constructs (Figure 13A). The 3'UTR of the *rbcL* gene in each construct is from the white poplar plastome and therefore the primers will not amplify the *rbcL* gene from *N. tabacum*. For a positive control, transgene expression was compared to *FaeG* expression. *FaeG* is a high-accumulating recombinant protein previously produced using the same transformation cassette (Kolotilin et al., 2012). STEC gene expression was normalized to *aadA* expression (Figure 13B). A Welch's ANOVA revealed that the expression levels of the transgenes are significantly different [ $F(2, 3.76) = 24.49$ ,  $p = 0.007$ ] (Figure 13C). A Games-Howell pairwise comparison demonstrated that the *EspD* construct has significantly higher mRNA levels compared to *EspA* and *Tir*. *FaeG* mRNA levels are higher than *EspD* levels but this difference cannot be confirmed statistically as only two biological replicates were analyzed for the *FaeG* samples. STEC RNA expression was also normalized to *rbcS*, *PsbA*, and *16S RNA* reference genes

(Figure 13B). Although graphically similar to the expression levels when normalized to *aadA*, STEC expression levels are not significantly different [ $F(2, 3.08) = 5.69, p = 0.092$ ].

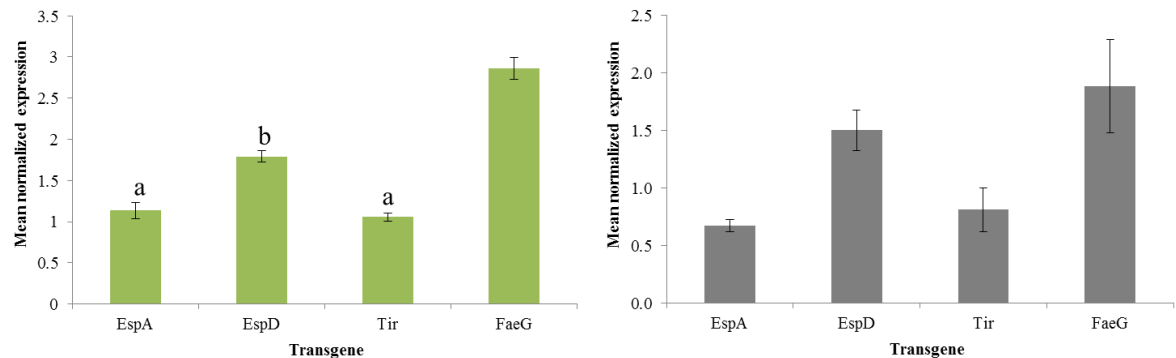
Despite there being slight differences in mRNA levels between the transgenes, the results presented here suggest that transcription and/or RNA stability are not the main determinants for STEC protein accumulation. *EspD* mRNA levels are close to those of *FaeG*, a protein that accumulates to 10% of TSP (Kolotilin et al., 2012), despite the fact that the *EspD* construct does not accumulate any detectable protein. As well, *EspD* has higher mRNA levels compared to *EspA* even though *EspA* is the only STEC construct accumulating recombinant protein. Since these results fail to provide direct evidence for a correlation between mRNA levels and STEC protein accumulation, it can be proposed that post-transcriptional mechanisms are primarily responsible for STEC protein production by the chloroplasts.



A)



B)



**Figure 13. Relative STEC expression of each transplastomic construct.** A) Schematic showing where primers for the qRT-PCR bind relative to the *GOI*. These primers bind to the 3' UTRs within the expression cassette and were used for all samples. B) Left: STEC expression normalized to *aadA* mRNA. Right: STEC expression normalized to *rbcS*, *PsbA*, and *16S RNA* mRNA. Expression levels were determined by quantitative RT-PCR and compared to *FaeG* expression. Each column represents the mean expression values calculated from the normalized expression values from three biological replicates. *FaeG* expression was calculated using two biological replicates and was not included in the statistical analysis. Error bars represent the standard error of the mean. Left: Means that do not share a letter are significantly different [ $F(2, 3.76) = 24.49, p = 0.007$ ]. Right: Means are not significantly different [ $F(2, 3.08) = 5.69, p = 0.092$ ].

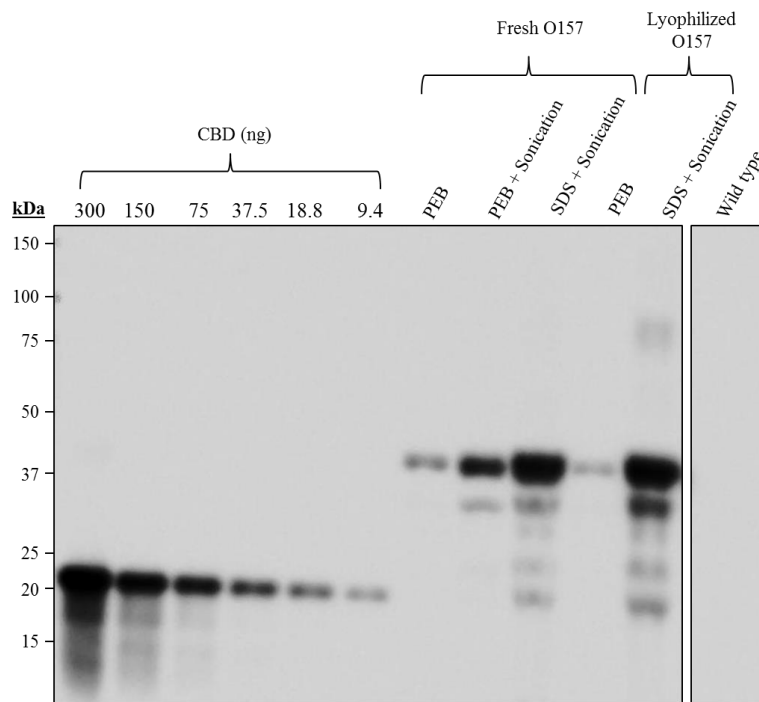
### **3.9 EspA accumulation in fresh and lyophilized *N. tabacum* transplastomic leaf tissue**

Leaf tissue from transplastomic EspA plants was collected and lyophilized for room-temperature storage and formulation for the animal trials. To determine if EspA is stable in lyophilized leaf tissue stored at room temperature, TSP was extracted from fresh leaf tissue and from milled, lyophilized leaf tissue from EspA transplastomic plants (Figure 14). EspA accumulated in the lyophilized samples to 48.1 mg/kg compared to 22.8 mg/kg in the fresh sample which is expected as water accounts for most of the fresh leaf weight and is absent in the lyophilized samples (Table 3). When comparing protein accumulation levels as a percentage of TSP, EspA accumulation in the lyophilized sample was roughly half of that in the fresh tissue (Table 3).

To determine if the extraction technique influences the amount of EspA extracted from the leaf tissue, different extraction protocols were employed on fresh and lyophilized leaf tissue. It was hypothesized that only a portion of EspA protein was being extracted using the regular extraction buffer and protocol, and some EspA protein could be trapped in the insoluble protein fraction. To investigate this, TSP was extracted from fresh EspA tissue following our standard TSP extraction protocol but with the additional step of sonicating the samples after PEB was added and the samples were vortexed. The additional sonication step was hypothesized to better disrupt the chloroplasts releasing more EspA protein. Conversely, total protein was extracted from fresh and lyophilized samples using a 10 mM tris buffer, pH 8.0, containing 2% (w/v) SDS, which was then followed by sonication. Remarkably, immunoblotting the samples revealed a drastic increase in EspA extraction in the PEB + sonication and SDS buffer + sonication samples

(Figure 14). The sonication step extracted roughly 6.25 times more EspA protein while the SDS extraction buffer with sonication extracted 9.7 times more protein (Table 3). Using the 2% SDS (w/v) extraction buffer, it was found that EspA accumulates to 220.7 mg/kg of fresh leaf weight or 1,952.9 mg/kg of dry leaf tissue compared to 22.8 mg/kg and 48.1 mg/kg respectively, for the samples extracted with regular PEB and with no sonication.

These results have two implications. First, EspA was detected in lyophilized leaf tissue stored at room temperature for several weeks and therefore is stable in lyophilized tissue. Less EspA accumulated in the lyophilized tissue (as a percentage of TSP), but this can be attributed to variation between plants used for the fresh and lyophilized samples. Tissue collected for the fresh samples came from younger plants which, as previously demonstrated, accumulate more EspA. This can account for the decrease in recombinant protein extraction from the lyophilized samples. Furthermore, this is evident when comparing accumulation levels in Table 2 and 3; the accumulation levels reported in Table 2 are much lower than reported in table three as older plants were sampled for the earlier EspA quantification. Second, it appears that EspA accumulates to a much higher level than previously reported and EspA extraction from transplastomic tissue can be greatly increased using sonication and using a 2% (w/v) SDS buffer.



**Figure 14. EspA accumulation in fresh and lyophilized *N. tabacum* transplastomic leaf tissue and a comparison of different protein extraction techniques.** An

immunoblot of protein extracted from fresh or lyophilized EspA O157:H7-O26:H11 tissue. Different extraction conditions were employed to maximize protein detection. TSP was extracted as previously described using regular plant extraction buffer (PEB) or with an additional sonication step. Total protein was also extracted using at 10 mM Tris 2% SDS extraction buffer with sonication. 10  $\mu$ g of TSP was loaded or 4  $\mu$ l of extract was loaded for the SDS samples. Protein was extracted from pooled leaf tissue from three plants.

**Table 3. EspA accumulation in fresh and lyophilized *N. tabacum* transplastomic leaf tissue and a comparison of different protein extraction techniques.**

Tissue type	Extraction Conditions	Accumulation as a percentage of total soluble protein	Accumulation in mg/kg of fresh leaf tissue	Accumulation in mg/kg of lyophilized leaf tissue
Fresh	PEB	0.14	22.8	
Fresh	PEB with Sonication	0.79	142.5	
Fresh	SDS buffer with Sonication		220.7	
Lyophilized	PEB	0.07		48.1
Lyophilized	SDS buffer with Sonication			1,952.9

N/A, data is not available. Total protein was extracted for these samples.

Different extraction conditions were employed to maximize protein detection. TSP was extracted as previously described using regular plant extraction buffer (PEB) or with an additional sonication step. Total protein was also extracted using at 10 mM Tris, 2% SDS extraction buffer with sonication. Protein was extracted from pooled leaf tissue from three plants.

## 4 DISCUSSION

### 4.1 STEC vaccine design

The goal of this study is to produce a plant-made, subunit vaccine candidate to immunize ruminants against multiple STEC serotypes. Previous vaccination efforts have mainly been focused on proteins secreted by the type III secretion pathway. LEE-encoded Esps and Tir knockouts determined that these proteins are required for STEC colonization (DeVinney et al., 1999; Naylor et al., 2005) and immunization with Tir, EspA and other type III secreted proteins caused a reduction in *E. coli* O157:H7 shedding in cattle feces (Potter et al., 2004). Since then, there have been several studies evaluating the efficacy of type III protein-containing vaccines and a systematic review of all STEC vaccine trials from 1990-2010 revealed that type III protein vaccines can cause a significant reduction of *E. coli* O157:H7 faecal prevalence in cattle (Snedeker et al., 2012). Because of this literature, I focused on T3SS proteins and other secreted effector proteins to design the vaccine for this study.

Asper and colleagues (2011) identified Tir, EspA, EspD, EspB, and NleA secreted proteins as reacting significantly to sera of *E. coli* O157:H7-infected or vaccinated cattle. In this study, NleA was the only non-LEE encoded effector protein reported to be highly immunogenic. EspB was omitted from my vaccine design, but remains a future candidate for plant-based production. The Stx2b subunit was included in my vaccine design because the complete Shiga toxin could be toxic to plant cells. Consequently, the STEC proteins EspA, EspD, Tir, NleA, and Stx2b were used for vaccine production *in planta*.

## 4.2 Transient production of STEC antigens

EspA, NleA, and Stx2b accumulated to quantifiable levels when transiently produced in *N. benthamiana* plants. One of the major findings from my transient assays is that the addition of an ELP tag increased antigen accumulation dramatically. EspA and NleA accumulated the highest when targeted to the ER and fused to an ELP tag. Also, Stx2b only accumulated to a detectable level when fused to an ELP tag. ELP tags increase the stability of recombinant proteins as they are not susceptible to hydrolysis or protease cleavage (Conley et al., 2009a) and may protect the protein through the formation of protein bodies (Conley et al., 2009b; Saberianfar et al., 2015). My findings are consistent with other recombinant proteins expressed in tobacco and *N. benthamiana* (Conley et al., 2009a; Floss et al., 2008). Addition of the hydrophobin tag also increased protein accumulation of EspA, but not to the extent of the ELP tag. It has been shown that hydrophobins increase protein accumulation when coupled to recombinant proteins (Joensuu et al., 2010; Linder et al., 2004) through a similar protein body-inducing mechanism as ELPs (Conley et al., 2011a). However, based on my findings, this mechanism appears to be protein specific as only EspA accumulated to a noticeably higher level when fused to HFBI.

No clear conclusions can be made on which intracellular organelle accumulates the most recombinant protein in this thesis. Overall, protein accumulation of the STEC antigens is low in all intracellular compartments tested, unless the protein is fused to an ELP tag. Targeting proteins to different intracellular compartments can cause significant variation in protein accumulation. For example, the B subunit of the heat-labile toxin I (LTB) produced stably in corn accumulates more than four orders of magnitude

depending on which subcellular location the recombinant protein is targeted (Streatfield et al., 2003). Each subcellular compartment or organelle has a unique biochemical environment, protease content, and physical size - all factors capable of influencing protein accumulation levels (Pillay et al., 2014; Streatfield, 2007). Recombinant proteins generally accumulate poorly when targeted to the cytoplasm (Conrad and Fiedler, 1998), due to the lack of co- or post-translational modifications or because of degradation by cytosolic proteases (Benchabane et al., 2008). Therefore it is not surprising that proteins such as EspA failed to accumulate highly when targeted to this organelle. Because of this, many high-accumulating recombinant proteins have been targeted to the ER due to its low hydrolytic activity, its innate plasticity, the presence of chaperones assisting with protein folding, and its oxidizing environment (Vitale and Pedrazzini, 2005). EspA, NleA, and Stx2b did accumulate the highest when targeted to the ER, but only when fused to an ELP tag. These results suggest that fusion tags have a greater influence on protein accumulation compared to the organelle to which the protein is targeted. It is probable that recombinant proteins must accumulate to a certain level in order for the organelle, to which the protein is targeted, to have a significant effect on protein accumulation. The STEC proteins may not simply accumulate to a high enough level for these effects to be observed.

STEC proteins were targeted to the chloroplasts to determine if these organelles are suitable for protein accumulation, and therefore to potentially screen for expression by the plastid genome. This is because FaeG was shown to accumulate to a very high level when targeted to the chloroplasts and consequently, accumulated to a high level when transplastomically produced (Kolotilin et al., 2012). Both EspA and NleA



accumulated when targeted to the chloroplasts albeit their accumulation levels were low. Because of this, these proteins were the most promising candidates for transplastomic production and it is not surprising that *EspA* was the only transplastomic construct to accumulate any protein. I speculate that NleA would also accumulate when transplastomically produced despite the fact that no positive transformants could be generated as of writing this thesis.

Why Tir, EspD, and the Colicin do not accumulate transiently in *N. benthamiana* remains unknown. Examining the level of gene expression, all constructs were driven by the double-enhanced viral promoter to ensure high transcription rates and these vectors have been previously shown to accumulate recombinant proteins to high levels and in several compartments (Pereira et al., 2014).

These recombinant proteins could be degraded by endogenous plant proteases targeting recombinant proteins that have not properly folded or been correctly synthesized (Desai et al., 2010). There are potentially hundreds of genes coding for proteases in plants (Benchabane et al., 2008) and there are at least 43 currently characterized proteases in *N. tabacum* (Rawlings et al., 2014). One strategy to increase transient accumulation is to reduce the proteolytic capacity of plant cells by using RNA-silencing strategies or co-transforming protease inhibitors along with the recombinant protein of interest (Benchabane et al., 2008). The latter was demonstrated when recombinant antibodies secreted from tobacco roots exhibited less degradation and accumulated to a higher level when co-expressed with a strong serine protease inhibitor (Komarnytsky et al., 2006). The cysteine protease inhibitor, *SICY8*, has been shown to increase recombinant protein accumulation up to 40% when co-infiltrated in *N.*

*benthamiana*, though the effect is confined to younger leaves where *SICY8* expression is the highest (Robert et al., 2013). Recently, silencing the cysteine protease CysP6 was shown to increase accumulation levels of the human therapeutic, interleukin (IL)-10, in nuclear-transformed tobacco plants (Duwadi et al., 2015). In addition, recombinant proteins can be fused to protease inhibitors which have been shown to stabilize and increase protein accumulation levels 25-fold (Sainsbury et al., 2013). Strategies such as these could be applied to better stabilize and protect the transiently-produced STEC antigens. For example, when targeted to the ER and fused to an ELP tag, EspA showed a notable degradation pattern by Western blot indicating that it could be improperly folded and targeted by proteases. An interesting experiment could be conducted by co-infiltrating EspA with, or fusing it to, a protease inhibitor to see if there is less degradation and potentially higher accumulation of this protein.

EspA, NleA, and Stx2b accumulated up to 87.8 mg/kg, 1.3 mg/kg, and 28 mg/kg of fresh leaf weight, respectively, when fused to an ELP tag and expressed transiently in *N. benthamiana*. These levels are comparable to reports of various STEC antigens being produced by stable integration into the nuclear genome. A chimeric EspA-Intimin-Tir protein was produced in tobacco and canola plants using *A. tumefaciens*-mediated nuclear transformation. This protein fusion accumulates on average to 0.2% of TSP in tobacco and 0.3% of TSP in canola, and was shown to reduce the duration of *E. coli* O157:H7 shedding when administered to mice (Amani et al., 2011). Intimin stably produced in tobacco cv. Bright Yellow 2 (BY2) cells accumulated to 13 µg/g (13 mg/kg) of plant material and was shown to reduce *E. coli* O157:H7 colonization in mice (Judge et al., 2004). Plant-optimized Stx2a and Stx2b genes were stably produced in tobacco cells

accumulating up to 8.2  $\mu\text{g/g}$  (8.2 mg/kg) of tobacco cells and induced the production of protective anti-Stx2 antibodies (Wen et al., 2006). These reports emphasize the validity of the accumulation levels described in this thesis and further strengthen the promise of a viable STEC-vaccine plant bioreactor.

Although these transient results are very encouraging, nuclear production of the STEC antigens may not be optimal due to the potential addition of *N*- and *O*- glycans to the recombinant proteins when targeted to the secretory pathway (Gomord et al., 2010). Indeed, Judge and colleagues found that nuclear-transformed Intimin became glycosylated when targeted to the ER and elicited a different antibody response in mice compared to Intimin produced in bacterial cells (2004). It is possible that the transiently-produced STEC antigens are glycosylated when targeted to the ER, which unfortunately is the compartment accumulating the most protein. Several potential glycosylation sites are predicted for the STEC constructs (Appendix I), which could alter the immune response to these antigens when transiently produced in *N. benthamiana* as these proteins are not glycosylated in STEC. A further argument against transient production of the STEC antigens is the fact that large-scale transient production would require the growth and maintenance of large volumes of *A. tumefaciens* cultures which would certainly increase production costs. Because of these important considerations, we elected to produce the antigens in the chloroplast genome of *N. tabacum* due to the lack of glycosylation, the stable inheritance of transplastomic genes eliminating the need for *A. tumefaciens*, and, most importantly, the fact that the highest recombinant protein levels are generally reported in transplastomic plants.

### 4.3 Generation of transplastomic bioreactors

STEC genes were transformed into the chloroplast genome of *N. tabacum* for high-yield, stable antigen production. Overall, transformation frequency was low and only EspA, EspD, and Tir transplastomic plants were generated. Southern blot analysis confirmed that the transplastomic lines were homoplasmic for the transgenes. Despite this, EspA was the only protein to accumulate to a detectable level, accumulating up to 220.7 mg/kg of fresh leaf weight. While there are many reports of recombinant proteins accumulating to levels much higher than this when produced transplastomically (Bock, 2014a), the accumulation of EspA is still a substantial achievement and lyophilized tissue containing EspA will be administered to a sheep ruminant model for the immunological assays. To strengthen this argument and as a comparison, a subunit vaccine against Crimean-Congo Hemorrhagic Fever Virus only accumulated to 1.8 µg/g (1.8 mg/kg) in stably-transformed tobacco and yet still generated an immune response in orally-administered mice (Ghiasi et al., 2011). The envelope glycoprotein 5 (GP5) from porcine reproductive and respiratory syndrome virus was produced in tobacco and accumulated only to 110 ng/g or 0.11 mg/kg of fresh leaf weight and was able to generate both IgG and IgA immune responses when only 50 grams of leaf tissue was orally administered to pigs (Chia et al., 2010). In another study, LTB was expressed in tobacco and accumulated only to 14 µg per gram of total soluble protein, and when orally administered, induced an immune response in mice (Haq et al., 1995). LTB accumulated to 300 mg/kg of lyophilized leaf material when expressed in *N. benthamiana* and *Petunia parodii* (petunia) plants and when this leaf tissue was orally administered to sheep in four 5 mg doses, LTB-specific IgA and IgG immune responses were observed (Pelosi et

al., 2012). The dosage reported in this last study will serve as the basis for our immunological assays as it is one of the few reports of a successful plant-made vaccine being orally administered to a sheep ruminant model.

It can be anticipated that an increase in antigen accumulation will lead to an increase in immune response, as well as allowing a reduction in the amount of lyophilized tissue needed to be administered, directly reducing the vaccine cost. A major challenge to mucosal antigen delivery is overcoming antigen degradation by the digestive process in ruminants (Shewen et al., 2009). Although plant tissue may bioencapsulate and provide partial protection from the harsh conditions of the ruminant digestive tract, protein accumulation should be maximized to ensure that sufficient vaccine is delivered to the mucosal immune sites. Therefore, investigating how to improve accumulation levels in the transplastomic plants remains of utmost importance to increase vaccine efficacy and to ensure vaccine production is economically feasible.

It was proposed that RNA accumulation may account for the low or absent recombinant protein levels. There are very few reports of RNA stability influencing recombinant protein accumulation in chloroplasts. An enzyme involved in carotenoid biosynthesis, lycopene  $\beta$ -cyclase, failed to accumulate when transformed into the tomato plastid genome (Wurbs et al., 2007). This was attributed to observed instabilities of the corresponding RNA transcripts. A qRT-PCR of the *EspA*, *EspD*, and *Tir* lines revealed that all of the constructs have comparable expression levels to the transplastomically-produced FaeG. While *EspA* and *Tir* mRNA levels are similar, *EspD* transcript levels are higher which is interesting as *EspD* did not accumulate any protein. This suggests that

post-transcriptional processes are primarily influencing protein levels and not transcription or RNA stability.

Translational errors could be responsible for the antigen accumulation levels. The chloroplast translation machinery is similar to *E. coli* and efficiency is dependent on translation initiation, elongation, and termination (Lithwick and Margalit, 2003; Sugiura, 2014). The *psbA* 5' UTR was included upstream of all constructs to confer mRNA stability and initiate translation by providing a correct reading frame and start codon (Kolotilin et al., 2013; Sugiura, 2014). As in transient production, the rate of translational elongation in the chloroplasts is dependent on the codon composition of the transgene. Rare codons used by the expression host are deleterious to recombinant protein yields (Gustafsson et al., 2004) and should be replaced with more frequently used synonymous codons. For example, human growth factor  $\beta$ 3 produced transplastomically in tobacco, accumulated 75-fold higher when codon optimized for chloroplast expression compared to the native coding sequence (Gisby et al., 2011). Due to codon usage differences between *E. coli* and *N. tabacum* plastids, all transplastomic STEC constructs were codon-optimized for expression by the chloroplast genome of tobacco. However, it should be noted that codon usage does not necessarily correlate with translational efficiency in chloroplasts. Translational efficiency is reported to vary dramatically between synonymous codons despite their similar usage (Nakamura and Sugiura, 2011). This questions the efficacy of our so-called codon optimization and an experiment comparing non-codon optimised and codon optimised STEC constructs is underway.

Polysome profiling would be a direct experiment to determine the translational efficiency of the STEC constructs. With this technique, polysomes can be isolated using

sucrose density centrifugation and transcripts associated with the polysomes can be subjected to a Northern blot to determine if ribosomes are binding to the STEC mRNA (Klein et al., 1988).

While transcription and translation can influence protein accumulation, protein stability is arguably the largest determinant of recombinant protein accumulation in the chloroplasts (Bock, 2014b). After synthesis, peptides must fold into a stable, three-dimensional state to avoid protein aggregation and degradation by proteases. Chloroplasts contain at least eleven protease families encoded by more than 50 genes, the majority of which are bacterial in ancestry (Sakamoto, 2006). One function of these proteases is to degrade proteins that are generated in excess, damaged, or incorrectly synthesised (Sakamoto, 2006). There are several reports of recombinant proteins accumulating to low levels when produced transplastomically due to peptide instabilities. The storage protein,  $\beta$ -Zein, failed to accumulate either when targeted to the chloroplasts or when transplastomically produced in tobacco (Bellucci et al., 2005). Low accumulation levels of a rotavirus protein, VP6, produced transplastomically in tobacco were attributed to protein instability and degradation (Birch-Machin et al., 2004). VP6 accumulation declined in older leaves, similar to my observations. EspA accumulation declined in older leaves of the same plant suggesting that the protein is not stable and is degraded in older leaf tissues.

The identity of the amino acid residues in the N-terminus of proteins correlates with the half-life of the protein, known as the N-end rule (Varshavsky, 1996). Both eukaryotes and prokaryotes operate under the N-end rule and it appears that the N-end rule exists in chloroplasts (Apel et al., 2010). Apel and colleagues determined that protein

stability is largely governed by the penultimate residue in chloroplasts and were able to boost accumulation with N-terminal, but not C-terminal fusions to GFP (2010). This proved to be an effective strategy to boost recombinant protein accumulation in chloroplasts. The HIV fusion inhibitor, Cyanovirin-N, does not accumulate to detectable levels when expressed in the chloroplasts of tobacco. However, fusing N-terminal peptides of high-accumulating proteins such as GFP to Cyanovirin-N, increased protein accumulation and interestingly, also increased mRNA stability (Elghabi et al., 2011). It is possible that EspD or Tir may have unstable N-terminal residues and could be fused to a more stable protein to increase protein accumulation. This was attempted with the fusion of the *T7g10* translational enhancer with a downstream box to the 5' end of the STEC constructs. Despite the low transformation success, T7g10-EspD plants were generated with no difference in protein accumulation. In future work, different N-terminal fusions could be fused to the recombinant proteins to attempt to better stabilize the peptides.

Another strategy to increase recombinant protein accumulation in the chloroplasts is to target proteins to the thylakoid lumen instead of targeting to the stroma. The bovine pancreatic trypsin inhibitor, aprotinin, was produced transplastomically in tobacco and was successfully targeted to the chloroplast thylakoid lumen (Tissot et al., 2008). This strategy also proved effective when producing single-chain, VHH antibodies transplastomically. Accumulation of the VHHs increased significantly when targeted to the thylakoid lumen using a signal peptide from *E. coli* (Lentz et al., 2012). The thylakoid lumen may contain protein chaperones, have a different set of proteases, and have a higher redox potential more appropriate for expressing proteins requiring disulfide-bonds (Bally et al., 2008). To target STEC proteins to the thylakoid lumen,



signal peptides targeting the twin-arginine translocation (Tat) pathway or the general secretion (Sec) pathway can be fused to the constructs and could also potentially stabilize the N-terminus.

Interestingly, the VHHs were believed to be toxic to the chloroplasts, reportedly causing difficulties regenerating homoplasmic plants (Lentz et al., 2012). I also had this problem generating NleA and Stx2b transplastomic plants as NleA transplastomic lines lost the transgene during the regeneration rounds on selective media, and Stx2b bombarded tissue did not regenerate. It is possible that these genes may be toxic in the chloroplasts, by for instance, interfering with photosynthesis, causing selection against transformed plastid genomes and eventual transgene loss. EspA transplastomic plants were noticeably paler in colour and had a reduced growth rate compared to wild type tobacco. It is conceivable that EspA may also be toxic to the plastids by reducing the photosynthetic capacity of these plants, which would explain the slow growth rates of these plants.

Many proteins require molecular chaperones to prevent protein aggregation during folding and to maintain a conformationally active state (Hartl et al., 2011). It was reported that EspA, EspD, and Tir require chaperones to properly fold and to prevent proteolysis. Several chaperones of STEC type III proteins have been identified to date and their absence in the chloroplast is a likely reason for the low accumulation levels. CesT has been identified as a chaperone of Tir as it promotes Tir stability and is involved in its secretion (Elliott et al., 1999). CesT is believed to be a global type III chaperone as it interacts with and promotes the secretion of Tir and many other STEC effector proteins including NleA (Thomas et al., 2005). Likewise, EspD fails to accumulate intracellularly

in *E. coli* and is not secreted when its chaperone, CesD, is knocked out (Wainwright and Kaper, 1998). A second chaperone, CesD2, of EspD was identified and also contributes to EspD stability and secretion (Neves et al., 2003). CesAB was identified as a chaperone for EspA and EspB, as it is required for secretion of both Esps and stabilizes EspA (Creasey et al., 2003). It appears that EspA additionally requires another two chaperones for stabilization, CesA2, and the gene product of gene *l0017* (Ku et al., 2009; Su et al., 2008).

Co-expressing EspA, EspD, or Tir with their appropriate chaperones may promote correct protein folding and increase accumulation. Many plastid genes are co-transcribed and this characteristic of the chloroplasts can be utilized to stack multiple transgenes together in an operon. This strategy was used to express the *Bacillus thuringiensis cry2Aa2* operon in tobacco chloroplasts. Both *cry2Aa2* and its chaperone were expressed causing *cry2Aa2* to accumulate up to 46.1% of TSP (De Cosa et al., 2001). In future experiments, STEC genes and their respective chaperones could be cloned as a polycistron in the chloroplast expression cassettes for plastid transformation to determine if chaperones increase STEC antigen accumulation in tobacco. Preliminary work transiently co-infiltrating chloroplast-targeted CesT and Tir in *N. benthamiana* has shown that Tir accumulates higher and to detectable levels in chloroplasts (Jacqueline MacDonald, Personal Communication). Ongoing work to nuclear transform the Tir transplastomic plants with chloroplast-targeted CesT is underway which may increase Tir accumulation. As an alternative strategy, synthetic constructs could be designed including only the epitopes of EspA, EspD, and Tir which may negate the need for chaperones to ensure proper folding.

One of the major findings from this research is that the amount of recombinant protein extracted is largely determined by the extraction technique used. Both sample sonication and extraction using an SDS buffer dramatically increased the amount of EspA extracted from the transplastomic tissues. Therefore it appears that previous quantifications of protein accumulation levels using standard PEB underestimate the amount of protein present in the tissues. It can be postulated that sonication and/or the presence of SDS helps lyse the chloroplasts releasing more recombinant protein or breaks up insoluble protein aggregates. How much of this extra insoluble protein that is released and still antigenically functional is debatable and can be addressed in the future.

Examining the literature, there is some variation in extraction buffers, but most are PBS, Tris, or HEPES based with various protease inhibitors (Table 4). It is probable that differences in the extraction buffer influence the amount of soluble protein extracted, however, it cannot be expected that these differences will cause an increase in the reported accumulations levels of EspA on par to what has been published (Table 4). Even extracting total protein with SDS only led to an increase in EspA to 220 mg/kg, a far cry from the 2 g/kg of soluble FaeG extracted using standard PEB (Kolotilin et al., 2012). Therefore, although the extraction buffer should be optimized in future experiments to maximize protein extraction, the differences in the accumulation levels between EspA and other high accumulating transplastomic proteins is most likely due to the specific protein in question rather than how it is extracted.

**Table 4. Comparison of different buffers used to extract and quantify vaccine antigens produced transplastomically.**

Authors	Plant Bioreactor	Recombinant Protein	Accumulation Level	Extraction Buffer
Miletic, 2015	Transplastomic tobacco	EspA fusion	0.04-0.14% of TSP	PEB (PBS pH=7.5, 1% Tween-20, 1 mM EDTA, 2% PVPP, 1% PMSF and 0.1% leupeptin) or 10 mM Tris with 2% SDS
Karimi et al., 2013	Transplastomic tobacco	EspA-Intimin-Tir	1.3-1.4% of TSP	50 mM HEPES/KOH, pH 7.5, 1 mM EDTA, 10 mM potassium acetate, 5 mM magnesium acetate, 2 mM PMSF, and 30 mM $\beta$ -mercaptoethanol
Kolotilin, et al., 2012	Transplastomic tobacco	F4 fimbrial adhesin FaeG	11.3% of TSP	PEB
Davoodi-Semirami, et al., 2010	Transplastomic tobacco and lettuce	CTB-apical membrane antigen-1 and CTB-merozoite surface protein-1	13.17% and 10.11% TSP in tobacco, 7.3% and 6.1% TSP in lettuce	100 mM NaCl, 10 mM EDTA pH 8, 200 mM Tris-HCl, pH 8, 0.05% Tween-20, 0.1% SDS, 14 mM $\beta$ -mercaptoethanol, 200 mM sucrose, 2 mM PMSF and EDTA-free protease inhibitor cocktail
Rosalés-Mendoza et al., 2009	Transplastomic tobacco	Heat-labile toxin (LT) fused to and the Heat-stable toxin (ST)	2.3% of TSP	0.05% Tween-20 in PBS with protease inhibitor cocktail
Arlen, et al., 2008	Transplastomic tobacco	Fraction 1-V antigen	14.8% of TSP	100 mM NaCl, 10 mM EDTA pH 8, 200 mM Tris-HCl, pH 8, 0.05% Tween-20, 0.1% SDS, 14 mM $\beta$ -mercaptoethanol, 200 mM sucrose, and 2 mM PMSF
Ruhlman, et al., 2007	Transplastomic tobacco and lettuce	CTB-Proinsulin	16% of TSP in tobacco, 2.5% TSP in lettuce	50 mM HEPES/KOH, pH 7.5, 1 mM EDTA, 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, and 2 mM PMSF
Koya, et al., 2005	Transplastomic tobacco	Anthrax protective antigen (PA)	14.2% of TSP	200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, and 2 mM PMSF
Glenz, et al., 2005	Transplastomic tobacco	Lyme Disease 1 lipoprotein OspA, OspA-Truncated	1% of TSP and 10% of TSP	0.3% Triton X-114, and 1 mM PMSF in PBS
Tregoning, et al., 2005	Transplastomic tobacco	Tetanus toxin Fragment C	18-27% and 7-10% of TSP	50 mM HEPES/KOH, pH 7.5, 1 mM EDTA, 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM or 10 mM dithiothreitol, and 2 mM PMSF
Birch-Machin, et al., 2004	Transplastomic tobacco	Rotavirus VP6	3% of TSP	50 mM HEPES/KOH, pH 7.5, 1 mM EDTA, 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, and 2 mM PMSF

#### **4.4 Transplastomically-produced EspA as a vaccine against STEC**

In this study, I was able to transplastomically produce an EspA fusion protein which is a potential vaccine candidate against STEC colonization in ruminants. Tobacco leaf tissue containing the vaccine candidate was lyophilized and will be administered via fistulation to a sheep ruminant model. Finely-ground plant tissue containing a 5 mg dose of EspA will be administered to sheep three times over a six week period. EspA-specific IgG and IgA antibody levels will be measured in the sheep to determine the efficacy of this vaccine. Animals will then be challenged with STEC to determine if immunization with the EspA fusion causes a reduction in STEC shedding and colonization. These experiments will be conducted over the summer of 2015 at Agriculture and Agri-Food Canada's facility in Lethbridge, Alberta by Dr. Tim McAllister and his colleagues.

Although the efficacy of this vaccine candidate will not be elucidated until these future experiments are complete, several considerations of this vaccine can be postulated and may need to be addressed in the future. One of the disadvantages of soluble antigens for use as oral vaccines is their general inability to induce a protective immune response (Kolotilin et al., 2014). The intestinal immune system displays local and systemic immunological tolerance, known as oral tolerance, to antigens encountered regularly in food or present on commensal bacteria. This is accomplished to avoid wasteful and potentially harmful immune responses against harmless proteins (Mowat, 2003). Because of this phenomenon, it is possible that EspA may not generate a sufficient immune response to be protective when administered orally to ruminants. For instance, mice orally immunized with purified EspB alone did not generate any serum IgG or fecal IgA immune responses, but EspB was found to be immunogenic when expressed in *Lactococcus lactis* bacterial delivery system (Ahmed et al., 2014). Conversely, a chimeric

EspA-Intimin-Tir fusion antigen produced in tobacco induced both IgA and IgG responses when orally administered to mice, though the IgG response was higher when a combination of oral and subcutaneous administrations was used (Amani et al., 2011). Also, a plant-made EspA-Intimin-Tir fusion was shown to reduce *E. coli* O157:H7 shedding in mice when orally administered (Karimi et al., 2013). Based on these studies, oral tolerance appears to operate in an antigen-specific or administration-specific manner, but remains a potential concern for an EspA oral vaccine.

If there is no immune response in the sheep to EspA, adjuvants can be included in the vaccine formulation to help activate the immune system (De Magistris, 2006). The cholera toxin (CT) and the heat-labile toxin I (LT) are both effective mucosal adjuvants and when orally co-administered with an antigen of interest, induce the production of antigen-specific IgG and IgA antibodies, effectively suppressing oral tolerance (Clements et al., 1988; Elson and Ealding, 1984). However, these toxins can be potentially harmful to the host and therefore the B subunits from LT and CT have been explored for their adjuvant activity. Indeed, CTB can have immunoadjuvant activity when orally co-administered with antigens (Tochikubo et al., 1998), but it is reported to induce a better immune response when coupled either chemically or genetically to the antigens of interest (McKenzie and Halsey, 1984; Sanchez et al., 1990). Coupling antigens to CTB or LTB increases antigen uptake across the mucosal barrier and increases antigen presentation to dendritic cells and macrophages (Holmgren et al., 2005). This strategy was used to create two vaccine candidates against both cholera and malaria by fusing malarial antigens to CTB produced transplastomically in tobacco and lettuce (Davoodi-Semiromi et al., 2010). In another example, tuberculosis antigens fused to CTB have

been produced transplastomically in tobacco and were shown to retain functionality (Lakshmi et al., 2013). As previously mentioned, Pelosi and colleagues orally administered a plant-produced LTB subunit vaccine to sheep and reported antigen-specific immune responses in mucosal secretions (2012). These studies suggest that adjuvants can be co-administered with, or coupled to, EspA to ameliorate the intestinal immune response in animals.

Originally, all of the antigens were to be fused with the Stx2b subunit as Stx2 is reported to promote intestinal colonization in cattle (Baines et al., 2008a) and therefore the B subunit was speculated to enhance antigen immunogenicity. Indeed, a Stx2b-Tir-Stx1b subunit vaccine fused to *Zonula occludens* toxin, induced IgG and IgA production and reduced *E. coli* O157:H7 shedding in goats (Zhang et al., 2012). Additionally, Gao and colleagues constructed a Stx2b-Stx1b fusion protein and saw high levels of IgG production when intraperitoneally administered in mice (2009). An EspA-Stx2A1 fusion was subcutaneously administered to mice and induced both EspA and Stx2A1-specific antibodies (Cheng et al., 2009). Despite these studies, it is reported that cattle lack the globotriaosylceramide (Gb3) receptor in the gastrointestinal tract (Pruimboom-Brees et al., 2000) or it is at least confined to epithelial crypt cells (Hoey et al., 2002), explaining the absence of symptoms in cattle from STEC infection. Also, there is evidence for Shiga toxins having immunosuppressive effects in cattle (Hoffman et al., 2006). Because of this, I decided to express the STEC antigens without Stx2b fusions to prevent any potential loss in protein function and immunogenicity. However, fusion to or co-administration with either Stx2b or CTB/LTB remains a potential strategy to increase immunogenicity and can be explored in future experiments.

#### 4.5 Conclusion and future perspectives

In conclusion, several vaccine candidates against STEC were designed and produced transiently in *N. benthamiana* and transplastomically in *N. tabacum*. One STEC antigen, an EspA fusion of two different serotypes, accumulated up to 220mg/kg of fresh leaf weight in the chloroplasts of tobacco. Therefore, I was able to create a transplastomic tobacco lines which stably produce a vaccine candidate against STEC colonization in ruminants. Thus, the results presented in this thesis fulfill the objectives of this study. The generated transplastomic lines from this study can potentially be grown in greenhouses or in farm fields across Canada to produce this recombinant protein on a large-scale level. Tissue could then be regularly administered to cattle herds throughout North America to provide multi-serotype protection against STEC and reduce contamination in our food supply.

Despite this success, there remain several questions to be addressed in both the fields of chloroplast biotechnology and ruminant immunology. Foremost, why did the STEC antigens accumulate to low levels when transplastomically produced in tobacco? The addition of N-terminal fusions or co-expression with chaperones and/or protease inhibitors may address these questions. Regarding vaccine administration, will the antigens be protected from the harsh conditions of the ruminant digestive tract? Will the vaccine dosage be sufficient or will adjuvants be required to induce a protective immune response? Many of these questions will hopefully be answered soon during our immunological experiments.

Using plants as production platforms is still a relatively new field of research and has increasing potential. Unfortunately, industry has been slow to adopt these technologies, preferring cell culture-based expression platforms which are capable of



high protein yields, maintain protein activity, and satisfy current biocontainment requirements. Plants are complex organisms which are still not completely understood, and consequently, will not produce every recombinant protein to an economically-feasible level. However, research has progressed significantly over the last decade with higher accumulation levels being reported than thought possible and given enough time, plants may become a competitive alternative to traditional production platforms. It is unknown if the tobacco bioreactors generated for this thesis accumulate EspA to a level feasible for large-scale protein production. Nevertheless, given enough time and experimentation, through the careful manipulation of the gene constructs, or with the addition of protein chaperones, antigen accumulation will inevitably increase making a low-cost, high output, plant bioreactor achievable in the future.

## REFERENCES

- Ahmed, B., Loos, M., Vanrompay, D. and Cox, E. (2014) Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization. *Vaccine* **32**, 3909-3916.
- Amani, J., Mousavi, S.L., Rafati, S. and Salmanian, A.H. (2011) Immunogenicity of a plant-derived edible chimeric EspA, Intimin and Tir of *Escherichia coli* O157:H7 in mice. *Plant Sci* **180**, 620-627.
- Apel, W., Schulze, W.X. and Bock, R. (2010) Identification of protein stability determinants in chloroplasts. *Plant J* **63**, 636-650.
- Armstrong, G.L., Hollingsworth, J. and Morris, J.G., Jr. (1996) Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. *Epidemiol Rev* **18**, 29-51.
- Arthur, T.M., Ahmed, R., Chase-Topping, M., Kalchayanand, N., Schmidt, J.W. and Bono, J.L. (2013) Characterization of *Escherichia coli* O157:H7 strains isolated from supershedding cattle. *Appl Environ Microbiol* **79**, 4294-4303.
- Asper, D.J., Karmali, M.A., Townsend, H., Rogan, D. and Potter, A.A. (2011) Serological response of Shiga toxin-producing *Escherichia coli* type III secreted proteins in sera from vaccinated rabbits, naturally infected cattle, and humans. *Clin Vaccine Immunol* **18**, 1052-1057.
- Asper, D.J., Sekirov, I., Finlay, B.B., Rogan, D. and Potter, A.A. (2007) Cross reactivity of enterohemorrhagic *Escherichia coli* O157:H7-specific sera with non-O157 serotypes. *Vaccine* **25**, 8262-8269.

- Baines, D., Erb, S. and McAllister, T. (2008a) Stx2 from enterohemorrhagic *Escherichia coli* O157:H7 promotes colonization in the intestine of cattle. *Can J Anim Sci* **88**, 581-584.
- Baines, D., Lee, B. and McAllister, T. (2008b) Heterogeneity in enterohemorrhagic *Escherichia coli* O157:H7 fecal shedding in cattle is related to *Escherichia coli* O157:H7 colonization of the small and large intestine. *Can J Microbiol* **54**, 984-995.
- Bally, J., Paget, E., Droux, M., Job, C., Job, D. and Dubald, M. (2008) Both the stroma and thylakoid lumen of tobacco chloroplasts are competent for the formation of disulphide bonds in recombinant proteins. *Plant Biotechnol J* **6**, 46-61.
- Barta, A., Sommergruber, K., Thompson, D., Hartmuth, K., Matzke, M.A. and Matzke, A.J. (1986) The expression of a nopaline synthase-human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. *Plant Mol Biol* **6**, 347-357.
- Bellucci, M., De Marchis, F., Mannucci, R., Bock, R. and Arcioni, S. (2005) Cytoplasm and chloroplasts are not suitable subcellular locations for beta-zein accumulation in transgenic plants. *J Exp Bot* **56**, 1205-1212.
- Benchabane, M., Goulet, C., Rivard, D., Faye, L., Gomord, V. and Michaud, D. (2008) Preventing unintended proteolysis in plant protein biofactories. *Plant Biotechnol J* **6**, 633-648.
- Besser, T.E., Hancock, D.D., Pritchett, L.C., McRae, E.M., Rice, D.H. and Tarr, P.I. (1997) Duration of detection of fecal excretion of *Escherichia coli* O157:H7 in cattle. *J Infect Dis* **175**, 726-729.

- Beutin, L., Geier, D., Steinruck, H., Zimmermann, S. and Scheutz, F. (1993) Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J Clin Microbiol* **31**, 2483-2488.
- Bock, R. (2014a) Engineering chloroplasts for high-level foreign protein expression. In: *Chloroplast Biotechnology* (Maliga, P. ed) pp. 93-106. Springer Science+Business Media.
- Bock, R. (2014b) Engineering Plastid Genomes: Methods, Tools, and Applications in Basic Research and Biotechnology. *Annu Rev Plant Biol* **66**, 3.1-3.31.
- Bock, R. (2014c) Genetic engineering of the chloroplast: novel tools and new applications. *Curr Opin Biotechnol* **26**, 7-13.
- Boynton, J.E., Gillham, N.W., Harris, E.H., Hosler, J.P., Johnson, A.M., Jones, A.R., Randolph-Anderson, B.L., Robertson, D., Klein, T.M., Shark, K.B. and et al. (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* **240**, 1534-1538.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.
- Brooks, J.T., Sowers, E.G., Wells, J.G., Greene, K.D., Griffin, P.M., Hoekstra, R.M. and Strockbine, N.A. (2005) Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *J Infect Dis* **192**, 1422-1429.
- Brown, C.A., Harmon, B.G., Zhao, T. and Doyle, M.P. (1997) Experimental *Escherichia coli* O157:H7 carriage in calves. *Appl Environ Microbiol* **63**, 27-32.

- Brown, T. (1998) Southern Blotting. *Curr Protoc Protein Sci*, 4:A.4G.1-A.4G.8.
- Callaway, T.R., Stahl, C.H., Edrington, T.S., Genovese, K.J., Lincoln, L.M., Anderson, R.C., Lonergan, S.M., Poole, T.L., Harvey, R.B. and Nisbet, D.J. (2004) Colicin concentrations inhibit growth of *Escherichia coli* O157:H7 in vitro. *J Food Prot* **67**, 2603-2607.
- Cheng, Y., Feng, Y., Luo, P., Gu, J., Yu, S., Zhang, W.J., Liu, Y.Q., Wang, Q.X., Zou, Q.M. and Mao, X.H. (2009) Fusion expression and immunogenicity of EHEC EspA-Stx2A1 protein: implications for the vaccine development. *J Microbiol* **47**, 498-505.
- Chia, M.Y., Hsiao, S.H., Chan, H.T., Do, Y.Y., Huang, P.L., Chang, H.W., Tsai, Y.C., Lin, C.M., Pang, V.F. and Jeng, C.R. (2010) Immunogenicity of recombinant GP5 protein of porcine reproductive and respiratory syndrome virus expressed in tobacco plant. *Vet Immunol Immunopathol* **135**, 234-242.
- Chilton, M.D., Drummond, M.H., Merio, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P. and Nester, E.W. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11**, 263-271.
- Cleary, T.G. (2004) The role of Shiga-toxin-producing *Escherichia coli* in hemorrhagic colitis and hemolytic uremic syndrome. *Semin Pediatr Infect Dis* **15**, 260-265.
- Clements, A., Young, J.C., Constantinou, N. and Frankel, G. (2012) Infection strategies of enteric pathogenic *Escherichia coli*. *Gut Microbes* **3**, 71-87.
- Clements, J.D., Hartzog, N.M. and Lyon, F.L. (1988) Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. *Vaccine* **6**, 269-277.

- Cobbold, R.N., Hancock, D.D., Rice, D.H., Berg, J., Stilborn, R., Hovde, C.J. and Besser, T.E. (2007) Rectoanal junction colonization of feedlot cattle by *Escherichia coli* O157:H7 and its association with supershedders and excretion dynamics. *Appl Environ Microbiol* **73**, 1563-1568.
- Conley, A.J., Joensuu, J.J., Jevnikar, A.M., Menassa, R. and Brandle, J.E. (2009a) Optimization of elastin-like polypeptide fusions for expression and purification of recombinant proteins in plants. *Biotechnol Bioeng* **103**, 562-573.
- Conley, A.J., Joensuu, J.J., Menassa, R. and Brandle, J.E. (2009b) Induction of protein body formation in plant leaves by elastin-like polypeptide fusions. *BMC Biol* **7**, 1-18.
- Conley, A.J., Joensuu, J.J., Richman, A. and Menassa, R. (2011a) Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants. *Plant Biotechnol J* **9**, 419-433.
- Conley, A.J., Zhu, H., Le, L.C., Jevnikar, A.M., Lee, B.H., Brandle, J.E. and Menassa, R. (2011b) Recombinant protein production in a variety of *Nicotiana* hosts: a comparative analysis. *Plant Biotechnol J* **9**, 434-444.
- Cooling, L.L., Walker, K.E., Gille, T. and Koerner, T.A. (1998) Shiga toxin binds human platelets via globotriaosylceramide (Pk antigen) and a novel platelet glycosphingolipid. *Infect Immun* **66**, 4355-4366.
- Covey, S.N., Lomonosoff, G.P. and Hull, R. (1981) Characterisation of cauliflower mosaic virus DNA sequences which encode major polyadenylated transcripts. *Nucleic Acids Res* **9**, 6735-6747.

- Cray, W.C., Jr. and Moon, H.W. (1995) Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Appl Environ Microbiol* **61**, 1586-1590.
- Creasey, E.A., Friedberg, D., Shaw, R.K., Umanski, T., Knutton, S., Rosenshine, I. and Frankel, G. (2003) CesAB is an enteropathogenic *Escherichia coli* chaperone for the type-III translocator proteins EspA and EspB. *Microbiology* **149**, 3639-3647.
- Davoodi-Semiromi, A., Schreiber, M., Nalapalli, S., Verma, D., Singh, N.D., Banks, R.K., Chakrabarti, D. and Daniell, H. (2010) Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. *Plant Biotechnol J* **8**, 223-242.
- De Block, M., Herrera-Estrella, L., Van Montagu, M., Schell, J. and Zambryski, P. (1984) Expression of foreign genes in regenerated plants and in their progeny. *EMBO J* **3**, 1681-1689.
- De Cosa, B., Moar, W., Lee, S.B., Miller, M. and Daniell, H. (2001) Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nat Biotechnol* **19**, 71-74.
- De Magistris, M.T. (2006) Mucosal delivery of vaccine antigens and its advantages in pediatrics. *Adv Drug Deliv Rev* **58**, 52-67.
- Dean-Nystrom, E.A., Bosworth, B.T., Cray, W.C., Jr. and Moon, H.W. (1997) Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. *Infect Immun* **65**, 1842-1848.
- Delahay, R.M., Knutton, S., Shaw, R.K., Hartland, E.L., Pallen, M.J. and Frankel, G. (1999) The coiled-coil domain of EspA is essential for the assembly of the type

- III secretion translocon on the surface of enteropathogenic *Escherichia coli*. *J Biol Chem* **274**, 35969-35974.
- Desai, P.N., Shrivastava, N. and Padh, H. (2010) Production of heterologous proteins in plants: strategies for optimal expression. *Biotechnol Adv* **28**, 427-435.
- DeVinney, R., Stein, M., Reinscheid, D., Abe, A., Ruschkowski, S. and Finlay, B.B. (1999) Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. *Infect Immun* **67**, 2389-2398.
- Donnenberg, M.S., Kaper, J.B. and Finlay, B.B. (1997) Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends Microbiol* **5**, 109-114.
- Drechsel, O. and Bock, R. (2011) Selection of Shine-Dalgarno sequences in plastids. *Nucleic Acids Res* **39**, 1427-1438.
- Durso, L.M., Bono, J.L. and Keen, J.E. (2005) Molecular serotyping of *Escherichia coli* O26:H11. *Appl Environ Microbiol* **71**, 4941-4944.
- Duwadi, K., Chen, L., Menassa, R. and Dhaubhadel, S. (2015) Identification, characterization and down-regulation of cysteine protease genes in tobacco for use in recombinant protein production. *PLoS One* **10**, e0130556.
- Dziva, F., van Diemen, P.M., Stevens, M.P., Smith, A.J. and Wallis, T.S. (2004) Identification of *Escherichia coli* O157 : H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis. *Microbiology* **150**, 3631-3645.



- Elder, R.O., Keen, J.E., Siragusa, G.R., Barkocy-Gallagher, G.A., Koohmaraie, M. and Laegreid, W.W. (2000) Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc Natl Acad Sci USA* **97**, 2999-3003.
- Elghabi, Z., Karcher, D., Zhou, F., Ruf, S. and Bock, R. (2011) Optimization of the expression of the HIV fusion inhibitor cyanovirin-N from the tobacco plastid genome. *Plant Biotechnol J* **9**, 599-608.
- Elliott, S.J., Hutcheson, S.W., Dubois, M.S., Mellies, J.L., Wainwright, L.A., Batchelor, M., Frankel, G., Knutton, S. and Kaper, J.B. (1999) Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. *Mol Microbiol* **33**, 1176-1189.
- Elson, C.O. and Ealding, W. (1984) Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J Immunol* **132**, 2736-2741.
- Fischer, R., Stoger, E., Schillberg, S., Christou, P. and Twyman, R.M. (2004) Plant-based production of biopharmaceuticals. *Curr Opin Plant Biol* **7**, 152-158.
- Fivaz, M. and van der Goot, F.G. (1999) The tip of a molecular syringe. *Trends Microbiol* **7**, 341-343.
- Floss, D.M., Sack, M., Stadlmann, J., Rademacher, T., Scheller, J., Stoger, E., Fischer, R. and Conrad, U. (2008) Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. *Plant Biotechnol J* **6**, 379-391.

- Fuchs, S., Muhldorfer, I., Donohue-Rolfe, A., Kerenyi, M., Emody, L., Alexiev, R., Nenkov, P. and Hacker, J. (1999) Influence of RecA on in vivo virulence and Shiga toxin 2 production in *Escherichia coli* pathogens. *Microb Pathog* **27**, 13-23.
- Garmendia, J., Phillips, A.D., Carlier, M.F., Chong, Y., Schuller, S., Marches, O., Dahan, S., Oswald, E., Shaw, R.K., Knutton, S. and Frankel, G. (2004) TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell Microbiol* **6**, 1167-1183.
- Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiol Mol Biol Rev* **67**, 16-37.
- Ghiasi, S.M., Salmanian, A.H., Chinikar, S. and Zakeri, S. (2011) Mice orally immunized with a transgenic plant expressing the glycoprotein of Crimean-Congo hemorrhagic fever virus. *Clin Vaccine Immunol* **18**, 2031-2037.
- Gisby, M.F., Mellors, P., Madesis, P., Ellin, M., Lavery, H., O'Kane, S., Ferguson, M.W. and Day, A. (2011) A synthetic gene increases TGFbeta3 accumulation by 75-fold in tobacco chloroplasts enabling rapid purification and folding into a biologically active molecule. *Plant Biotechnol J* **9**, 618-628.
- Gomord, V., Fitchette, A.C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D. and Faye, L. (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnol J* **8**, 564-587.
- Griffin, P.M. and Tauxe, R.V. (1991) The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* **13**, 60-98.

- Gruenheid, S., Sekirov, I., Thomas, N.A., Deng, W., O'Donnell, P., Goode, D., Li, Y., Frey, E.A., Brown, N.F., Metalnikov, P., Pawson, T., Ashman, K. and Finlay, B.B. (2004) Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic *Escherichia coli* O157:H7. *Mol Microbiol* **51**, 1233-1249.
- Gu, J., Liu, Y., Yu, S., Wang, H., Wang, Q., Yi, Y., Zhu, F., Yu, X.J., Zou, Q. and Mao, X. (2009) Enterohemorrhagic *Escherichia coli* trivalent recombinant vaccine containing EspA, intimin and Stx2 induces strong humoral immune response and confers protection in mice. *Microbes Infect* **11**, 835-841.
- Guarner, F. and Malagelada, J.R. (2003) Gut flora in health and disease. *Lancet* **361**, 512-519.
- Gupta, R., Jung, E. and Brunak, S. (2004) Prediction of N-glycosylation sites in human proteins. *unpublished*.
- Gustafsson, C., Govindarajan, S. and Minshull, J. (2004) Codon bias and heterologous protein expression. *Trends Biotechnol* **22**, 346-353.
- Gyles, C.L. (2007) Shiga toxin-producing *Escherichia coli*: an overview. *J Anim Sci* **85**, E45-62.
- Hale, C.R., Scallan, E., Cronquist, A.B., Dunn, J., Smith, K., Robinson, T., Lathrop, S., Tobin-D'Angelo, M. and Clogher, P. (2012) Estimates of enteric illness attributable to contact with animals and their environments in the United States. *Clin Infect Dis* **54 Suppl 5**, S472-479.

- Haq, T.A., Mason, H.S., Clements, J.D. and Arntzen, C.J. (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* **268**, 714-716.
- Hartl, F.U., Bracher, A. and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteostasis. *Nature* **475**, 324-332.
- Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of antibodies in transgenic plants. *Nature* **342**, 76-78.
- Hilborn, E.D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L. (1999) A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch Intern Med* **159**, 1758-1764.
- Hoey, D.E., Currie, C., Else, R.W., Nutikka, A., Lingwood, C.A., Gally, D.L. and Smith, D.G. (2002) Expression of receptors for verotoxin 1 from *Escherichia coli* O157 on bovine intestinal epithelium. *J Med Microbiol* **51**, 143-149.
- Hoffman, M.A., Menge, C., Casey, T.A., Laegreid, W., Bosworth, B.T. and Dean-Nystrom, E.A. (2006) Bovine immune response to Shiga-toxicogenic *Escherichia coli* O157:H7. *Clin Vaccine Immunol* **13**, 1322-1327.
- Holmgren, J., Adamsson, J., Anjuere, F., Clemens, J., Czerkinsky, C., Eriksson, K., Flach, C.F., George-Chandy, A., Harandi, A.M., Lebens, M., Lehner, T., Lindblad, M., Nygren, E., Raghavan, S., Sanchez, J., Stanford, M., Sun, J.B., Svennerholm, A.M. and Tengvall, S. (2005) Mucosal adjuvants and anti-infection

- and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunol Lett* **97**, 181-188.
- Horsch, R.B., Fraley, R.T., Rogers, S.G., Sanders, P.R., Lloyd, A. and Hoffmann, N. (1984) Inheritance of functional foreign genes in plants. *Science* **223**, 496-498.
- Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**, 379-433.
- Huub, J.M. and Van Loon, L.C. (1991) Pathogenesis-related proteins of plants. *Cr Rev Plant Sci* **10**, 123-150.
- Ivarsson, M.E., Leroux, J.C. and Castagner, B. (2012) Targeting bacterial toxins. *Angew Chem Int Ed Engl* **51**, 4024-4045.
- Jacewicz, M.S., Mobassaleh, M., Gross, S.K., Balasubramanian, K.A., Daniel, P.F., Raghavan, S., McCluer, R.H. and Keusch, G.T. (1994) Pathogenesis of *Shigella* diarrhea: XVII. A mammalian cell membrane glycolipid, Gb3, is required but not sufficient to confer sensitivity to Shiga toxin. *J Infect Dis* **169**, 538-546.
- James, C. (2013) Global status of commercialized biotech/GM crops: 2013. In: *Executive summary: Brief 46*. ISAAA.
- Janssen, B.J. and Gardner, R.C. (1990) Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. *Plant Mol Biol* **14**, 61-72.
- Jeon, S.J., Elzo, M., DiLorenzo, N., Lamb, G.C. and Jeong, K.C. (2013) Evaluation of animal genetic and physiological factors that affect the prevalence of *Escherichia coli* O157 in cattle. *PLoS One* **8**, 1-9.

- Joensuu, J.J., Conley, A.J., Lienemann, M., Brandle, J.E., Linder, M.B. and Menassa, R. (2010) Hydrophobin fusions for high-level transient protein expression and purification in *Nicotiana benthamiana*. *Plant Physiol* **152**, 622-633.
- Judge, N.A., Mason, H.S. and O'Brien, A.D. (2004) Plant cell-based intimin vaccine given orally to mice primed with intimin reduces time of *Escherichia coli* O157:H7 shedding in feces. *Infect Immun* **72**, 168-175.
- Kaper, J.B., Nataro, J.P. and Mobley, H.L. (2004) Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2**, 123-140.
- Karimi, F., Mousavi, A., Salmanian, A., Alizadeh, H. and Rafati, S. (2013) Immunogenicity of EIT chimeric protein expressed in transplastomic tobacco plants towards development of an oral vaccine against *Escherichia coli* O157:H7. *Plant Biotechnol Rep* **7**, 535-546.
- Keene, W.E., McAnulty, J.M., Hoesly, F.C., Williams, L.P., Jr., Hedberg, K., Oxman, G.L., Barrett, T.J., Pfaller, M.A. and Fleming, D.W. (1994) A swimming-associated outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7 and *Shigella sonnei*. *N Engl J Med* **331**, 579-584.
- Kenny, B., DeVinney, R., Stein, M., Reinscheid, D.J., Frey, E.A. and Finlay, B.B. (1997) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91**, 511-520.
- Kenny, B. and Finlay, B.B. (1995) Protein secretion by enteropathogenic *Escherichia coli* is essential for transducing signals to epithelial cells. *Proc Natl Acad Sci USA* **92**, 7991-7995.

- Kim, J., Thanabalasuriar, A., Chaworth-Musters, T., Fromme, J.C., Frey, E.A., Lario, P.I., Metalnikov, P., Rizg, K., Thomas, N.A., Lee, S.F., Hartland, E.L., Hardwidge, P.R., Pawson, T., Strynadka, N.C., Finlay, B.B., Schekman, R. and Gruenheid, S. (2007) The bacterial virulence factor NleA inhibits cellular protein secretion by disrupting mammalian COPII function. *Cell Host Microbe* **2**, 160-171.
- Klein, R.R., Mason, H.S. and Mullet, J.E. (1988) Light-regulated translation of chloroplast proteins. I. Transcripts of *psaA-psaB*, *psbA*, and *rbcL* are associated with polysomes in dark-grown and illuminated barley seedlings. *J Cell Biol* **106**, 289-301.
- Knutton, S., Rosenshine, I., Pallen, M.J., Nisan, I., Neves, B.C., Bain, C., Wolff, C., Dougan, G. and Frankel, G. (1998) A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* **17**, 2166-2176.
- Kolotilin, I., Kaldis, A., Devriendt, B., Joensuu, J., Cox, E. and Menassa, R. (2012) Production of a subunit vaccine candidate against porcine post-weaning diarrhea in high-biomass transplastomic tobacco. *PLoS One* **7**, 1-12.
- Kolotilin, I., Kaldis, A., Pereira, E.O., Laberge, S. and Menassa, R. (2013) Optimization of transplastomic production of hemicellulases in tobacco: effects of expression cassette configuration and tobacco cultivar used as production platform on recombinant protein yields. *Biotechnol Biofuels* **6**, 1-15.
- Kolotilin, I., Topp, E., Cox, E., Devriendt, B., Conrad, U., Joensuu, J., Stoger, E., Warzecha, H., McAllister, T., Potter, A., McLean, M.D., Hall, J.C. and Menassa,

- R. (2014) Plant-based solutions for veterinary immunotherapeutics and prophylactics. *Vet Res* **45**, 1-12.
- Komarnytsky, S., Borisjuk, N., Yakoby, N., Garvey, A. and Raskin, I. (2006) Cosecretion of protease inhibitor stabilizes antibodies produced by plant roots. *Plant Physiol* **141**, 1185-1193.
- Krenek, P., Samajova, O., Luptovciak, I., Doscocilova, A., Komis, G. and Samaj, J. (2015) Transient plant transformation mediated by *Agrobacterium tumefaciens*: Principles, methods and applications. *Biotechnol Adv* **In press**, 1-19.
- Ku, C.P., Lio, J.C., Wang, S.H., Lin, C.N. and Syu, W.J. (2009) Identification of a third EspA-binding protein that forms part of the type III secretion system of enterohemorrhagic *Escherichia coli*. *J Biol Chem* **284**, 1686-1693.
- Kulasekara, B.R., Jacobs, M., Zhou, Y., Wu, Z., Sims, E., Saenphimmachak, C., Rohmer, L., Ritchie, J.M., Radey, M., McKeivitt, M., Freeman, T.L., Hayden, H., Haugen, E., Gillett, W., Fong, C., Chang, J., Beskhlebnaya, V., Waldor, M.K., Samadpour, M., Whittam, T.S., Kaul, R., Brittnacher, M. and Miller, S.I. (2009) Analysis of the genome of the *Escherichia coli* O157:H7 2006 spinach-associated outbreak isolate indicates candidate genes that may enhance virulence. *Infect Immun* **77**, 3713-3721.
- Kuroda, H. and Maliga, P. (2001) Complementarity of the 16S rRNA penultimate stem with sequences downstream of the AUG destabilizes the plastid mRNAs. *Nucleic Acids Res* **29**, 970-975.
- Lai, L.C., Wainwright, L.A., Stone, K.D. and Donnenberg, M.S. (1997) A third secreted protein that is encoded by the enteropathogenic *Escherichia coli* pathogenicity



island is required for transduction of signals and for attaching and effacing activities in host cells. *Infect Immun* **65**, 2211-2217.

- Lakshmi, P.S., Verma, D., Yang, X., Lloyd, B. and Daniell, H. (2013) Low cost tuberculosis vaccine antigens in capsules: expression in chloroplasts, bio-encapsulation, stability and functional evaluation in vitro. *PLoS One* **8**, 1-15.
- Lentz, E.M., Garaicoechea, L., Alfano, E.F., Parreno, V., Wigdorovitz, A. and Bravo-Almonacid, F.F. (2012) Translational fusion and redirection to thylakoid lumen as strategies to improve the accumulation of a camelid antibody fragment in transplastomic tobacco. *Planta* **236**, 703-714.
- Linder, M.B., Qiao, M., Laumen, F., Selber, K., Hyytia, T., Nakari-Setälä, T. and Penttilä, M.E. (2004) Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems. *Biochemistry* **43**, 11873-11882.
- Lithwick, G. and Margalit, H. (2003) Hierarchy of sequence-dependent features associated with prokaryotic translation. *Genome Res* **13**, 2665-2673.
- Louise, C.B. and Obrig, T.G. (1995) Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. *J Infect Dis* **172**, 1397-1401.
- Majowicz, S.E., Scallan, E., Jones-Bitton, A., Sargeant, J.M., Stapleton, J., Angulo, F.J., Yeung, D.H. and Kirk, M.D. (2014) Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. *Foodborne Pathog Dis* **11**, 447-455.

- Matthews, L., Reeve, R., Gally, D.L., Low, J.C., Woolhouse, M.E., McAteer, S.P., Locking, M.E., Chase-Topping, M.E., Haydon, D.T., Allison, L.J., Hanson, M.F., Gunn, G.J. and Reid, S.W. (2013) Predicting the public health benefit of vaccinating cattle against *Escherichia coli* O157. *Proc Natl Acad Sci USA* **110**, 16265-16270.
- McKenzie, S.J. and Halsey, J.F. (1984) Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. *J Immunol* **133**, 1818-1824.
- McNeilly, T.N., Mitchell, M.C., Rosser, T., McAteer, S., Low, J.C., Smith, D.G., Huntley, J.F., Mahajan, A. and Gally, D.L. (2010) Immunization of cattle with a combination of purified intimin-531, EspA and Tir significantly reduces shedding of *Escherichia coli* O157:H7 following oral challenge. *Vaccine* **28**, 1422-1428.
- Mele, C., Remuzzi, G. and Noris, M. (2014) Hemolytic uremic syndrome. *Semin Immunopathol* **36**, 399-420.
- Menassa, R., Ahmad, A. and Joensuu, J. (2012) Transient expression using agroinfiltration and its applications in molecular farming. In: *Molecular farming in plants: recent advances and future prospects* (Wang, A. and Ma, S. eds), pp. 183–198. New York: Springer Science and Business Media.
- Menassa, R., Nguyen, V., Jevnikar, A. and J., B. (2001) A self-contained system for the field production of plant recombinant interleukin-10. *Mol Breeding* **8**, 177-185.
- Montenegro, M.A., Bulte, M., Trumpf, T., Aleksic, S., Reuter, G., Bulling, E. and Helmuth, R. (1990) Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. *J Clin Microbiol* **28**, 1417-1421.

- Mowat, A.M. (2003) Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* **3**, 331-341.
- Munro, S. and Pelham, H.R. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907.
- Murai, N., Kemp, J.D., Sutton, D.W., Murray, M.G., Slightom, J.L., Merlo, D.J., Reichert, N.A., Sengupta-Gopalan, C., Stock, C.A., Barker, R.F. and Hall, T.C. (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. *Science* **222**, 476-482.
- Murray, L.J., Habeshaw, J.A., Wiels, J. and Greaves, M.F. (1985) Expression of Burkitt lymphoma-associated antigen (defined by the monoclonal antibody 38.13) on both normal and malignant germinal-centre B cells. *Int J Cancer* **36**, 561-565.
- Nakamura, M. and Sugiura, M. (2011) Translation efficiencies of synonymous codons for arginine differ dramatically and are not correlated with codon usage in chloroplasts. *Gene* **472**, 50-54.
- Naylor, S.W., Low, J.C., Besser, T.E., Mahajan, A., Gunn, G.J., Pearce, M.C., McKendrick, I.J., Smith, D.G. and Gally, D.L. (2003) Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. *Infect Immun* **71**, 1505-1512.
- Naylor, S.W., Roe, A.J., Nart, P., Spears, K., Smith, D.G., Low, J.C. and Gally, D.L. (2005) *Escherichia coli* O157 : H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. *Microbiology* **151**, 2773-2781.

- Neves, B.C., Mundy, R., Petrovska, L., Dougan, G., Knutton, S. and Frankel, G. (2003) CesD2 of enteropathogenic *Escherichia coli* is a second chaperone for the type III secretion translocator protein EspD. *Infect Immun* **71**, 2130-2141.
- Obrig, T.G., Louise, C.B., Lingwood, C.A., Boyd, B., Barley-Maloney, L. and Daniel, T.O. (1993) Endothelial heterogeneity in Shiga toxin receptors and responses. *J Biol Chem* **268**, 15484-15488.
- Omisakin, F., MacRae, M., Ogden, I.D. and Strachan, N.J. (2003) Concentration and prevalence of *Escherichia coli* O157 in cattle feces at slaughter. *Appl Environ Microbiol* **69**, 2444-2447.
- Pelosi, A., Piedrafita, D., De Guzman, G., Shepherd, R., Hamill, J.D., Meeusen, E. and Walmsley, A.M. (2012) The effect of plant tissue and vaccine formulation on the oral immunogenicity of a model plant-made antigen in sheep. *PLoS One* **7**, 1-9.
- Pereira, E.O., Kolotilin, I., Conley, A.J. and Menassa, R. (2014) Production and characterization of in planta transiently produced polygalacturanase from *Aspergillus niger* and its fusions with hydrophobin or ELP tags. *BMC Biotechnol* **14**, 1-11.
- Perna, N.T., Mayhew, G.F., Posfai, G., Elliott, S., Donnenberg, M.S., Kaper, J.B. and Blattner, F.R. (1998) Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* **66**, 3810-3817.
- Pillay, P., Schluter, U., van Wyk, S., Kunert, K.J. and Vorster, B.J. (2014) Proteolysis of recombinant proteins in bioengineered plant cells. *Bioengineered* **5**, 15-20.
- Potter, A.A., Klashinsky, S., Li, Y., Frey, E., Townsend, H., Rogan, D., Erickson, G., Hinkley, S., Klopfenstein, T., Moxley, R.A., Smith, D.R. and Finlay, B.B. (2004)

Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins. *Vaccine* **22**, 362-369.

Pruimboom-Brees, I.M., Morgan, T.W., Ackermann, M.R., Nystrom, E.D., Samuel, J.E., Cornick, N.A. and Moon, H.W. (2000) Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. *Proc Natl Acad Sci USA* **97**, 10325-10329.

Puhar, A. and Sansonetti, P.J. (2014) Type III secretion system. *Curr Biol* **24**, R784-791.

Rabinovitz, B.C., Gerhardt, E., Tironi Farinati, C., Abdala, A., Galarza, R., Vilte, D.A., Ibarra, C., Cataldi, A. and Mercado, E.C. (2012) Vaccination of pregnant cows with EspA, EspB, gamma-intimin, and Shiga toxin 2 proteins from *Escherichia coli* O157:H7 induces high levels of specific colostral antibodies that are transferred to newborn calves. *J Dairy Sci* **95**, 3318-3326.

Rawlings, N.D., Barrett, A.J. and Bateman, A. (2014) Using the MEROPS database for proteolytic enzymes and their inhibitors and substrates. *Curr Protoc Bioinformatics* **48**, 1.25.21-21.25.33.

Robert, S., Khalf, M., Goulet, M.C., D'Aoust, M.A., Sainsbury, F. and Michaud, D. (2013) Protection of recombinant mammalian antibodies from development-dependent proteolysis in leaves of *Nicotiana benthamiana*. *PLoS One* **8**, 1-9.

Saberianfar, R., Joensuu, J.J., Conley, A.J. and Menassa, R. (2015) Protein body formation in leaves of *Nicotiana benthamiana*: a concentration-dependent mechanism influenced by the presence of fusion tags. *Plant Biotechnol J.*

- Sainsbury, F., Varennes-Jutras, P., Goulet, M.C., D'Aoust, M.A. and Michaud, D. (2013) Tomato cystatin *SlCYS8* as a stabilizing fusion partner for human serpin expression in plants. *Plant Biotechnol J* **11**, 1058-1068.
- Salvadori, M.I., Sontrop, J.M., Garg, A.X., Moist, L.M., Suri, R.S. and Clark, W.F. (2009) Factors that led to the Walkerton tragedy. *Kidney Int Suppl*, S33-34.
- Sanchez, J., Johansson, S., Lowenadler, B., Svennerholm, A.M. and Holmgren, J. (1990) Recombinant cholera toxin B subunit and gene fusion proteins for oral vaccination. *Res Microbiol* **141**, 971-979.
- Sandford, J.C. (2000) The development of the biolistic process. *In Vitro Cell Dev Biol - Plant* **36**, 303-308.
- Scharff, R.L. (2012) Economic burden from health losses due to foodborne illness in the United States. *J Food Prot* **75**, 123-131.
- Schmidt, T.G., Koepke, J., Frank, R. and Skerra, A. (1996) Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin. *J Mol Biol* **255**, 753-766.
- Schuller, S., Heuschkel, R., Torrente, F., Kaper, J.B. and Phillips, A.D. (2007) Shiga toxin binding in normal and inflamed human intestinal mucosa. *Microbes Infect* **9**, 35-39.
- Shewen, P.E., Carrasco-Medina, L., McBey, B.A. and Hodgins, D.C. (2009) Challenges in mucosal vaccination of cattle. *Vet Immunol Immunopathol* **128**, 192-198.
- Shinozaki, K. and Sugiura, M. (1982) The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Gene* **20**, 91-102.

- Siegler, R.L., Obrig, T.G., Pysher, T.J., Tesh, V.L., Denkers, N.D. and Taylor, F.B. (2003) Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. *Pediatr Nephrol* **18**, 92-96.
- Silhavy, D., Molnar, A., Luciola, A., Szitty, G., Hornyik, C., Tavazza, M. and Burgyan, J. (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *EMBO J* **21**, 3070-3080.
- Smith, J.L., Fratamico, P.M. and Gunther IV, N.W. (2014) Shiga toxin-producing *Escherichia coli*. *Adv Appl Microbiol* **86**, 145-197.
- Snedeker, K.G., Campbell, M. and Sargeant, J.M. (2012) A systematic review of vaccinations to reduce the shedding of *Escherichia coli* O157 in the faeces of domestic ruminants. *Zoonoses Public Hlth* **59**, 126-138.
- Stirling, J., Griffith, M., Dooley, J.S., Goldsmith, C.E., Loughrey, A., Lowery, C.J., McClurg, R., McCorry, K., McDowell, D., McMahan, A., Millar, B.C., Rao, J., Rooney, P.J., Snelling, W.J., Matsuda, M. and Moore, J.E. (2008) Zoonoses associated with petting farms and open zoos. *Vector Borne Zoonotic Dis* **8**, 85-92.
- Streatfield, S.J. (2007) Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnol J* **5**, 2-15.
- Streatfield, S.J., Lane, J.R., Brooks, C.A., Barker, D.K., Poage, M.L., Mayor, J.M., Lamphar, B.J., Drees, C.F., Jilka, J.M., Hood, E.E. and Howard, J.A. (2003) Corn as a production system for human and animal vaccines. *Vaccine* **21**, 812-815.
- Strockbine, N.A., Marques, L.R., Newland, J.W., Smith, H.W., Holmes, R.K. and O'Brien, A.D. (1986) Two toxin-converting phages from *Escherichia coli*

- O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. *Infect Immun* **53**, 135-140.
- Su, M.S., Kao, H.C., Lin, C.N. and Syu, W.J. (2008) Gene l0017 encodes a second chaperone for EspA of enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiology* **154**, 1094-1103.
- Sugiura, M. (2014) Plastid mRNA translation. *Methods Mol Biol* **1132**, 73-91.
- Svab, Z., Hajdukiewicz, P. and Maliga, P. (1990a) Stable transformation of plastids in higher plants. *Proc Natl Acad Sci USA* **87**, 8526-8530.
- Svab, Z., Harper, E.C., Jones, J.D. and Maliga, P. (1990b) Aminoglycoside-3'-adenyltransferase confers resistance to spectinomycin and streptomycin in *Nicotiana tabacum*. *Plant Mol Biol* **14**, 197-205.
- Takao, T., Tanabe, T., Hong, Y.M., Shimonishi, Y., Kurazono, H., Yutsudo, T., Sasakawa, C., Yoshikawa, M. and Takeda, Y. (1988) Identity of molecular structure of Shiga-like toxin I (VT1) from *Escherichia coli* O157:H7 with that of Shiga toxin. *Microb Pathog* **5**, 57-69.
- Thanabalasuriar, A., Koutsouris, A., Weflen, A., Mimee, M., Hecht, G. and Gruenheid, S. (2010) The bacterial virulence factor NleA is required for the disruption of intestinal tight junctions by enteropathogenic *Escherichia coli*. *Cell Microbiol* **12**, 31-41.
- Thomas, N.A., Deng, W., Puente, J.L., Frey, E.A., Yip, C.K., Strynadka, N.C. and Finlay, B.B. (2005) CesT is a multi-effector chaperone and recruitment factor required for the efficient type III secretion of both LEE- and non-LEE-encoded effectors of enteropathogenic *Escherichia coli*. *Mol Microbiol* **57**, 1762-1779.



- Tissot, G., Canard, H., Nadai, M., Martone, A., Botterman, J. and Dubald, M. (2008) Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. *Plant Biotechnol J* **6**, 309-320.
- Tobe, T., Beatson, S.A., Taniguchi, H., Abe, H., Bailey, C.M., Fivian, A., Younis, R., Matthews, S., Marches, O., Frankel, G., Hayashi, T. and Pallen, M.J. (2006) An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proc Natl Acad Sci USA* **103**, 14941-14946.
- Tochikubo, K., Isaka, M., Yasuda, Y., Kozuka, S., Matano, K., Miura, Y. and Taniguchi, T. (1998) Recombinant cholera toxin B subunit acts as an adjuvant for the mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin. *Vaccine* **16**, 150-155.
- Tuttle, J., Gomez, T., Doyle, M.P., Wells, J.G., Zhao, T., Tauxe, R.V. and Griffin, P.M. (1999) Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol Infect* **122**, 185-192.
- Twyman, R.M., Stoger, E., Schillberg, S., Christou, P. and Fischer, R. (2003) Molecular farming in plants: host systems and expression technology. *Trends Biotechnol* **21**, 570-578.
- van Diemen, P.M., Dziva, F., Abu-Median, A., Wallis, T.S., van den Bosch, H., Dougan, G., Chanter, N., Frankel, G. and Stevens, M.P. (2007) Subunit vaccines based on intimin and Efa-1 polypeptides induce humoral immunity in cattle but do not

- protect against intestinal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7 or O26:H-. *Vet Immunol Immunopathol* **116**, 47-58.
- Varshavsky, A. (1996) The N-end rule: functions, mysteries, uses. *Proc Natl Acad Sci USA* **93**, 12142-12149.
- Verma, D. and Daniell, H. (2007) Chloroplast vector systems for biotechnology applications. *Plant Physiol* **145**, 1129-1143.
- Verma, D., Samson, N.P., Koya, V. and Daniell, H. (2008) A protocol for expression of foreign genes in chloroplasts. *Nat Protoc* **3**, 739-758.
- Vitale, A. and Pedrazzini, E. (2005) Recombinant pharmaceuticals from plants: the plant endomembrane system as bioreactor. *Mol Interv* **5**, 216-225.
- Voinnet, O., Rivas, S., Mestre, P. and Baulcombe, D. (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**, 949-956.
- Wainwright, L.A. and Kaper, J.B. (1998) EspB and EspD require a specific chaperone for proper secretion from enteropathogenic *Escherichia coli*. *Mol Microbiol* **27**, 1247-1260.
- Warawa, J., Finlay, B.B. and Kenny, B. (1999) Type III secretion-dependent hemolytic activity of enteropathogenic *Escherichia coli*. *Infect Immun* **67**, 5538-5540.
- Wells, J.G., Davis, B.R., Wachsmuth, I.K., Riley, L.W., Remis, R.S., Sokolow, R. and Morris, G.K. (1983) Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J Clin Microbiol* **18**, 512-520.

- Wen, S.X., Teel, L.D., Judge, N.A. and O'Brien, A.D. (2006) A plant-based oral vaccine to protect against systemic intoxication by Shiga toxin type 2. *Proc Natl Acad Sci USA* **103**, 7082-7087.
- Wong, A.R., Pearson, J.S., Bright, M.D., Munera, D., Robinson, K.S., Lee, S.F., Frankel, G. and Hartland, E.L. (2011) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. *Mol Microbiol* **80**, 1420-1438.
- Wong, C.S., Jelacic, S., Habeeb, R.L., Watkins, S.L. and Tarr, P.I. (2000) The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *N Engl J Med* **342**, 1930-1936.
- Wu, K., Malik, K., Tian, L., Hu, M., Martin, T., Foster, E., Brown, D. and Miki, B. (2001) Enhancers and core promoter elements are essential for the activity of a cryptic gene activation sequence from tobacco, tCUP. *Mol Genet Genomics* **265**, 763-770.
- Wurbs, D., Ruf, S. and Bock, R. (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. *Plant J* **49**, 276-288.
- Xu, J., Dolan, M.C., Medrano, G., Cramer, C.L. and Weathers, P.J. (2012) Green factory: plants as bioproduction platforms for recombinant proteins. *Biotechnol Adv* **30**, 1171-1184.
- Yamada, M., Ebina, Y., Miyata, T., Nakazawa, T. and Nakazawa, A. (1982) Nucleotide sequence of the structural gene for colicin E1 and predicted structure of the protein. *Proc Natl Acad Sci* **79**, 2827-2831.

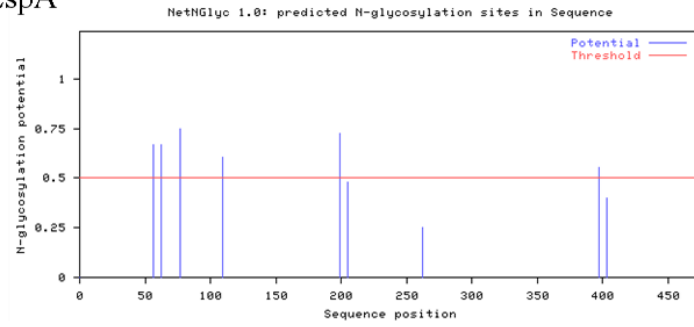
- Zhang, X., McDaniel, A.D., Wolf, L.E., Keusch, G.T., Waldor, M.K. and Acheson, D.W. (2000) Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. *J Infect Dis* **181**, 664-670.
- Zhang, X.H., He, K.W., Zhao, P.D., Ye, Q., Luan, X.T., Yu, Z.Y., Wen, L.B., Ni, Y.X., Li, B., Wang, X.M., Guo, R.L., Zhou, J.M. and Mao, A.H. (2012) Intranasal immunisation with Stx2B-Tir-Stx1B-Zot protein leads to decreased shedding in goats after challenge with *Escherichia coli* O157:H7. *Vet Rec* **170**, 178.
- Zhou, F., Karcher, D. and Bock, R. (2007) Identification of a plastid intercistronic expression element (IEE) facilitating the expression of stable translatable monocistronic mRNAs from operons. *Plant J* **52**, 961-972.

## **APPENDIX**

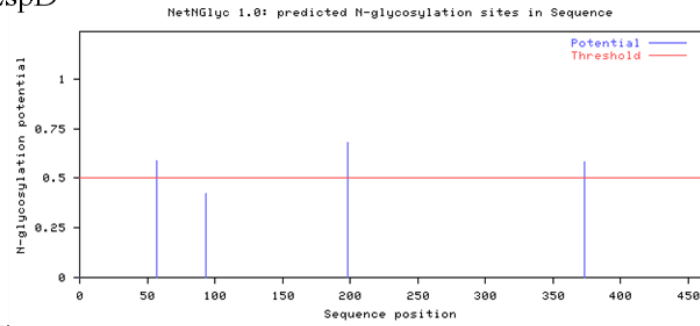
### **Appendix I**

The graphics are output formats generated from the NetNglyc server (Gupta et al., 2004), illustrating the predicted N-glycosylation sites across each of the ER targeted, transient STEC constructs. The X-axis represents the protein length from N- to C-terminal and blue vertical lines crossing the threshold value of 0.5 (red line) represent potential glycosylation sites. No N-glycosylation sites were predicted for Stx2b.

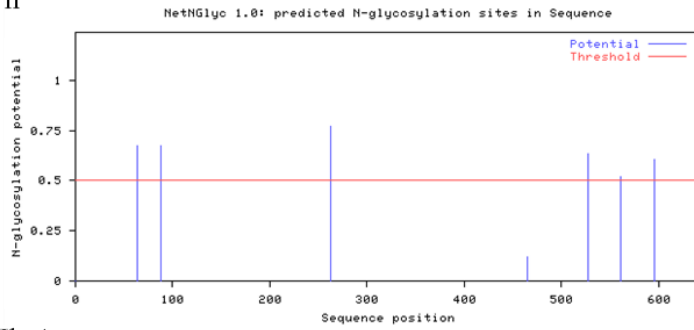
EspA



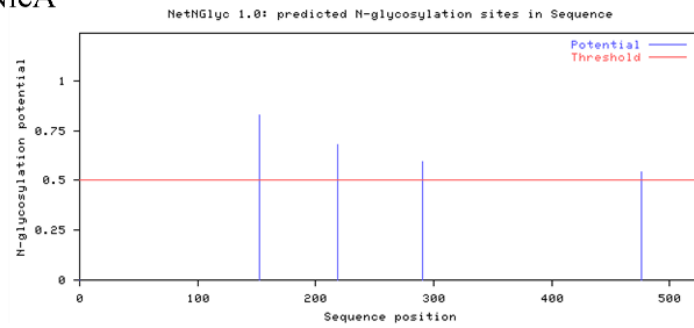
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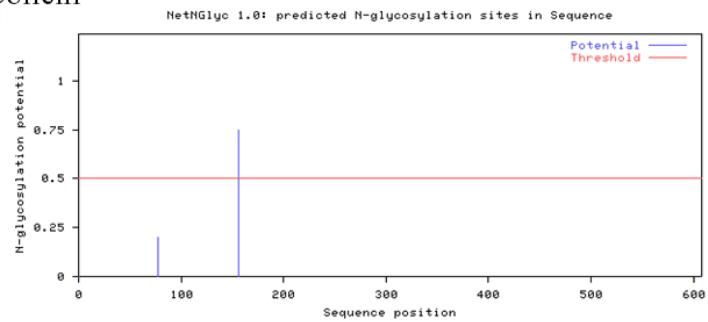
Tir



NleA



Colicin



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- 2013 **Plant-made Vaccines as an Alternative to Antibiotics: Countering Antibiotic Resistance and Ensuring Human Food Safety Workshop.** London, Ontario, Canada. September 23rd-25th. Presentation: Plant-made antibiotic alternatives: Vaccines targeting Shiga toxin-producing *Escherichia coli*.
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- 2014 **Third place in Three Minute Thesis Competition.** The University of Western Ontario.
- 2013 **Western Science Entrance Scholarship.** The University of Western Ontario.
- 2013 **Best-voted 4999E thesis presentation in genetics.** The University of Western Ontario.
- 2009 **The University of Western Ontario Scholarship.** The University of Western Ontario.



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