Production of a Candidate Recombinant Protein Vaccine for Mannheimia haemolytica in Lettuce and Tobacco Chloroplasts

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Graduate Program in Biology

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Abstract

The cattle industry worldwide is greatly impacted by bovine respiratory disease (BRD), a bacterial disease caused by *Mannheimia haemolytica*. Recent efforts to design vaccines against *M. haemolytica* focus on a virulence factor, leukotoxin, in addition to surface lipoproteins. Plant-based protein production is a safe and inexpensive alternative to traditional methods. Edible vaccines deliver antigens to pharyngeal tissues, which can provide local immunization against *M. haemolytica* prior to its progression into the lungs. In this project, a chimeric protein containing *M. haemolytica* antigens was produced in tobacco chloroplasts as a candidate edible vaccine for BRD. Attempts were made to transform lettuce chloroplasts as an alternative production platform, including extensive genetic and procedural modifications. This endeavor necessitated the optimization of lettuce growth, regeneration and transformation and will thereby support future work with transplastomic lettuce.

Keywords

Plant biotechnology, chloroplast transformation, agroinfiltration, lettuce, tobacco, *M. haemolytica*, bovine respiratory disease, vaccine.
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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAP</td>
<td>Benzyaminopurine</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRD</td>
<td>Bovine Respiratory Disease</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower mosaic virus</td>
</tr>
<tr>
<td>CEC4</td>
<td>Chloroplast expression cassette</td>
</tr>
<tr>
<td>CPL</td>
<td>Cholera toxin B, <em>Pasteurella</em> lipoprotein E, leukotoxin</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera Toxin B</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphate</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DPI</td>
<td>Days post-infiltration</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELP</td>
<td>Elastin-like polypeptide</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh weight</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM</td>
<td>Genetically Modified</td>
</tr>
<tr>
<td>GOI</td>
<td>Gene of interest</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>hr</td>
<td>Hours</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>Immunoglobulin G</td>
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<td>IL-8</td>
<td>Interleukin 8</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
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<tr>
<td>kb</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KDEL</td>
<td>ER retrieval signal peptide</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NAA</td>
<td>Naphthaleneacetic acid</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>nos</td>
<td>Nopaline synthase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>p19</td>
<td>Tomato bushy stunt virus suppressor of gene silencing</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline with 0.1% (v/v) tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylefluoride</td>
</tr>
<tr>
<td>Pr1b</td>
<td>Pathogenesis-related protein 1b of tobacco</td>
</tr>
<tr>
<td>psbA</td>
<td>Chloroplast photosystem 2 D1 protein</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PVPP</td>
<td>Polyvinylpolypyrrolidone</td>
</tr>
<tr>
<td>rbcL</td>
<td>RuBisCo large subunit</td>
</tr>
<tr>
<td>recA</td>
<td>DNA recombination and repair protein</td>
</tr>
<tr>
<td>rev/min</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RTX</td>
<td>repeats-in-toxin</td>
</tr>
<tr>
<td>SD</td>
<td>Shine Dalgarno</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SLIC</td>
<td>Sequence- and ligation-independent cloning</td>
</tr>
<tr>
<td>T7g10</td>
<td>5′-translational enhancer element of bacteriophage T7</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid, EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>tCUP</td>
<td>Tobacco cryptic upstream promoter</td>
</tr>
<tr>
<td>Ti</td>
<td>Tumour-inducing</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer DNA</td>
</tr>
<tr>
<td>TSP</td>
<td>Total soluble protein</td>
</tr>
<tr>
<td>TLP</td>
<td>Total leaf protein</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>trnI</td>
<td>Transfer RNA of isoleucine in chloroplast</td>
</tr>
<tr>
<td>trnA</td>
<td>Transfer RNA of alanine in chloroplast</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>x g</td>
<td>Factor of earth’s gravitational acceleration</td>
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</table>
1 Introduction

1.1 *M. haemolytica* and bovine respiratory disease

Ruminants such as cattle rely heavily on a diverse community of bacterial endosymbionts for digestion and other processes (Halliwell, 1957; Hungate, 1966). However, cattle also face a vast array of infectious bacteria, a challenge greatly worsened by modern farming practices. One of the most dangerous is *Mannheimia haemolytica*, which colonizes the upper respiratory tract of ruminants (Carter et al., 1995; Frank, 1989). While some strains of *M. haemolytica* reside within the upper respiratory tract unnoticed, others opportunistically infect the lungs of immunocompromised calves, particularly during shipping of calves from farms to feedlots. This has earned the resulting illness the appropriate names “shipping fever” and “bovine respiratory disease” (BRD) (Rice et al., 2007; Yates, 1982). Cattle suffering from BRD exhibit devastating symptoms: lobar pneumonia deep within the lungs, accompanied by lung exudates containing immune cells and the clotting protein fibrin (Allan, 1978; Hoerlein et al., 1961; Schiefer et al., 1978; Thomson et al., 1975; Yates, 1982). These exudates cause pleural inflammation that exerts pressure upon the lungs, greatly hindering respiration (Rice et al., 2007; Yates, 1982). The economic impact of BRD is large, as symptoms can often lead to death. A 2010 survey of the United States cattle industry found that 26.5% of all non-predator-related deaths, a total of 460 000 cattle, resulted from BRD (USDA, 2011a). Not surprisingly, BRD is the most common and costly disease in North American feedlot cattle (Smith, 1998). Economic losses as a result of BRD in North American feedlots have been approximated at $500 million (Miles, 2009) to $1 billion per year (Whiteley et al., 1992).

Efforts to combat infection by *M. haemolytica* often employ antibiotics, which are expensive and applied non-specifically at high dosages (Rice et al., 2007; Zecchinon et al., 2005). This practice has produced antibiotic-resistant strains of *M. haemolytica*, (Highlander, 2001; Kehrenberg et al., 2001) with over 40% of strains in some areas resistant to five antibiotics (Hörmansdorfer et al., 1996). Physical interventions such as
isolation of infected calves are difficult, as diagnostic tests are labour-intensive, expensive (Fulton et al., 2012) or not sensitive enough to detect infected calves (McCorkell et al., 2014).

*M. haemolytica*, previously known as *Pasteurella haemolytica*, are non-motile, gram-negative, coccobacilli (Hirsch et al., 1999; Quinn, 1994) that primarily inhabit the nasopharynx and tonsils of ruminants (Babiuk et al., 1984; Frank et al., 1992; 1995). Of the 12 *M. haemolytica* serotypes, the majority of BRD cases are associated with serotypes A1 (Allan et al., 1985; Frank et al., 1983a) and A6 (Al-Ghamdi et al., 2000; Ewers et al., 2004). Not unexpectedly, the A1 and A6 serotypes possess highly similar surface molecules (Davies et al., 1996; Morton et al., 1996). Affecting approximately 16% of feedlot cattle in the United States in 2011 (USDA, 2011b), the A1 *M. haemolytica* serotype is the predominant cause of BRD (Allan, 1978; Frank et al., 1983b; Whiteley et al., 1992). Consequently, efforts to understand and prevent this illness have been strongly directed towards serotype A1 (Brogden et al., 1995; Conlon et al., 1993; Gatewood et al., 1994; Lee et al., 2001; 2003; Lo et al., 1985; 1987).

*M. haemolytica* accomplish strong virulence primarily through exploitation of vulnerable calves. During the shipping process, calves face immune suppression due to physical factors including cold-stress (Slocombe et al., 1984) and transportation-associated stress (Aich et al., 2009; Filion et al., 1984; Jericho, 1979). As they enter feedlots, calves from multiple farms are mixed (Ribble et al., 1995) and are thereby introduced to a battery of viruses and bacteria (Rice et al., 2007) that contribute to the suppression of innate immunity by altering calf mucous secretions, anionic peptides and β-defensins (Brogden et al., 1998). While many calves are exposed to some strain of *M. haemolytica* prior to shipping, it is during the shipping process that virulent strains, particularly A1, spread to vulnerable calves. Unfortunately, prior exposure to less-aggressive strains such as A2 does not confer protection against the A1 strain (Purdy et al., 1998). Healthy calves can effectively expel *M. haemolytica* from the lungs: in four hours (hr), 90% of administered bacteria can be removed (Lillie et al., 1972). However, infection by synergistic viruses impedes this process: bovine viral diarrhea virus, parainfluenza virus 3 (PI-3), and bovine herpes virus 1 inhibit ciliary activity of tracheal epithelial cells (Rossi et al., 1977). PI-3
also has been demonstrated to inhibit pulmonary expulsion of *M. haemolytica* in calves (Lopez et al., 1976).

In addition to their exploitative nature, the strong pathogenicity of *M. haemolytica* is thought to be owed to a virulence factor named leukotoxin (Lkt). Lkt is produced by *M. haemolytica* during lag- and log-phase growth, likely following progression into the uncolonized lower respiratory tract (Shewen et al., 1985). Lkt ultimately causes lysis of leukocytes and platelets (Atapattu et al., 2005; Baluyut et al., 1981; Clinkenbeard et al., 1989b; 1991). Lkt is a part of the repeats-in-toxin (RTX) family of cytotoxins, specifically the pore-forming RTX toxins, and is closely related to the α-haemolysin of *Escherichia coli* (Davies et al., 2001; Lo, 1990; Strathdee et al., 1987). Lkt has detrimental effects on macrophages and other leukocytes, but has more severe effects on neutrophils (Clarke et al., 1998; Clinkenbeard et al., 1989a, 1989b; O’Brien et al., 1987). Macrophages from young calves are more susceptible to Lkt than those of adult cattle (O’Brien et al., 1987). The result of Lkt exposure in bovine leukocytes is dose-dependent. At low concentrations, Lkt initiates neutrophil degranulation, followed by the generation of free radical molecules, and release of cytokines (Singh et al., 2011), leading to inflammation (Czuprynski et al., 1991; Maheswaran et al., 1992; Zecchinon et al., 2005). At relatively moderate concentrations, Lkt promotes apoptosis of leukocytes (Lally et al., 1999; Stevens et al., 1996; Sun et al., 1999). At even higher concentrations, Lkt causes the lysis of neutrophils via pore-formation within neutrophil plasma membranes (Clarke et al., 1998; Clinkenbeard et al., 1989a, 1989b). The mechanism of Lkt activity has been thoroughly-studied. A nonapeptide repeat on the C-terminus of Lkt (Coote, 1992) binds to CD18, a component of the lymphocyte function associated-1 (LFA-1) receptor on the surface of bovine leukocytes (Arnaout, 1990; Jeyaseelan et al., 2000; Li et al., 1999; Mentzer et al., 1987). The LFA-1 receptor is an integrin molecule involved in activation of the cell-stress caspase-9-dependent apoptosis pathway (Atapattu et al., 2005). This initiates a signal cascade involving G proteins that causes the opening of voltage-gated calcium channels, leading to a dose-dependent elevation of intracellular calcium concentration (Gerbig et al., 1989; Hsuan et al., 1998; 1999; Jeyaseelan et al., 2001a; 2001b; Ortiz-Carranza et al., 1992). This influx of calcium from the extracellular fluid leads to apoptosis via the NF-KB pathway, cytolysis (Gerbig et al., 1989), and the
production of pro-inflammatory cytokines including IL-8, IL-1β and, TNF-α (Hsuan et al., 1999; Sang-Yoo et al., 1995). Together, these responses lead to mobilization of neutrophils, exacerbation of inflammation, and continually worsening symptoms of *M. haemolytica* infection (Slocombe et al., 1985; Weiss et al., 1991).

To avoid antibiotic overuse, an attractive preventative strategy against *M. haemolytica* infection is vaccination. The first vaccines against BRD were created over 100 years ago, and comprised killed or attenuated *M. haemolytica* cultures (bacterins) (Mosier et al., 1989). Unfortunately, these vaccines have either proved detrimental (Farley, 1932), or did not show any protective benefit (Mosier et al., 1989). Despite continued development, more recent bacterin-based *M. haemolytica* vaccines appear to be either ineffective (Hamdy et al., 1965; Martin, 1983), or harmful to calves (Friend et al., 1977; Highlander et al., 2000; Schipper et al., 1971; Wilkie et al., 1980). Similarly, vaccines comprising live bacteria are not consistently protective (Mosier et al., 1998; Purdy et al., 1986; Smith et al., 1985), and are unamenable to usage in feedlots, where antibiotics are prevalent (Hjerpe, 1990; Rice et al., 2007; Zecchinon et al., 2005). Another type of commercially available vaccine is formulated from supernatants of log-phase *M. haemolytica* cell cultures, shown by ELISA (Enzyme-linked immunosorbent assay) and agglutination assays to contain secreted Lkt and cell surface molecules including capsular polysaccharide and iron regulated outer membrane proteins (Shewen et al., 1985; Srinand et al., 1996). One commercial vaccine, Presponse, has some efficacy, reducing pneumatic tissue by 56% in one experimental trial (Conlon et al., 1995), and initiating antibody responses against Lkt and capsular polysaccharide (Srinand et al., 1996). However, the results of field trials conducted using this vaccine were inconsistent (Bateman, 1988; Jim et al., 1988), with one study of 1192 calves revealing no distinguishable difference between vaccinated and unvaccinated calves (Thorlakson et al., 1990). Supplementation of cell culture supernatant vaccines with recombinant Lkt led to improved clinical scores following experimental challenge with *M. haemolytica*. However, it is notable that administering Lkt alone does not appear to provide any detectable level of protection against *M. haemolytica* (Conlon et al., 1991a).
The relative success of protein-based cell-free vaccines has led to the development of *M. haemolytica* protein subunit vaccines, which comprise partial antigens, intended to elicit specific antibody responses. Subunit vaccine design has been guided by knowledge of molecular interactions between *M. haemolytica* proteins and the host immune system. Effective host defense functions primarily through two mechanisms: neutralization of active Lkt (Pandher et al., 1999), and destruction of *M. haemolytica* by immune cells through agglutination, complement-mediated killing, and phagocytosis (Czuprynski et al., 1987; MacDonald et al., 1983; Pandher et al., 1998). These activities are supported by host antibody binding to both *M. haemolytica* cell surfaces and secreted Lkt (Confer et al., 1985; Pandher et al., 1998). Antibodies capable of neutralizing Lkt activity have been revealed to bind to an epitope near the C-terminus, within a region known as nLkt (Lainson et al., 1996; Rajeev et al., 2001). A well-studied *M. haemolytica* surface lipoprotein, *Pasteurella* lipoprotein E (plpE), has been demonstrated to provide protection as a vaccine against *M. haemolytica* (Confer et al., 2003; 2006). The most highly antigenic epitope of PlpE has been mapped to a region near the N-terminus, R2, which comprises eight non-identical hexapeptide repeats (Ayalew et al., 2004). Cattle administered a portion of PlpE containing the R2 region (rPlpE) in addition to the commercial Presponse vaccine were significantly more resistant to *M. haemolytica* A1 and A6 infection than those administered Presponse alone (Confer et al., 2003; 2006).

Using this knowledge, vaccines composed of nLkt and rPlpE have been designed and tested experimentally (Ayalew et al., 2008). These tests demonstrated the production of antibodies that neutralized Lkt, as well as those that bound PlpE to facilitate complement-mediated bacteriolysis and phagocytosis (Ayalew et al., 2008). This vaccine, composed of two copies of each rPlpE and nLkt, in tandem as nLkt-rPlpE-nLkt-rPlpE, improved calf resistance against *M. haemolytica* infection, reducing pleural lesion scores by 75% when co-administered with a bacterin, or by 35% when administered alone (Confer et al., 2009a). For comparison, lesion scores were reduced by 36% when bacterins were administered alone, suggesting that protective effects may be additive.

Attempts to optimally administer protein-subunit vaccines have been directed by knowledge of specific host-pathogen interactions. As commensal bacteria,
*M. haemolytica* initially colonize the tonsillar and nasal mucosae (Babiuk et al., 1984) before progressing to the lungs where they cause damage leading to the symptoms of BRD (Grey et al., 1971). Current commercially available vaccines aim to prepare calf immune systems to combat *M. haemolytica* infection once it has reached the lungs. This is accomplished via induction of systemic immunity against *M. haemolytica* through subcutaneous or intramuscular parenteral administration of the vaccine (Confer et al., 2003; 2006; Thorlakson et al., 1990; Tucci et al., 2016). Internal injection of vaccines leads to the induction of a pro-inflammatory response throughout the body, ultimately causing the generation of IgG antibodies, which circulate in the blood and extracellular space (Junqueira, 2003; Schur, 1987). Alternatively, recently developed vaccines aim to prepare calf immune systems to neutralize *M. haemolytica* bacteria prior to their entry into the lungs through induction of mucosal immunity. Mucosal immunity is triggered by the recognition of potentially harmful antigens by the mucosa-associated lymphoid tissue, which ultimately leads to the production of secretory IgA antibodies (sIgA), secreted into the mucous. As *M. haemolytica* infection occurs following its progression past mucosal surfaces, mucosal immunization against this bacterium is particularly attractive.

In hopes of inducing mucosal immunity, many recombinant vaccines in development are intended for nasal or oral delivery (Ayalew et al., 2009; Confer et al., 2009a; 2009b; Lee et al., 2001; 2003; 2008). Although mucosal immunization is particularly advantageous for generation of sIgA antibodies, this method can also induce systemic immune responses (Garulli et al., 2004; Staats et al., 1994). IgG antibodies against both Lkt and PlpE were generated in calves following nasal (mucosal) administration of the aforementioned nLkt-rPlpE-nLkt-rPlpE vaccine (Confer et al., 2009b).

Induction of mucosal immunity can be enhanced by coupling antigens to the B-subunit of the cholera toxin (CTB), improving antigen reception by dendritic cells and macrophages (Holmgren et al., 2005). CTB binds to the mammalian cell surface receptor GM1 ganglioside, found on mucosal cells of the upper respiratory tract and intestines, triggering uptake of CTB (de Haan et al., 1998; Spangler, 1992) along with any coupled molecules present (Holmgren et al., 2005). Additionally, CTB functions as an adjuvant due to a strong immunogenic effect in mucosal tissues, triggering an immune response.
and promoting the development of antibodies against co-administered antigens. Expectedly, CTB has been incorporated into many vaccines administered either orally or nasally (Davoodi-Semiromi et al., 2010; Hamorsky et al., 2013; Holmgren et al., 2005; Lee et al., 2014; Ruhlman et al., 2007; Sheoran et al., 2002). Nasal administration of a recombinant vaccine against *M. haemolytica* in combination with CTB as a protein adjuvant led to the generation of nasal anti-Lkt and anti-PlpE antibodies (Confer et al., 2009b). Another vaccine developed by the same group consisted of Lkt and PlpE covalently linked to CTB, rather than supplementing the vaccine with the CTB protein (Ayalew et al., 2009). This vaccine was tested alongside the group’s previously-designed vaccines: nLkt-rPlpE-nLkt-rPlpE alone and supplemented with CTB, and calves were vaccinated then challenged with *M. haemolytica*. This experiment revealed that the two vaccines containing CTB offered the most protection against the bacteria, resulting in better clinical scores for calves receiving those vaccines. Additionally, the CTB-linked vaccine produced the highest level of anti-PlpE antibodies in serum, suggesting that CTB-linkage could provide systemic benefits as well (Ayalew et al., 2009).

While recombinant vaccines are a promising option, inefficiencies in production can make them expensive and inaccessible to farmers (Daniell et al., 2009). Recombinant vaccines require extensive processing prior to delivery. This includes purification, formulation with adjuvants, and injection (Davoodi-Semiromi et al., 2010), which must be performed under cold and sterile conditions (Daniell et al., 2009; Lee et al., 2008). Processing of pharmaceutical proteins by current production methods through bacterial, mammalian, or insect cell cultures accounts for up to 80% of productions costs (Chen, 2011). Conversely, plants require much simpler growth conditions and do not require management of risks such as potential human pathogens, which do not infect plants. In addition, plant tissues can be fed directly to recipients, eliminating the need for purification. These benefits together can reduce total production and purification costs by 90% (Daniell et al., 2005). Foreign proteins can also be safely stored or shipped within dried plant tissue at room temperature under non-sterile conditions following drying (Boyhan et al., 2011; Lee et al., 2003). Lkt specifically has been demonstrated to be stable in plant cells for at least one year after oven-drying (Lee et al., 2003). In addition to being edible, plant cells are also amenable to oral delivery for their cell walls, which
can protect recombinant antigens from stomach acids and enzymes that may otherwise quickly degrade them (Kwon et al., 2013). The efficacy of plant-based orally-delivered vaccines targeting the respiratory mucosa has been demonstrated by oral-administration of plant-produced avian influenza haemagglutinin, which triggered the production of sIgA in pleural secretions of mice, conferring protection against challenge with influenza virus (Lee et al., 2015). In ruminants such as cattle, cellulosic cell walls are degraded gradually through rumination, a process involving regurgitation, which facilitates repeated exposure of tonsillar lymphoid tissue of the pharynx to released antigens (Lee et al., 2003; 2008; Shewen et al., 2009). Exposure of the tonsils to M. haemolytica triggers antibody responses in the nasal mucosa (Frank et al., 1992), and similarly tonsillar exposure to M. haemolytica through feeding effectively prevents colonization (Briggs et al., 1999; 2012), making the tonsils a prime target for local immunization through edible vaccines.

1.2 Using plants to produce recombinant proteins

The ability to produce foreign proteins within plants is a relatively recent advancement regarding plant genetic modification (GM). The vast majority of plant GMs thus far have been conducted through selective breeding, and focused on the enhancement of nutrition, stress tolerance, growth rates, and amenability to use as crops. Recently, owing to advancements in the knowledge and tools of molecular biology, plant GM has become much faster. In 1983, the first GM plants were created: sunflower and tobacco, transformed with a bean phaseolin gene (Murai et al., 1983) and bacterial antibiotic-resistant genes (De Block et al., 1984; Horsch et al., 1984), respectively. The ability to produce foreign proteins in plants for human use was later realized and exploited, as the first pharmaceutical protein, human growth hormone (hGH), was produced in plants (Barta et al., 1986). Successful production of hGH among other proteins such as a functional mouse IgG (Hiatt et al., 1989) encouraged further developments by scientists attempting to produce valuable pharmaceuticals in plants. The field has since grown tremendously, and been given the name “molecular farming” as scientists endeavour to farm pharmaceutical and other biologically-active molecules (Xu et al., 2012).
Transformation of plants was first, and is still conducted using the bacterium *Agrobacterium tumefaciens*. *A. tumefaciens* naturally transfers tumour-inducing genes to plant cells, ultimately causing crown-gall disease (Chilton et al., 1977). These tumour-inducing genes are located upon a tumour-inducing (Ti) plasmid within the transfer DNA (T-DNA), flanked by left and right border sequences that facilitate T-DNA excision. Virulence proteins enable T-DNA transfer to the plant cell nucleus, where it integrates into the nuclear genome and is expressed by the host cell (Gelvin, 2003). With knowledge of this process, scientists adapted the natural genetic engineer for human use, creating new versions of the Ti plasmid capable of receiving foreign genes for rapid delivery to plant cells (Krenek et al., 2015). Further exploration of the *A. tumefaciens* transformation system revealed that transgene expression exhibits temporal variation. Plant cell nuclear expression rises two to four days post-infection, drops significantly, and rises once more roughly 14 days post-infection (Janssen et al., 1990). This phenomenon has since been explained: many T-DNA copies are delivered to the host cell nucleus and expressed but do not integrate within the genome. These copies are transiently-expressed to produce the initial expression peak, but are degraded thereafter (Janssen et al., 1990). Some copies of the T-DNA stably insert into the genome over time, and expression of these copies leads to a later rise at roughly 14 days, continuing thereafter due to insertion stability (Janssen et al., 1990). Scientists have made use of both of these expression peaks following *A. tumefaciens*-mediated transformation for the production of foreign proteins in plants (Ahmad et al., 2010; Daniell et al., 2009).

To generate stably-transformed plants requires regeneration while applying selection for transgene insertion (Gelvin, 2003). However, it is also necessary to screen for single insertion of the transgene within the genome, otherwise transgene silencing may act to abrogate foreign protein production entirely (Gelvin, 2003; Rajeev et al., 2015). Following this, plants must be self-fertilized, and offspring screened to ensure homozygosity (Visser et al., 1989). These necessary processes together make the procedure of generating stable, nuclear-transformed plants long and labour-intensive (Fischer et al., 2004). While stable nuclear transformation is useful for many studies, the technique is inefficient with regard to protein accumulation, as low levels of protein can
accumulate in these plants compared to those produced by other methods (Schillberg et al., 2003; Wroblewski et al., 2005).

Another method of producing recombinant proteins is through transient expression of DNA, thus avoiding silencing associated with multiple transgene insertions. Transiently-expressed DNA is present in many copies, and therefore high levels of mRNA expression and protein accumulation can be achieved (Daniell et al., 2009). Transient expression is also relatively simple and fast to conduct: plants are grown to near maturity, then “agroinfiltrated”: infiltrated (injected) with Agrobacterium carrying an expression construct with a gene of interest (GOI) using a syringe or vacuum (Menassa et al., 2012). Plants are then grown for several days while accumulating the protein product. Transient expression has been conducted in many different plants (Sohi et al., 2005; Wroblewski et al., 2005; Yasin et al., 2010; Zottini et al., 2008), although the most amenable of these is the close tobacco relative, Nicotiana benthamiana (Menassa et al., 2012). N. benthamiana has a short life cycle, small size, and is easily agroinfiltrated using either syringe or vacuum (Conley et al., 2011). Using N. benthamiana, several biotechnology companies have been able to industrialize the agroinfiltration process, moving from handheld syringe infiltration to large-scale automated vacuum infiltration (Fischer et al., 2004). This has allowed the efficient production of large quantities of pharmaceuticals and biologicals and is a continuously expanding industry.

Another attractive method of producing recombinant proteins in plants is through transformation of the chloroplast genome. Chloroplasts were first transformed with non-plant genes in 1988 using particle bombardment (Boynton et al., 1988). In this method, also known as biolistic (biological, ballistic) transformation, DNA is coated onto micro particles and bombarded into plant tissue, reaching nuclear and organelle genomes (Sanford, 2000). Particle bombardment has since been widely adopted as the method of choice for chloroplast transformation, enabling scientists to conduct this process that would otherwise be prohibitively cumbersome (Daniell et al., 2002; Maliga, 2002).

Impressively, the highest accumulation level of foreign protein in a plant, 70% of total soluble protein (TSP), was accomplished through chloroplast transformation of tobacco
with the gene encoding protein antibiotic (Oey et al., 2009). High accumulation levels of this antibiotic protein among other proteins are owed to several factors, including the prokaryotic translational machinery of the chloroplast (Sugiura et al., 1998), an endosymbiotic organelle of prokaryotic origin (Gray, 1993), the lack of silencing mechanisms (Fischer et al., 2004), and the high ploidy of the chloroplast genome (Daniell et al., 2009). To add to this, most plants have a high number of chloroplasts per cell, and the chloroplast genome contains only roughly 130 genes (Bock, 2014b). Therefore, genes inserted into the chloroplast are well-represented within the total genome and are expressed at high levels. These inserted genes can also be easily directed to any locus in the chloroplast genome, as transformation occurs by homologous recombination (Bock, 2014b). Owing to their cyanobacterial origin, chloroplasts contain genes arranged in operons, allowing transgenes to be inserted in sequence for co-expression from the same promoter (Bock, 2015). Chloroplast transformation also offers biocontainment of transgenes. In the vast majority of angiosperm species, chloroplasts are maternally inherited and are not present in pollen, and therefore the potential for outcrossing with native plants or nearby crops is significantly diminished (Bock, 2014b; Daniell, 2007).

One of the first successfully transformed plants, tobacco is the most popular and well established model plants for transgene expression (Fischer et al., 2004). Likely owing to tobacco’s popularity as a model plant, foreign proteins have thus-far been produced to the highest levels of all plants in tobacco (Oey et al., 2009). Tobacco is also attractive for its high biomass and relatively fast growth, which provide scalability for industrial production (Fischer et al., 2004). Since tobacco is not used for human food or animal feed, there is decreased risk of transgenic material entering food supplies (Stoger et al., 2002). However, tobacco is high in toxic alkaloids such as nicotine, which can prove harmful to consumers and can therefore hamper edible vaccine-development (Davoodi-Semiromi et al., 2010). To overcome this hindrance, some scientists have begun using low-alkaloid tobacco cultivars (Gutiérrez et al., 2013; Kenward et al., 1999; Kolotilin et al., 2013; Menassa et al., 2007), and alternative production platforms such as alfalfa, lettuce and tomato were developed (D’Aoust et al., 2004; Dong et al., 2014; Kanagaraj et al., 2011; Su et al., 2015; Wroblewski et al., 2005).
One edible plant currently gaining attention as an alternative production platform is lettuce. Lettuce is capable of yielding high biomass in a relatively short time, roughly six weeks after planting (Davoodi-Semiromi et al., 2010; Mackowiak et al., 1994; Su et al., 2015), and produces few alkaloids or phenolic compounds (Lai et al., 2012). Lettuce also has generally regarded as safe status (GRAS) under the United States Food and Drug Administration, and can be cultivated throughout all seasons in greenhouses, with existing systems permitting safe large-scale production and distribution (Lai et al., 2012). Bio-containment beyond that offered by greenhouse growth and chloroplast transformation is provided by late-flowering of lettuce (Daniell, 2002; Weaver, 1997). Both transient expression by agroinfiltration (Wroblewski et al., 2005) and chloroplast transformation by particle bombardment (Kanamoto et al., 2006) have been adapted for use in lettuce. It has been determined that lettuce chloroplasts are capable of accumulating high levels of foreign proteins, with one group achieving 36% TSP (Kanamoto et al., 2006) and another up to 53% of total leaf protein (TLP) (Boyhan et al., 2011). Lettuce’s safety for growth and consumption, in combination with foreign protein-production capabilities make it a strong candidate for the production of recombinant vaccines.
1.3 Hypothesis and Objectives

In this project, I endeavored to produce an edible recombinant vaccine against *M. haemolytica* in lettuce and tobacco, through both transient expression and chloroplast transformation. In doing so, I hypothesize that a vaccine against *M. haemolytica* with evidence of efficacy can be produced to appreciable levels in lettuce and tobacco. The objectives of this study were:

1. Optimization of lettuce regeneration, transformation, and growth in the lab.

2. Design and assembly of genetic constructs for lettuce chloroplast transformation and vaccine expression.

3. Transient expression of constructs in lettuce and *N. benthamiana* and assessment of subcellular localization of proteins expressed in lettuce.

4. Transformation of lettuce and tobacco chloroplasts to produce a subunit vaccine for BRD.
2 Methods and Materials

2.1 L. sativa growth, regeneration, and analysis

2.1.1 L. sativa growth using multiple fertilizers and environments

2.1.1.1 L. sativa growth conditions

Seeds of 4 lettuce cultivars were purchased from Canadian suppliers: Simpson Elite (Lindenberg Seeds Ltd.), Iceberg (Lindenberg Seeds Ltd.), Romaine (Lindenberg Seeds Ltd.), Rouge d’hiver (West Coast Seeds). Plants were grown from seeds in Pro-Mix BX soil (Premier Tech) in a growth room, growth chamber, or greenhouse. Growth experiments were conducted on Simpson Elite lettuce only. The growth room and growth chamber were set to a temperature of 22°C during a 16 hr light period and 18°C during an 8 hr dark period, 55% relative humidity. In the growth room, light intensity averaged 100 μE. The greenhouse was set to a 16 hr light/8 hr dark cycle, with day time temperature of 25°C and night time temperature of 18°C. The greenhouse control system senses and augments incoming light to provide 100 mM of light per day, at 55% relative humidity. In the growth room and greenhouse, three different fertilizers were applied, with percentage by weight of nitrogen-phosphorus-potassium of: 20-20-20 Classic (Plant-Prod, Brampton, ON), 20-8-20 Forestry Special (Plant-Prod), or Plant-Prod Solutions 19-8-13 Total Plus. Fertilizer type and concentration were adjusted as independent variables. In the growth chamber, plants were watered exclusively with 1 g/L 20-20-20 fertilizer, and light intensity was altered as an independent variable. Plants were consistently harvested 7 weeks after seeding, and above-ground tissue was removed, weighed and recorded. Above-ground tissue was then frozen at -80°C for a minimum of 16 hr, then lyophilized using a FreeZone 18 Liter Console Freeze Dry System (Labconco, Kansas City, USA). Lyophilized tissue was weighed once more to determine the dry weight (DW) of each plant.
2.1.1.2  *L. sativa* characteristic analysis

At the time of harvest, three samples consisting of three leaf discs each were collected from leaves of differing ages from each plant using a 7 mm cork borer. Each sample was weighed using a Sartorius (Göttingen, Germany) B 120S fine balance, placed in 2 mL tubes with three, 2.3 mm ceramic beads, and flash frozen in liquid nitrogen and stored at -80°C. To determine total soluble protein (TSP), proteins were extracted from each sample and Bradford assays (Bradford, 1976) were conducted. Briefly, to extract proteins, frozen leaf discs were homogenized for two intervals of one minute (min) each using a TissueLyser (Qiagen, Venlo, Netherlands). Samples were then centrifuged at 3000 x g for one min and 200 μL of plant protein extraction buffer was added to each sample. This protein extraction buffer consists of 1X PBS, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.1% (v/v) Tween-20, 100 mM ascorbic acid, 1 mM of phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 μg/mL leupeptin. Tubes were vortexed for five seconds (sec) both upright and inverted, then centrifuged at 4°C for 10 min at 20,817 x g. Two microliters of supernatant was collected from each tube and used in a Bradford assay, as described in section 2.1.1.2. Protein concentration was determined using a Bio-Rad iMark™ Microplate Reader.

Differences between above-ground fresh weights (FW), DW, and total soluble protein content per DW or FW of plants treated differently were compared via statistical analysis. Comparisons between groups were conducted using a one-way analysis of variance (ANOVA), and a Levene’s test was used to determine whether treatment groups had equal variances. Following determination of statistical significance via ANOVA, differences between groups with equal variances were compared using a Tukey Pairwise Comparison. Differences between groups with unequal variances were compared by a Games-Howell Pairwise Comparison. All statistical analyses were conducted using the Statistical Software Package for Social Sciences (SPSS) (IBM, North Castle, New York, USA).
2.1.2 Assay of protein content in *L. sativa* cultivars, *N. benthamiana* and *N. tabacum*

*L. sativa* plants of each of the 4 cultivars were grown from seeds as described (2.1.1.1) for growth experiments, and watered daily with 1.5 g/L of 20-20-20 fertilizer. At age 49 days, lettuce plants were harvested as described in section 2.1.1.1. *N. benthamiana* plants were grown for 49 days and watered with 0.5 g/L 20-8-20 fertilizer prior to sampling. *N. tabacum* were grown from regenerated calli, and sampled approximately five weeks after transfer to soil. Bradford assays (described in section 2.1.1.2) were conducted to determine the TSP content of all plants.

2.1.3 *L. sativa* flowering

*L. sativa* plants of each of the 4 cultivars were grown in the greenhouse from seeds as described in section 2.1.1.1, and watered daily with 1 g/L of 20-20-20 fertilizer. Plants were inspected twice weekly until flowers and seeds were observed. Plants were grouped according to cultivar, and the average age of each cultivar at its time of flowering and producing seeds was compared as described in section 2.1.1.2.

2.1.4 *L. sativa* regeneration

*L. sativa* seeds of all four cultivars: Simpson Elite, Rouge d’hiver, Iceberg, and Romaine were sterilized and grown in the same manner. Seeds were wrapped in pouches formed from folded and tied KimWipes, immersed in 70% (v/v) ethanol for 30 sec, immersed in sterile, filtered, deionized MilliQ (Merck Millipore, Darmstadt, Germany) water for 30 sec, then immersed in a 10% bleach (0.525% sodium hypochlorite), 0.2% Tween-20 solution, followed by three additional washes in sterile MilliQ water. Sterile seeds were grown in sterile Millipore-Sigma Phytatray™ containers on sterile basal media, (4.43 g/L Murashige & Skoog Basal Medium with vitamins, 30 g/L sucrose, 7 g/L agar, and 500 mg/L polyvinylpyrrolidone). Seeds were grown in a Conviron® (Winnipeg, Canada) TC16 growth cabinet at 22°C with 16 hr light and 8 hr dark conditions for six weeks or until fully expanded leaves, approximately 4 cm² in size, appeared.
Fully expanded leaves of 28 day-old plants were divided into 0.5 cm\(^2\) pieces and spread onto deep-dish petri dishes containing \textit{L. sativa} regeneration media composed of basal media with added synthetic plant hormones: 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA). The concentration of NAA varied from 0.05 mg/L to 0.2 mg/L, while the concentration of BAP varied from 0.1 mg/L to 0.4 mg/L. Explants were divided such that 12 explants were placed on each dish. Regenerating explants were monitored weekly for new shoots, and the number of shoots present on each plate was recorded each week. For each cultivar, regenerability under each hormone combination was compared. Also, each cultivar’s most efficient regeneration in any medium was compared to that of each other cultivar statistically, as described in section 2.1.1.2.

### 2.2 Transformation vector synthesis and cloning

#### 2.2.1 Gene synthesis

The BRD candidate vaccine gene to be expressed was designed using information gleaned from published studies and was composed of subunits of three genes: CTB, PlpE, and Lkt (Ayalew et al., 2008; 2009; Confer et al., 2006; 2009a; 2009b), and was named CPL, as it will be referred to hereinafter. The sequences of each gene correspond to serotype A1, and were retrieved from the National Center for Biotechnology Information (NCBI) database using accession numbers: ABQ65166 (CTB), ABB20693.1 (PlpE), M20730 (Lkt). DNA sequences encoding each subunit were submitted to BioBasic (Markham, Ontario) and were designed to be easily assembled using GoldenGate cloning (Engler et al., 2009). Subunit coding sequences were ordered as native sequences or were codon-optimized for nuclear expression in \textit{L. sativa}. For gene expression in \textit{L. sativa} chloroplasts, a vector with a GoldenGate-compatible insertion site for protein-coding genes was synthesized (BioBasic) (Figure 1). The 3’ end of the insertion site sequence: GGTCTCTGAGC, was composed of the \textit{BsaI} sequence GGTCTC, followed by any nucleotide (T), then the designed sticky-end GAGC. Sequences were therefore ordered with 3’ sequence GAGCTGAGACC to facilitate GoldenGate cloning. The 5’ insertion
site sequence: CAAGTGAGACC, contains the reverse-complement of the BsaI site, facilitating cutting in the other direction to generate a CAAG sticky-end. Sequences were therefore also ordered with 5’ sequence GGTCTCTCAAG to facilitate GoldenGate cloning. This chloroplast expression construct designated pLSCTGG contained a C-terminal c-Myc tag translationally fused to coding sequences within the insertion site, which allowed detection of fusion proteins using Western blot. Another L. sativa chloroplast transformation construct was generated using DNA sequences donated by Dr. Heribert Warzecha (Technische Universität Darmstadt, Germany) via a combination of GoldenBraid and Sequence- and Ligation-Independent Cloning (SLIC) assembly methods (Li et al., 2007b; Sarrion-Perdigones et al., 2011). GoldenBraid cloning makes use of the GoldenGate principles, alternating usage of restriction sites BsaI and BsmBI in destination vectors to facilitate iterative and potentially infinite addition of new sequences. SLIC makes use of 5’ complementary overhangs on both strands of sequences to be joined, added to each sequence by PCR using long primers. Annealing of complementary sequences is facilitated by DNA recombination and repair protein, RecA.

2.2.2 GoldenGate cloning

Genes to be expressed either transiently or transplastomically were cloned into destination vectors using GoldenGate technology. This technology utilizes BsaI sites flanking each protein-coding gene, which cleave DNA outside enzyme recognition sites upstream and downstream of each gene (Figure 1). This digestion generates DNA fragments with compatible ends that allow ligation in a predetermined order. Specifically, 200 ng of each plasmid carrying a gene fragment was mixed with 200 ng of vector, 2 μL of CutSmart buffer (NEB), 2 μL of 10 mM ATP, and 1 μL of BsaI high-fidelity enzyme (NEB), and 1 μL of t4 DNA ligase (NEB). The volume of this reaction mixture was adjusted to 20 μL with MilliQ water, incubated for 16 hr at 37°C, then ethanol-precipitated.
The BsaI enzyme recognizes the sequence GGTCTC (orange), and cuts one nucleotide downstream of the recognition sequence on the top strand (as displayed), and five nucleotides downstream on the bottom strand (scissor locations), generating an overhang (green). Grey sequences are irrelevant to the assembly process. Arrows with plus signs represent ligation of sequences with complementary overhangs. Longitudinally-split arrows point to resulting ligation products.

A) The GOI (purple) is liberated from the donor vector via BsaI digestion, and BsaI sites are not present in the fragment removed. B) The fragment containing the BsaI sites is removed from the recipient vector via BsaI digestion. C) Through ligase activity, one new product containing all BsaI sites can form, as can the original donor and recipient vector with their fragments (A and B). However, these contain BsaI sites and can therefore be re-cut. D) The intended product of Golden Gate assembly is formed from the insert and the destination vector. This product does not contain any BsaI sites, and will therefore predominate as BsaI continues to digest other sequence combinations.

**Figure 1. GoldenGate Cloning.**

The BsaI enzyme recognizes the sequence GGTCTC (orange), and cuts one nucleotide downstream of the recognition sequence on the top strand (as displayed), and five nucleotides downstream on the bottom strand (scissor locations), generating an overhang (green). Grey sequences are irrelevant to the assembly process. Arrows with plus signs represent ligation of sequences with complementary overhangs. Longitudinally-split arrows point to resulting ligation products.

A) The GOI (purple) is liberated from the donor vector via BsaI digestion, and BsaI sites are not present in the fragment removed. B) The fragment containing the BsaI sites is removed from the recipient vector via BsaI digestion. C) Through ligase activity, one new product containing all BsaI sites can form, as can the original donor and recipient vector with their fragments (A and B). However, these contain BsaI sites and can therefore be re-cut. D) The intended product of Golden Gate assembly is formed from the insert and the destination vector. This product does not contain any BsaI sites, and will therefore predominate as BsaI continues to digest other sequence combinations.
2.2.3 GoldenBraid and sequence- and ligation-independent cloning

Gene sequences received from Dr. Warzecha (Darmstadt, Germany) were assembled to form the *L. sativa* chloroplast transformation construct pHeri through GB cloning and SLIC. Prior to GB cloning, expression elements were assembled alongside a selectable marker, and flanking sequences were placed into GB-compatible vectors using GoldenGate cloning as described in section 2.2.2. GoldenBraid cloning was then conducted to assemble the upstream flanking sequence with the expression elements and the selectable marker. GoldenBraid cloning used the same reaction recipe as GoldenGate cloning described in section 2.2.2, but 1 μ *BsmBI* high-fidelity enzyme (NEB) was also added to the reaction mixture. The mixture was incubated for 4 hr at 55°C, then 1 μL of T4 DNA ligase (NEB) was added and the reaction was incubated at an alternating 37°C and 16°C for 10 min per cycle for 30 cycles. To assemble the downstream flanking sequence with the other sequences combined by GoldenBraid, sequence- and ligation-independent cloning was conducted, as follows. For this, 2 μg of pUC57 vector was digested with 1 μL of *Bam HI* restriction enzyme for 16 hr, separated on a 1% agarose gel, and then the linearized vector was gel-purified using the QIAquick Gel Extraction Kit (Qiagen). The GoldenBraid-assembled sequences and the downstream flanking sequence were each amplified by PCR using primers immediately adjacent to the GOI, designed with 5’ overhangs identical to the ultimately adjacent region of the other gene. To each of the two PCR products, 1 μL of *Dpn I* was added to digest template DNA, then each was purified using the QIAquick Gel Extraction Kit (Qiagen). To each of the two purified amplicons and purified vector, 1 μL of T4 DNA polymerase (NEB) as an exonuclease and 2 μL of buffer 2.1 (NEB) were added, and the reaction was incubated for 45 min at room temperature. One-tenth of each reaction volume of dCTP was added to halt exonuclease activity, the two amplicons and vector reactions were combined, then 2 μL T4 DNA ligase buffer (NEB) and 1 μL of recA (NEB) were added to facilitate DNA annealing.
2.2.4 Subcloning of CPL

The CPL sequence was transferred from the L. sativa chloroplast expression construct to a previously generated chloroplast expression cassette (CEC) for tobacco chloroplast transformation (Kolotilin et al., 2013). In this procedure, alternate restriction enzymes were employed to either retain or eliminate the t7g10 downstream box from the CEC. Succinctly, CPL was amplified from pLSCTGG using a forward primer to add either a Nhe I or Sap I restriction site and a reverse primer that annealed downstream of a Not I site immediately following the c-Myc tag. For 16 hr, 2 μg of CEC was digested with 1 μL of Not I and 1 μL of either Nhe I or Sap I to generate fragments with and without a t7g10 downstream box, respectively. Additionally, 3 μg of amplified CPL was digested with enzymes corresponding to the sites added on the forward primer. The resulting fragments were separated on a 1% (w/v) agarose gel, excised, and gel-purified using the QIAquick Gel Extraction Kit (Qiagen). Purified DNA fragments were ligated together by combining 500 ng of the CEC with 200 ng of digested CPL amplicon, 1 μL of T4 DNA ligase (NEB), and 2 μL of 10 mM ATP. The volume of this reaction mixture was adjusted to 20 μL with MilliQ water and incubated for 16 hr at 16°C.

To generate the vector pLSCTGG-P, the N. tabacum PsbA sequence from the CEC vector was used to replace the intercistronic expression element (IEE) sequence originally present within the pLSCTGG vector. This was conducted by cutting both vectors with Bpi I and Pvu II restriction enzymes to excise the desired fragments. Briefly, at 10% (v/v) NEB buffer 2.1, 2 μg of pLSCTGG was cut overnight with 0.05 μL of Pvu II and 1.5 μL Bpi I, while 4 μg CEC was cut with 1 μL of Pvu II and 2 μL of Bpi I. The products were separated on a 1% agarose gel, and the desired linear fragments were excised and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen, Catalogue Number: 28704). Then 150 ng of each fragment was mixed with 2 μL of T4 DNA ligase (NEB), 2 μL T4 DNA ligase buffer, and 2 μL of 10 mM ATP. The volume of this reaction mixture was increased to 20 μL and incubated for 16 hr at 16°C.
2.2.5  *E. coli* transformations

All constructs generated through cloning procedures were subsequently ethanol-precipitated then transformed into electro-competent *E. coli* DH5α, with the exception of the SLIC-generated pHeri and restriction-ligation-modified pLSCTGG-P, which were transformed into chemically-competent *E. coli*. Briefly, ethanol precipitation was conducted by adding sodium acetate (pH 5.2) to a final concentration of 0.3 M to the sample. Half the sample volume of -20°C isopropanol was added, then the sample was mixed and placed at -80°C for one hour. The sample was removed and centrifuged at 4°C and 20 817 x g for ten min, the isopropanol removed, and 300 μL of -20°C 70% ethanol was added. The sample was mixed and centrifuged at 20 817 x g for two min, then ethanol removed, and lyophilized until dry. The sample was then resuspended in 10mM Tris buffer, pH 8.0, generally to a volume of 10 μL. Each ethanol-precipitated reaction was combined with 50 μL of *E. coli* and electroporated via the Gene Pulser II system (Bio-Rad Laboratories Inc., Hercules, U.S.A.).

Chemical transformation was conducted by adding the entire reaction to 100 μL of cells, incubating on ice for 15 min, then incubating at 42°C for 1 minute. Following transformations, *E. coli* were diluted in 1 mL of lysogeny broth (LB) and incubated for one hour at 37°C, shaking at 250 rpm (Innova® 42 Incubator, Eppendorf, Hamburg, Germany), then 100 μL and 950 μL were spread onto LB plates with 50 μg/mL of kanamycin or 100 μg/mL ampicillin.

Successfully transformed *E. coli* colonies were identified by PCR using primers specific to genes inserted/combined within the recipient vector. PCR products were visualized on a 1% agarose gel to confirm expected sizes. Colonies carrying the intended products were grown in 15 mL LB with 50 μg/mL of kanamycin or 100 μg/mL ampicillin for approximately 16 hr. To extract plasmid DNA from each culture, the QIAprep® Spin Miniprep Kit (Qiagen, Venlo, Netherlands) was utilized according to the manufacturer’s protocol.
2.2.6 Sequencing

All altered regions of constructed vectors were sequenced prior to their use in either infiltration for transient expression (section 2.3 and 3.3) or biolistic transformation (section 2.4, 3.4, and 3.5). Sequencing reads were taken using primers that facilitated coverage of a region adjacent to, and including the insertion site, demonstrating that the correct insert was present. Sequencing was conducted by a technician at Agriculture and Agri-food Canada’s London Research and Development Centre using an ABI PRISM 3130 xl Genetic Analyzer.

2.3 Transient Expression Assays

2.3.1 A. tumefaciens transformation

Transformation of electro-competent A. tumefaciens EHA105 was conducted as described for E. coli (section 2.2.5) with the variation that A. tumefaciens were incubated at 28°C shaking at 250 rpm after electroporation, and spread on LB plates with 50 μg/mL kanamycin and 10 μg/mL rifampicin for a two-day incubation period at 28°C. A. tumefaciens colonies were screened for successful transformants as described for E.coli (section 2.2.5).

2.3.2 Transient expression in L. sativa and N. benthamiana plants

A. tumefaciens carrying transformed plasmids were inoculated into 3 mL of LB with 50 μg/mL kanamycin and 10 μg/mL rifampicin and grown for 16 hr at 28°C, shaking at 250 rpm. A 1/8500 dilution of these cultures, or a 1/4250 dilution of A. tumefaciens carrying the p19 suppressor of gene silencing was then used to inoculate a 17 mL infiltration culture composed of the same medium with added 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) at pH 5.6 and 100 μM acetosyringone. Infiltration cultures were incubated at 28°C shaking at 250 rpm for 16 hr or until they reach an optical density (OD) of 0.5-1.0, measured at 600 nm (OD$_{600}$) using a Thermo Scientific Nanodrop 2000c spectrophotometer. Infiltration cultures were then centrifuged at 6000 x g for 10 min, resuspended to the desired OD in Gamborg’s solution: 3.2 g/L Gamborg’s B5 with
vitamins, 20 g/L sucrose, 10 mM MES (pH 5.6) and 200 μM acetosyringone, then incubated with gentle agitation for 1 hr at room temperature. Cultures of A. tumefaciens were either infiltrated alone or co-infiltrated alongside an A. tumefaciens culture harbouring a construct for the suppressor of post-transcriptional gene silencing from the cymbidium ringspot tombusvirus, p19 (Silhavy et al., 2002). A. tumefaciens in Gamborg’s solution was infiltrated into 6-7 week old L. sativa grown in the greenhouse as described in section 2.1.1.1, watered with an optimized fertilizer concentration of 1.5 g/L 20-20-20 fertilizer. Infiltration was conducted by injecting each A. tumefaciens suspension into the abaxial side of a leaf, using one leaf per treatment per plant, and a minimum of three plants. Plants were returned to the greenhouse after infiltration and sampled four days-post-infiltration (DPI). Tissue collection, total soluble protein extraction, and quantification of total soluble protein were conducted as described previously (section 2.1.1.2). Insoluble proteins were extracted from tissue samples following extraction of soluble proteins, using the pellet remaining after withdrawal of the supernatant fraction as the final step in soluble protein extraction. To extract insoluble proteins the extraction buffer used was composed of 50 mM Tris-HCl pH 8.0, 1% sodium dodecyl sulfate (SDS) (v/v), and 20 mM dithiothrietol (DTT).

2.3.3 Quantification of recombinant protein

Immunoblots featuring a standard curve of known protein concentrations were employed to determine recombinant protein accumulation levels. Prior to immunoblotting, protein extracts were mixed with a sample buffer to a final concentration of 1X, containing 0.06 M Tris-HCl pH 8.0, 1% (w/v), SDS, 2% (v/v) glycerol, 20mM DTT, and 0.01% (w/v) Phenol Red. Samples were then heated to 95°C for ten min, and loaded into a GenScript ExpressPlus™ 4-20% (w/v) polyacrylamide gradient alongside a dilution series of a standard protein produced our lab called eGEHK composed of GFP with elastin-like polypeptide (ELP), hydrophobin (HFBI), a polyhistidine (His)-tag, and a C-myc tag. To size-separate proteins, gels were run at 140 V for 80 min using a Bio-Rad® Power Pac 3000. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane using a Bio-Rad Trans-Blot® Transfer System at 25 V for seven min, then blocked overnight in a 5% (w/v) skim milk powder in phosphate-buffered saline with
0.1% (v/v) Tween-20 (PBS-T). To detect CPL with a c-Myc tag, the GenScript One-hour Western™ Detection System kit was used. A solution of mouse anti-c-Myc antibody was first mixed with a solution of rabbit anti-mouse secondary antibody, conjugated to horseradish peroxidase (HRP). This mixture was then applied to the membrane in a 1:1000 dilution, which was incubated at room temperature with gentle agitation for 1 hr. Membranes were thrice-washed for ten min with gentle agitation in TBS-T. Membranes were then each incubated for five min in a 5 mL mixture of 2.5 mL Bio-Rad Clarity™ Western ECL peroxide and 2.5 mL Bio-Rad Clarity™ Western ECL enhancer. Membranes were placed between clear plastic GEMEX report covers, and imaged using a DNR Bio-Imaging Systems MicroChemi 4.2 operated through DNR Bio-Imaging Systems Image Capture software. Quantities of recombinant protein in each lane of the blot were determined by comparison of band intensities to those of the eGEHK standard curve using Total lab TL100 software (Nonlinear Dynamics, Durham, USA). The quantity of recombinant protein was then compared to both the total soluble protein, determined by Bradford Assay as described in section 2.1.1.2, and the FW of leaf tissue in each sample. The quantities of recombinant proteins, expressed as a percentage of total soluble protein or mg/kg of fresh leaves, were compared amongst treatment groups as described in section 2.1.1.2.

2.3.4 Recombinant protein characterization

To assay for glycosylation of CPL, enzymatic deglycosylation with PNGase-F was employed. To remove the glycan side chains, 35 μL of protein extract from infiltrated leaves was mixed with 10 μL of 250 mM disodium phosphate pH 7.5 and 2.5 μL 2% SDS, 1M β-mercaptoethanol, then heated to 100°C for five min. To this mixture, 2 μL of PNGase-F enzyme (NEB) and 2.5 μL of 15% Triton X-100 were added. The reaction was incubated at 37°C for 40 hr, then was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described in section 2.3.2.1. A positive control protein, known to be glycosylated in other experiments, was treated in the same way, and analyzed alongside CPL on a Western blot.
2.3.5 Protein subcellular localization

Confocal microscopy was used to localize transiently-expressed GFP within *L. sativa* epidermal cells. Leaf discs 5 mm in diameter were collected at 4 DPI from *L. sativa* cv. Simpson Elite or Rouge d’Hiver, and placed abaxial (infiltrated) side up on a glass microscope slide on top of one drop of MilliQ water. Each leaf disc slide was visualized using a Leica TCS SP2 inverted confocal microscope with a 63X water immersion objective lens. For visualization of GFP, an argon laser at wavelength 488 nm was used to excite GFP, while light emitted at wavelength 500-525 nm was monitored. To visualize chloroplasts, the same excitation wavelength was employed, and light emitted at wavelength 640-700 nm was monitored. Images taken using the two emission wavelengths were overlaid using the Leica TCS SP2 software to create merged images.

2.4 Generation of transplastomic plants

2.4.1 *L. sativa* chloroplast transformation

2.4.1.1 *L. sativa* biolistic transformation

*L. sativa* seeds of each of the four *L. sativa* cultivars were sterilized and grown as explained in section 2.1.4. Seeds were grown for six weeks or until fully expanded leaves, approximately 4 cm² in size, appeared, then harvested. Biolistic transformation was conducted as described previously for tobacco (Verma et al., 2008), with several modifications. Briefly, 50 μL of 50 mg/mL 0.6 μm diameter gold particles were coated with 20 μg of 1 μg/μL chloroplast transformation vector by mixing under agitation the gold, vector, 50 μL of 2.5 M CaCl₂, and 20 μL of 0.1 M spermidine. This mixture was incubated for 15 min with agitation, and centrifuged prior to each wash. The pellet was washed with 70% (v/v) ethanol, centrifuged once more, then with 100% (v/v) ethanol, and resuspended in 50 μL of 100% (v/v) ethanol. The DNA-coated gold particles were dispensed 10 μL each onto five macrocarriers and allowed to dry prior to each of five bombardments. Fully expanded leaves were placed adaxial side up on a petri dish filled with regeneration media (section 2.1.4) lacking spectinomycin. Leaves were bombarded
using a Bio-Rad PDS-1000 / He™ Biolistic Particle Delivery System, using 1100 psi 
rupture discs, under 29 mm Hg vacuum at a target distance of 9 cm. Following 
bombardment, leaves were incubated in petri dishes of regeneration media composed of 
*L. sativa* basal media with 0.2 mg/L BAP and 0.1 mg/L NAA lacking spectinomycin in 
complete darkness at room temperature for two days, maintaining sterility. Leaves were 
then divided and regrown as described in section 2.1.4, with the bombarded adaxial side 
contacting the regeneration media containing spectinomycin. Leaves in petri dishes were 
then incubated in conditions identical to those of seed growth until shoots were visible or 
leaves appeared brown and dead after approximately 12 weeks. Calli with shoots were 
divided and transferred to new dishes containing the same type of media.

### 2.4.1.2 Modifications to procedure

The biolistic transformation procedure was also conducted with *L. sativa* leaves placed 
abaxial side up during bombardment and contacting the media following explanting. 
Separately, target distance was altered from 9 cm to 11 cm, 6 cm, or 3 cm. In 
combination with target distance alterations, three leaves were stacked on each petri dish 
containing media. All three leaves were then divided and placed onto new plates. The 
first-described procedure was also conducted with media used for regeneration following 
bombardment containing 30 mg/L rather than 50 mg/L of spectinomycin. Also using the 
same procedure, leaves of age 4-7 weeks were bombarded. Separately, the biolistic 
apparatus was adjusted such that the macrocarrier was closer to the rupture disc.

Following a protocol provided by Dr. Fernando Bravo-Almonacid at the Institute for 
Research on Genetic Engineering and Molecular Biology (Ingebi Conicet), Argentina, 
some aspects of the chloroplast transformation protocol were altered. Prior to 
bombardment, explanted leaves were placed adaxial side upward on petri dishes of the 
basal media with 0.1 mg/L BAP and 0.1 mg/L NAA, held in darkness for one day. When 
preparing DNA-coated gold particles, 3 mg of gold in 50 μL were mixed with 20 μg of 
plasmid DNA, 50 μL of 2.5 M calcium chloride, and 20 μL of 0.1 M spermidine. This 
mixture was agitated at room temperature for 20 min, and then washed by adding 200 μL 
of 100% ethanol, vortexing for five sec, and centrifuging for 30 sec at 800 x g. Washing
was repeated six times before the gold particles were spread onto five macrocarriers and bombardment was conducted as previously mentioned.

### 2.4.1.3 Alternate *L. sativa* chloroplast transformation vectors

Two alternative *L. sativa* transformation vectors were generated. One vector consisted of the original synthesized vector, with *N. tabacum* PpsbA cloned upstream of aadA, the spectinomycin resistance gene (process described in 2.2.4). The PpsbA sequence replaced the IEE originally present within the pLSCTGG vector to form the vector pLSCTGG-P. The GFP gene was cloned into each the synthesized pLSCTGG and pLSCTGG-P by GoldenGate cloning, as described (2.2.2). The second alternative vector consisted of sequences donated by Dr. Heribert Warzecha and was assembled using GoldenBraid and SLIC cloning technologies, as previously described (2.2.4). Each of these vectors was employed in the unmodified biolistic transformation procedure for *L. sativa* using for a minimum of 40 bombardments.

### 2.4.1.4 Assays for successful *L. sativa* transformants

Tissue was collected from putative transformants by taking three leaf discs, 9 mm in diameter in a 2 mL tube with three silicon beads. These were flash frozen and homogenized using a TissueLyser (Qiagen), then centrifuged at 4000 x g for one minute. To the tube, 200 μL of DNA extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 7.5) was added. The tube was vortexed twice, inverting between each application, then centrifuged at 18000 x g at 4°C for two min. The supernatant was transferred to a 1.5 mL tube, one volume of ice cold isopropanol was added, the sample was mixed by inverting, then incubated at -20°C for 30 min. The sample was centrifuged at 18000 x g at 4°C for five min, the supernatant was discarded, 200 μL 70% ethanol was added to the tube. The pellet was dislodged by flicking the tube. The sample was centrifuged at 18000 x g at 4°C for five min, and the supernatant discarded. The sample was dried using an Eppendorf Vacufuge then resuspended in 50 μL of 10 mM Tris-HCl. DNA from each putative transformant was PCR-amplified using gene-specific primers and primers that annealed to native sequence outside the transgene insertion location.
PCR was conducted for 35 cycles using a denaturation temperature of 94°C for 30 sec, an annealing temperature of 55°C for 30 sec, and an extension temperature of 72°C for two min. The plasmid used for biolistic transformation was amplified using the same primers and compared to amplicons from putative transformants to assess whether transformation had occurred.

2.4.2  N. tabacum chloroplast transformation

2.4.2.1  N. tabacum biolistic transformation and regeneration of transplastomic plants

Sterilization of 81V9 N. tabacum seeds was conducted as mentioned for L. sativa seeds (section 2.1.4), except no wash with bleach and Tween-20 was used. Seeds were grown in the same basal medium used for L. sativa, for six weeks, or until fully-expanded leaves appeared, roughly 10 cm² in size, then biolistic transformation was conducted. Transformation of 81V9 N. tabacum was conducted as first described for L. sativa, except leaves were placed with adaxial side contacting the media during bombardment and following explanting. The media used in N. tabacum regeneration consisted of basal media with 1 mg/L BAP, 0.1 mg/L NAA, 1 mg/L thiamine hydrochloride, and 0.1 g/L myo-inositol. Following three rounds of regeneration, each comprising explanting of leaf tissue followed by callus-generation and growth of new shoots, meristems with attached shoots were transferred to basal media. Meristems with shoots were grown until they reached the top of their container and had noticeable root development, permitted by growth in basal media lacking hormones. Plantlets were then transferred to soil and grown in the greenhouse under the same conditions as greenhouse-grown lettuce. Growth continued until plants flowered and seeds were produced. Those seeds were collected and stored for later growth of transplastomic plants.

2.4.2.2  Confirmation of homoplasmy in transplastomic N. tabacum

Once all transplastomic plants containing CPL were growing in soil lacking selection, and some plants had begun to flower, a Southern blot (Southern, 1975) was conducted to evaluate homoplasmy. The Southern blot makes use of a detectable probe complementary
to regions flanking the insertion site, and allows detection of DNA with or without insertions, which is transformed or wild type, respectively. For use in this Southern blot, a DIG-labelled probe was prepared previously by PCR using primers that annealed within the trnI and trnA flanking regions of the insertion site (Figure 2) (Kolotilin et al., 2012). This PCR utilized digoxigenin (DIG)-labelled nucleotides (Roche DIG High Prime DNA Labeling and Detection Starter Kit II). Following PCR, the probe was denatured at 95°C for 5 min, then frozen at -20°C until use. Ten leaf discs were collected from each plant using a 7 mm cork borer and flash frozen in liquid nitrogen. DNA was extracted from these leaf discs using the Qiagen DNeasy plant mini kit, according to the manufacturer’s instructions, and DNA was quantitated by the Nanodrop instrument (manufacturer, location). A 0.8% agarose, 0.5X TAE (Tris base, acetic acid, EDTA) gel was used to separate 5 μg of each sample to assess purity. 10 μg of each sample was digested at 37°C overnight using 20 units of RsrII in a total volume of 20 μL at 1X cutsmart buffer. To each sample, 4 μL of 6X bromophenol blue loading buffer was added, and 2 μL of sample ran on a 0.8% agarose, 1X TAE gel to assess digestion. The remainder of each sample was loaded onto a 0.8% agarose, 1X TAE gel alongside a DIG-labelled molecular weight marker from the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Basel, Switzerland) and ran at 70 V for three hours. The gel was depurinated in 500 mL of 0.2 M HCl, shaking for 30 min, then denatured while shaking for 30 min in 500 mL of 0.5 M NaOH, 1.5 M NaCl. The gel was neutralized while shaking for 30 min in 500 mL of 1 M Tris-Cl, 1.5 M NaCl, pH 8.0, then incubated for 30 min while shaking in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate). DNA was transferred from the gel to a Hybond N+ nylon membrane by placing the membrane on top of the gel, and pressing the gel and membrane together using glass plates and a 0.5 kg weight atop the upper plate. DNA was crosslinked to the membrane using a Thermo Fisher Scientific FB UVXL-1000 UV crosslinker set to 1200 units, ran for one min. The blot was then rinsed with MilliQ water, then with 6X SSC and transferred to a hybridization tube, to which 20 mL of pre-hybridization solution containing 15.6 g of DIG Easy Hyb Granules (Roche cat# 11 796 895 001) was added. The blot within the tube was incubated at 50°C for one hour, rotating at 8 revolutions/minute (rev/min). The DIG-labelled probe in pre-hybridization solution was defrosted at 68°C for 10 min then
Figure 2. Targeted integration sites within the *L. sativa* and *N. tabacum* chloroplast genomes.

All constructs target both regions between the repeated *trnI* and *trnA* genes with the exception of pHeri, which targets the *rcbL-accD* region. LSC: Long single copy region, SSC: Short single copy region, IR A/B: Inverted repeat region A and B, 16S rRNA: 16S ribosomal RNA, *trnI*: transfer RNA isoleucine, *trnA*: transfer RNA alanine, *Ls rbcL*: *L. sativa* chloroplast RuBisCo Large chain, *Ls accD*: *L. sativa* acetyl-coenzyme A carboxylase subunit B.
pre-hybridization solution was removed from the hybridization tube and replaced with the probe solution. The tube with the probe and membrane was incubated at 50°C rotating at 8 rev/min overnight. The hybridization solution was removed from the tube and the membrane was washed twice at room temperature for 15 min each in 100 mL 0.1% SDS, 2X SSC, rotating at 10 rev/min. The membrane was then washed thrice for 1.5 hr each at 68°C in 100 mL 0.1% SDS, 0.5X SSC, rotating at 10 rev/min. The membrane was then washed in 0.3% tween 20, maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) at 48°C for five min, rotating at 8 rev/min. The membrane was blocked overnight at 48°C in 100 mL blocking buffer (1% Roche Blocking Reagent in maleic acid buffer), and 24 mL of 48°C anti-DIG antibody solution (24 mL blocking buffer with 1.714 μL anti-DIG antibody) was added prior to incubation for one hour at 48°C, and the blot washed twice for 30 min at 48°C in maleic acid buffer with 0.3% Tween-20. To the hybridization tube, 20 mL of detection buffer (0.1M Tris-HCl, 0.1 M NaCl, pH 9.5) was added, and the blot was incubated at room temperature for 10 min. The blot was removed from the tube, placed between clear plastic sheets, and 1 mL of Chloro-5-substituted adamantyl-1, 2-dioxetane phosphate solution (Roche, SKU 11655884001) was added directly to the membrane surface. The membrane was incubated at room temperature for five min, then sealed between the sheets and incubated for ten min at 37°C. The sealed membrane was imaged using a DNR Bio-Imaging Systems (Jerusalem, Israel) MicroChemi 4.2 imager to visualize bands corresponding to wild type and transformed chloroplast genomes.

2.4.2.3 Assessment of developmental effects of transplastomic recombinant protein gene

Transplastomic N. tabacum were transferred from sterile basal media to soil following a minimum of three rounds of regeneration. Wild type plants were grown in sterile media to the same size as regenerated transplastomic N. tabacum, and transferred to soil simultaneously. Transplastomic and wild type N. tabacum were each measured weekly to determine their height, the length and width of the largest two leaves present, and the total number of non-chlorotic leaves longer than 2 cm growing from the main stem. Measurements continued until seeds could be collected, at which point the plant was
discarded. Sizes of transplastomic and wild type plants according to each measured variable at each time point were compared by Student’s t-test. Lines of best fit were developed for each variable using Excel regression function to produce a fourth-order polynomial equation.

2.4.2.4 Assessment of developmental dependence of recombinant protein accumulation

Once each transgenic tobacco plant had 10 leaves over 15 cm in length, near the time of flowering, tissue was sampled from each leaf. Soluble and insoluble proteins were extracted from each sample as previously described (section 2.1.1.1 and 2.3.2). The accumulation of the recombinant protein in each leaf was determined by Western blotting as described in section 2.3.2.1. Recombinant protein in each leaf was compared as described in section 2.1.1.2.

2.4.2.5 Quantification of recombinant protein accumulation in transplastomic N. tabacum

Using data acquired from developmental dependence experiment, recombinant protein accumulation in soluble and insoluble fractions was compared. The amount of recombinant protein in each fraction was averaged across the ten sampled leaves, and the two fractions were compared and added to calculate total average recombinant protein accumulation throughout the plant. The amount of protein in each soluble and insoluble fraction of six plants was compared via Student’s t-test.

2.4.2.6 Stability of transplastomically-produced recombinant protein following lyophilization

Plants were sampled by taking eight leaf discs, 9 mm in diameter, in a 2 mL tube with 3 silicon beads. Eight additional discs were taken from a location adjacent to the first, and placed in another tube. Both samples were flash frozen, and one was lyophilized using the FreeZone 18 Liter Console Freeze Dry System. Samples were homogenized for two 1-minute intervals using a TissueLyser and extraction from both lyophilized and fresh
leaf tissue was conducted as previously described using the SDS-based protein extraction buffer previously defined for insoluble protein extraction. An extraction buffer volume of eight times the original sample mass (measured prior to lyophilization or freezing) was used for extraction of recombinant protein. This was intended to extract total protein, soluble and insoluble, in one buffer. Tissue within tubes lacking beads was ground by hand using a mortar and pestle after extraction buffer was added. Proteins were separated by SDS-PAGE and analyzed by western blot, using known amounts of a standard protein to quantify each sample, as previously described in section 2.3.2.1. The amount of protein in fresh and freeze-dried samples was compared via Student’s t-test.
3 Results

3.1 Improvement of *L. sativa* as a laboratory model

3.1.1 First evaluation of fertilizer dependence of lettuce growth

To determine the best fertilizer, and concentration of that fertilizer, that could be used to produce the largest and highest protein-content in lettuce cv. Simpson Elite, experiments were conducted to compare fertilizers and multiple concentrations. In the first experiment, plants were watered with 1 g/L, 1.5 g/L, or 2.0 g/L of fertilizers of 20-20-20, 20-8-20 or 19-8-13 percentage of nitrogen to phosphorus to potassium. As controls, plants were also grown in the growth room with no fertilizer. Plants were grown in a growth room or a greenhouse. Greenhouse-grown plants were significantly larger than those grown in the growth room, averaging 111 g FW, compared to 29 g FW for the growth room-grown plants watered with the same fertilizer, 1 g/L of 20-8-20 (Figure 3a). Among the growth room-grown plants, few significant differences exist between treatment groups (plants grown with different fertilizer type or concentration). However, plants watered with 1.5 g/L or 2.0 g/L 19-8-13 fertilizer had significantly lower FW than those watered with any concentration of 20-20-20 or 20-8-20 fertilizer, except those watered with 2.0 g/L of 20-20-20 fertilizer. The best growing group with regard to FW was 1 g/L 20-8-20, although this was only significantly higher than the 19-8-13 fertilizer groups and the unfertilized plants. The unfertilized plants were significantly smaller than all fertilized plants. Protein content per FW was not significantly different among any of the growth room-grown groups, and greenhouse-grown plants had significantly higher protein than 1.5 g/L and 2.0 g/L 19-8-13 plants. Measurements of DW and protein per DW (Figure 3b) revealed few statistically significant differences in plant performance, with the exception of plants fertilized with 19-8-13 fertilizer, which have generally lower protein content than those watered with the other two fertilizers. Analysis of TSP per plant displayed similar trends to FW (Figure 3c), with greenhouse-grown plants significantly higher than all others, and 19-8-13 plants generally accumulating less soluble protein, with significant differences between 19-8-13 plants and some other groups. From this experiment, it was concluded that greenhouse growth
Figure 3. First evaluation of fertilizer dependence of *L. sativa* growth.

*L. sativa* cv. Simpson Elite plants grown in a growth room or a greenhouse. Four plants were grown under each treatment. Error bars represent standard error of the mean. Treatments that are not labelled with the same letter are significantly different. The Y axes are colour coded, with corresponding colour bars. Measurements displayed in one colour were compared only to measurements of the same trait, displayed in the same colour.

A) Fresh weight and protein content per fresh weight of *L. sativa*. B) Dry weight and protein content per dry weight. C) Total soluble protein content per plant.
of lettuce plants is likely superior to growth within a growth room, and the 19-8-13 fertilizer was discarded from further tests as plants watered with that fertilizer generally performed worse than those watered with the other two fertilizers.

3.1.2 Light intensity dependence of *L. sativa* growth

Hypothesizing that greenhouse-grown *L. sativa* plants may be larger due to a higher light intensity, an experiment was conducted to investigate light intensity effects on *L. sativa* growth. Based on the greenhouse light intensity of roughly 200 μM/m²/s, this experiment compared plants that received light at an intensity above and below that experienced in the greenhouse, at intensities that could be made approximately equal for multiple plants under each treatment using the lighting available. The lower light intensity was intended to be closer to that of the greenhouse, at an average of 173 μM/m²/s, while the higher light intensity was above that of the greenhouse, at an average of 260 μM/m²/s. Eight plants were grown in each group, and all were watered with 1 g/L 20-20-20 fertilizer. No significant differences were found between the two treatments in any of the variables tested: FW (Figure 4A), DW (Figure 4B), protein content per FW (Figure 4A) or DW (Figure 4B), or total protein content per plant (Figure 4C). Therefore, at the levels tested, it could not be concluded that light intensity has any effect on plant size or protein content. Following these results, plants were grown exclusively in the greenhouse, as growth in that environment could not be replicated within growth chambers or growth rooms.

3.1.3 Evaluation of fertilizer dependence of lettuce growth in the greenhouse

The first fertilizer growth experiment was repeated in the greenhouse to determine whether differences exist between plants grown in that location. Six plants were used per treatment as biological replicates. Of the six groups tested (Figure 5), the 1.5 g/L 20-20-20 group tended to yield the highest measurements, although no significant differences exist between this group and the 1 g/L 20-20-20 group. Measurements of FW and DW showed the fewest significant differences between test groups, indicating only that the 0.5
g/L groups performed worse than higher fertilizer concentrations, without pointing to any specific fertilizer type or concentration as most effective. However, differences in protein content, measured as protein either per weight or per plant (Figure 5C), and were significant, with the 1 g/L and 1.5 g/L 20-20-20 fertilizer outperforming all other groups, although no significant differences existed between the two best-performing groups. It was concluded from this experiment that 1.5 g/L of 20-20-20 fertilizer should be used to maximize Simpson Elite lettuce growth and protein content, although 1 g/L 20-20-20 fertilizer may be substituted to reduce fertilizer costs.

3.1.4 Comparison of protein content in *L. sativa*, *N. benthamiana*, and *N. tabacum*

To determine which *L. sativa* cultivar is most suitable for recombinant protein production, the total soluble protein content of four cultivars was evaluated. As a prediction of recombinant protein accumulation in *L. sativa* relative to other production platforms, protein content of the *L. sativa* cultivars was compared to that of *N. benthamiana* and *N. tabacum*. It was determined (Figure 6) that *L. sativa* cv. Romaine and Rouge d’Hiver have a higher protein content than the Simpson Elite cultivar. It was also determined (Figure 6) that *N. tabacum* cv. 81V9 has a higher protein content than the other two species analyzed, and that *N. benthamiana* has a similar protein content to Simpson Elite lettuce. Since the Simpson Elite cultivar was ultimately used in future experiments, recombinant protein accumulation levels were expected to be similar to those found in *N. benthamiana* and below those found in *N. tabacum*.

3.1.5 Initiation of *L. sativa* flowering

To develop a simple protocol to collect seeds for storage and future analysis, each cultivar of *L. sativa* was induced to flower and to produce seeds. It was determined that growth in the greenhouse (described in section 2.1.1.1) for an extended period of time, up to 95 days, can cause each cultivar to flower. According to cultivar, *L. sativa* plants were found to require different lengths of time to flower and produce seeds (Figure 7). It was
Figure 4. Light-intensity dependence of *L. sativa* growth.

*L. sativa* cv. Simpson Elite plants grown in a growth cabinet with 1 g/L 20-20-20 fertilizer. Six plants were grown under each treatment. Red bars represent protein content and green bars represent plant weight. Error bars represent standard error of the mean. Treatments that are not labelled with the same letter are significantly different. Groups represented by bars labelled with capital letters were compared only against other groups with capital letters, and those with lower-case letters compared only against others with lower-case letters.

A) Fresh weight and protein content per fresh weight. B) Dry weight and relative protein content. C) Total soluble protein content.
Figure 5. Second evaluation of fertilizer dependence of *L. sativa* growth.

*L. sativa* cv. Simpson Elite plants grown in a greenhouse. Six plants were grown under each treatment. Red bars represent protein content and green bars represent plant weight. Error bars represent standard error of the mean. Treatments that are not labelled with the same letter are significantly different. Groups represented by bars labelled with capital letters were compared only against other groups with capital letters, and those with lower-case letters compared only against others with lower-case letters.

A) Fresh weight and protein content per fresh weight. B) Dry weight and protein content per dry weight. C) Total soluble protein content.
Figure 6. Comparison of protein content in *L. sativa*, *N. benthamiana*, and *N. tabacum*.

The protein content of two species of *Nicotiana* and four cultivars of *L. sativa*. Error bars represent standard error of the mean. Treatments that are not labelled with the same letter are significantly different. Three *L. sativa* of each cultivar, four *N. benthamiana*, and seven *N. tabacum* plants were analyzed.
Figure 7. Initiation of flowering and seed production of four cultivars of *L. sativa*.

A) Pictured left to right (age 65 days): Rouge d’hiver, three Romaine plants, two Simpson Elite plants. Only the first three plants show flowers. B) Flowering time of four lettuce cultivars. C) Time until first appearance of seeds of four lettuce cultivars. Eight plants of each cultivar were measured. Error bars represent standard error of the mean. Treatments that are not labelled with the same letter are significantly different.
found that *L. sativa* cv. Simpson elite and Iceberg take longer to flower and produce seeds than Romaine and Rouge d’Hiver cultivars.

### 3.1.6 Regeneration of *L. sativa*

To determine the optimal hormone combinations for regeneration of each of the four *L. sativa* cultivars considered in this study, regeneration experiments were conducted. Each of the four cultivars responded differently to different hormone concentrations, and there are inherent differences in regenerability (Figure 8). The Simpson Elite cultivar (Fig. 8 B) regenerates more efficiently than Iceberg (Fig. 8A), Romaine (Fig. 8C), and Rouge d’Hiver (Fig. 8D), as measured by number of shoots present five weeks after explanting. The cultivars other than Simpson Elite regenerate at similar efficiencies (Fig. 8E). The Simpson Elite cultivar regenerates most efficiently under the 0.1 mg/L NAA, 0.2 mg/L BAP, 0.1 mg/L NAA, 0.3 mg/L BAP, and 0.2 mg/L NAA, 0.4 mg/L BAP combinations. That 0.1 mg/L NAA, 0.2 mg/L BAP and 0.2 mg/L NAA, 0.4 mg/L BAP yield similar results suggests that regeneration may be dependent upon hormone ratio rather than quantity. However, as 0.05 mg/L NAA, 0.1 mg/L BAP led to less efficient regeneration suggests that some minimal hormone concentration at an equal NAA:BAP ratio is necessary for efficient regeneration. From these results, it appears 0.1 mg/L NAA, 0.2 mg/L BAP is sufficient for maximal regeneration of *L. sativa* cv. Simpson Elite. Other cultivars displayed similar trends, with the same three hormone combinations eliciting similar high regeneration efficiencies, although these were lower than those observed in Simpson Elite (Figure 8). The results of this experiment led to adoption of Simpson elite as the main cultivar for this study, as successful chloroplast transformation is heavily dependent upon plant regeneration.

### 3.2 *M. haemolytica* vaccine design

Experimentally effective subunit vaccines for *M. haemolytica* have been designed and sequence information made available (Ayalew et al., 2008; Ayalew et al., 2009; Confer et al., 2009a; 2009b). These vaccines comprise two *M. haemolytica* proteins: outer membrane lipoprotein E (plpE) and leukotoxin (Lkt), and a protein subunit of the cholera toxin originating from *Vibrio cholerae*, cholera toxin B (CTB). Vaccines containing CTB
Figure 8. Regeneration of four cultivars of *L. sativa* under different hormone concentrations.

A total of 120 explants per treatment for each cultivar regenerated for six weeks with varying concentrations of BAP and NAA. Error bars represent standard error of the mean. Treatments that are not labelled with the same letter are significantly different. A star indicates the optimal regeneration medium for each cultivar, as defined by the highest number of shoots per plate. The optimal medium may not be statistically different from some other media.

translationally fused to Lkt and plpE are more effective than those composed of Lkt and plpE alone (Ayalew et al., 2009). Therefore, a vaccine composed of CTB alongside immunogenic, neutralizing target regions of Lkt (nLkt) and the antigenic r2 epitope of plpE (rPlpE) was designed. As in the published sequence of an experimentally effective vaccine, CTB was placed at the N-terminus, nLkt at the C-terminus, and rPlpE between the other two protein fragments (Ayalew et al., 2009). Each fragment was separated by a flexible glycine-proline-glycine-proline linker to allow independent folding and activity. The sequences of Lkt and plpE represent serotype A1, the most common strain of M. haemolytica associated with BRD. These sequences were synthesized in their native form for chloroplast expression, and were codon-optimized for expression from the lettuce nucleus for transient expression.

3.3 Transient production of recombinant proteins

3.3.1 Genetic constructs for transient expression of recombinant protein

The CPL chimeric recombinant protein was produced transiently as a representation of accumulation levels in the chloroplasts of transplastomic plants. It is hypothesized that proteins expressed from the nucleus that accumulate well when transported into the chloroplasts will also accumulate well when produced within the chloroplasts, as accumulation is largely dependent upon the stability of each protein in its subcellular location (Bock, 2015). Transient expression of CPL also served as a potential production method. The protein subunits of CPL codon-optimized for L. sativa nuclear expression were cloned into the destination vectors pCLGG-CHL and pCLGG-ER (Figure 9) for trafficking of the protein to the chloroplast stroma and endoplasmic reticulum, respectively. This trafficking was accomplished by use of signal peptides translationally-fused to the recombinant protein that target the protein to different intracellular organelles. Proteins were targeted to the chloroplast using an N-terminally fused rubisco small subunit transit peptide (rbcS), which interacts with the translocons of the outer and inner membrane of the chloroplast to facilitate ATP and GTP-dependent import (Chen et al., 1999). Proteins were targeted to the ER through use of a C-terminally-fused ER
retrieval tetrapeptide (KDEL), which facilitates retrieval of peptides from the Golgi apparatus to the ER (Munro et al., 1987).

In addition to these targeting sequences, constructs each contain left and right T-DNA border sequences to allow A. tumefaciens–mediated transformation of plant cells. Expression of foreign proteins is driven by a double-enhanced 35S promoter from cauliflower mosaic virus (2X35S) (Covey et al., 1981) and translation efficiency improved by the tCUP translational enhancer, a cryptic gene activation sequence of tobacco (Wu et al., 2001). In the ER-targeting vector the recombinant protein has an N-terminally-fused pathogenesis-related-1b protein (PR1b) signal peptide (Linthorst et al., 1991). A c-Myc tag is translationally fused to the C-terminus of the protein of interest in each vector, upstream of the KDEL tag in the ER-targeting vector. Transcripts encoding the GOI with translational fusions are transcriptionally-terminated and stabilized by the terminator and 5’ UTR of the nopaline synthase gene (nosT) (Figure 9).

Since there is extensive evidence that post-transcriptional gene silencing leads to foreign transcript degradation (Voïnnet, 2001), the cultures of A. tumefaciens harboring foreign gene expression constructs were co-infiltrated alongside an A. tumefaciens culture harbouring a construct for the suppressor of post-transcriptional gene silencing, p19 (Silhavy et al., 2002). As a negative control, the A. tumefaciens strain carrying p19 was infiltrated in the absence of A. tumefaciens carrying gene-expression constructs.

Following infiltration, leaves of L. sativa or N. benthamiana plants were faintly yellowed, with a ring of visible necrosis at the precise location of infiltration (data not shown), presumably due to infiltration pressure. Infiltrated leaf tissue was sampled at 4 DPI and proteins were extracted then analyzed by Western blot to quantify protein accumulation.

3.3.2 Quantification of transiently-produced recombinant protein

Western blots conducted using protein extracts from L. sativa and N. benthamiana plants transiently expressing the CPL protein revealed that the protein in some cases accumulates as a larger molecule than the expected 35.5 kDa, with size differences depending on the compartment to which the protein is targeted (Figure 10).
**Figure 9. Genetic constructs for transient expression of recombinant proteins.**

A schematic displaying two gene constructs used for transient expression of CPL in *L. sativa* and *N. benthamiana*. 2x P35S: double-enhanced 35S promoter from cauliflower mosaic virus, tCUP TE: cryptic gene activation sequence or translational enhancer of tobacco, Xpress: tag for detection, CPL: candidate vaccine coding sequence, c-Myc: tag for detection, T-NOS: nopaline synthase terminator.

Figure 10. Quantification of transiently-produced recombinant protein.

Four plants were infiltrated for each treatment with *A. tumefaciens* carrying each of the constructs shown in Figure 9 co-infiltrated with p19, or with p19 alone (shown in lanes labelled p19). S: soluble proteins, I: insoluble proteins. eGEHK: protein standard used for quantification.

A) Protein accumulation in *L. sativa*. B) Protein accumulation in *N. benthamiana*. C) Accumulation of CPL protein per FW in different fractions within different compartments of *N. benthamiana* and *L. sativa*. Accumulation in soluble and insoluble fractions was combined to calculate total accumulation. Error bars represent standard error of the mean. Means that are not labelled with the same letter are significantly different. Bars labelled with lower-case letters represent extract fractions, and were not compared against bars labelled with capital letters, which represent extract totals.
In *L. sativa* (Figure 10A), the CPL protein accumulates only within the chloroplasts as an insoluble fraction of size 37 kDa, roughly equal to the expected 35.5 kDa. In *N. benthamiana* (Figure 10B), the soluble form of CPL targeted to the chloroplast accumulates at roughly the expected size, but also as a larger molecule than expected, at approximately 43 kDa. One potential explanation of this size increase is the presence of the rubisco transit peptide, which is roughly 6 kDa, and is assumed to be cleaved off in the chloroplast (explored in section 4.3). In the ER, CPL accumulates as an even larger molecule, approximately 47 kDa. This size increase may be explained by protein post-translational modifications (explored in section 4.3). Statistical analysis by t-test revealed that CPL accumulates in the chloroplast to significantly higher levels in an insoluble than in a soluble form. Conversely, CPL accumulates in the ER to significantly higher levels in a soluble than in an insoluble form. No significant difference was found between total accumulation in the chloroplast and the ER. Since the soluble, monomeric form of the CPL is most likely to expose immunologically important epitopes, ER-targeting of this recombinant protein may be more useful than chloroplast-targeting. However, the increase in size of the ER-targeted protein may be caused by protein modifications that could potentially obstruct epitopes, thereby decreasing candidate vaccine efficacy. Which cellular compartment is most suitable for candidate vaccine production would be best determined by a vaccination study in animals.

### 3.3.3 ER-targeted CPL is not glycosylated

Hypothesizing that increases in size of the ER-targeted CPL protein were caused by N-glycosylation, an experiment to reveal the presence of N-glycosylation was conducted. This experiment made use of the enzyme PNGase-F, which removes N-glycans (Tarentino et al., 1994; Tarentino et al., 1989). Western blots of proteins treated with PNGase-F compared to untreated proteins showed no detectable size shift of the CPL band (Figure 11). However, PNGase-F treatment did reduce the size of a positive control protein, which is a longer length plpE fragment produced in our lab, previously shown to be glycosylated by PNGase-F treatment (data not shown). Therefore, it was concluded that the ER-targeted CPL protein is not glycosylated following transient expression in plant cells, and the visible size increases must be due to other reasons.
**Figure 11. ER-targeted CPL protein is not glycosylated.**

Soluble protein extracts from infiltrations with ER-targeted CPL expression constructs were pooled from four plants and treated with PNGase-F to remove N-glycans. A positive control plpE fragment previously shown to be glycosylated was used for comparison. +: PNGase-F treated, -: not PNGase-F treated, I: insoluble fraction.
3.3.4 Protein subcellular localization in *L. sativa*

To verify that protein tags previously designed for *N. benthamiana* are functional in *L. sativa* cells, CPL was replaced with GFP in the transient expression constructs. *A. tumefaciens*-mediated transient expression was conducted using GFP with an N-terminally fused RbcS transit peptide for chloroplast-targeting, or Pr1b and KDEL for ER-targeting. This allowed subcellular localization of GFP, and assessment of the functionality of transit peptides. One reason transient expression was conducted using vectors that target CPL to the chloroplast was to determine whether CPL could accumulate well in that compartment. Subcellular localization allows verification that proteins fused to the RbcS transit peptide indeed enter the chloroplast, and therefore can be used as a representation of protein stability in that compartment. Confocal microscopy revealed that GFP targeted to either the chloroplast or ER does accumulate within the targeted subcellular compartment, and is not detectable elsewhere in the cell (Figure 12). This can be visualized in the chloroplast by co-localization with the red autofluorescence signal emitted by chlorophyll pigment under UV light. However, not all chloroplasts within a given cell exhibit green fluorescence, and some chloroplasts fluoresce more brightly than others. This can be explained by unequal reception of GFP from the cytoplasm: by chance, some chloroplasts are more accessible than others, or possess more active import systems, and therefore accumulate more GFP. Additionally, some cells show less or no fluorescence at all. This can be explained by imperfect infection by *A. tumefaciens*, which does not infect every epithelial cell, and can transfer different numbers of T-DNA to different cells (Gelvin, 2003). It is not known which of the chloroplasts shown in Figure 12 are within the same cell. It is possible that fluorescing chloroplasts are within an infected cell, and non-fluorescing chloroplasts are within an uninfected cell.

3.4 Attempts to generate transplastomic *L. sativa*

3.4.1 Genetic constructs for generation of transplastomic *L. sativa*

Multiple vectors were designed for transformation of lettuce chloroplasts (Figure 13). To facilitate homologous recombination with the plastid genome, the first cassette
**Figure 12. Protein subcellular localization in *L. sativa*.**

*L. sativa* expressing GFP targeted to either the chloroplast or ER and was then viewed using a confocal microscope. GFP was visualized at a wavelength of 500-525 nm, and chloroplast auto-fluorescence was visualized at a wavelength of 640-700 nm. Fluorescence of GFP was captured and is shown in these images as green colour, while chloroplast autofluorescence under UV light was captured and is shown as red colour. The two images were merged to show whether GFP was co-localization with autofluorescing chloroplasts. GFP expression constructs were co-infiltrated with p19. A) Chloroplast-targeted GFP. B) ER-targeted GFP. C) p19 without GFP.
Figure 13. *L. sativa* chloroplast transformation and expression constructs.

Genes *CPL* and *GFP* were cloned into the first-designed transformation vector (A), and modifications were made to produce subsequent vectors (B and C). The final vector was assembled separately, and each vector was used in minimally 40 bombardments.


(Figure 13A), termed pLSCTGG, contains upstream flanking sequences of 16S ribosomal rna (rrn16) then transfer RNA isoleucine (trnI) and downstream transfer RNA alanine (trnA) then 23S ribosomal rna (rrn23) to facilitate cassette integration between trnI and trnA genes of the ribosomal RNA (rRNA) operon within the inverted repeat regions of the L. sativa chloroplast genome (Figure 2). The targeted integration site of this vector lies within the ribosomal operon, which has read-through transcription, allowing production of trnI, trnA, and any genes between them without a dedicated promoter. Genes transcribed together in this manner, share a single mRNA transcript. Within the flanking sequences, from furthest upstream to downstream, pLSCTGG contains an IEE from N. tabacum. The IEE present on pLSCTGG facilitates cleavage of the inserted sequences from the native ribosomal operon sequences to promote efficient transgene expression as a separate transcript (Zhou et al., 2007). Downstream of the IEE lies the aminoglycoside-3′-adenyltransferase (aadA) gene, which confers spectinomycin resistance (Svab et al., 1990). The aadA gene is expressed in transgenic plants due to read-through transcription of the rRNA operon, and a Shine-Dalgarno sequence is included immediately before the aadA gene to facilitate ribosome binding. Transcription of the aadA gene is terminated and the transcript stabilized by the terminator and 3′ untranslated region (3′ UTR) of photosystem II chlorophyll protein (CP) (TpsbC) of poplar. Expression of the GOI is driven by the L. sativa plastid promoter of photosystem II protein D1 (PpsbA) (Kanamoto et al., 2006). Stability of the GOI is provided by the 5′ UTR of the E. coli phage 7 gene 10 (T7g10). The GOI insertion location lies after the PpsbA sequences and allows insertion by golden-gate cloning. Translationally fused to the C-terminus of the GOI is a c-Myc tag for protein detection via Western blot. Transcription of the GOI is terminated and transcripts are stabilized by the terminator and 3′ UTR of the white poplar chloroplast RuBisCo Large subunit (TrbcL), (Kolotilin et al., 2013; Shinozaki et al., 1982). The candidate gene CPL was inserted into the vector pLSCTGG using GoldenGate cloning (Figure 13B). To reveal potential detriments caused by CPL expression, green fluorescence protein gene (GFP) was also inserted into the GOI site in place of CPL using GoldenGate cloning (Figure 13C).

The vector pLSCTGG was modified such that the IEE was removed and the N. tabacum psbA promoter was added in its place, with the intention of increasing aadA expression to
confer strong spectinomycin resistance upon transformants. These modifications generated a new vector, termed pLSCTGG-P (Figure 13D). A minimal vector, pHerei (Figure 13E) was designed and assembled with the intention to avoid any unidentified complications of other vectors that may affect transformation. This vector consists of partial *L. sativa* chloroplast rubisco large chain (*rbcL*) and acetyl-coenzyme A carboxylase subunit B (*accD*) gene sequences flanking the insert sequences to facilitate recombination. Between the flanking sequences lies the *N. tabacum* ribosomal operon promoter (*Prrn*), which drives expression of *aadA*. The *aadA* transcript is stabilized by the *L. sativa* ribosomal protein S16 terminator (*Trps16*). No other elements were included within the minimal vector, pHerei.

### 3.4.2 Procedural modifications conducted in attempts to generate transplastomic *L. sativa*

As described in section 2.4.1.2, approaches used to generate transplastomic lettuce were altered in many ways. Despite their demonstrated ability to regenerate on a media lacking antibiotic selection of transformation, bombarded explants of *L. sativa* were generally unable to regenerate shoots on selective media. However, in several occasions shoots could be visualized regenerating from bombarded tissue that had begun to form callus. These shoots were a lighter green colour than the original leaves from which they were generated, and eventually lost all green colour to appear a yellow-brown-white. These shoots would eventually die and appear to be viable only for a short time on selective media. This was true for all except one occasion, when an antibiotic-resistant lettuce callus grew on selective media, and maintained a light yellow-green colour following transfer to new selective media. PCR was conducted to corroborate the transplastomy of this callus. Following gel electrophoresis, no bands of the expected transplastomic size could be produced using transplastomic gene-specific primers, as was successful for transplastomic tobacco (described in section 3.5). Although transplastomic *L. sativa* could not be produced, as a negative result this study may serve future researchers, providing a record of procedures and modifications to those procedures that will not produce transplastomic lettuce. The potential reasons for this outcome are explored in section 4.4.
3.5 Generation of transplastomic *N. tabacum* for recombinant protein production

Transplastomic *N. tabacum* was generated as an alternate CPL production platform to transplastomic *L. sativa*. Transplastomic *N. tabacum* has better-established chloroplast transformation procedures, which allow chloroplast transformation where transformation of *L. sativa* was unsuccessful. Transplastomic proteins have also been produced to extremely high levels in *N. tabacum* chloroplasts, up to 70% TSP (Oey et al., 2009), highlighting the potential for efficient protein production in *N. tabacum*. The candidate vaccine protein produced transplastomically used native-encoded DNA sequences, fused to a c-Myc tag for protein detection via Western blot. This gene was assembled using GoldenGate technology and ligated into two previously-constructed chloroplast expression cassettes (Kolotilin et al., 2013). These CECs (Figure 14) comprise many of the same components as the first-designed *L. sativa* transformation vector (Figure 13A), with a few key differences. Compared to *L. sativa* vectors, the *N. tabacum* CECs contain shorter flanking sequences of *trnI* and *trnA* only, which are *N. tabacum* sequences rather an *L. sativa* sequences. Similarly, the expression of the GOI is driven by the *PpsbA* of *N. tabacum* (Verma et al., 2007) rather than that of *L. sativa*. The 5’ UTR of these CECs also originates from *N. tabacum* *psbA*. The second cassette (Figure 14b), pCEC4, is identical to pCEC5, but contains the downstream box from *E. coli* phage 7 gene 10 (T7g10) immediately upstream of the recombinant protein coding sequence. The downstream box, nine amino acids translationally fused to the N-terminal end of the recombinant protein, improves translation and protein stability (Kuroda et al., 2001).

Bombardment was conducted on *N. tabacum* cv. 81V9, a low alkaloid cultivar, using pCEC5 and pCEC4 carrying the candidate vaccine gene. Use of the 81V9 cultivar diminishes the levels of alkaloids that are undesirable in orally-administered vaccines (Menassa et al., 2001). The initial transformation event does not transform all plastids or genomes within a plant cell and thereby creates a heteroplasmic state, in which there are
Figure 14. *N. tabacum* chloroplast transformation and expression constructs.


A) pCEC5CPL: the first construct used for transformation, which was successful in generating transformants. B) pCEC4CPL: the second construct used for transformation, which was not successful in generating transformants. T7g10 5’+DB: *E. coli* phage 7 gene 10 5’ UTR and downstream box. Schematic is not to scale.
both transgenic and native plastids present within the first-transformed cell. To select for transformed plastids, the antibiotic spectinomycin was applied within regeneration media. Spectinomycin activity is detoxified by the AadA protein encoded within the transformation cassette. As an adenyltransferase, the AadA protein confers spectinomycin resistance by catalyzing the adenylation of a hydroxyl group of the spectinomycin molecule (Wright, 1999). The selection process is necessary to ensure that all plastids and all copies of the genome within each polyploid plastid are transgenic. Following the selection process, carried out over three rounds of regeneration, the plant is usually homoplasmic: all genomes within all plastids in all cells of the plant are identical, and a stable transformant has been generated (Bock, 2015). Following regeneration, transplastomic *N. tabacum* 81V9 plants grew successfully in soil in the greenhouse until flowering and producing seeds. Seeds of self-pollinated plants were collected for storage and growth of future plants of this strain.

### 3.5.1 Determination of homoplasmy in transplastomic lines

Homoplasmy in transplastomic lines precludes the loss of transgenes from the plastid genome via genome segregation during plastid division, and maximizes transgene copy number, thereby maximizing transgene expression and ultimately transgenic protein accumulation (Bock, 2015). A Southern blot was therefore conducted to verify that transplastomic plants were homoplasmic. A probe for this Southern blot was generated previously (Kolotilin et al., 2012) and anneals within the *N. tabacum* *trnI* - *trnA* flanking regions, facilitating binding of the probe to DNA from the pCEC5 vector carrying CPL (pCEC5CPL), transplastomic genome sequences, and wild type genome sequences (Figure 15A, B). In the Southern blot conducted by digesting total genomic DNA with the restriction endonuclease *RsrII* and separating the digested DNA by agarose gel electrophoresis (Figure 15C), all 5CPL plants were confirmed to be homoplasmic. Only one band, of size 3502 bp, corresponding to the size of inserted DNA was detected in all 5CPL lanes and in the positive control consisting of pCEC5CPL plasmid (Figure 14A, 15B) used to transform *N. tabacum* plants. This suggests that 5CPL plants are transplastomic, carrying the insert from the pCEC5CPL construct. The transplastomic plants lack the smaller molecular weight band of 1054 bp, indicating that they lack wild
A) Wildtype genome

Expected band size: 1054 bp

B) Transformed genome

Expected band size: 3502 bp

C) pCEC5CPL N. clones

Transplastomic

Wild type
Figure 15. Determination of homoplasmy in transplastomic lines.

A southern blot was conducted on seven transplastomic plants, one wild type negative control, and the pCEC5CPL plasmid as a positive control. A) A section of the wild type genome, showing the insertion site (arrow) of pCEC5 and pCEC4 between trnI and trnA and the annealing location of the probe (horizontal bar). The scissors indicate the digestion sites of the RsrII endonuclease. B) A section of the transformed genome, with transplastomic sequences integrated between flanking sequences as intended. C) A Southern blot conducted showing that transplastomic tobacco lines are homoplastic. The pCEC5CPL plasmid was used as a positive control (+) to display the band size of transformed chloroplasts: 3502 bp. A wild type N. tabacum plant was used as a negative control (WT), and displays a band of size: 1054 bp.
type DNA. A larger molecular weight band is visible in the pCEC5CPL plasmid lanes as a result of incomplete digestion of the pCEC5CPL plasmid, producing linearized plasmid DNA with the flanking regions and plasmid backbone. The linearized plasmid has an expected size of 7252 bp. Smaller bands are also present in plasmid lanes, which may represent multiple conformations of supercoiled plasmid DNA, which flows through a gel matrix faster than linear or circular forms, and may persist following incomplete digestion.

3.5.2 Developmental dependence of recombinant protein accumulation

To determine the most effective time to harvest *N. tabacum* leaves for processing prior to vaccine delivery, CPL accumulation in leaves of multiple ages was compared (Figure 16A). This measurement may also provide information regarding the stability of CPL, as more stable proteins will likely accumulate to higher levels in older leaves. Once a plant had 10 leaves that were all at least 15 cm in length, they were sampled and proteins extracted. A Western blot (Figure 16B) using an anti-c-Myc antibody was conducted to reveal protein accumulation in the various leaves. Alongside the extracted plant proteins, increasing known amounts of the eGEHK standard protein were loaded, and used to calculate the accumulation of CPL in the leaves. Six independent transplastomic plants were analyzed in this manner, and used as biological replicates. This experiment demonstrated (Figure 16C) that CPL accumulates to the highest levels in the third, fourth, fifth, sixth and seventh leaves. Of these leaves, the fifth leaf appears to have a non-significantly higher accumulation level than the other leaves. Specifically, CPL accumulates to 17.1 ± 2.1 mg/kg (mean ± standard deviation of the mean) in the first leaf, rises to 33.0 ± 2.7 mg/kg in the fifth leaf, and decreases gradually to 7.7 ± 0.9 mg/kg in the tenth leaf. As with transiently expressed CPL (section 3.3.2), transplastomic CPL is expected to accumulate as a 35.5 kDa molecule. The blot in Figure 16B shows bands of roughly 35.5 kDa, 70 kDa, 140 kDa, and larger sizes. It is hypothesized that these bands represent dimers, tetramers, and multimers of CPL. The lower 35.5 kDa band and the 70 kDa band were added to assess protein accumulation.
Figure 16. Developmental dependence of protein accumulation.

A) A schematic of the *N. tabacum* plants, with leaves used for analysis numbered from youngest (1) to oldest (10). B) A Western blot of proteins extracted from each of the 10 leaves sampled from one plant during this experiment, shown as a representation of the six plants sampled. eGEHK: protein standard for quantification. C) The accumulation level of CPL in each of the 10 leaves sampled. Six plants were sampled and considered in this analysis. Error bars represent standard error of the mean. Means that are not labelled with the same letter are significantly different. Means were compared using ANOVA followed by Games-Howell pairwise comparisons.
3.5.3 Quantification of recombinant protein accumulation

CPL was quantified in transplastomic lines to assess whether the lines could produce commercially viable levels of this protein. To assess accumulation within the entirety of each plant, quantifications from the developmental dependence experiment were used. The protein levels in each leaf were averaged to estimate the average protein level within a single plant. This protein level was averaged across six plants to reveal that the recombinant protein accumulates to an average of 23.0 ± 5.6 mg/kg FW (Figure 17). Analysis of sequentially-extracted soluble and insoluble proteins from the same tissue revealed that the protein accumulates mainly as an insoluble form, accumulating to 7.4 ± 3.8 mg/kg FW as a soluble form, and 15.6 ± 4.6 mg/kg FW as an insoluble form.

3.5.4 Developmental effects of transplastomic recombinant protein

To accurately assess the phenotypic effects of the genotypic changes implemented in this study, the development of transplastomic N. tabacum was monitored (Figure 18). Measurements of plant height, the length and width of the largest two leaves present, the total number of leaves longer than 2 cm, and of flowering time demonstrated that transplastomic plants carrying CPL had significantly reduced growth and flowered and produced seeds after a longer period of time (Figure 19). It is visibly noticeable (although less obvious in print) that the transplastomic plants generated are of a lighter shade of green than wild type plants (Figure 19). While transplastomic plants grow slower in terms of height, and reach flowering stage later (Figure 19A), neither the size of the largest leaves nor the number of leaves appears to be affected (Figure 19B, C, D, E). The time to
Figure 17. Quantification of recombinant protein accumulation in transplastomic N. tabacum.

The average accumulation levels in N. tabacum was determined by averaging the protein accumulation levels each of 10 sampled leaves, and averaging these levels across the six sampled plants. Error bars represent standard error of the mean of plants. Bars that are not labelled with the same letter are significantly different.
Figure 18. Visualization of developmental effects of CPL expression from the chloroplast genome.

Transplastomic CPL plants grown alongside wild type plants under identical growth conditions display differences in phenotype, including height and leaf color.
Figure 19. Developmental effects of CPL expression from the chloroplast genome.

Seven transplastomic plants and five wild type plants were utilized in this experiment. All error bars represent standard error of the mean. Stars are placed above data points to indicate significant difference between the 5CPL and wild type group at that point in time. A) Height and flowering time. B) Average length of longest two leaves. C) Average width of longest two leaves. D) Average leaf area of longest two leaves, modelled as ellipses. E) Number of non-chlorotic leaves arising from main stem.
reach a height of 80 cm significantly differed between transgenic and wild type plants, with transplastomic plants reaching 80 cm at $8 \pm 0.45$ weeks, and wild type plants at only 5 weeks, with all plants flowering within the same week (Figure 19A). Flowering time was the most affected variable measured, with transplastomic plants flowering at 8.3 weeks after transplanting, and wild type plants at only 5 weeks. This difference was statistically significant. The transplastomic plants produced seeds that appear indistinguishable from wild type seeds and are therefore presumed to be fertile, although this has not yet been verified experimentally.

3.5.5 Recombinant protein accumulation in fresh and lyophilized *N. tabacum* leaf tissue

To determine whether CPL can survive lyophilization, which is generally implemented for tissue storage prior to vaccine delivery, transplastomic *N. tabacum* leaf tissue was lyophilized. One leaf was collected from four different plants, and protein accumulation was measured before and after lyophilization. For this, total protein was extracted from each lyophilized and frozen tissue and quantified using Western blot. This test revealed that CPL persists at $46.9 \pm 10.3\%$ of its original level following lyophilization (Figure 20A), constituting an average of $48.0 \pm 21.7$ mg/kg DW, up to 84 mg/kg DW in the leaves sampled. Comparatively, CPL accumulated to an average of $18.7 \pm 9.9$ mg/kg FW in the unlyophilized tissue sampled. The CPL protein appears to persist in an intact form of size equal to that of CPL extracted from non-lyophilized tissue. No degradation products were visible on Western blots conducted with extracts from lyophilized tissue (Figure 20B).
Figure 20. Recombinant protein accumulation in fresh and lyophilized *N. tabacum* leaf tissue.

A) Recombinant protein accumulation in four leaves collected from four separate plants, and an average of the recombinant protein accumulation those four leaves in each fresh and lyophilized tissues. Values for fresh tissue are calculated per fresh weight, while values for lyophilized tissue are calculated per dry weight. B) Western blot showing recombinant protein accumulation in each of the four sampled transplastomic leaves, alongside a wild type negative control. eGEHK is a protein standard used for quantification.
4 Discussion

4.1 M. haemolytica vaccine design

Multiple vaccines against *M. haemolytica* infection have been developed, originally composed of cell culture supernatants or killed *M. haemolytica*. More recent efforts have focused on transgenically-produced *M. haemolytica* antigens as vaccines, delivered either subcutaneously or nasally (Ayalew et al., 2008; 2009; Confer et al., 2009a; 2009b). These vaccines are composed of two *M. haemolytica* proteins: *Pasteurella* lipoprotein E (PlpE) and leukotoxin (Lkt). PlpE is a surface lipoprotein of *M. haemolytica*. Antibodies against PlpE facilitate complement-mediated bacteriolysis and phagocytosis of the bacterium (Ayalew et al., 2008). Lkt is a secreted toxic molecule that binds to the LFA-1 receptor of leukocytes (Atapattu et al., 2005), leading to caspase 9-dependent apoptosis and ultimately inflammation and damage to alveolar tissues (Zecchinon et al., 2005).

Inclusion of Lkt epitopes in vaccines has been demonstrated to be highly beneficial but not sufficient for robust protection against *M. haemolytica* infection (Conlon et al., 1991b). One effective vaccine against *M. haemolytica* was therefore designed as a recombinant protein comprising neutralizing and immunogenic epitopes of Lkt and plpE, specifically the neutralizing epitope of Lkt: nLkt, and the r2 region of plpE: rPlpE.

Efficacy of nasally-administered *M. haemolytica* vaccines has been improved through the addition of a protein subunit originating from *V. cholerae*, CTB (Confer et al., 2009b). This improvement is strengthened by linking the CTB protein directly to the vaccine (Ayalew et al., 2009). CTB forms a pentamer which binds the GM1 ganglioside and triggers uptake of the subunit into epithelial cells (Spangler, 1992). CTB acts as a strong mucosal adjuvant and also presumably facilitates uptake of translationally-fused recombinant protein into epithelial cells and later recognition and response by the immune system, leading to the general improvement in efficacy of vaccines linked to CTB (Holmgren et al., 2005; Sheoran et al., 2002). This vaccine, designed and tested by Confer, Ayalew et al. (2009b), appears in the literature to be the most effective subunit vaccine developed thus far. The major goal of this study was to produce a similar vaccine in plant tissue for oral delivery to cattle.
4.2 Transient production of CPL in *L. sativa*

The CPL protein accumulated to detectable levels in *L. sativa* only when targeted to the chloroplast and extracted using an insoluble protein extraction buffer. Differences in protein accumulation are expected across cellular compartments, as each compartment has different biochemical composition and protease content (Pillay et al., 2014; Streatfield, 2007). Accumulation in *L. sativa* was accomplished after some improvement of infiltration techniques, and by the same infiltration technique on the same day that led to high accumulation levels in *N. benthamiana*. The cause of these low accumulation levels is not known. It is presumed that errors in infiltration would be minimal as the same infiltration technique, used on the same occasion, led to high accumulation levels in *N. benthamiana*. One potential explanation for low accumulation levels in *L. sativa* is poor infection by *A. tumefaciens*. It is notable that infiltration techniques, while adjusted for *L. sativa* expression based on available literature, have been optimized for *N. benthamiana* expression, with *L. sativa* receiving significantly less attention in the literature and no previous testing in our lab. The OD used in this infiltration was 0.7, a compromise of ODs employed by others in the literature (Chen et al., 2016; Joh et al., 2005; Li et al., 2007a; Negrouk et al., 2005; Wroblewski et al., 2005).

*A. tumefaciens* strain may also play a role in infection success, and should therefore be considered (Li et al., 2007a). While the EHA105 strain employed in this study has been confirmed to successfully infect *L. sativa* (Lupan et al., 2010; Zhao et al., 2017), it is uncommon for this purpose, and the efficiency of *L. sativa* infection by agrobacterium EHA105 strain is not known. Two other laboratory strains, GV3101 and C58C1, predominate in literature regarding transient protein expression in *L. sativa* (Chen et al., 2016; Joh et al., 2005; Li et al., 2007a; Negrouk et al., 2005; Wroblewski et al., 2005). The C58C1 strain has been demonstrated to infect an average of 80% of cells in one study (Wroblewski et al., 2005), and the GV3101 strain produced 50 mg/kg FW of antibody protein in another study, confirming that these two strains are sufficiently capable of lettuce cell infection. It is therefore possible that *A. tumefaciens* strain could be responsible for low accumulation levels, with *L. sativa* possessing some degree of resistance, or poor amenability to infection by *A. tumefaciens* EHA105. This has been
observed for other strains, which infect *N. benthamiana* but not *L. sativa* (Wroblewski et al., 2005). This would be most easily tested by comparing protein accumulation between plants or leaves infiltrated with the three different strains carrying identical constructs.

Another explanation is that transient expression from the delivered plasmid was insufficient. This could be explored using quantitative real-time PCR (qRT-PCR) to analyze foreign transcript levels. However, it is expected that expression from the double-enhanced CaMV 35S promoter should be sufficient for protein accumulation, as this promoter has routinely been used to drive relatively high-level expression of transiently-produced proteins in *L. sativa* (Joh et al., 2005; Li et al., 2007a; Liu Clarke et al., 2017; Negrouk et al., 2005; Sohi et al., 2005; Wroblewski et al., 2005). The 35S promoter is also exceedingly the most common promoter employed in transient expression in lettuce (Dong et al., 2014). Another promoter occasionally used in place of 35S is the ubiquitin promoter (Dong et al., 2014). In one study wherein a taste-modifying protein, miraculin, was produced in lettuce, the ubiquitin promoter and terminator were compared to the 35S promoter and NOS terminator. It was demonstrated by qRT-PCR and Western blot that the miraculin RNA and protein respectively were present at higher levels under the ubiquitin promoter and terminator than 35S and NOS (Hirai et al., 2011). Another study, in which CTB was produced by nuclear transformation and expressed from the ubiquitin promoter and terminator, found the CTB gene to be expressed and the protein to accumulate to 0.24% TSP. While this constitutes a successful employment of the ubiquitin promoter, it was not compared with the 35S promoter, and therefore no definite conclusions can be made. If low transcript levels in infected cells could be identified, use of the ubiquitin terminator may serve as a potential solution.

It is not expected that inefficient translation from produced transcripts led to low accumulation, as these genes were optimized for expression from the lettuce nucleus and should therefore be translated efficiently. However, it is possible that the optimization was misinformed and sequences used are not necessarily those best suited for rapid translation. While codon optimization follows trends in codon usage bias of the target genome, translational efficiency is not consistently correlated with codon usage.
Similarly-frequent codons can exhibit large differences in translational efficiency (Nakamura et al., 2011).

While CPL is surely less stable than GFP, which accumulated to detectable levels in both the chloroplast and ER of lettuce, the protein is sufficiently stable to accumulate to levels as high as 200 mg/kg in *N. benthamiana*. However, these two plants are not closely related and therefore a protein may be stable within the cells of *N. benthamiana* but not those of *L. sativa*. While infection efficiency is similarly high in *L. sativa* compared to *N. benthamiana* using *A. tumefaciens* C58C1 (Wroblewski et al., 2005), accumulation levels of the same protein in the two species are not well-known. This study may therefore serve as one of the first comparisons of protein stability within the two species.

### 4.3 Transient production of CPL in *N. benthamiana*

The CPL protein accumulated in *N. benthamiana* to levels significantly higher than those observed in *L. sativa*. The protein accumulated to high levels in both the chloroplasts and the ER, with accumulation in the ER somewhat higher, albeit not significantly, than within the chloroplasts. This trend could be attributed to the suite of chaperones available within the ER, its low hydrolytic activity, or its oxidizing environment (Vitale et al., 2005). For its general amenability to foreign protein accumulation, many recombinant proteins have been produced to high levels through targeting to the ER (Vitale et al., 2005). While CPL accumulates to high levels within the ER, the ER-targeted CPL protein appears to be modified, as it shows a larger size than expected when assayed via Western blot. Since a similar protein produced transiently in our lab, comprising a larger region of PlpE than produced in this project, was shown to be glycosylated, it was expected that CPL may be glycosylated, leading to molecular weight increases. Additionally, the CTB protein has been demonstrated to be glycosylated following plant nuclear expression (Matoba, 2015). This encoded glycosylation site was not removed from CPL as the glycosylation was found not to affect immunogenicity in an animal model (Matoba, 2015). A glycan side chain would therefore be expected to be present on ER-targeted CPL, although the identified glycan common in plant-produced CTB is only 3 kDa in size (Mishra et al., 2006), much smaller than the increase in size of CPL observed here.
Speculating that glycosylation may be responsible for observed size increases, a de-glycosylation assay was conducted. The results of a de-glycosylation assay suggested that the soluble form of CPL within the ER is not glycosylated. However, this result was not conclusive as banding was faint for de-glycosylated CPL extracts, and not especially clear for a positive control. It is therefore unknown what post-translational modifications (PTMs) might have led to this increase in molecular weight. There exists a multitude of different post-translational modifications that could lead to this size increase (Friso et al., 2015). One potential modification that is sufficiently large to cause the size change observed is the addition of a small ubiquitin-like modifier, or SUMOylation. SUMOylation entails the binding of an approximately 12 kDa protein to the lysine residues of the target (Friso et al., 2015). The observed size increase in the ER is roughly 12 kDa, and CPL contains four potential SUMOylation motifs (Zhao et al., 2014), making SUMOylation a potential cause of this increase. There is some potential that post-translational modifications to proteins targeted to the ER could interfere with vaccine epitope exposure. Therefore, ER-targeting is not preferred, especially if accumulation is sufficiently high when targeting to other compartments.

The CPL protein was also targeted to the chloroplast, first as a potential production method, and secondly as a representation of accumulation levels in transplastomic N. tabacum. The CPL protein is stable within the chloroplasts, albeit at somewhat lower levels than within the ER. The chloroplast-targeted CPL protein accumulated mainly as an insoluble form, which appeared to contain no significant PTMs, as it is of the expected molecular size, 35 kDa. However, the soluble form, similar to ER-targeted CPL, comprises both 35 kDa molecules and larger molecules similar in size to those of ER-targeted CPL. The origin of this size increase is unknown. Proteins must be trafficked along the secretory pathway to undergo glycosylation (Friso et al., 2015), and therefore this is not a possibility. It is assumed that the rubisco transit peptide used for trafficking to the chloroplast is cleaved off after CPL reaches its destination. However, it may be possible that the transit peptide is not cleaved properly, and remains attached to CPL. The rubisco transit peptide used in this study is approximately 6 kDa in size and could therefore explain the size increase observed. It is notable that the modification of CPL observed in the chloroplast is associated with solubility: Roughly half the molecules of
the soluble form are modified, while those of the insoluble form are not. Modifications may therefore be involved in improving solubility or are perhaps only possible for soluble molecules, with insoluble ones inaccessible to post-translationally-modifying enzymes.

It is hypothesized that proteins expressed from the nucleus that accumulate well when transported into the chloroplasts will also accumulate well when produced within the chloroplasts, as accumulation is largely dependent upon the stability of each protein in its subcellular location (Bock, 2015). This trend has been observed for proteins such as a fimbrial protein from enterotoxic E. coli, FaeG and a xylanase from Aspergillus niger (Kolotilin et al., 2012; Kolotilin et al., 2013). It was therefore expected that CPL accumulation levels within transplastomic N. tabacum would be quite high. Transplastomic production also requires neither A. tumefaciens culturing nor infiltration procedures, which are intensive and expensive. For these reasons, along with the aforementioned lack of PTMs on chloroplast-produced proteins, I attempted to produce CPL in lettuce and tobacco chloroplasts an alternative production platform to transient expression.

4.4 Attempts to generate transplastomic L. sativa

To generate transplastomic L. sativa, a straightforward protocol was attempted, which was demonstrated to be successful in producing transplastomic tobacco (Kolotilin et al., 2012). Many modifications to this protocol were gleaned from published studies that reported successful transformation of L. sativa chloroplasts. The cultivar Simpson Elite was acquired as one group has shown repeated success in transforming and generating this cultivar (Boyhan et al., 2011; Kanagaraj et al., 2011; Ruhlman et al., 2007; Su et al., 2015). Regeneration experiments were conducted to disprove the hypothesis that chloroplasts may be transformed but unable to multiply within plant tissue that does not regenerate. These experiments also served to optimize regeneration conditions. The optimized hormone concentration for shoot proliferation of 0.2 mg/L BAP and 0.1 mg/L NAA was used for regeneration of all bombarded explants, the majority of which originated from the Simpson elite cultivar.
I hypothesized that perhaps CPL itself was produced within transformed chloroplasts, but imposed such detriments on transformed chloroplasts that they could not survive. It has been demonstrated that some proteins confer significant negative health effects on plants, specifically chloroplasts (Lentz et al., 2012). This causes the resulting transplastomic plants to be a lighter green colour and to grow slowly (Kolotilin et al., 2013; Miletic et al., 2017). Indeed, this effect was observed in *N. tabacum* expressing the recombinant protein in this study. To disprove this hypothesis, GFP was used. GFP is known to be innocuous and to express well in many biological systems, including plant chloroplasts. Therefore, GFP was cloned into the *L. sativa* chloroplast transformation construct in place of CPL. This construct was used in 65 bombardments and did not yield any transformants.

There exist multiple reports of *L. sativa* chloroplast transformation with physical conditions of transformation often differing between reports. In attempts to generate transformants, variables that differed between reported transformations were altered and tested at each possible configuration. These include leaf orientation, which is adaxial side upward in many successful reports (Boyhan et al., 2011; Harada et al., 2014; Kanagaraj et al., 2011; Kanamoto et al., 2006; Lim et al., 2011; Maldaner et al., 2013; Ruhlman et al., 2007; Su et al., 2015), but abaxial side upward in *N. tabacum* transformation (Boyhan et al., 2011; Kolotilin et al., 2012; Kolotilin et al., 2013; Ruhlman et al., 2007). Target distance, the distance between the leaves and the macrocarrier, through which the gold particles travel, also varies between reports, with some using 6 cm (Ruhlman et al., 2007) and others 9 cm (Lim et al., 2011; Tabar et al., 2013). Both of these distances were employed in bombardments. Other possible distances, 3 cm and 12 cm were also used. Leaves were also stacked on top of one another in another trial as it was hypothesized gold may travel through the first leaf and penetrate into the chloroplasts of the second or third. Another protocol similar to that of the Menassa lab was acquired by request from another lab attempting produce transplastomic *L. sativa*. This protocol recommended beginning leaf regeneration one day prior to conducting the transformation procedure, cutting leaves prior to bombardment, and using a 0.1 mg/L BAP, 0.1 mg/L NAA regeneration medium. This protocol was precisely followed, although notably this regeneration medium is sub-optimal for all of the cultivars used in this study.
I then hypothesized that transcription of the \textit{aadA} gene in the original chloroplast transformation construct may be insufficient to confer strong spectinomycin resistance. The original chloroplast transformation construct relied upon read-through transcription from the rRNA operon, which begins upstream of the \textit{trnI} - \textit{trnA} insertion location. While this locus offers sufficient transcription for inserted \textit{aadA} in \textit{N. tabacum} to confer spectinomycin resistance, it is unknown whether the same is true for \textit{L. sativa}. To test this hypothesis, the promoter from \textit{N. tabacum psbA} was inserted immediately upstream of the \textit{aadA} gene in efforts of improving transcription. Additionally, explants were regenerated on media containing 30 mg/L spectinomycin rather than 50 mg/L. These modifications did not yield any transformants either.

After extensive modifications to procedure and constructs, I hypothesized that an error in the design of the pLSCTGG construct may have produced a flaw, present in all subsequent constructs, which abrogated \textit{L. sativa} chloroplast transformation. To refute this hypothesis, a minimal construct for \textit{L. sativa} chloroplast transformation was assembled, which comprised \textit{rbcL} and \textit{accD} flanking sequences. Between flanking sequences, a \textit{N. tabacum rrn} promoter drives \textit{aadA} expression and the \textit{L. sativa rps16} terminator and 3'UTR halt transcription and stabilize the \textit{aadA} transcript. No unnecessary genes or associated genetic elements were included in this construct to reduce the possibility that any component may interfere with transformation or proliferation of transformed chloroplasts. This construct did not produce any transplastomic lettuce either.

It is also notable that although a \textit{N. tabacum} transformant was identified for the pCEC5, transformation efficiency for \textit{N. tabacum} in this study is still far below expectations (Ruhlman et al., 2010). It was therefore hypothesized that transformation efficiency for \textit{L. sativa} may be excessively low, such that transformation is possible but unlikely. The total number of bombardments used to generate one \textit{N. tabacum} transformant in this study using the pCEC5 construct was 40. Comparatively, over 200 bombardments were conducted on \textit{L. sativa} and no transformants could be identified, despite regenerability. It is therefore possible that an unidentified factor reduced transformation efficiency in both cases. This reduction could have compounded with the lowered transformation efficiency
of *L. sativa*, which is not optimized in our lab, to reduce efficiency to levels too low for a transformation event to be likely within 200 bombardments.

4.5 Generation of transplastomic *N. tabacum*

One transplastomic *N. tabacum* line expressing CPL was produced. Transformation efficiency was low, and therefore no pCEC4 transformants were generated. Only one transformant was generated using a similar construct, pCEC5, and this transformant was regenerated into seven clones. Southern blots demonstrated that these clones were homoplasmic. Despite homoplasmy, the recombinant protein did not accumulate to exceptionally high levels in any of the clones. This is not uncommon, as accumulation of different proteins often varies despite expression from identical constructs. The low level of protein produced is best described relative to other transplastomically-produced proteins. For instance, EspA, a protein from shiga-toxin producing *E. coli* produced using this same expression cassette accumulated to 220.7 mg/kg of dried leaf tissue, although two other genes in the same construct did not produce any detectable level of protein (Miletic et al., 2017). A xylanase produced using the same expression cassette accumulated to much higher levels: 1.3 g/kg (Kolotilin et al., 2013) and FaeG accumulated to 2.0 g/kg leaf tissue. There is evidently a large degree of variation between the accumulation levels of different proteins produced transplastomically (Bock, 2014a), and it is therefore important to consider the causes of these differences to develop potential strategies to increase protein accumulation levels.

Transcript levels, influenced by both transcription rates and RNA stability, could ultimately affect protein accumulation. In one report wherein the enzyme lycopene B-cyclase was expressed within transplastomic tomato, RNA transcript levels were found to be excessively low, leading to low protein accumulation levels (Wurbs et al., 2007). As conducted in that study, qRT-PCR could be employed to assess RNA levels within the tissue of transplastomic *N. tabacum* plants generated in this study. It is notable that three studies have demonstrated variable protein accumulation levels while expressing different genes using the pCEC5 vector (Kolotilin et al., 2012; Kolotilin et al., 2013; Miletic et al., 2017).
Another factor affecting transgenic protein levels is translational efficiency. As with all organisms, chloroplasts display biases towards using certain codons over others. Codons that are less common tend to result in lower foreign protein yields (Gustafsson et al., 2004). In one study, accumulation of the human growth factor B3 was demonstrated to increase 75-fold in *N. tabacum* chloroplasts following codon-optimization of the sequence (Gisby et al., 2011). However, it is notable that this difference resulted from altering codon usage from that of a mammal to that of a chloroplast, two organisms that are only distantly related. Conversely, in this study prokaryotic genes were transformed into chloroplasts, both of which have prokaryotic codon usage, which could be expected to exhibit higher similarity to each other than to animals. Therefore, in this study, codon usage of the recombinant candidate vaccine gene was not modified when producing transplastomic constructs, which were intended for expression by both *L. sativa* and *N. tabacum*. As detailed previously, there is also evidence that the codon usage bias of an organism may not necessarily correlate directly to translational efficiency (Nakamura et al., 2011). It may therefore be possible that current codon-optimization schemes do not necessarily lead to increases in protein levels, especially when genes are transferred between related species.

Protein stability is likely the largest contributor to foreign protein accumulation levels within chloroplasts (Bock, 2015). The chloroplast genome encodes 11 or more protease families, which are mainly of bacterial origin (Sakamoto, 2006). These proteases function to eliminate proteins that have been overproduced, or display an incorrect shape due to errors in synthesis, folding, or damage thereafter. Unstable proteins are more likely to misfold, potentially forming aggregates, and triggering degradation by native proteases. It is therefore paramount that designed recombinant proteins are as stable as possible to avoid protein degradation. One of the primary factors affecting the half-life of proteins within cells is the sequence of amino acids found at the N-terminus of the protein. This correlation, termed the N-end rule, applies to prokaryotes and eukaryotes alike (Varshavsky, 1996), and not surprisingly, seems to apply to chloroplasts as well (Apel et al., 2010). It was concluded that in chloroplasts of *N. tabacum*, the second residue in a protein sequence had a great impact on protein accumulation. This effect was most pronounced when the second amino acid was changed to glutamic acid, and therefore
glutamic acid was included as the second amino acid in the *L. sativa* expression constructs designed in this study. Researchers have also observed increases in protein accumulation after translationally fusing foreign proteins to the N-terminus of GFP, suggesting other determinants of protein stability also seem to be found elsewhere within the N-terminal region of proteins (Apel et al., 2010). Similarly, the HIV protein cyanovirin-N, was not detectable in transplastomic tissue when transformed alone, but accumulated to detectable levels when GFP was fused to the N-terminus. In this case, mRNA stability was also observed to increase following this fusion (Elghabi et al., 2011). It might therefore be predicted that the recombinant protein produced in this study could be stabilized by CTB, which has accumulated to high levels as a component of other recombinant proteins (Davoodi-Semiromi et al., 2010; Ruhlman et al., 2007).

Accumulation levels of transiently-produced proteins targeted to the chloroplasts can serve as a prediction of transplastomic accumulation levels. When the gene for an ER-accumulating storage protein from maize was expressed transplastomic *N. tabacum*, it did not accumulate to detectable levels within the chloroplasts (Bellucci et al., 2005). The storage protein also did not accumulate when produced through nuclear transformation and targeted to the chloroplast, although transcript levels appeared reasonable by Northern blot. It was therefore concluded that accumulation defects were due to protein instability within chloroplasts. The converse situation was demonstrated by the aforementioned expression of FaeG and a xylanase, both of which accumulated to high levels within the chloroplasts following infiltration, and in transplastomic plants (Kolotilin et al., 2012; Kolotilin et al., 2013). By this logic it is mysterious that accumulation levels of transiently produced CPL in this study were comparable between the ER and chloroplast targeted proteins at 235 mg/kg FW and 163 mg/kg FW respectively, although far less protein accumulated in transplastomic CPL-expressing plants, averaging 23 mg/kg FW.

A final factor affecting the level of detected recombinant protein, but not accumulation levels, is the extraction buffer used. In this project, two extraction buffers were employed to assess the amount of soluble and insoluble protein. It was revealed that roughly one-third of the CPL accumulated in soluble form and two-thirds in an insoluble form. The
insoluble form of CPL forms multimers, as demonstrated by western blot in Figure 15B. These multimers are likely caused by association of multiple CTB subunits, which form pentamers in their native form (Spangler, 1992). Since CTB interacts with its target receptor as a pentamer (Spangler, 1992), it is beneficial that CTB exists in multimeric forms, although it is not known whether insoluble multimers would be liberated during digestion and regurgitation in ruminants from the membranes they seem to bind.

4.6 Transplastomic CPL as a vaccine against *M. haemolytica*

In this project, a candidate vaccine against BRD was designed and produced to an appreciable level. While other proteins have accumulated to considerably higher levels than CPL, it is notable that such high accumulation levels are not invariably necessary for use of plant material as an oral vaccine. For example, both IgA and IgG responses were triggered in pigs orally administered 50 grams of fresh *N. tabacum* leaf tissue containing 0.11 mg/kg fresh leaf weight of vaccine comprising the porcine reproductive and respiratory syndrome virus envelope glycoprotein 5 (Chia et al., 2010). Similarly, in another study, an immune response could be produced by feeding mice tobacco tissue containing a subunit vaccine comprising antigens of Crimean-Congo Hemorrhagic Fever Virus, which accumulated to only 1.8 mg/kg of fresh leaf tissue (Ghiasi et al., 2011). In addition, another study demonstrated that tobacco-produced LTB could induce immune responses in mice after orally administering fresh tissue that contained only 0.0014% TSP of LTB (Ghiasi et al., 2011). The demonstrated success of low-accumulating transgenic plants as oral vaccines suggests it may be possible to produce an oral vaccine from tissue of *N. tabacum* generated in this study.

It is also useful to consider how much candidate vaccine protein total should be administered when speculating on the necessary accumulation levels and therefore amount of administered leaf tissue. In the study that demonstrated efficacy of the subunits produced in this project, 112 µg of a similarly-designed vaccine was administered nasally to calves. This led to significant IgA responses against whole cells and Lkt, and the vaccine offered protection against *M. haemolytica* bacteria (Ayalew et al., 2009). In
comparison, to administer 112 μg of the vaccine produced in this study would require only 5 g of lyophilized plant tissue. It is of course expected that significantly less of the recombinant protein would reach immune cells than if a purified protein were administered. However, it would be substantially less intensive to deliver leaf tissue than purified protein, despite the significantly higher dose necessary. As opposed to nasal delivery, oral delivery poses the risk of antigen degradation within the ruminant digestive tract. The recombinant protein must remain intact for some period within the rumen to be exposed to tonsillar and nasal mucosae upon regurgitation (Shewen et al., 2009). It is not known to what extent the protein produced is stable within the rumen environment, and therefore it is difficult to predict how much plant tissue is required for an effective dose of vaccine.

To identify an effective dose of the CPL candidate vaccine and to determine whether CPL is efficacious as a vaccine when administered in this manner, a feeding trial must be conducted. While not the ultimate goal of this work is to administer CPL to cattle, oral immunogenicity could be first tested in small mammals such as mice or rabbits. In such an experiment, the recipient animal would be tested for neutralizing antibodies against *M. haemolytica* and the leukotoxin. In one study, wherein a GFP-Lkt fusion vaccine was produced in white clover, it was demonstrated that rabbits that had been injected with a protein extract developed antibodies capable of neutralizing native Lkt molecules (Lee et al., 2001). The same group produced a 50 kDa truncated form of Lkt in alfalfa, termed Lkt50, and challenged calves with *M. haemolytica* infection following immunization with their vaccine. The vaccinated calves possessed anti-Lkt IgG and IgA antibodies before challenge with *M. haemolytica*, rising significantly in titer following challenge, conferring strong resistance against the pathogen. Calves that did not receive the vaccine developed lower titers of anti-Lkt antibodies following challenge, and were severely affected by the disease. In that study, calves were fed 300 g of dried plant tissue each day for five days, and this was repeated two weeks later. With accumulation levels of roughly 90 mg/kg DW, calves received 27 mg of vaccine protein per day, a total of 270 mg over the ten feeding days (Shewen et al., 2009). Comparably, CPL accumulated at up to 84 mg/kg DW in transplastomic tobacco, and therefore a slightly larger dose of 322 g DW per day would be required to achieve the same vaccine dose. It is also worth noting
that Lkt50 contains only the Lkt protein, whereas the CPL vaccine produced in this project contains epitopes of CTB, PlpE, and Lkt. The inclusion of CTB is expected to boost immunogenicity, while PlpE epitopes are expected to facilitate destruction of the bacteria. At a molecular weight of only 35 kDa, compared to the 50 kDa of Lkt50, the CPL candidate vaccine would also provide more vaccine molecules if a comparable weight of protein were delivered. One might therefore hypothesize that the CPL vaccine would be more efficacious and require a lower dose than this alfalfa-produced vaccine. To corroborate this hypothesis would necessitate animal trials.

Since animal trials with large animals such as calves are expensive, it would be judicious to first conduct such a trial with a smaller mammal model, as has been performed for another *M. haemolytica* vaccine. The *M. haemolytica* surface protein Gs60 produced in transgenic alfalfa was administered to rabbits both orally and via injection. Both administration methods triggered immune responses and the production of anti-Gs60 antibodies that recognized genuine Gs60 in extracts of *M. haemolytica* (Lee et al., 2001). It is likely that vaccines that are immunogenic and elicit antibody production in small mammal models could evoke similar responses in calves, considering that calves ruminate, and repeatedly pass antigens over the mucosal tissues of the tonsils and adenoids (Shewen et al., 2009). Therefore, following positive results in small mammals, CPL could later be used in a challenge experiment, wherein it would be orally administered to calves, followed by challenge with *M. haemolytica*. While experiments such as these have been performed with a highly similar vaccine following purification from bacteria (Ayalew et al., 2009), and a less-similar vaccine produced in alfalfa (Shewen et al., 2009), and shown promising results, this would be the first animal test of this antigen combination produced in plants.
5 Conclusion and future perspectives

In this project, I ultimately intended to generate a plant-based production system for a vaccine against *M. haemolytica*. In this respect, the project was certainly successful, as protein production appears to rival that of other labs producing similar vaccines that have progressed to animal trials. However, the specific objectives of this study must be addressed. While a considerable amount of effort was directed toward developing transgenic *L. sativa* as a production platform, transplastomic *L. sativa* could not be generated, and little accumulation of foreign protein was observed. I have speculated on the potential causes of these issues, making them extremely difficult to overcome. It is unfortunate that the extensive optimization of *L. sativa* growth and regeneration in our lab therefore cannot be applied. Optimistically, it is possible that future efforts to transform *L. sativa* chloroplasts or to transiently produce proteins will be more successful, allowing application of the optimizations conducted here.

Similar to much of the work in this thesis, the future performance of the CPL candidate vaccine is unpredictable. It is not known whether CPL will survive the rumen environment in a sufficient extent to deliver antigens to immune cells. If CPL is sufficiently stable, it must also provoke an effective immune response to function as a vaccine. This will likely require liberation of insoluble CPL, which may or may not occur within the rumen. These uncertainties can only be addressed through continued experimental analysis.

While CPL accumulated in transplastomic *N. tabacum* to levels comparable to those of another lab, it is not known whether these levels will be feasible for commercial production of CPL as a vaccine. The field of plant biotechnology is still relatively young, with chloroplast biotechnology specifically much smaller and younger. While protein production in transgenic plants, and particularly within chloroplasts, has proven extremely effective and adaptable (Ahmad et al., 2010), there is still much to be learned. However, chloroplast biotechnology is advancing quickly, and it is hoped that incoming knowledge from new research will contribute to the production of biologically important proteins such as CPL produced in this study. Through thoughtful integration and
application of knowledge of plant biotechnology and immunology, it is my hope that in the near future bovine respiratory disease, the devastating outcome of *M. haemolytica* infection can be restrained.
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