The Expression of Complex Proteins in Plant Systems for Industrial and Biomedical Applications

by

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ABSTRACT

The expression of complex proteins was studied in multiple plant systems. Recombinant spider silk, which could be utilized for biomedical applications such as coatings or doped into silk fibers, was successfully expressed in Nicotiana. benthamiana wild type and GnGn glycoengineered transgenic plants and purified from endogenous plant proteins which could be utilized for biomedical applications such as coatings or doped into silk fibers. However, the purification process requires further optimization to result in commercialized production of recombinant spider silk. Green fluorescent protein and Norovirus virus-like particles were expressed in multiple plant systems including alfalfa, beets, lettuce, and spinach, in addition to N. benthamiana, to determine the ability of these plant expression systems to produce vaccine candidates for edible vaccine applications in the agricultural sector as well as low-to-middle income countries. It was determined that alfalfa, beets, and lettuce are potential high production expression systems for edible vaccines however they require further optimization to be commercialized. Lastly, novel virus-like particles and antigen presenting nanoparticles based on the bacteriophage AP205 coat protein and norovirus capsid proteins fused to human papillomavirus L2 protein segments (S and P) were expressed in N. benthamiana and utilized to vaccinate mice against the L2 capsid protein (aa14-38x2 and aa14-122) of Human Papillomavirus 16 to study a potential boosting effect of the Recombinant Immune Complex vaccine platform upon prime-boost dosing with the virus-like particle being the prime and the Recombinant Immune Complex being the boost in this vaccine schema.

DEDICATION

This Dissertation is dedicated to all the people who labored not knowing what work that future would bring.

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To my brother who supported all my good and bad decisions, for the most part, over the years.

To my mom who said I shouldn't do a PhD but was supportive when I did.

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CHAPTER 1

A REVIEW OF PLANT BIOTECHNOLOGY AND IT'S BIOMEDICAL APPLICATIONS

Plant Biotechnology: From Then to Now

Plant biotechnology began in earnest with the production of the first genetically modified, truly transgenic plant produced in the 1980s (Bevan et al., 1983; De Block et al., 1984; Fraley et al., 1983; Herrera-Estrella et al., 1983; Horsch et al., 1984). While experimentation with plants, taking the form of a cellular biology approach akin to the culturing of mammalian cells, began in the mid 1950s, it wasn't until the discovery of the *Agrobacterium tumefaciens* tumor inducing (Ti) plasmid that large scale genetic modification began to be studied (Chilton et al., 1977, 1980; Willmitzer et al., 1980). This Ti plasmid allows, in nature, the Agrobacterium to hack the plant genome to produce sugars to eat, however in plant biotechnology this Ti plasmid is utilized to deliver foreign DNA in order to force the plant to create any protein of interest. Into the modern era, the legacy of both Agrobacterium mediated gene editing and the use of tobacco as an early test subject for plant cell culture continues to impact the modern, cutting edge research of plant biotechnology (Diamos, Hunter, et al., 2020).

After the first transgenic plant was produced the production of resistant crops, typically to herbicides, boomed in just over a decade. Almost five million acres of modified crops were planted in 1996 in the United States, with over 1.7 billion total acres being planted worldwide in the following decade (Vasil, 2008). In the modern day the number of genetically modified crops utilized is staggering, in fact current cultivation of genetically

modified crops is equal to the surface area of Mexico (Turnbull et al., 2021)**.** While there have been increased regulation around the use of genetically modified crops worldwide, especially in the European Union, they have become a pervasive part of the modern era and the number of gene edited crops continues to increase worldwide (Turnbull et al., 2021). This is due, in large part, to a secondary key factor in the history of plant biotechnology, the sequencing of plant genomes. Knowledge of the genomes of plants has allowed for highly directed genetic editing as well as the adoption of genetic elements from various plant origins into modern biotechnology applications (Diamos, Hunter, et al., 2020; Diamos & Mason, 2018a).

While crop production has been a key product from plant biotechnology, there are a wide array of applications. One is the production of biologics such as antibodies, enzymes, and vaccines (Diamos, Hunter, et al., 2020; Diamos, Pardhe, et al., 2020). The other primary application is the creation of biomaterials such as biopolymers (Singh et al., 2023). In 1990, the first pharmaceutical protein, human serum albumin, was produced in transgenic tobacco plants (Sijmons et al., 1990). This was quickly followed by the production of a hepatitis B surface antigen in transgenic tobacco plants in 1992 marking one of the first vaccines produced in transgenic plants (Mason et al., 1992). Nearly two decades later, the first plant-made biologic, β-glucocerebrosidase, marketed as ELELYSO, was developed by Protalix Biotherapeutics and approved for human use (Wolfson, 2013). It was a long wait between the first produced pharmaceutical protein and the first major step into the realm of high potential biopolymers with the production of spider silk proteins in both tobacco and potato plants in 2001 (Scheller et al., 2001). At a certain point, the

production of biologics in plants has shifted from transgenic - meaning the protein of interest is produced by the plant in low quantities constantly and this expression is passed down to progeny - to transient expression - meaning the *Agrobacterium* is used to produce protein in large amounts in a short amount of time (typically 3-5 days) with the expression not being passed down to progeny - a major boon for modern plant-expressed biologics and biomaterials.

The legacy of plant biotechnology in these fields is still blooming into modern progress in various fields. For instance in 2020 spider silk fibroin was produced in rice seeds (W. T. Yang et al., 2020), while in 2023 tobacco plants have been utilized to create a vaccine which may have the potential to protect against Herpes Simplex Virus (HSV) type 1 and type 2 (Diamos et al., 2023). While genetically modified plants have been used to great effect in the production of crops there has been less success in the promised simple, cost-effective, high throughput production of plant produced biologics and biopolymers (LeBlanc et al., 2021). The first approved biologic is produced in carrot cell culture, a non-typical expression vector in the plant biotechnology community. *Nicotiana benthamiana*, a cousin of *Nicotiana tobacum*, known colloquially as the tobacco plant, is one of the most common plant biotechnology production systems, namely for transient rather than transgenic expression of materials (Moon et al., 2020). In transient expression, the protein of interest is expressed over the course of several days at high quantities and the genetic modifications cannot be passed down via pollination of plants. Whereas in transgenic expression the plant nuclear or chloroplast genome is permanently modified and the genetic modification can potentially be passed from one plant to another; a major

issue for both regulators and companies alike (Cullet, 2005). Transgenic expression, while being highly popular for crops, is less optimal for biologic production due to its overall low yield by comparison. That is not to say that there is a blanket solution to be found in transiently expressed material. In the most optimal conditions transient expression of protein can reach ~5mg per gram of leaf tissue however this is often approximately 1.5mg per gram of leaf tissue for biologics such as antibodies (Diamos, Hunter, et al., 2020; Diamos & Mason, 2018a). Yet, that does not mean the protein produced is always easily extracted and purified from plant tissues. Many proteins are highly onerous not in their overall expression levels but in their difficult extraction from plant material, leaving one of the major hurdles of commercial production to be how extraction can be performed not only at a large scale, but in a way that leads to FDA and other regulatory approval of the biologics produced (Moon et al., 2020).

There are two major ways the production capacity of a plant expressed protein may be increased: 1) increase the overall expression of the protein in question, and 2) increase the percentage of the protein that is properly extracted from the plant material. As mentioned, transient expression already does much of the heavy lifting when it comes to increasing the overall expression of the protein in question if the system is optimized (Diamos & Mason, 2018a). However, this expression may be increased in various ways. One of the most important factors in the expression of proteins in any system is the expression machinery utilized. In plant biotechnology viral replication vectors are utilized. There are several viral-based vectors that may be used, the vector utilized for all experiments discussed herein are geminiviral expression vectors based on the bean

yellow dwarf virus (BeYDV), however vectors based on tobacco mosaic virus (TMV) and cowpea mosaic virus (CPMV) have also been highly popular for facilitation the plant-based expression of proteins.

Geminiviruses are single-strand DNA viruses that innately possess a small genome which can replicate in a highly extensive range of host plant species and are highly infectious due to the replication of these genomes to large copy numbers (Bhattacharjee & Hallan, 2022). The bean yellow dwarf virus utilized as the basis for the Geminiviral vectors herein infects the nucleus of plant cells, using the DNA synthesis machinery and the virally encoded replication initiator protein (Rep) to begin viral genome synthesis (Q. Chen et al., 2011). The expression vectors utilized herein, like most used in modern plant biotechnology, are deconstructed meaning they lack the majority of redundant plant virus proteins such as the coat protein (Q. Chen et al., 2011). Geminiviruses include a TAATATTAC sequence on a portion of the genome known as the long intergenic region (LIR) which initiates rolling circle replication, the process by which geminiviral vectors replicate (Q. Yang et al., 2017). The ability for geminiviruses to replicate makes them a powerful tool for plant biotechnology as a small amount of genomic material may be introduced into the plant cells resulting in a high number of expression vectors which in turn result in large amounts of protein. Benefits of this system include lower amounts of agrobacterium needed for infection of all plant cells, high amounts of expression vector mRNA in plant cells, potential for high amounts of protein expression, the potential ability for geminiviral vectors to be highly effective in a broad range of plant species, and the ability to produce several proteins in a single plant cell as they are included on the

same expression vector. Potential drawbacks include use only in transient rather than transgenic expression, potential limitations of genome size, and potential mutation of genetic sequences being replicated (Abrahamian et al., 2020; Bhattacharjee & Hallan, 2022; Q. Yang et al., 2017).

Tobacco mosaic virus (TMV) is a single-stranded RNA virus and has been widely utilized in the production of proteins in the N. *benthamiana* expression system. TMV expression vectors are divided into two modules, one containing the necessary genes for replication and the other containing genes of interest to be expressed. This allows for the expression of a large number of gene replicates. The MagnICON™ system, designed by Icon Genetics, allows for the infiltration of agrobacterium containing one of the two TMV expression cassettes into the intercellular space of leaves resulting in faster infection and expression compared to other systems at the time (Hefferon, 2012). An addition to this system was movement protein (MP) which allows for cell-to-cell movement of the expression vector from one plant cell to another increasing the infectivity rate of TMV vectors (Gleba et al., 2004). Benefits of the TMV system, namely the MagnICON™ system, include the ability to infect the majority of plant cells easily, have high expression of RNAs encoding a protein of interest, and potential for high production of proteins of interest. Drawbacks to this system are its need for separate non-competing replicons for co-expression of two proteins - such as antibodies - are to be produced, and the TMV system may not be able to express large proteins similarly to the geminiviral system. However, the major drawback of the TMV based expression vectors are the fact that vectors may be competing. For this reason some groups utilized both

TMV and other RNA virus expression vectors to produce complex proteins such as monoclonal antibodies (Q. Chen et al., 2011; Sainsbury et al., 2010). TMV is one of many RNA viruses that have been developed, others of which include those based on the potato virus X (PVX), cowpea mosaic virus (CPMV) and alfalfa mosaic virus (AIMV). However, these systems have the same drawbacks as TMV based expression vectors without many of the benefits due to the development of the MagnICON™ system.

In addition to the expression vectors which have been discussed there are also non-replicative systems such as one based on the Cowpea mosaic virus (CPMV) (Sainsbury et al., 2010). These systems are often able to reach similar expression levels of protein as their replicating counterparts, however, the amount of agrobacterium utilized is quite high, approximately 1.2 - as measured by OD_{600} - compared to approximately 0.3 as required by replicating vectors (Diamos & Mason, 2019; Sainsbury et al., 2009).

Another major way is the inclusion of various promoters, 5' untranslated DNA elements, 3' untranslated DNA elements, and performing various additional and less sophisticated methods such as incubating a N. benthamiana plant that has been infiltrated at 37C to increase the production of chaperone proteins due to heat shock response (Diamos et al., 2016; Diamos & Mason, 2018a; Norkunas et al., 2018; Rosenthal et al., 2018). While these methods have increased yields of various proteins there are still many additional factors that limit protein production. Namely optimal extraction methods. While it is not discussed herein, an additional consideration is the protein design itself, as some proteins are intrinsically less stable and more insoluble than others. An example of the latter is

full length spider silk proteins as the protein is natively insoluble and hydrophobic (Lentz et al., 2022).

Another issue of merit is the fact that proteins with various subunits or disulfide bonds may not fold correctly, this is doubly true if the protein is extracted under harsh conditions that may break the bonds keeping subunits/disulfides together (Ban et al., 2020). In fact, disulfides may become bound under incorrect pairing conditions resulting in insolubility of protein (Ban et al., 2020). Subunits can experience similar issues, if they are extracted under harsh conditions they may not be able to reform in their native conformations (Nedergaard Pedersen et al., 2020). Intense stress, such as centrifugation or protein concentration may result in protein conformation issues as well, such as the shearing of proteins (Maa & Hsu, 1996).

Plant protein extraction, in general, is a highly diverse field. Many approaches may be taken to extract naturally occurring plant proteins including use of alcohol, detergents, acids, alkalines, and salts in aqueous extraction methods (Kumar et al., 2021). Enzyme based extraction, microwave extraction, ultrasound/mechanical extraction, and high pressure water extraction may also be utilized (Kumar et al., 2021). In the extraction of biologics from transient plant expression systems it is typical to utilize acids, alkalines, salts, and mechanical extraction techniques to isolate the target protein (Kamzina et al., 2021; Schillberg & Finnern, 2021).

In brief, alkaline additives disrupt cystine (disulfide) bonds increasing solubility of many proteins, however this treatment risks damage to proteins with extensive cystine bonds,

such as antibodies (Ban et al., 2020; Kamzina et al., 2021; Kumar et al., 2021). High temperatures may also be used to increase solubility of proteins, however if proteins are not heat stable they are at risk for becoming improperly folded and degraded (Kumar et al., 2021). Acids can be utilized to make non-target proteins insoluble if the target protein is unaffected. Insolubility in this case occurs due to the deionization of amino acids under acidic conditions (Dewitt et al., 2002). Detergents are utilized at times for the extraction of proteins as the detergent degrades cellular membranes, releasing membrane bound proteins and disrupting protein-protein interactions (Kamzina et al., 2021; Kumar et al., 2021). By far the most common additive to any extraction protocol for plants transiently expressing protein are salts. Salts increase solubility of proteins by preventing non-specific binding of proteins to impurities or other proteins released during the extraction process. All of these previously mentioned techniques are combined with ultrasound/mechanical extraction techniques that tear the plant material apart, allowing for more efficient access of the extraction buffer components to the endogenous plant proteins and protein(s) of interest (Kumar et al., 2021).

Generally, difficulties in the extraction of a target protein can be overcome by modulating the extraction buffer used, temperature the extraction occurs at, and any additional steps, such as acid or base precipitation of non-target proteins. For highly insoluble proteins, such as recombinant spider silk, highly solubilizing extraction solutions may be used to remove a majority of the soluble plant proteins before harsher extraction methods are applied to the remainder of the non-soluble fraction to ultimately result in a soluble target protein that may then be purified. As each protein will have a different optimized

extraction process there can be an extensive learning curve associated with this portion of the production process.

A Brief History of Vaccines: From Smallpox to Modern Platforms

There are many types of vaccines both commercially available and in consistent development around the world. However the first vaccines to be produced were live attenuated vaccines, meaning whole pathogens were taken and, through various processes, their infectivity was reduced. The first live attenuated vaccine was against smallpox, invented in 1798, followed by a vaccine for Rabies in the year 1885 nearly a century later (Lombard et al., 2007). After the rabies vaccine was made, another approach to creating vaccines was utilized to make less dangerous vaccines that could not infect or cause extensive disease in the patient receiving them. These vaccines are known as inactivated vaccines and include both a Typhoid and Cholera vaccine produced in 1896 (S. Plotkin, 2014)**.** It wasn't until the 1920s that the third type of vaccine was created, toxoid vaccines, with Diphtheria toxoid and Tetanus toxoid vaccines being produced in 1923 and 1926 respectively. These vaccines marked the first use of an individual protein to create a protective immune response in patients and were a boon as they were much safer than other approaches to vaccination which could in and of themselves cause disease (Minor, 2015)**.**

As a general trend, vaccines have become increasingly safe in their design. Where a live attenuated vaccine had the potential to cause disease in some individuals, toxoid vaccines (falling under the subunit vaccine category) may cause symptomatology associated with

the toxin used but cannot cause systemic disease (Lombard et al., 2007). A push for safe vaccines has led to many current vaccines utilizing extensive genetic engineering. However, genetically engineered vaccines may fall under most of the major vaccine categories put forward by the Centers for Disease Control and World Health Organization which include live attenuated vaccines, inactivated vaccines, toxoid vaccines, subunit, recombinant, polysaccharide, and conjugate vaccines, as well as mRNA vaccines and viral vector vaccines. The majority of modern vaccines are recombinant subunit vaccines, recombinant conjugate vaccines, polysaccharide vaccines, mRNA vaccines, and viral vectored vaccines (Excler et al., 2021; Meissner, 2022). As mRNA and viral vector vaccines simply produce/introduce a protein of interest in a unique way to the immune system their final product can theoretically fall under the subunit, conjugate, and/or polysaccharide vaccine categories which are discussed herein.

It wasn't until 1986 that the first genetically engineered vaccine, and first virus like particle (VLP) vaccine was produced. hepatitis B surface antigen was utilized to create a viral particle that was non-infectious and produced a robust immune response in individuals vaccinated with it (Zeltins, 2013). This opened a floodgate of scientific investigation into many additional virus-like particle vaccines. For example, both the Human papillomavirus (HPV) and meningitis B VLP vaccines are currently approved (Aston-Deaville et al., 2020; Kim et al., 2022; Yousefi et al., 2022). Various virus-like particles including those made from noroviruses, bacteriophages, modified hepatitis B core and surface antigen VLPs, and many others that have been investigated by researchers for decades to either protect against an individual disease or to be used as a

platform base which may be used to prevent any disease or even act as drug carriers (Nooraei et al., 2021). Protein-protein conjugate vaccines and protein-polysaccharide vaccines have also become popular in research; a successful example of protein conjugate vaccines are the various Pneumococcal and Meningococcal vaccines on the market, while these are not recombinant they are highly successful examples of conjugate vaccines (Rappuoli et al., 2019). An example of a protein-polysaccharide conjugate vaccine is the Multiple Antigen Presenting System (MAPS) platform developed by Affinavax and sold, via whole company acquisition, to GSK undergoing Phase 2 clinical trials (Cieslewicz et al., 2022). There are many novel platforms for vaccine development currently under investigation both at universities, large pharmaceutical companies, and small R&D companies. Two highly promising examples of novel technologies studied extensively at Arizona State University are the Recombinant Immune Complex (RIC) platform and the recombinant hepatitis B Core antigen (HBc) VLP platform (Aston-Deaville et al., 2020; Diamos et al., 2019).

The RIC platform consists of an antibody-antigen fusion that, through antibody-epitope interactions to an epitope tag on the antigen portion of the fusion, leads to the formation of immune complexes (Diamos et al., 2023; Mason, 2016). Immune complexes are naturally forming during the course of infection and are a primary stimulator of both innate and adaptive immune responses leading to protection. There are many applications of RICs and, while they're likely never going to reach the multi-valency levels of the MAPS platform, are highly stable and can potentially provide protection from disease in a single dose (Diamos et al., 2023; Diamos, Pardhe, et al., 2020).

The HBc VLP platform consists of two hepatitis core antigens connected via a short peptide linker. One antigen has no insertion into its loop region while the other allows for antigen insertion into this loop region resulting in highly stable particles even when large antigen insertions are utilized (Diamos et al., 2019; Z. Huang et al., 2008; Peyret et al., 2015). While it is not multivalent the HBc VLP is extremely optimized resulting in high expression/purification levels with ease, simple editing, and a consistent performance across various diseases.

While there are many other vaccine platforms that could be discussed, these three have been selected to showcase the various differentiators between potential approaches to vaccination and illustrate the fact that there is no one size fits all approach to vaccines even in the modern age.

Vaccines and Their Various Routes of Administration

In 1796, Edward Jenner discovered that infection of the cowpox virus, administered via a bifurcated needle scratching the skin, prevented smallpox infection (Lombard et al., 2007). This discovery led to the first ever modern vaccine effort in which many citizens of the newly United States of America and Europe lined up to be stuck by the cowpox inoculated needles (Mark & Rigau-Pérez, 2009). Many centuries before a much different technique called variolation, in which smallpox virulence is attenuated by leaving infected pox scabs out in the elements for a certain amount of time before being blown up the noses of individuals, was utilized in China to stem the spread of smallpox (Leung, 2011). Where Edward Jenner's technique has a very low chance of causing death,

variolation would generally result in a mortality rate of ~10% whereas smallpox itself would result in a mortality rate of 30% or more (Leung, 2011). It is not only the prevention of smallpox, but the safety of the vaccines administration, that has resulted in Edward Jenner becoming the forefather of vaccinology.

In the modern day there are many vaccines that are administered in a variety of ways. Often, the way a vaccine is administered can determine its performance as much as the formulation itself. There are five primary routes of administration for any given vaccine: subcutaneous (s.c.) administration where the vaccine is injected into the subcutaneous tissue below the dermis and above the muscle, intramuscular (i.m.) administration where the vaccine is injected into the muscle tissue, intradermal (i.d.) administration where the vaccine is injected between the epidermis and dermis (resulting in a bubble of vaccine liquid forming), oral administration, via the ingestion of a vaccine containing substance which is exposed to the mucosal membranes in the mouth and intestines, and intranasal vaccines (i.n.) which are also exposed to mucosal membranes via the nasal passages generally via a spray as is the case in the nasal flu vaccine (Yusuf & Kett, 2017; Zhang et al., 2015).

By far the most widespread route of administration for vaccines is the i.m. route (Griesenauer & Kinch, 2017). This is due to several reasons one of which is the ease of administration by non-intensively trained personnel where as intradermal vaccine administration for example requires specialized training and personnel may become a bottleneck as was seen during the 2022/2023 MPox outbreak as the Janos smallpox

vaccine used to prevent MPox required i.d. administration to extend the vaccine supply (Roper et al., 2023).

Intramuscular administration of vaccines has many other benefits beyond its ease of use, these include rapid and uniform absorption of the vaccine, rapid onset of action, modified absorbance via an adjuvant such as Alum can provide slow, sustained release of the vaccine, abundance of antigen presenting cells (APCs) in muscle tissue, and decreased chance of severe reaction due to the aforementioned rapid and uniform absorption/uptake even in cases where adjuvants like Alum are utilized (Zuckerman, 2000).

The benefits of intradermal administration of a vaccine are many but revolve around the increased immunogenicity gained via i.d. administration due to uptake of the vaccine by the resident dendritic cells in the skin. This uptake and presentation pathway has allowed for lower doses of vaccines to be utilized than the i.m. route while resulting in the same overall immunogenicity outcomes (Whitaker et al., 2019). Improved efficacy results in lower dosages being necessary and can reduce the chance for vaccine shortages. It is also important to note that the i.d. route has a similar safety profile to i.m. administration due to the rapid uptake of the vaccine. However, a downside to i.d. administration is the fact that a specifically trained professional must be on site to make sure the vaccine is not accidentally administered subcutaneously which can result in subpar immune responses especially in immunocompromised individuals (Zuckerman, 2000).

The benefits of subcutaneous administration of vaccines are few and revolve around the fact that there is less vasculature in the subcutaneous fat tissue as compared to the muscle

or skin. This means any vaccine administered will be slowly, but consistently, delivered to the immune system which has been found to be appropriate for some live attenuated vaccines. However, this route of administration is not appropriate for many vaccines developed today as there is a subdued immune response compared to i.m. and i.d. routes of administration (Zuckerman, 2000).

The benefits of oral vaccine administration are many. Oral vaccines can be easier to manufacture, such as growing the vaccine in an edible plant for humans or livestock, easy to administer via the eating or spraying of minimally processed or purified vaccine respectively upon foodstuffs, and finally oral vaccines can potentially activate the mucosal immune cells that are not present elsewhere in the body stimulating IgA production, the immunoglobulin which is the first line of defense in the mucosa (Vela Ramirez et al., 2017). As many diseases infect first through the mucosa, a vaccine administered to preferentially prime those sites for defense is a potential major benefit. However, survival of the vaccine material through the stomach and gut microbiota pose a potential hurdle to vaccines administered in this way (Stafford et al., 2020). Another hurdle is the fact that dosing may be difficult to control for edible vaccines, and thus validated processing methods that insure stability of antigen and dosing are needed.

Finally vaccines may be administered intranasally, which retains many of the benefits of oral vaccination without the major hurdle of the material needing to survive the stomach and microbiota of the digestive system (Chavda et al., 2021). However, intranasal administration requires purified material unlike some oral vaccine approaches and therefore has a similar manufacturing cost to vaccines made for other routes of

administration. This means intranasal vaccines are created on a cost benefit analysis based mostly around the preference of individuals, mostly children, to get vaccinated without a needle stick.

Ultimately, vaccine administration route should be determined by rigorous scientific inquiry, however more research into alternate routes of administration - namely oral and intranasal - could greatly benefit global health over time.

CHAPTER 2

PRODUCTION AND PURIFICATION OF RECOMBINANT SPIDER SILK IN A PLANT EXPRESSION SYSTEM.

Introduction

Spider silk has three primary sector applications: 1) in biotechnology for the encapsulation and delivery of biologics via micellar-like structures or other micro-structures formed by recombinant spider silk proteins (Bakhshandeh et al., 2021), 2) in biomedical engineering as either a tissue replacement or coating to reduce microbial growth (Abraham et al., 2020; Bakhshandeh et al., 2021; Salehi et al., 2020), or 3) in large scale industrial applications where spider silk could be utilized as an extremely high-tensile strength fiber weave (Ramezaniaghdam et al., 2022). All three of these primary applications, while they're widely different, have similar bottlenecks. The most pervasive issue limiting the application of spider silk to multiple long-standing problems is a lack of affordable, high throughput, production capacity. In nature, it would require over a million spiders and 70 human workers to create a 3.4m textile from dragline silk at a cost of ~500,000 USD (Cuppoletti, 2011). While there is a possibility for recombinant spider silk production to be much more affordable in current expression systems including bacteria, yeast, plants, insect cells, and mammalian cells there has yet to be a major breakthrough in production capacity (Gomes & Salgueiro, 2022). This constraint remains due to three primary factors: 1) spider silk proteins are very large, limiting full protein translation/transcription in bacterial systems and reducing production capacity in higher order expression systems, 2) if expression of the protein is high, spider silk by nature is insoluble leading to issues extracting and purifying the final product and 3) any

spider silk produced, most likely recombinant, will require doping of the silk fiber which may not be easy, efficient, or lead to a fiber with the same properties as natural spider silks (Ramezaniaghdam et al., 2022).

In brief, doping of the spider silk occurs through the formation of micellar structures of the spider silk protein in the ampulla of the spider followed by a pulling or doping of the silk by the spider via its hind legs (Gomes & Salgueiro, 2022; Lentz et al., 2022). The micellar structures undergo a pH and ion change along a gradient as they're doped resulting in the proper formation of beta sheets and other secondary protein structures to create the mechanical properties of the highly desirable material. This effect has been replicated through the invention of mechanical doping systems that process soluble recombinant spider silk protein in a way analogous to the natural doping mechanisms the silk undergoes in spiders (Arndt et al., 2021).

The first step in addressing these bottlenecks is designing a spider silk protein that will be easy to express in a system, be easy to purify, and ultimately lead to a fiber which has similar strength profiles to the spider silk it is attempting to emulate whether it be dragline silk or another silk type. There are seven types of spider silk; dragline silk, minor ampullate silk, flagelliform silk, aggregate silk, pyriform silk, aciniform silk, and tubuliform silk. All of these silks have different properties associated with them. However, dragline silk is the most commonly studied as it has the most desirable material properties for a wide range of applications encompassing biotechnology, biomedical engineering, industrial, and military applications (Bittencourt et al., 2022; Cuppoletti, 2011).

Dragline silk is composed of two major proteins, major ampullate spidroin 1 (MaSp1) and major ampullate spidroin 2 (MaSp2) which are each composed of highly similar repeats in their long central regions (typically of GGX, GPGXX, PolyA and PolyAG motifs) which make up 90% of the protein, with non-repetitive amino acids making up both their N-terminal and C-terminal domains (Whittall et al., 2021). In natural silks, the MaSp1/MaSp2 proteins will interact to form fibers, however there has been a focus on the production of MaSp1 primarily in recombinant spider silk production. It is thought that MaSp2 increases extensibility of the fiber if incorporated which could explain why many different species of spiders have varying ratios of MaSp1 to MaSp2 in their silks (Malay et al., 2022). Of particular interest, there is a growing number of studies that have introduced a third subset, major ampullate spidroin 3 (MaSp3) to the mix (Malay et al., 2022). In fact, a study of *Trichonephila clavipes* resulted in the discovery of 8 distinct MaSp subtypes in its silk (Babb et al., 2017).

Genomic study of spider silk is highly complex and there is likely going to be an increased scope of understanding on the variations within silk proteins as research moves forward. However, focusing on the MaSp1 protein is still often done in modern recombinant spider silk production experiments (Babb et al., 2017; Li et al., 2022). In fact, it was not until recently that the importance of C-terminal and N-terminal elements of the spider silk proteins were known and incorporated into recombinant spider silk production efforts (Li et al., 2022; Rising et al., 2006; Xu et al., 2017). Today it is fairly well established that there are three things that increase a recombinant silk fibers

strength: 1) number of spider silk core protein consensus repeats utilized, 2) inclusion of the C-terminal domain, 3) inclusion of the N-terminal domain.

While major work is being pursued to further unravel the genetic nature of various spiders and their silks a simple approach was taken herein to test the expression potential and purification efficiency of a recombinant spider silk MaSp1 protein based on the commonly studied *Nephila clavipes* MaSp1 protein component of dragline silk which included the N-terminal and C-terminal domains of the native spider silk.

Methods

Vector Construction: The construction of plasmids pBYKEAM-BA6H-SS3X, utilized in the expression of spider silk in N. benthamiana, discussed herein (**Figure 1 and 2)** was cloned into plasmid pBYKEAM-BASP-6D8 (obtained from A. Diamos) by XbaI and SacI restriction digestion (RD) of the whole spider silk gene outlined in Figure 2 which contained XbaI and SacI sites at the N-terminus and C-terminus respectively followed by ligation of the resulting plasmid backbone fragment and whole spider silk gene restriction digest product. Spider silk repeats were increased to 3X via restriction digestion of the pBYKEAM-BA6H-SS1X plasmid and SS2X gene block with BsaI. BsaI digested products were ligated together resulting in plasmid pBYKEAM-BA6H-SS3X which was screened via restriction digestion and PCR to determine a successful clone. pBYKEAM-BA6H-SS3X was digested with XbaI and SacI restriction enzymes as was pBYKEAM-6D8 and pBYKEAM-TP-6D8 (containing the rbcS chloroplast transit peptide coding sequence). Backbone fragments from pBYKEAM-6D8 and pBYKEAM-TP-6D8 were ligated with the SS3X insert from pBYKEAM-BA6H-SS3X before being screened via restriction digestion and PCR to determine a successful clone. The pBYKEAM-BA6H-SS3X plasmid was utilize to create the PBYKEAM-BA6H-SS3Xdel via PCR site directed mutagenesis of the cysteine amino acid utilizing primers 5'-agcggagacgtg-3' and 5'-tcgcctctgcac -3' as previously described (Heydenreich et al., 2020).

Protein Production, Extraction, and Purification: *Agrobacterium tumefaciens* strain EHA105 was transfected with expression vectors via electroporation. Resulting transformed *Agrobacterium* strains were confirmed using PCR. PCR confirmed

Agrobacterium strains were grown overnight at 30°C in YENB media + 50 mg/L kanamycin and 2.5 mg/L rifampicin. *Agrobacterium* cells were pelleted for 10 minutes at 4,500 g. Pellets were resuspended in infiltration buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO4) to a final OD600 of 0.25-0.35. *Agrobacterium* suspensions were infiltrated using a syringe without needle into the leaves of 5 week old wild type (WT) N. benthamiana or glycoengineered (GnGn) N. benthamiana silenced for production of the plant-specific β1,2-linked xylose and α1,3-linked fucose (Castilho & Steinkellner, 2012). Infiltrated leaves were harvested 4 days post infiltration (DPI).

0.1g leaf tissue samples expressing ER targeted, chloroplast targeted, or cytosol targeted recombinant spider silk proteins were homogenized in 1:3 w/v extraction buffer (25 mM Tris-HCL, 125 mM NaCl, 3 mM EDTA, 8.0M urea, pH 8.2 with 50 mM sodium ascorbate, and 2 mM phenylmethylsulfonyl fluoride (PMSF) added before extraction). 12–14 ZnO beads, 2.0 mm (Fisher Scientific, Waltham, MA, United States), were added to 1.5 mL tubes containing leaf samples and extraction buffer. The tubes were bead beaten using a Bullet Blender machine (Next Advance, Averill Park, NY, United States) for two 5-min rounds with tubes being inverted twice between rounds. Homogenized leaf tissue was then rotated at 4°C for 20 minutes before samples were centrifuged at 13,000 g for 10 minutes at 4°C in a 5417R centrifuge (Eppendorf, Hauppauge, NY, United States). Once centrifuged the supernatant was transferred to a new 1.5mL tube. The samples were centrifuged again at 13,000 g for 5 min at 4°C to obtain a clarified extract free of major plant contaminants ready for use in SDS-PAGE and Western Blot analysis.

Four mL of Talon Complete metal affinity resin (Clontech/Takara Bio, Mountain View, CA, USA) was added to a metal affinity chromatography column with a total volume of 30 mL. The ethanol storage solution was allowed to flow out of the column resulting in 2 mL resin bed volume which was washed using 15 mL of water followed by an equilibration step with 15 mL of extraction buffer. A final wash of 30 mL of extraction buffer was performed before the filtered extract was passed through the column. Resin was washed with 60 mL of PBS buffer at pH 7.4. Elution of bound proteins was achieved using PBS + 150 mM imidazole at pH 7.4. Elution samples were dialyzed overnight in one liter of sterile PBS using a three mL 3,500 MWCO Slide-A-Lyzer G2 dialysis cassette (Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer's instructions. Elution concentrations were determined both before and after dialysis by an A280 absorption using PBS + 150 mM imidazole and PBS, respectively, as blank solutions.

SDS-PAGE and Western Blot Analysis: Clarified extracts were mixed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue, 0.5 M DTT) under reducing conditions, or non-reducing conditions with 0.5M DTT omitted. Samples were boiled for 10 minutes after the addition of sample buffer. Samples were separated on stain-free 4–15% polyacrylamide gels (Bio-Rad, Hercules, CA) then analyzed using Coomassie stain (Bio-Rad, Hercules, CA, United States) and Western blot.

Samples were electroblot transferred to PVDF membranes. PVDF membranes were blocked with 5% dry milk in PBST (PBS with 0.05% Tween-20) at 37°C for 1 h and

probed with polyclonal rabbit-anti Histidine 1:5000 in 1% PBSTM followed by goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (Sigma) diluted 1:5000 in 1% PBSTM as previously described (Diamos, Hunter, et al., 2020). Bound antibody was detected with luminol reagent (Santa Cruz Biotechnologies, Santa Cruz, CA).

Coomassie and Western blot samples were further analyzed to determine the expression levels of spider silk constructs in both WT and GnGn plants by utilizing the ImageJ application. ImageJ allows for the determination of a protein bands intensity by outlining the band. This relative intensity can be utilized to compare one band on the same gel to another. Coomassie gels were utilized to determine the overall protein expression by determining band intensity for all protein bands present in a sample. These were utilized to set a total protein standard for the same samples examined under Western blot procedures. Band intensity of spider silk protein as determined by anti-His antibody binding was divided by total protein band intensity to determine a relative expression amount compared to total protein. These standardized samples were then compared. In these comparison studies three pooled leaf samples were processed using this approach.

Results

Recombinant spider silk constructs were designed to contain the N-terminal region of the *N. clavipes* MaSp1 spider silk protein linked to three consensus repeats of the primary structural MaSp1 dragline silk protein from the same spider followed by linkage to the C-terminal region of the same MaSp1 silk protein **(Figure 1)**. This protein was tagged with 6-histidines at the N-terminal end of the recombinant spider silk protein and was targeted either to the endoplasmic reticulum (ER), the chloroplast stroma, or the cytosol. One of the two ER targeted constructs had a deletion of the only cysteine present in the C-terminal region of the construct **(Figure 2)**. Of note, the N-terminal and C-terminal domains of the recombinant spider silk proteins studied include more amino-acids than the triple repeat of spider silk consensus sequences utilized. Figure 2 shows the amino acid sequences of each section of the recombinant spider silk protein and highlights the cysteine bond that was removed in construct #2 in Figure 1. The four constructs created were tested for expression in *N. benthamiana* plants of which two varieties were used. First the wild type (WT) *N. benthamiana* was utilized followed by expression studies in the xylose and fucose glycosylation pathway silenced GnGn plant variants.

Figure 3 shows the results of a metal affinity column chromatography purification of cytosolically targeted recombinant spider silk proteins expressed in WT *N. benthamiana* leaves which were extracted utilizing standard salt extraction buffer with the addition of 8.0M urea diluted to 4.0M urea before addition to the metal affinity column. A plcC-NetB toxoid fusion protein with the same 6-histidine tag with known concentration was used as a standard to compare concentrations of eluted recombinant spider silk proteins.

The flow through and wash show no evidence of protein loss during the purification process. Elutions 2 through 6 show purified recombinant spider silk protein at the expected band size of ~36 kDa, however the reducing conditions show his-tagged protein products below this expected size. However, the Elution 3 sample in non-reducing conditions shows no similar small protein bands, instead having higher kDa bands that correspond to the size of dimers of the recombinant spider silk (~71 kDa). Both reducing and non-reducing conditions show a slightly smaller than expected protein band.

Following successful expression and purification of the recombinant spider silk protein extensive expression studies were performed of all four constructs outlined in Figure 1. These constructs were either expressed in WT *N. benthamiana* or GnGn *N. benthamiana*. A large scale comparison is presented in Figure 4. All four constructs, when expressed in WT plants showed markedly increased expression rates as opposed to those expressed in GnGn plants. Of additional interest, the ER targeted construct containing a cysteine deletion performed worse in expression studies than the ER targeted construct without the cysteine deletion indicating a potential stabilizing effect of this cysteine. While GnGn plants performed poorly compared to WT plants, both the cytosol targeted and chloroplast targeted constructs showed similar expression levels in WT plants.

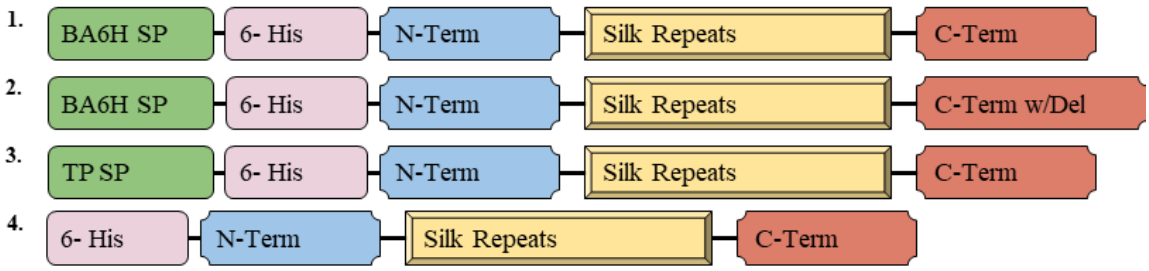


Figure 1. Construct Designs to Test Elements of Spider Silk Strength. BA6H-SP = Endoplasmic Reticulum (ER) signal peptide. TP SP = Chloroplast transit peptide. 6-His = 6-Histidine Tag. N-Term = N-Terminal element of dragline spider silk. Silk Repeats = three repeats of consensus spider silk core protein amino acid sequence based on N. clavipes MaSp1. C-Term = C-Terminal element of dragline spider silk. Construct 2 has a cystine deletion in the C-Terminal element theorized to increase solubility of the protein. Construct 4 has no signal peptide meaning it will be secreted into the cytosolic space of the plant cells. All constructs were testing for expression in both wild type and GnGn N. benthamiana plant expression systems.

Figure 2. Spider Silk Construct Base Elements with Associated Amino Acid and DNA Sequences Resulting in an Approximately 36 kDa Recombianant Spider Silk Protein. The N-Terminal element of the recombianant spider silk protein is based on the *N. clavipes* MaSp1 N-terminal domain. The Silk Repeats are based on a consensus sequence of the *N. clavipes* MaSp1 protein. Three repeats were chosen due to their average size. The C-Terminal element of the recombinant spider silk protein is based on the *N. clavipes* MaSp1 C-Terminal domain. The cystine present is highlighted and deleted in construct 2 presented in Figure 1.

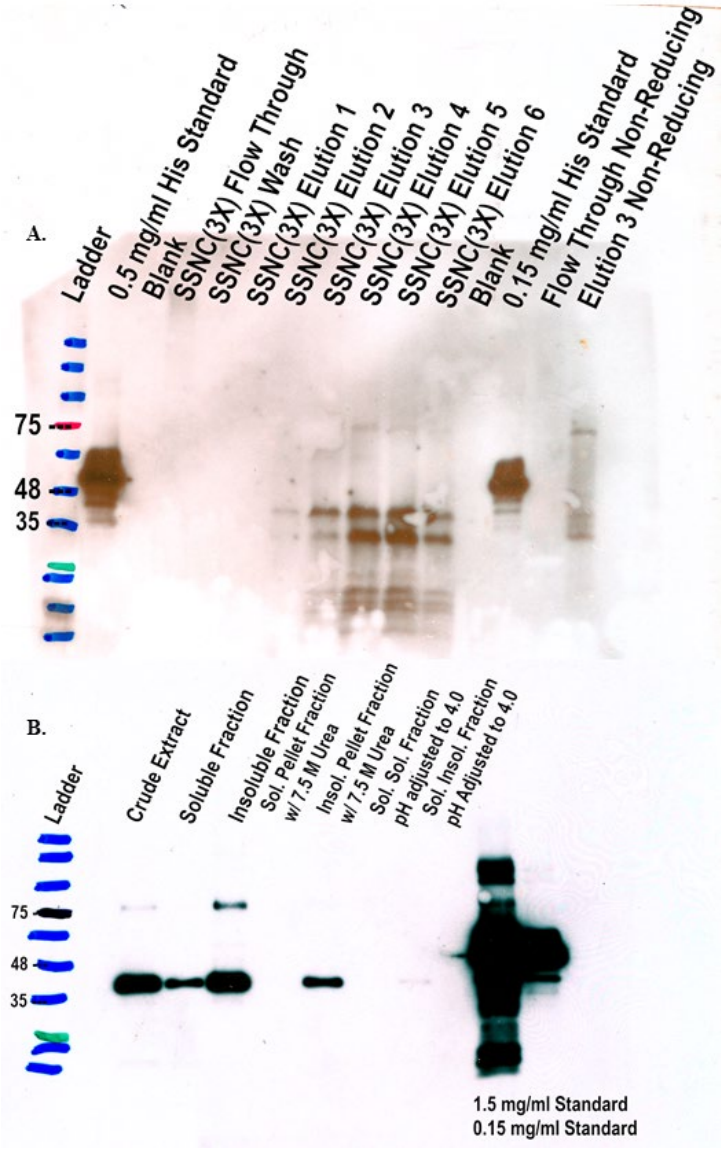


Figure 3. Expression and Purification of Recombinant Spider Silk from Wild Type *N. benthamian*. A. The recombinant spider silk protein was probed by mouse-anti-His antibody followed by secondary probe with donkey-anti-mouse HRP conjugate antibody. His-tagged protein can be seen in only the elutions, which follow a standard elution curve, and are generally at the expected size of the whole recombinant spider silk protein, 36kDa. Degradation products can be seen which retain the His-tag and were therefore purified. A non-reducing protein sample of elution 3 shows no degradation. B. Three 0.1g infiltrated leaf tissue samples were used for each extraction experiment. Crude extract, which includes soluble and insoluble material showed expression of recombinant spider silk at approximately 500µg/mL when 0.1g of leaf tissue was extracted in approximately 500µl of extraction buffer. The soluble fraction showed low extraction efficiency and the insoluble fraction retained the majority of recombinant spider silk protein. The insoluble fraction, when re-extracted with 7.5M urea showed increased solubility.

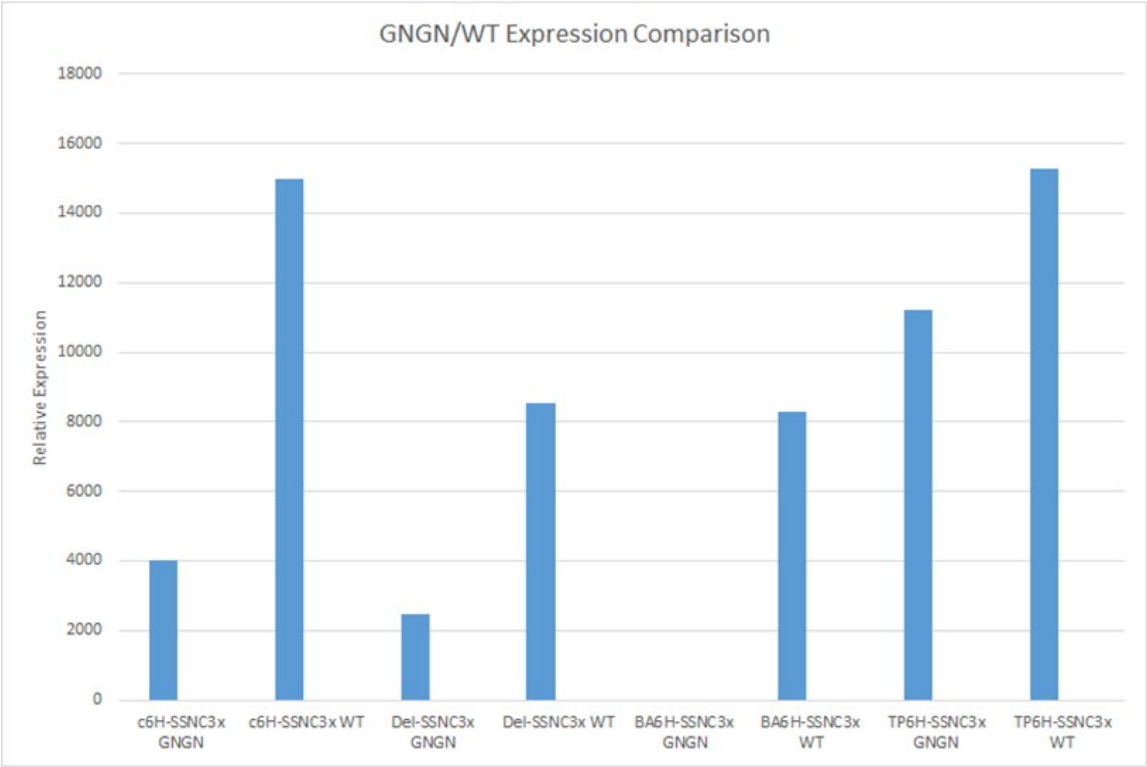


Figure 4. Expression Comparison of All Recombinant Spider Silk Constructs in WT and GnGn Plants. The recombinant spider silk protein was produced via expression of the four constructs outlined in Figure 1 in either Wild Type (WT) *N. benthamiana* plants or fucose and xylose glycosylation pathway silenced (GnGn) *N. benthamiana* plants. Expression values were determined by Western blot of pooled samples from three different leaves. Both chloroplast (TP6H) and cytosolic (c6H) targeted protein outperformed both ER targeted (BA6H) and C-terminal cysteine deletion (Del) constructs. Wild Type expression was higher than GnGn on average in for all constructs.

Discussion

Spider silk is one of the most sought-after biomaterials in the world much like silk was for over a millennia (Kluge et al., 2008). For this reason, many groups have devoted a wealth of resources to both the study of spider silk protein on a structural level and the production of such a protein on an industrial scale for more than twenty years (Heslot, 1998). Recombinant spider silk production fits into the niche of aiming to produce structurally similar or improved spider silk proteins while allowing for large scale protein production. Thus far however, there have been three major hurdles to successfully producing recombinant spider silk on an industrial scale: 1) producing full sized spider silk, or recombinant silk with the same structural properties as full sized spider silk, 2) maintaining a high expression level of the spider silk, recombinant or native, in any system of expression, 3) extracting and purifying the spider silk from any endogenous proteins and doping it into a functional fiber (Ramezaniaghdam et al., 2022).

In our studies we aimed to produce a small, recombinant spider silk protein that could potentially be expressed at high levels in a plant expression system, N. *benthamiana*. Previously, expression levels reaching 5mg/g protein to leaf weight have been shown for green fluorescent protein with more complex proteins such as antibodies reaching expression levels of ~1.5 mg/g protein to leaf weight (Diamos, Hunter, et al., 2020; Diamos & Mason, 2018a). Therefore, the N. *benthamiana* system was selected as an expression system with a high potential of success in producing the complex spider silk proteins especially as plant systems, namely tobacco which is utilized herein, have

previously been utilized in the production of recombinant spider silk (Heppner et al., 2016; Menassa et al., 2004; Scheller et al., 2001).

Figure 1 shows the various constructs expressed in the *N. benthaminana* plant expression system. Targeting to the various subprocessing compartments of the plant cell including the endoplasmic reticulum and chloroplast stroma, and the cytosol, were studied because the recombinant spider silk proteins are created in a highly specific environment within the spider's abdomen. All constructs have an N-terminal 6-His tag which allows for purification of the recombinant spider silk via metal affinity column chromatography (Rodriguez et al., 2020). Three repeats of a *N. clavipes* dragline silk consensus sequence were used, since the number of repeats increased the strength of fibers in other studies; in addition, an N-terminal and C-terminal end to the recombinant spider silk were added from the same spider species in their full native length as previous studies have shown that the inclusion of these regions allows for stronger spider silk fibers (Malay et al., 2022; Rising et al., 2006; Xu et al., 2017). In addition to the other constructs listed in Figure 1, there is an ER targeted construct with a deletion of the single cysteine within the C-terminal region of the recombinant spider silk protein. Figure 2 shows the amino acid sequences utilized from N. *clavipes* with the cysteine deletion in the C-terminal element highlighted.

Most proteins produced in the *N. benthamiana* plant expression system are biologics and are targeted to the ER. However, many other proteins are targeted to the cytosol to allow for the highest levels of expression such as fluorescent proteins for expression studies (Diamos et al., 2016). For this reason the cytosolically targeted recombinant spider silk

construct was utilized first in expression studies followed by large scale purification. Wild Type *N. benthamiana* plants are generally used for the expression of non-biologic proteins and were therefore utilized before other strains. A genetically modified N. *benthamiana* called GnGn with the fucose and xylose glycosylation pathways silenced is generally used for biologic protein production because these glycans are plant-specific and could potentially induce an immune response to the glycans. For the purification study presented in Figure 3 WT *N. benthamiana* plants were utilized.

Extraction of the recombinant spider silk protein was difficult due to the extreme insolubility of the protein. 8.0-8.5 M urea was used to extract and solubilize the protein which could be made soluble in any large quantity only under these extreme extraction conditions. The resulting extract was passed through a metal affinity chromatography column and the flow through and wash fractions captured all recombinant spider silk that was passed through the column indicating a highly effective protein capture protocol **(Fig. 3)**. These proteins were then eluted and had an end concentration of approximately 0.1 mg/ml. The total amount purified compared to total leaf weight utilized resulted in ~500µg/g protein to leaf tissue. While this is not an extremely low amount, and the purification was highly efficient, the amount of loss to insolubility of the protein.

Of note in Figure 3, the elution lanes, and all others except the elution 3 non-reducing lane, utilized DTT in the reducing buffer and, as a result, show various degradation products that are much smaller than expected for the recombinant spider silk expressed. Of additional note, elution 3 non-reducing shows a higher order band that is not seen in

the other elution lanes indicating a potential dimer of the spider silk protein being formed.

The successful purification demonstrated in Figure 3, though there is some degradation of the recombinant spider silk protein at least under reducing/boiling conditions, shows a pathway forward for large scale production and purification. This will require expression levels of the recombinant spider silk to be optimized and the extraction improved to solubilize the majority of insoluble material.

Figure 4 shows expression of all four constructs outlined in Figure 1 in both WT and GnGn *N. benthamiana* plants normalized utilizing the concentration of the abundant leaf protein rubisco as a measure of total protein concentration in each sample. In all cases WT plants out performed GnGn plants in expression levels with cytosolic targeted recombinant spider silk performing as well as chloroplast targeted spider silk. It was originally hypothesized that the chloroplast targeted protein may perform better as it is in a tightly compact cellular compartment whereas the cytosolically targeted protein was expected to potentially express at a high rate but be toxic to the plant cells leading to necrosis, however necrosis of plant leaves was observed at low levels in all constructs except ER targeted constructs where medium necrosis was observed at 4 days post infiltration (DPI). The observed necrosis as well as the low expression of ER targeted constructs in both WT and GnGn plants may indicate that glycosylation of the spider silk protein leads to an interaction which either reduces overall expression or increases protein degradation/insolubility, which would be in line with the increased observed necrosis of leaf tissue. Of interest is the fact that, even in the constructs targeted to the

cytosol which should not be glycosylated canonically, GnGn plants performed worse across all constructs when observing expression. There are several potential reasons for this. Firstly, there could be a native plant chaperone which is not properly glycosylated and, when it is properly glycosylated, leads to better assembly of recombinant spider silk. Secondly, there is the potential that an N-linked glycosylation site (NKS with N at aa78 in the provided sequence in Fig. 1) on the recombinant spider silk is still being glycosylated when not targeted to the ER, for which there are known mechanisms (West & Hart, 2017)**.** Lastly, GnGn plants could have dysregulated gene expression that is currently unknown, leading to lower expression of certain proteins that are generally not studied in plant expression systems. Of interest, the C-terminal cysteine deletion had no profound effect on expression, however, Ittah et. al. found that a conservative mutation to serine of this cysteine allows fiber formation yet a mutation to arginine prevents fiber formation (Ittah et al., 2007).

Moving forward, WT plants should be used as a basis to determine the expression of various recombinant spider silk conformations. While it was found that WT outperformed GnGn plants when expressing the constructs listed in Figure 1, it is possible that other recombinant spider silk proteins would either see no difference in expression or could potentially express better in another plant system altogether. However WT N. *benthamiana* plants would likely be the best starting point in any future studies.

Overall, recombinant spider silk was found to express fairly well, and be easily purified from soluble extracts using standard metal affinity column chromatography. Extraction of the insoluble recombinant spider silk proteins however remains the major hurdle for the

approach taken herein. While there are many different approaches and additives to protein extraction they are extensive and must be studied further to generate an extraction and purification pipeline that is mature enough for industrial applications. At this time, plant expression systems seem to be a viable option for expression of spider silk but the same hurdles that are seen in other systems still apply, namely the extraction of the majority of recombinant spider silk proteins from the plant tissue.

Further studies should be performed to determine the validity of the WT vs. GnGn expression data as there is only one potential N-linked glycosylation site present on the N-terminus of the recombinant spider silk protein investigated herein. In addition, increasing the size of the recombinant spider silk protein through addition of more repeats should be performed as the ability for plant expression systems to successfully produce large, complex proteins may make it an optimal and, perhaps more importantly, capital efficient system. Another major step that must be taken is the doping of the spider silk fiber, and while some groups claim to utilize seemingly easy techniques none of them were successfully applied to the purified recombinant spider silk proteins produced herein. Lastly, once doping of the spider silk fibers is performed, their structural mechanics must be tested.

While overall expression and purification levels of recombinant spider silk were low, the plant expression system utilized showed promise in its ability to produce complex spider silk proteins. While purification of the recombinant spider silk protein may not be commercially viable, another approach such as integration of such a protein into the proteins that make up plant-based fibers could be considered as it has the potential to lead

to an end product with enhanced physical properties, however such a product would by necessity need to be transgenically expressed rather than transiently expressed. This approach could potentially overcome the next hurdle which is not addressed by the research herein, doping of the recombinant spider silk fiber as the process can be extremely extensive, requiring specialized small scale equipment which replicates the doping process natural spider silk undergoes.

CHAPTER 3

EDIBLE VACCINES AND THE FUTURE OF GLOBAL HEALTH.

Introduction

While there are many types of vaccines, from live-attenuated to mRNA, their method of delivery is often injection into the muscle, dermis, or subcutaneous fat and, in some rare cases such as the nasal flu vaccine, mucosally delivered. While these modes of delivery are highly tested and globally implemented there are drawbacks. Production of vaccines must be performed in facilities often far away from the final destinations of the vaccine, leading to major logistical challenges for shipment, storage, and stockpiling especially in low-to-middle income countries (LMIC) (Kurup & Thomas, 2020; Lazarus et al., 2022). Injected vaccines also require needles, sterile water, and can be difficult to administer in large quantities quickly, this is doubly true when livestock are the target for vaccination rather than individual people (Farlow et al., 2023). Lastly, multiple doses are often required for vaccines however many individuals may receive only one dose in LMIC due to difficulty traveling to centralized vaccination hubs or the financial burden associated with such travel (Farlow et al., 2023).

Vaccine equity therefore has two primary issues which must be addressed 1) centralized production leading to long lead times and high logistical burden and, 2) ease of administration without dependence on local resources which may be unavailable at the time of vaccine administration.

Edible vaccines are able to address these two major issues easily. Firstly, edible vaccines whether they take the form of seeds, leafy greens, or partially processed/coated feedstock

are able to be produced locally, thus reducing the costs and logistical challenge associated with vaccinating large groups of animals or people (Gunasekaran & Gothandam, 2020; Kurup & Thomas, 2020; Wickramasuriya et al., 2021). Secondly, edible vaccines, as they are integrated into a plant in some way, are often highly stable while within the plant material and may be easily shipped short distances at local temperatures without reducing the efficacy of the vaccine; however, there is one major drawback to edible vaccines in this case, which is controlled dosing of the vaccine (Naik, 2022; Polshettiwar et al., 2022). Where vaccines are normally highly controlled, both in terms of manufacturing controls and validation and their doses, edible vaccines that are locally manufactured would need to undergo some form of processing to have the dosing be tested and normalized which may not be appropriate to rural areas lacking scientific equipment and personnel. This is a major barrier to orally vaccinating people. Yet, it may not be of concern when vaccinating livestock such as cows and chickens which can spread zoonotic diseases such as avian flu (Naik, 2022).

Therefore, the vaccination of livestock may be the primary application of edible vaccines until the technology and controls underlying the industry can undergo extensive field validation. Of note, there are also diseases that may not be properly protected against during mucosal immunization such as rabies. As the majority of LMIC countries still experience a high number of deaths from diarrheal diseases and/or respiratory diseases, the mucosal route of administration is likely a best route of administration for many of the highest impact diseases in these specific countries (Boutayeb, 2010). This, combined

with a potential pipeline of local vaccine production, could potentially reduce a high number of deaths attributed to very simple diseases each year.

There are many plants which offer various benefits when it comes to the production of edible vaccines. Tomatoes and potatoes are two primary systems which have been utilized in the past to great effect (Davod et al., 2018; P. Gupta et al., 2022). However, leafy greens such as spinach, alfalfa, beet leaves, and lettuce may provide an opportunity for more readily controlled administration based on expression averages within the material as it could be easily shredded and mixed before being administered en masse. Leafy greens could also benefit from the current transient expression technologies for rapid production of vaccines on a semi-local scale which would enable municipalities to defend against unforeseen disease threats such as an outbreak of Anthrax or Ebola. To this end, the expression of fluorescent protein in various plant expression systems was performed followed by the expression of norovirus VLPs in beet leaves.

Methods

Vector Construction: The construction of plasmids pPS-OGFP-EA, pPS-OGFP-3N, and pPS-OGFP-3N-NR utilized in the expression of GFP in all plant expression systems discussed herein (**Figure 5 and 6)** are previously described and were utilized without modification (Diamos et al., 2016).

The construction of plasmids pBYR2eK2Mc-IEU, pBYR2eK2Mc-IEU-35S, and pBYR2eK2Mc-35S-NbACT3 utilized in the expression of norovirus VLPs in beets (**Figure 7)** were previously detailed in and were utilized in the experiments herein without modification (Diamos & Mason, 2018b).

Protein Production, Extraction, and Purification: *Agrobacterium tumefaciens* strain EHA105, GV3101, or LBA4404 was transfected with expression vectors via electroporation. Resulting transformed *Agrobacterium* strains were confirmed using PCR and restriction digestion (RD) of purified plasmids. PCR and RD confirmed *Agrobacterium* strains were grown overnight at 30°C in YENB media + 50 mg/L kanamycin and 2.5 mg/L rifampicin. *Agrobacterium* was pelleted for 10 minutes at 4,500 g. Pellets were resuspended in infiltration buffer (10 mM 2-(N-morpholino) *ethanesulfonic* acid (MES), pH 5.5 and 10 mM MgSO4) to a final OD600 of 0.25-0.35. *Agrobacterium* suspensions were infiltrated using a syringe without needle into the leaves of 5 week old glycoengineered (GnGn) N. *benthamiana* silenced for production of the plant-specific β1,2-linked xylose and α1,3-linked fucose (Castilho & Steinkellner, 2012). Infiltrated leaves were harvested 4 days post infiltrations (DPI).

0.1g leaf tissue samples expressing GFP or norovirus VLPs were homogenized in 1:3 w/v extraction buffer (25 mM Tris-HCL, 125 mM NaCl, 3 mM EDTA, pH 8.0 with 50 mM sodium ascorbate, and 2 mM phenylmethylsulfonyl fluoride (PMSF) added before extraction). 12–14 ZnO beads, 2.0 mm (Fisher Scientific, Waltham, MA, United States), were added to 1.5 mL tubes containing leaf samples and extraction buffer. The tubes were bead beaten using a Bullet Blender machine (Next Advance, Averill Park, NY, United States) for two 5-min rounds with tubes being inverted twice between rounds. Homogenized leaf tissue was then rotated at 4°C for 20 minutes before samples were centrifuged at 13,000 g for 10 minutes at 4°C in a 5417R centrifuge (Eppendorf, Hauppauge, NY, United States). Once centrifuged the supernatant was transferred to a new 1.5mL tube. The samples were centrifuged again at 13,000 g for 5 min at 4°C to obtain a clarified extract free of major plant contaminants ready for use in SDS-PAGE, Fluorescence Imaging, and Western Blot analysis.

SDS-PAGE, Fluorescence Imaging, and Western Blot Analysis: Clarified extracts were mixed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) under either reducing (0.5 M DTT added) conditions for norovirus VLP samples and non-reducing conditions (no DTT added) for GFP samples. Reducing samples were boiled for 10 minutes while samples mixed with non-reducing buffer were not boiled, but instead incubated at room temperature for 10 minutes after the addition of sample buffer. Samples were separated on stain-free 4–15% polyacrylamide gels (Bio-Rad, Hercules, CA). Florescent protein samples run under non-reducing conditions on standard 4–15% polyacrylamide gels were visualized on a UV-

transilluminator and photographed for analysis while norovirus VLP samples were analyzed using Coomassie stain (Bio-Rad, Hercules, CA, United States) and Western blot.

Norovirus VLP samples were electroblot transferred to PVDF membranes. PVDF membranes were blocked with 5% dry milk in PBST (PBS with 0.05% Tween-20) at 37°C for 1 h and probed with polyclonal rabbit-anti GII.4 diluted 1:5000 in 1% PBSTM followed by goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (Sigma) diluted 1:5000 in 1% PBSTM as previously described (Diamos, Hunter, et al., 2020). Bound antibody was detected with luminol reagent (Santa Cruz Biotechnologies, Santa Cruz, CA).

Coomassie and Western blot samples were further analyzed to determine the expression levels of fluorescent proteins expressed in plants as well as norovirus VLP subunits by utilizing the ImageJ application. ImageJ allows for the determination of a protein bands intensity by outlining the band. This relative intensity can be utilized to compare one band on the same gel to another. Coomassie gels were utilized to determine the overall protein expression by determining band intensity for all protein bands present in a sample. These were utilized to set a total protein standard for the same samples examined under Western blot procedures. Band intensity of spider silk protein as determined by anti-his antibody binding was divided by total protein band intensity to determine a relative expression amount compared to total protein. These standardized samples were then compared. In these comparison studies three samples were processed using this

approach. Three leaf samples were processed in this way to calculate the averages presented in Figure 5 and Figure 6 while Figure 7 shows calculations made for each leaf sample individually.

Results

Four edible plant species were selected to test expression of GFP as a test protein and norovirus virus like particles (VLPs) as a potential edible vaccine in the optimal edible plant expression system. The species selected were beets, spinach, alfalfa, and lettuce as they are all produced in large quantities globally and are edible for humans and/or a wide range of livestock animals including cows, goats, and chickens in the case of alfalfa. Wild Type *N benthamiana* was utilized as a standard expression system as it has been highly optimized over several decades for production of complex proteins including vaccines, antibodies, and enzymes.

Two expression factors were tested first in all expression systems to determine an optimal system for further development. The first factor was the *Agrobacterium* strain used for DNA delivery. *Agrobacterium* cells were, transformed with the same plasmid expressing GFP, using the EA terminator (tobacco extensin terminator fused with an *N. benthamiana* actin terminator), bean yellow dwarf virus expression vector without the genetic machinery for replication (denoted as Rep, though this is not in reference to the Rep gene alone), and the CaMV 35S promoter. The *Agrobacterium* strains tested were LBA4404, GV3101, and EHA105, the latter of which is the standard for expression in *N. benthamiana*. The second factor tested was the terminator combination. *Agrobacterium*, EHA105 was used to deliver a GFP cassette having the EA terminator, the 3N terminator (35S terminator from cauliflower mosaic virus fused to the NOS terminator from *Agrobacterium*), or the 3N terminator in a non-replicating cassette without Rep genes (3N-NR), all of which used the CaMV 35S promoter. These variants were used to

determine the effect of terminator combinations and cassette replication on expression levels in all plant expression systems tested as the second factor.

EHA105 performed the best in all plant expression systems however GV3101 performed well in beets, lettuce, and nearly 50% better than LBA4404 in alfalfa (**Figure 5B)**. The EA terminator performed best in all expression systems however the 3N terminator was comparable in some cases. For overall expression alfalfa performed second to N. *benthamiana* with an average expression of GFP of approximately 1.25 mg/g leaf tissue when using EHA105 and the expression construct containing the EA terminator **(Figure 6)**. N. *benthamiana* had an average GFP expression of approximately 4 mg/g leaf tissue using the same construct. Both beets and lettuce performed similarly to each other with an average GFP expression of approximately 0.5 mg/g leaf tissue using the same parameters as both N. *benthamiana* and alfalfa. Spinach performed poorly with an average expression of half that of both beet leaves and lettuce. As Alfalfa is a product generally not eaten by humans, and Beet leaves were easier to infiltrate using the syringe infiltration technique, they were selected over other plant expression systems for further study. In addition to their ease of infiltration beets were selected as they are grown globally in many regions as a source of sugar and, as the root rather than the leaves are utilized for sugar processing, beets could potentially serve a dual purpose in a community as a vaccine expression system and source of high-calorie foodstuffs.

Figure 7A shows expression of a norovirus VLP in beet leaves using EHA105 agrobacterium with various terminators. The terminators used are IEU (tobacco extensin terminator), IEU-35S (tobacco extensin terminator fused to the cauliflower mosaic 35S

virus terminator), or 35S-NbACT3 (cauliflower mosaic virus terminator fused to the N. *benthamina* homolog of *Arabidopsis thaliana* actin 7 terminator). The 35S-NbACT3 terminator performed best in all three leaf samples. Norovirus expression was measured using SDS-PAGE coomassie stain **(Figure 7B)** with expression levels normalized to the amount of rubisco protein present. Norovirus VLP expression was confirmed by Western blot utilizing an anti-Norwalk (aka anti-norovirus) antibody. Of note, Figure 7C shows an additional band which is a potential dimer of the norovirus VLP even under reducing conditions, indicating potential assembly of capsomere dimers.

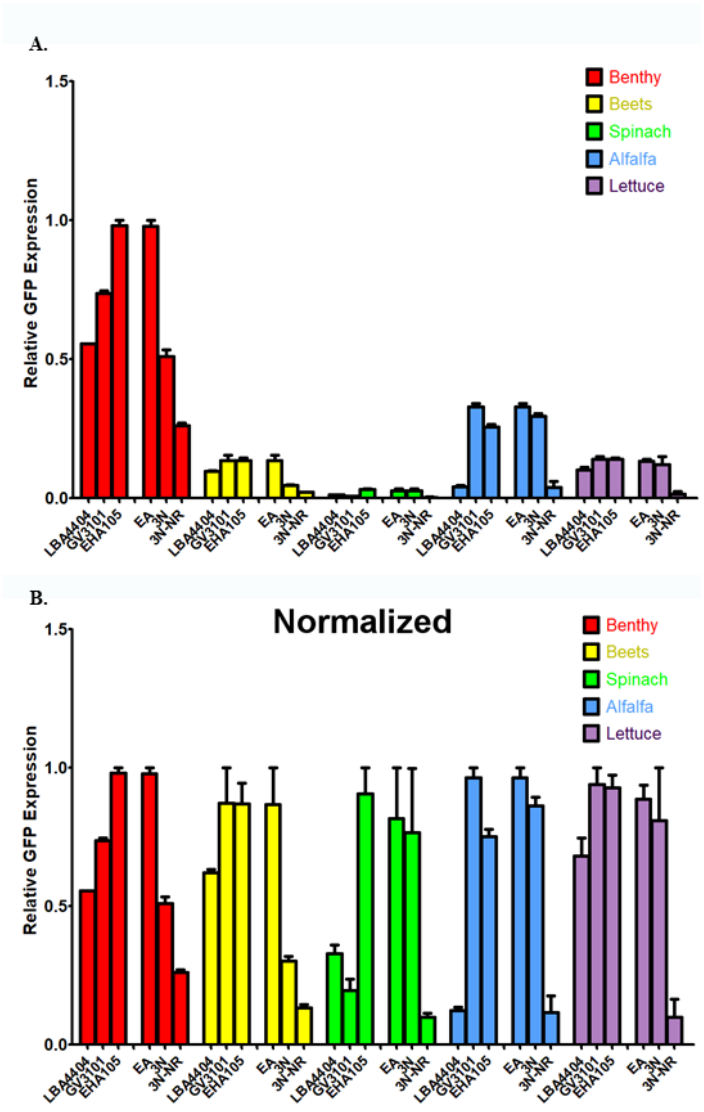


Figure 5. GFP Expression in Various Plant Expression Systems. Expression of GFP was determined by expression relative to Benthy (A) or normalized to the highest value for each plant (B). Expression was studied in *N. benthamiana* (Benthy), Beets, Spinach, Alfalfa, and Romain Lettuce using three *Agrobacterium* strains; LBA4404, GV3101, and EHA105. All constructs used to test *Agrobacterium* expression levels utilized the cauliflower mosaic virus (CaMV) 35S promoter, Rep for construct replication, and the EA terminator. Constructs with differentiated terminators in EHA105 *Agrobacterium* were studied, the terminators utilized were EA (tobacco extensin terminator fused with an *N. benthamiana* actin terminator), 3N (35S terminator from CaMV fused to the NOS terminator from *Agrobacterium*), and 3N-NR (3N terminator in a non-replicating plasmid). Data are means +/- standard error for three independent samples.

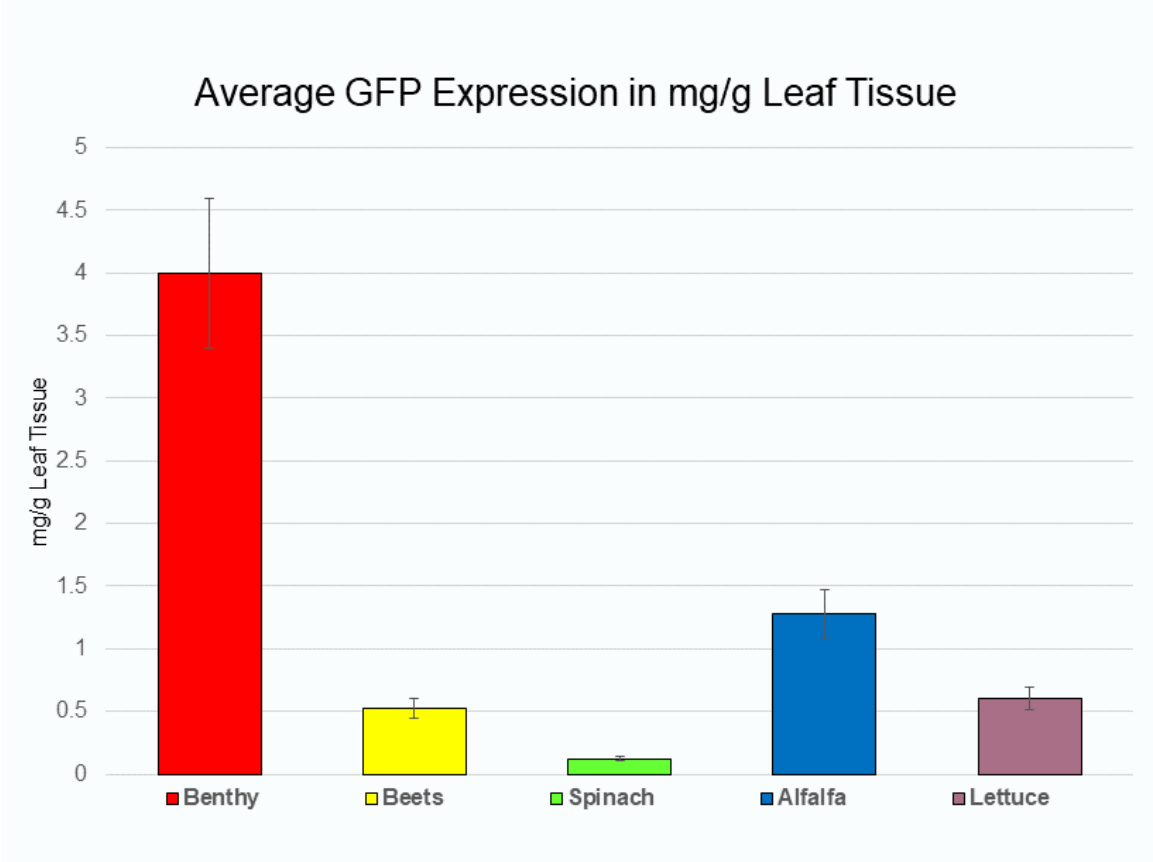


Figure 6. Average GFP Produced per Gram of Leaf Tissue in Various Plant Expression Systems. *N. benthamiana* (Benthy) was utilized as a baseline for optimal expression of GFP. Beeta, Spinach, Alfalfa, and Romaine Lettuce were compared based on relative GFP expression utilizing the optimal *Agrobacterium* and constructs determined in Figure 5; those being EHA105 and a replicating plasmid utilizing the EA terminator respectively. Alfalfa was the second best producer of GFP with an average production of approximately 1.25 mg/g. Both Beets and Lettuce performed well with an average production of approximately 0.5 mg/g. Spinach performed the worst with an average production below 0.25 mg/g. Data are means +/- standard error for three independent samples.

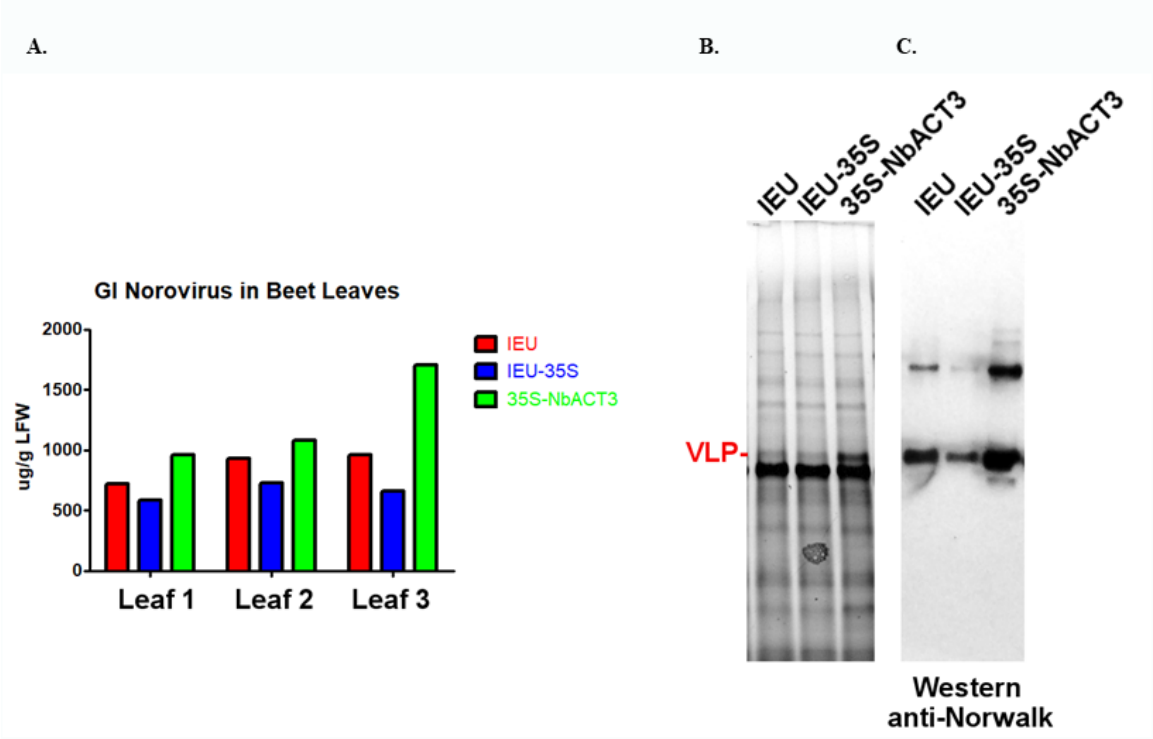


Figure 7. Expression of Norovirus Virus Like Particles in Beet Leaves Utilizing Various Terminators. Norovirus VLPs were expressed in beet leaves using the EHA105 *Agrobacterium* with constructs expressing either IEU (tobacco extensin terminator), IEU-35S (tobacco extensin terminator fused to the cauliflower mosaic virus terminator), or 35S-NbACT3 (cauliflower mosaic virus terminator fused to the *N. benthamina* homolog of *A. thalina* actin 7 terminator). Figure 7A shows µg/g leaf fresh weight (LFW) across three leaves which were spot infiltrated. Figure 7B shows expression of the Norovirus VLP compared to native plant proteins via SDS-PAGE stained for total protein. Figure 7C shows Norovirus VLP expression determined by Western blot analysis utilizing anti-Norwalk (aka anti-Norovirus) HRP conjugated antibodies. 35S-NbACT3 was found to be the highest expressing construct.

Discussion

Plants have been utilized for decades for the production of biologics and, more specifically, vaccines (Mason et al., 1992). While there are no currently commercialized vaccines, there are cases of approved and commercialized biologics made in plant systems including a Covid-19 vaccine made in N. *benthamiana* plants produced by Medicago in Canada before the company was closed down (Benvenuto et al., 2023; Hager et al., 2022). The production of biologics and vaccines in plants has been touted as a cheap alternative to highly expensive mammalian expression systems and could, theoretically, be utilized to promote equity and local production of highly needed biologic medicines (Nandi et al., 2016). While in theory local production of biologics, including vaccines, via small plant production facilities could potentially be created they would still require a highly educated technical workforce on site, something that many low-to-middle income countries simply cannot provide on a scale given thier socio-economic and education conditions (Murad et al., 2020). Therefore, plant based production of biologics may have to undergo a different pathway to provide the benefits of local, small scale production without the drawbacks. One approach that may lead to this outcome is the production of vaccines in edible plants that could be locally grown and doses standardized through simple processes, such as homogenization of large batches or freeze drying before being pressed into pill form, not requiring a highly educated workforce (Gunasekaran & Gothandam, 2020; P. Gupta et al., 2022; Kurup & Thomas, 2020).

With this in mind four edible plants were selected due to three factors: 1) the amount produced globally, 2) the global spread of consumption, and 3) their ability to be readily infiltrated with agrobacterium via a syringe infiltration technique. With these factors in mind, and *N. benthamiana* being utilized as an expression standard, beets, spinach, alfalfa, and romaine lettuce were selected for their potential use in either human or livestock oral vaccination via ingestion.

As it has previously been shown that the terminators of a gene play a large role in the overall expression of the encoded protein, three different terminators on the same GFP expression plasmid were tested in Figure 5 (Diamos & Mason, 2018b; Rosenthal et al., 2018). It was found that for all plant expression systems, including the *N. benthamiana* standard, the EA chimeric double terminator performed well. The 3N chimeric double terminator on the other hand did not perform well in some plant species but performed as well as the EA terminator in others. This indicates that the EA terminator is broadly applicable to many plant species but the 3N, or other terminators, may be optimal for specific species only. The 3N terminator construct lacking replication genes led to less overall expression in all plant systems indicating that replication of the expression cassette carrying the gene encoding the protein of interest is highly impactful to the overall level of expression (C. Huang et al., 2009).

Figure 5 also includes testing of different *Agrobacterium* strains all expressing the EA terminator construct. Of the three strains tested, EHA105, one of the most commonly used *Agrobacterium* strains, performed well in all plant species. Similarly to the 3N terminator the GV3101 *Agrobacterium* strain performed as well, or even slightly better

than, EHA105 in beets, alfalfa, and lettuce though it performed far worse in spinach. The third *Agrobacterium* strain tested, LBA4404, performed worse in every plant species than EHA105. Therefore, EHA105 utilizing the EA terminator with replication genes and the CaMV 35S promoter was determined to be the best construct and *Agrobacterium* combination of all studied in Figure 1. The variability seen in both *Agrobacterium* and terminators utilized indicates that a high level of optimization may be done to boost the level of protein expression in the plant species studied herein. However, due to their ease of syringe infiltration, large leaves, and ability to be eaten by both humans and livestock, as well as the fact that they're grown in many regions globally, and could potentially provide a sugar source in addition to a vaccine source, beets were selected for further expression studies over both lettuce and alfalfa. beets may also represent the best plant species for further development due to its high production amount (~270 million tonnes globally compared to spinach at ~32 million tonnes and lettuce at ~27 million tonnes). However, if a biologic was being designed for consumption by animal species only alfalfa may offer a better alternative as its highest expression of GFP was seen at 1.25 mg/g protein to leaf weight while beets and lettuce were seen to have GFP expression levels of only 0.5 mg/g protein to leaf weight (**Figure 6).**

Figure 7A shows additional expression studies in beets utilizing three terminators. It was determined that the 35S-NbACT3 terminator performed the best when expressing norovirus virus-like particles at 1-1.5 mg/g protein to leaf tissue weight. The IEU terminator performed fairly well but had slightly lower expression levels than 35S-

NbACT3. Of interest, the double terminator IEU-35S performed worse than the IEU terminator.

Norovirus virus-like particles were seen to express in high levels on SDS-PAGE gels (**Figure 7B)** which was confirmed with anti-norwalk (anti-norovirus) antibody Western blot in Figure 7C. Norovirus VLPs were selected as the optimal vaccine subject for edible vaccines as they can survive the harsh environment and low pH of the stomach (Roth & Karst, 2016). Norovirus infection is also one of the leading causes of disease and death in LMIC due to the fact that many in these countries cannot access the simple healthcare resources necessary to turn norovirus from a deadly disease into a merely extremely unpleasant one (Devant et al., 2019; Mans, 2019).

These results show that norovirus VLPs may be readily produced in beets as a case study for potential edible plant expression systems, namely for biologics such as vaccines. The fact that expression was nearly doubled when comparing GFP to norovirus VLPs by changing only the terminator shows that expression in this system has the potential to be highly optimized. beets should also be further examined as a potential two-in-one crop for production of biologics in the leaves and production of sugar using the bulb as is done on a massive scale worldwide. Of note, beets are highly distributed throughout the world according to analysis by the Food and Agriculture Organization of the United Nations with 66.8% being grown in Europe, 13.5% being grown in Asia, 13.2% being grown in the Americas, and 6.5% being grown in Africa whereas spinach and lettuce production is focused primarily in Asia.

These results may serve as a basis for future development of edible vaccines in terms of selecting both the expression system utilized and the vaccine target. However, next steps in development should be informed by key opinion leaders in the regulatory agencies which would ultimately approve the vaccines as well as key opinion leaders in the national and local governments which would be responsible for the oversight of vaccination programs utilizing the edible vaccine approach studied herein.

CHAPTER 4

POTENTIAL FOR A UNIVERSAL HPV VACCINE UTILIZING VARIOUS VACCINE PLATFORM TECHNOLOGIES

Introduction

Human Papillomavirus (HPV) is an expansive family of viruses containing over 200 strains, some of which are oncogenic causing cervical, head, neck, esophageal, penile, vaginal, anal, and other cancers as well as warts. Currently Gardasil-9 is the best in class HPV vaccine providing a high level of protection against seven strains of HPV which cause the majority of cervical cancer and two HPV strains which cause genital warts. However, there are many strains of HPV with over 25 known to be of risk many of which are cancer causing, with others of the 200 strains being wart causing or of an undetermined level of risk to health (M. Chen et al., 2021; R. Gupta et al., 2020). As there are shortcomings of the current vaccines on the market to prevent all cancers associated with HPV it is important to find a viable and competitive solution. While all current vaccines against HPV target the L1 capsid protein of HPVs, which differs for each strain, there is another option in the form of the highly conserved L2 capsid protein. L2 capsid protein is mostly buried beneath the L1 capsid structure, therefore, does not generally produce neutralizing antibodies during the course of typical infection. However, when used as a singular antigen in a vaccine the L2 capsid protein provides neutralizing antibodies, especially against the highly conserved RG1 epitope which has been shown to protect against 25 strains of HPV (Huber et al., 2021).

The L2 capsid protein is approximately 55kDa and just under 500 amino acids in length (Wang & Roden, 2013). However, the neutralizing epitopes that have been discovered are the RG1 epitope (aa17-36), the Mab5 epitope (aa69-81), and the Mab13 epitope (aa108-120) (Huber et al., 2021; Olczak & Roden, 2020). All of these epitopes are on the N-terminal end of L2, which is the portion of L2 responsible for furin cleavage leading to endosomal escape of the virus in infected cells (Wang & Roden, 2013). Therefore, the section of L2 containing these three cross-neutralizing epitopes (aa14-122) has been utilized in various vaccines, as has the RG1 epitope alone and full length L2 protein (Huber et al., 2021). These three protein antigens may be utilized for study of vaccine platforms such as virus like particles (VLPs) or Recombinant Immune Complexes (RICs). An overview of all three L2 cross neutralizing epitopes is provided in Figure 8.

Vaccine platforms such as VLPs and RICs are highly desirable due to the fact that they may be easily modified and utilized not only for existing diseases of interest, but as of now unknown diseases. While there are many platforms, VLPs are a common solution as they are often easily produced and purified due to self-assembly of the particles. These VLPs can often have protein insertions made into their structure without affecting assembly due to steric hindrance and are thus a vehicle for the delivery of antigens such as the L2 antigens discussed herein. Sometimes the virus-like particle, VLP, term is utilized to describe what may more properly be called an antigen presenting nanoparticle (APN). APNs are clusters of proteins that self-assemble like VLPs, and present antigens like VLPs, but are not composed of the same overall viral capsid structure seen in the self-assembled virus-like particles. Rather they have variable structures depending on

their protein base (Tan & Jiang, 2019). VLPs and APNs are often created from highly studied viruses, such as the Acinetobacter phage discussed herein and norovirus, a genus based on the original Norwalk virus.

Phages have been studied for decades, in large part due to their potential application as an antibacterial agent which could be administered as a fully functional virus to kill bacteria responsible for infections in a highly targeted manner. However, the in-depth study of their genomics, and their lack of infectivity of mammalian cells, makes them prime targets for VLP development. In the case of the Acinetobacter phage, the coat protein was utilized to create VLPs with antigen insertions at either the N-terminal or C-terminal ends as has been previously described (Tissot et al., 2010). These AP205 VLP vaccines have been used to create vaccines against HPV, malaria, and COVID-19 (Janitzek et al., 2019; Liu et al., 2021).

Norovirus is an extremely infectious and hearty virus that can survive extremely acidic pH as it must survive the stomach to infect the digestive tract. This makes it a prime candidate for study as a VLP vaccine as it has the potential not only for typical administration but also mucosal administration through either a nasal spray or edible vaccine. There are two primary domains that form the norovirus Viral Protein 1 (VP1) capsid, an S domain at the N-terminus of VP1 and a P domain at the C-terminus of VP1 separated by a hinge at aa218-225 (Tan & Jiang, 2019). The S domain, when expressed alone forms a nanoparticle highly reminiscent of a typical VLP (called S60), while the P domain expressed alone, forms a nanoparticle without interior space to encapsulate

proteins, DNA, or RNA (called P24) (Tan & Jiang, 2019). Both these nanoparticles have been used previously in vaccine studies to good effect and are prime candidates for APNs utilized in future vaccine studies.

The AP205 VLP along with the S60 and P24 APNs represent three potentially effective vaccine platforms that may be easily modified to present an antigen from various diseases to the immune system via typical vaccination routes and, in the case of the APNs potentially via the oral route of vaccine administration due to its basis on the acid stable norovirus. Similarly, the hepatitis B core antigen (HBc) VLP is an additional VLP platform that has been highly studied and shown to be capable of initiating an immune response against various disease antigens (Aston-Deaville et al., 2020). As it has been studied for so many years, the HBc VLP is a prime positive control in any vaccine study utilizing other VLP platforms or any other vaccine platform for that matter such as the APNs and Recombinant Immune Complex platform discussed below.

In addition to these VLP and nanoparticles, there are many other vaccine platforms. However, one with a high degree of novelty is the Recombinant Immune Complex, or RIC. This platform consists of an antibody which binds to a cognate epitope from Ebola virus GP1 known as 6D8. The expression construct of the RIC is designed so an antigen of interest from any pathogen may be included at either the C-terminal or N-terminal ends of the antibody heavy chain, with the 6D8 epitope "tag" being expressed on the C-terminal end. This epitope tag allows for the assembly of the antibody-antigen-tag construct into immune complexes which trigger the immune system through activation of

complement and Fcgamma receptor activation, leading to heightened uptake and presentation via antigen presenting cells (APCs) (Diamos et al., 2023).

In the present study, these aforementioned vaccine platforms were expressed, purified, and utilized in the vaccination of mice using either the L2 aa14-122 antigen or RG1 epitope of L2. Tests were performed in a prime-boost fashion as recent studies of Covid-19 vaccinations showed that being vaccinated with one vaccine, followed by another, increased responses above being vaccinated with a single vaccine twice (Larkin, 2022). In addition, it was previously discovered that when an RIC is co-administered with a VLP there is a synergistic increase in immunity (Diamos et al., 2019, 2023). It is important to discover if this synergy is seen with a prime-boost model of vaccination similar to Covid-19 vaccine population studies as this is a more likely use case for a future HPV vaccine since many individuals have already received at least one dose of currently marketed HPV VLP vaccines. Therefore VLPs and APNs were utilized in dose 1 with the RIC platform being utilized in dose two, with mice vaccinated receiving 2µg of total antigen.

Methods

Vector Construction: The construction of plasmid pBYKEAM-YFP has previously been described (Diamos, Hunter, et al., 2020). Construction of plasmids pBYKEAM-AP205-L2(14-38x2), pBYKEAM-P24-L2(14-38x2), and pBYKEAM-S60-L2(14-38x2) was performed via digestion of either AP205-L2(14-38x2) whole gene, P24-L2(14-38x2) whole gene, or S60-L2(14-38x2) whole gene (synthesized by Integrated DNA Technologies) and plasmid pBYKEAM-YFP with XbaI and SacI restriction enzyme digest. Fragments from whole genes were ligated with the backbone fragment of the pBYKEAM-YFP plasmid resulting in the aforementioned plasmids.

The construction of plasmids and pBYR2eK2M-HBche encoding the HBc heterodimer VLP without antigen insertion and pBYR2eK2M-HBcheL2ic encoding the HBc heterodimer VLP with HPV16 L2 aa14-122 antigen insertion have been previously described (Diamos et al., 2019). The construction of plasmid pBYR2eK2M-HBche-L2(14-38x2) was performed via the digestion of plasmids pBYR2eK2M-HBche and pBYKEAM-S60-L2(14-38x2) with BamHI and SpeI restriction enzymes. The resulting backbone from pBYR2eK2M-HBche and L2(14-38x2) antigen insertion fragment were ligated resulting in plasmid pBYR2eK2M-HBche-L2(14-38x2). All plasmids were confirmed via PCR and restriction digest.

The construction of plasmid pBYR11eMa-h6D8-L2 encoding the Recombinant Immune Complex displaying the HPV16 L2 aa14-122 antigen has been previously described (Diamos et al., 2019). An overview of vector construction is provided in Figure 9.

Protein Production, Extraction, and Purification: *Agrobacterium tumefaciens* strain EHA105 was transformed with expression vectors via electroporation. Resulting transformed *Agrobacterium* strains were confirmed using PCR and restriction digestion of purified plasmids. Confirmed *Agrobacterium* strains were grown overnight at 30°C in YENB media + 50 mg/L kanamycin and 2.5 mg/L rifampicin. *Agrobacterium* cells were pelleted for 10 minutes at 4,500 g. Pellets were resuspended in infiltration buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO4) to a final OD600 of 0.2-0.3. *Agrobacterium* suspensions were infiltrated using a syringe without needle into the leaves of 5 week old glycoengineered (GnGn) *N. benthamiana* silenced for production of the plant-specific β1,2-linked xylose and α1,3-linked fucose (Castilho & Steinkellner, 2012). Infiltrated leaves were harvested 4-5 days post infiltrations (DPI).

0.1g leaf tissue samples expressing VLPs displaying either the HPV16 L2 aa14-38x2 antigen or HPV16 L2 aa14-122 antigen were homogenized in 1:3 w/v extraction buffer (25 mM Tris-HCL, 125 mM NaCl, 3 mM EDTA, pH 8.0 with 50 mM sodium ascorbate, and 2 mM phenylmethylsulfonyl fluoride (PMSF) added before extraction). 12–14 ZnO beads, 2.0 mm (Fisher Scientific, Waltham, MA, United States), were added to 1.5 mL tubes containing leaf samples and extraction buffer. The tubes were bead beaten using a Bullet Blender machine (Next Advance, Averill Park, NY, United States) for two 5-min rounds with tubes being inverted twice between rounds. Homogenized leaf tissue was then rotated at 4°C for 20 minutes before samples were centrifuged at 13,000 g for 10 minutes at 4°C in a 5417R centrifuge (Eppendorf, Hauppauge, NY, United States). Once

centrifuged the supernatant was transferred to a new 1.5mL tube. The samples were centrifuged again at 13,000 g for 5 min at 4°C to obtain a clarified extract ready for use in SDS-PAGE and Western Blot analysis.

Large scale purification of leaf tissue samples expressing VLPs displaying either the HPV16 L2 aa14-38x2 antigen or HPV16 L2 aa14-122 antigen was performed via homogenization in 1:3 w/v extraction buffer (25 mM Tris-HCL, 125 mM NaCl, 3 mM EDTA, pH 8.0 with 50 mM sodium ascorbate, and 2 mM phenylmethylsulfonyl fluoride (PMSF) added before extraction) in a blender on high speed for 1 minute. Extract was transferred to a beaker and stirred at 4°C for 30 minutes before being filtered through miracloth. Filtered extract was centrifuged at 13,000 g for 10 minutes at 4°C in a 5417R centrifuge (Eppendorf, Hauppauge, NY, United States) to remove particulate material. Supernatant was transferred to a new 1.5 mL tube and centrifuged again at 13,000 g for 5 min at 4°C to obtain a clarified extract. Clarified extract was transferred to a new 1.5 mL tube and centrifuged again at 17,000 g for 24 hours at 4°C to obtain a pellet containing VLPs. The pellet was resuspended in Tris-Buffered Saline (TBS) before a final centrifugation at 13,000 g for 5 min at 4°C to remove all insoluble material leaving VLPs in the supernatant which was transferred to a new 1.5mL tube and ready for use in SDS-PAGE and Western Blot analysis.

Recombinant Immune Complex extraction and purification was performed as previously described (Diamos et al., 2019).

RICs utilized in Dose 1 of the vaccine schema presented in Figure 10 were concentrated utilizing a 10kDa molecular weight cutoff protein concentrator following manufacturer specification (Pierce™ Protein Concentrators, Thermo Scientific). RICs utilized in Dose 2 of the vaccine schema presented in Figure 10 were not concentrated.

AP205, S60, and P24 virus like particles were purified utilizing metal affinity chromatography. In brief, four mL of Talon Complete metal affinity resin (Clontech/Takara Bio, Mountain View, CA, USA) was added to a metal affinity chromatography column with a total volume of 30 mL. The ethanol storage solution was allowed to flow out of the column resulting in 2 mL resin bed volume which was washed using 15 mL of water followed by an equilibration step with 15 mL of extraction buffer. A final wash of 30 mL of extraction buffer was performed before the filtered extract was passed through the column. Resin was washed with 60 mL of PBS buffer at pH 7.4. Elution of bound proteins was achieved using PBS + 150 mM imidazole at pH 7.4. Elution samples were dialyzed overnight in one liter of sterile PBS using a three mL 3,500 MWCO Slide-A-Lyzer G2 dialysis cassette (Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer's instructions. Elution concentrations were determined both before and after dialysis by an A280 absorption using PBS + 150 mM imidazole and PBS, respectively, as blank solutions.

SDS-PAGE and Western Blot Analysis: Purified extracts of either RIC or VLPs were mixed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) under reducing (0.5 M DTT added) and non-reducing conditions

(no DTT added). RIC samples and VLP samples probed with antibody for Western Blot were Reduced. Reducing samples were boiled for 10 minutes while samples mixed with non-reducing buffer were not boiled, but instead incubated at room temperature for 10 minutes after the addition of sample buffer. Samples were separated on stain-free 4–15% polyacrylamide gels (Bio-Rad, Hercules, CA) then analyzed using Coomassie stain (Bio-Rad, Hercules, CA, United States) and Western blot.

RIC and VLP samples were electroblot transferred to PVDF membranes. PVDF membranes were blocked with 5% dry milk in PBST (PBS with 0.05% Tween-20) at 37°C for 1 h. VLP samples were probed with polyclonal rabbit-anti His1:5000 in 1% PBSTM followed by goat anti-rabbit IgG antibody-horseradish peroxidase conjugate (Sigma) diluted 1:5000 in 1% PBSTM as previously described (Diamos, Hunter, et al., 2020). RIC samples were probed with donkey anti-human IgG antibody-horseradish peroxidase conjugate. Bound antibody was detected with luminol reagent (Santa Cruz Biotechnologies, Santa Cruz, CA).

Vaccination: 6 week old Balb/C mice were vaccinated according to the schema outlined in Figure 10. In brief, 8 total groups of 8 mice (6 mice for PBS control) were vaccinated with 2μg equivalent antigen of either HVP16 L2(aa14-38x2) - denoted by -38 on all graphs - or HPV16 L2(aa14-122) - denoted by -122 on all graphs - on day 0 via the subcutaneous route of administration. All RICs used expressed the aa14-122 antigen of HPV16 L2. The groups are as follows, in order: HBc-122, HBc-38, S60-38, P24-38, AP205-38, HBc-122+RIC, RIC, and PBS control. A cheek bleed was taken from mice at

day 0 before vaccination. On day 21 an additional cheek bleed was taken before a second dose of 2µg equivalent antigen was administered via the subcutaneous route of administration, however all groups received an RIC dose to study the potential of a prime-boost effect of the RIC. On day 42 a terminal bleed was performed and fecal pellets taken from group cages.

The amount of antigen delivered to mice was determined as follows. The extinction coefficient of the protein was determined utilizing the Expasy ProtParam tool. Once the extinction coefficient was determined the overall percentage of antigen present in the construct was determined. Purified samples were first run on an SDS-PAGE gel before their percent contamination was observed. This percent contamination was determined by utilizing the band intensity as determined by ImageJ analysis. In brief, each lane was gated with the overall intensity in that lane observed. Then, the band corresponding to the protein of interest was gated and that result divided by the total protein intensity band. Once this percentage of contamination was determined in this way, the A280 value of the purified protein suspension was determined. The A280 was determined, multiplied by the percentage purity of the sample, and then multiplied by the percent of the antigen present. The final antigen concentration was set to approximately 2µg by dilution of the protein sample with PBS. It is important to note that molar equivalents were not utilized in this study.

Enzyme-Linked ImmunoSorbent Assay (ELISA): A 96-well plate was coated with HPV16 L2 protein (aa14-122) produced in *N. benthamiana* plants using the construct

pBYe3R2K2Mc-L2(14-122)6H (H. Mason) and purified by metal affinity column chromatography as previously described (Diamos et al., 2019). The capture antigen was incubated on the plate in PBS for 1 hour at 37°C. The plate was washed three times with PBST. Pooled sera, or individual mouse sera, from each mouse group was administered to the plate, in 1% PBSTM, after blocking with 5% PBSTM for 1 hour at 37°C in the following dilutions: 1:50, 1:250, 1:1250, and 1:6250. The ELISA plate(s) was/were incubated for 1 hour at 37°C. Plates were washed three times with PBST before being probed with 1:2000 dilution goat-anti-mouse IgG Heavy chain/Light chain HRP conjugate antibody in 1% PBSTM incubated either overnight at 4°C - in the case of pooled sera samples utilized in Figure 11 and Figure 12 - or 1 hour at 37°C - in the case of individual sera samples and pooled samples utilized in Figure 13 and Figure 14 - before being washed four times with PBST. Once thoroughly washed ELISA plates had 50µl of TBM solution added and, once a shift in solution to blue could be recognized as not progressing further over the course of approximately 2-3 minutes, stopped with 1M phosphoric acid. The OD450 of the plate was read using a multi-mode plate reader (BioTek Synergy H1) and results recorded before being analyzed and graphed utilizing the GraphPad program. Background cutoff was determined by 2x the OD450 of blank wells. Samples with an OD450 within 50% of the OD450 of PBS control samples were determined to be non-responders in this trial when serial dilutions graphs were performed. For GMT antibody titer calculations, mouse group sera were pooled and ELISAs run in replicates of four pooled sera samples diluted following the dilution schema outlined above utilizing the 2x OD450 of blank wells as a cutoff. Dilutions were determined and graphed. A one-way ANOVA statistical method was performed with

Tukey post-hoc test to analyze the data seen in Figure 14. Only results with a p-value less than or equal to 0.0001 were analyzed.

Results

Figure 8 shows the three cross-neutralizing epitopes of the HPV L2 protein. These epitopes have been found to provide protection against infection and, in the case of the RG1 epitope, been used in clinical studies to prevent infection of a broad range of HPVs with over 20 HPV types being neutralized using pseudovirion assays (Ahmels et al., 2022; Huber et al., 2021). While the RG1 epitope is used in the majority of HPV L2 vaccines, the amino acid segment 14-122 contains all three cross-neutralizing epitopes and, therefore, could potentially provide higher efficacy when protection against variable HPV strains as there are over 200 known strains of HPV.

Figure 9A. shows the amino acid sequence of the AP205 bacteriophage coat antigen based VLP. This VLP has been shown in previous studies to be immunogenic and easily assembled whether a singular coat protein is used or a dimer is used (Janitzek et al., 2019; Liu et al., 2021). Figure 9B shows the amino acid sequence of the P24 APN which is, theoretically, an optimized antigen presenting system for vaccination. This construct includes a stabilizing domain that had previously been shown to increase nanoparticle stability without reducing expression levels (Tan et al., 2011; Tan & Jiang, 2019). Figure 9C shows the amino acid sequence of the S60 APN which, much like the P24 nanoparticle, is an optimized antigen presenting system for vaccination (Tan & Jiang, 2019; Xia et al., 2022). This construct contains several amino-acid substitutions which had previously been shown to increase particle stability (Tan et al., 2011; Xia et al., 2018). Of all the virus-like particles/antigen presenting nanoparticles produced the S60 nanoparticle was the only one which had expression levels rivaling the highly optimized

HBc VLP. However, further studies to optimize extraction protocols of P24 and AP205 were not performed where as they were with S60.

Figure 10 shows the vaccine schema utilized during the course of this vaccine trial. In brief, 8 mixed sex, 6 week old Balb/C mice (6 mixed sex mice in the case of the PBS control) were vaccinated with dose 1 on day 0, dose 2 on day 21, and were bled on days 0, 21, and 42 (terminal bleed). Fecal samples were also collected on day 42. All vaccine doses were given at 2µg equivalent antigen. The RIC vaccine used in dose 1 had to be concentrated using a 10 kDa MW protein concentrator.

Figure 11 shows the antibody titers of each mouse group vaccinated with 2µg equivalent HPV16 L2(aa14-38x2) antigen, denoted by the VLP/APN name and a "-38". Of note, the AP205-38 VLP out performed the historically highly immunogenic HBc-38 VLP while both the P24-38 and S60-38 nanoparticles did not elicit an antibody response as compared to the PBS control group.

Figure 12 shows the antibody titers of each mouse group vaccinated with 2µg equivalent HPV16 L2(aa14-38x2) antigen or the HPV16-L2(aa14-122) antigen, denoted by the VLP/APN name and a "-122". The RIC expressed HPV16-L2(aa14-122) antigen and is not denoted as such as it's the only version of the platform used in this study. Of note, the HBc-122 VLP and HBc-122 VLP/RIC elicited the highest antibody titers with the sole HBc-122 VLP eliciting the highest antibody titers of any group. The AP205-38 VLP elicited the third highest overall antibody titers. Of note, the RIC alone group elicited

antibody titers that were slightly heightened over the PBS control group, but not statistically significant over the PBS group.

Figure 13 shows dose 2 antibody titers of all groups, each of which was vaccinated with 2µg equivalent HPV16 L2(aa14-122) antigen presented by the RIC platform; each group is denoted by its dose 1 label plus (+) RIC-122. The boost dose induced high antibody titers in the HBc-122 + RIC-122 group as well as the HBc-122/RIC-122 + RIC-122 group in which HBc-122 and RIC-122 were originally co-delivered with each presenting 1µg of antigen. Both the HBc-38 + RIC-122 and RIC-122 + RIC-122 groups had a middling response. Of note, AP205-38 + RIC-122 performed at approximately the same level as the S60-38 + RIC-122 and P24-38 + RIC-122 groups.

Figure 14 shows dose 2 GMT antibody titers for all groups, each of which was vaccinated with 2µg equivalent HPV16 L2(aa14-122s) antigen presented by the RIC platform, each group is denoted by its dose 1 label plus (+) RIC-122. These results reflect those in Figure 13. Of interest the HBc-122 + RIC-122 and HBc-122/RIC-122 + RIC-122 groups both performed similarly while the HBc-38 + RIC-122 group did not perform as well as either of those groups. S60-38 + RIC-122, P24-38 + RIC-122 and AP205-38 + RIC-122 did not have statistically different GMT antibody titers. The RIC-122 + RIC-122 group performed as well as the HBc-38 + RIC-122 group even though the original dose of RIC-122 did not show a high level of immune response in dose 1 as seen in Figure 12. All groups were statistically significant when compared to the PBS control.

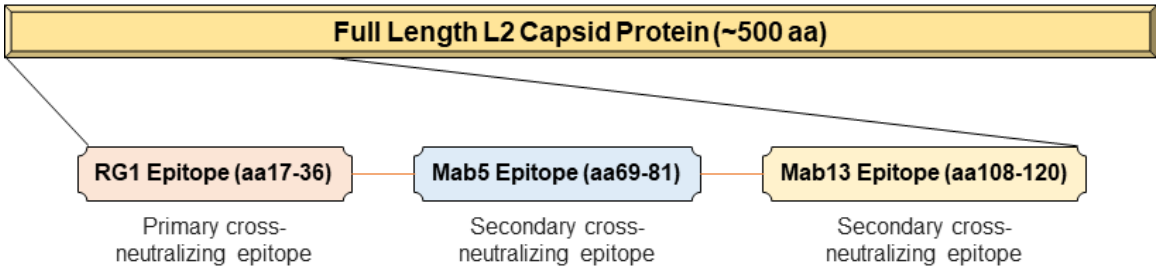


Figure 8. Cross Neutralizing Epitopes of the HPV L2 Capsid Protein. RG1 is canonically the cross-neutralizing epitope with the highest effective neutralization and consists of L2 capsid protein amino acids 17-36. The Mab5 and Mab13 cross neutralizing epitopes, corresponding to L2 capsid protein amino acids 69-81 and 108-120 respectively, have been shown to have neutralizing activity over several HPV types but are less potent than the RG1 epitope.

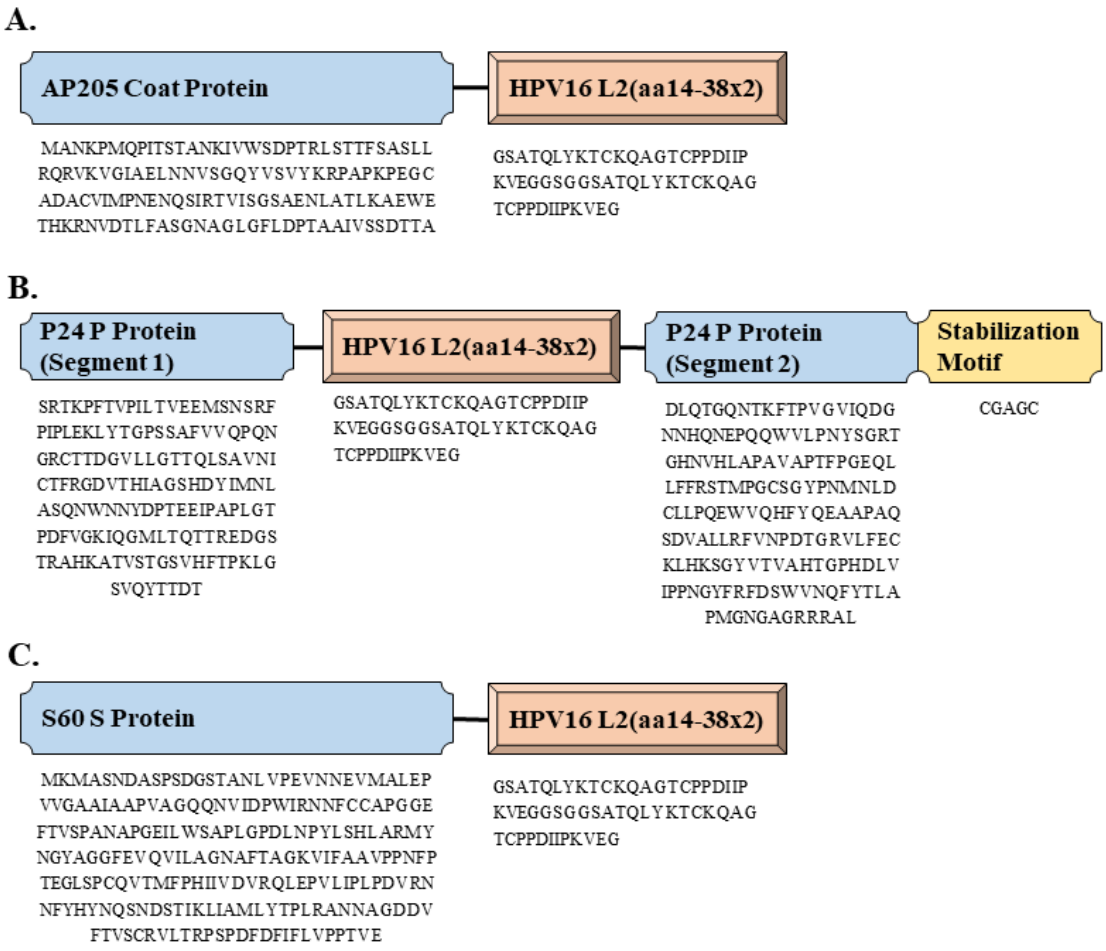
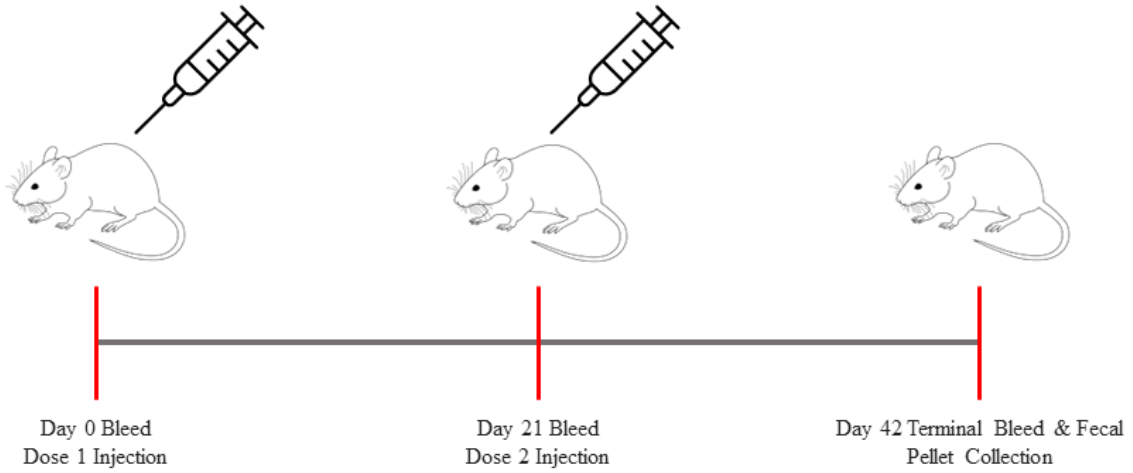


Figure 9. Virus-like Particle Constructs Utilized to Vaccinate Against HPV16 L2(aa14-38x2). A. A diagram of the AP205-HPV16 L2(14-38x2) construct with amino acid sequences listed. The black line between major segments represents a GGSGGS linker. Not shown is a C-terminal 6-His tag. B. A diagram of the P24-HPV16 L2(14-38x2) construct with amino acid sequences listed. The black lines between major segments represent a GGSGGS linker. A stabilization motif is directly linked to the P24 P Protein second segment. Not shown is a C-terminal 6-His tag. C. A diagram of the the S60-HPV16 L2(14-38x2) construct with amino acid sequences listed. The black lines between major segments represent a GGSGGS linker. Multiple amino-acid substitutions were made including (R69A, V57C, Q58C, S136C) when reading the beginning segment as amino acid 1. Not shown is a C-terminal 6-His tag.

Table 1. DNA Sequences of Novel VLPs and APNs Without Antigen Insertion	
AP205 DNA Sequence Without Insert	atggcgaacaagccaatgcaacccatcacctctactgccaacaaaatagtttggtccgatccaactagact ttctactacttttctgcttccttactcagacagagagtgaaggttggcattgctgaattgaacaacgtgtctg gtcaatacgtttctgtatacaagagacctgctcccaagcctgagggttgcgccgatgcttgtgtaattatgc ccaatgagaatcagtctatcagaacagttatttctggatctgccgagaacctcgccacacttaaagctgagt gggaaacacataagagaaatgtagatactttgttcgcctctggaaacgctggtcttggtttcttggacccca ctgccgcaattgtttcttccgacactaccgccggaggatctggaggaggatctgggggatccgcaactca gttgtataagacttgcaaacaagccggtacttgcccaccagacatcatccctaaagttgagggaggatctg ggggatctgccactcaactttacaagacctgcaaacaagctggtacttgcccacccgacattattcccaag gttgagggaggttcttaa
S60 DNA Sequence Without Insert	atgaagatggcttccaatgacgcttctccctcagacggttccactgccaaccttgttcctgaggtcaataac gaggttatggctcttgagccagttgttggagctgctattgcagctccagttgccggtcagcaaaacgttatc gacccttggatcagaaacaatttctgttgcgctcctggaggagagtttaccgtatctccagccaatgcccc gggcgagatcttgtggtcagccccacttgggccggacttaaatccttacctttcacaccttgcacgtatgta caacggatatgccggaggatttgaggttcaagttatccttgctggtaacgcttttactgctggcaaggtaat ctttgccgctgttccgccaaactttccaactgaagggttgtccccttgtcaagttacaatgtttccacacataa tcgtggatgttagacagttggagccagtgcttataccacttccagatgttagaaacaacttctatcattacaa ccagtcaaatgactctactatcaagcttatcgccatgctttataccccacttagagcgaataacgccggtga cgacgttttcaccgtttcctgtagagttcttaccagaccatctccagacttcgatttcatcttcttggttccacc aactgttgagggaggatctggaggatccgccactcagttgtacaaaacatgcaagcaagctggaacttg cccaccggacatcatcccaaaagttgaaggaggatctggaggttctgctactcagttatacaaaacctgc aagcaagctggaacttgtcctcccgacatcatccctaaagttgagggataa
P24 DNA Sequence Without Insert	atgtcccgtactaagccattcactgttccaatccttactgttgaggaaatgtcaaactccagattccctatccc attggagaagctttatactggtccctcttctgccttcgtagttcagccacagaacggtagatgcaccacaga tggggtacttcttggaactactcagttgtcagctgtgaatatctgtacttttagaggagacgttactcacatcg cgggttctcacgactacatcatgaacttggcatctcagaactggaacaactacgatccaactgaagagatc cctgctcctcttggcacaccagatttcgttgggaagatccaaggaatgcttactcagacaaccagagagg acggttccactagagcacacaaagcgactgtgtccactggatctgttcactttactcccaagcttggctctg ttcagtacaccacagatacaaatgggggttccggtggatccgctactcaattatacaagacctgcaagca agcgggaacctgcccgccggacatcatccccaaggtcgaggggggatctggaggatctgctactcagc tctacaaaacctgcaagcaagccggtacttgcccaccagacatcatccccaaagttgagggcactagtg gaggatctgggggttcagatctccagactggccagaacaccaagtttactcctgttggagttattcaagac ggaaacaaccaccagaacgagccacagcagtgggtccttccgaattactctggaaggactggacacaa cgttcaccttgccccagctgttgctcctactttccccggagagcaattgcttttcttcagatctaccatgcctg gttgttctggttacccaaacatgaacttggattgccttcttccccaagagtgggtccagcacttttaccaaga ggccgcgccagcccagtccgatgttgctttgttgaggttcgtgaaccccgatactggtagagtcctctttga gtgcaagttgcacaaatctggatacgtcaccgtcgctcacactgggccacacgaccttgttatcccaccaa acggatacttccgtttcgattcttgggttaaccagttctacactcttgctccaatgggtaacggcgccggaa gaagaagagctttgtgcggtgccggatgttaa



Group #	Dose 1 (2ug Total Antigen)	Dose 2 (2ug Total Antigen)
1	HBc-L2-122 (aa14-122)	RIC-L2-122 (aa14-122)
2	HBc-L2-38 (aa14-38x2)	RIC-L2-122 (aa14-122)
3	S60-L2-38 (aa14-38x2)	RIC-L2-122 (aa14-122)
4	P24-L2-38 (aa14-38x2)	RIC-L2-122 (aa14-122)
5	AP205-L2-38 (aa14-38x2)	RIC-L2-122 (aa14-122)
6	HBc-L2-122 (aa14-122) + RIC-L2-122 (aa14-122)	RIC-L2-122 (aa14-122)
7	RIC-L2-122 (aa14-122)	RIC-L2-122 (aa14-122)
8	PBS	PBS

Figure 10. Vaccination Schema to Test Prime-Boost of VLP Vaccines with the RIC Platform. A. On day 0 cheek bleeds were taken from 6-week-old Balb/c mice before being vaccinated with a VLP, RIC, or VLP+RIC. All VLP expressed HPV16 L2 (aa14-38x2), and RIC expressed HPV16 L2(aa14-122) as the antigen of interest. All mice were vaccinated with 2µg equivalent L2(aa14-38x2) or L2(aa14-122) on day 0. On day 21 cheek bleeds were taken from mice and all groups were vaccinated with RIC expressing HPV16 L2(aa14-122) at an antigen concentration of 2µg. On day 42 terminal bleeds were taken from mice and fecal pellets collected to test IgG and IgA antibody responses respectively.

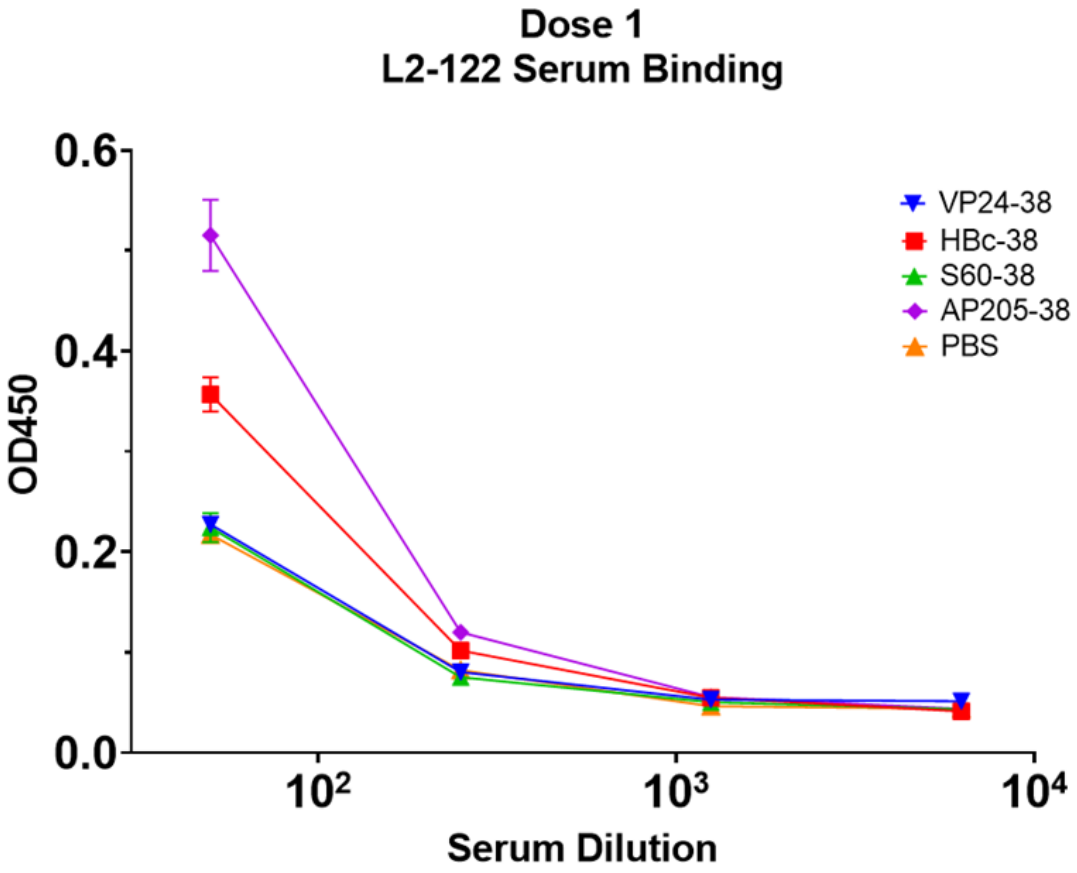


Figure 11. Binding of Mouse Sera from HPV16 L2(aa14-38x2) Vaccinated Groups to HPV16 L2(aa14-122) Protein as Measured by ELISA. P24, S60, AP205, and HBc VLPs or nanoparticle antibody response was measured utilizing ELISA. P24-38, S60-38, AP205-38, as well as one of the HBc-38 VLPs/nanoparticles in this graph expressed the HPV16 L2(aa14-38x2) antigen. AP205 VLP elicited the highest order antibody response with the HBc VLP being the second highest. The P24 and S60 nanoparticles did not elicit an antibody response above background as measured by the PBS control group. Data are means +/- standard error for 8 different mice in each group.

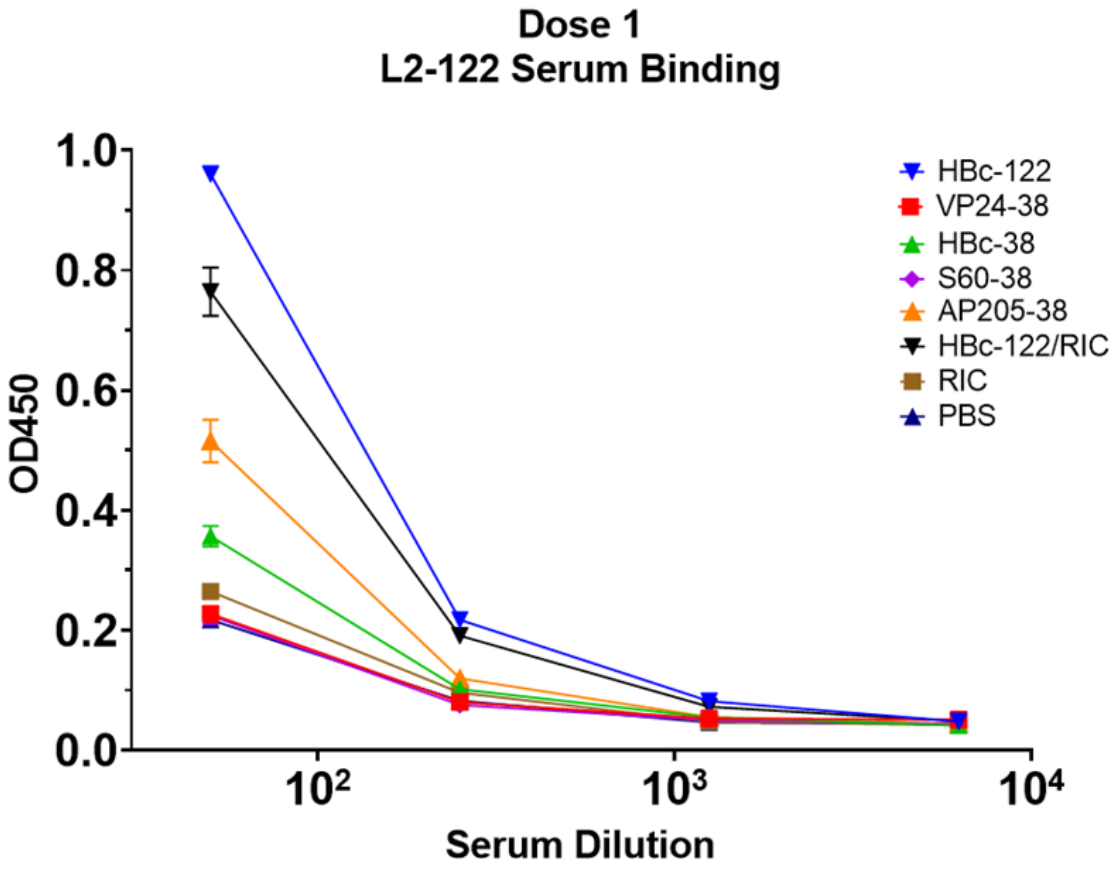


Figure 12. Binding of Mouse Sera from HPV16 L2(aa14-38x2) and HPV16 L2(aa14-122) Vaccinated Groups to HPV16 L2(aa14-122) Protein as Measured by ELISA. P24, S60, AP205, and HBc VLPs or nanoparticle antibody response was measured utilizing ELISA as were RIC antibody responses. P24-38, S60-38, AP205-38, as well as one of the HBc-38 VLPs/nanoparticles in this graph expressed the HPV16 L2(aa14-38x2) antigen. The RIC, HBc-122, and HBc-122/RIC groups expressed the HPV16-L2(aa14-122) antigen. Both the HBc-122, and the HBc-122/RIC had higher antibody titers than all other groups, however the HBc-122/RIC group was lower. The AP205-38 VLP outperformed the HBc-38 VLP and but was not equivalent to either of the groups which included the HBc-122 VLP. The RIC alone group showed a slight, but statistically insignificant, increase over background as measured by the PBS control group. Data are means +/- standard error for 8 different mice in each group.

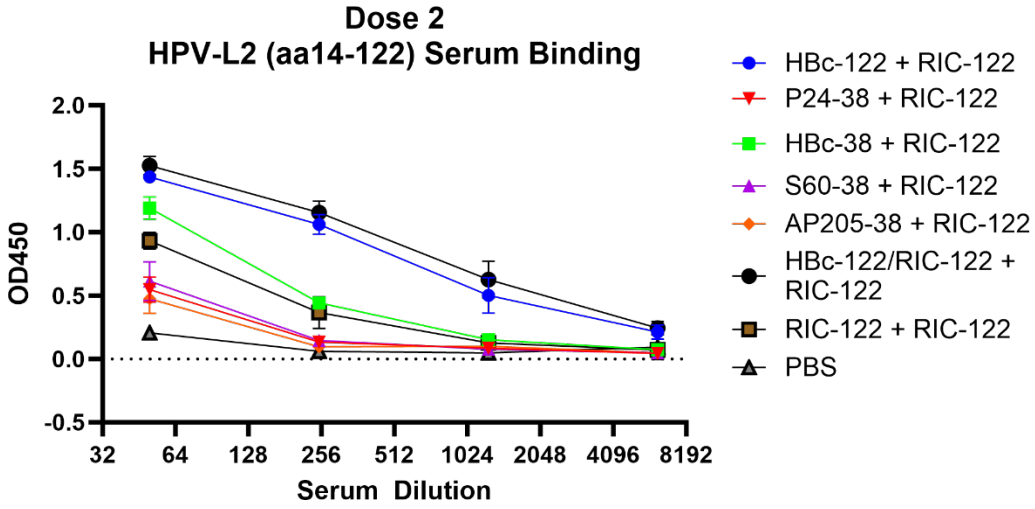


Figure 13. Binding of Mouse Sera from Dose Two of Vaccinated Groups to HPV16 L2(aa14-122) Protein as Measured by ELISA. P24, S60, AP205, and HBc VLPs or nanoparticles and RIC antibody responses were measured after dose 1 groups were boosted with 2µg of HPV16 L2(aa14-122) presented on a RIC. Both the HBc-122 + RIC-122, and the HBc-122/RIC + RIC-122 had higher antibody titers than all other groups, with the HBc-122/RIC group matching the former. The AP205-38 + RIC-122 group has similar titers to the P24-38 + RIC-122 and S60-38 + RIC-122 groups while the HBc-38 + RIC-122 and RIC-122 + RIC-122 groups performed similarly well but not at the same level as the HBc-122 + RIC-122 and HBc-122/RIC-122 + RIC-122 groups. All groups outperformed the PBS control group. Data are means +/- standard error for 8 different mice in each group.

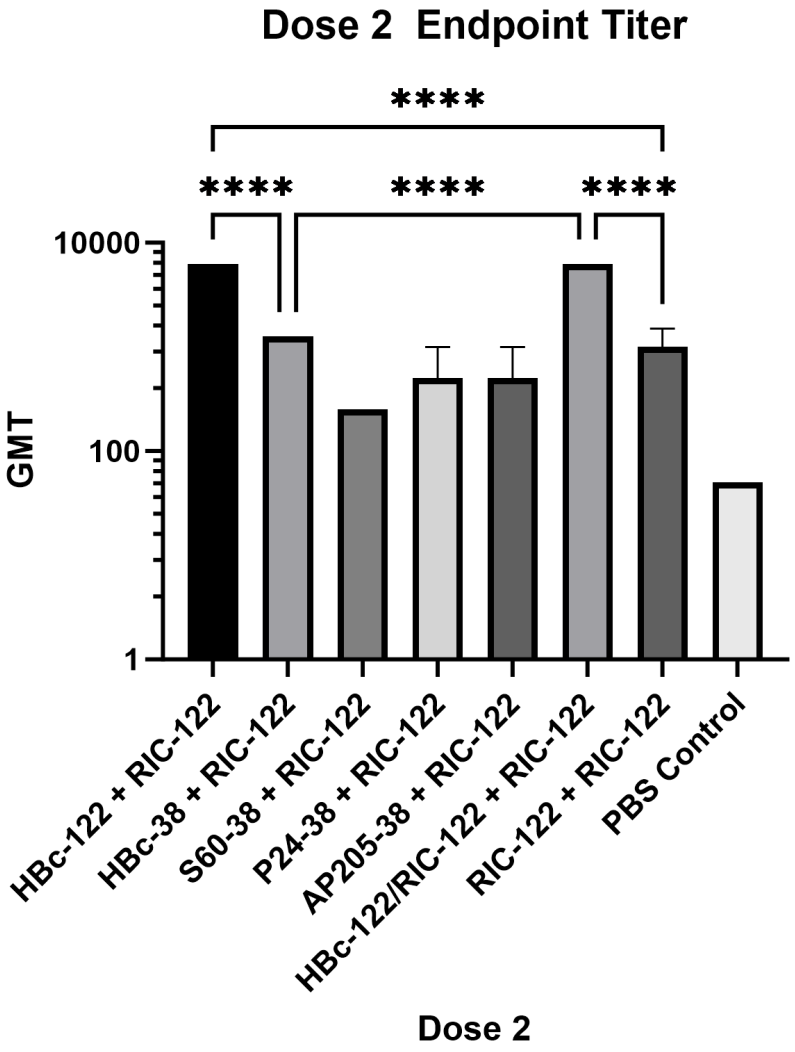


Figure 14. End Point Titer of Dose 2 Vaccinated Groups as Measured by ELISA. P24, S60, AP205, and HBc VLPs or nanoparticles and RIC antibody responses were measured after dose 1 groups were boosted with 2µg of HPV16 L2(aa14-122) presented on a RIC. Data are means +/- standard error for four individual samples of mouse group pooled serum. HBc-122 + RIC-122, and HBc-122/RIC + RIC-122 groups had the highest antibody titers. Both these groups were statistically significant in their difference from all lower titer groups but not one another. The HBc-38 + RIC-122 and RIC-122 + RIC-122 groups performed similarly, not statistically different, but were statistically lower when compared to the HBc-122 + RIC-122 and HBc-122/RIC-122 + RIC-122 groups. The AP205-38 + RIC-122 group has similar titers to the P24-38 + RIC-122 and S60-38 + RIC-122 groups and were not statistically different when compared to one another. All groups were statistically significant when compared to the PBS control group indicating an antibody response against HPV L2. All groups showed an increase in antibody titers as compared to the first dose.

Discussion

Extensive research into the cross-neutralizing potential of the HPV L2 protein has been performed, with recent experimentation and commercialization focusing solely on the RG1 epitope rather than all three cross neutralizing epitopes (Figure 8) (Ahmels et al., 2022; R. Gupta et al., 2020; Huber et al., 2021). One of the aims of this study was to determine the difference in antibody response to the RG1 epitope vs. an antigenic sequence containing all three cross-neutralizing epitopes. This was performed by constructing a hepatitis B Core antigen VLP expressing two repeats of the HPV16 L2(aa14-38) segment which contains RG1, labeled HBc-38, and a HBc VLP expressing all three cross neutralizing epitopes by expressing the HPV16 L2(aa14-122) segment containing RG1, Mab5, and Mab13 epitopes, labeled HBc-122.

It was also the aim of this study to identify novel virus-like particle/antigen presenting nanoparticle platforms that may be utilized in future vaccination efforts.

Three novel platforms were selected for study. First the bacteriophage based VLP AP205 was selected due to its simplicity and extensive use as a VLP platform showing promise against malaria, flu, and COVID-19 among other diseases (Janitzek et al., 2019; Liu et al., 2021). As Norovirus (aka Norwalk virus) has been extensively studied in the Hugh Mason lab at Arizona State University, two APN vaccine platforms based on the Norovirus VP1 protein were selected for use. Extensive work had previously been done by other groups to create stable nanoparticle vaccine candidates using the P-domain and S-domain of the norovirus VP1 protein to create the P24 and S60 nanoparticles used in

this study. Major enhancements include a stabilizing domain included in P24 and various amino acid substitutions which stabilized the S60 nanoparticle, all of which is outlined in Figure 9 (Tan & Jiang, 2019; Xia et al., 2018). Even with their increased stability, and the simplicity of the AP205 VLP and P24/S60 APNs, these vaccine platforms were difficult to purify in large quantities via a metal affinity column chromatography extraction/purification method. However, upon extraction and purification of the S60 nanoparticle following the same schema used to purify HBc VLPs a marked increase in purified sample was seen. This indicates that expression and purification may be similarly optimized for the AP205 and P24 vaccine platforms. In the future, GFP will be utilized to determine the overall expression levels of the P24, S60, and AP205 vaccine platforms, as well as their optimal extraction/purification conditions. Once this is completed it will be simple to determine which of the platforms is the best to utilize in future studies from a manufacturing perspective.

Figure 11 shows a marked increase in the efficacy of the AP205-38 VLP over the highly studied HBc-38 VLP when it comes to stimulating an antibody response after a single dose while both P24 and S60 nanoparticles failed to produce an immunization response when compared to the PBS control group. This indicates that the AP205 VLP may be highly immunogenic after a single dose when compared to other VLP and APN platforms, however the dose two data demonstrates that it's immunogenicity may be limited. It is important to note that the P24 and S60 nanoparticles may have failed to stimulate a robust immune response during the first dose due to a lower response to the platform and a low overall dose as the total amount of antigen presented was only 2µg of

HPV16 L2(14-38x2) administered subcutaneously without adjuvant, two factors that may preclude a notable response after a single dose (Zuckerman, 2000). When the second dose of RIC-122 was administered the P24 and S60 nanoparticle dose 1 groups saw L2 specific antibody titers reaching that of the AP205-38 + RIC-122 group. This may indicate that there was a stimulatory effect from the first dose of P24-38 and S60-38 but it did not promote a robust antibody response after just a singular dose. Additional studies, either using a different antigen or the same antigen at a higher concentration, or administered intramuscularly/intradermally, should be performed to determine if the APN platforms are able to stimulate an effective immune response after a single dose. As they are produced in a plant expression system in the case of this study, unlike all previous studies using the P24 and S60 APNs, there may be specific interactions during expression that truncated efficacy as well.

Figure 12 shows all vaccine groups antibody responses to a boost with HPV16 L2 aa14-122 RIC. HBc-122 elicited the highest antibody titers of any group, followed by the HBc-122/RIC group. However, it is important to note that the antibody amount may be expected to be higher in these groups, as L2 aa14-122 was utilized as a capture antigen for the ELISA this data is based on. Given that AP205-38 outperformed the HBc-38 in Figure 11 it is valid to speculate that, if AP205-122 had been used during vaccination, it may have outperformed HBc-122. A peptide of only the RG1 epitope is planned to be used in a future ELISA to determine if the response to AP205-38 is greater than HBc-122 under that specific circumstance, however a direct comparison should also be performed in a future mouse trial.

Figure 13 shows dose 2 GMT antibody titers of all groups, each of which was booster vaccinated with 2µg equivalent HPV16 L2(aa14-122) antigen presented by the RIC platform. The boost dose induced high antibody titers in the HBc-122 + RIC-122 group as well as the HBc-122/RIC-122 + RIC-122 and were found to have statistically undifferentiated antibody titers in Figure 14. In Figure 12 these two groups had a similar dose curve, however the 122/RIC-122 did slightly underperform the HBc-122 group after dose 1. This is likely due to the fact there may have been an issue with the RIC-122 preparation due being processed with a protein concentrator. If this is the case, or the RIC-122 used in dose 1 simply didn't provide an extensive immune response due to only 2µg being used the second dose of RIC-122 provided a large boost to both groups in terms of antibody titers.

Both the HBc-38 + RIC-122 and RIC-122 + RIC-122 groups had a middling response in Figure 13 with neither being statistically different from one another in Figure 14. As the dose 1 RIC-122 showed almost no response above the PBS control and was designated as most likely being a non-responder it is an interesting result to see RIC-122 + RIC-122 performing as well as it is. One explanation is that the dose 1 RIC-122 was flawed and the RIC-122 performed extremely well in what is essentially a first dose. Another explanation is that the 2µg of antigen presented on the RIC was not enough to create a primary immune response but acted as a priming dose which was then boosted by the second RIC-122 dose. While HBc-38 + RIC-122 performed as well as RIC-122 + RIC-122 it did not perform as well as other groups containing HBc. This may be because the

initial antibodies made after dose 1 were to the RG1 epitope and a second dose of RIC-122 could have increased the potency of those antibodies as it contains that epitope as well while also producing new antibodies against the additional epitopes present on HPV L2(14-122). If the majority of antibodies were initially against the RG1 epitope this may explain the underperformance as compared to the other HBc containing groups as they all contained HBc-122. This would also explain the difference seen in Figure 12.

Of note, AP205-38 + RIC-122 performed at approximately the same level as the S60-38 + RIC-122 and P24-38 + RIC-122 groups in both Figure 13 and Figure 14. This is of interest as the AP205-38 VLP outperformed both the S60-38 and P24-38 groups after only one dose (Figure 11). It is not clear why the AP205-38 + RIC-122 group did not see a boost similar to the HBc-38 + RIC-122 group in Figure 13 and 14, as AP205-38 performed as well as HBc-38 as can be seen in Figure 11. This may indicate that the RIC-122 boosting synergized with some VLPs/APNs better than others as the S60-38 and P24-38 groups performed very poorly until boosted with the RIC-122.

The AP205 VLP producing a highly effective immune response in dose 1 followed by a middling response after being boosted with the RIC-122 is of great interest as the VLP is simple and seemed to have promising initial results followed by poor performance. It may be that the synergy documented previously between RICs and HBc VLPs does not apply to all VLPs or that the AP205 VLP response is very short lived. Further study of the AP205 VLP should be performed to determine if there are innate immunological reasons for the lack of response in dose 2 or if the low amount of antigen may be part of

the cause. Further study into other bacteriophage based VLPs should be performed as there are many bacteriophages which could have potentially wide applications such as those extremophiles which reside in highly acidic conditions being utilized to create a VLP platform which remains intact through the stomach. Such a VLP would be optimal for use in edible vaccines and could be utilized in low-to-middle income countries to great effect.

The fact that the boosting dose of RIC-122 was able to increase the antibody titers of the RIC-122 dose 1 group to be similar to the HBc-38 + RIC-122 groups show the vast potential of this platform in inducing a robust immune response. However, it does not seem like this is true in all cases. While a low dose of antigen may be to blame the S60-38 + RIC-122 and P24 + RIC-122 groups did not perform extremely well when compared to the RIC-122 + RIC-122 group, the same can be said of the AP205-38 + RIC-122 group.

Both HBc-122 + RIC-122 and HBc-122/RIC-122 + RIC-122 performing very well in this vaccine study indicates that there is still a potential synergy between these VLPs and the RIC platform when a prime boost schedule is implemented. It will be important to test this potential more rigorously in the future by having direct comparisons of co-delivered and prime-boost groups. While the AP205 VLP and S60/P24 APNs did not perform well, their further study has merit as the dose utilized in this vaccine trial was low when compared to similar trials which may have resulted in underperformance of these vaccine platforms.

Overall, these results demonstrate the ability for various vaccine platforms to be produced in plants, purified by various methods, and result in an immune stimulating effect in the majority of cases.

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