HARNESSING THE MANUFACTURING POWER OF THE PLANT SYSTEM: PRODUCTION OF RECOMBINANT HUMAN INTERLEUKIN-2 IN *NICOTIANA BENTHAMIANA*

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ABSTRACT

HARNESSING THE MANUFACTURING POWER OF THE PLANT SYSTEM: PRODUCTION OF RECOMBINANT HUMAN INTERLEUKIN-2 IN *NICOTIANA BENTHAMIANA*

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Various expression systems are employed for the mass production of recombinant proteins to be used for pharmaceutical, diagnostic and industrial purposes. In this thesis, I have explored the feasibility of producing recombinant human interleukin-2 (IL-2) in the green tissues of transgenic *Nicotiana benthamiana* (tobacco). IL-2 is a biopharmaceutical of great importance as it is the standard immunotherapeutic treatment for late-stage metastatic melanoma and renal cell cancers. Using the whole-plant expression system, rather than relying on the current production platform of microbes would not only be cheaper, but the IL-2 might show greater biological activity due to processing in a eukaryotic host. The human IL-2 gene was codon optimized to maximize expression in this host system. Five DNA constructs were developed using this optimized gene and used to create stable transgenic plants. Each of the five constructs targeted IL-2 accumulation to different sub-cellular compartments. An additional construct fusing red fluorescent protein to the IL-2 protein was developed and used for a transient expression assay. The correct folding of this IL-2 fusion protein was confirmed via confocal microscopy. Western blotting of the stably transformed lines demonstrated maximum accumulation of the appropriately sized protein in the endoplasmic reticulum and chloroplasts. Protein extract samples were tested against murine splenic CD4+ T-cells from C57BL/6 mice and were shown to have specific biological activity. This research has demonstrated the efficacy of using tobacco as an expression system for the production of human IL-2.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Biopharmacology is a branch of science that studies the use of biotechnology to develop novel methods of drug production. The biopharmaceutical compounds produced using these methods are obtained from engineered biological sources that are not the native host for these compounds. To produce these compounds, the genomes of biological organisms are transformed by stable insertion of genes of interest. The transgene can come from any source and modern technology allows for insertion of genes into the genomes of many types of hosts.

The global market sector for biopharmaceutical drug proteins was reported in 2007 to be worth $86.8 billion and predicted to almost double by 2013 (Brock, 2008). Developing better and more efficient strategies to produce recombinant biopharmaceutical proteins will not only will be lucrative to the production companies, but also will help to make these drugs more freely available to poorer segments of the world's population.

Since its inception, this practice of harnessing biological sources as factories of protein production has expanded to produce a broad range of materials. Although a major focus of this practice has been on mass producing biopharmaceutical compounds including human enzymes, vaccines and antibodies, many commercial products such as industrial enzymes, plastics, nutritional supplements and diagnostic proteins also are actively pursued in this field (Lau and Sun, 2009; Sharma and Sharma, 2009).

USE OF PLANTS AS BIOREACTORS

1) Background. For years, plants have been used for food, feed, fiber, fuel, shelter, lumber, raw materials and as medicinal remedies. Being sessile, plants have evolved to produce a number of

metabolites to aid in their defense against biotic and abiotic stresses. Many plant-produced compounds (plant natural products) have served to aid humans because a number of them can produce therapeutic and medicinal effects.

Since the late '70s, with the advances in *in vitro* culture and genetic engineering, entirely new uses of plants have emerged. Genetic engineering has provided the means of transforming a plant into a biological factory for the mass production of novel materials. A gene of interest from any organism is cloned and inserted into the genome of a plant, allowing that plant to produce the protein(s) coded by such gene. This protein accumulates in the plant tissue, is harvested as crude cell lysate, purified and marketed for a designated purpose. This practice, termed "molecular farming," has a number of applications, and has proven quite promising in recent years because almost every aspect of the process has been optimized.

Transgenic plants are not the only system available for molecular farming. Many other systems including bacteria, fungi, mammalian cells, insect cells, and animals have been transformed to produce hererologous proteins and polymers. Every system has its own advantages and limitations associated with producing various transgene products. Microbes, for example, lack the necessary molecular machinery to process and express certain eukaryotic proteins properly. Mammalian and insect cell cultures are widely used for these purposes since they are eukaryotic; however, they can be costly to maintain and cannot easily be scaled up (Kusnadi et al., 1997). Fungi cultures also are widely used due to relatively simple purification steps necessary to isolate secreted proteins; however, hyper-glycosylation can occur in this system (Hood, 2002).

Effective and low-cost extraction methods are requirements for economic viability of any molecular farming operation, but are difficult to accomplish when using any type of production

platform. The more purity required, the higher the associated cost inevitably will be. For this reason, commercial production of antigens for oral animal vaccines and industrial enzymes are the main plant-produced transgenic proteins currently seen on the market, since oral vaccines do not require extraction and industrial enzymes do not need to be as pure as pharmaceutical compounds (Streatfield, 2006).

2) Benefits of using plants as bioreactors. Using plant systems has several economic advantages over other production platforms. For example, plants use the energy from the sun rather than energy input from other sources. Cell culture of any type often requires optimal environmental conditions, growth chambers, gasses, growth factors, nutrients, media, etc. As opposed to cell culture systems, plants also require less hands-on care, less attention, and less training to be cultivated successfully, so the personnel costs also are lower. When considering a field crop, the scale at which plants can be grown is almost unlimited. There is virtually no difference between growing transgenic versus non-transgenic plants, other than the federal and state regulatory aspects. Once the transgenic crop is created, it can be propagated like normal plants. Large scale production, processing, storage, and distribution of crops are already common practices. Therefore, once regulatory aspects are in hand, scaling up for commercialization of transgenic crops will be quite easy and affordable. Plants also are an attractive production platform due to the large biomass that can be generated as well as the conserved molecular machinery which allows folding of complex proteins from other eukaryotic proteins. Aside from differences in glycosylation patterns, protein processing in plants is similar to that of animals. Plants even possess the ability to assemble complex, multi-component proteins such as antibodies (Hein et al., 1991).

There also are several biological advantages to a plant production system. The use of plants to produce pharmaceuticals greatly reduces the risk of contaminating the protein of interest with human or animal pathogens since these would not be infecting the plant host organism (Lau and Sun, 2009). In addition, the biological activity of eukaryotic proteins might be sustained better by producing them in a eukaryotic system, rather than using a fermentation platform and conducting re-folding steps after extraction. The ability of plants to produce human proteins that retain biological activity has been well documented (Sharma and Sharma, 2009).

Current knowledge will only allow the transformation of a certain number of plant species. Some species are more difficult to transform than others. Although still relatively genotype-specific, at present, the most routinely transformed plant genera include maize, rice, tobacco, banana, soybean, alfalfa, pea, potato, and tomato (Sharma and Sharma, 2009). Selection of the best plant host for a particular molecular farming project depends largely on the objective of the project and specifications of the compound being produced. Thus the crop of choice should be decided on a case-by-case basis. For example, if an edible vaccine is being designed, a food crop should be utilized. If containment is a critical issue, a self-pollinating plant might be preferred. If the crop is to be grown in a particular climate, a crop that is acclimated to that climate should be used.

One of the major limitations of molecular farming is the ability to produce successfully a substantial amount of heterologous product to make this practice economically feasible and competitive with a bacterial fermentation production system. The amount of recombinant protein that needs to be produced to make this practice economic has a lot of flexibility depending on the value of the protein and the efficiency of recovery after purification. Many researchers have produced demonstrations which far surpassed the threshold of feasibility,

showing substantial economic gains when compared to other molecular farming platforms. Transgenic corn expressing heat-labile toxin (LT) reached expression levels of 1.8% TSP and then was increased five-fold through breeding methods (Streatfield et al., 2002). The majority of heterologous proteins expressed in plants are not this high. However, luckily there are many ways to increase the heterologous product yield. These methods are aimed at either increased transcription, increased translation or increased stability of the final heterologous product.

Concerning transcription, often a constitutive promoter is chosen rather than a native promoter, and this can have a tremendous impact on yield. One of the most widely used promoters is from the cauliflower mosaic virus 35S gene (CaMV35S). However, there also are many plant proteins that are continually expressed such as ubiquitin and actin, so their promoters are utilized as well (Sharma and Sharma, 2009). Experiments also have demonstrated that co-transformation with a transcription factor that drives the expression of the transgene can increase protein expression by almost four-fold (Yang et al., 2001). It is intuitive to think that if one were to increase the copy number, the heterologous protein yield also would be increased. However, copy number does not always have a direct correlation to expression, due to several factors. Mainly, transgenes present in multiple copies interact with the RNA silencing pathway. However, silencing is less of an issue if copy number is increased in increments like in breeding several independent transgenic lines, as opposed to creating a transgenic plant containing several copies from the start (Streatfield, 2006).

Concerning translation, specific viral 5' untranslated regions (UTR) can elevate protein expression levels (Pooggin and Skryabin, 1992) as well as codon optimization to align more closely the codons of the gene with the codon usage profile of the host. In regard to accumulation, targeting the same heterologous protein product to multiple different sub-cellular

compartments can increase the overall production (Hyunjong et al., 2006). Creation of transgene fusions with elastin-like polypeptides also has shown to not only increase accumulation of certain transgene products, but also aid in the ease of heterologous protein extraction (Patel et al., 2007). Several more approaches to boost heterologous product yield can be found in Table 2 of *Approaches to achieve high-level heterologous protein production in plants* (Streatfield, 2006).

3) Drawbacks of using plants as bioreactors. The major hurdle to overcome with this area of research is biological containment of genetically engineered organisms. The United States government has strict no-tolerance policies when it comes to regulation of genetically modified (GM) crops. This is not to say that GM crops all are hazardous to human health. Despite the public stigma about GM crops, they are found readily in the local grocery store. These crops have undergone intensive characterization to verify their safety for human consumption. These GM crops are modified to increase yield or afford the plant resistance to some type of stress, whether biotic or abiotic, and they have been adopted readily by American growers. In 2010, it was reported that of Americas soybean, cotton and corn crops, 93%, 78% and 70%, respectively were transgenic herbicide tolerant varieties (Fernandez-Cornejo, 2010). The main concern is GM crops not meant for consumption mixing with our food crops. In this situation, the transgenic plant would not be cleared for safety and there is a potential threat of detrimental effects on the consumer. Several incidents have been reported in which transgenic plants not meant for consumption (or that are, but have not yet received all the necessary clearance) have appeared in the human food chain. These inevitably result in major losses for the company that developed the plant. One of the earliest incidents occurred in 2000 when StarLink, a type of BT corn (expressing *Bacillus thuringiensis* toxin) produced by Syngenta, was found in the human food chain. This corn was meant only to be used as animal feed and was not approved for

human consumption (Harl et al., 2000). Another incident occurred in 2002. Volunteer transgenic corn was found growing in a soybean field in Nebraska that ProdiGene had used previously for testing of a crop they were developing. The transgenic corn was genetically modified to produce a vaccine for pigs. As a result of this discovery, 500,000 bushels of soybeans and 155 acres of corn were required to be destroyed. The cost of reparations came to about $3 million (Goldenberg, 2002). Even though there an extremely small amount of transgenic material was present and it posed no real risk to consumers, these actions were required by law.

Any transgene placed in a crop that also is used for human consumption has the potential to enter the human food chain. This may occur through pollen spread, accidental seed mixing or even the growth of volunteers. Despite the potential legal ramifications of contamination, several companies still are actively pursuing the use of food crops such as maize, rice, barley, wheat and soybean in molecular farming (Murphy, 2007). Examples include Planton (GmbH, Kiel, Germany, http://www.planton.de/) who currently uses potato as their main platform, and ORF Genetics (Reykjavik, Iceland, www.orfgenetics.com) who uses barley. Domesticated species often are bred for biomass, and are adapted to thrive in a number of different geographical locations. These species have gone through extensive breeding to become high yielding and rapidly growing. For these species, transformation methods are established and routinely performed (Ramessar et al., 2008). On the other hand, wild species offer only the benefit of having an appearance dissimilar to domestic species, which reduces the possibility of their accidental usage as a food crop (Sharma and Sharma, 2009). Several non-food crops, such as tobacco and several grasses, also are bred for biomass and can be a suitable alternative to reduce the possibility of food chain contamination.

Containment can be achieved through several methods when dealing with plants used for molecular farming. When dealing with a smaller scale operation, some crops are grown easily in greenhouses; however, many crops are more practically grown in field conditions. Containment is more difficult to achieve in these situations, and often is done in remote locations, far from other fields. Using species that typically are self pollinating will help to minimize the chance of propagating transgenic material into the wild through pollen spreading (Sharma and Sharma, 2009). One way to completely eliminate transgene contamination via pollen spread is chloroplast transformation. Chloroplasts can express several fully functional foreign proteins ranging in size, post-transcriptional modification requirements and even proteins composed of several subunits (Daniell, 2006). Additionally, expression levels of foreign proteins in plants with transformed chloroplasts have ranged much higher, from levels similar to nuclear transformants, up to 47% of total protein (Kumar and Daniell, 2004). Although chloroplast production sounds promising, it is not applicable to all tansgene products and to all crop species. Certain products require assembling and processing that can only be performed by the plant cell as a whole, and therefore research for molecular farming of nuclear transformed plants is still of the utmost importance.

Another alternative to reduce the chance of human consumption of transgenic material not approved for human consumption is through the use of tissue-specific promoters. There are several reasons why one would want to express a protein in a tissue or organ-specific manner. For example, expression in all tissues could be deleterious to the normal development of the host plant, or perhaps only one portion of the plant can be processed easily, and expression in other tissues is not necessary (Sharma and Sharma, 2009). Because of the concern of crop-mixing and possible introduction into food supplies, many transgenes are specifically produced in green

tissues (excluding seeds and fruit). The Plant Research International of Wageningen University and Research Center (Wageningen, The Netherlands, http://www.pri.wur.nl/UK/products/ImpactVector/) has developed a series of transformation vector plasmids (ImpactVectors^{TM}) containing the light-regulated Rubisco promoter. These ImpactVectors^{TM} have been used, for example, to produce biologically active microbial endo-cellulase in the green tissues of maize (Mei et al., 2008). Production of heterologous proteins in green tissues such as leaves and stems is an attractive option, especially when considering biomass; however, these organs are not the most ideal environment for accumulation, due to the high water content which can result in low protein stability. Stability and storage is a key factor that must be considered when thinking about large scale production and what the requirements will be for the harvesting operation (i.e. if the material must be transported or stored for processing). For this reason, many researchers have turned to seeds for a solution since these organs are designed for long term storage of proteins. There have been several successful demonstrations of the seed-targeted transgenes, such as human growth hormone (Leite et al., 2000) and single chain antibodies (Fiedler et al., 1997). A comprehensive table comparing the advantages and disadvantages of various plant production systems can be found in Table 1 within the article, "Maize plants: An ideal production platform for effective and safe molecular pharming" (Ramessar et al., 2008).

4) Examples of Efficacy.

4.1) Commercialization of plant derived recombinant proteins. ProdiGene Inc. (College Station, Texas, www.prodigene.com) was the first company to market commercially recombinant proteins derived from a plant system. Their initial products in the late '90s both were from corn. Avidin, a diagnostic reagent, is produced at 1.5-3% TSP within the seed of their transgenic lines and is physically and functionally identical to native avidin (Elizabeth E. Hood,

1997). This avidin was produced at approximately $50/kG. A press release revealing this technology quotes the president of Stauffer Biotech (a partner of ProdiGene) as saying "To produce the same amount with traditional methods would cost the equivalent of $1000 per kilogram." (SeedQuest, 1998) Their other initial product was β-glucuronidase, also produced within seed and found to be similar to the native protein. The expression did not exceed 0.7% TSP, but even at these levels, this proved to be an economically viable system for commercial production (Witcher et al., 1998). Since the release of these products, ProdiGene has filed numerous patents on related technologies, and has put new recombinant products on the market.

Several other companies also actively are pursuing plant production platforms for molecular farming purposes. Ventria Bioscience (College Station, Texas, www.ventria.com) specializes in proteins targeted for accumulation in rice grain. Using this platform, currently they market recombinant human lactoferrin and lysozyme, under the names Lacromin and Lysobac, respectively. Both of these compounds are available from Sigma-Aldrich (Louis, MO, USA, www.sigmaaldrich.com). Ventria Bioscience also is progressing on the commercialization of rice-derived recombinant human transferrin.

SemBioSys Genetics Inc. (Calgary, Canada, www.sembiosys.com) has developed a system to easily extract and purify proteins of interest using the plant's natural oil-bodies. Their host plant is the safflower due to its natural oil production. Their method involves creating a fusion protein of their product of interest with oleosin, a protein that resides on the surface of oil-bodies. Not only is the product produced and accumulated in large quantities, but due to its location, it can be extracted and purified at a relatively low cost. SemBioSys has demonstrated the efficacy of this system with a variety of proteins including safflower-produced chymosin which is an industrial enzyme used in cheese making. The two main pharmaceuticals they

currently are producing with this platform are insulin and Apo AI_{Milano}. Both drugs have shown to be bioequivalent to their recombinant counterparts currently used for treatment, and the company is actively seeking partnership with manufacturing companies to commercialize these compounds.

4.2) Use of recombinant plants in vaccine production. In particular, plants have been viewed as a highly desirable system for vaccine production. One side of the spectrum of this research is edible oral vaccines from transgenic plants made to express certain pathogenic antigens in their edible tissues. These plants then are consumed, resulting in a mucosal immune response at the epithelial membranes along the digestive tract (Mason and Arntzen, 1995). This system is cheaper than, and just as effective as, many oral vaccines designed to invoke a mucosal immune response. Scientists have demonstrated that within mice, only four feedings of 5g transgenic potato tubers expressing a subunit of heat-labile enterotoxin (LT) were necessary to evoke a humoral immune response (Mason and Arntzen, 1995). Although this vaccination method holds much potential, especially for developing countries, there still is a great deal of optimization required to make this system effective. One of the main concerns is controlling dosage so that the patient ingests enough antigen to elicit an immune response, but not so much that the body becomes tolerant as it does with proteins that are part of the normal diet (Mason and Arntzen, 1995; Phoolcharoen et al., 2011). A major benefit of this system is that there is no need for extraction or purification, significantly reducing the cost.

The other side is using plants to mass produce these antigens that then are injected subcutaneously with other adjutants. Early studies investigating the immunogenic response elicited from plant-derived recombinant hepatitis B surface antigen, as compared with the already used yeast-derived antigen. These studies reported equivalent antigenic responses from

immunized mice and confirmed that antigen from the plant system preserved B- and T-cell epitopes necessary to elicit an adaptive immune response (Thanavala et al., 1995).

It is quite clear that there is a tremendous amount of potential in these types of recombinant protein production systems. For my thesis research, I chose to express human interleukin-2 (IL-2) in the green tissues of tobacco plants. There were several reasons why IL-2 was chosen. IL-2 is a small, simple protein with only one disulphide bond. Previous work in our laboratory regarding a protein with several disulphide bonds suggested that proper folding of this protein was challenging. Additionally, there was prior evidence that IL-2 could properly be folded by this host system (Magnuson et al., 1998). Also, this protein is used for treatment in a clinical setting. Since the production cost and demand for this protein is relatively high, developing a method of lowering the cost of production should make this biopharmaceutical more readily accessible to a larger number of patients who would benefit from IL-2 treatment. A comprehensive explanation of IL-2 structure, function and clinical uses can be found below.

INTERLEUKIN-2

1) Structure and Function. Many immunological functions within humans are regulated by a group of protein hormones known as cytokines. Each of these proteins has a unique role in controlling a diverse array of functions including inflammation, tissue repair, and protection from both domestic and foreign threats to the body. Each cytokine is secreted by a subset of immune cells in particular circumstances to evoke an immune response specific for the situation the body has detected. Most cytokines have several roles and not only direct the actions of immune cells, but also are important in directing lineage from progenitor cells to these final effecter forms (Kelso, 1989).

One family of cytokines is known as interleukin. There are 35 known interleukin proteins, many of which have been well characterized in structure and function. Interleukins, as a whole, play a role in directing numerous physiological responses of the immune system. Some functions are shared between many ILs such as immunoglobulin synthesis and class switching, T- and B-cell expansion and cytokine synthesis (Kelso, 1989). Other functions are restricted to just one or a few ILs such as angiogenesis (Langowski et al., 2006), osteoclastogenesis (Wei et al., 2005) and acute phase protein production (Wigmore et al., 1997), just to name a few. IL-2 is one of the interleukins that has a significant amount of clinical relevance.

IL-2 first was called T-cell growth factor because of its ability to sustain activated T-cells in culture (Gillis et al., 1978). Although it is produced mainly by activated helper T-cells, it also is secreted in part by cytotoxic T-cells, medullary thymocytes and certain large granular lymphocytes (Pontzer et al., 1992). Its target cells include T-cells, B-cells, natural killer (NK) cells, monocytes, and macrophages. These cells have surface receptors for IL-2, although different subunits with varying affinities are expressed under different circumstances (stimulated vs. resting). The high affinity receptor seen on activated cells is composed of three subunits, α, β, and γ, specific for the IL-2 molecule. The α component is responsible for initial binding of IL-2 to the receptor and the β component serves to hold the IL-2 and lock it in place. The γ subunit is also a common component of receptors for other interleukins, as well as other cytokines (Stauber et al., 2006). The ability to vary receptor expression allows the immune system to fine-tune its responses to stimuli.

IL-2 has a different effect on each of the cell types with which it interacts. It is most notable for its originally discovered function which is its ability to provide T-cells with the signal necessary to proliferate. Without this necessary signal transduction, mitosis would not occur and

T-cells would remain in the G0 or G1 phase. When a T-cell receptor is stimulated, it becomes activated and proceeds from G0 to G1. There is a G1 arrest that does not allow the cell to proceed further. It is a signal cascade provided by the stimulation of the IL-2 receptor which allows the T-cell to bypass G1 arrest and proceed through the cell cycle. Several factors determine whether a T-cell will be committed to divide. These include the IL-2 concentration of the environment, the length of time the cell interacts with IL-2 and the density of receptors present on the cell (Pontzer et al., 1992). It is an all-or-nothing response, so conditions must be right for proliferation to occur.

One of the biochemical responses of a stimulated T-cell is to secrete IL-2 which functions in an autocrine fashion to allow this stimulated T-cell to proliferate. IL-2 also functions in a paracrine fashion to stimulate surrounding immune cells. Upon binding, the ligand receptor complex is internalized. I diagram showing the primary roles of IL-2 in immune regulation can be found in Figure 1.

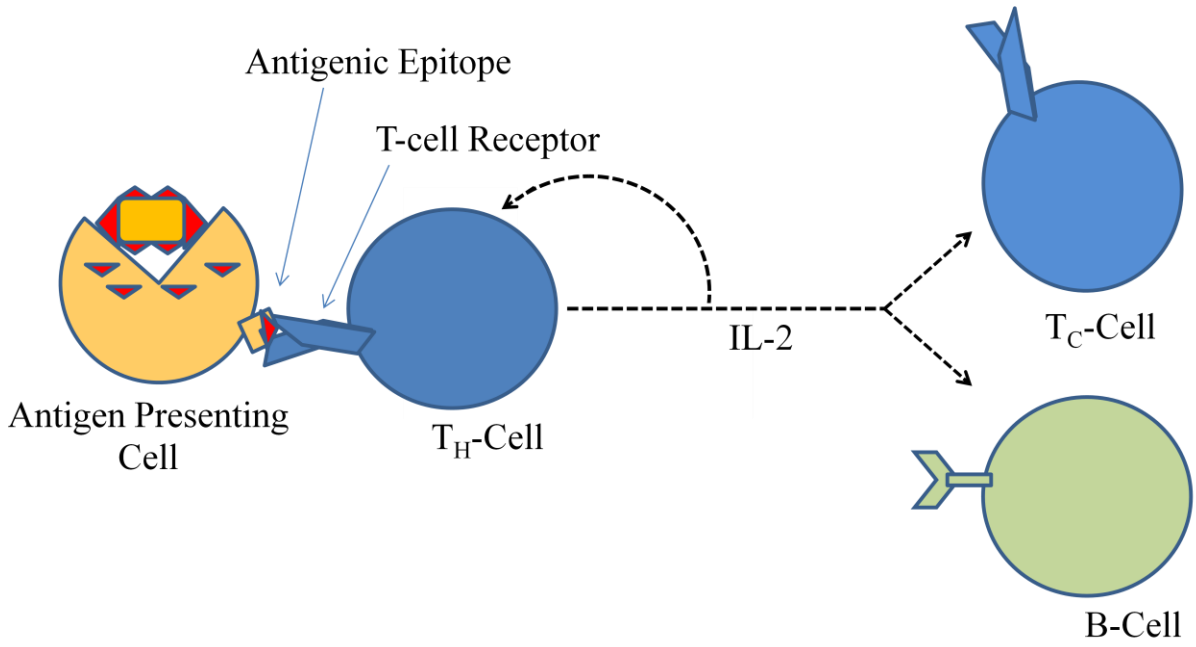

Figure 1. Diagram depicting the roll of IL-2. Here we can see an antigen presenting cell presenting an antigenic epitope to a T helper (T_H) cell. The stimulation of the T-cell receptor induces IL-2 secretion. This IL-2 functions in an autocrine fashion to stimulate the T_H cell to divide, as well as a paracrine fashion to activate various other immune cells. The targets shown here are a cytotoxic T-cells (T_c) cell as well as a B-cell. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis

In humans, IL-2 is a 4intron-3exon gene that is translated as a 153 amino acid peptide. The first 20 amino acids are a signaling peptide to direct the peptide into the secretory pathway. This leader sequence is cleaved to form a mature 133 amino acid protein. Production in its native host results in a glycosylated protein, although glycosylation is not required for biological activity (Pontzer et al., 1992). There are three cystine residues with one disulphide bond between residues 58 and 105. This bond is required for biological activity and demonstrated by mutation studies (Pontzer et al., 1992). The tertiary structure is composed of six α-helices, four of which form a bundle.

2) Clinical Applications. The ability of high-dose subcutaneous IL-2 injections to reduce tumor burden and prevent metastasis first was demonstrated in mice (Rosenberg et al., 1985). The results from these initial studies led to clinical trials in humans. The initial clinical trials to determine efficacy of IL-2 treatment for late-stage metastatic melanoma in humans were conducted from 1985 - 1993. Eight clinical trials were carried out over this period of time including a total of 270 patients. These patients all had reached stage III or IV which typically is too far along for curative surgery and is associated with a 95% mortality rate (Atkins et al., 1999). The regimen included 600,000 or 720,000 IU/kg administered every eight hours for five days. This was followed by about one week of rest due to the toxicity of IL-2, and repeated for a second round of IL-2 treatment. Each block of treatment was repeated 6-12 weeks. These clinical trials reported an overall response rate of 16%. This included 17 individuals who showed no sign of remaining tumors and 26 individuals who showed over 50% tumor reduction (Atkins et al., 1999). Based on the data collected in these clinical trials, the Food and Drug Administration (FDA) approved IL-2 for the treatment of end-stage metastatic melanoma in 1998 (Mouawad et al., 2010). IL-2 was the first and only FDA-approved immunothereputic drug for melanoma until 13 years later in March of 2011 (Diehl, 2011). At this time, the FDA approved a new drug developed by Bristol-Meyers Squibb (New York, NY, USA, www.bms.com) called Yervoy. This is a recombinant human-monoclonal antibody against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (RxList, 2011). This antigen is found on T-cells and serves an inhibitory roll. Yervoy functions by blocking CTLA-4 ability to send inhibitory signals to the T-cell, resulting in sustained T-cell activity.

The same IL-2 regimen used for treatment of melanoma patients also was carried out in 255 patients with renal cell carcinoma. This disease also is lethal, with a median survival of one

year. These clinical trials reported 12 (5%) patients showing no sign of remaining tumors and 24 (9%) showing over 50% tumor reduction (Fyfe et al., 1995). Although the response rate of patients in these studies is lower than the 15-20% reported from clinical trials of other treatments, the responses from IL-2 are longer lasting (Fyfe et al., 1995). This statement also holds true for treatment regimes for metastatic melanomas (Mouawad et al., 2010).

Although case studies of IL-2 treatment in other diseases have been documented, to date, metastatic melanoma and renal cell cancer are the only two diseases in which large scale clinical trials of IL-2 therapy have been performed. Some of these other small-scale case studies have demonstrated potential efficacy of IL-2 treatment in other diseases such as leukemia (Maraninchi et al., 1991), but larger studies are necessary before efficacy can be confirmed.

IL-2 often is administered along with other cytokines that work synergistically. One study of mice injected with Friend leukemia cells (infected with the murine Friend leukemia virus) concluded that treatment with IL-2 in conjunction with IL-1β produces a stronger anti-tumoral response than treatment with IL-2 alone (Belardelli et al., 1989). Other cytokines such as interferon alpha (IFN α) and tumor necrosis factor (TNF) also produce a much stronger anti-tumoral response when combined with IL-2 treatment than either treatment alone (Pontzer et al., 1992). The response rates of 425 patients with metastatic renal cell carcinoma receiving a regimen of either IL-2, IFNα-2a, or both were 6.5%, 7.5% and 18.6%, respectively (Negrier et al., 1998).

In comparison with results associated with chemotherapy alone, response rates for immunotherapies are not better. However, those who do respond to IL-2 treatment exhibit median response times measured in years, compared with the response times exhibited by chemotherapy patients measured in months (Mouawad et al., 2010). Immunotherapies also have

proven to provide a much higher curative rate than alternative strategies. Additionally, much optimization still is underway with immunotherapies. The combinations of drugs, dosages, and treatment regimes for immunotherapies are still being studied and fine tuned. The progress in clinical research has produced a positive trend in complete responses, and we are discovering ways of lowering much of the toxcicity associated with immunotherapies (Atkins et al., 1999), allowing them to be used on a larger subset of patients. There still is no set standard for treatment of metastatic disease since every case is unique; however, most scientists agree that a combination of treatment strategies will prove to be the most effective. Therefore, it is critical that we continue to pursue this type of immunotherapeutic research.

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CHAPTER 2

PRODUCTION OF RECOMBINANT HUMAN INTERLEUKIN-2 IN *NICOTIANA BENTHAMIANA*

INTRODUCTION

The current production platform used for human IL-2 is recombinant *E. coli.* This product was developed by Novartis Pharmaceuticals and released commercially under the name Proleukin (Aldesleukin) in 1998. Proleukin is regularly used in clinical settings to treat patients with metastatic melanoma and renal cell cancers. It is also used in research laboratories for the proliferation and maintenance of T-cells in vitro. The standard IL-2 immunotherapy regimen requires administration of 600,000 IU/kg (0.036mg/kg) IL-2 with each injection. These injections are provided every 8 hours for a maximum of 14 consecutive doses. This regimen is then repeated after 9 days of rest, completing one course of treatment. Following treatment, patients are reevaluated. If the patient is responding to treatment, an additional course of treatment can begin at least 7 weeks from completion of the last course (Proleukin - aldesleukin injection, 2009). The average cost of IL-2 alone for each course of treatment was reported to be about $11,400 USD (MTRAC, 1996). Reducing the cost of IL-2 production will not only benefit the manufacturer, but can drive down the cost of IL-2 therapy, allowing it to be more accessible to patients that require treatment.

The research described here details the steps taken to develop a more economic production platform for IL-2. A plant production system was favored due to the wealth of benefits attributed to this system discussed in Chapter 1. Three different types of plant expression systems have already been studied for the production of human IL-2. Two of the projects showed accumulation of biologically active IL-2 in plant organs that are used for food: potato tubers (Park and Cheong, 2002) and tomato fruits (Cui et al., 2008). The third project

showed accumulation of biologically active IL-2 in tobacco cell culture (Magnuson et al., 1998). To date, there have been no demonstrations of IL-2 production in a whole field crop, making use of the entire biomass of the plant.

The green tissue of *Nicotiana Benthamiana* (tobacco) was chosen as the expression platform due to 1) ease of transformation 2) substantial biomass 3) non-food crop 4) extensive demonstrations of biologically active recombinant protein production 5) low cost of production and 6) interest of tobacco growers for new uses of this crop.

MATERIALS AND METHODS

1) Construct Design.

1.1) Interleukin-2 gene analogue. Human IL-2 is a 153 amino acid molecule that is processed as it passes through the endoplasmic reticulum and golgi to yield a mature protein of 133 amino acids. The first 20 residues of the N terminus comprise an endogenous secretory signal that are cleaved upon entry into the secretory pathway. Shown below is the 399 nucleic acid sequence and the 133 amino acid sequence of the mature protein.

GCACCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACTGGAGCATTTACTGCTG
GATTTACAGATGATTTTGAATGGAATTAATAATTACAAGAATCCCAAACTCACCAGG
ATGCTCACATTTAAGTTTTACATGCCCAAGAAGGCCACAGAACTGAAACATCTTCAG
TGTCTAGAAGAAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGCAA
AAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAATAGTTCTGGA
ACTAAAGGGATCTGAAACAACATTCATGTGTGAATATGCTGATGAGACAGCAACCA
TTGTAGAATTTCTGAACAGATGGATTACCTTTTGTCAAAGCATCATCTCAACACTGA
CTTGA

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL
EEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNR
WITFCQSIISTLT

Even though the native host of this IL-2 gene and expression host are both eukaryotic, codon optimization was performed on the coding sequence in order to optimize translation in the

expression systems. The Genscript rare-codon-analysis-tool, available at http://www.genscript.com/cgi-bin/tools/rare_codon_analysis, was utilized to predict expression efficiency of this gene within the expression hosts of interest. This tool also compares these parameters to those of the optimized gene sequence that is generated from their algorithm. Since this gene was designed to be used for corn transformation as well, an optimized sequence was developed for expression primarily in *Zea mays* (corn) and secondary expression in *Nicotiana benthamiana* (tobacco). A section of this Genscript report showing alignments of the native and optimized sequence can be found in Appendix B. This section of the report also shows specifically which nucleotides were changed and provides a protein alignment to verify that the correct polypeptide sequence will be produced.

Both the native gene as well as the optimized gene are given a Codon Adaptation Index (CAI) on a scale of 0-1. This CAI score is calculated based on the frequency at which optimal codons for the expression system are used, with 1 being perfect for the chosen expression host. For expression in corn, the native gene has a CAI of 0.60, while the optimized gene has a CAI of 0.71. For expression in tobacco, the native gene has a CAI of 0.75, while the optimized gene has a CAI of 0.74. As apparent from the report, the native sequence is predicted to express well in tobacco, but poorly in corn. The optimized version is predicted to show increased expression in corn, and shouldn't significantly affect expression in tobacco. Therefore, this optimized version was used for transformations. Shown below is the optimized DNA sequence, coding for the same polypeptide.

GCACCCACTTCAAGCTCTACAAAAAAGACTCAACTTCAACTGGAACATCTGCTGCTG
GACCTTCAAATGATACTCAACGGGATTAATAACTACAAGAATCCAAAACTGACTAG
GATGCTTACATTCAAGTTCTACATGCCTAAGAAAGCAACTGAACTCAAGCACCTGCA
GTGCCTTGAGGAAGAGTTGAAACCGCTCGAAGAGGTCCTCAATCTGGCTCAATCTAA
GAACTTCCATTTGAGGCCAAGGGATCTCATATCAAATATCAACGTGATCGTTCTTGA

ATTGAAAGGCTCCGAGACTACTTTCATGTGTGAATACGCTGACGAGACCGCCACGAT
AGTTGAGTTCCTTAACCGCTGGATTACCTTTTGTCAGAGTATCATTAGCACACTTACC

Flanking restriction sites were added to allow for cloning into vectors. The vector series that was used has four restriction sites to choose from at the multiple cloning site. The two restriction sites at the outermost edges were chosen in order to eliminate excess residues. These restriction sites are NcoI and BglII. Shown below is the sequence of the gene with restriction sites. Two residues were added between the restriction sequence and the gene to maintain the proper codon frame. The sequence of this oligo is shown below. The restriction sites are capitalized, the residues to maintain reading frame are italicized and the sequence coding for mature IL-2 with no stop codon is in lower case letters.

CCATGG *CT* gcacctactt caagttctac aaagaaaaca cagctacaac tggagcattt actgctggat ttacagatga
ttttgaatgg aattaataat tacaagaatc ccaaactcac caggatgctc acatttaagt tttacatgcc caagaaggcc acagaactga
aacatcttca gtgtctagaa gaagaactca aacctctgga ggaagtgcta aatttagctc aaagcaaaaa ctttcactta
agacccaggg acttaatcag caatatcaac gtaatagttc tggaactaaa gggatctgaa acaacattca tgtgtgaata
tgctgatgag acagcaacca ttgtagaatt tctgaacaga tggattacct tttgtcaaag catcatctca acactgact *GC*
AGATCT

1.2) Expression Cassette.

1.2.1) Stable Transformation Vectors. There were several requirements to keep in mind when considering the expression cassette. First, it was decided that a tissue specific promoter should be utilized. There are several reasons why one would want to express a protein only in certain plant organs. Expression in all tissues could be deleterious to the normal development of the plant. Perhaps only one portion of the plant can easily be processed, and expression in other tissues will not be necessary (Sharma and Sharma, 2009). Because of the concern of crop-mixing and possible introduction into food supplies, many transgenes are specifically produced in green tissues (excluding seeds and fruit). For example, should transgenic corn seed not meant for consumption get mixed in with seed for a food crop, no recombinant protein will be produced

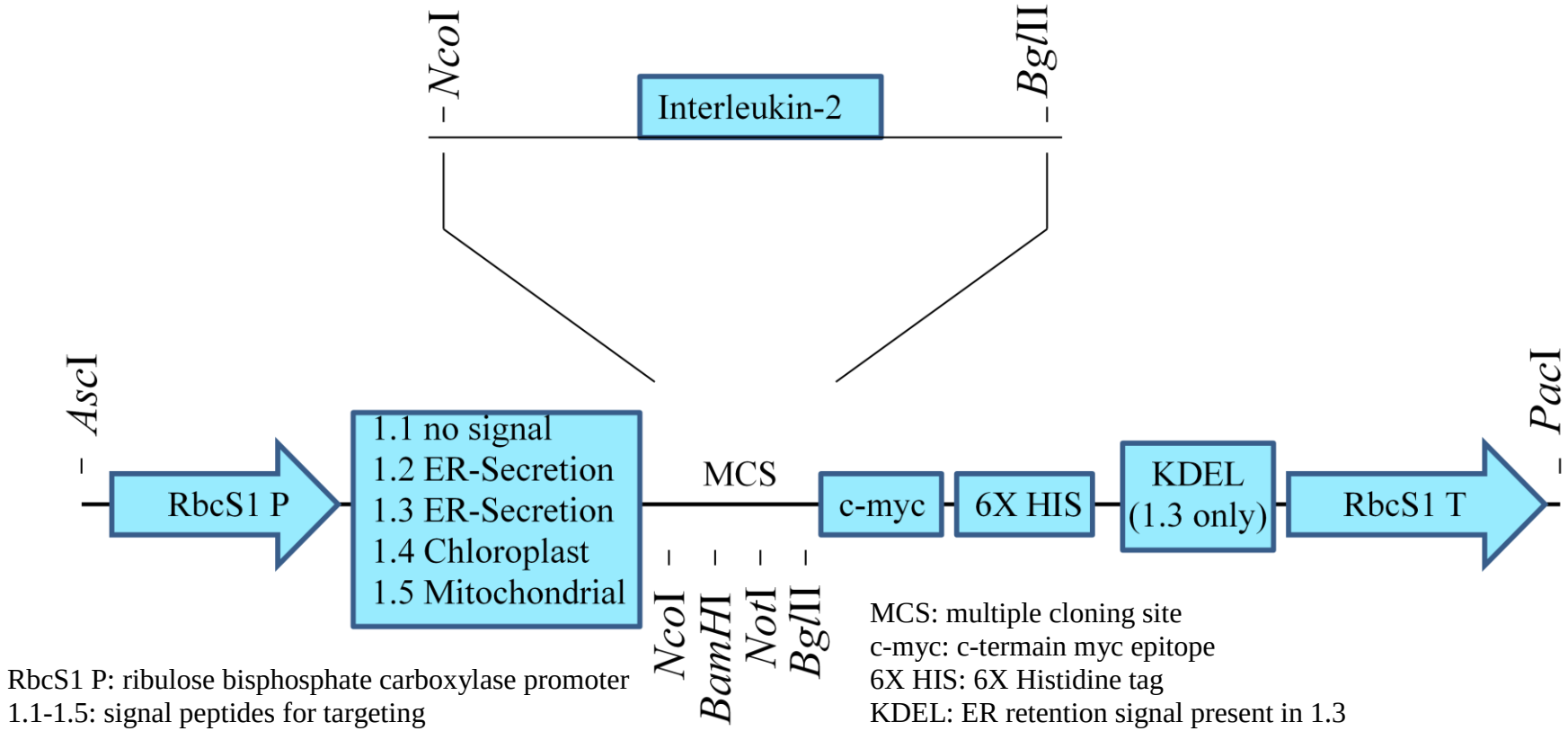
in the seed and therefore there will be no risk of accidental ingestion of the recombinant protein. Tobacco is not an edible crop, but the constructs used here were also used to transform corn, so this concern was still valid.

Second, the heterologous protein is to be targeted to several different sub-cellular compartments, which means at least five different vectors must be utilized with various targeting peptides. Third, the protein eventually must be extracted from the total soluble protein present in the tissue. For this to be easily accomplished, a histidine tag should be added to the C-terminus of the peptide, allowing for binding and elution from a nickel column.

Plant Research International of Wageningen University and Research Center has developed a series of transformation vector plasmids which fit all of these requirements (see: http://www.pri.wur.nl/UK/products/ImpactVector/). In this Impact Vector (I.V.) series, expression is driven by the light-regulated ribulose bisphosphate carboxylase small subunit (RbcS1) promoter and terminator from *Asteraceous chrysanthemum*. Rbc is involved in carbon fixation and therefore produced solely in photosynthetic tissues such as leaves and stems.

Each of the five vectors allows for targeting to a different location within the cell. I.V. 1.1 contains no signaling peptide, allowing for cytosolic accumulation. I.V. 1.2 contains a signaling peptide from sea anemone equistatin which allows the protein to be secreted to the apoplast. I.V. 1.3 is the same as I.V. 1.2, however a KDEL retention signal was added to the C terminus, allowing proteins to accumulate in the endoplasmic reticulum. I.V. 1.4 harbors a signal peptide from *Chrysanthemum morifolium* small subunit which targets the protein to the stroma of the chloroplast. Finally, I.V. 1.5 utilizes the yeast CoxIV secretion signal to target the protein to the mitochondrial matrix.

This vector series also contains a segment downstream that, when cloned in frame with the gene insert, provides the addition of a C-myc epitope and poly histidine tag to the C-terminus of the protein. The c-myc epitiop provides a peptide section for which antibodies are readily available and the poly histidine tag allows for easy extraction of the protein from cell extracts. These vectors harbor a gene for ampicillin resistance. A depiction of the expression cassette can be seen in Figure 2.



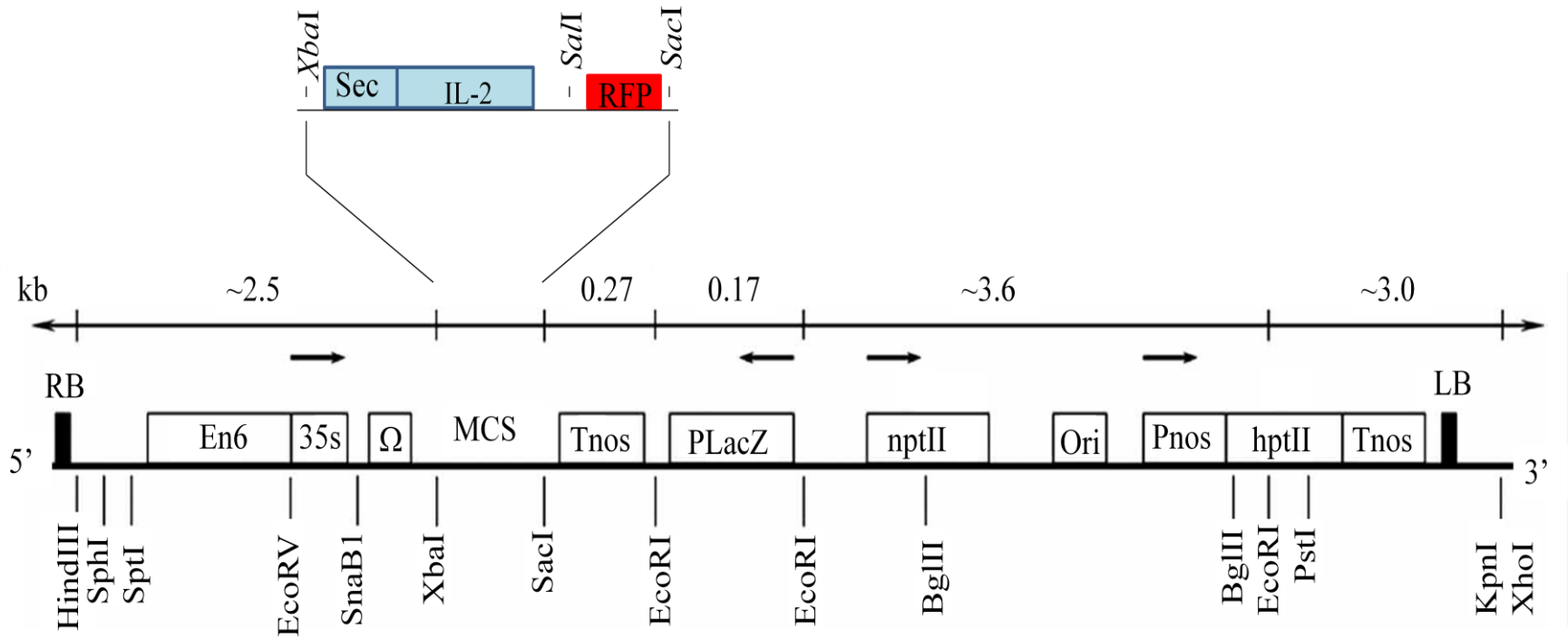
RbcS1 P: ribulose bisphosphate carboxylase promoter
1.1-1.5: signal peptides for targeting

MCS: multiple cloning site
c-myc: c-termain myc epitope
6X HIS: 6X Histidine tag
KDEL: ER retention signal present in 1.3
RbcS1 T: ribulose bisphosphate carboxylase terminator

Figure 2. Expression cassette from the gene constructs used to transform tobacco plants. Expression is driven by the ribulose bisphosphate carboxylase promoter (and terminator from *Asteraceous chrysanthemum.* A series of five vectors was used, each with a different (or no) signaling peptide. The IL-2 gene was cloned in frame with C-myc and 6X HIS tags to allow for easy protein extraction. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

1.2.1) Transient Transformation Vector. The requirements for the transient expression cassette were different. This construct did not require tissue specific expression, but did require a fusion protein that would allow for visualization of heterologous protein accumulation. Plasmid pVKH18En6 35s St-m-RFP suits this requirement and was kindly provided by Dr. Federica Brandizzi (Michigan State University, Plant Biology Department). This is a binary vector which allows for a gene to be inserted upstream of the gene for red fluorescent protein, resulting in a fusion protein. This vector also affords kanamycin resistance for selective purposes.

To create the insert, PCR was performed using specially designed primers with impact vector 1.2 containing the optimized IL-2 gene as a template. The forward primer sequence had a leader sequence, an XbaI restriction site, two nucleotides to maintain the proper reading frame, and finally the first 15 nucleotides of the secretory signal peptide. The primer sequence is 5'CAGGACG-TCTAGA-TG-TCTCTTAGCCAGAAC3', with dashes indicating the different sections of this primer. The reverse primer was designed with a leader sequence, a SalI restriction site, two nucleotides to maintain reading frame, and the last 15 nucleotides of the coding region. The sequence is 5'CATGACC-GTCGAC-AT-GTGATGGTGATGGTG3', with dashes indicating the different sections of this primer. This insert was then cloned into pVKH18En6 35s St-m-RFP in-frame with the RFP so that the coding region will contain the IL-2 gene (including tags) with an n-terminal secretory signal and a c-terminal RFP fusion. Figure 3 shows a depiction of this construct with the restriction sites necessary to perform the necessary cloning.



RB: T-DNA right border
En6 35s: 6X enhanced CaMV 35s promoter
Ω: TMV 5' leader
MCS: multiple cloning site
Tnos: nos terminator
PLacZ: LacZ promoter
nptII: kanamycin resistance marker
Ori: ColE1 origin of replication
Pnos: nos promoter
hptII: hygromycin resistance marker
Tnos: nos terminator
LB: T-DNA left border

Figure 3. Expression cassette for the gene construct used for the transient expression assay (pVKH18En6 35s St-m-RFP). This image shows a modified portion of Figure 2 in the paper discussing the development of this vector and demonstrating its capabilities (Sparkes et al., 2006). A 6X cauliflower mosaic virus 35S promoter is used to drive the expression of secreted IL-2 fused to red fluorescent protein.

2) Transient Transformation of Tobacco.

2.1) Infiltration. ElectroMAX *A. tumefaciens* strain LBA4404 from Invitrogen (Carlsbad, CA, USA, www.invitrogen.com) was transformed via electroporation with pVKH18En6St-m-RFP containing the IL-2 gene analogue. Additionally, this *A. tumefaciens* strain was transformed with pVKH18En6St-m-RFP empty vector. The cells were handled as indicated by the manufacturer's instructions. The instrument used to carry out electroporation was a BTX ECM 630 and the parameters were as indicated; Mode: 2.5kV/RESISTANCE High Voltage; Capacitance: 50μF; Resistance: 129 ohms; Chamber Gap: 1mm; Charging Voltage: 1.44kV; Desired Field Strength: 14.4kV/cm; Desired Pulse Length: 5.0 msec.

Cells that had undergone electroporation were incubated in liquid YM media for three hr with shaking at 30°C, followed by plating on YM containing 100mg/L kanamycin. The NPTII gene on the plasmid confers kanamycin resistance which was used to select for transformants. After two to three days of incubation at 30°C, individual colonies were isolated, cultures were inoculated, and plasmid was isolated for sequencing to ensure the construct was correct. Transformation of tobacco plants was carried out once sequence information was confirmed.

Infiltration was the method used to transiently infect tobacco leaves of the Samsun variety. *A. tumefaciens* strain containing the binary vectors of interest (pVKH18En6St-m-RFP containing the IL-2 gene analogue as well as pVKH18En6St-m-RFP empty vector) were cultured overnight at 28°C in liquid LB media containing gentamicin (25mg/L), rifampicin (50mg/L) and kanamycin (100mg/L). One mL of culture was transferred to an Eppendorf tube and centrifuged for five min at 8K RPM. Infiltration media (IF) was prepared as specified in Table 1.

Table 1. Components necessary to prepare infiltration media used for transient transformation.

COMPONENT	AMOUNT
MES 500mM pH5.6	2mL
Na_3PO_4 20mM	2mL
D-Glucose	0.1g
Acetosyringone 200mM in DMSO	20uL
Water	-
TOTAL	20mL

The supernatant was then removed from the pellet, and the pellet was re-suspended in 1mL of IF. This cell suspension was then centrifuged, the pellet re-suspended in fresh IF media and centrifuged once more as a washing step. The final re-suspension in 1mL of IF was our stock to be used for infiltration. A 1:5 dilution of this stock was prepared by mixing 200μL stock with 800μL of fresh IF media. The optical density (OD) of this dilution was measured and recorded at 600nm. A value between 0.0 and 0.6 is good. Optimally it will be around 0.2-0.3. The OD measurement was used to create a 0.05% solution of *A. tumefaciens* in a final volume of 0.5mL of IF. The following equation was used to determine the volume of cell suspension needed in the final volume of 0.5mL.

Equation 1: $\frac{0.5 \cdot 0.05}{\mathrm{OD}}$ = volume of cell suspension needed in 0.5mL

The plants used for infiltration were four weeks old, and had been maintained in a growth room at room temperature under a 16L/8D photoperiod. Infiltration was performed on leaves that had reached a length of four inches. Sterile 1mL syringes without the needle were filled with the cell suspension and pressed against an adaxial leaf vein that had been nicked. The syringe plunger was slowly depressed, holding the leaf from the abaxial side. The airspace

within the leaf tissue of the designated section was filled with the cell suspension, and the encompassing area was marked with a waterproof marker. The plants were returned to the growth room.

2.2) Fluorescence Confocal Microscopy. Two days post infiltration, microscopy was performed to visualize protein expression and accumulation. The facility used for this experiment was Michigan State University's Center for Advanced Microscopy. The instrument utilized was the Olympus FluoView FV1000 Confocal Laser Scanning Microscope (Center Valley, PA) with a 60X PlanApo N oil objective. A small section of leaf tissue from each infiltrated area was excised and mounted on a microscope cover slip. There were three samples: 1) pVKH18En6St-m-RFP with the IL-2 analogue 2) pVKH18En6St-m-RFP empty vector as a localization control and 3) WT tobacco as a negative control. Two representative sections from each sample were photographed, and two images were taken of each of these sections. The first was a brightfield image to visualize the epidermal cells. For the second image, the sample was excited with a 559nm diode laser line, and the fluorescence emission was recorded at 570-620nm (RFM emission) and 655-755nm (chlorophyll emission). These images were then overlaid to determine localization of accumulation.

3) Stable Genetic Transformation and Regeneration of Tobacco Plants. *A. tumefaciens* mediated transformation was the method used for stable transformation of tobacco. The expression cassette from each of the 5 Impact Vectors (1.1-1.5) was cloned into a pBINPLUS binary vector using standard cloning techniques. The expression cassette was cleaved with AcsI and PacI and ligated to the pBINPLUS backbone that was also cleaved with these restriction enzymes. These five vectors were then used to transform ElectroMAX *A. tumefaciens* strain LBA4404 via electroporation. Electroporation, selection, and sequencing were carried out in the

same manner as described for the transient expression vector. The *A. tumefaciens* strains were then used for transformation of tobacco via a leaf-disk method. The selection for transformed cells was accomplished by adding kanamycin (100mg/L) to the media since the nptII gene on the binary vector affords kanamycin resistance to transformed cells. Transformation was carried out according to the protocol available from Dr. Jurgen Deneche's lab at the University of Leeds. (http://www.plants.leeds.ac.uk/jd/Protocols.html). The components of the media required for each step is listed in Table 2.

Several young leaves from tobacco plants grown in vitro were collected under aseptic condition in a laminar flow hood. These leaves were cut into 0.5-1cm^2 squares. About 15 leaf squares were set to float in 10mL of A10 per Petri dish. This A10 was supplemented with acetosyringone to achieve a final concentration of 0.1mM. Six Petri dishes were prepared in this manner. One dish was used for each of the five vectors and a negative control that was not treated with *A. tumefaciens.* Seventy-five μL of overnight cultures of *A. tumefaciens* were added to the Petri dishes and gently swirled to mix the bacteria evenly. These Petri dishes then were parafilmed and incubated for three days at room temperature under low light density by placing a piece of paper over them.

After this incubation, a film of *A. tumefaciens* was noted at the bottom of each dish, excluding the negative control with no treatment. Wash steps were performed to remove the bacteria. The leaf squares were transferred to a new Petri dish with 10mL of fresh A10 and swirled gently for 10-15 min. The media was replaced with fresh media and swirled gently three times over 10 min. The wash process was continued twice more and the media used for the last wash was supplemented with 500μg/uL cefotaxime. A maximum of 10 leaf squares were transferred to Petri dishes containing solid A11 and pressed gently to the media to ensure

selective pressure and adequate nutrition. Petri dishes were maintained in the growth room for seven days under a 16L/8D photoperiod, followed by transfer to fresh A11 plates. After 10 days, leaf cultures were sub-cultured to freshly made A11 media.

At this point, calli had formed around wound sites (Figure 4, Panel 1). Those that had grown to1-2mm were transferred to A12 plates at a density of about 50 calli/plate. Petri dishes were maintained in the growth room for two weeks. After this incubation, calli that had matured to about 5mm (Figure 4, Panel 2) were transferred to Magenta boxes containing 50-100mL of A13 and maintained under the same conditions for another two weeks. After this incubation, calli that had not developed shoots were transferred to new A12 media for additional incubation time. Calli that had formed shoots were removed and the shoot was excised at the base with a scalpel. These shoots were transferred to Magenta boxes containing MS media supplemented with 0.2μg/mL IAA (Figure 4, Panel 3). Three shoots were placed in each box to ensure sufficient nutrition. Although selective pressure was not necessary at this stage, the MS media was supplemented with 100μg/L kanamycin to avoid growth of non-transgenic escapes.

Table 2. Recipes for media used in stable tobacco transformation. Components listed below the bold line should be added after autoclaving the media at 121°C and 15 PSI for 30 min.

	A10	A11	A12	A13	
B5*	+	+	+	+	2.48g
NH_4NO_3 (250mg/L)	+	+	+	+	200mg
MES (500mg/L)	+	+	+	+	400mg
Glucose (2%)	+	+	+	+	16g
Agar (0.75%)	-	+	+	+	6g
Adenine (40mg/L)	-	+	+	+	32mg
BAP**	1μg/mL	1μg/mL	1μg/mL	1μg/mL	
NAA***	0.1μg/mL	0.1μg/mL	0.1μg/mL	-	
Cefotaxime	-	500μg/mL	200μg/mL	200μg/mL	
Kanamycin	-	100μg/mL	100μg/mL	100μg/mL	
TOTAL					800mL

*B5 = Gambor's B5 complete Media
**BAP = 6-benzylamino purine
***NAA = α-naphthalene acetic acid
MS media contained 4.43g MS w/ vitamins and 2.5G Gerlite Gellan Gum per liter.

Plantlets were transferred to small (4in x 4in) soil pots once they had a visibly well-developed root structure (Figure 4, Panel 4). Potted plantlets were maintained in plastic bags for three days to expose the plantlets to high humidity, similar to the humidity of in vitro cultures. Afterwards, the bags were opened up 1/3 of the way each day thereafter until the plants were well acclimated to ambient conditions, at which point they were removed from the plastic bags completely. These plants were transferred to a greenhouse where they were maintained under high intensity lamps (400 watt sodium lamps, GE lighting) at a 16L/8D photoperiod. The greenhouse temperature controls did not allow the temperature to drop below 15°C or rise above 32°C. Once plants were identified as expressing IL-2, they were transferred to larger pots to allow for larger growth (Figure 4, Panel 5).

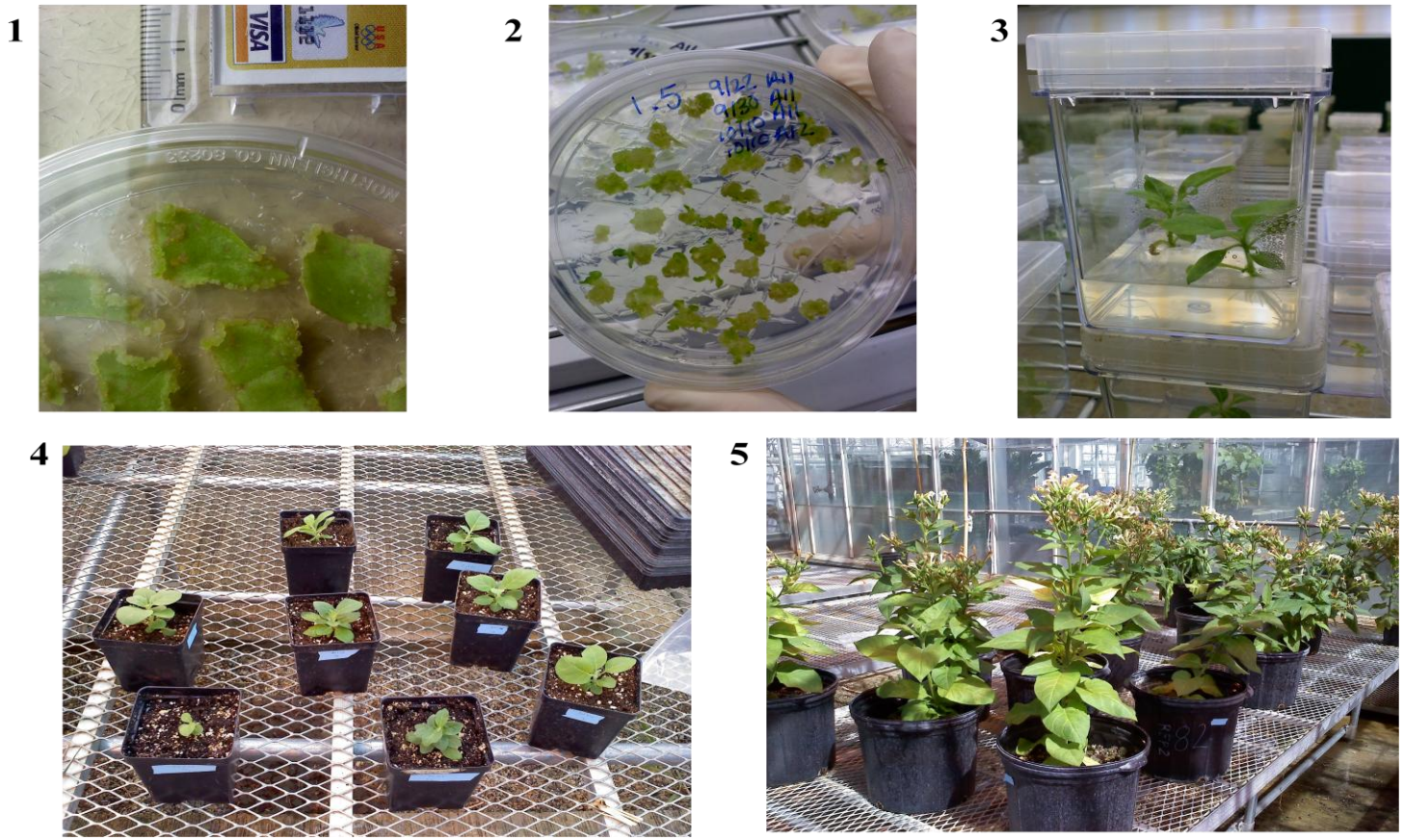


Figure 4. Images of plant material at various stages of stable transgenic line development. Panels 1 and 2 show callus formation from *A. tumefaciens* infected leaf squares. Panel 3 shows excised stems that have been placed in rooting media. Panel 4 shows plantlets transferred to soil. Panel 5 shows adult plants that had been identified to have good IL-2 protein expression and transferred to larger pots.

4) Polymerase Chain Reaction. DNA extraction was performed using a version of the CTAB method (Edwards et al., 1991). Five leaf disks excised with a standard 6mm diameter 1-hole punch were placed in a 1.5mL Eppendorf tube and placed on ice until all samples were collected. The end of the tube was dipped in liquid nitrogen to allow material to freeze, which was subsequently disrupted using a blue plastic pestle available from Sigma-Aldrich (CN: Z359947). Four hundred μL of CTAB extraction buffer, supplemented with 1% β-mercaptoethanol, was added to the leaf tissue powder and further ground to achieve homogeneity. The CTAB extraction buffer components can be found in Table 3.

Table 3. Components present in CTAB extraction buffer.

COMPONENT	AMOUNT
CTAB	6.0g
5M NaCl	80.0mL
0.5M EDTA pH 8.0	12.0mL
1M TRIS-HCL pH 8.0	30.0mL
Water	121.0mL
TOTAL	300mL

This mixture was incubated in a 65°C water bath for 20 min, and inverted twice during the incubation. The mixture was allowed to cool and 400 μL of 24:1 chloroform: isoamyl alcohol was added to each tube and shaken for 15 min. The samples then were centrifuged at 13,000 RPM for 5 min to separate the layers. The top aqueous layer was transferred to a new tube with the 400 μL of cold isopropanol. This was mixed by 4 gentle inversions and incubated at -20°C for 1 hr. After incubation, the samples were centrifuged at 13,000 RPM for 5 min. The

supernatant was removed, being careful not to disrupt the pellet, and the pellet was left to dry in a laminar flow hood.

Once dried, the pellets were re-suspended in 100uL of autoclaved water, and 4μL of RNAse A was added to each suspension. This was left at room temperature for 15 min, followed by the addition of 500μL of cold 100% ethanol. Tubes were mixed by gentle inversion and incubated at -20°C for 1 hr. Samples then were centrifuged at 13,000rpm for 5 min to pellet the DNA and the supernatant was removed. The pellets were left to dry in the laminar flow hood, and subsequently re-suspended in 100uL of autoclaved water.

Some samples produced colored pellets that would not fully re-suspend in water. These samples were incubated at room temperature for up to 30 min, followed by centrifugation to pellet the material that would not re-suspend. The supernatant was removed and used for later steps. DNA was quantified using an Eppendorf Biophotometer plus (Hauppauge, NY, www.eppendorfna.com).

PCR reactions typically were set up in 15μL volumes. The thermocycler used was a Perkin Elmer GeneAmp CR System 9600 manufactured in Livonia, MI. Table 4 shows the components added to each reaction.

Table 4. Components present in PCR reactions.

COMPONENT	AMOUNT (μL)	FINAL CONC.
10x buffer	1.5	1x
10mM dNTP	0.3	0.2mM
10μM F primer	0.3	0.2μM
10μM R primer	0.3	0.2μM
Template DNA	1	~100ng total

Table 4 cont'd.

Taq Polymerase	0.3	100 units/mL
DMSO	0.75	5%
H_2O	10.55	
TOTAL	15	

The primers used to screen for the IL-2 transgene were designated IL-2OPT_F1 and IL-2OPT_R1. The sequences of these primers are CATGGCGGCACCCACTTCAA and GGCGGTCTCGTCAGCGTATT, respectively. They amplify a 343bp segment of the IL-2 transgene. The thermocycler was programmed to go through the following cycles: 94°C for 1 min (1 cycle), 94°C for 30s (35 cycles), 62°C for 30s (35 cycles), 72°C for 45s (35 cycles), 72°C for 5 min (1 cycle), 4°C indefinitely. The positive control used for these reactions was purified Impact Vector 1.1 containing the IL-2 analogue. Each sample contained about 100ng of DNA.

5) Protein Extraction/Quantification. Protein extraction for western blot identification of lines that express well was carried out with a TRIS grinding buffer: 50mM Tris HCl ph8.0, 1mM EDTA, 10mM diethyldithio carbamic acid, 0.5% Tween 20. This was supplemented with 2X protease inhibitor cocktail for plant cell extracts purchased from Sigma (CN: P9599). This extraction buffer met the two important criteria: 1) the ability to maintain IL-2 integrity as confirmed by western results; 2) does not react with coomassie brilliant blue which is used to quantify protein concentrations. Quantification was necessary for normalization of protein concentrations.

The expression of rubisco is under circadian regulation, with highest transcription and translation in the morning (Outchkourov et al., 2003). Being that recombinant IL-2 is driven by

the rubisco promoter, protein samples were collected prior to 10 A.M. To prepare protein extracts for western blotting, 100μL of extraction buffer was added to 1.7mL Eppendorf tubes. From fresh sample tissue, 2 leaf disks were removed with a standard 1-hole punch (0.6cm diameter) and submerged in the extraction buffer on ice. Immediately after collection, small blue tip pestles were used to disrupt the tissue by grinding with a rotary instrument. All work was done on ice. When all samples were processed, samples were spun at 12,000 RPM for 10 min in a table top centrifuge set to 4°C. Supernatant was removed to a new tube and quantified.

Protein quantification was performed using the Coomassie Plus (Bradford) Protein Assay reagent available from Thermo Scientific (CN: 23238) to prepare the samples and a BioPhotometer Plus from Eppendorf to read absorbencies. A standard curve was set up using albumin aliquots that come with the Bradford reagent. The curve was set up according to the recommended specification of the company with three replicates of nine different concentrations on a range from 0ng/μL to 2000ng/μL. Any samples that measured outside of this range were subsequently diluted in grinding buffer and re-sampled. Samples were prepared by combining 15μL of protein extract with 500μL of Bradford reagent and incubating at room temperature for 10 min. Each sample was replicated three times and the concentration was determined by taking the average of these three readings.

6) Western Blot Screening. Western blotting first was carried out to screen for heterologous protein expression and to compare protein expression levels in different transgenic lines. Crude protein samples were extracted and quantified the previous day and frozen overnight at -20°C. Expression of the transgene was under the rubisco promoter which is regulated in a circadian fashion. Transcription of rubisco peaks just after dawn (Pilgrim and McClung, 1993), so protein samples were always collected prior to 10 A.M. Twenty kanamycin resistant plants were

selected from each of the five Impact Vectors for testing, totaling 100 plants. Each plant was assigned a unique label that identified the vector type (1.1-1.5) and distinguished individuals of the same vector type (a-t). Each of the western blots run to look for expressing lines had three lanes in common. The first lane contained MagicMark XP Western Protein Standard from Invitrogen. The second lane contained 10ng of recombinant human IL-2 (CN: 202-IL) purchased from R & D systems (Minneapolis, MN, USA, www.rndsystems.com) as a positive control. The third lane contained protein extracted from wild type tobacco as a negative control. Several gels also contained a sample of 1.3a since this plant was identified early on as having a good expression of IL-2. Each time leaf samples were processed, a fresh 1.3a sample was processed to serve as a sampling control. A 1.3a sample was run for each batch of samples, though not on every gel. The NuPAGE system from Invitrogen was used to run the western. The NuPAGE Novex 10% BIS-TRIS gels with 10 wells (CN: NP0301BOX) were used rather than the 15-well gels due to better band resolution.

For each sample, 15μg of total protein was added to 2.4μL of 10X NuPAGE reducing agent, six μL of 4X Loading Dye and enough double distilled water to bring the final volume to 24μL. The mixture was incubated for five min at 80°C and then loaded into the gel. The inner chamber of the western apparatus was filled with 200mL of 1X NuPAGE running buffer supplemented with 0.5mL of NuPAGE Antioxidant. The outer chamber was filled to capacity with 1X NuPAGE running buffer. Gels were run at 200V for 30 min, or until the lower dye band reached the bottom of the gel. Gels were carefully removed from the casting cassette and a square of PVDF membrane (Polyscreen PVDF Transfer membrane, NEF1002001PK, Perkin Elmer, Waltham, MA, USA) was placed on top. This membrane was activated previously by soaking in methanol for one minute followed by rinsing with transfer buffer. This then was

sandwiched between Whattman paper and sponges and then placed in the transfer cassette. This cassette was filled with 1X NuPAGE transfer buffer and placed in the transfer apparatus. The apparatus was filled with water which acted as a heat-sink, and a voltage of 30V was maintained for 1 hr.

The membrane was removed from the transfer cassette and rocked for 45 min in 20mL of blocking buffer composed of 1X PBS, 0.1% TWEEN 20 and 5% (w/w) non-fat dry milk. The blocking buffer then was removed and the primary antibody solution was added and rocked for 1.5 hrs. The primary antibody solution was 20mL of 1X PBS with 0.2 μg/mL of polyclonal goat IgG specific for IL-2 (R&D systems AF-202-NA). The membrane was washed with 1X PBS with 0.1% TWEEN 20. Once the primary antibody solution was removed, 20 mL of wash buffer was added and rocked for 10 min. This buffer was changed twice more over 30 min of rocking. Once the final wash buffer was removed, the secondary antibody solution was applied to the membrane and rocked for 1.5 hrs. The secondary antibody was HRP conjugated donkey anti-goat IgG (R & D HAF109). The antibody solution was a 1:1000 dilution of this antibody in blocking buffer. This incubation also was followed by the wash steps with three changes of wash buffer over 30 min.

The SuperSignal West Pico Kit (CN # 34079) from Thermo Scientific was used for visualization. The procedure was performed as indicated by the manufacturer's instructions. In brief, membranes were blotted dry from the underside and laid on plastic wrap. One mL of Stable Peroxidase Buffer was spread evenly over the gel, followed by 1mL of Luminol/Enhancer. The membrane was wrapped to allow the liquid to spread evenly over the entire surface, and incubated in the dark for 5 min. Following incubation, membranes were blotted dry from the underside, wrapped in new plastic wrap, and taped to an exposure cassette.

The cassette was taken to the dark room, where autoradiography film (Premier Autoradiography Film, E3018, Denville Scientific Inc, Metuchen, NJ) was placed in the kit for 45-90s followed by development.

7) Biological Activity Assay.

7.1) Sample Preparation. Three different methods were used to prepare samples for biological activity testing.

7.1.1) Ammonium Sulfate Precipitation Sample. The first of these methods was extraction of crude total soluble protein via ammonium sulfate precipitation, followed by dialysis to remove residual ammonium sulfate. In this method, the TRIS protein grinding buffer used for protein preparation for western screening was substituted with 100mM sodium phosphate buffer pH 5.8. This substitution was made because the pH of phosphate buffer is less affected by temperature change than TRIS. In addition, using a phosphate buffer allowed for a lower pH of 5.8 to be achieved. The stability of IL-2 increases as pH decreases from 7 to 4 (Ricci et al., 2003). It was assumed that increased stability should translate to a higher IL-2 concentration in the sample; therefore, it was optimal to maintain samples at a lower pH. This buffer also was supplemented with 1mM EDTA, 10mM Diethydithio carbamic (DIECA) acid, 0.5% Tween 20 and 2X protease inhibitor cocktail for plant cell extracts (CN # P9599).

Six-and-a-half grams of plant leaf tissue were ground in liquid nitrogen to a fine powder. Twenty mL of phosphate grinding buffer (pH 5.8) was added and ground further on ice. Once the sample was adequately homogenized, it was alliquoted into 1.5mL Eppendorf tubes and centrifuged at 12,000 RPM. The supernatant then was removed and 15mL was placed in a beaker and stirred on a stir plate in a cold room at 4°C. A total of 8g ammonium sulfate was added slowly to achieve 80% saturation (Mertelsmann et al., 1983). This mixture was allowed to

equilibrate for 1 hr, and subsequently alliquoted into new Eppendorf tubes and centrifuged once again at 12,000 RMP. This time, the supernatant was discarded and the pellet was saved since the pellet is the protein that had precipitated from the sample. These pellets were pooled and re-suspended in 1.5 mL of phosphate buffer (pH 5.8) with all components mentioned above, except for Tween 20. This concentrated protein suspension then was dialyzed using a 3mL Slide-A-Lyzer G2 Dialysis Cassette from Thermo Scientific (CN #87718). This cassette has a 2,000 membrane molecular-weight cutoff (MWCO) which will allow for the retention of the 18.3 kDa IL-2 and the removal of residual ammonium sulfate, as well as any alkaloid toxins and other small weight molecules that may remain in the sample. The dialysis cassette was set up as specified by the manufacturer's instructions. The sample was dialyzed against a total of 250 times the sample volume. Two buffer changes were performed with the first change after two hrs of incubation, and the second after another two hrs of incubation. The final buffer change was allowed to incubate overnight and the sample was collected from the cassette in the morning. Protease inhibitor cocktail was added to achieve a 2X concentration.

7.1.2) Desalted Sample. The second sample preparation method was using a desalting column to remove small molecules and exchange the buffer. Six-and-a-half grams of leaf tissue also were ground in the same phosphate buffer described for the ammonium sulfate precipitation and centrifuged in the same manner. An additional step of clarification was necessary for running a sample through this column, so prior to loading, the sample was passed through a filter with 0.22μm pores. The buffer used to pass the sample through the column was 20mM citrate buffer pH 4.5. This more acidic buffer was preferred to increase stability, and its weakness was preferred so that it would have minimal effect when added to the T-cell cultures. The resulting

fraction was then concentrated using a Vivaspin 5000 MWCO centrifuge tube from Fisher Scientific (CN # VS0611) to 1.2mL and placed at -20°C.

7.1.3) Histidine Affinity Gel. The third and final sample preparation method utilized HIS-Select HF Nickel Affinity Gel from Sigma (CN # H0537) to isolate the recombinant IL-2 from plant cell extracts. The crude extract used for this method was prepared differently due to specifications required for incubation with the affinity gel. A pH between 7-8 was necessary, so a 100mM sodium phosphate buffer (pH 7.5) was used. Phosphate was the preferred buffer in this situation because TRIS has a slight tendency to bind metal ions, and this could disrupt the Ni ions on the affinity gel. This buffer was supplemented with 0.5% Tween 20, but no EDTA or DIECA was added due to their chelating function which also could strip the affinity gel of Ni ions. A special protease inhibitor cocktail from Sigma (CN # P 8849) specifically designed for use in nickel column chromatography was used to supplement this buffer at a 2X concentration.

Grinding of 4.5g of tissue in liquid nitrogen followed by centrifugation and recovery of supernatant was carried out in the same manner as the other sample preparation methods. An additional step of clarification also was performed by passing the sample through a 0.22μM membrane to ensure that the solution was free of debris. One mL of HIS affinity gel was used as described by the manufacturer's protocol for batch preparation. In brief, affinity gel was centrifuged at 5,000 G for 5 min and the supernatant was removed. The gel was re-suspended and washed in 10 mL of equilibration buffer [50mM sodium phosphate (pH8), 0.3M sodium chloride, 10mM imidazole]. After recovery by centrifugation, the gel was added to the 15mL of IL-2 sample and rocked at 4°C for 15 min. The gel was recovered by centrifugation at 5,000 G for 5 min and washed 3 times with equilibration buffer by re-suspension, centrifugation and re-suspension in fresh equilibration buffer. Upon the final centrifugation recovery of the gel, 2mL

of elution buffer (50mM sodium phosphate (pH8), 0.3M sodium chloride, and 250mM imidazole) was added and shaken at 4°C for 10 min. Centrifugation was performed to pellet the gel and recover the supernatant containing the IL-2 protein. This elution step was carried out two additional times and pooled. Necessary wash steps were performed to recondition the HIS affinity gel, and another round of extraction was performed using the same sample extract used in the first extraction. The elutions from each round of extraction were pooled and concentrated to 1.5mL using the same Vivaspin columns. This 1.5mL sample then was dialyzed in the same manner as in the ammonium phosphate sample preparation to remove the remaining imidazole.

7.2) IL-2 Quantification. Small alliquotes of samples prepared using each of the described methods were frozen separately to allow for later analysis and quantification. Quantification was performed by western blotting. Each gel had a control lane of 10ng of recombinant IL-2 protein to serve as a standard. Serial dilutions of each sample then were loaded in decreasing amounts (15, 7.5, 3.75, 1.87 and 0.94μL). Quantity One 1-D Analysis Software from Bio-Rad was used to quantify the intensity of each band and these values were used to generate a standard curve (logarithmic). The intensity measured of positive controls in each of the gels then was used along with the equation of each generated curve to determine the concentrations of each sample.

7.3) T-cell Proliferation Assay. (Written by Dr. Beau Carson, Edited by Jason Matakas) For analysis of the *in vitro* proliferative potential of the IL-2 preparations, a standard CD4+ T-cell proliferation assay was utilized. Briefly, splenic $CD4^{+\,+}$ T-cells from C57BL/6 mice at 8-10 weeks of age (Taconic Farms, Germantown, NY, USA) were isolated utilizing magnetic bead separation (Miltenyi Biotech, Auburn, CA, USA), according to the manufacturer's protocol. Purified CD4+ T-cells were plated in flat-bottom 96-well plates that were pre-coated with one μg/ml α-CD3 (BD Biosciences, San Jose, CA, USA), and IL-2 preparations were added to the

cell culture medium at the indicated concentrations. Cells were cultured in RPMI 1640 (Mediatech, Herndon, VA, USA) supplemented with 10% FBS (Atlas Biologicals, Ft. Collina, CO, USA), penicillin/streptomycin, L-glutamine, MEM-non-essential amino acids, Na-pyruvate (Lonza, Basel, Switzerland) and 2-ME (Sigma-Aldrich, St. Louis, MO, USA), and were incubated with the indicated stimuli for a period of three or four days. During the final six hours of culture, cells were labeled with one μCi/well of ^{3}H-thymidine. After six hours of incubation with radiolabeled thymidine, cells were harvested onto glass filters and analyzed using a beta scintillation counter (Becton-Dickinson, Franklin Lakes, NJ, USA).

RESULTS AND DISCUSSION

1) Transient Transformation. A representative sample of the images taken using the fluorescence confocal microscope is shown in Figure 5. In this figure, each panel shows the overlay of the brightfield image with the images recorded at 570-620nm and 655-755nm which shows RFP emission and chlorophyll emission, respectively. Panel A displays WT tobacco epidermal tissue. A slight amount of red is seen due to the natural occurring compounds that can be excited at our designated wavelength. No excitation is seen in the apoplast or golgi. Panel B displays tobacco epidermal tissue that was infected by *A.tumefaciens* harboring the empty vector pVKH18En6St-m-RFP. This vector has a golgi targeting signal so accumulation was expected to be noted in the golgi. As is apparent from the image, this was seen. The golgi should appear as small dots. Since the vacuole of plant cells is so large, they should be pressed up against the interior of the cell. This empty vector control shows that 1) the RFP is being produced and 2) it is not secreted to the apoplast. Panel C shows tobacco epidermal tissue that was infected with *A. tumefaciens* harboring pVKH18En6St-m-RFP containing the IL-2 analogue with a secretory signal. It is apparent from the image that the heterologous IL-2 protein is accumulating in the

apoplast as expected. The final destination of proteins trafficked for secretion is the apoplast. Proteins first must pass through the endoplamic reticulum (ER) which is equipped with many types of chaperones that aid in proper folding. If the host cell is capable of properly folding the protein, it will be successfully trafficked through the secretory pathway. If, however, the host cell is unable to fold the protein properly, it will be retained within the ER and degraded (Vitale and Denecke, 1999). Since the IL-2 is secreted properly, this implies the host cell can fold this protein properly.

Proper folding is required for biological activity, and thus folding ability is a key feature of the expression system of interest. If a system, for example *E. coli*, is unable to fold the protein of interest properly, more downstream processing is required to refold the protein. This adds cost to the production platform and also results in loss of material. Each additional processing step results in a certain amount of loss; therefore, the goal is to reach the end product with the fewest number of steps possible.

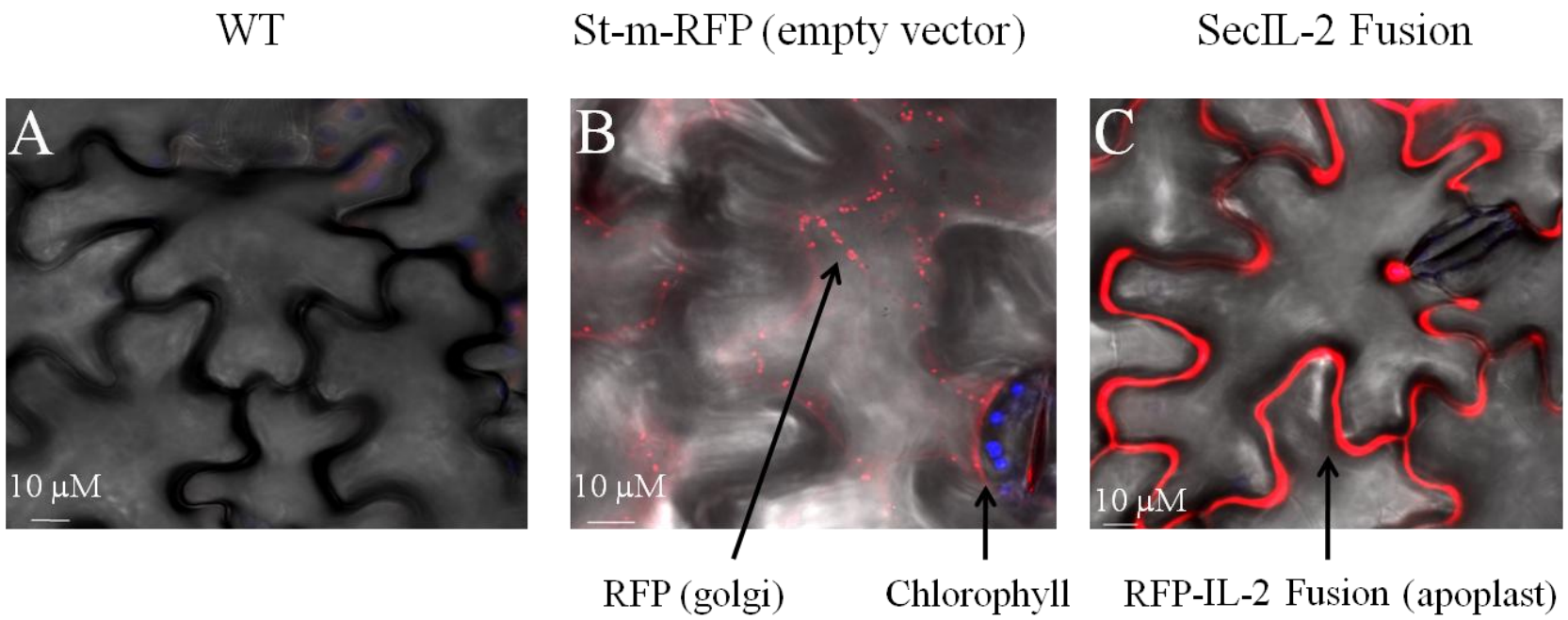


Figure 5. Transient Expression Assay. Brightfield images of tobacco epidermal cells with confocal fluorescence overlay recorded at 570-620nm and 655-755nm. Wild type tobacco leaf tissue is seen in panel A and transgenic leaf tissue is seen in panels B and C. Panel B shows cells transformed with the empty vector which targets red fluorescent protein (RFP) to the Golgi apparatus. Panel C shows cells transformed with the construct in which the Golgi targeting sequence has been removed and human interleukin-2 with an N terminal secretory signal has been added. The blue signal seen in the first two panels is emission from chlorophyll.

2) Stable Genetic Transformation.

2.1) Morphology. Within the T0 population, an albino characteristic was noted from a subset of Impact Vector 1.4 (chloroplast targeting) transformed plants. From seedling to mature plant, the leaves maintained either a bleached or spotty appearance. These plants appeared healthy and grew at the same rate as the rest of the population. All but one plant (1.4o) set seed. Figure 6 shows this observed phenotype compared with wild type.

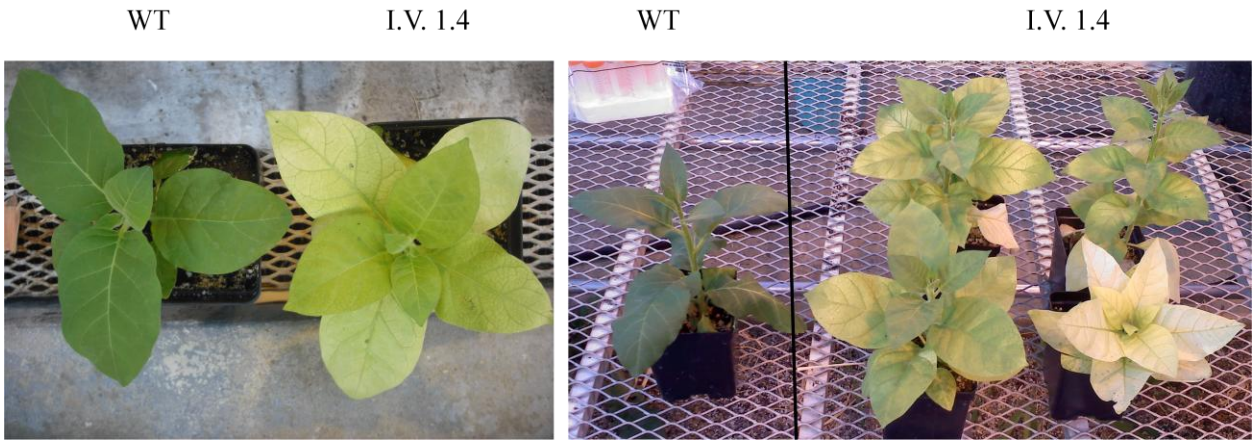


Figure 6. Albinism observed in a subset of the I.V. 1.4 transformed plants.

Interestingly, western blot screening revealed that all 6 out of 20 plants with albino morphology showed expression of the IL-2 protein. None of the other 14 out of 20 I.V. 1.4 plants with normal pigmentation were found to express IL-2 protein. Each plant was an independent transgenic line. Dr. Robert Larkin (Plant Biology, Michigan State University) noted that the phenotype observed here is similar to that of chlorophyll biosynthesis mutants; however, determining the exact cause of the chlorophyll deficiencies seen here would require further investigation that is outside the scope of this project.

2.2) Polymerase Chain Reaction. Figure 7 shows a gel image of PCR results from 4 plants from each expression vector. As seen in the figure, no bands are seen in the wild type or water

lane. A band of the appropriate size (343bp) is seen in the positive lane, as well as the lanes of each of the samples tested.

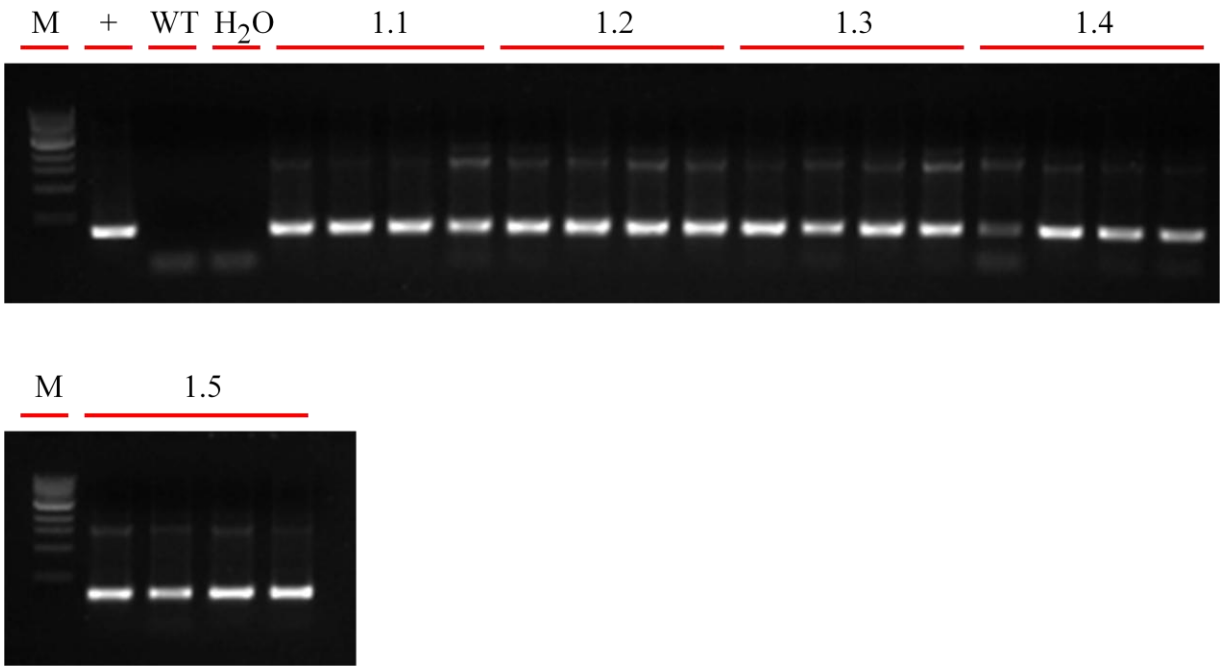


Figure 7. Polymerase chain reactions of four samples from each of the five expression vectors.

2.3) Western Blot Screening. Blotting for 20 plants per impact vector required that at least three gels to be run per vector. The recombinant human IL-2 used as a positive control in each gel is 15kDa. This is the mature protein of 133 amino acids with an additional N-terminal methionine and no glycosylation.

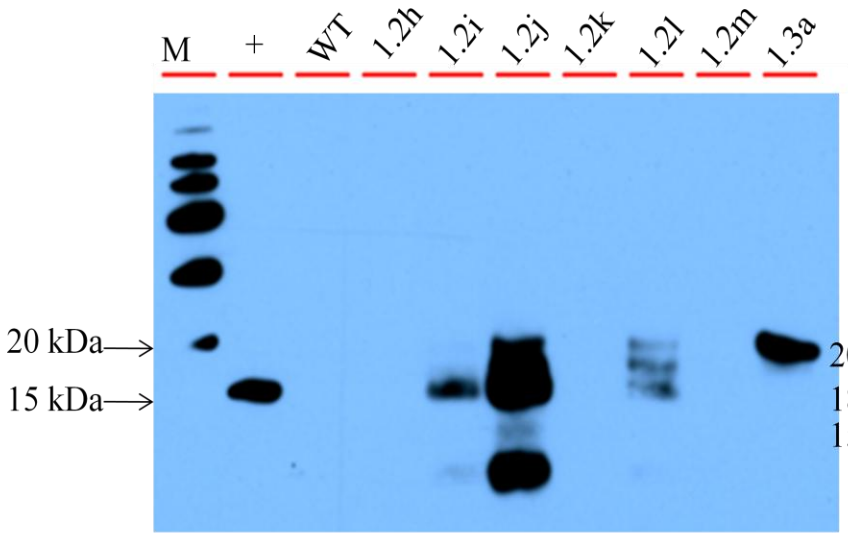
Using this system, no expression was detected for Impact Vectors 1.1 (cytoplasmic targeted) and 1.5 (mitochondrial matrix targeted). Not all compartments were expected to be conducive to IL-2 stability and thus we did not expect to see accumulation at each location. Each microenvironment has unique characteristics with varying degrees of protease pressures, pH and hydrolytic activity, all of which play a role in protein stability (Grignon and Sentenac, 1991; Voet et al., 2006).

Representative western blot images for expression screening of vectors 1.2 (apoplast targeted), 1.3 (endoplasmic reticulum targeted) and 1.4 (chloroplast stroma targeted) can be seen in Figure 8. The expected size of the IL-2 protein with the addition of a c-myc and 6X HIS tag is ~17.8kDa. This prediction was made using the online tool available at the EXPACY website (http://ca.expasy.org/cgi-bin/pi_tool). Blotting vector 1.2 samples consistently resulted in multiple bands ranging from this to smaller sizes. The last lane of the gel image of 1.2 samples shows a 1.3 sample to allow for comparison. It is not certain what is causing this banding pattern, but one possibility is that each band is a specific cleavage product, possibly due to extracellular protease activity. Due to this multiple banding pattern, it was determined that the apoplast was not a suitable location for stable IL-2 accumulation. On the other hand, other transgene products are well suited for this location. There have been many reports of successfully producing functional proteins in this location (Schillberg et al., 1999).

Western blotting of vector 1.3 and 1.4 plants consistently resulted in a single band of the appropriate sizes. For vector 1.3, the addition of the C-myc, 6X HIS tag, and KDEL sequence produced a molecule predicted to be 18.3kDa. As apparent from Figure 8, the band seen is of the correct size. The lowest band in the marker lane is 20kDa, and the band in the sample lanes is just below this. The band might be a bit higher due to glycosylation. In addition to the c-myc and 6X HIS tag, vector 1.4 adds an 11 amino acid sequence to the N-terminus to allow for targeting to the stroma of the chloroplast. This tag is not cleaved in vivo, and results in a molecule predicted to be 18.9kDa (Wong et al., 1992). This size also corresponds to the band size seen in the western blot image.

From these results, it was decided that IL-2 from vector 1.3 would be studied further for biological activity. This protein more closely represents the native protein than the protein

produced from vector 1.4. The 1.3 protein has additions to the C-terminus, whereas 1.4 has additions to both the N- and C-terminus. No cleavage sites were engineered into this construct to remove these tags once the protein is recovered.



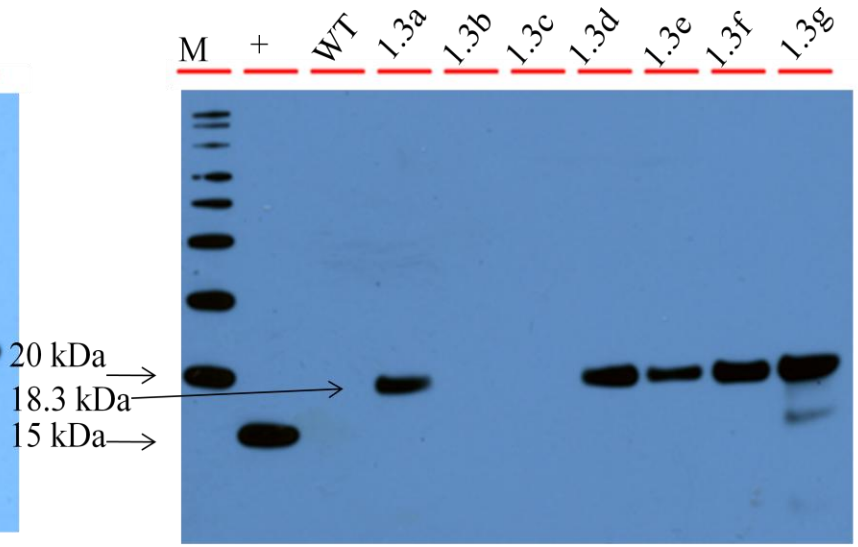
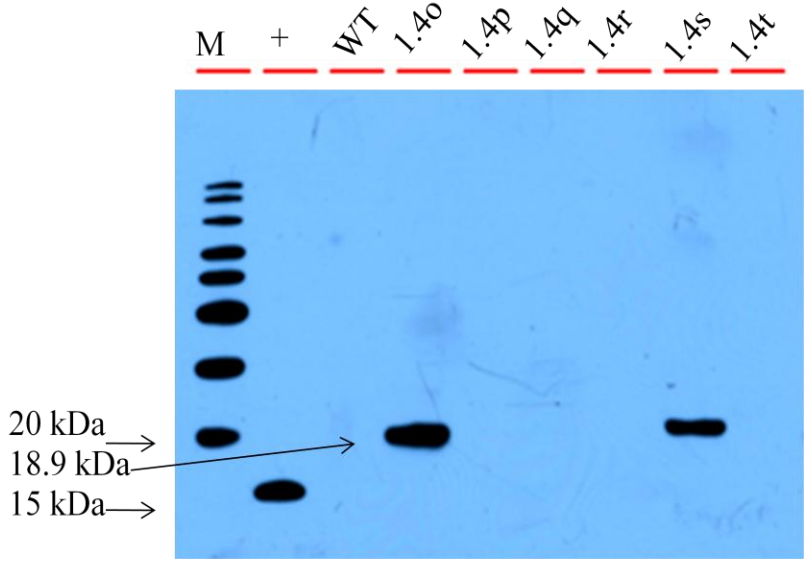


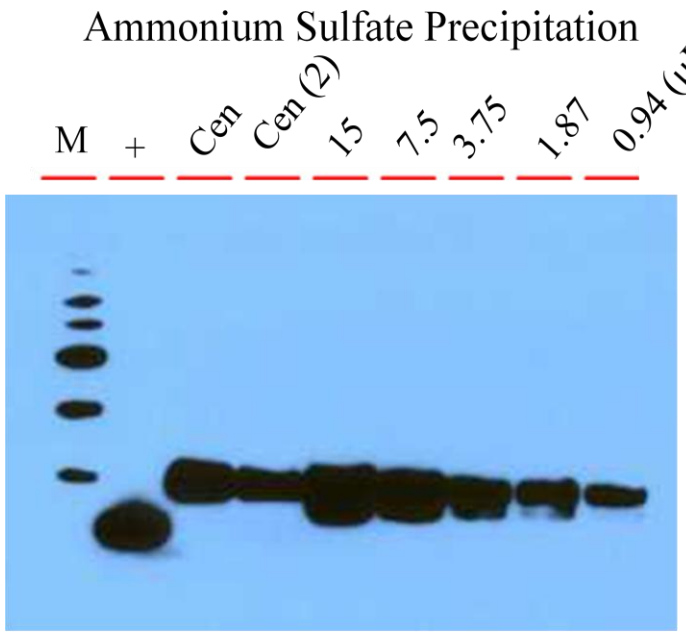
Figure 8. Western Blots. Shown here is a subset of western blots analyzing IL-2 expression in transgenic tobacco plants. Each of the five targeted compartment is represented by a different number (1.1-1.5), and each of the 20 independent lines is represented by a letter (a-t). The recombinant human IL-2 positive control is about 15kDa, and the predicted size of our product (considering the c-myc epitope and HIS tag) is about 18.3kDa. Blotting of 1.2 (apoplast) consistently showed multiple bands of lower-than-expected sizes, while blotting of 1.3 (endoplasmic reticulum) and 1.4 (chloroplast) samples showed a single band of correct size in plants that produced IL-2 above detectable levels.



2.4) Biological Activity Test.

2.4.1) Sample Quantification. The standard test for biological activity of recombinant IL-2 is a T-cell proliferation assay. Most of the literature on projects producing recombinant IL-2 describe the direct use of supernatant from media when IL-2 is secreted in cell culture (Sjogren-Jansson et al., 1994; Magnuson et al., 1998) and crude cell lysate when expressed in tissue (Park and Cheong, 2002) for this type of test. However, in the present research, crude cell lysate should not be used due to the fact that IL-2 is being expressed in tobacco, which is a highly toxic plant. The researchers that produced IL-2 in tobacco cell culture found that even though there was 10-fold greater IL-2 produced intracellularly, the crude cell lysate produced a highly diminished proliferative effect when applied to T-cells (Magnuson et al., 1998). Most likely this was due to the inhibitory effect of the cell lysate.

Tobacco is a member of the *Solanaceae* family. Several members of this family, including tobacco, are known for toxicity due to rich alkaloid content. One of the most well known of these toxins is nicotine which can account for 0.6-3% of tobacco leaf dry weight (Fu et al., 2010). Among the hazards that nicotine poses is oxidative stress and DNA damage. To remove these alkaloids, as well as other potentially inhibitory small molecules, sample preparation was carried out as described in the methods section. Figure 9 shows the western images used for quantification of IL-2 in each of these samples. Raw band intensity data can be found in Appendix B. Figure 10 shows the graphs generated from these data with their respective trend line equations. The concentrations were determined to be 1.47ng/uL for the ammonium sulfate sample, 2.53 ng/uL for the desalt sample, and 1.52ng/uL for the HIS affinity gel sample.



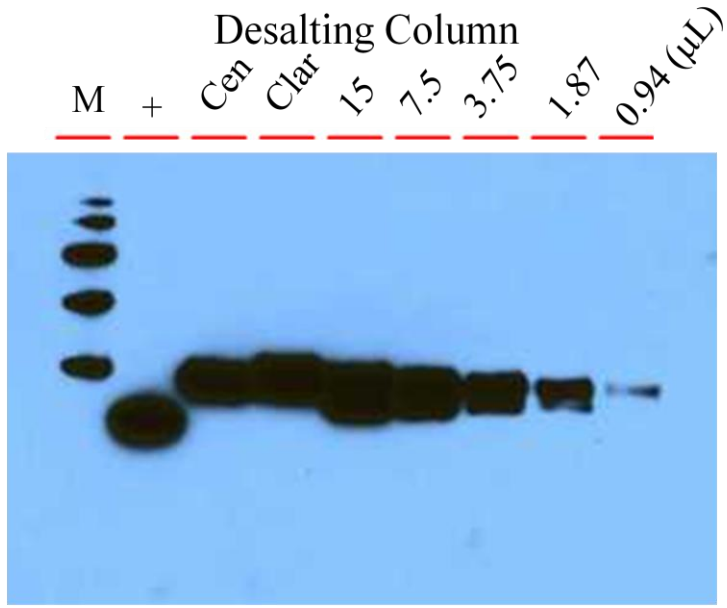
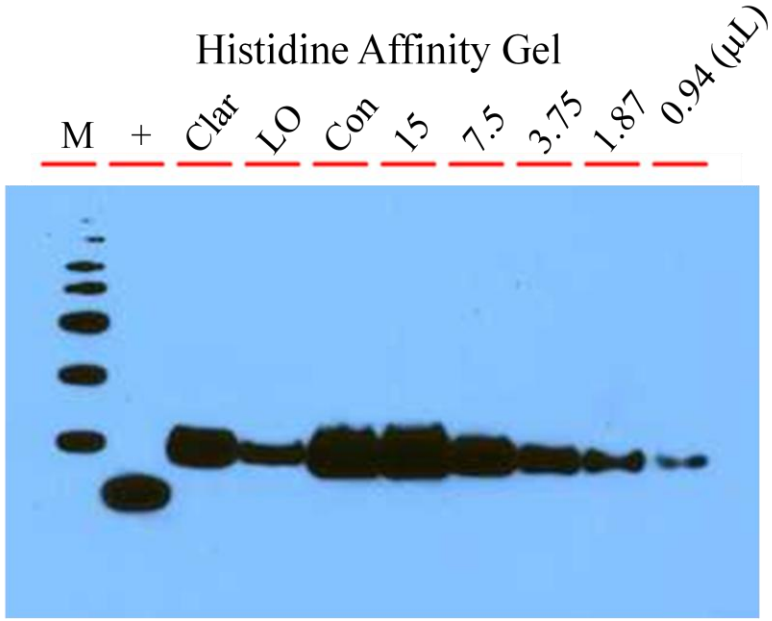
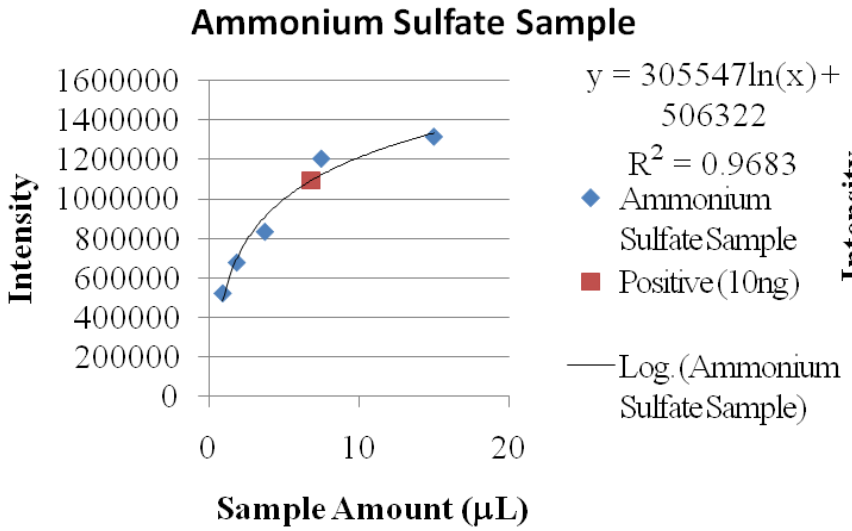
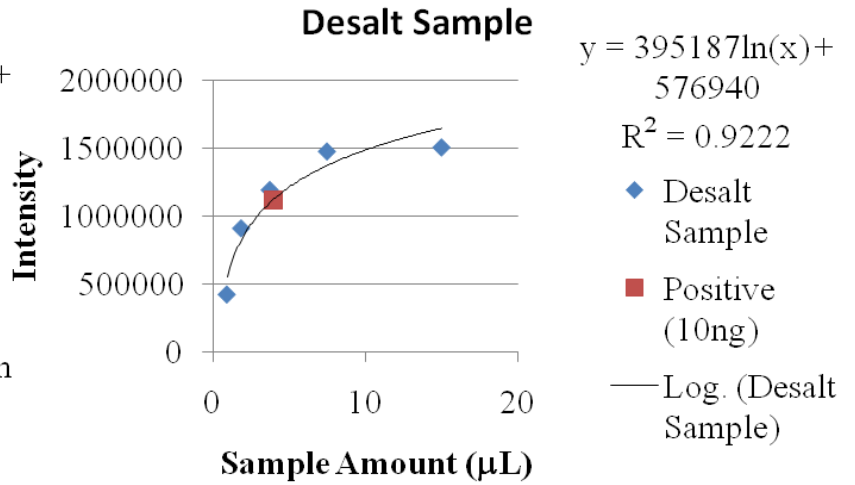


Figure 9. Western blots of protein extract samples. Serial dilutions of prepared samples were blotted to determine approximate concentrations. Each image is labeled with the method of preparation and the first two lanes are the molecular marker and 10ng of positive control, respectively. The last five lanes in each gel are labeled with the amount (µL) of sample added. Other lanes are samples taken at various times during sample prep; Cen = after centrifugation, Cen (2) = after freezing and additional centrifugation to remove precipitant, Clar = after clarification with 0.2 µm filter, LO = left-over supernatant after HIS gel was passed through, Con = Eluted sample after concentration, before dialysis.







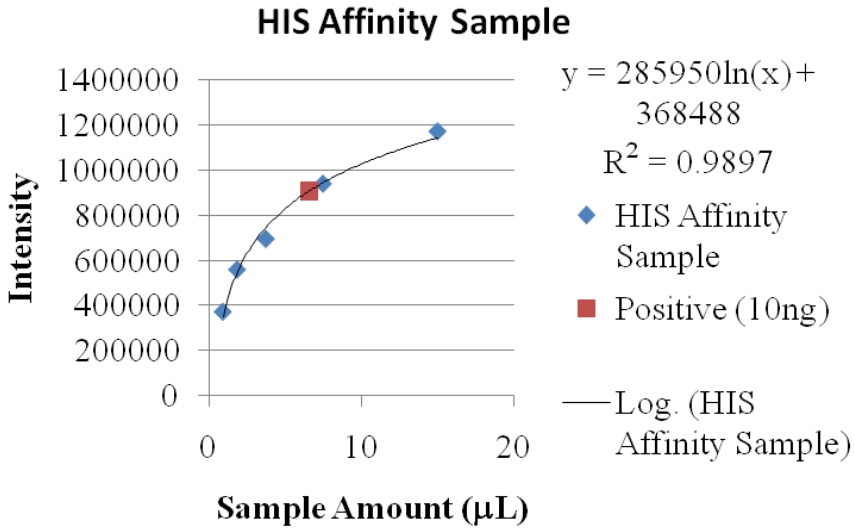


Figure 10. Quantification of recombinant IL-2 present in each sample. Band intensity was measured for each dilution shown in Figure 9. and a trend line was established. The positive control intensity was plotted on this line to correlate intensity with amount of protein, and this information was used to determine the concentrations of each sample.

2.4.2) Proliferation Assay Results. The specific activity of the protein describes how many units of activity the protein elicits per weight amount. The international standard for one unit of activity is the weight of IL-2 per mL that produces half-maximal proliferation in a given system. With the current data, we cannot assess specific activity of the recombinant IL-2 produced here; however, at low concentrations, we are able to compare percent activity with the positive control.

The results from the first set of cultures can be seen in Figure 11. These cultures were set up using whole splenocytes ($1x10^6$ cells per well) which contain resting T-cells. Resting T-cells were preferred over activated T-cells since activation induces IL-2 secretion. We were concerned that IL-2 secretion from the T-cell cultures could interfere with the ability to measure the biological activity of the recombinant IL-2 samples. In Figure 11 it can be seen that, at all concentrations, WT protein extract did not exhibit any proliferative effect, while increasing amounts of purified, *E.coli* derived IL-2 resulted in increasing proliferation measured by counts per minute (CPM). At 5 ng/mL, T-cell cultures from all samples exhibited some level of proliferation, although not as high as the positive control. Except for the desalt sample, this proliferative effect disappeared when the concentration was elevated to 10ng/mL IL-2. The proliferative effect of the desalt sample also was significantly diminished once a concentration of 50ng/mL IL-2 was achieved, while at this concentration, the proliferation of T-cells treated with purified IL-2 spiked dramatically.

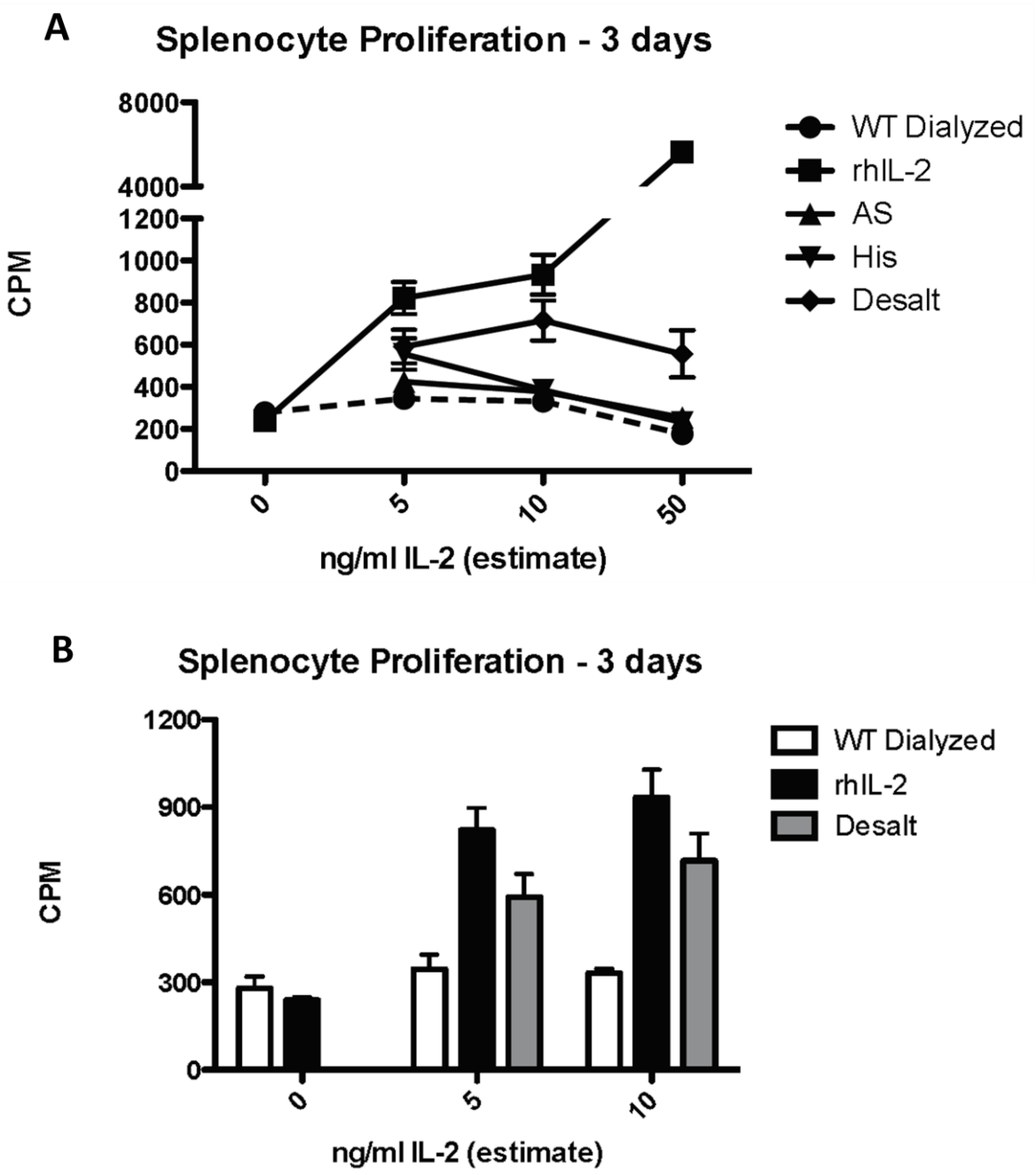


Figure 11. Results from IL-2 biological activity test using whole splenocyte cultures. Panel A is a line graph of proliferative results from WT-protein (negative control), purified rIL-2 (positive control) and all three samples at estimated concentrations of 0,5,10 and 50ng/mL IL-2. Panel B shows a bar graph of the positive control, negative control and desalt sample at concentrations of 0,5 and 10 ng/mL IL-2. All error bars from this point onward represent standard error.

Since, for all samples, the activity drops as the concentration is increased, it is very possible that there is some unknown inhibitory substance in the samples which is more potent at higher concentrations. Of all of the samples, the desalt is the most concentrated and therefore at each dosage, this was the sample of which the least amount was added to the T-cell cultures. This sample showed the most activity at all dosages. At 5ng/mL, the positive control registered 820 counts per minute (CPM) while the desalt sample registered 592 CPM. The background (WT protein extract) registered 344 CPM. Correcting for background results in values of 476 CPM for the positive control and 248 for the desalt sample. This means that the plant-derived IL-2 is roughly 52% as active as the positive control. At 10ng/mL, the plant-derived IL-2 is roughly 64% as active as the positive control.

The second set of cultures contained only purified splenic CD4+ T-cells ($5x10^4$ cells per well) that were activated using an anti-CD3 antibody. The purpose of using activated T-cells as opposed to resting T-cells is that they are much more sensitive to IL-2 and thus should exhibit greater proliferation at lower concentrations of IL-2. This decreased need for IL-2 means that a smaller amount of sample should produce a greater proliferative effect than seen in the previous cultures. Adding a smaller amount of sample should minimize the effect of inhibitory substances that could be present in the samples. Although these cultures did secrete their own IL-2, this did not affect our ability to measure the biological activity of the recombinant IL-2 since we still were able to see the cultures respond in a dose-dependent manner.

Figure 12 displays the results from the second set of cultures. Again, the negative control shows no proliferation at any dosages. Maximum proliferation is seen in the positive control at 1ng/mL IL-2 and diminishes at higher doses. This is normal since over-activation of immune cells tends to result in apoptosis. The ammonium sulfate precipitation sample still exhibits no

proliferative activity. However, although lower than the positive control, both the desalt sample and the HIS affinity gel sample show proliferative activity trending similarly to that seen in the positive control.

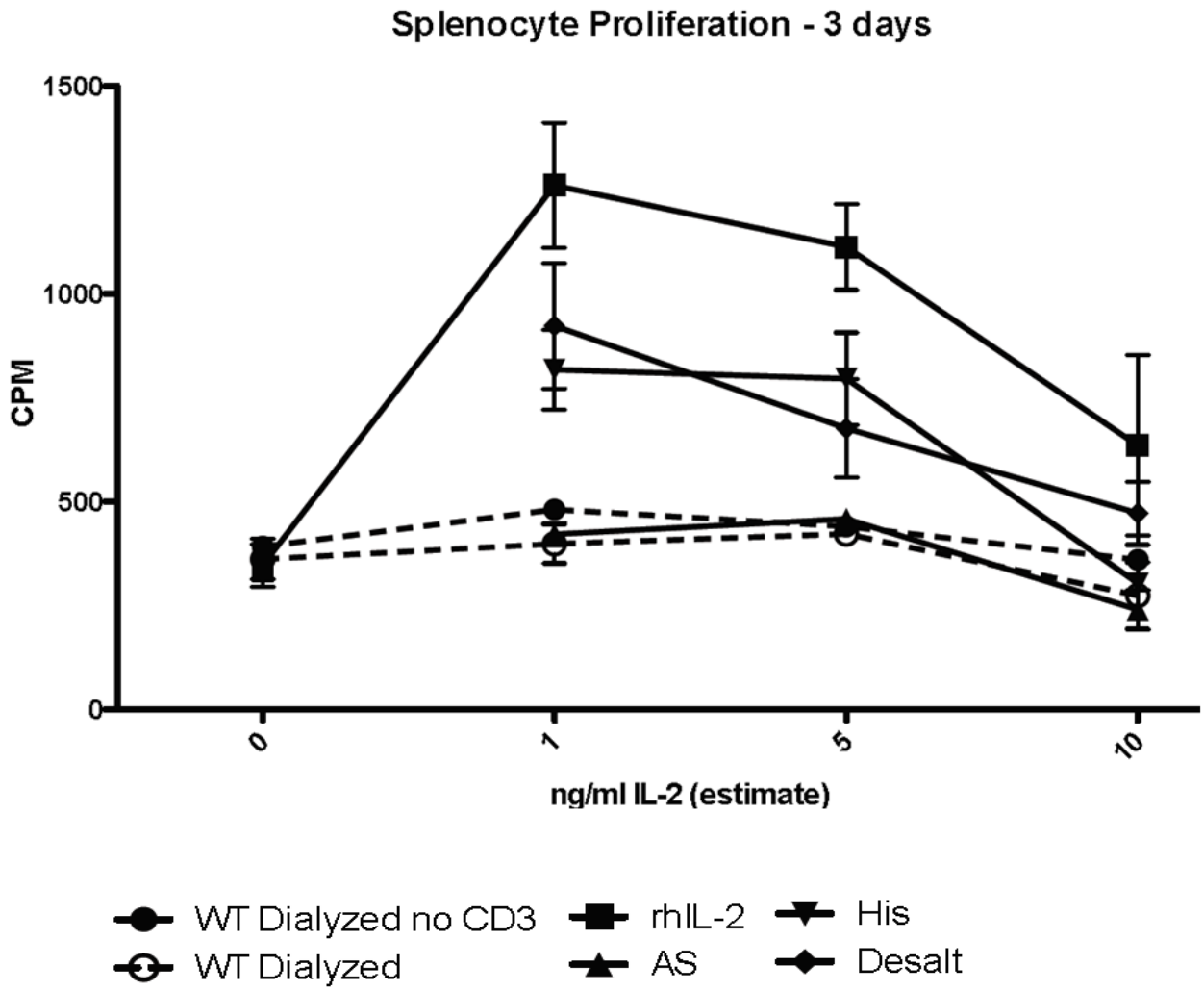


Figure 12. Results from the second set of cultures testing IL-2 biological activity.

From these results, it was apparent that using stimulated CD4+ T-cells was the preferred method to get accurate functional data at lower doses of IL-2. A third set of cultures was prepared to repeat the last experiment; however, this time the cell number was increased to $3x10^5$ cells per well and the cultures were incubated for four days instead of three. The results from the third set of cultures can be found in Figure 13.

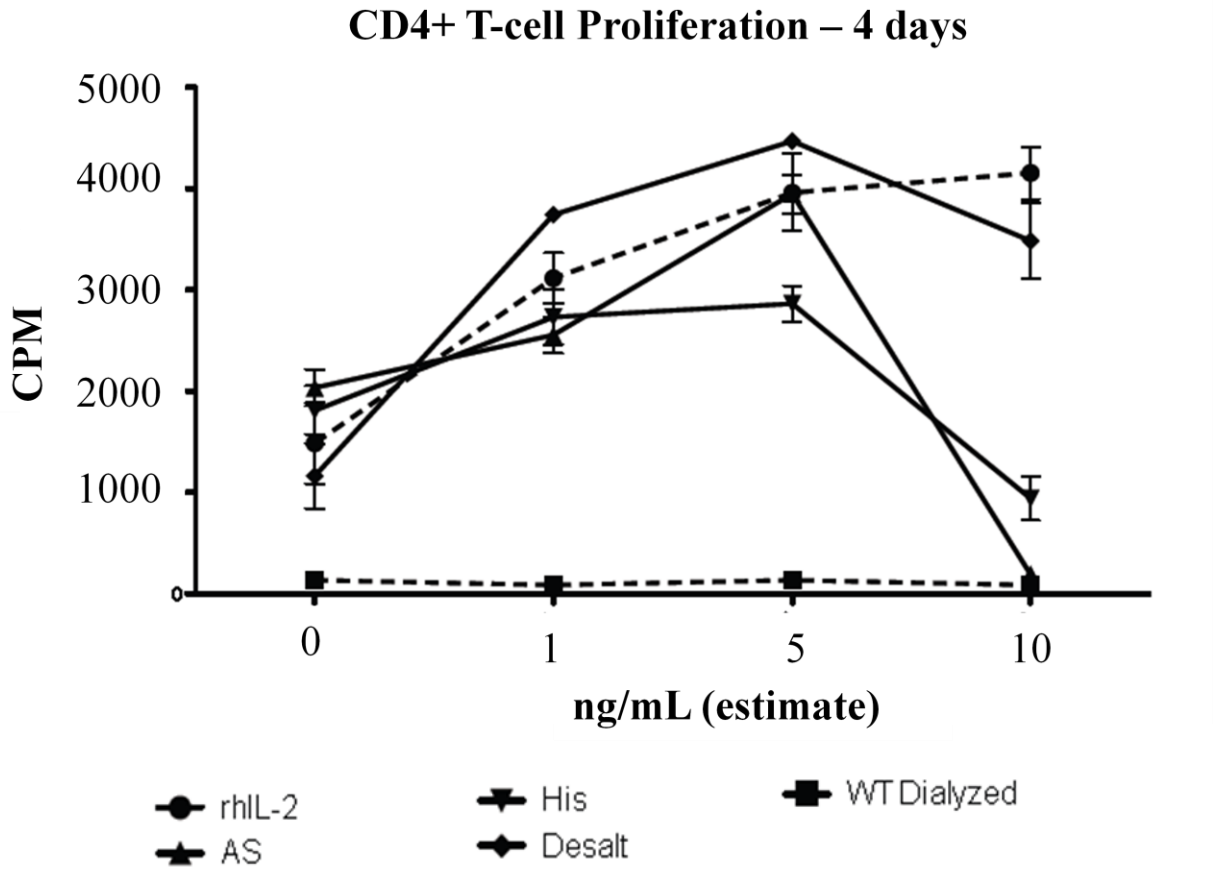


Figure 13. Results from the third set of cultures testing IL-2 biological activity.

In this third set of cultures, it is apparent that all three samples are showing biological activity above baseline. From the 0-5 ng/mL range, both the desalt and the ammonium sulfate sample show activity at or above that of the positive control. With all samples, again we see a drop in activity when this concentration is exceeded.

To demonstrate that it was a property of the samples which had an inhibitory effect, a fourth set of cultures was prepared. These cultures also were prepared from isolated CD4+ T-cells that were activated with anti-CD3 antibody, and each well contained $2x10^5$ cells. One set of wells was treated with purified IL-2, another set was treated WT protein extract (volumes equal to the amount that would be added of the ammonium sulfate sample to deliver the

appropriate dosage of IL-2) and a third set was treated with the same volumes of WT protein extract with the addition of purified IL-2. The results from these cultures can be found in Figure 14.

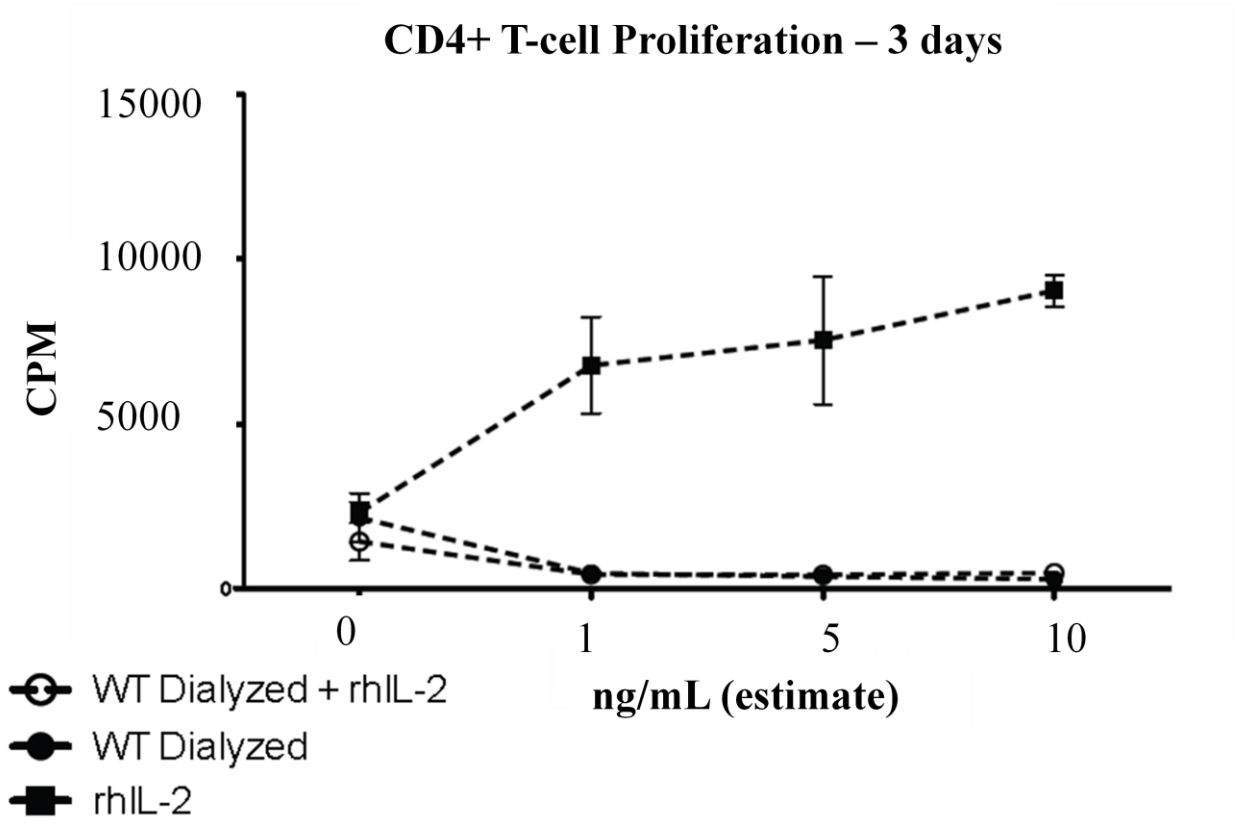


Figure 14. Results from the fourth set of cultures which tested for inhibitory properties of the samples.

The results seen in Figure 14 demonstrate clearly that there is some property of the samples that has an inhibitory effect on T-cell proliferation. Since this inhibitory effect is present in all samples, even the sample prepared using histidine affinity gel which should be relatively pure of other tobacco extract components, it seems likely that it is a property of the sample preparation and not a cell extract component that is inducing this inhibitory effect. The most likely explanation is pH. Dr. Carson reported seeing a significant color change of the media (a pH color indicator was present in the media) when he reached the 10ng/mL

concentrations. In vitro cell cultures can be very sensitive to even small changes in pH, and this stress can have significant impacts on cellular function.

Student t-tests were performed to verify that the data is statistically relevant. The data collected for 1 and 5ng/mL was studied because these concentrations did not appear to have an inhibitory effect on T-cell proliferation. The raw data for these calculations, as well as the calculated t values, can be found in Appendix C.

First, right-tailed t-tests were used to verify that the CPM values of the positive control and the three samples were significantly higher than those of the negative control. Each culture was done in triplicate, resulting in two degrees of freedom. An alpha of 0.05 was used. The t values calculated for each of these points was well over the cutoff t value from the distribution table ($t_{0.05,\ df=2} = 2.92$), allowing for the rejection of the null hypothesis in each case (H_0: $\mu \leq \mu_{WT\ Extract}$; H_a: $\mu > \mu_{WT\ Extract}$). There is sufficient evidence to suggest that the effects of the positive control and each of the three samples are greater than that of the negative control. Second, two-tailed t-tests were performed to evaluate whether or not the CPM values from the samples were significantly different from the CPM values of the positive control. The same values from the triplicate cultures were utilized as well as an alpha of 0.05. The cutoff t value from the distribution table ($t_{0.025\ df=2} = 4.303$) was greater than the absolute value of the t statistic calculated in each case, except for the desalt sample at both concentrations. Therefore, for the ammonium sulfate and HIS affinity samples, the null hypothesis is not rejected (H_0: $\mu_{sample} = \mu_{rhIL\text{-}2}$; H_a: $\mu_{sample} \neq \mu_{rhIL\text{-}2}$). There is not sufficient evidence to suggest that the values from the ammonium sulfate and HIS affinity samples are different from the average value of the positive control (rhIL-2). However, the t values calculated from for the desalt sample are

well over 4.303, meaning that there is evidence that the proliferative effect induced by these samples is different than that produced by the positive control. Looking at the data, it is apparent that proliferation using the desalt sample is actually greater than that of the positive control.

2.5) Inheritance. Seeds were collected from the selfed T0 population. Only nine of the 13 IL-2-expressing plants produced seeds. All plants that did not produce seeds appeared healthy and did flower. Of the seed-bearing plants, a portion of the seeds collected were germinated and grown in a growth room maintained at room temperature with a photoperiod of 16L/8D. Two independent progeny from each of the nine seed-producing lines were chosen at random for to verify transmission of the transgene to the next generation. CTAB extraction of genomic DNA and PCR was carried out as described in the methods section. The results of these PCR reactions can be found in Figure 15. As shown in this figure, all tested plants appear to be transgenic, inferring the gene is stably inherited.

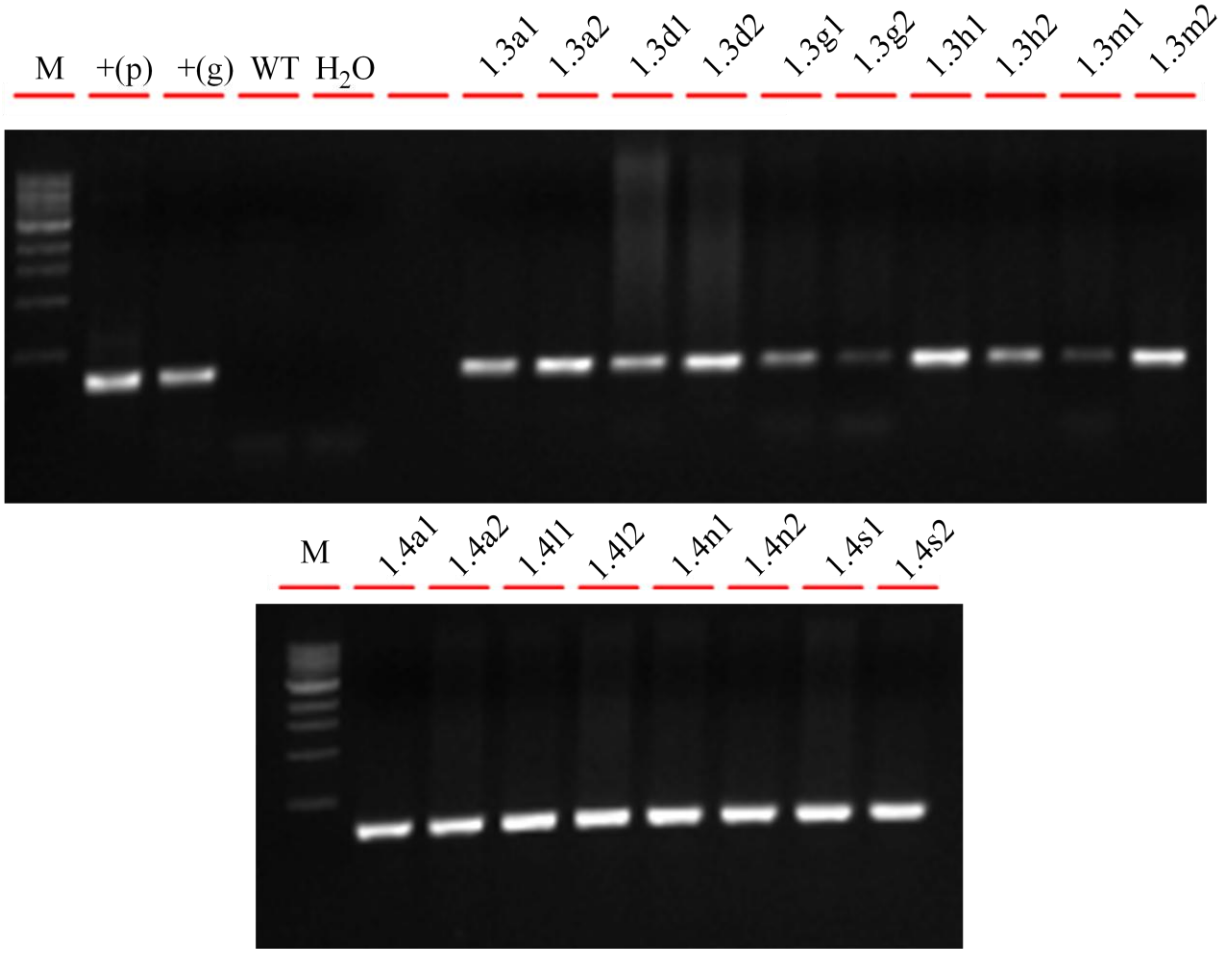


Figure 15. Polymerase chain reactions for the T1 progeny. Lane 1 contains 1kB marker. Lane 2 and 3 are positive controls with plasmid and T0 DNA, respectively. Lane 4 contains WT template DNA. Lane 5 contains no template DNA. The other lanes containing T1 gDNA samples are identified in the figure.

CONCLUSION AND FUTURE PROSPECTIVES

The present research has demonstrated the feasibility of producing biologically active human IL-2 in the green tissues of tobacco. It is difficult to determine precisely how much recombinant IL-2 is produced with this system. A good estimate, however, can be deduced from the western blot screening since the amount of protein added was controlled carefully. When

visually comparing the band sizes of certain samples, such as samples from independent transgenic lines 1.3g and 1.3h, to the positive control lane, they are very similar. Estimating that there were 10ng of IL-2 in the loaded transgenic plant leaf crude extract and knowing the volume of sample loaded, the volume of the total sample prepared and the weight of tissue used to prepare the sample, it was possible to estimate amount of IL-2 production. For sample 1.3h, 8.9 of 100μL total prepared sample was loaded to produce a band that is visually similar to the 10ng positive control. This translates to about 111.5ng of IL-2 in the total sample, which was prepared from two leaf disks of approximately 0.0055g each. This translates to about 10.1μg IL-2 per gram of fresh tissue, or 10.1g IL-2 per ton of fresh tissue. However, this figure alone does not fully describe the value of this system. The potency of the IL-2 produced is just as important. As demonstrated in Figure 13, the recombinant protein produced here appears to have potency similar to that of the commercially available recombinant IL-2 derived from *E. coli*. The volume at which IL-2 can be produced and the cost-effectiveness of this system might make this production platform preferential over the currently used platform.

To get an accurate measurement of biological activity of plant-produced IL-2, one must isolate the plant-produced IL-2 prior to testing and also utilize a more accurate method of quantification, such as high-performance liquid chromatography (HPLC). Additionally, the isolated protein should be constituted in a weak buffer as not to have a significant impact on cell culture pH when testing is performed.

Although the expression level of tobacco leaf-produced IL-2 is relatively modest, much can be done to improve the IL-2 purity and yield. To improve purity, alternative methods of extraction and purification could be used for higher-quality IL-2. To improve yield, the independent transgenic lines created here could be self-bred to establish homozygosity which has

been shown to result in increased expression (Zhong et al., 1999). Also, these lines could be crossed to slowly increase copy number which has also demonstrated increased expression when performed and monitored appropriately (Streatfield, 2006). In addition, the IL-2 yield and biological activity might be increased when IL-2 is produced in different or in multiple sub-cellular compartments. The biological activity of chloroplast targeted IL-2 has not been evaluated. Multiple compartment targeting can be accomplished either via re-transformation of transgene constructs, co-transformation of multiple transgene constructs or via cross breeding of IL-2 transgenic plants.

If this project were to be repeated, it might behoove the researcher to redesign the DNA construct to include a peptide cleavage site on the C-terminal end of the gene which would place it upstream of the C-myc and HIS tag. Even though biological activity has been demonstrated for the protein including these tags, regulations might require that the tags be removed before use in humans.

It probably will take pyramiding of several approaches to increase the IL-2 yield (see Chapter 1) in order to manufacture this valuable biotech drug at commercial level. To date, there are many examples of approaches to increase yield of a recombinant plant-produced biopharmaceutical being used on an individual basis, but very few examples taking advantage of multiple yield-augmenting methods. The pyramiding of yield-enhancing methods will become increasingly important in the coming decades. As a global community, we have only limited technologies that can be utilized to produce biopharmaceuticals and limited resources at our disposal. With the strain on current pharmaceutical production systems, it will become necessary to use recombinant plant production systems to manufacture biopharmaceuticals in a more effective manner. If a field of food crops, such as corn, that originally was grown solely to

produce feed for livestock also now can produce valuable co-products, such as IL-2, in its non-edible tissues, the value of this field could be greatly augmented. Additionally, a significant amount of processed stover will be left over after IL-2 extraction and can be used for biofuel production, thus making use of every part of the plant (Sticklen, 2009). There is tremendous "green" potential in this type of work and that is why I chose to pursue production of the high value biopharma IL-2 in plants as my thesis research. Considering that this technology is relatively new, I am sure that we are just beginning to scratch the surface of what plant production platforms have to offer, and will see tremendous progression in the next few decades.

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LITERATURE CITED

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APPENDICES

APPENDIX A

Shown here are screenshots of certain sections of the IL-2 codon optomization report provided by GenScript. An alignment of the optimized and native gene is shown with red nucleotides highlight the sections of the gene that were altered. Below this is a protein alignment showing that both the native as well as the optimized gene translate the same protein.

DNA Alignment

Figure 16. Image from the GenScript report showing the nucleotide alignment of the native IL-2 gene and the codon optimized version.

Protein Alignment

```
Optimized     1     AAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL
Original      1     AAPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL

Optimized    61     EEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLN
Original     61     EEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLN

Optimized   121     RWITFCQSIISTLT
Original    121     RWITFCQSIISTLT
```

Figure 17. Image from the GenScript report showing the amino acid alignment of the peptide produced from the native and codon optimized version of the IL-2 gene.

APPENDIX B

Table 5. Raw Data from intensity quantification of western blot bands using Bio-Rad Quantity One 1-D Analysis Software.

	Intensity (INT)		
Sample Volume (μL)	Ammonium Sulfate	Desalt	HIS Affinity Gel
15	1315861.4	1504600.5	1171193.3
7.5	1204375.5	1475126.4	938623.5
3.75	833156.2	1190490.3	694076.5
1.88	676797.7	907269.4	557691.2
0.94	520706.6	418915.3	370632.8
Positive Control (10ng)	1093160.5	1120423.4	907912.5

APPENDIX C

Table 6. Raw data from the T-cell cultures shown in Figure 13. All values represent CPM unless otherwise noted.

ng/mL IL-2	rhIL-2			Ammonium Sulfate		
0	2285	1191	988	2370	1756	1976
1	2716	3057	3573	2912	2415	2338
5	4672	3370	3834	3861	3664	4315
10	4558	4235	3672	222	164	186
	HIS Affinity			**Desalt**		
0	1697	2272	1464	590	1697	1201
1	2709	2272	3214	3742	3737	3749
5	2749	2629	3214	4444	4476	4483
10	1353	858	615	3263	4204	2986
	WT protein					
	82	197	132	128	74	63

Table 7. Statistical data for CPM readings of T-cell cultures shown in Figure 13. Only data from 1 and 5 ng/mL IL-2 were analyzed.

ng/mL IL-2	rhIL-2					Ammonium Sulfate				
	Average	Stdev	StErr	t value (Rt-tailed)	t value (2-tailed)	Average	Stdev	StErr	t value (Rt-tailed)	t value (2-tailed)
1	3115.333	431.468	249.108	**12.506**	**0.000**	2555.000	311.559	179.879	**14.204**	**-1.798**
5	3958.667	659.892	380.989	**10.391**	**0.000**	3946.667	333.848	192.747	**20.476**	**-0.036**
	HIS Affinity					**Desalt**				
	Average	Stdev	StErr	t value (Rt-tailed)	t value (2-tailed)	Average	Stdev	StErr	t value (Rt-tailed)	t value (2-tailed)
1	2731.667	471.409	272.168	**10.037**	**-0.814**	3742.667	6.028	3.480	**1075.447**	**104.075**
5	2864.000	308.990	178.396	**16.054**	**-3.543**	4467.667	20.793	12.005	**372.162**	**24.480**

APPENDIX D

Transformation and regeneration of *Zea Mays* with recombinant human interleukin-2.

Introduction

Zea Mays also was transformed to produce recombinant human IL-2. The same five expression cassettes used to transform tobacco were used here. The Impact Vectors were used directly without the need to excise the expression cassette and ligate it to an alternative backbone because transformation was carried out via particle bombardment. Particle bombardment was the method of transformation used since corn is a monocot and not readily infected by *Agrobacterium*. Our laboratory has developed a system of co-bombardment to achieve transformation. In this method, the microcarriers are coated with a mixture of DNA containing the plasmid containing the gene of interest as well as a plasmid containing a selectable marker. The plasmid used for selection was JS101 which contains a BAR gene affording resistance to an herbicide (Bialaphose). The selectable marker allowed for the selection of transformants which then were screened via PCR and western blotting to identify plants containing the gene of interest. The theory behind this method is that plants that are transformed with one of the plasmids will be more likely also to have been transformed with the second plasmid. The protocol used for transformation was developed by Thang Nguyen.

Materials and Methods

Stable Genetic Transformation and Regeneration of Corn Plants.

1) Preparation for Bombardment. The microcarriers used were tungsten particles. The microcarrier preparation for a single bombardment was carried out by making a mixture containing7μL of 50μg/mL tungsten, 1.5μg of each of the plasmid constructs, 7.5μL of 2M calcium chloride and 3μL of Spermidine. This recipe was multiplied to allow for five individual bombardments to be carried out. Components were added in the order listed and the mixture was

vortexed vigorously after the addition of each component. Once this mixture was prepared, it was centrifuged briefly for 10s to allow the tungsten particles to collect at the bottom of the tube. The supernatant was discarded and the pellet was washed with 70% EtOH. After this EtOH was removed, the pellet was resuspended in 6μL of 100% EtOH for each volume of tungsten particles being prepared.

The instrument used for bombardment was a Biolistic Particle Delivery System from Dupont (Model number: PSD-1000). Before use, the bombardment chamber as well as an appropriate number of macrocarriers, stopping plates and rupture disks were sterilized with 70% EtOH. The entire process was carried out in a laminar flow hood.

For each of the five constructs, five plates of calli were bombarded. The corn calli were generated from HiII immature embryos (Plant Transformation Center at Iowa State University). Each Petri dish contained calli spread in a circle of about three centimeters in the center of the Petri dish containing N6OSM. These calli had been subcultured to N6OSM media at least four hrs prior to bombardment.

2) Bombardment. Six μL of the bombardment suspension containing the DNA coated microcarriers was spread onto the surface of the macrocarrier, allowed to dry slightly and placed in the appropriate section of the chamber. A Petri dish containing N6OSM media with calli in the center was placed at the bottom of the chamber. The recipe for each type of media can be found in Table 6. The chamber was sealed and evacuated by vacuum. Compressed CO_2 was allowed to build up in pressure behind the rupture disk until a pressure of 1100PSI was reached. At this point, the rupture disk broke, releasing the compressed air which accelerated the macrocarrier. The stopping plates utilized here were mesh screens to stop the macrocarriers from reaching the calli, while allowing the microcarriers to reach them.

Following bombardment, the vacuum was released and the Petri dish was covered, sealed with parafilm and incubated for 16 hrs in darkness at room temperature. Calli from each Petri dish then were separated into four equal sections and subcultured onto N6E media for five days.

3) Selection. After this five-day recovery period, calli were subcultured onto N6S selection media and maintained in darkness at room temperature. Calli were subcultured every 15 days to new N6S for 10-12 weeks. During these weeks, brown tissue was removed and white, fast-growing tissue was transferred to new N6S to progress it forward. Good contact with the media was ensured to allow for proper selection.

4) Regeneration. Healthy calli were transferred to R1 media and placed in a photoperiod of 16L/8D under low light density. This was accomplished by placing them in the vicinity of a shelf with lighting, but not directly underneath it. This incubation was maintained for seven days and green spots were noted on many of the calli. Calli with green spots were transferred to R2 media and maintained under full light. Calli that remained were subcultured after two weeks to fresh R2, while calli that had regenerated into immature seedlings were transferred to magenta boxes containing R3 media. Seedlings were placed four to a box and maintained under the same light and temperature conditions. To accommodate for seedling height, a second magenta box was inverted and sealed to the top. Once seedlings had developed a significant root system, they were rinsed to remove residual media and transferred to 4 x 4in soil pots. These pots were maintained in bags to simulate the high humidity of the magenta box environment, and slowly opened over a period of several days until the plants were acclimated to ambient conditions. The plants then were transferred to the greenhouse and potted in larger containers. Both watering and fertilization were carried out regularly.

Table 8. Recipes for media used in stable corn transformation. These are the amounts necessary for the preparation of one liter.

	N6E	N6OSM	N6S	R1	R2	R3
N6 S+V	4.00g	4.00g	4.00g			
MS S+V				4.43g	4.43g	2.21g
2,4D	2mg	2mg	2mg	0.25mg		
Myo-inositol	100mg	100mg	100mg	100mg	100mg	
Sucrose	30g	30g	30g	30g		15g
Proline	2.76g	0.69g				
Sorbitol		36.4g				
Mannitol		36.4g				
Maltose					60g	
Casein hyd	100mg	100mg				
pH	5.8	5.8	5.8	5.8	5.8	5.8
Gelrite	2.5g	2.5g	2.5g	2.5g	2.5g	2g
$AgNO_3$ (25μM)	1mL	1mL	200μL			
BAP				1mg		
IBA						1mg
Bialaphos			2mg			1mg

Discussion

Several hundred plants, a varying number for each construct, were regenerated using this method. Since the selectable marker was not linked to the gene of interest, it was very important that PCR be carried out to identify plants transformed with the IL-2 gene. CTAB extraction for this number of plants would require an exorbitant amount of time, so a quicker kit method was chosen.

The initial PCRs to identify plants transformed with the IL-2 gene were conducted using the REDExtract-N-Amp Plant Kit from Sigma (CN: XNAP2). The procedure was carried out according to the manufacturer's instructions. Several attempts were made at extraction and amplification using 5-10 plants for each attempt. Unfortunately, no bands could be detected in any of these plants (data not shown). The thermocycling temperatures and primers were the

same used successfully for tobacco. The inability to identify IL-2 transformed plants could have been due to a low efficiency of co-transformation, or the DNA extracted using this kit may have been of such poor quality that PCR was failing.

At this point many of the plants in the greenhouse became very unhealthy. Spider mites continued to cause much damage despite two rounds of pesticides having been sprayed. Many of the plants also appeared diseased, having very short stalks that became soft and curled. About 70 plants still appeared healthy and were transferred to a different greenhouse. Despite the lack of PCR data, western blotting was carried out on 56 of these plants. Western blotting was conducted in the same manner as it was for tobacco. None of these samplings showed any IL-2 expression. Efforts are still underway to obtain reliable PCR data. CTAB extraction is being performed to obtain high quality DNA and eliminate the variable of the kit.