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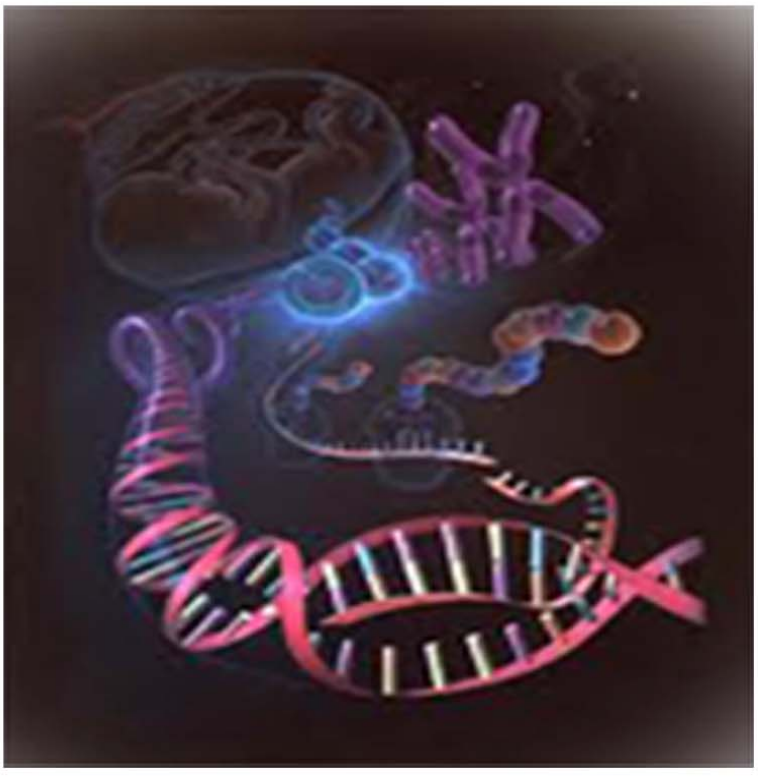

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Research Paper

PLANT-PRODUCED RECOMBINANT HUMAN INTERLEUKIN-2 AND ITS ACTIVITY AGAINST SPLENIC CD4 + T-CELLS

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Recombinant human interleukin-2 (rhIL-2) is a biopharmaceutical protein of great importance, as it is the standard FDA-approved immunotherapeutic treatment for end-stage metastatic melanoma and renal cell cancer. In this study, we explored the feasibility of producing biologically active rhIL-2 in the green tissues of transgenic tobacco (*Nicotiana benthamiana*). Production of rhIL-2 proteins in whole-plant expression system will be more economical when compared to the current *E. coli* based expression system. The human rhIL-2 gene was codon optimized to maximize plant host system expression. A construct fusing red fluorescent protein to rhIL-2 was developed, and confocal microscopy was utilized to verify the targeted rhIL-2 accumulation, and its proper folding in this system. Five additional plant-specific transgene constructs were developed for stable expression rhIL-2 with targeting to each sub-cellular compartment. Western blotting of the stably transformed lines demonstrated accumulation of the appropriately sized rhIL-2 protein in the endoplasmic reticulum and chloroplasts. The plant-produced rhIL-2 was purified, and its biological activity was compared with that of commercially available *E. coli* produced rhIL-2 on murine splenic CD4+ T-cells from C57BL/6 mice. This research demonstrated the efficacy of using tobacco as an expression system for the production of biologically active rhIL-2.

Keywords: Recombinant DNA Technology, Biopharmaceutical Production, Interleukin-2, Tobacco plant

INTRODUCTION

Plant genetic engineering technology developed in the early 1980s has help to use plant as a host of making biomolecules. Further advances in plant genetic engineering have provided the means of transforming a plant into a biological factory for the mass production of novel materials including recombinant proteins. This practice, termed "molecular farming", has a number of applications, and has proven quite promising in the recent years.

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The plant system has several economic and biological advantages over other production platforms and is rapidly becoming the platform of choice for many compounds (Obembe *et al*., 2011; Sirko *et al*., 2011). As compared to microbes, plants offer the advantages of (1) using solar energy rather than energy input from other sources; (2) requiring less hands-on care, less attention, and less training for successful cultivation; (3) virtually unlimited and easy scale-ups; (4) common practices for large scale production, processing, storage, and distribution; and (5) often host large biomass production; and (6) having conserved molecular machinery which usually allows folding of complex proteins similar to the protein processing in animals, including humans. For example, plants usually possess the ability to assemble complex, multi-component proteins such as antibodies (Hein *et al*., 1991).

Glycosylation is the major factor to keep under consideration when producing recombinant proteins in *E.coli* based expression system. The correct glycosylation is necessary for many proteins to fold properly, playing a crucial role in protein stability and turnover rates (Gomord *et al*., 2010). Non-native glycan structures can be immunogenic. However, recombinant proteins targeted for retention within the Endoplasmic Reticulum (ER) of plant cells will only receive the addition of high-mannose-type N-glycans, which are structurally similar in plants and animals. It isn't until further processing in the secretary pathway that immunogenic complex N-glycans and O-glycans result. Several studies have demonstrated the production of recombinant proteins tagged for ER retention that primarily have high-mannose-type N-glycans (Ko *et al*., 2003; Triguero *et al*., 2005).

The production cost and demand for many conventionally produced biopharmaceutical proteins is high. Developing strategies to produce biologically active recombinant pharmaceutical proteins in plants will not only lucrative to the production companies, but also might help to make these therapeutic protein more affordably available to poorest segments of the world population. One of these key biopharmaceutical proteins is human IL-2.

IL-2 is a cytokine which regulates many immunological processes and possesses a significant amount of clinical relevance. In 1992, the Food and Drug Administration (FDA) approved *E. coli*-produced rhIL-2 for the treatment of end-stage metastatic melanoma and renal cell cancer. The rhIL-2 was the first and only FDA-approved immunotherapeutic drug for melanoma until 2011 (Diehl, 2011). This rhIL-2 was developed by Novartis Pharmaceuticals and released commercially under the name Proleukin (Aldesleukin). Proleukin is regularly used in clinical settings to treat patients with metastatic melanoma and renal cell cancers, as well as used in research laboratories for the *in vitro* proliferation and maintenance of T-cells *in vitro*.

The research described here given details about the steps taken to develop a potentially low cost production platform for rhIL-2. Previous studies have demonstrated rhIL-2 production in potato tuber (Park and Cheong, 2002), fruits of tomato (Cui *et al.*, 2008) and tobacco cell culture (Magnuson *et al.,* 1998). However, this is the first study to demonstrate the feasibility of utilizing the green tissues of a non-food crop tobacco plant (*N. Benthamiana*) for the production of rhIL-2.

In this study, we describe codon optimization of the human IL-2 gene to match the codons of this plant, the successful folding and production

of rhIL-2 protein in the ER and chloroplast of green tissues of plants, followed by the purification and biological activity of the plant-produced rhIL-2 and compared with commercially available *E. coli* produced rhIL-2 against marine splenic CD4+ T-cells from C57BL/6 mice.

MATERIALS AND METHODS

Construct Design

IL-2 Gene Analogue

The Genscript rare-codon-analysis-tool, available at http://www.genscript.com/cgi-bin/tools/rare_codon_analysis, was utilized to determine a sequence of codons that would afford optimal expression of the human IL-2 gene in *N. benthamiana*. Flanking restriction sites (NcoI and BglII) were added to allow for cloning into a specific plant expression vectors. This oligo was purchased from GeneScript. The gene sequence, as well as the corresponding amino acid translation, could be seen in the supplemental Figures 5.

Transient Expression Vector

Plasmid pVKH18En6 35s St-m-RFP is a binary vector which allows codon optimized human IL-2 to be inserted upstream of the gene red fluorescent protein, resulting in a fusion protein. This vector also affords kanamycin resistance for chemical selection purposes. The development of this vector is described in Sparkes *et al.* (2006).

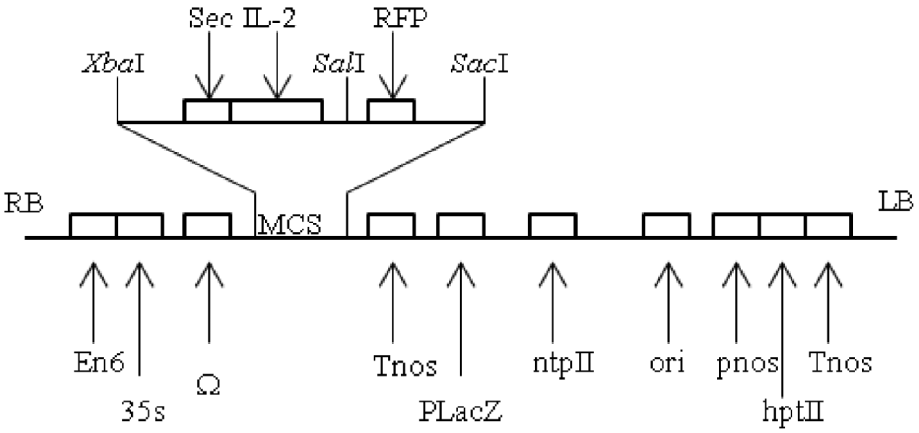
Polymerase Chain Reaction (PCR) was performed to create an insert containing the optimized human IL-2 gene fused to the secretory signal of an ER-targeted vector. The insert contained an upstream XbaI and a downstream SalI restriction sites, as well as two nucleotides between each restriction site and the gene to maintain reading frame. This insert was cloned into pVKH18En6 35s St-m-RFP in-frame with the RFP so that the coding region will contain the human IL-2 gene (including tags) with an n-terminal secretory signal and a c-terminal RFP fusion. A 6X cauliflower mosaic virus 35S promoter drove expression of secreted rhIL-2-RFP. Figure 1 shows a diagram of this construct with the restriction sites necessary to perform necessary cloning.

Stable Expression Vectors

Plant Research International of Wageningen University and Research Center has developed a series of five transformation vector plasmids named Impact Vector series (see: http://www.pri.wur.nl/UK/products/ImpactVector/) which fit all of requirements of this research. In these vectors (Figure 2), the transgene expression is driven by the light-regulated ribulose bisphosphate carboxylase small subunit (RbcS1) promoter and terminator from *Asteraceous chrysanthemum*. The ribulose bisphosphate carboxylase is involved in carbon fixation and produced solely in photosynthetic tissues such as leaves and stems.

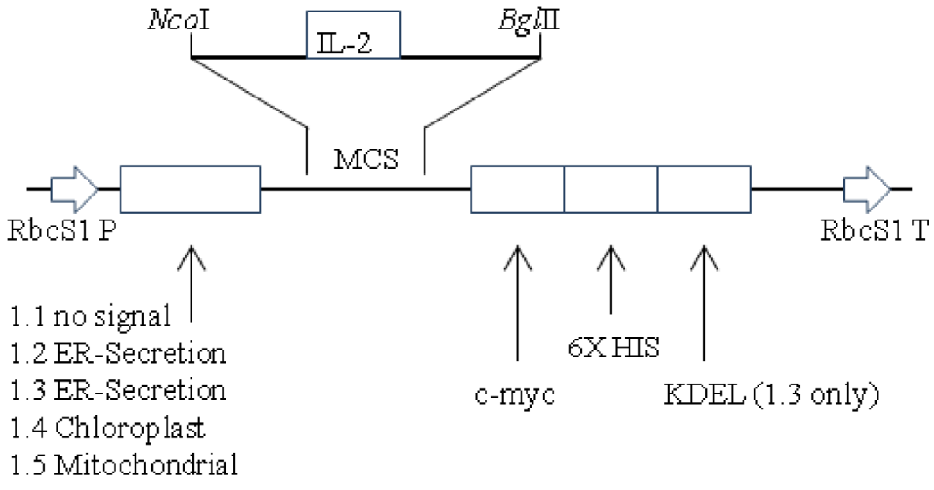
Each of the five vectors allows for targeting of the rhIL-2 to a particular host plant sub-cellular compartment within the plant cell. The vector 1.1 contains no signaling peptide, allowing for cytosolic accumulation. The vector 1.2 contains a signaling peptide from sea anemone equistatin which allows the rhIL-2 to be secreted into the apoplast. The vector 1.3 is the same as vector 1.2; however a KDEL retention signal has been added to the C terminus, allowing the rhIL-2 to accumulate into the ER. The vector 1.4 harbors a signal peptide from *Chrysanthemum morifolium* small subunit which targets the rhIL-2 into the

Figure 1: Expression cassette for the gene construct used for the transient expression assay. This image depicts the DNA construct created by inserting the IL-2 analogue gene into pVKH18En6 35s St-m-RFP vector (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



Abbreviations: RB : T-DNA right border; En6 35s : 6x enhanced CaMV 35s promoter ; Ω: TMV 5' leader ; MCS : multiple cloning site; Sec : secretory peptide ; RFP : red fluorescent protein ; Tnos : nos terminator ; PLacZ: LacZ promoter; nptII: kanamycin resistance marker; Ori: ColE1 origin of replication; Pnos: nos promoter; hptII: hygromycin resistance marker; LB: T-DNA left border.

Figure 2: Expression cassette from the gene constructs used to stably transform tobacco plants. This image depicts the DNA construct created by inserting the IL-2 analogue gene into a set of five vectors from the Impact Vector series. Each vector has a different (or no) signaling peptide. Expression is driven by the ribulose bisphosphate carboxylase promoter (and terminator) from *Asteraceous chrysanthemum.* The IL-2 gene was cloned in frame with C-myc and 6X HIS tags to allow for easy protein extraction



Abbreviations: RbcS1 P: ribulose bisphosphate carboxylase promoter; 1.1-1.5: signal peptides for targeting ; MCS: multiple cloning site; c-myc: c-termainal myc epitope ; 6X HIS: 6X Histidine tag ; KDEL: ER retention signal present in 1.3; RbcS1 T: ribulose bisphosphate carboxylase terminator.

stroma of the chloroplast. Finally, the 6 vector 1.5 utilizes the yeast CoxIV secretion signal to target the rhIL-2 into the mitochondrial matrix.

The Impact Vector series also contain a segment downstream that, when cloned in frame with the gene inserts provides the addition of a C-myc epitope and a poly histidine tag to the C-terminus of the rhIL-2 protein. The c-myc epitope 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.

TRANSFORMATIONS

Transient Transformation of Tobacco

ElectroMAX *A. tumefaciens* strain LBA4404 from Invitrogen (Carlsbad, CA, USA, www.invitrogen.com) was transformed via electroporation with pVKH18En6St-m-RFP containing the codon-optimized human IL-2 gene analogue. Additionally, this *A. tumefaciens* strain was transformed with pVKH18En6St-m-RFP empty vector. The NPTII gene on the plasmid confers kanamycin resistance used to select for transformants. The infiltration method set forth by Sparks *et al*. was used to transiently transform tobacco leaves of the Samsun variety (Sparkes *et al*., 2006).

Stable Genetic Transformation and Plant Regeneration

The expression cassette from each of the five vectors (1.1-1.5 in Figure 2) was cloned into a pBINPLUS binary vector using the AcsI and PacI restriction sites. These five vectors were then used to transform ElectroMAX *A. tumefaciens* strain LBA4404 via electroporation. 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 (100 mg/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 reported from Dr. Jurgen Deneche's lab at the University of Leeds (http://www.plants.leeds.ac.uk/jd/Protocols.html).

Transgenic plantlets were transferred to a greenhouse where they were maintained under high intensity lamps (400 W sodium lamps, GE lighting) at a 16L/8D photoperiod. The greenhouse 7 temperature was controlled at about 30„aC with a photoperiod of 16L/8D. Once plants were identified as expressing rhIL-2, they were transferred to larger pots and kept under the same greenhouse conditions to allow for further growth and maturation. Stable transformation was confirmed via PCR. DNA extraction was performed using a version of the CTAB method (Edwards *et al*., 1991).

EVALUATION OF TRANSGENIC PLANTS

Fluorescence Confocal Microscopy

Microscopy was performed on plant material that had been transformed with the transient expression construct. This was carried out two days post infiltration to visualize protein expression and accumulation. The instrument utilized was the Olympus FluoView FV1000 Confocal Laser Scanning Microscope (Center Valley, PA) with a 60X PlanApo N oil objective. Brightfield images with confocal fluorescence overlay were recorded at 570-620 nm and 655-755 nm.

Total Soluble Protein Extraction/ Quantification (for Western Blots)

Fresh leaf samples were homogenized in phosphate grinding buffer: 100 mM sodium phosphate pH 5.8, 1 mM EDTA, 10 mM diethyldithio carbamic acid, and 0.5% Tween 20. A 2X protease inhibitor cocktail (CN: P9599; Sigma-Aldrich; St. Louis, MO 63103) was added to the buffer to prevent proteases degrading the rhIL-2 during extraction. The protein quantification was performed using the Coomassie Plus (Bradford) Protein Assay reagent available from Thermo Scientific (CN: 23238). All work was conducted on ice.

Western Blot Screening

Western blotting was carried out to screen for the plant-produced rhIL-2 protein expression in stably transformed plants. This is also allowed us to comparison the expression levels between different transgenic lines. Twenty kanamycin-resistant plants were selected from each of the five expression Vectors (Figure 2) for testing, totaling 100 plants. Each plant was assigned a unique label that identified its used vector type (1.1, 1.2, 1.3, 1.4 and 1.5), and distinguished individual plants among the same vector type (from a to t). All of the western blots run had three lanes in common. The first lane contained the MagicMark XP Western Protein 8 Standard from Invitrogen (Grand Island, NY 14072). The second lane contained 10 ng of commercially available *E. coli*-produced rhIL-2 (CN: 202-IL) purchased from R & D systems (Minneapolis, MN, USA), used as a positive control. The third lane contained the wild-type plant total soluble protein, used as a negative control. The NuPAGE system from Invitrogen was used to run the western blotting. The NuPAGE Novex 10% BIS-TRIS gels with 10 wells (CN: NP0301BOX) were used due to its band resolution. 15 μg of total soluble protein was loaded in each well to test samples.

Plant Progeny Inheritance

Seeds were collected from the self pollinated to plant population. Only nine of the 13 rhIL-2-expressing plants produced seeds. All plants that did not produce seeds appeared healthy, but did produce 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 human IL-2 transgene to the next generation. CTAB extraction of genomic DNA and PCR was carried out as described in the procedures section. As shown in this figure, all tested plants appear to be transgenic, inferring the gene is stably inherited.

EVALUATION OF PLANT DERIVED RHIL-2

Sample Preparation for T-Cell Proliferation Assay

Leaf tissues were ground to fine powder in liquid nitrogen, and further ground to a homogenous solution using protein extraction buffer, clarified, and filtered through a 0.22 μm PVDF filter from Millipore (Billerica, MA, USA). This extract was passed through a HisPrep 26/10 desalting column (CN: 17-5087-01; purchased from GE Healthcare, Port Washington, NY, USA) to remove small molecules and exchange the buffer, and reconstituted in a 20 mM citrate buffer pH 4.5. The resulting fraction of interest was then concentrated using a Vivaspin 5000 MWCO centrifuge tube from Fisher Scientific (CN # VS0611) to 1.2 mL and placed at -20 ^{o}C.

Plant-Produced rhIL-2 Quantification

Quantification was performed by western blotting. Each gel had a control lane of 10 ng of the positive control *E. coli*-produced rhIL-2 protein to serve as a standard. Serial dilutions of each sample were loaded in decreasing amounts (15, 7.5, 3.75, 1.87 and 0.94 μL). Quantity One 1-D Analysis Software from Bio-Rad (Hercules, CA 94547) was used to quantify the intensity of each band and these values were plotted as a function of sample volume to generate a logarithmic curve. 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.

T-cell Proliferation Assay

The standard CD4 + T-cell proliferation assay was utilized for analysis of the *in vitro* proliferative potential of the rhIL-2 preparations. 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 manufacturers protocol. Purified CD4+ T-cells were plated (3 x 105 cells/well) in flat-bottom 96-well plates that were pre-coated with one μg/ml ±α-CD3 (BD Biosciences, San Jose, CA, USA), and the rhIL-2 samples 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 City, 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 four days. During the final 6 h of culture, cells were labeled with one μCi/well of 3H-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).

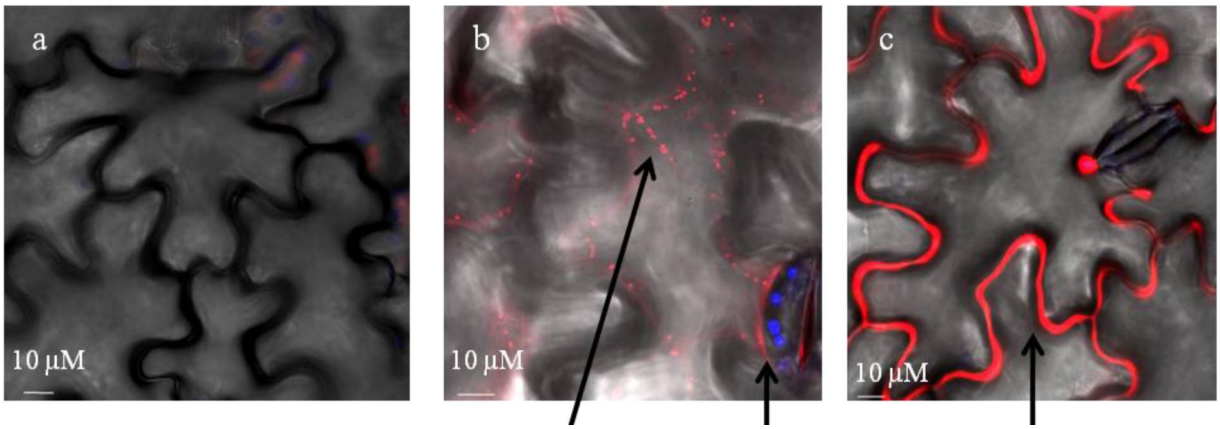
RESULTS AND DISCUSSION

Microscopy of Transiently Transformed Plant Tissue

Panels a, b and c of Figure 3 show brightfield images overlain with the fluorescence images recording emission at 570-620 nm and 655-755 nm. These fluorescence images will show emission from red fluorescent protein (RFP) and chlorophyll, respectively. Panel a displays the Wild-Type (WT) tobacco epidermal tissue. A slight amount of red is seen in this panel due to the 10 naturally occurring compounds that auto-fluoresce when excited. In this panel, no excitation is seen in the apoplast or Golgi. Panel b displays tobacco epidermal tissue that was infected with *Agrobacterium tumefaciens* harboring the empty vector pVKH18En6St-m-RFP. This vector has a Golgi targeting signal and, as apparent from the image, accumulation is noted in the Golgi. 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. As seen in the image, the IL-2-RFP accumulates in the apoplast as expected, because the final destination of proteins trafficked for secretion is the apoplast.

Proteins first must pass through the ER which is equipped with chaperones that aid in proper protein folding. If the host cell is capable of properly folding the protein, it will be successfully trafficked through the secretory pathway. However, if 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 tobacco-produced

Figure 3: 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.



rhIL-2 is secreted properly, this implies that the tobacco host cell can fold this protein properly.

Western Blot Screening of Stably Transformed Plants

No expression was detected for constructs created using vectors 1.1 and 1.5 (cytoplasmic and mitochondrial matrix targeting, respectively). Not all compartments were expected to be conducive to rhIL-2 stability and thus we did not expect to see accumulation of the rhIL-2 in each sub-cellular location. Each micro environment 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). These factors might have affected the accumulation of rhIL-2 in these two sub-cellular compartments.

Representative western blot images for expression screening of vectors 1.2, 1.3 and 1.4 (apoplast, ER and chloroplast stroma targeting, respectively) are shown in Figure 4. The expected size of the rhIL-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).

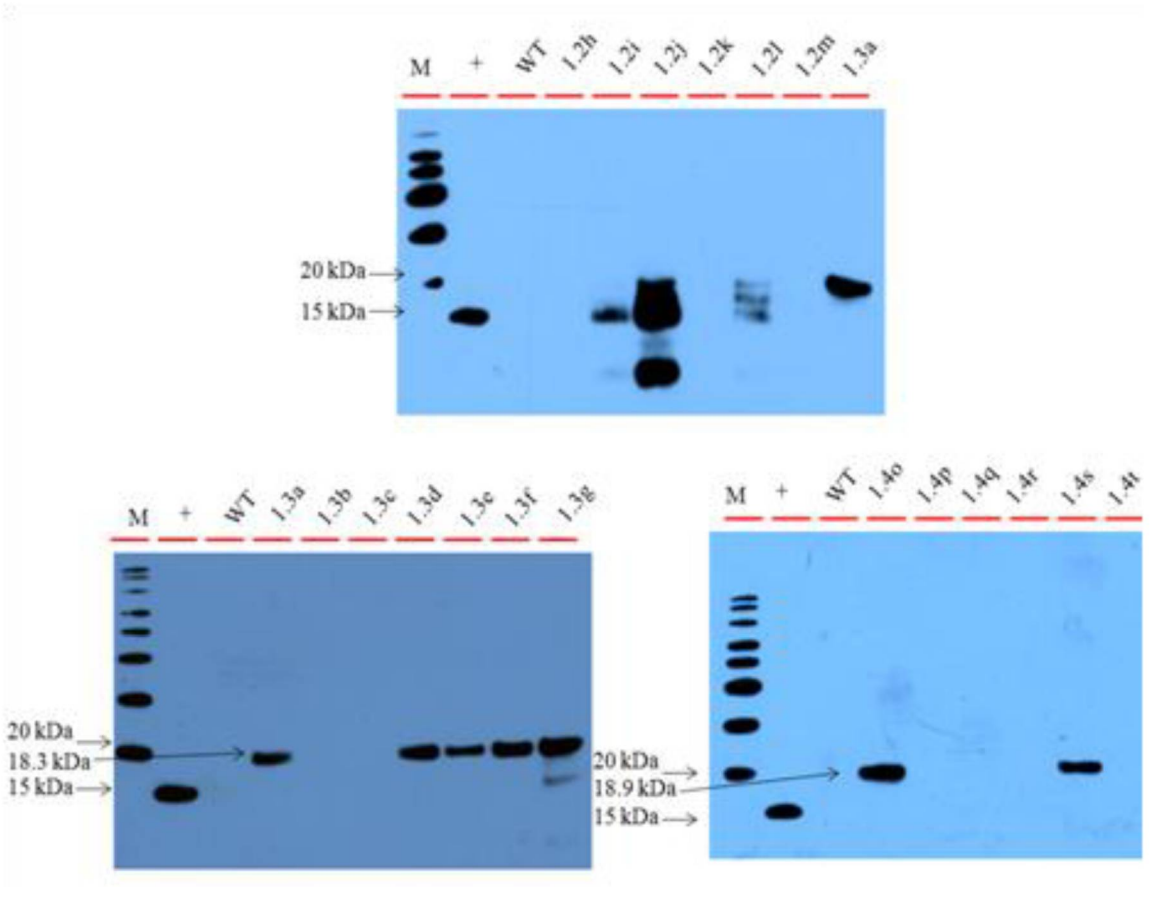
Western blotting of apoplast targeted rhIL-2 samples consistently resulted in multiple bands ranging from the expected ~17.8kDa to smaller sizes. It is not certain what might have caused this banding pattern, but one possibility is that each band is a specific cleavage product, possibly due to extracellular protease activity. Because of this, it was determined that the apoplast was not a suitable location for stable recombinant IL-2 accumulation.

Western blotting of vector 1.3 and 1.4 (ER and chloroplast matrix targeting, respectfully) samples consistently resulted in a single band of the appropriate sizes. In vector 1.3, the addition of the C-myc, 6X HIS tag, and KDEL sequence produced a molecule predicted to be 18.3 kDa.

As apparent from Figure 4, the band seen is of the correct size. Glycosylation might cause this product to run slightly higher. In addition to the c-myc and 6X HIS tag, the chloroplast matrix-targeted rhIL-2 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 has not been cleaved *in vivo*, and results in a molecule predicted to be 18.9 kDa (Wong *et al.*, 1992). This size also corresponds to the band size seen in the western blots shown in Figure 4.

From these results, the rhIL-2 produced via the use of vector 1.3 was selected to be studied further in biological activity tests. This recombinant protein more closely represents the native protein than the protein produced from vector 1.4 since 1.3 has additions to only the C-terminus, whereas 1.4 has additions to both the N- and C-terminus. Unfortunately, no cleavage sites were initially engineered into this construct to remove these tags once the protein is recovered.

Figure 4: Western Blots. Shown here is a subset of western blots analyzing the rhIL-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 transgenic 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 targeted) consistently show multiple bands of lower-than-expected sizes, while blotting of 1.3 (ER targeted) and 1.4 (chloroplast stroma targeted) samples show a single band of correct size in plants that produced rhIL-2 above detectable levels



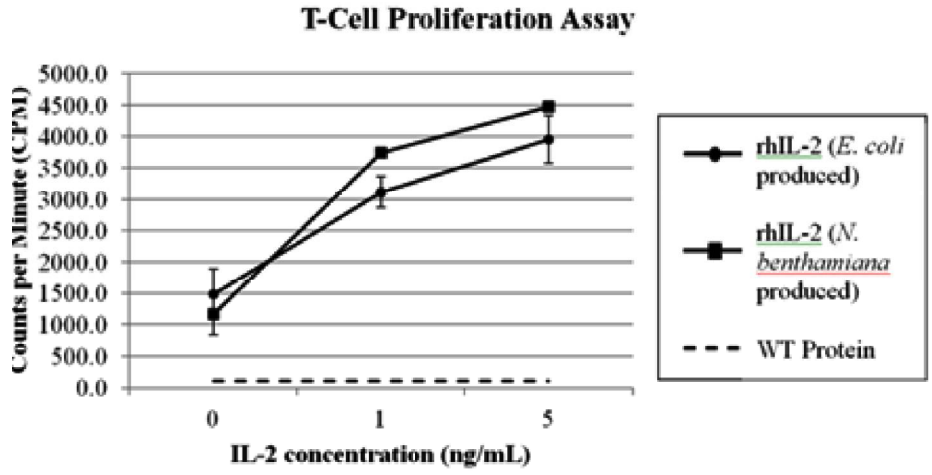
Cell Proliferation Assay

The T-cell cultures contained only purified splenic CD4+ T-cells, 3 x 10^5 cells per well, that were activated using an anti-CD3 antibody. Activated T-cells were used as opposed to resting T-cells because they are much more sensitive to IL-2 and thus should exhibit greater proliferation at lower concentrations of rhIL-2. Although these cultures did secrete their own IL-2, this did not affect the researchers' ability to measure the biological activity of the rhIL-2 as the cultures responded to the addition of rhIL-2 in a dose-dependent manner.

The biological activity of plant-produced rhIL-2 was found to be on par with that of commercially available *E. coli*-produced rhIL-2 as shown by the results in Figure 5. It is apparent that plant derived rhIL-2 is demonstrating biological activity above baseline, to the same magnitude as that of the commercially available rhIL-2. When student t-tests were performed to confirm whether the data is statistically relevant, the "Right-tailed *t*-tests" verified that the CPM values of the positive control and the experimental sample were significantly higher than those of the negative control. Each culture was tested in triplicate, resulting in two degrees of freedom. An alpha of 0.05 was used. The calculated t values 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: $\mu \leq \mu$WT Extract). Therefore there is sufficient evidence to suggest that the effects of the *E. coli* produced rhIL-2 (positive control) and each of the three Plant-produced rhIL-2 samples are greater than that of the wild-type plant negative control.

In addition, two-tailed *t*-tests were performed to evaluate whether or not the CPM values of the sample was significantly different from the CPM

Figure 5: T-Cell Proliferation Assay. The incorporation of tritiated thymidine, reflecting the degree of T-cell survival and proliferation, is measured in counts per minute (CPM). The measured intensity is plotted against the concentration of rhIL-2 supplementing the media (estimated concentration of N. benthamiana produced rhIL-2, known concentration for the commercially available E. coli produced IL-2). Error bars designate standard error. The amount of the wild type (WT) tobacco protein extract (negative control) added to the media was equivolume with the amount of desalt sample added to achieve a concentration of 5 ng/mL IL-2. Six replicates of WT protein cultures were performed and the average CPM was calculated to produce the WT protein line.



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 less than the absolute value of the t statistically calculated for the desalt sample at both concentrations. Therefore, the null hypothesis is rejected (H_0: μsample = μrhIL-2 ; H_a: μsample $\neq$ μrhIL-2).

The *t* values calculated 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. The proliferation using the desalt sample is greater than that of the positive control. However, since the quantification of rhIL-2 within the sample is only an estimate, no conclusion on the relative biological activity between these two rhIL-2 types can be drawn.

CONCLUSION

The present research was intended to be a proof-of-principle study aimed at determining if biologically active rhIL-2 could be produced in tobacco leaves and stems (green tissues). This research has demonstrated the feasibility of producing biologically active rhIL-2 in the green tissues of tobacco. The recombinant protein produced here appears to have potency similar to that of the commercially available rhIL-2 produced in *E. coli.* More research and economic analysis are needed to identify the volume at which rhIL-2 can be produced per ton of tobacco biomass and the cost-effectiveness of this system as a platform preferential over the currently used *E. coli* platform.

Although the plant produced rhIL-2 yield was not measured, the independent transgenic lines created here could be self-bred towards homozygosity, which has been shown to result in increased protein expression yield (Zhong *et al.*, 1999). Also, these independen transgenic lines could be bred to slowly increase copy number which has also demonstrated increased expression when performed and monitored appropriately (Streatfield, 2006). In addition, the rhIL-2 yield and biological activity could be increased when rhIL-2 is produced in multiple sub-cellular compartments. Multiple compartment targeting can be accomplished either via re-transformation of transgene constructs, co-transformation of multiple transgene constructs or via cross breeding of the rhIL-2 transgenic plants that have the protein targeted into their different sub-cellular compartments. It probably will take pyramiding of the aforementioned approaches to increase the rhIL-2 yield before it could be manufactured as valuable biotech drug at a commercial level.

With the limitations of current pharmaceutical production systems, it is prudent to use recombinant plant production systems to manufacture biopharmaceuticals in a more effective manner. This practice has the potential to benefit all parties involved in both humanitarian and economic aspects. With the correct implementation of these systems, the value of tobacco crops used for recombinant protein production could far surpass the revenues generated from using them for the production of cigarettes (Sticklen, 2009).

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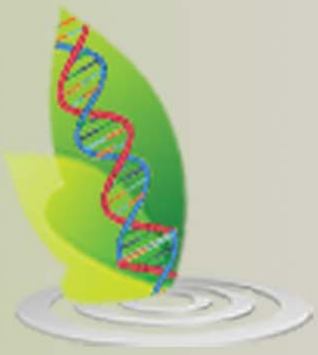

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