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Comparison of expression systems for the production of human interferon-α2b

Research Article

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Abstract: The production of human interferon alpha2b (IFN-α2b) in two expression systems, tobacco (*Nicotiana tabaccum*) and *Escherichia coli*, was compared in various aspects such as safety, yield, quality of product and productivity. In the *E. coli* system, IFN-α2b was expressed under a pelB signal sequence and a T7*lac* promoter in a pET 26b(+) vector. The same gene was also cloned in expression plant vector (pCAMBIA1304) between cauliflower mosaic virus promoter (CaMV35S) and poly A termination region (Nos) and expressed in transgenic tobacco plants. The expression of protein in both systems was confirmed by western immunoblotting and the quantity of the protein was determined by immunoassay. The amount of periplasmic expression in *E. coli* was 60 μg/L of culture, while the amount of nuclear expression in the plant was 4.46 μg/kg of fresh leaves. The result of this study demonstrated that IFN-α2b was successfully expressed in periplasm of bacterial and plant systems. The limitations on the production of IFN-α2b by both systems are addressed and discussed to form the basis for the selection of the appropriate expression platform.

Keywords: *Expression platform • Interferon-α2b • Escherichia coli • Transgenic tobacco*

1. Introduction

Human interferon alpha2b (IFN-α2b) is expressed by cells in response to a variety of infectious agents [1,2]. Although originally discovered for its broad spectrum antiviral properties, IFN-α2b has antiproliferative and immunomodulatory effects on a variety of cell types. The practical importance of these pleiotropic activities is shown by the approval of recombinant IFN-α2b for the treatment of more than 14 diseases world-wide, including hairy-cell leukemia, condyloma acuminatum, Kaposi's sarcoma and hepatitis [3,4].

At present, several heterologous protein expression systems are available for the production of therapeutic proteins for use in human healthcare. Each of these systems offers distinct advantages in terms of protein yield, ease of manipulation and cost of production [5]. The choice of an expression system for the high-level production of recombinant proteins depends on many factors. These include growth characteristics of cells, expression levels, intracellular and extracellular expression, post-translational modifications and biological activity of the protein of interest as well as regulatory issues in the production of therapeutic proteins [5-7]. In addition, the selection of a particular expression system requires a cost breakdown in terms of process,

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design and other economic considerations. The relative merits of bacterial, fungal, insect, mammalian and plant expression systems have been previously reported [5].

The many advantages of using an *Escherichia coli* expression system have ensured that it remains a valuable microorganism for the high-level production of recombinant proteins [7,8]. The profound genetic and physiological characterization, a short generation time, the ease of handling, an established knowledge of the fermentation and finally the high capacity to accumulate foreign proteins (more than 20% of the total cellular protein content) have made *E. coli* the most widely used production host [9-11]. However, in spite of the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this microorganism [5]. Unique, subtle structural features of the gene sequence, the stability and translational efficiency of the mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in codon usage between the foreign gene and native *E. coli*, and the potential toxicity of the protein to the host could all effect the ability of the organism to express the gene [10,12]. The major drawbacks of *E. coli* as an expression system include the inability to perform many of the post-translational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium, the inability to produce high amounts of protein in the soluble form and the limited ability to facilitate extensive disulfide bond formation [12]. However, many eukaryotic proteins retain their full biological activity in an unglycosylated form and, therefore, can be produced in *E. coli* [5,13]. In addition, some progress, for example, periplasmic expression, has been made to increase the amount of protein in soluble form and to produce the protein with the correct disulfide bond formation in bacteria [14,15].

Plants have been used in medicine for many centuries but it is now possible to exploit plants as bioreactors for the production of therapeutic human proteins. Plants are ideal candidates as host systems because of several features including, ease of transformation, low cost of investment, dispersed capital requirements, rapid scale-up, high control of the level of expression and capability of performing post-translational modification [16]. In 1986, it was shown that tobacco plants and sunflower calluses could express recombinant human growth hormone [17]. Since then, a diverse range of plant systems has been used for the production of pharmaceuticals. Plants provide a safe method of delivery of recombinant proteins for therapeutic use, they are also easy to store and distribute. In addition, there are less concerns about product safety and public acceptance, making plants the potentially preferred system as factories for production of recombinant proteins [18-20]. A tobacco farm could produce up to 100 tonnes of leaf biomass per hectare each year and each tobacco plant could produce up to one million seeds [16]. The use of plants such as tobacco, which are not grown for human or animal consumption, have the benefits of reducing the likelihood of transgenic material contaminating the food chains [21] and avoids the conflict of utilizing a food crop for recombinant protein production. Therefore, tobacco is suitable as a recombinant host from the view point of biosafety.

The main objective of this study was to develop IFN-α2b expression system in two platforms based on bacterial (*E. coli*) and transgenic plant (*Nicotiana tabaccum*) systems. The productivity of the two systems will then be compared, along with other factors that could influence the choice of a suitable expression platform for industrial use.

2. Experimental Procedures

2.1 *E. coli* expression system

2.1.1 Characteristics of the plasmid

E. coli expression vector pET-26b(+) was chosen for the expression of IFN-α2b in *E. coli*. The vector pET 26b(+) contains T7*lac* promoter for higher transcription, kanamycin resistance marker for plasmid stability and pelB signal sequence for periplasmic expression.

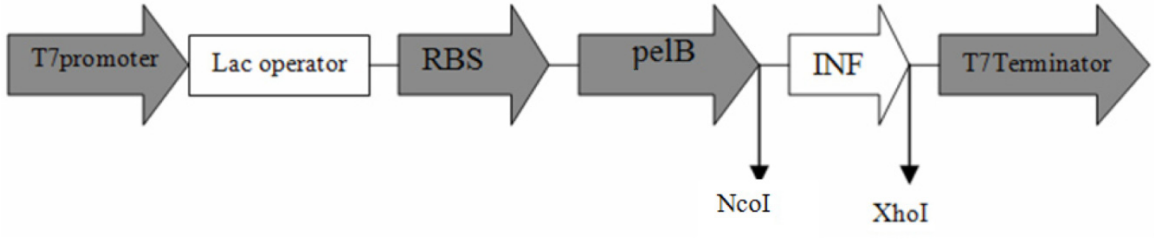

Figure 1. Construct of *E. coli* expression vector, pET-IFN.

2.1.2 Construction of the *E. coli* expression vector pET-IFN

The vector pET-IFN was prepared by amplifying the coding sequence of IFN-α2b from the source vector (pALCA1SIFN from ATCC 53369) using specific forward primer (IFN-FP) 5'GAATGGTCTCTCATG TGTGATCTGCCTCAA3' and reverse primer (IFN-RP) 5'GCAGTCCTCGAGTTATTCCTTACTTCTTAAAC3'. The amplified gene was double digested using *BsaI* and *XhoI* restriction enzymes. The overhang prepared was subcloned in pET 26b(+) between *NcoI* and *XhoI* restriction sites (Figure 1). The recombinant plasmid DNA was then transformed into *E. coli* strain Rosetta gami 2(DE3) [RG 2(DE3)]. The resulting clone was verified by colony PCR amplification and sequencing. RG 2(DE3) contains the plasmid carrying the genes for seven codons, rarely used in *E. coli.* This is useful for increasing the expression level of heterologous proteins by mitigating codon bias [22,23].

2.1.3 Fermentation

A single colony of recombinant *E. coli*, grown on Luria Bertani (LB) agar was inoculated into LB broth along with antibiotics (34 mg/L of chloramphenicol and 30 mg/L of kanamycin). The culture was incubated at 37°C in a rotary shaker (Certomat® BS-1 B. Braun, Germany), agitated at 2.13 x g for 8 h and used as an inoculum for fermentation. The fermentation medium consisted of 60 g/L of overnight express instant terrific broth (ITB; auto-induction medium, Merck). ITB works on the principle of utilizing the primary carbon source (eg: glucose or glycerol) for high cell density growth and then utilizing the secondary carbon source (lactose) for expressing the target gene [24]. Sterile glycerol (1 mL) was added to the sterile culture medium (100 mL) in a 500 mL flask, along with the antibiotics as mentioned previously. The flask was inoculated with a 1% (v/v) inoculum and incubated at 37°C in a rotary shaker, agitated at 2.13 x g. The fermentation was carried out in triplicates. During the fermentation, samples were withdrawn at 2 h intervals for analysis.

The culture samples were centrifuged at 8000 x g for 10 min to harvest the cells. The collected cell pellets were treated with osmotic shock to release periplasmic proteins [25].

2.2 Plant expression system

2.2.1 Characteristics of the plasmid

The plant binary expression vector pCAMBIA1304 was chosen for expression of IFN-α2b in tobacco plants. This expression vector retains a high copy number in *E. coli* for high DNA yields and a pVS1 replicon for high stability in *Agrobacterium*. Plant selection genes in the pCAMBIA vectors are driven by a double-enhancer version of the CaMV35S promoter and terminated by the CaMV35S polyA signal.

2.2.2 Construction of the plant transformation binary vector (pCAMBIA-IFN)

DNA fragments encoding the IFN-α2b gene were arranged into a pCAMBIA1304 binary vector as follows. The IFN-α2b gene was amplified using specific forward primer (IFN-pFP) 5'CATGCCATGGCACATCATCAT CATCATCATCAACAATGTGATCTGCCTCAAACC3' and reverse primer (IFN-pRP) 5'CATCAGGGTCACCC TATTATTCCTTACTTCTTAAACTTTC3'. The amplified gene was replaced in GUS-GFP region under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter and a NOS terminator. The resulting cassette was denoted as pCAMBIA-IFN (Figure 2).

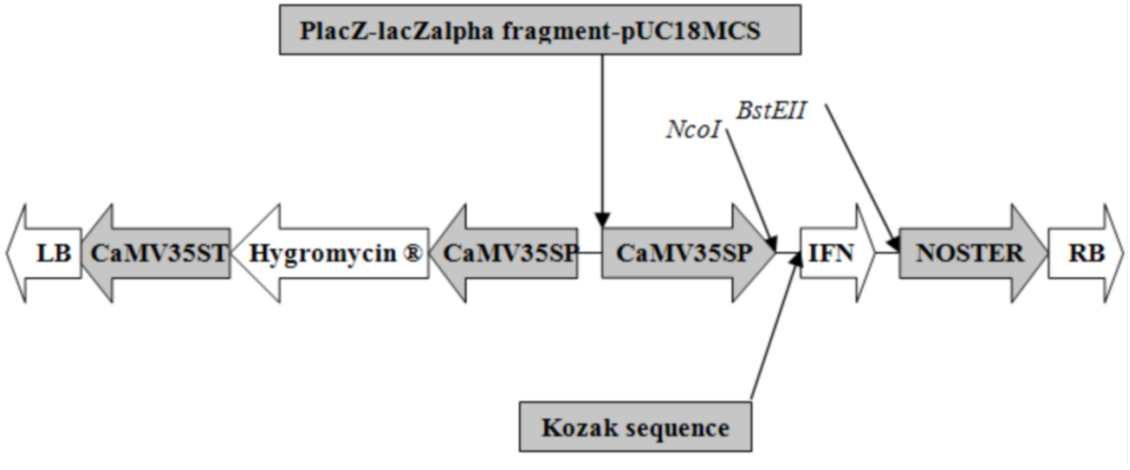

Figure 2. Generation of expression plant binary vector, pCAMBIA-IFN.

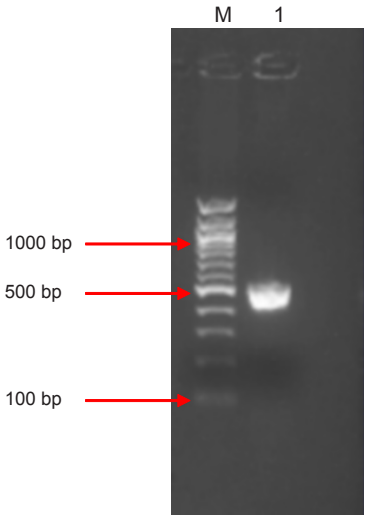

Figure 3. PCR analysis of *E. coli* transformation. M - 100 bp DNA marker; 1 - PCR product by using the IFN-α2b gene specific primers, namely IFN FP and IFN RP, with the cells transformed with the clone pET-IFN as DNA template.

2.2.3 Agrobacterium transformation

The pCAMBIA-IFN binary vector construct was transformed to agrobacterium (*Agrobacterium tumefaciens* strain LBA 4404) and the kanamycin (50 mg/L) resistant transformants were used for digestion. Digestion reaction was conducted by *Bst*EII, *Nco*I in NEB buffer 3. The digestion product was extracted from the gel using a QIA GEN kit, ligated by T4 DNA ligase and the results were then confirmed by sequencing.

2.2.4 Plant transformation

Tobacco (*Nicotiana tabacum* cv. *Xanthi*) leaf pieces were used for agrobacterium mediated transformation (*Agrobacterium tumefaciens* strain LBA 4404). After transformation, leaf pieces were transferred to Murashige and Skoog based medium containing 6-benzylaminopurine (1 mg/L), naphthaleneacetic acid (0.1 mg/L), cefatoxim (200 mg/L), and hygromycin (15 mg/L). The regenerated plants were transferred to vermiculite (100%) and then planted in soil. The plant genomic nuclear DNA was then isolated according to the method as described by Saghai-Maroof *et al.* [26]. In brief, freeze-dried leaves (0.75 g, dry weight) were powdered, dispersed in 15 mL of extraction buffer (50 mM Tris pH 8.0, 0.7 M NaCl, 10 mM EDTA, 1% hexadecyltrimethylammonium bromide, 0.1% 2-mercaptoethanol) and shaken at 60°C for 60 min. Ten mL of a solution of chloroform:isoamylalchol at a ratio of 24:1 (v/v) was mixed with the buffer solution and centrifuged at 6000 x g for 10 min at room temperature. The aqueous phase was removed and then isopropanol (2:3 of whole volume) was added to precipitate the DNA from the solution. The precipitated DNA was lifted out with a glass hook, transferred to a tube containing 20 mL of 70% ethanol/10 mM ammonium acetate for 20 min, and then dissolved in 1.5 mL of a 10 mM ammonium acetate, 0.25 mM EDTA solution. PCR amplification of genomic DNA by specific primers was performed to detect the presence of the IFN-α2b gene.

2.2.5 Plant protein extraction

Fresh leaves were ground to a fine powder in liquid nitrogen with a pestle and mortar. The protein was extracted with the addition of an equal weight of powdered leaves per volume of extraction buffer (10 mM, Tris-HCl pH 8.0, 2 mM Phenylmethanesulfonyl Fluoride (PMSF), 0.5 M NaCl, 5 mM DTT and 5 mM EDTA). After vortexing, the samples were put on ice for one hour and pellets were separated by two rounds of centrifugation (20,000 × g, 30 min, 4°C). The supernatant was used for detection and quantification of total protein and IFN-α2b.

2.3 Analytical procedures

Cell concentration of *E. coli* was determined using filtration and oven drying method. The culture sample (5 mL) was filtered through the predetermined weight of cellulose nitrate filter paper with a pore size of 0.2 µm and then dried overnight in an oven at 70°C to obtain the dry weight of the cells. Cell concentration was expressed as dry cell weight per unit volume of sample (g DCW/L).

Western blot analysis for the proteins extracted from *E. coli* cells and transgenic plant leaves was performed by running electrophoresis at 130 V in Tris-glycine buffer with 20% gel concentration using mini Protean 3 apparatus (Bio-Rad). Separated proteins were transferred onto a polyvinyldifluoride (PVDF) membrane for 45 min at 15 V. The transferred membrane was incubated firstly with 2% bovine serum albumin (BSA) in phosphate buffered saline/Tween 20 (PBST) for one hour, followed by primary antibody (Anti-α-IFN Mouse mAb, Calbiochem) in 1% BSA/PBST for one hour and then with secondary antibody (Goat Anti-Mouse Total Ig Peroxidase conjugate, Calbiochem) in 1% BSA/PBST for 30 min. The membrane was washed with PBST for three times after each incubation period. The blot was then developed using diaminobenzidine (DAB) tetrahydrochloride and hydrogen peroxide.

The quantity of IFN-α2b extracted from *E. coli* and transgenic plant was quantified using a biosensor chip in Biacore3000 [25].

3. Results

3.1 Periplasmic expression of IFN-α2b in *E. coli*

The recombinant plasmid (pET-IFN) was verified by colony PCR digestion and sequencing (Figure 3). Western blot analysis was carried out for the periplasmic fraction of pET-IFN. RG 2(DE3) containing pET-26b(+) was grown using a similar protocol to that used to grow RG 2(DE3) containing pET-IFN and its periplasmic fraction was used as a negative control, while an IFN-α2b standard was used as a positive control. The presence of IFN-α2b in RG 2(DE3) was detected using western blot analysis (Figure 4).

3.2 Growth profile of *E. coli* and IFN-α2b production

The growth profile of *E. coli* and IFN-α2b production is shown in Figure 5. A stationary growth phase with the maximum cell concentration of 11.77 g/L was attained after 15 h of fermentation. The expression of IFN-α2b was started when cell growth reached the stationary phase. The highest production of IFN-α2b (60 µg/L) was achieved after 25 h of fermentation and the specific product yield was found to be 5.2 µg/g cell.

3.3 Production of transgenic lines and expression of IFN-α2b in plant cells

PCR amplification of pCAMBIA-IFN showed a fragment of approximately 550 bp.

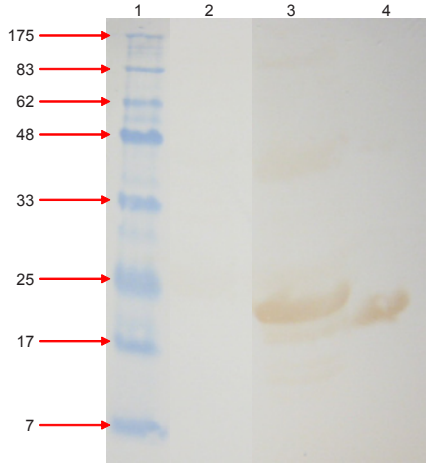

Figure 4. Western blot analysis for the extracted protein from *E. coli*. 1 - Prestained protein marker (7-175 kDa broad range protein marker, P-7708S, New England Biolabs); 2 - Periplasmic fraction of pET 26b(+) in RG 2(DE3); 3 - Periplasmic fraction of pET-IFN in RG 2(DE3); 4 - IFN-α2b standard.

The pCAMBIA-IFN cassette was transferred into tobacco plants by the agrobacterium-mediated transformation method. The tobacco leaves that were inoculated by agrobacterium with pCAMBIA-IFN showed organogenesis on tissue culture medium containing hygromycin (15 mg/L) and cefatoxim (200 mg/L). However, no control plants regenerated, plants infected with agrobacterium containing no pCAMBIA-IFN. PCR analysis using specific primers for extracted genomic DNA of 8 normal regenerated plants showed a 550 bp band in transformants. However, no band was observed in wild type plants as a control, showing that the transgenic plants have received at least one copy of the IFN-α2b gene (Figure 6). The presence of IFN-α2b in transgenic lines was detected in western blot analysis (Figure 7). The biosensor analysis showed that the average amount of expression of IFN-α2b in 1 mL of extracted total protein solution from 1 g fresh leaves was 4.46 ng/µL or 4.46 µg/kg.

4. Discussion

The different factors to be considered for the selection of an expression platform are summarized in Table 1. The prime decision criteria for the choice of an expression system in pharmaceutical production are the safety of the yielded protein in context with the post-translational modification pattern, followed by overall cost, production timescale, scale up capacity, product quality and storage cost [10,12,27-29]. All plant expression platforms, including whole plants systems, have safety benefits over expression platforms based on microbial cells. Endotoxins are a major concern in bacterial systems, plants, however, do not harbor human pathogens or produce endotoxins [19]. The USA Food and Drug Administration (FDA) as well as the European Agency for the Evaluation of Medicinal Products (EMEA) have published draft guidance documents addressing the issue [30,31].

The quantity of expressed protein is another challenge. In our study, we used a pET system for bacterial expression, which is one of the most powerful systems that has been developed for the cloning and expression of recombinant proteins in *E. coli* and IFN-α2b gene was cloned under control of strong bacteriophage T7 expression signals. The final periplasmic IFN-α2b protein obtained in recombinant *E. coli* was lower than expected. A lower level of expression can be attributed to various factors, for example, codon bias, media and culture condition as well as stability of the mRNA transcript and its secondary structure. Transfer ribonucleic acids (tRNAs)

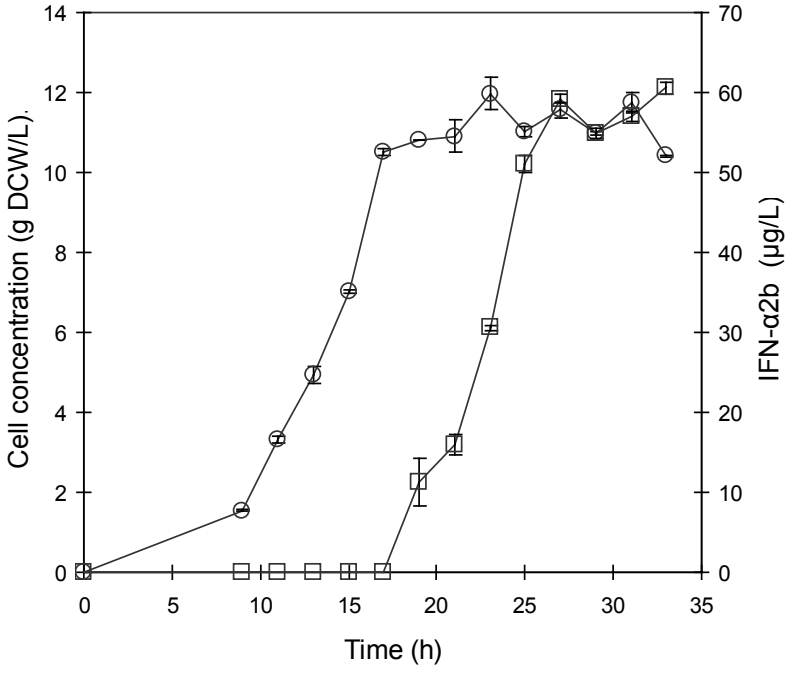

Figure 5. Growth profile of recombinant *E. coli* and IFN-α2b production in shake flask fermentation using ITB medium. (○) cell concentration (g/L); (□) IFN-α2b production (µg/L). The error bar shows the standard deviation.

of some codons corresponding to arginine, isoleucine, leucine and proline are found to be limited in *E. coli* [22]. An attempt to express a gene in *E. coli* containing these rare codons would directly reduce the level of expression. The problem could be elevated if transcripts containing rare codons in clusters, such as doublets and triplets, accumulate in large quantities [22]. In IFN-α2b, 10 out of 15 rare codons are for arginine and the clusters in doublets are in the positions 13 and 23 (data not shown here). The optimization of these rare codons resulted in high level expression of IFN-α2b as inclusion bodies [32] but not as a soluble form in the periplasm [33]. The effect of the rare codons in this study was mitigated by selecting the RG 2(DE3), which could supply the tRNAs for the rare codons.

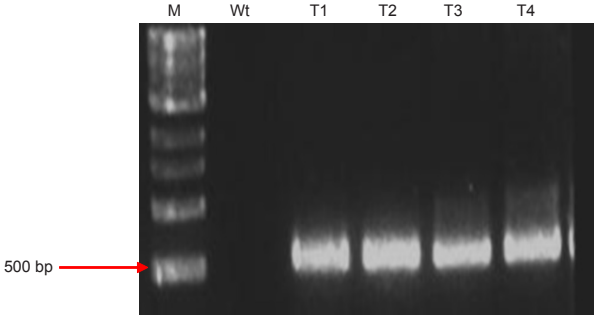

Figure 6. PCR analysis of transgenic plants. M - O'RangeRuler™ 500 bp DNA Ladder; Wt - wild type plant; T1, T2, T3 and T4: transgenic plant.

Medium composition and culture condition also plays a major role in periplasmic protein expression. ITB medium is optimized to produce high cell density and to trigger high level of protein expression during the stationary growth phase [24]. However, permeability of the inner cell wall varies with the different phases of cell growth and the translocation efficiency is reduced in the stationary growth phase [34]. Culture conditions such as temperature may affect the rate of protein expression and the formation of inclusion bodies, which in turn, will affect the soluble periplasmic protein expression. Furthermore, the mRNA stability [22] and its secondary structure [35] also have been shown to affect the rate of protein expression. The effect of media and culture condition on the genomic and proteomic level of the expression is currently under investigation in our laboratory, with the aim to optimize of the rate of translocation of product across the membrane.

In the plant expression system, pCAMBIA vector was used which drove IFN-α2b gene by a double-enhancer version of the strong CaMV35S promoter and terminated by the CaMV35S polyA signal. The amount of protein obtained in this plant system was low at 4.46 ng/µL of extracted soluble protein or 4.46 µg/kg of fresh leaves, in previous studies with transgenic rice

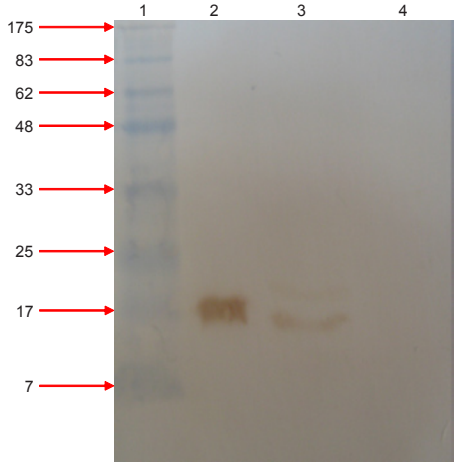

Figure 7. Western blot analysis for the extracted protein from transgenic plant leaves. 1 - Prestained protein marker (7-175 kDa broad range protein marker, P-7708S, New England Biolabs); 2 - IFN-α2b standard; 3 - extracted protein from transgenic plant leaves; 4 - extracted protein from non-transgenic plant leaves.

the yield of IFN-α2b was 2.5 to 10 µg/kg of fresh leaves [36]. The results obtained from these studies strongly indicate that nuclear transformation would lead to low expression level for this protein.

The level of expression can be increased by the gene gun method of transformation, which would increase the copy number of plasmids. However, higher-copy-number or overexpression of nuclear genes may result in gene silencing, which would also reduce protein expression. Gene silencing may be alleviated by incorporating the foreign DNA into the nuclear genome [37]. Limitations such as codon usage also need to be considered. Modifying codon usage in some genes may increase the protein synthesis as well as eliminating the instability of sequences [38]. A possible strategy is to introduce inducible and tissue specific promoters using the chloroplast expression system. The use of the chloroplast expression system is currently being investigated in our laboratory to overcome the limitation of nuclear expression in producing soluble protein.

The other difference between the two expression systems is the quality of the protein expressed. Post-translational modification such as disulfide bond formation is complete in plant. In contrast, correct disulfide bonds formations occur mainly in the periplasmic fraction of bacterial expression. The insoluble fraction needs to be unfolded and then refolded in the correct arrangement, which is a costly process. In this study, the proteins expressed by the *E. coli* and tobacco-plant systems were confirmed to be IFN-α2b using Western Blotting. Future biological assays shall be conducted to determine the bioactivity of the protein when it is expressed in the plant system. The bioactivities of various recombinant proteins expressed in plant cells system have been previously reported [36,39,40].

The comparatively low cost of large-scale protein production is a major advantage for using transgenic plants in the pharmaceutical industry. Skilled personnel to run complicated equipment such as bioreactors are not required for the production of protein using transgenic plants, so the capital and running costs are significantly lower than for bacterial cell-based production systems [21]. Table 1 also shows the estimated cost for the production of 1 mg IFN-α2b using *E. coli* fermentation and transgenic tobacco plant systems. Production of IFN-α2b using *E. coli* is calculated based on two different working volumes of bioreactor (2.9 m^3 and 36.2 m^3) according to the method of Ernst *et al*. [41]. Production of IFN-α2b using transgenic plants, however, is calculated based on Burley tobacco using the method of Foreman [42]. In both systems, the costs are only estimated for the production of biomass containing the recombinant protein without taking into account the cost of purification. Information and data to enable the calculation of the purification costs are not available in the literature. The estimated cost for the

Factors	*E. coli* periplasm	Plant
Amount of protein expressed	60 µg/L of fermentation broth	4.46 µg/kg of fresh leaves
Time of production	< 1 day	>9 months
Production cost for 1 mg of IFN-α2b	[a]2.9 m^3 (USD $6,356) [a]36.2 m^3 (USD $11,906)	[b]USD $755
Capital cost	Moderate	Very low
Upstream operating cost	Moderate	Very low
Downstream operating cost	Low	High
Scalability	Moderate	High
Safety	Moderate	High

Table 1. Comparison of *E. coli* periplasm and plant systems for their suitability as an expression platform.

[a]*Calculated based on two working volumes of bioreactors according to the method of Ernst et al. [41].*
[b]*Calculated based on Burley tobacco according to the method of Foreman [42].*

production of 1 mg IFN-α2b using recombinant *E. coli* and transgenic tobacco plant system is USD $63,565 for the working volume of 2.9 m^3 and USD $11,906 for the working volume of 36.2 m^3. The estimated cost for the production of 1 mg IFN-α2b using the transgenic tobacco plant system is only USD $755. Percentage of the production cost using transgenic tobacco plant with respect to the cost of production using recombinant *E. coli* fermentation is 1.2% and 6.3% for the working volume of 36.2 m^3 and 2.9 m^3, respectively. Twyman *et al*. [21] estimated that the cost for the production of recombinant proteins using plants was only about 2 to 10% of the cost of microbial fermentation systems, depending on the product yield. These values are comparable to the values reported in this study (1.2 to 6.3%).

In contrast to the product yield, protein purification from large amounts of plant leaves is still based predominantly on laboratory-developed procedures that are often not directly scalable because of the high costs of the chemicals used, the difficulties in controlling foaming, and pumping problems related to non-homogeneity and viscosity. The bioseparation step in the bacterial system is significantly lower than plant system [43]. The low upstream cost of production of recombinant protein in plants will not counterbalance the higher downstream processing costs associated with plant tissue if the efficiency of purification from plants is not increased dramatically.

Time is another important factor, it takes a longer time (more than 9 months in our study) to regenerate a whole plant in comparison with the proliferation of recombinant bacteria, which normally take only a few hours. However, the tobacco plant is capable to produce up to one million seeds per plant, which can easily compete with fast growing recombinant bacteria in a bioreactor. Both systems have this advantage over other recombinant production systems, for example, transgenic animal or mammalian cell culture systems.

The production of medicinal recombinant protein in plants can compete with conventional production systems employing recombinant bacteria. However, a number of problems in the production of plant-derived proteins such as higher cost of purification and low expression capability have to be addressed and strategies have to be developed to overcome the problems. The most prominent and critical subject is to identify which system can provide safer, faster and cheaper pharmaceutical products.

5. Acknowledgements

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