Using Agro-infiltration for production of Biopharmaceuticals in plants

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Abstract
Molecular farming in plants can be achieved by stable or transient expression of a recombinant protein. Transient expression of recombinant proteins in plants can rapidly provide large amounts of the proteins for detailed characterization. The systems for transient expression of foreign genes in plants, via agroinfiltration or viral vectors, are characterized by the fast, easy and low-cost production of macro molecules. Using agroinfiltration, we have transiently expressed human growth hormone (hGH) in tobacco plants. cDNA of hGH was cloned into binary vectors that were transferred into PGV 3850 of Agrobacterium strain and introduced to tobacco leaves. Expression of human growth hormone in protein extracts was quantified by immunoblot analysis. Also western blot analysis was confirmed that hGH was produced. The presence impurities in pellet of extracts was more. The amount of purified protein was near 1mg/ml compared with standard human growth hormone. The advantage of this system are that the experimental procedure dose not require sophisticated equipment and it could be equal from economical point of view or even inexpensive compared with Fermantors for microbial production especially in developing countries.

Keywords: Transient Expression, Molecular Farming, Agroinfiltration, Biopharmaceuticals

Introduction:
Molecular farming of pharmaceuticals in plants has the potential to provide almost unlimited amounts of recombinant proteins for use in disease diagnosis and therapy (Daniell 2001). Transgenic plants are attracting interest as bioreactors for the inexpensive production of large amounts of safe, functional, recombinant macromolecules, such as blood substitutes, vaccines and antibodies. Disadvantage of the transgenic plants include the long development time required to establish a producer line, and, of more relevance here, the bio saftey issue surrounding transgene integration and expression. In response to such concerns, alternative systems that reduce the likelihood of transgene spread in the environment have been established. These include the use of transplastomic plants (in which the chloroplast genome rather than the nuclear genome is transformed) and transient gene expression (Fischer and Emans2000, Schillberg etal 2002). It seems that protein production can be increased by upscaling by using transient-expression systems. Various approaches, including biolistic delivery of naked DNA, infiltration with recombinant Agrobacteria (agroinfiltration) and infection with modified viral vectors now are used for transient expression. In agroinfiltration evacuated plant leaves take up large amounts of recombinant Agro-bacteria upon rapid vacuum release (Fischer,1999). Here we concentrate on using agro infiltration as a way for expression of human growth hormone in different types of plant leaves. The feasibility of producing sufficient
quantities of recombinant human growth hormone with the agro infiltration system for further purification and biochemical analysis will be reported.

**Materials and methods**

**Construction of plant expression vector**

A pBin19hgh was constructed as follows. cDNA of human growth hormone was cloned into pUC 18 at the *BamHI* site (provided by Dr. Adeli, Windsor university, Canada). The clone was digested with BamHI and the resulting fragment was ligated into the *BamHI* site of pRTL plasmid (Gifted by Dr. Carrington), that contains a 35S CaMV dual-enhancer promoter followed by tobacco etch virus (TEV) leader (Fig 1). The orientation of fragment (in correct form 5’-3’) was checked by *BglII* and *EcorV* sites. Expression cassettes containing CaMV 35S promoter, TEV leader fragment, cDNA of human growth hormone and the nopaline synthase terminator was digested by *HindIII* and was ligated into the same site of pBIN19 to create pBin19hgh.

**Explant preparation**

*Nicotiana tabacum* c.v Xanti was grown in greenhouse in standard soil and intact leaves of 1 month old plants was used.

**Preparation of agrobacterium for infiltration**

To assess the expression of transgenes in plants, they were transiently expressed by infiltrating tobacco and lettuce leaves (*Nicotinia tabacum* cv. Xanthi) with *A. tumefaciens* cultures containing the hGH expression construct as previous described with some modification. In brief Plasmid of pBin19 hgh was transformed into *Agrobacterium tumefaciense* pGV3850 by the freeze-thaw method. Growth of recombinant agrobacterium and vacuum infiltration of plant leaves was performed as described with some modification. Agrobacterium was inoculated into 100 ml LB medium and grow the culture overnight to logarithmic phase (O.D. 600 near 0.6) at 28°C. Bacteria was centrifuged and resuspended in half volume of infection medium containing MS Salt (Murashige and Skoog 1962), 10mM MES, 20g/l sucrose and 200µm acetosyringone to a final O.D. 600. the suspension was kept at 22°C for 1 hour and then used for infiltrations.

**Transient expression in tobacco by agroinfiltration.**

Three or four leaves were put in the jar containing the bacterial suspension and jar was put in a vacuum chamber. Leaves were swirl gently during the application of vacuum. A continuous vacuum in the range of 1 to 0.1 mbar was applied for different time 30, 40 and 60 minutes in each experiment. After that vacuum was broken rapidly. After infiltration, leaves were rinsed in sterile water and keep on a Whatman paper No.40 with adaxial side facing up and put in sealed trays (22°C /16h photoperiod). After 60 hr leaves were frozen in liquid nitrogen and stored at -80°C until analyzed.

**Protein extraction from infiltrated leaves and agrobacterium**

For the purification of transiently expressed recombinant hGH approximately 20 gr of each type of leaves was used. Infiltrated leaves were ground in liquid nitrogen to a fine powder with a mortar and pestle and extracted with 1x w/v extraction buffer (10mM Tris-HCl- PH 8, 2mM PMSF, NaCl 0.5 M, 5mM, DTT and 5mM EDTA ). Cell debris was separated by two rounds of centrifugation (20,000g, 30 min, 4°C), and the supernatant was used for expression analysis. A sample of Bacterial cell was also purified as the same way.
Analysis of rhGH by SDS page, dot blot and western blot

We evaluated expression level of rhGH by dot blot, SDS/PAGE (10% polyacrylamid gel) and Western blotting. In Western Blotting an aliquot of sample containing 30µg of total soluble protein assay was separated by 10% sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) at 100 v for 1.5-2 hr in Tris- Glycin buffer. rhGH on Blots was detected with an anti hGH polyclonal antibody (Padatan ELM, Iran). The blot was washed and incubated with 4-chloro1-naphtol which was used as the chromogenic substrate. Commercial Human growth hormone (Novo Company, Denmark) was used as standard in this assay.

Results:

Our aim was to express human growth hormone in tobacco leaves using agrobacterium – mediated transient expression system. Previous work demonstrated that the tobacco etch virus (TEV) leaders sequence enhances in vitro translation of mRNA in a cap independent manner. The two regulatory elements in this 143 base pair leader sequence function optimally in a 5' proximal position. Therefore we used an expression vector containing CaMV 35S promoter and TEV leader sequence (Fig1). Direction of hGH in the construct was detected by BglII and EcorV restriction enzymes. The 700 and 400 bp fragments were produced respectively for sense and antisense direction of hGH coding sequence (Fig2). The Binary vector containing the construct of hGH in right direction was made and transiently expressed in tobacco upon vacuum infiltration with recombinant agrobacterium.

Detection and Quantification infiltrated leaves of rhGH in tobacco

rhGH was expressed in tobacco leaves after infiltration with recombinant agrobacterium. Soluble protein extract for analysis were prepared from tobacco leaves after 60hr (Fig 3, 4). Expression level of rhGH was detected in compare with serial dilution of standard human growth hormone. Immunoblot analysis was showed that expression level of the rhGH in tobacco leaves was high and rhGH accumulated to near 1mg/ml in compare with Standard human growth hormone (Fig 5).

We carried out the Western blot analysis for both part of pellet and supernatant result from protein extraction of tobacco leaves. The pellet section contained several band. In pellet the amount of 22kDa form was lower. The main band migrated with an apparent molecular mass of 22kDa, corresponding to the predicted size of rhGH. The another bands, probably correspond to dimmer form of rhGH (Fig 6).

Discussion

Transient expression is generally used to verify transformation construct activity and to validate small amounts of recombinant protein. The systems for transient expression of foreign genes in plants, via agroinfiltration are characterized by the fast, easy and low-cost production of macromolecules. Moreover, in some cases transient expression of proteins gives higher yields than transgenic plants. With some limitations, these systems can be scaled up to field-scale production. Generally, transient gene expression is a convenient test for the synthesis and characterization of a recombinant protein before the time-consuming generation of transgenic plants for large-scale, long-term, protein production. Here the capability of agro-infiltration system for production of recombinant human growth hormone in tobacco leaves was analysis.
Our results showed using of different inoculation time was showed that this factor has no effect on expression level of rhGH. Therefore we continue the experiments with 35 minutes as a best inoculation time. The amount and form of protein in supernatant and pellet of extracts were different. Although supernatant has lower expression but the different form of rhGH as dimmer or incomplete fragment was lower. Therefore the fraction was used for purification could be important and in each plant considering the impurities of extract needs more purification steps. The amount of the recombinant hGH reached near 1mg/ml.

Recently several reports have described how this agroinfiltration could be scaled up more efficiently. Researchers in Medicago Inc Have described how the agroinfiltration of alfalfa leaves can be scaled up to 7500 leaves per week, producing micrograms of recombinant protein. In another report similarly was shown that up to 100 kg of wild type tobacco leaves could be processed by agroinfiltration, resulting in the production of several hundred milligrams of protein (R Fischer, S Schillberg unpublished.) Moreover, in some cases transient expression of proteins gives higher yields than transgenic plants (Twyman, 2003). Considering the other transient expression in plants and and biosafety regulation in virus infected plants techniques and regulatory compliance for transgenic plants agroinfiltration could be as a suitable way for production of biopharmaceuticals that especially important in therapy even in low dose. The advantage of this system are that the experimental procedure dose not require sophisticated equipment and it could be equal from economical point of view or even inexpensive compared with Fermentors for microbial production especially in developing countries.

Fig1: Schematic representation Of pRTLhGH plasmid

Fig2: Restriction analysis for determining the orientation
Lane1: uncut plasmid
Lane 2: pRTLhGH digested with BglII and EcoRV (700bp sense)
Lane 3: pRTLhGH digested with BglII and EcoRV (400bp antisense)
Lane 4: 100bp Ladder:
Fig 3: Vacuum Jar for Agroinfiltration

Fig 4: Tobacco leaves after Infiltration

Fig 5: Dot Blot with serial dilution of protein extract
1=1.3 mg/ml of standard human growth hormone
2=0.13 mg/ml of standard human growth hormone
3=0.013 mg/ml of standard human growth hormone
4=0.0065 mg/ml of standard human growth hormone
5=Sample1
6=Sample2

Fig 6: Western Blot Analysis of protein extracts of tobacco leaves
Lane 1=Standard Human growth hormone
Lane 2,3: Supernatant of protein extract
Lane 4,5: Pellet of protein extract

References: