Transient expression of human growth hormone in potato (*Solanum tuberosum*), tobacco (*Nicotiana tobacum*) and lettuce (*Lactuca sativa*) leaves by agroinfiltration

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Abstract
Using agro-infiltration technique, we have transiently expressed human Growth Hormone (hGH) in tobacco (Nicotiana tobacum), potato (Solanum tuberosum) and lettuce (Lactuca sativa) leaves. Out of three different inoculation times used for infiltration in our study, it was seen that highest level of hGH expression was achieved when leaves were infiltrated for 35 min. The presence of biologically active hGH was detected in leaf extract by western blotting and ELISA. The highest expression was measured in tobacco was found to be1.5-3 hGH mg/kg leaves.
Keywords: *Transient Expression, Molecular Farming, Agro-infiltration, Biopharmaceuticals.*

INTRODUCTION

In the past decade, plant-based expression system has emerged as a serious competitive force in the large-scale production of recombinant proteins. The first pharmaceutically relevant protein made in plants was human Growth Hormone (hGH), which was expressed in transgenic tobacco seeds and sunflower callus in mid eighties (Barta, 1986; Leite *et al*., 2000) Nowadays, several plant–derived biopharmaceutical proteins are reaching at an advanced stage for commercial production. For such products, plants offer practical and safety advantages as well as lower production costs compared with traditional systems which are based on production in microbial or animal cells, or transgenic animals (Gidding *et al*., 2000).

However, transgenic plants have some disadvantages such as the extent of time required to establish a producer line, transformation of recalcitrant cultivar and public concerns about transgene escape in environment (Miele, 1997). In response to such concerns, alternative systems have been established. These include, production of transplastomic plants in which the chloroplast genome rather than the nuclear genome is transformed (Daniell *et al*., 2001) and transient gene expression (Fischer *et al*., 1999). Transient expression can be used to verify expression of constructs and produce small amounts of product for functional analysis before proceeding to development of transgenic plants. Various approaches, including biolistic delivery of naked DNA, infiltration with recombinant Agrobacteria (agro-infiltration) and infection with modified viral vectors are now being used it (Fisher *et al.,* 1999). In agro-infiltration evacuated plant leaves take up large amounts of recombinant Agrobacteria upon rapid vacuum release. Agro-infiltration targets many cells in a leaf and the T-DNA harboring the gene of interest is actively transferred into the nucleus by the bacterial proteins (Kapilla *et al.*, 1997 and Fischer, 1999). Recombinant protein production can be increased by up-scaling using transient-expression systems. Considering other transient expression systems for plants and biosafety regulation that must be considered in virus infected plants techniques as well as regulatory compliance for transgenic plants, agro-infiltration can be used as a suitable technique for pro-

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duction of biopharmaceuticals important for therapeutic uses especially in low dose. In the present work, we have studied the capability and efficiency of potato, tobacco and lettuce leaves for the expression of human Growth Hormone as a recombinant protein.

MATERIALS AND METHODS

Construction of plant expression vector: A binary vector, pBin19hGH, containing coding sequence of human Growth Hormone was constructed as follows: Clone phGH 107 (cDNA of human growth hormone provided by Dr. K. Adeli ATCC Cat. # 31538) was digested with *Bam*HI and resulting fragment (660 bp) was ligated into the *Bam*HI site of pRTL plasmid. This plasmid contains a CaMV 35S promoter followed by tobacco etch virus (TEV) leader. Orientation of fragment in correct direction was determined by *Bgl*II and *Eco*RV restriction digestion of construct. Expression cassettes containing CaMV 35S promoter, TEV leader fragment, cDNA of human growth hormone and the nopaline synthase terminator was digested by *Hind*III and was introduced into the same site of pBin19 to create pBin19 hGH (Fig. 1).

Plant material: Tobacco (*Nicotiana tobacum* cv. Xanthi), Potato (*Solanum tuberosum* cv. Agria) and lettuce (*Lactuca sativa*) were grown in green house (photoperiod: natural daylight, Temperature regime: 22/25°C day-night). Intact young leaves were used for agro-infiltration.

Transient expression assay in vacuum infiltrated leaves: Preparation of Agrobacterium for infiltration of plant leaves were performed as described before by Kapilla *et al*. (1997) with some modifications. Plasmid pBin19 hGH was transformed into *Agrobacterium tumefaciense* strain pGV3850 (Km^R Rif^R). Preculture (2 ml) of Agrobacterium was inoculated into 100 ml LB medium supplemented with 50 mg/l of Kanamycin and grown overnight to logarithmic phase (OD_{600} = 0.6) at 28°C. Bacteria were centrifuged and resuspended in half volume of infection medium (Murashig and Skoog (MS) basal medium (pH 5.5) containing 5.0% sucrose, 10 mM MES , and 200 mM acetosyringone) and grow at 28°C for 2-3h to a final OD_{600} = 0.6). A continuous vacuum in the range of 0.5-1 mbar was applied for 0.5 g intact leaves. For analysis of the effect of infiltration time tobacco leaves were infiltrated infiltrated for 15, 25 and 35 minutes. Best result was used for infiltration of tobacco , potato and lettuce leaves. After infiltration vacuume was broken rapidly leaves were rinsed in sterile water, kept on a Whatman paper # 40 with adaxial side facing up and put in sealed trays (16/8 h photoperiod, 25°C) for 60-72h. Leaves were used directly for further steps or frozen in liquid nitrogen and stored at -80°C until they were analyzed.

Protein extraction from infiltrated leaves: For protein extraction, 0.5 g infiltrated leaves were ground in liquid nitrogen to a fine powder with a mortar and pestle and extracted with 1x w/v 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), 500 µl buffer/ 0.5 g leaves. After vortexing, the samples were put on ice for 1h and pellets were separated by two rounds of centrifugation (20,000×g, 30 min, 4°C). The supernatant was used for immunoblot and ELISA analysis. The concentration of extracted total soluble protein (TSP) in each sample was determined by Lameli assay (Lameli, 1970).

Detection of expressed recombinant hGH and its biological activity: Transformed leaf tissues were analyzed by immunoblot detection methods for the presence of hGH gene expression. Thirty µg from each sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) carried out at 100 volts for 2-3h in Tris-glycine buffer. Two µl of hGH standard (Novo Nordisk A/S Denmark 1.3 mg/ml) was used as positive control Separated bands of proteins were transferred from gel to membrane (Nitrocellulose) by electro blotting. The presence of hGH on blots was detected by using the anti-hGH monoclonal antibody (Padtan Elm, Iran).

Expression of biologically active hGH was also analyzed by ELISA kit (Diagnostic systems Laboratories, Inc, Germany). In this method only the hGH molecules that has biologically active form and poses both receptor binding sites could be detected.

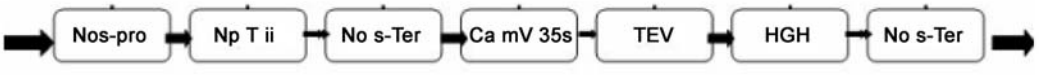

Figure 1: Schematic representation of Binary vector derived from pBIN19 used for the transformation of tobacco, potato and lettuce leaves containing the hGH cDNA under the control of CaMV35S promoter and translational enhancer from Tobacco Etch Virus (TEV).

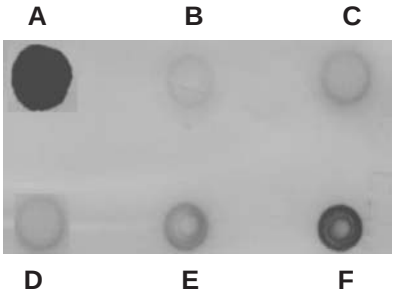

Figure 2: hGH expression level at different infiltration times on tobacco leaves. Five µl of protein extract was used, A: hGH standard from Novo company (1.3 mg/ml), B: untransformed plant, C-F: preparation at 10, 15, 25 and 35 minutes.

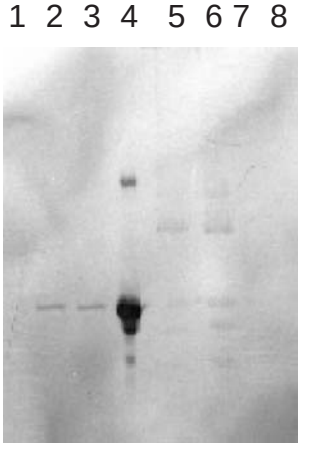

Figure 3. Immuno Blot of hGH protein in transformed leaves. Similar amount of total soluble protein 30 µg was loaded per lane. Lane 1-3: upper phase of protein extract for lettuce, potato and tobacco. Lane 4: hGH Standard (1.3 µg/µl, Novo Co.). Lane 5-7: lower phase of protein extract for tobacco, potato and lettuce respectively. Lane 8: control of non-infiltrated leaves.

Therefore, absorbance is directly proportional to the concentration of biologically active intact hGH.

Aliquots of the extracted protein (50 µl) were diluted with 100 µl of phosphate buffer-saline (pH 7.0) and added to an ELISA plate coated with monoclonal anti-hGH. Similarly, a protein sample obtained from a untransformed plant was treated and considered as negative control. The plates were incubated for 3h at 37°C, washed 3 times and incubated with the biotinylated human growth hormone binding protein (GHBP) for 16 to 24h at 2-8°C. The binding of GHBP to captured hGH molecules was monitored by addition of streptavidine labeled with the enzyme horseradish peroxidase (HRP). After incubation with the substrate tetramethylebenzidine (TMB), the degree of enzyme turn over was determined by dual wavelength measurements at 450 nm and 620 nm.

RESULTS

Effect of infiltration time on expression of hGH in tobacco leaves: Role of inoculation time during agro-infiltration process on tobacco leaves as a model showed that this factor plays an important role in expression level of recombinant protein in leaves. We have designed an experiment to study different inoculation time *viz*., 15, 25 and 35 min in agro-infiltration on tobacco leaves. Analysis of immunoblot showed that by increasing infiltration time highest expression level could be achieved (Fig. 2). This result was also confirmed by ELISA (Data not shown). Based on result, 35 min inoculation time was selected for further experiments with other types of leaves.

Detection and Quantification of expressed hGH in tobacco, potato and lettuce leaves: Western blot analysis of protein extracted from each type of leaves showed difference in amount and purity of expressed protein for each type of leaves. The major band migrated with an apparent molecular mass of 22 kDa, corresponding to the predicted size of hGH (Fig. 3) recombinant protein was detected in potato and tobacco in both phase but not in lettuce leaves. Our result also showed that the amount of impurities or dimmer band of hGH was higher in lower phase of protein extract. This factor could be important for purification process especially at the industrial scale.

ELISA was carried out for measurement of expressed biologically active recombinant hGH in tobacco, potato and lettuce leaves. The amount of active hGH as constitutive of total soluble plant protein (TSP) was calculated by dividing the amount of hGH detected based on ELISA by the TSP measured in plant tissue. As shown in table 1, tobacco and lettuce leaves had highest and lowest expression of biologically active hGH respectively. Potato leaves expressed recombinant protein 10 times lower than tobacco leaves.

DISCUSSION

The objective of this study was to evaluate the capacity of different plant leaves for the expression of hGH, by agro-infiltration. As efficiency of agro-infiltration is highly depend on ability of bacterial penetration inside the leaf tissue (Fischer *et al*., 2004), it seems by increasing infiltration time, there is more chance for passing the epidermis barrier and infecting the neighboring cell and transferring T-DNA containing the desired gene into the nucleus. While standardizing for maximum expression level at different inoculation time *viz.*, 15, 25 and 35 min, we found that after 35

Table1. Quantification of total soluble proteins extracted from tobacco, potato and lettuce after 35 min.

Leaf	% hGH of total soluble protein	hGH (mg /kg leaves)
Tobacco	0.001-0.002	1.5- 3
Potato	0.0001-0.00015	0.17-0.26
Lettuce	0.00001-0.00003	0.024-0.072

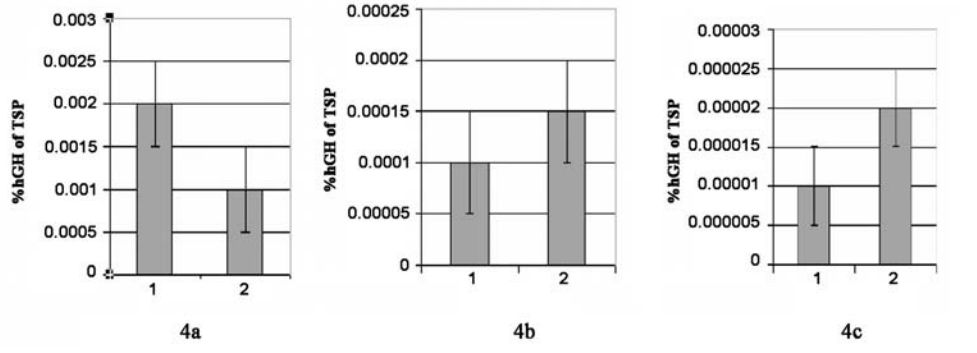

Figure 4. hGH protein levels determined by ELISA in agro-infiltrated leaves [4a (Tobacco), 4b (Potato), 4c (Lettuce)] after 35 min**.** Lanes 1 and 2 show repeat of the experiment. The absorbance values were compared with a human growth hormone standard curve to determine hGH expression level in leaves.

min infiltration time, highest expression was achieved. Therefore, 35 minutes time for infiltration experiments was used for further study. As prolonged exposure to vacuum could be rapidly decreased the temperature of bacterial suspension which may have additionally reduced the expression (Wroblewski *et al.,* 2005). Therefore we didnot use longer inoculation time.

It seems that type of leaves could be an effective factor for production level and down stream processing of recombinant protein. The level of recombinant proteins synthesized in transgenic plants in general range is from 0.0001 to 0.3% of total soluble protein (Arakawa *et al.,* 1998). In our expriment tobacco and potato leaves showed good expression level of biologically active hGH, but as tobacco produces high level of toxic alkaloids and phenolic substance that are released during grinding and protein extraction which can interfere with downstream processing might be not a suitable candidate for agro-infiltration system (Fisher *et al*., 2004). Leafy crops such as lettuce could be a suitable choice, as they have less organic substance that interfere with purification process. A very low amount of hGH in lettuce (Table 1 and Fig. 4) may be caused by synthesis of low yield but it could be managed by increasing the amount of leaves. For example researcher at Medicago Inc. have described how the agro-infiltration of alfalfa leaves can be scaled up to 7500 leaves per week producing micrograms of recombinant protein (Fischer *et al.,* 2004).

Therefore the amount of desired fraction and purity could be another important for further purification steps that could be crucial in large-scale production. Our results suggest that potato leaves could be ideal for transient expression system, by this reality that potato leaves are not used in industry. The agro-infiltration system allows scaling up and comparable to transgenic plants in terms of quantity and quality. Therefore, considering some limitations for production at commercial scale, it could be possible to choose suitable plant and optimized condition, agro-infiltration could be equal from economical point of view or even inexpensive and a suitable replacement especially in developing countries that has undeveloped biosafety regulation for releasing of transgenic plants.

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