Transgenic Research **13:** 245–259, 2004.
© 2004 *Kluwer Academic Publishers. Printed in the Netherlands.*

Recombinant protein expression plasmids optimized for industrial *E. coli* fermentation and plant systems produce biologically active human insulin-like growth factor-1 in transgenic rice and tobacco plants

Mitra Panahi[1], Zaman Alli[1], Xiongying Cheng[1], Loubaba Belbaraka[2], Jaafar Belgoudi[2], Ravinder Sardana[1], Jenny Phipps[2] & Illimar Altosaar[1,*]
[1]*Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, K1H 8M5, Canada*
[2]*Steacie Institute for Molecular Sciences, National Research Council of Canada, Ottawa, Ontario, K1A OR6, Canada*

Received 4 March 2002; accepted 13 December 2003

Key words: codon-optimization, human insulin-like growth factor-1 (hIGF-1), maize zeamatin protein, recombinant protein expression, SH-SY5Y neuroblastoma cell line, transgenic rice, transgenic tobacco

Abstract

Human insulin-like growth factor-1 (hIGF-1) is a growth factor with clinical significance in medicine. The therapeutic potential of recombinant hIGF-1 (rthIGF-1) stems from the fact that hIGF-1 resembles insulin in many aspects of physiology. The expression of hIGF-1 in transgenic tobacco and rice plants using different expression cassettes is reported here. In the present study, two coding sequences were tested, one with the original human sequence, but partially optimized for expression in *E. coli* and the other with a plant-codon-optimized sequence that was expected to give a higher level of expression in plant systems. Three different hIGF-1 recombinant expression constructs were generated. All expression constructs utilized the maize ubiquitin 1 promoter with or without a signal sequence. Analyses conducted using a hIGF-1 specific ELISA kit showed all transgenic plants produced hIGF-1 and the accumulated hIGF-1 increased from the *E. coli* codon bias to higher levels when the hIGF-1 coding sequence was codon-optimized to match that of the maize zeamatin protein – the most transcribed gene in maize endosperm suspension cells. Further analyses that compared the functionality of the bacterial signal peptide Lam B in plants showed that this leader peptide led to lower expression levels when compared to transgenic plants that did not contain this sequence. This indicated that this expression construct was functional without removal of the bacterial signal sequence. The maize ubiquitin 1 promoter was found to be more active in rice plants than tobacco plants indicating that in this case, there was a class preference that was biased towards a monocot host. Biological analyses conducted using protein extracts from transgenic plants showed that the rthIGF-1 was effective in stimulating the *in vitro* growth and proliferation of human SH-SY5Y neuroblastoma cells. This indicated that the plant-produced rthIGF-1 was stable and biologically active. As some plants have been reported to express an endogenous insulin-like protein, we also looked for any effect of the human growth factor in transgenic plants, but no developmental or morphological differences with wild type tobacco or rice plants were detected. Since insulin and hIGF-1 share some overlapping roles, hIGF-1 may become a substitute therapeutic agent in subjects with certain defects in their insulin receptor signaling. Hence, if the full beneficial potential of rthIGF-1 is achieved, it is expected that in the future the demand will likely increase significantly.

Abbreviations: ELISA – Enzyme linked immunosorbent assay; Nos-TER – Nopaline synthase sequence for termination of transcription; NT – Non-transformed plant; rth – Recombinant human.

Introduction

Human insulin-like growth factor-1 (hIGF-1) is essential for normal fetal growth and development. It is known to stimulate proliferation and survival of many cell types but can only promote differentiation of a limited number of cells (Jones & Clemmons, 1995). The mature hIGF-1 is a single-chain peptide of 70 amino acid residues that shares 50% homology with human insulin (Daughaday & Rotwein, 1989). The

*Author for correspondence *E-mail:* altosaar@uottawa.ca

synthesis of hIGF-1 occurs mainly in the liver and is regulated by several factors. It has been shown that human growth hormone (GH) can stimulate the synthesis and secretion of hepatic IGF-1 (Schwander et al., 1983), which in return regulates pituitary GH secretion by feedback inhibition (Berelowitz et al., 1981). In addition to GH regulation, IGF-1 synthesis is also stimulated by insulin (Winter et al., 1979). Lack of insulin as seen in type I diabetes is accompanied by reduced IGF-1 levels, although GH secretion is elevated (Lanes et al., 1985). In recent years, it has been shown that IGF-1 is beneficial clinically in some situations. Since insulin and IGF-1 have their own receptors, IGF-1 may become a substitute therapeutic agent in subjects with functional defects in their insulin receptor. For example, hIGF-1 is considered a therapeutic agent for the following conditions: growth hormone-sensitive (Laron-type) syndrome (Carel et al., 1996), type A insulin resistance syndrome (Schoenle et al., 1991), diabetes (Cusi & DeFronzo, 1995), osteoporosis (Hussain & Froesch, 1995) and acquired immunodeficiency syndrome (AIDS) (Hirschfeld, 1996). IGF-1 increases insulin sensitivity and possesses hypoglycemic effects similar to insulin (Zapf et al., 1981). Hence, if the full beneficial potential of IGF-1 is achieved, it is expected that in the future the demand for IGF-1 is likely to increase.

Recombinant hIGF-1 has been expressed in several different host–vector systems including yeast (Gill et al., 1999), *E. coli* (Joly et al., 1998) and transgenic rabbits (Brem et al., 1994; Zinovieva et al., 1998). However, these expression systems have several limitations that hinder maximum output of biologically active and safe therapeutic agents. For example, the following disadvantages have been reported: (1) formation of inclusion bodies in bacteria, (2) formation of non-native proteins having different biological activities in yeast, (3) low transgene expression levels, (4) transgene induced instability of certain cell lines in mammalian cell cultures, and (5) contamination of animal-based products with human pathogens. Such shortcomings invite alternative methods of production to ensure the safety and economical supply of recombinant therapeutic proteins (Cramer et al., 1999; Gill et al., 1999). We are interested in investigating plant-based genetic systems as vehicles for the biological assembly and production of blood proteins and vaccines (Ganz et al., 1995; Alli et al., 2000a, b, 2001a, 2002; Alli, 2001; Panahi, 2002; Sardana et al., 2002; Panahi et al., 2003). The production of recombinant proteins in plant expression systems has many potential advantages for generating biopharmaceuticals relevant for the successful outcomes in clinical treatments. These include minimal risk of contamination by pathogenic microorganisms, the ability to effect post-translational modifications resulting in authentic products, high yields of stable products, and low scale-up costs (Fischer & Emans, 2000). Stable transgenic lines are possible when recombinant expression constructs are introduced into host plant genomes via the particle gun (Christou, 1995; reviewed by Christou, 1997) or *Agrobacterium tumefaciens* (Sardana et al., 2002).

Yields of recombinant proteins have been increased by addition of plant signal sequences, increased G + C content of the coding sequences, and through the use of endoplasmic reticulum retention sequences (Alli et al., 2002). Edible tissues can be obtained from a host of different plants including (1) cassava and potato tubers, (2) banana and tomato fruits, and (3) corn and rice grains. These can be targeted for precise tissue-specific accumulation of recombinant proteins. Plant tissues are acquiescent to assembly of recombinant therapeutics in different subcellular compartments without loss of stability (Cramer et al., 1999; Alli et al., 2002; Lee et al., 2003). Not surprisingly, there are many examples of successful expression of human recombinant proteins in transgenic plants (Cramer et al., 1996; Ganz et al., 1996; Dieryck et al., 1997; Torres et al., 1999; Alli et al., 2000a, b, 2001a, 2002; Leite et al., 2000; Stoger et al., 2000, 2002; Alli, 2001; Farran et al., 2002; Huang et al., 2002; Sardana et al., 2002). For example, antibodies, human enzymes, cytokines and antigens have been harvested from different plants. The present study reports the feasibility of producing recombinant and biologically active hIGF-1 in transgenic tobacco and rice plants. Human IGF-1 was expressed in the leaves of transgenic tobacco and rice initially to test the suitability of the existing industrial constructs to produce hIGF-1 in plants. These experiments are part of our pipeline analyses. We have adopted rice as the model edible plant for all future work. Eventually, the recombinant protein will be restricted to the protein bodies in seeds of transgenic rice using the glutelin promoter and its associated signal sequence (Sardana et al., 2002).

Choosing the appropriate regulatory elements in expression constructs can enhance the level to which a recombinant protein accumulates in a particular expression system. Controlling the destination of the protein within the cell has a large effect on protein

stability and expression level (Alli et al., 2002; Stoger et al., 2002). In the current work, a rice prolamin signal sequence was included in one construct to target the protein to the endoplasmic reticulum in transgenic rice that was predicted to result in augmented expression. In another construct, an N-terminal signal sequence from bacteria preceded the coding sequence to test its functionality in plants. Therefore, if this experiment were to prove fruitful, it will be possible to express a bacterial protein in plants without prior removal of the signal sequence. Another important factor for the expression of a gene is the choice of a suitable promoter that will result in the desired amount, location and timing of transgene expression (Schenk et al., 1999). Often, a strong constitutive promoter is required to ensure sufficient expression of the transgene throughout the plant. In this paper, the maize ubiquitin 1 promoter was used in both tobacco and rice to obtain initial information on expressing hIGF-1 in plants. Promoters from viral or plant origins have also been reported to be active in several plants (Alli et al., 2002; Sardana et al., 2002). For example, the Cauliflower Mosaic Virus 35S promoter is active in both monocots and dicots while monocot promoters from the glutelin multigene family were found to be active in dicots too. However, the level of expression due only to the strength of the promoter can be significantly enhanced if the plant promoter is from the same class as the transgenic plant line.

Plant-codon optimized sequences also play an important role in enhancing the recombinant protein expression levels (Perlak et al., 1991; Alli et al., 2001a, 2002). For example, when the hepatitis B core particle was codon-optimized to match that of the *Arabidopsis thaliana* codon usage, higher levels of recombinant protein expression were achieved. In the present study, two coding sequences were tested, one with the original human sequence, but partially optimized for expression in *E. coli* (to test feasibility of foreign protein expression in plants without codon modifications), and the other with a plant-codon-optimized sequence which was expected to give a higher level of expression in plant systems (Williams et al., 1992). Leaf extracts from the engineered tobacco and rice plants were shown to contain the rthIGF-1 proteins. The level of rthIGF-1 increased from the partially codon optimized *E. coli* sequence to higher levels when the hIGF-1 coding sequence was codon-optimized to match that of the maize zeamatin protein. Further analyses that compared the functionality of a bacterial signal sequence in plants showed that the Lam B signal sequence led to lower levels of rthIGF-1 when compared to plants that were transformed with the same expression construct that did not contain the Lam B signal sequence. The leaf extracts of transgenic plants were able to actively support the proliferation and growth of a hIGF-1 dependent cell line, SH-SY5Y, indicating that the rthIGF-1 products of transgenic plants were biologically active.

Materials and methods

DNA cloning and assembly of expression constructs

The plasmid (pBKIGF-2B) containing the Lam B-IGF-1 coding sequence that was partially optimized for expression in *E. coli* was obtained from Genentech (South San Francisco, CA). The pBKIGF-2B plasmid was cut with *Xba* I and *Hpa* I and the fragment of interest was subcloned into the multiple cloning site of a pGEM4Z plasmid (Promega). The resulting plasmid was called pGIGF-1. The plasmid pAHC25 (US Department of Agriculture) containing the maize ubiquitin 1 promoter was cut with *Sma* I and *Sac* I to remove the *gusA* coding sequence. The pGIGF-1 plasmid was cut with *Xba* I and *Hpa* I to release the Lam B-IGF-1 coding sequence. The Lam B-IGF-1 DNA was cloned into the above treated pAHC25 plasmid to generate a construct containing the maize ubiquitin 1 promoter, Lam B-IGF-1 coding sequence and Nos-TER sequence. This construct was designated pULam B-IGF-1 (Figure 1, Panel A). When the pGIGF-1 plasmid was cut with *Nco* I and *Sac* I, the IGF-1 fragment was released. This fragment was cloned into the pAHC25 plasmid that was predigested with *Sma* I and *Sac* I. The new construct containing the maize ubiquitin 1 promoter, hIGF-1, and Nos-TER sequences was designated pUIGF-1 (Figure 1, Panel B). Plasmids pULam B-IGF-1 and pUIGF-1 were cleaved by *Hin*d III digestion and ligated with the dephosphorylated pKHG4 (INRA, France) binary vector (Cheng et al., 1998). To obtain higher levels of rthIGF-1 expression in plants, the plant-codon optimized hIGF-1 coding sequence (Figure 1) along with 66 bp that encoded the rice 13 kDa prolamin signal sequence was ligated into pAHC25 (digested with *Sac* I and *Sma* I). The resulting construct containing the maize ubiquitin 1 promoter, rice prolamin signal sequence, plant-codon optimized hIGF-1 coding sequence, and Nos-TER sequence was designated as pUSYN-IGF-1 (Figure 1,

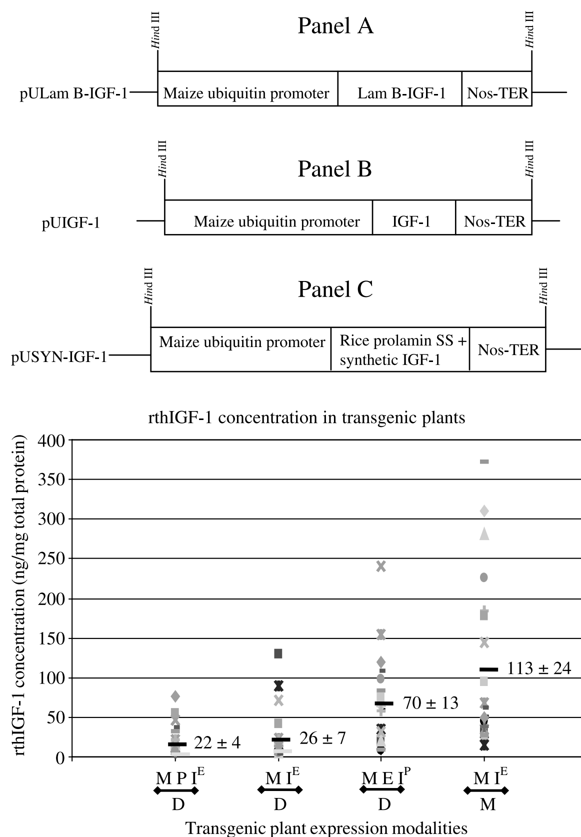


Figure 1. The rthIGF-1 expression constructs used for generation of transgenic tobacco and rice plants. The gene constructs containing the hIGF-1 sequences (210 bp) were placed under the control of the maize ubiquitin 1 promoter (Panels A–C). The construct pULam B-IGF-1 contained the *E. coli* Lam B signal sequence (75 nt) fused in-frame with the mature human IGF-1 coding sequence (Panel A) but the Lam B signal sequence was absent from the pUIGF-1 expression construct (Panel B). The construct pUSYN-IGF-1 encoded the prolamin signal sequence from rice (66 nt) and was fused in frame with the plant codon-optimized hIGF-1 sequence (Panel C). All three expression constructs were used for *Agrobacterium tumefaciens* mediated transformation of tobacco and rice tissues (maps not to scale). *Graph*: Human IGF-1 specific enzyme linked immunosorbent assays conducted on protein extracts derived from transgenic tobacco and rice leaves. The quantitative data obtained for representative plants are shown in this graph. Leaf extracts were tested for the presence of the rthIGF-1 protein using an Active IGF-1 ELISA kit from Diagnostic Systems Laboratories Inc. Microplates were pre-coated with monoclonal IGF-1 antibody. Diluted aliquots of plant leaf extracts were transferred into the wells in duplicate. After the incubation period was complete, the unbound substrates were washed away. The amount of rthIGF-1 was quantitated using a horseradish peroxidase (HRP) conjugated secondary antibody and a substrate solution. A standard curve was calibrated using commercially derived rthIGF-1 from *E. coli* and the results of the unknowns were determined. The graph shows the results as ng of rthIGF-1/mg of total protein in each plant extract. The group means calculated in ng of IGF-1/mg total protein for each transgenic line were: tobacco plants transformed with the pULam B-IGF-1 (M P I^E/D) expression construct, 22 ± 4; tobacco plants transformed with the pUIGF-1 (M I^E/D) expression construct, 26 ± 7; tobacco plants transformed with the pUSYN-IGF-1 (M E I^P/D) expression construct, 70 ± 13; rice plants transformed with the pUIGF-1 (M I^E/M) expression construct, 113 ± 24. The ELISA value obtained for NT plants for each transgenic group was zero. X-axis labels: The *in planta* expression modalities assigned to the upper 'construct' numerator terms were: M, monocot maize ubiquitin 1 promoter; P, prokaryotic derived signal sequence Lam B; E, eukaryotic derived plant signal sequence from rice prolamin protein; I, hIGF-1 coding sequence that was either partially codon-optimized for *E. coli* expression (^{E}) or for plant expression (^{P}). The *in planta* expression modalities assigned to the lower 'host' denominator term were: D, dicot host or M, monocot host. The monocot promoter/monocot host (M/M) modality favored a maximum expression of 371 ng/mg total protein or about 0.037% human protein.

Panel C). For all the cloning and sequencing work, standard techniques were followed (Sambrook et al., 1989). Oligonucleotides (oligos) were prepared by Eppendorf using the dimethoxytrityl nucleoside solid support approach (Urbina et al., 2000).

Tobacco and rice transformations

The engineered pKHG4-IGF-1 plasmids were used to transform the *Agrobacterium* strain LBA4404 using a standard protocol (Horsch et al., 1985). For tobacco transformation, *Agrobacterium* cells were used to transform *Nicotiana tabacum* cultivar Xanthi using the transformation and regeneration procedures that were previously published (Horsch et al., 1985, Ganz et al., 1996). After maturation of the regenerated tobacco plants, they were transferred into the greenhouse and maintained to maturity. For rice transformation, the pKHG4 plasmids containing the recombinant expression constructs were used to transform the *Agrobacterium* strain LBA4404. Transformation and regeneration of Japonica rice (*Oryza sativa* cultivar 93VA) were performed using *Agrobacterium* cells as previously described by Cheng et al. (1997) and modified to use 40-day old rice tissues by Alli (unpublished data).

Southern analysis, PCR and RT-PCR

Genomic DNA was extracted from transgenic plants according to Albani et al. (1992). About 10 µg of DNA was digested with *Hin*d III and Southern analysis was performed according to previously published standard procedures (Sambrook et al., 1989). Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments were performed to detect the presence of full-length IGF-1 mRNA. Total RNA was isolated

from leaves of transgenic plants using the QIAGEN kit (Mississauga, ON) in which RNase-free DNase digestion was included. Approximately 40–100 ng total RNA was used for RT-PCR reactions using the Advantage™ RT-for-PCR kit from CLONTECH (Palo Alto, CA). Polymerase chain reactions were conducted as follows: one cycle of 93°C for 3 min, 52°C for 45 s, 72°C for 1 min followed by 35 cycles of 94°C for 30 s, 52°C for 45 s and 72°C for 1 min. In the final cycle, the extension time at 72°C was increased to 5 min.

ELISA and western blotting

Total soluble protein was extracted from tobacco and rice leaves (500 mg) by homogenization in 500 μl of extraction buffer (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10% glycerol, 20 mM DTT, 5 mM NH_4Cl, 2% Protease Inhibitor Cocktail from Sigma). The extracts were centrifuged at 4°C (14,000*g*) and the supernatant transferred to new tubes. The clear extracts were used for measuring the amounts of rthIGF-1 using an active hIGF-1 ELISA kit from Diagnostic Systems Laboratories, Inc. (Montreal, Quebec). The ELISA kit is a hIGF-1 specific immunoassay kit. All samples including standards were assayed in duplicates. Diluted aliquots of commercial rthIGF-1 and of leaf extracts were transferred into the wells of microplates. After incubation for 2 h at room temperature, the unbound substances were washed away. This was followed by addition of anti-hIGF-1 antibody conjugated to HRP and further incubation at room temperature. The substrate solution resulted in the formation of a colored product that allowed for quantification of the rthIGF-1 concentration. The optical densities were determined using a microtitre plate reader set at 450 nm. Standard curves were generated for each assay using commercial IGF-1 (*E. coli*-derived) to derive the rthIGF-1 concentrations in the plant extracts. Total protein concentrations were determined by the method of Bradford (1976). For western blots, 1 mg each of total protein extracts was run on 20% SDS polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (0.2 μ). After blocking with 0.25% gelatin in Tris base saline (10 mM Tris pH 7.4, 0.9% NaCl, 0.1% Triton X-100, 0.02% SDS), the blots were probed with a 1:500 dilution of a polyclonal antibody to IGF-1 (Genentech, South San Francisco, CA). After washing, the membranes were incubated with a 1:2000 diluted alkaline phosphatase conjugated goat anti-rabbit IgG. Blots were developed using the NBT/BCIP substrate system (Fisher Scientific, Ottawa) as described by the manufacturer.

IGF-1 biological activity assay

The SH-SY5Y (Pahlman et al., 1991; Zeidman et al., 1999) cells obtained from the National Research Council of Canada were grown as suspension cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were washed twice with 1 × PBS and were resuspended in RPMI 1640 medium containing 10% fetal bovine serum. The cells (1×10^4) were transferred to the wells of a tissue culture plate. An aliquot of 0.5 ml RPMI medium with 10% fetal bovine serum containing one of the following samples at a time was added to each of the wells: 10 ng/ml commercial IGF-1 (*E. coli*-derived), transgenic leaf extract containing 10 ng/ml of rthIGF-1, leaf extract from a non-transformed plant (NT) at equivalent protein concentration, leaf extract from an NT plant combined with 10 ng/ml *E. coli*-derived rthIGF-1, protein extraction buffer alone. The experiments were done in triplicate and under sterile conditions. Cellular proliferation and differentiation were monitored at 24, 48, and 72 h. The live cells were counted using haemocytometry/trypan blue exclusion.

Results and discussion

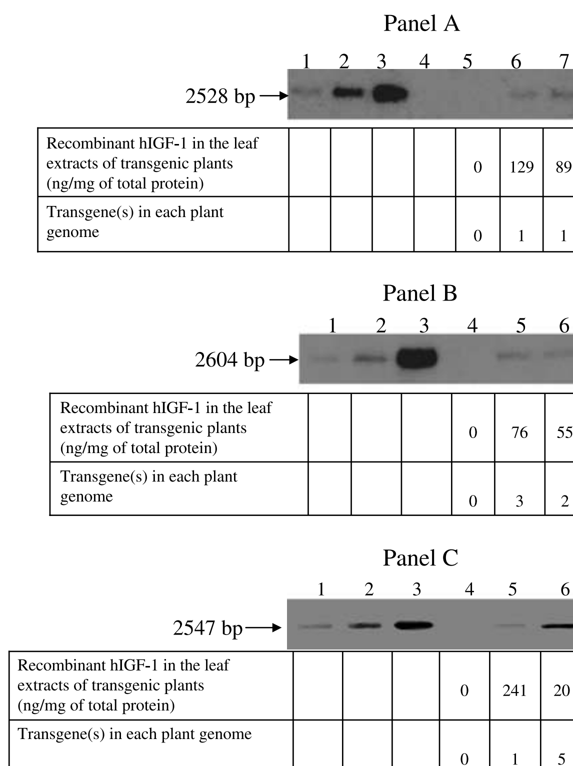
IGF-1 specific ELISA on transgenic plants

The *Agrobacterium* cells containing the chimeric pKHG4 vectors were used to transform tobacco and rice tissues. Approximately 20 independent transgenic plants were generated for each transgenic line and screened by ELISA (Figure 1, Graph). All transgenic plants analyzed produced rthIGF-1 up to a level of 241 ng/mg of total protein in tobacco and 371 ng/mg of total protein in rice. The sensitivity of the assay was 0.03 ng/ml of hIGF-1. The rthIGF-1 range and group means observed for the three groups of tobacco plants (pUIGF-1, pULam B-IGF-1, pUSYN-IGF-1) were as follows: 4–129 ng/mg total protein (group mean = 26 ± 7 ng of rthIGF-1/mg total protein), 5–73 ng/mg total protein (group mean = 22 ± 4 ng of rthIGF-1/mg total protein) and 8–241 ng/mg total protein (group mean = 70 ± 13 ng of rthIGF-1/mg total protein), respectively. However, the rthIGF-1 range observed for the pUIGF-1 rice plants was 15–371 ng/mg total protein (group mean = 113 ± 24 ng of rthIGF-1/mg total protein). The ELISA data

gathered for the transgenic pUIGF-1 tobacco and rice plants indicate that although these plants were transformed with the identical expression construct, there were monocot biases that lead to higher levels of rthIGF-1 in transgenic rice plants when compared to transgenic tobacco plants. Transgenic plants that showed the highest expression levels were chosen for further studies. The transgenic nature of all plants was verified by performing Southern analyses.

Southern, transgene copy reconstruction, transcriptional analyses

Purified genomic DNA was isolated and digested with *Hin*d III, fractioned by agarose gel electrophoresis, transferred onto nylon membrane and probed with radiolabelled fragments containing the hIGF-1 sequence. Only the expected fragment was seen for rthIGF-1 transgenic tobacco and rice plants. Figure 2 shows the data obtained for representative transgenic tobacco plants that produced high levels of rthIGF-1. The data in Panels A–C were obtained for the pUIGF-1, pULam B-IGF-1 and pUSYN-IGF-1 transgenic tobacco plants, respectively. Only the predicted size fragments were seen on all Southern blots. This suggests the absence of any rearrangement of the hIGF-1 constructs in these plants. No bands were observed for the NT. To interpret the Southern hybridization patterns that may lead to high expression of the transgene(s), more information was obtained by conducting transgene copy reconstruction experiments for tobacco and rice plants. Four different transgene copy reconstruction experiments were conducted using DNA standards of 1, 5 and 10 transgene copies on the Southern blots. The results of representative plants are shown in Figures 2 and 3. For transgenic tobacco plants #4 and #11, (Figure 2, Panel A), these bands correspond to one transgene copy. In Figure 2, Panel B the bands seen for transgenic tobacco plants #34 and #37 represent three and two transgene copies respectively. For transgenic tobacco plant #7 (Figure 2, Panel C), one transgene copy was detected. However, transgenic tobacco plant #19 which produced a low amount of rthIGF-1, showed at least five transgenes (Figure 2, Panel C). A representative Southern blot of four transgenic rice plants is shown in Figure 3, Panel A. All four transgenic rice plants analyzed showed bands at 2528 bp. No other bands were present on the blot and hence, no rearrangements of the transgenes had occurred. To further quantify the number of transgenes present in the best transgenic rice plant #9, transgene copy reconstruction was conducted using standards of 1, 5 and 10 gene copies (Figure 3, Panel B). Three transgene copies were detected in this



Panel A

	1	2	3	4	5	6	7
Recombinant hIGF-1 in the leaf extracts of transgenic plants (ng/mg of total protein)					0	129	89
Transgene(s) in each plant genome					0	1	1

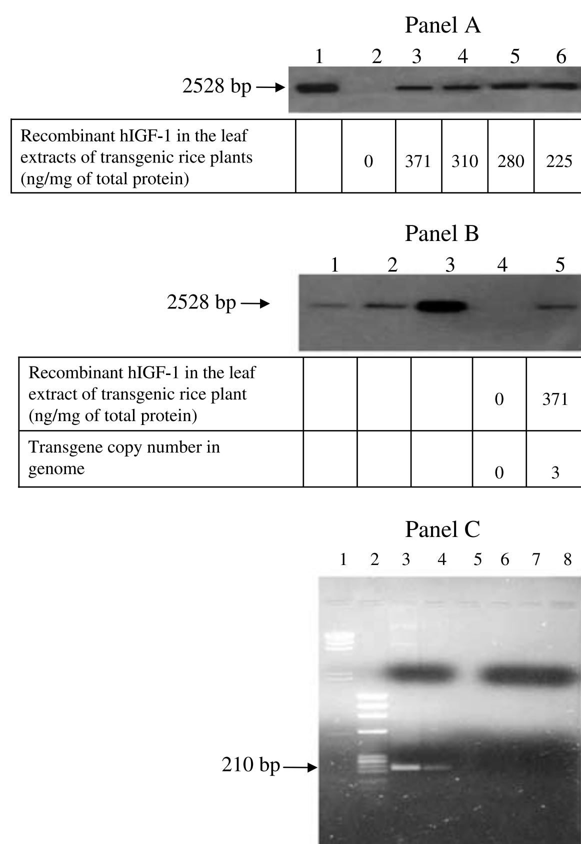
Panel B

	1	2	3	4	5	6
Recombinant hIGF-1 in the leaf extracts of transgenic plants (ng/mg of total protein)				0	76	55
Transgene(s) in each plant genome				0	3	2

Panel C

	1	2	3	4	5	6
Recombinant hIGF-1 in the leaf extracts of transgenic plants (ng/mg of total protein)				0	241	20
Transgene(s) in each plant genome				0	1	5

Figure 2. Southern blots and transgene copy reconstruction experiments on three different lines of transgenic tobacco plants. The experiments were conducted as described in the Materials and methods section. *Panel A*: Lanes 1–3: The *Hin*d III cleaved pUIGF-1 (maize ubiquitin 1 promoter + IGF-1 + Nos-TER) plasmid to release the complete expression construct and reconstituted as 1, 5 and 10 transgene copies, respectively; lane 4: empty; lane 5: DNA from a non-transformed tobacco plant cleaved with *Hin*d III; lanes 6 and 7: DNA from two independent pUIGF-1 transgenic plants #4 and #11 cleaved with *Hin*d III. The rthIGF-1 ELISA data for each plant is also shown along with the deduced number of transgene. *Panel B*: Lanes 1–3: The *Hin*d III insert released from the plasmid pULam B-IGF-1 (maize ubiquitin 1 promoter + Lam B signal sequence-IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 transgene copies, respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hin*d III; lanes 5 and 6: DNA from two independent pULam B-IGF-1 transgenic plants #34 and #37 cleaved with *Hin*d III. The rthIGF-1 ELISA data for each plant is also shown along with the deduced number of transgenes. *Panel C*: Lanes 1–3: The *Hin*d III insert released from the plasmid pUSYN-IGF-1 (maize ubiquitin 1 promoter + rice prolamin signal sequence + synthetic IGF-1 sequence + Nos-TER) and reconstituted as 1, 5, 10 transgene copies respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hin*d III; lane 5: DNA from a pUSYN-IGF-1 transgenic plant #7 cleaved with *Hin*d III; lane 6: DNA from a pUSYN-IGF-1 transgenic plant #19 cleaved with *Hin*d III. The rthIGF-1 ELISA data for each plant is also shown along with the deduced number of transgenes.



	1	2	3	4	5	6
Recombinant hIGF-1 in the leaf extracts of transgenic rice plants (ng/mg of total protein)		0	371	310	280	225

	1	2	3	4	5
Recombinant hIGF-1 in the leaf extract of transgenic rice plant (ng/mg of total protein)				0	371
Transgene copy number in genome				0	3

Figure 3. Southern blot, transgene copy reconstruct and RT-PCR analyses on representative transgenic rthIGF-1 rice plants. The experiments were conducted as described in the Materials and methods section. *Panel A*: Southern blot analysis on DNA extracted from the leaves of representative transgenic rice plants. Lane 1: the *Hin*d III insert released from the pUIGF-1 expression construct (maize ubiquitin 1 promoter + IGF-1 + Nos-TER); lane 2: DNA from a non-transformed rice plant cleaved with *Hin*d III; lanes 3–6: DNA from transgenic rice plants #9, #10, #12 and #15 cleaved with *Hin*d III. The rthIGF-1 ELISA data for each plant is also shown. *Panel B*: Transgene copy reconstruction on a representative rice plant. The analysis utilized DNA extracted from the leaves of transgenic rice plant #9. Lanes 1–3: the *Hin*d III insert released from the pUIGF-1 expression construct (maize ubiquitin 1 promoter + IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 transgene copies, respectively; lane 4: DNA from a non-transformed rice plant cleaved with *Hin*d III; lane 5: DNA from transgenic rice plant #9 cleaved with *Hin*d III. The rthIGF-1 ELISA data for rice #9 is also shown along with the deduced transgene number. *Panel C*: Human IGF-1 transcripts detected via RT-PCR analysis in a representative transgenic rice plant. Lane 1: Lambda DNA *Hin*d III-digested marker; lane 2: Phi-X174 DNA *Hae* III-digested marker; lane 3: a 210 bp fragment amplified from the positive control plasmid; lane 4: a 210 bp fragment amplified from the reverse transcribed total RNA derived from transgenic plant #9; lane 5: empty; lane 6: reverse transcribed total RNA derived from a NT; lane 7: H_2O used as a negative control template; lane 8: total RNA from transgenic plant without the reverse transcription step.

plant genome (Figure 3, Panel B). It is noteworthy to mention that the transgenic plants that showed low numbers of transgene copies had higher expression levels than the plants with greater than three transgenes. For example, the pUIGF-1 transgenic tobacco plants, #4 and #11, with one transgene copy produced 129 and 89 ng of rthIGF-1/mg total protein while the transgenic tobacco plant that produced the lowest level (4 ng of rthIGF-1/mg total protein) of rthIGF-1 in this pUIGF-1 group showed at least four transgene copies (data not shown). Similar data were obtained for the other transgenic tobacco and rice plants within each group (Figure 2). The transgene reconstruction experiments to some extent mimic the results reported by Alli (2001) where transgenic plants that produced the highest level of recombinant human (rth) Granulocyte Macrophage Colony Stimulating Factor (hGM-CSF) were shown to carry three transgene copies. No phenotypic abnormalities were observed for any of the regenerated plants. This may indicate that the insertion site(s) of the rthIGF-1 expression constructs did not disrupt any genes that were responsible for the normal phenotype characteristics of the host plants. Further, the transgenes did not have a detrimental physiological effect on the transgenic plants. These results contrast sharply with the expressed bovine growth hormone that resulted in severe morphological changes in transgenic tobacco roots (Oh et al., 2003).

To determine if the transgenes were expressed, total RNA was extracted from the leaves of transgenic plants. The presence of hIGF-1 mRNA was verified by reverse transcriptase-polymerase chain reaction amplifications using the hIGF-1 sequence-specific primers. The amplified fragment is shown for a representative hIGF-1 rice plant (Figure 3, Panel C, data not shown for tobacco plants). The results show an amplified band of 210 bp for the rice plant. The data suggest that the transgenes were being actively transcribed in the leaves of these transformed plants. Without the RT step or when negative controls were used (NT tobacco or NT rice plant RNAs or water as template), no PCR amplified fragments were detected. Thus the results indicate that the RT steps were true and DNA did not contaminate the RNA preparations.

Recombinant hIGF-1 synthesis in plants

The successful production of foreign proteins in a variety of plants has been reported with varying levels of expression. When the codon usage was changed to a plant-preferred codon bias, expression was in-

creased (Perlak et al., 1991; Alli et al., 2001b, 2002). Many plant RNA-processing signals, in particular those for polyadenylation, mRNA decay, and splicing, are AT rich. Therefore, increasing the GC content of the genes may eliminate potential RNA-processing signals (Vaeck et al., 1987). To quantify rthIGF-1 produced in transgenic plants, leaf protein extracts from transgenic and NT plants were assayed using a hIGF-1 specific immunoassay (Active hIGF-1 ELISA, Diagnostic Systems Laboratories, Inc.). According to the ELISA results, the leaves of all transformed plants produced rthIGF-1 (Figure 1, Graph). This indicated that every transgenic plant had the capacity to accumulate the human growth factor. The leaf extracts from comparable NT plants showed no immunoreactive material. The rthIGF-1 (ng/mg of total protein) group mean obtained from transgenic tobacco was significantly higher for the pUSYN-IGF-1 plant group (70 ± 13 ng/mg, plant-codon optimized hIGF-1) when compared to two other groups of tobacco plants that did not have the plant-preferred codon bias {(26 ± 7 ng/mg, pUIGF-1 group ($P < 0.006$) and 22 ± 4 ng/mg, pULam B-IGF-1 group ($P < 0.001$)}. This statistically significant 3-fold difference may result from using the maximum GC content possible for the pUSYN-IGF-1 group. In our approach, we eliminated the maximum number of AT rich cryptic processing signals. Interestingly, we used the maximum GC content possible for this protein of 70%, without encountering codon usage problems. In fact, plant genes with such high GC content are known (e.g., maize zeamatin). Specifically, the codons present in the hIGF-1 coding sequence were redesigned to match that of the plant zeamatin cDNA. The zeamatin coding sequence has a GC content of about 70% and represents the most highly transcribed mRNA in maize endosperm suspension cells.

The plant-codon optimized group showed a general trend towards higher expression levels than that of the non-codon optimized groups where the codon usage had been left in the *E. coli* 'industrial' state. The GC content of hIGF-1 is 58% and the *E. coli* optimized hIGF-1 is 52%. Therefore, the AT content of the *E. coli* optimized hIGF-1 may lead to poor expression of these constructs in transgenic plants. These results are in agreement with the results of Perlak et al. (1991), Sardana et al. (1996), Cheng et al. (1998), Horvath et al. (2000) and Alli et al. (2002) where it has been shown that plant-codon optimized sequences supported higher expression levels. The higher expression level observed for the plant-codon optimized hIGF-1 group was also seen when the best rthIGF-1 plants from two groups were compared (241 ng/mg of total protein, vs. 129 ng/mg of total protein, Figure 1, Graph). Transgenic tobacco plant #7 showed the highest rthIGF-1 level and contained the pUSYN-IGF-1 expression construct.

Further, the group mean for the transgenic rice plants was significantly higher ($P = 0.001$) than the group mean for the same type of transgenic tobacco plants (Figure 1, Graph: 26 ± 7 ng/mg, pUIGF-1 tobacco group; 113 ± 24 ng/mg, pUIGF-1 rice group). These data indicate that the maize ubiquitin 1 promoter showed stronger activity in a host plant that is a monocot instead of a dicot non-native host plant. The group mean for the transgenic rice plants showed a significant 4-fold (ng of rthIGF-1/mL protein extract) increase over that of the tobacco plant group. The transgenic leaf extracts contained rthIGF-1 up to a level of 0.01% (corresponding to 129 ng of rthIGF-1) for the pUIGF-1 tobacco group and up to 0.02% (corresponding to 241 ng of rthIGF-1) for the pUSYN-IGF-1 tobacco group and 0.03% (corresponding to 371 ng of rthIGF-1) for pUIGF-1 rice group.

Western blots

The soluble protein extracts from leaves of transgenic plants, which contained the highest levels of rthIGF-1 proteins were prepared and subjected to denaturing polyacrylamide gel electrophoreses. Proteins were transferred to nitrocellulose membranes and probed with anti-hIGF-1 antibody (Figure 4, Panels A and B). A protein band of the expected size (7.5 kDa) was only detected in the extracts of transgenic tobacco and rice plants. No band of this size was detected in the NT plant lanes. In addition, the intensity of the band of *E. coli*-derived rthIGF-1 incubated with leaf extract from an NT plant was the same as that of the *E. coli*-derived rthIGF-1. This indicated that nothing from the leaf extracts was interfering with the rthIGF-1 detection. All other transgenic tobacco and rice plants also produced rthIGF-1 proteins as assessed by ELISA (Figure 1, Graph) and western blots (Figure 4, Panels A and B). It should be noted that the *E. coli*-derived rthIGF-1 appeared as two bands on western blots. Other researchers have also reported additional bands for rthIGF-1 (Brem et al., 1994), bovine cholesterol esterase (Vakos et al., 1997) and rthGM-CSF (Sardana et al., 2002). For example, dimerization of rthGM-CSF and bovine cholesterol esterase has been observed on SDS-PAGE gels.

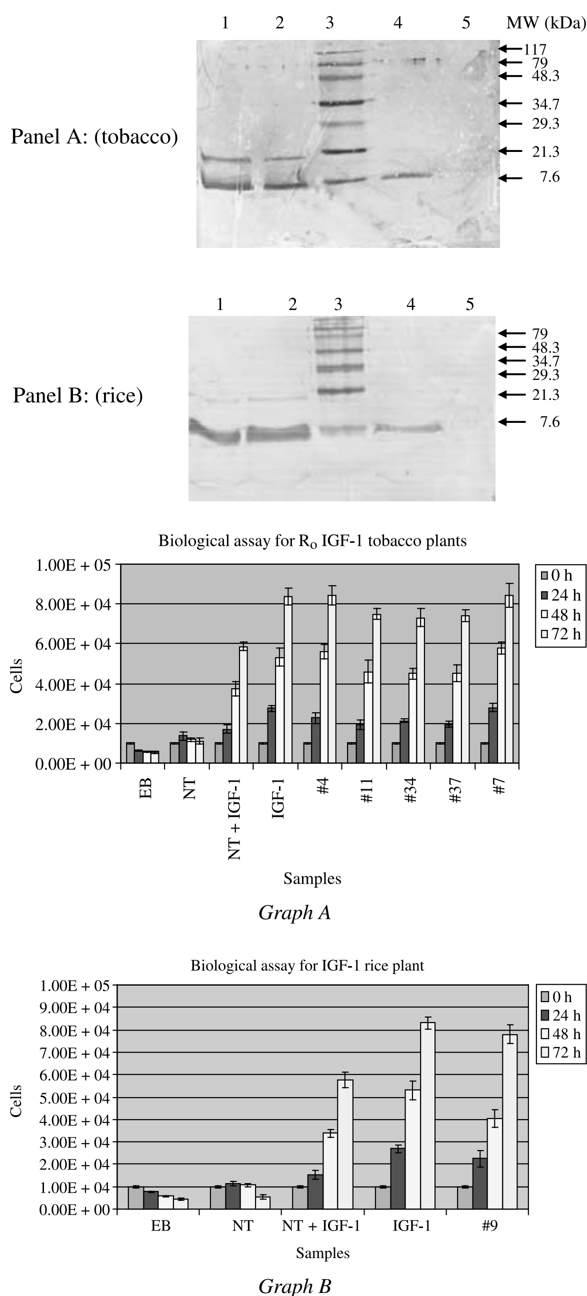


Figure 4. Detection of the rthIGF-1 protein in transgenic tobacco and rice plants via immunoblot analyses. Details of the experiment can be found in the Material and methods section. *Panel A*: Detection of the tobacco transgene product via western blot analysis. Lane 1: *E. coli*-derived rthIGF-1 (7.5 kDa); lane 2: *E. coli*-derived rthIGF-1 added to tobacco leaf extract from a NT; lane 3: prestained SDS-PAGE molecular weight standards from BIO-RAD; lane 4: leaf extract from a transgenic plant transformed with the expression construct that contained the maize ubiquitin 1 promoter, the hIGF-1 coding sequence and the Nos-TER sequence; lane 5: leaf extract from a NT. *Panel B*: Detection of the rice transgene product via western blot analysis. Lane 1: *E. coli*-derived rthIGF-1 (7.5 kDa); lane 2: *E. coli*-derived rthIGF-1 added to leaf extract from a NT; lane 3: prestained SDS-PAGE molecular weight standards from BIO-RAD; lane 4: leaf extract from transgenic plant #9 transformed with the expression construct that contained the maize ubiquitin 1 promoter, the hIGF-1 coding sequence and the Nos-TER sequence; lane 5: leaf extract from a NT. *Graph A*: Determination of the biological activity of the rthIGF-1 protein derived from the leaves of transgenic tobacco lines expressing three different construct modalities. The bioassays were performed on human SH-SY5Y neuroblastoma cells. The results shown represent the proliferation of cells as measured at 24, 48, and 72 h. The cells were grown as suspension cultures and pipetted in duplicate into the wells of a culture plate. The cells were incubated in the presence or absence of aliquots of leaf extracts from transformed and NTs, and *E. coli*-derived hIGF-1. All wells containing rthIGF-1 had a final concentration of 10 ng/ml of rthIGF-1. Cell number and viability were determined using haemocytometry/trypan blue exclusion. EB: extraction buffer; NT: non-transformed leaf extract; plants #4 and #11 contained leaf extracts from independent transgenic tobacco plants transformed with the hIGF-1 expression construct, pUIGF-1; plants #34 and #37 contained the leaf extracts from independent transgenic tobacco plants transformed with the hIGF-1 expression construct, pULam B-IGF-1; plant #7 contained the leaf extract from a transgenic tobacco plant transformed with the hIGF-1 expression construct, pUSYN-IGF-1. *Graph B*: Determination of the biological activity of the rthIGF-1 protein derived from the leaves of a pUIGF-1 transgenic rice plant. The bioassays were performed on human SH-SY5Y neuroblastoma cells. The results shown represent the proliferation of cells as measured at 24, 48, and 72 h. The cells grown as suspension cultures were pipetted in duplicate into the wells of a culture plate. The cells were incubated in the presence or absence of aliquots of leaf extracts from transformed and NTs, and *E. coli*-derived hIGF-1. All wells containing rthIGF-1 had a final concentration of 10 ng/ml of rthIGF-1. Cell number and viability were determined using haemocytometry/trypan blue exclusion. EB: extraction buffer; NT: non-transformed leaf extract; plant #9: leaf extract from transgenic rice plant transformed with the hIGF-1 expression construct, pUIGF-1.

Promoter choice

The ability to express foreign genes in plants depends on many factors including the chosen promoter and the position of insertion of the recombinant expression construct within the plant genome. Active chromatin has decreased methylation (5-methylcytosine), which allows for accessibility by transcription factors as well as RNA polymerase (reviewed by Jones & Baylin, 2002). Demethylation involving the removal of 5-methylcytosine residues and acetylation of core histones facilitate accessibility of transcription factors to the DNA binding sites in the active chromatin. Condensed and silenced chromatins have hypermethylation of cytosine residues that help them to remain inactive and may result in gene silencing. In the present work, the maize ubiquitin 1 promoter was used to generate dicot transgenic tobacco and monocot transgenic rice plants. The expression constructs containing

the maize ubiquitin 1 promoter were used in tobacco to initially test the functionality of the rthIGF-1 expression constructs as part of our 'recombinant protein pipeline'. The rthIGF-1 group mean for the pUIGF-1 construct in rice was 4-fold that of the pUIGF-1 tobacco group (113 ± 24 ng/mg in rice vs. 26 ± 7 ng/mg in tobacco). This 4-fold difference is significant ($P = 0.001$) and may be attributed to better recognition of the maize ubiquitin 1 promoter in rice, which is also a monocot. Similarly, experiments were conducted using monocot-derived promoters in the rapid test plant – tobacco (Leite et al., 2000; Alli, 2001; Sardana et al., 2002). In those experiments, the rice glutelin-1 and glutelin-3 monocot promoters were both found to be active in transgenic dicot plants. For example Alli (2001) reported the use of the above-mentioned rice derived promoters in tobacco that resulted in up to 0.03% expression of the rthGM-CSF in tobacco seeds. Further, the data of Sardana et al. (2002) would suggest that in a large group of transgenic plants, the positional effects of transgenes may be considered constant and the strength of the promoters may be determined directly from the group means. That is precisely what the present study has adapted since the transgene positional effects in the two compared groups of transgenic plants will be 'factored-in' during statistical analyses and will not result in a biased group mean.

In this work, the ELISA data showed that the expression level of the *E. coli*-optimized rthIGF-1 transgenic rice group was higher than the same tobacco transgenic group (113 ± 24 ng/mg for rice vs. 26 ± 7 ng/mg for tobacco). This 4-fold difference ($P = 0.001$) may be due to the 'native host phenomena' of the maize ubiquitin 1 promoter in rice as opposed to the dicot tobacco plant. Further, *trans* acting factors that bind to enhancer elements located on the maize ubiquitin 1 promoter could be present in the 'native' rice plant but may be absent or have reduced binding affinity for the same promoter in tobacco.

Signal peptide effect

To see whether a prokaryotic signal peptide could function directly in plants the bacterial Lam B signal sequence was retained in one expression construct. If this case were to prove feasible, it would be possible to directly express industrial *E. coli* host-optimized constructs in plants without plant-optimized modifications. There are several reports of transformation of plant cells with genes of bacterial, plant or mammalian origin. Inclusion of a plant or sometimes a bacterial signal sequence in the chimeric gene constructs has resulted in the secretion of heterologous proteins from plant cells. Tobacco cells have been successfully transformed to secrete (1) bacterial alpha-amylase using the signal sequence of the same gene (Pen et al., 1992), (2) human monoclonal antibody IgG using barley alpha-amylase signal peptide (During et al., 1990) and (3) human serum albumin using tobacco pathogenesis-related protein signal peptide (Sijmons et al., 1990). Mori and Cline (1998) used a signal peptide from *E. coli* that directed targeting to the thylakoid (chloroplast inner envelope membrane) in plants and suggested that the alternate targeting pathway in prokaryotes and plant thylakoids are analogous.

The results reported here indicate that the rthIGF-1 construct containing the bacterial signal sequence also was successfully expressed in tobacco plants but the expression levels were slightly lower than the transgenic rthIGF-1 group that did not carry the Lam B signal peptide. The two group means were found to be 26 ± 7 ng/mg (Figure 1, Graph: pUIGF-1 construct without a signal sequence) and 22 ± 4 ng/mg (Figure 1, Graph: pULam B-IGF-1 group that possessed the *E. coli* Lam B signal sequence). The general trend observed for these two groups of plants tend to indicate an inclination towards similar expression levels of the proteins whether or not the bacterial signal sequence is present or absent and this indicates the usefulness of plant bioreactor systems. Perhaps the presence of the Lam B bacterial signal sequence in the plant's protein translocation system is the reason for the lower group mean observed for the rthIGF-1 plants having the Lam B signal sequence. Further analysis should provide a better idea of this phenomenon, for example, if the bacterial signal sequence was properly cleaved or incompletely processed by the signal recognition particle. If the signal sequence was not cleaved, it may affect the level of protein accumulation in secretory vesicles and perhaps the rthIGF-1 biological activity. Since the plant-derived rthIGF-1 was found to be biologically active as discussed below, the signal sequence is predicted to have been cleaved. The constructs containing the bacterial signal sequence led to detectable levels of rthIGF-1 protein albeit at a lower level than the group devoid of the Lam B peptide ($P = 0.6248$). Therefore, we accept the plant host system as a potential bioreactor in which to express foreign proteins without prior codon modifications.

To better mimic protein targeting in plants, one signal sequence was based on a cDNA encoding

the rice 13-kDa prolamin (Masumura et al., 1990) (pUSYN-IGF-1, Figure 1, Panel C). Use of such a monocot signal peptide should facilitate transport into the secretory pathway and enhance protein deposition into the storage vesicles of rice endosperm cells. Such transgenic rice plants will eventually be harvested in order to test for the expression of rthIGF-1 in rice seeds as future work. Pre-commercial feasibility production via field trials could assess the logistical advantages of launching fermentor-type constructs in the field instead.

Plant-derived rthIGF-1 is maintained in a stable-biologically active form

To assess the functionality of the rthIGF-1 from transgenic plants, biological assays were conducted using the human neuroblastoma cell line SH-SY5Y (Zeidman et al., 1999; Zumkeller and Schwab, 1999). The SH-SY5Y cell line is known to proliferate and differentiate only in the presence of hIGF-1 (or insulin). For the biological assay, cells were dispensed in triplicate into a tissue culture plate and incubated in the presence or absence of commercially available rthIGF-1 or aliquots of transgenic protein extracts from leaf tissue. Before addition to the medium, the concentrations of rthIGF-1 in different leaf extracts were determined and aliquots containing equal amounts of leaf-derived IGF-1 were used. The viability of the cells was quantified using vital staining (trypan blue exclusion).

Over the periods of 24, 48 and 72 h, neither the leaf-extract from a NT nor the protein extraction buffer (EB) added to the assay medium supported cellular proliferation of the SH-SY5Y cells (Figure 4, Graphs A and B). For example, Figure 4, Graph A shows that the original 1.0×10^4 cells progressively decreased to 0.54×10^4 cells by 72 h when the EB was added to the medium. When the non-transformed tobacco extract was added, the number of cells observed for the assay period was approximately constant, indicating no effect on cellular proliferation. However, the protein extracts of transgenic tobacco or rice plants that contained the rthIGF-1 or the *E. coli*-derived rthIGF-1, when added to the medium separately (final concentration of rthIGF-1 in the medium 10 ng/ml), supported proliferation of the cells as assessed at 24, 48 and 72 h. For example, Figure 4, Graph A shows the results of the biological assay. When hIGF-1 was present in the medium, cellular proliferation progressively increased for the assay period. The number of cells observed at 72 h increased to 8.5×10^4 and 8.4×10^4 when the assay medium contained transgenic extracts from plants #4 and #7 of different lines respectively or to 8.4×10^4 when the *E. coli*-derived rthIGF-1 was added to the medium. Similarly, the number of cells observed at 72 h increased to 7.5×10^4, 7.3×10^4 and 7.4×10^4 when the assay medium contained transgenic extracts from plants #11, #34 and #37, respectively (Figure 4, Graph A). Similar results were observed for the *E. coli*-derived rthIGF-1 and the transgenic rice extract derived from plant #9 (Figure 4, Graph B). For example, at 72 h the number of cells observed was 8.3×10^4 and 7.8×10^4 for the medium supplemented with *E. coli*-derived rthIGF-1 and transgenic plant extract, respectively. However, this effect was lower for the medium supplemented with the *E. coli*-derived rthIGF-1 along with non-transgenic rice plant extract. The EB also showed an inhibitory effect on cellular proliferation.

The combination of *E. coli*-derived rthIGF-1 and non-transformed tobacco extract led to increased cellular proliferation but the number of viable cells was 30% lower at 72 h when compared to the assay medium that contained only the *E. coli*-derived rthIGF-1 (Figure 4, Graph A). This effect is known to occur due to the noxious nature of tobacco (Sardana et al., 2002). A similar inhibitory effect was obtained for the rice leaf extract when combined with the *E. coli*-derived rthIGF-1 in the assay medium (Figure 4, Graph B). A 31% decrease in the cell number was observed at 72 h when compared to the assay medium that contained only the *E. coli*-derived rthIGF-1. Therefore, the plant-produced rthIGF-1 may have acquired the ability to remain in a quaternary structure that is conducive to human neuroblastoma cell growth. Indeed, both insulin and IGF-1 are known mitogens that induce SH-SY5Y cellular proliferation at low concentrations (Pahlman et al., 1991). Hence, the rice and tobacco derived rthIGF-1 may function therapeutically in medicine.

To further confirm that the SH-SY5Y cells were actually proliferating because of the supplemented rthIGF-1, the concentration of rthIGF-1 was increased in the assay. When the final concentration of rice leaf derived rthIGF-1 was increased from 10 to 50 ng/ml, cellular proliferation ceased and cellular differentiation was initiated (Figure 5). In this experiment, protein extracts containing the rthIGF-1 from transgenic rice plant #9 or *E. coli*-derived rthIGF-1 were used to supplement the assay medium. Figure 5, Panels A and C represent the proliferation profile

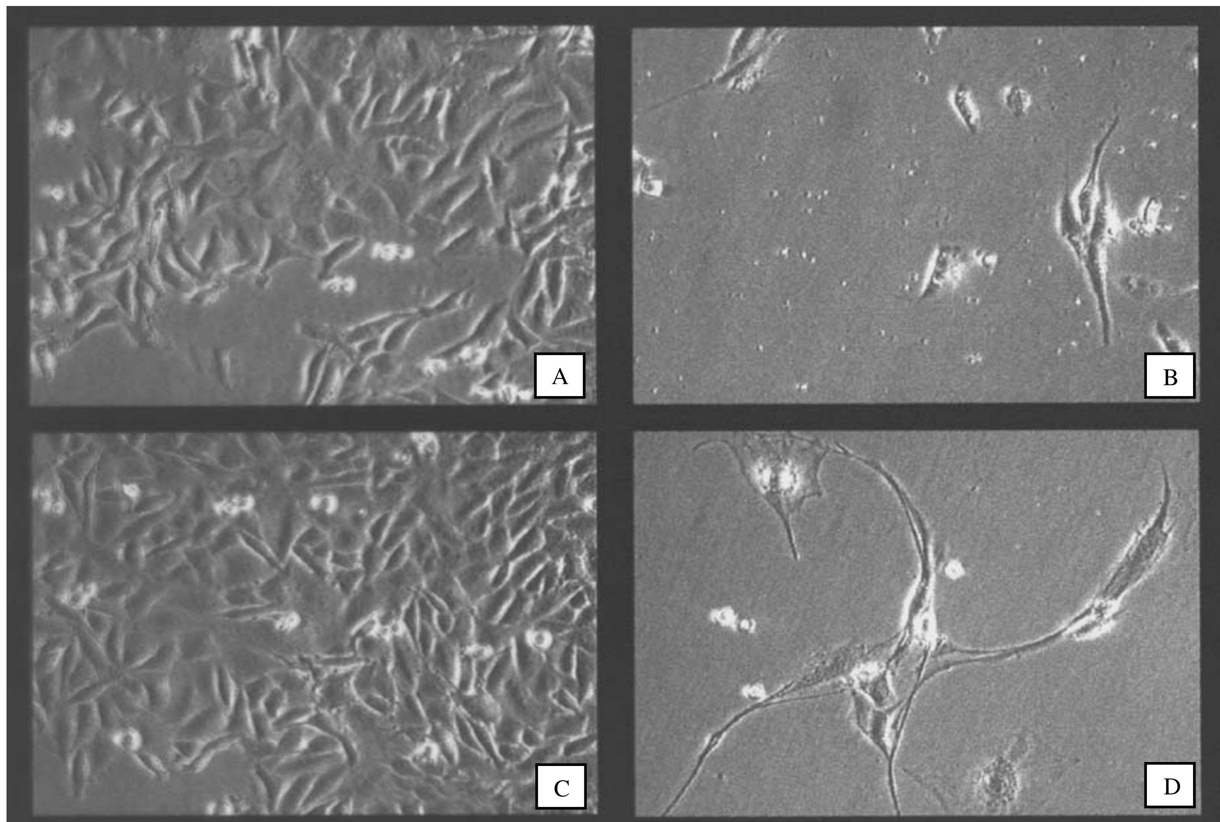


Figure 5. Plant-derived rthIGF-1 protein is functional and influences the cellular proliferation and differentiation of neuroblastoma cells. Protein extracted from the leaves of transgenic rice plant #9 was tested for its ability to stimulate the proliferation and differentiation of the SH-SY5Y neuroblastoma cell line in biological assays. *Panel A*: *E. coli*-derived rthIGF-1 standard, 10 ng/ml led to proliferation of cells; *Panel B*: NT extract at equivalent protein concentration did not lead to proliferation or differentiation of cells; *Panel C*: protein extract from transgenic rice plant #9 that contained the rthIGF-1, 10 ng/ml led to proliferation of cells; *Panel D*: protein extract from transgenic rice plant #9 that contained the rthIGF-1, 50 ng/ml led to differentiation but not proliferation of cells. Additional details can be found in the Materials and methods section.

of SH-SY5Y cells when 10 ng/ml of rthIGF-1 derived from *E. coli* or transgenic plant extract was present in the assay medium. However, when no rthIGF-1 was used to supplement the assay medium (Figure 5, Panel B), no SH-SY5Y cells differentiated or proliferated. The protein extract from a NT was used to supplement the assay medium shown in Figure 5, Panel B. Figure 5, Panel D shows the neuronal differentiation profile of SH-SY5Y cells when 50 ng/ml of rthIGF-1 derived from transgenic plant extract was present in the assay medium. Similar data were obtained for the rthIGF-1 derived from tobacco leaf extracts but are not presented here. These results were expected for the biologically active hIGF-1 in promoting cellular proliferation and differentiation of SH-SY5Y cells when low and high concentrations were used respectively (Pahlman et al., 1991). In the case of differentiated SH-SY5Y cells, the IGF-1 receptor remains expressed while neuronal marker proteins are upregulated including the growth-associated protein 43 (Pahlman et al., 1991). Recently, Lopez-Carballo et al. (2002) suggested that all-*trans*-retinoic acid (RA) might play a critical role in neuronal survival by up-regulating certain differentiation-promoting genes. This is accomplished through signaling via the phosphatidylinositol 3-kinase/Akt pathway. Others have shown that in the presence of vitamin D3 and IGF-1, human HL-60 promyeloid cells differentiate into macrophages at a very high rate $78 \pm 5\%$ (Liu et al., 1998). Further, Edsjo et al. (2003) showed that all-*trans*-RA and brain-derived neurotrophic factor (BDNF) can also lead to the expression of growth-associated protein 43 which is associated with the neuronal differentiation pathway. In addition, phorbol 12-myristate 13-acetate can also influence positively the effects of IGF-1 on SH-SY5Y differentiation (Pahlman et al., 1991).

These effects of IGF-1 occur via signaling through the MAP kinase pathway (Kurihara et al., 2000). Therefore, our prediction is that the plant-derived hIGF-1 bound its receptor, which led to the activation of the SH-SY5Y cellular differentiation pathway resulting in the observed neuronal phenotype.

These data suggest that the rthIGF-1 produced and stably stored in plant cells was functional and maintained in a biologically active conformation. In addition, it was observed that leaf extracts from an NT plant inhibited the proliferation of SH-SY5Y cells. For example, with the addition of both *E. coli*-derived rthIGF-1 and NT leaf extract to the same medium, a decrease rate of proliferation was observed (Figure 4, Graphs A and B). This suggests that some component in the NT leaf extracts may inhibit the proliferation and differentiation of these SH-SY5Y cells. This may mean that the biological activity observed for the plant-derived hIGF-1 is also affected similarly and hence the number of cells observed for the proliferation assays should be higher in theory. This phenomenon was also reported by Alli (2001) for the seed extracts from transgenic tobacco plants that contained hGM-CSF (see also Sardana et al., 2002).

Summary

These results show that non-plant-optimized sequences from bacteria can be expressed *in planta*. Transgenic plants therefore are useful tools to express foreign proteins for further clinical applications. It is now clear that where high amounts of the rth protein are in demand, greenhouse production or field grown crop plants will be preferred (Stoger et al., 2002). By engineering expression cassettes with strong plant promoters, signal sequences and proper codon choice, it is possible to produce large amounts of rth proteins in plants. Chloroplast transformation strategy is also an alternative to nuclear transformation to obtain higher expression levels in plants (Reddy et al., 2002). In summary, we demonstrated the feasibility of producing biologically active rthIGF-1 protein over one generation of transgenic tobacco plants using an *E. coli* derived unmodified expression construct. Our data indicate that the *E. coli* expression construct containing the bacterial signal sequence – Lam B – showed lower protein yields for this sequence when it was present in tobacco plants. By increasing the GC content of the hIGF-1 coding sequence from 52 to 70% and addition of a plant signal sequence, improved recombinant protein accumulation in transgenic plants was observed. When compared to its expression in tobacco, the maize ubiquitin 1 monocot promoter in a 'native' monocot plant led to significantly higher yields of rthIGF-1.

Acknowledgements

The authors thank all those who technically assisted the project. This work was supported by the Natural Sciences and Engineering Research Council of Canada and a grant from Genentech Inc. The input and constructive advice of Gian Polastri, James R. Swartz and Rob Arathoon at Genentech is greatly valued. Peter Quail kindly supplied the pAHC25 from the USDA Plant Gene Expression Center (Albany, CA).

References

Albani D, Sardana R, Robert LS, Altosaar I, Arnison PG and Fabijanski S (1992) A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is specifically expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic tobacco plants. *Plant J* **2**: 332–343.

Alli Z (2001) Expression of biologically active human granulocyte macrophage colony stimulating factor in the seeds of transgenic tobacco. Masters Thesis, University of Ottawa.

Alli Z, Sardana RK, Andonov A, Robert LS, Schernthaner JP and Altosaar I (2000a) Biological assembly of truncated hepatitis core antigen in transgenic plants: towards oral delivery of low-cost vaccines. Seventh Annual National Conference and Exhibition, Ottawa, Canada, 4–6 December 2000, 31 pp. Ottawa Life Sciences Council, Ottawa, Canada.

Alli Z, Sardana RK, Dudani A, Tackaberry E, Ganz PR, Panahi M et al. (2000b) Harvesting of human serum cytokines from endosperm expression systems: evaluation of stability of antecedent and *E. coli* derived cytokines in biological assays. Seventh Annual National Conference and Exhibition, Ottawa, Canada, 4–6 December 2000, 25 pp. Ottawa Life Sciences Council, Ottawa, Canada.

Alli Z, Sardana RK, Andonov A, Robert LS, Schernthaner JP and Altosaar I (2001a) Plant codon optimized hepatitis B core particle gene increases assembly of viral particles in transgenic plants: a technological advancement towards increased antibody titre from oral vaccines. Eighth Annual National Conference and Exhibition, Ottawa, Canada, 5–7 November 2001, 27 pp. Ottawa Life Sciences Council, Ottawa, Canada.

Alli Z, Sardana RK, Andonov A, Robert LS, Schernthaner JP and Altosaar I (2001b) β-Glucuronidase protein expressed in three different bioreactor lines of transgenic carrot: towards augmented, therapeutic peptide yields from edible plant tissues. Eighth Annual National Conference and Exhibition, Ottawa, Canada, 5–7 November 2001, 25 pp. Ottawa Life Sciences Council, Ottawa, Canada.

Alli Z, Sardana R, Pierre B, Andonov A, Robert LS, Schernthaner JP et al. (2002) Pharming vaccines for hepatitis and cytomegalovirus: towards the development of multivalent and subunit vaccines for oral delivery of antigens. *Phytochem Rev* **1**: 55–66.

Berelowitz M, Szabo M, Frohman LA, Firestone S and Hintz RL (1981) Somatomedin-C mediates growth hormone negative feedback by effects on both hypothalamus and the pituitary. *Science* **212**: 1279–1281.

Bradford MM (1976) Rapid and quantitative method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**: 248–252.

Brem G, Hartl B, Besenfelder U, Wolf E, Zinovieva N and Pfaller R (1994) Expression of synthetic cDNA sequences encoding human insulin-like growth factor-1 (IGF-1) in the mammary gland of transgenic rabbits. *Gene* **149**: 351–355.

Carel JC, Chaussain JL, Chatelain P and Savage MO (1996) Growth hormone insensitivity syndrome (Laron syndrome): main characterization and effects of IGF-1 treatment. *Diabetes Metab* **22**: 251–256.

Cheng X, Sardana R and Altosaar I (1997) Rice transformation by *Agrobacterium* infection. In: Cunningham C and Porter A (eds), Methods in Biotechnology. Vol. 3 Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds (pp. 1–9). Humana Press Inc., Totowa, NJ.

Cheng X, Sardana R, Kaplan H and Altosaar I (1998) *Agrobacterium*-transformed rice plants expressing synthetic *cry1A(b) and cry1A (c)* genes are highly toxic to yellow stem borer and striped stem borer. *Proc Natl Acad Sci USA* **95**: 2767–2772.

Christou P (1995) Particle bombardment. *Methods Cell Biol* 50: 375–382.

Christou P (1997) Rice transformation: bombardment. *Plant Mol Biol* **35**: 197–203.

Cramer CL, Weissenborn DL, Oishi KK, Grabau EA, Bennett S, Ponce E et al. (1996) Bioproduction of human enzymes in transgenic tobacco. *Ann New York Acad Sci* **792**: 62–71.

Cramer CL, Boothe JG and Oishi KK (1999) Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. *Curr Topics Microbiol Immunol* **240**: 95–118.

Cusi K and DeFronzo RA (1995) Treatment of NIDDM, IDDM, and other insulin-resistant states with IGF-1. *Diabetes Rev* **3**: 207–236.

Daughaday WH and Rotwein P (1989) Insulin-like growth factors I and II: peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev* **10**: 68–91.

Dieryck W, Pagnier J, Poyart C, Marden MC, Gruber V, Bournat P et al. (1997) Human haemoglobin from transgenic tobacco. *Nature* **386**: 29–30.

During K, Hippe S, Kreuzaler F and Schell J (1990) Synthesis and self assembly of a functional antibody in *Nicotiana tabacum*. *Plant Mol Biol* **15**: 281–293.

Edsjo A, Lavenius E, Nilsson H, Hoehner JC, Simonsson P, Culp LA et al. (2003) Expression of trkB in human neuroblastoma in relation to MYCN expression and retinoic acid treatment. *Lab Invest* **83**: 813–823.

Farran I, Sanchez-Serrano JJ, Medina JF, Prieto J and Mingo-Castel AM (2002) Targeted expression of human serum albumin to potato tubers. *Transgenic Res* **11**: 337–346.

Fischer R and Emans N (2000) Molecular farming of pharmaceutical proteins. *Transgenic Res* **9**: 279–299.

Ganz PR, Dudani AK, Tackaberry ES, Sardana R, Sauder C, Cheng X et al. (1995) Plants as factories for producing human blood proteins. *Can Soc Trans Med Bull* **7**: 112–121.

Ganz P, Dudani AK, Tackaberry ES, Sardana R, Sauder C, Cheng X et al. (1996) Expression of human blood proteins in transgenic plants: the cytokine GM-CSF as a model protein. In: Owen MRL and Pen J (eds), Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins (pp. 281–297). Wiley, New York.

Gill R, Verma C, Wallach B, Urso B, Pitts J, Wollmer A et al. (1999) Modeling of the disulphide-swapped isomer of human insulin-like growth factor-1: implications for receptor binding. *Protein Eng* **2**: 297–303.

Hirschfeld S (1996) Use of human recombinant growth hormone and human recombinant insulin-like growth factor-1 in patients with human immunodeficiency virus infection. *Hormone Res* **46**: 215–221.

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG and Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* **227**: 1229–1231.

Horvath H, Huang J, Wong O, Kohl E, Okita T, Kannangara CG et al. (2000) The production of recombinant proteins in transgenic barley grains. *Proc Natl Acad Sci USA* **97**: 1914–1919.

Huang J, Wu L, Yalda D, Adkins Y, Kelleher S, Crane M et al. (2002) Expression of functional recombinant human lysozyme in transgenic rice cell culture. *Transgenic Res* **11**: 229–239.

Hussain MA and Froesch ER (1995) Clinical uses of insulin-like growth factor I. *Adv Endocr Metabol* **6**: 143–165.

Joly JC, Leung WS and Swartz JR (1998) Overexpression of *Escherichia coli* oxidoreductases increases recombinant insulin-like growth factor-1 accumulation. *Proc Natl Acad Sci USA* **95**: 2773–2777.

Jones JH and Clemmons DR (1995) Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* **16**: 3–10.

Jones PA and Baylin SB (2002) The fundamental role of epigenetic events in cancer. *Nat Rev Genet* **3**: 415–428.

Kurihara S, Hakuno F and Takahashi S (2000) Insulin-like growth factor-I-dependent signal transduction pathways leading to the induction of cell growth and differentiation of human neuroblastoma cell line SH-SY5Y: the roles of MAP kinase pathway and PI 3-kinase pathway. *Endocr J* **47**: 739–751.

Lanes R, Recker B, Fort P and Lifshitz F (1985) Impaired somatomedin generation test in children with insulin-dependent diabetes mellitus. *Diabetes* **34**: 156–160.

Lee RWH, Pool AN, Ziauddin A, Lo RYC, Shewen PE and Strommer JN (2003) Edible vaccine development: stability of *Mannheimia haemolytica* A1 leukotoxin 50 during post-harvest processing and storage of field-grown transgenic white clover. *Mol Breed* **11**: 259–266.

Leite A, Kemper EL, Da Silva J, Luchessi AD, Siloto MP, Bonaccorsi ED et al. (2000) Expression of correctly processed human growth hormone in seeds of transgenic tobacco plants. *Mol Breed* **6**: 47–53.

Liu Q, Ning W, Dantzer R, Freund GG and Kelley KW (1998) Activation of protein kinase C-zeta and phosphatidylinositol 3′-kinase and promotion of macrophage differentiation by insulin-like growth factor-I. *J Immunol* **160**: 1393–1401.

Lopez-Carballo G, Moreno L, Masia S, Perez P and Barettino D (2002) Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. *J Biol Chem* **277**: 25297–25304.

Masumura T, Hibino T, Kidzu K, Mitsukawa N, Tanaka K and Fujii S (1990) Cloning and characterization of a cDNA encoding a rice 13 kDa prolamin. *Mol Gen Genet* **221**: 1–7.

Mori H and Cline K (1998) A signal peptide that directs non-sec transport in bacteria also directs efficient and exclusive transport on the thylakoid delta pH pathway. *J Biol Chem* **273**: 11405–11408.

Oh K, Cheon BY, Cho SH, Truong HQ, Ok SH, Jeung JU et al. (2003) Expression of the bovine growth hormone alters the root morphology in transgenic tobacco plants. *Transgenic Res* **12**: 363–367.

Pahlman S, Meyerson G, Lindgren E, Schalling M and Johansson I (1991) Insulin-like growth factor I shifts from promoting cell division to potentiating maturation during neuronal differentiation. *Proc Natl Acad Sci USA* **88**: 9994–9998.

Panahi M (2002) Biological activity of recombinant prohormone precursor insulin-like growth factor-1B (IGF-1B) and the mature form of IGF-1 expressed in transgenic plants. Doctoral Thesis, University of Ottawa.

Panahi M, Cheng X, Alli Z, Sardana R, Callaghan M and Altosaar I (2003) Plant-derived recombinant human insulin-like growth factor precursor prohormone IGF-1B caused differentiation of human neuroblastoma cell lines SH-SY5Y. *Mol Breed* **12**: 21–31.

Pen J, Molendijk L, Quax WJ, Sijmons PC, Ooyen AJJ, Van den Elzen PJM et al. (1992) Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefaction. *Bio/Technology* **10**: 292–296.

Perlak FJ, Fuchs RL, Dean DA, McPherson SL and Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control genes. *Proc Natl Acad Sci USA* **88**: 3324–3328.

Reddy V, Leelavathi S, Selvapandiyan A, Raman R, Giovanni F, Shukla V et al. (2002) Analysis of chloroplast transformed tobacco plants with *cry*1Ia5 under rice *psb*A transcriptional elements reveal high level expression of Bt toxin without imposing yield penalty and stable inheritance of transplastome. *Mol Breed* **9**: 259–269.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Sardana R, Dukiandjiev S, Giband M, Cheng X, Cowan K, Sauder C et al. (1996) Construction and rapid testing of synthetic and modified toxin gene sequences *Cry IA* (b & c) by expressing in maize endosperm culture. *Plant Cell Reports* **15**: 677–681.

Sardana R, Alli Z, Dudani A, Tackaberry E, Narayanan M, Panahi M et al. (2002) Biological activity of human granulocyte-macrophage colony stimulating factor is maintained in a fusion with seed glutelin peptide. *Transgenic Res* **11**: 521–531.

Schenk P, Sagi L, Remans T, Dietzgen R, Bernard M, Graham M et al. (1999) A promoter from sugarcane bacilliform badnavirus drives transgene expression in banana and other monocot and dicot plants. *Plant Mol Biol* **39**: 1221–1230.

Schoenle EJ, Zenobi PD, Toressani T, Werder EA, Zachman M and Froesch ER (1991) Recombinant human insulin-like growth factor I (rhIGF-1) reduces hyperglycemia in patients with extreme insulin resistance. *Diabetologia* **34**: 675–679.

Schwander JC, Hauri C, Zapf J and Froesch ER (1983) Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. *Endocrinology* **113**: 297–305.

Sijmons PC, Dekker BMM, Schammeijer B, Verwoed TC, Van den Elzen PJM and Hoekema A (1990) Production of correctly processed human serum albumin in transgenic plants. *Bio/Technology* **8**: 217–222.

Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J et al. (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol Biol* **42**: 583–590.

Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM et al. (2002) Practical considerations for pharmaceutical antibody production in different crop systems. *Mol Breed* **9**: 149–158.

Torres E, Vaquero C, Nicholson L, Sack M, Stoger E, Drossard J et al. (1999) Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. *Transgenic Res* **8**: 441–449.

Urbina G, Schirmeister-Tichy H, Schernthaner J, Taralp A and Pfleiderer W (2000) Direct immobilization of oligonucleotides on polymer supports. Use of FMOC derivatives in DNA/RNA synthesis. Methods for using immobilized oligonucleotides. In: Epton R (ed), Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Libraries (41 pp.). Collected papers, Sixth International Symposium, York, England, UK.

Vaeck M, Reynaerts A, Hofteh H, Jansens S, De Beuckeleer M, Dean C et al. (1987) Transgenic plants protected from insect attack. *Nature* **328**: 33–37.

Vakos HT, Burton GW and Kaplan H (1997) Taurocholate-induced dimerization of bovine cholesterol esterase in sodium dodecylsulfate. *Biochim Biophys Acta* **1342**: 103–108.

Williams S, Friedrich L, Dincher S, Carozzi N, Kessmann H, Ward E et al. (1992) Chemical regulation of *Bacillus thuringiensis* delta-endotoxin expression in transgenic plants. *Bio/Technology* **10**: 540–543.

Winter RJ, Phillips LS, Klein MN, Traisman HS and Green OC (1979) Somatomedin activity and diabetic control in children with insulin-dependent diabetes. *Diabetes* **28**: 952–954.

Zapf J, Schoenle E, Waldvogel M, Sand I and Froesch ER (1981) Effect of trypsin treatment of rat adipocytes on biological effects and binding of insulin and insulin-like growth factors. Further evidence for the action of insulin-like growth factors through the insulin receptor. *Eur J Biochem* **113**: 605–609.

Zeidman R, Pettersson L, Sailaja PR, Truedsson E, Fagerstrom S, Pahlman S et al. (1999) Novel and classical protein kinase C isoforms have different functions in proliferation, survival and differentiation of neuroblastoma cells. *Int J Cancer* **81**: 494–501.

Zinovieva N, Lassnig C, Schams D, Besenfelder U, Wolf E, Muller S et al. (1998) Stable production of human insulin-like growth factor 1 (IGF-1) in milk of hemi- and homozygous transgenic rabbits over several generations. *Transgenic Res* **7**: 437–447.

Zumkeller W and Schwab M (1999) Insulin-like growth factor system in neuroblastoma tumorigenesis and apoptosis: potential diagnostic and therapeutic perspectives. *Hormone Metabol Res* **31**: 138–141.