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**Biological activity of recombinant prohormone
precursor insulin-like growth factor-1B (IGF-1B) and the
mature form of IGF-1 expressed in transgenic plants**

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Thesis submitted to the Department of
Biochemistry, Microbiology and Immunology
in partial fulfillment of the requirements for the
Ph.D. degree

University of Ottawa
Ottawa, Ontario, Canada

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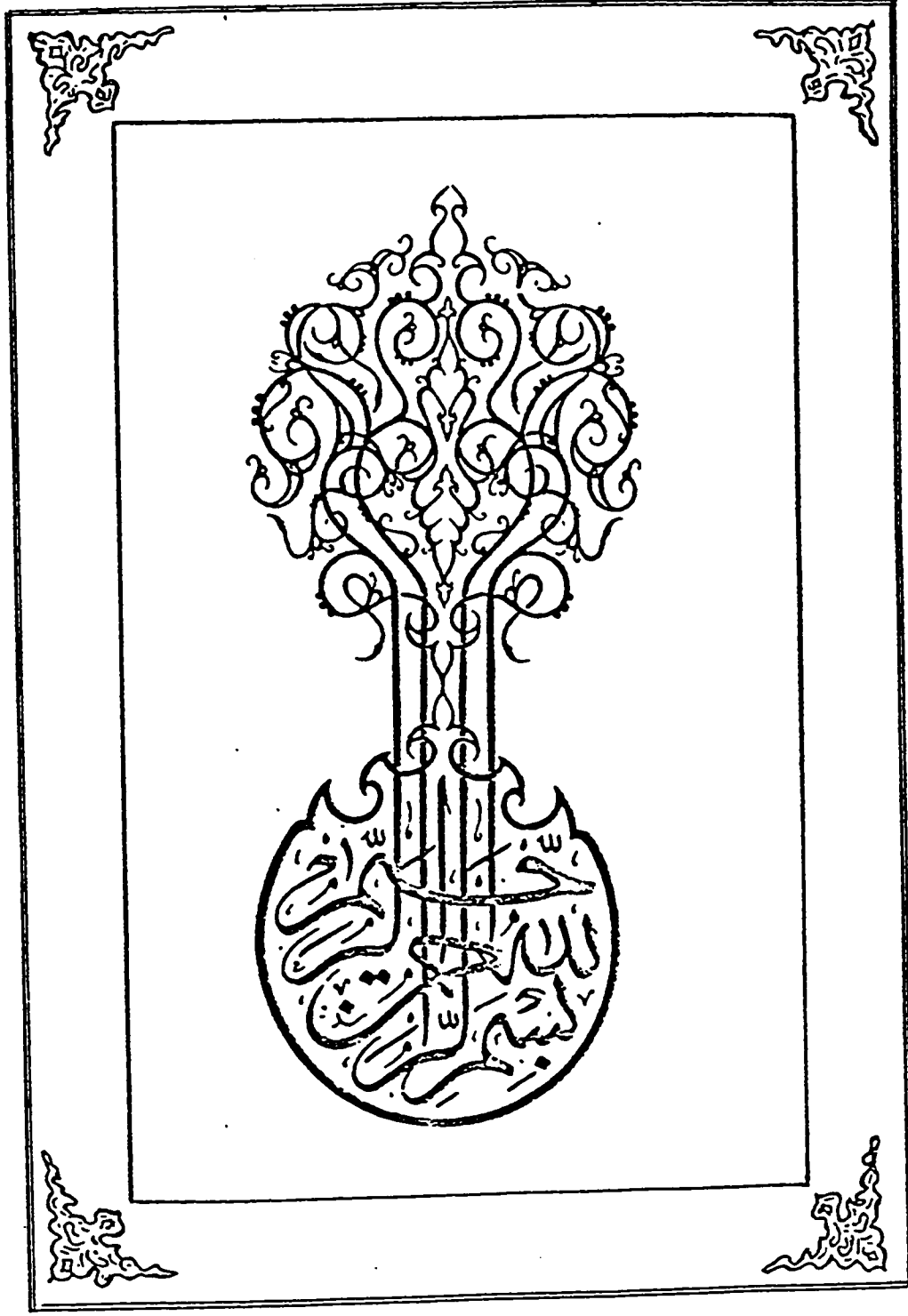
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Abstract

Recombinant DNA technology was used in an effort to produce insulin-like growth factor-1 (IGF-1) in large amount for therapeutic purposes. Therapeutic potential of human recombinant IGF-1 stems from the fact that IGF-1 resembles insulin in many aspects. Therefore, IGF-1 is considered as a promising agent in treatment of some types of diabetes, especially in cases of insulin receptor defects. To manufacture large amounts of IGF-1, others have expressed human IGF-1 in bacteria and yeast systems. In both those foreign host cells, most of the recombinant protein was unable to fold properly and often accumulated to form inactive inclusion bodies. Also, together with the important limitations of other expression systems like mammalian cell cultures and transgenic animals, plants remained to be studied as a potential transgenic production system of choice for the expression of human recombinant IGF-1. Transgenic plants are potentially one of the most economical systems for large scale production of recombinant proteins for industrial and pharmaceutical uses. Low production costs and possibility of administering transgenic plants orally are some of the advantages of plant-based production (i.e. "protein farming"). In view of these advantages, human IGF-1 was expressed in transgenic tobacco and transgenic rice. Furthermore, the role of IGF-1 precursor (IGF-1B) in growth and differentiation is not clear. Despite some reports concerning the mitogenic activity of some parts of the E-peptide domain of this protein, no recombinant form of the full-length prohormone had been reported. In this thesis, IGF-1B was expressed in transgenic tobacco plants and showed biological activity comparable to that of recombinant IGF-1.

Enzyme-linked immunosorbent assay (ELISA) data from leaves of transgenic plants detected higher expressing plants. These plants producing higher recombinant IGF-1 and IGF-1B levels were used for further studies.

Southern analysis of transgenic plants detected the expected DNA fragment corresponding to the foreign expression construct. The copy reconstruction experiments showed

that in most of the transgenic plants one copy of the transgene was incorporated. PCR and RT-PCR experiment data indicated recombinant IGF-1 and IGF-1B transgenes and transcripts were present in transgenic plants but absent in non-transformed plants. Western blot conducted on protein extracts from leaves of transgenic plants confirmed the expression of the recombinant proteins as being the same size as the standards.

Biological assays showed the mitogenic effects of recombinant IGF-1 expressed in transgenic plants on a human cell line (human neuroblastoma cell line SH-SY5Y). Addition of non-transgenic plant extracts to the assay containing the *E. coli* recombinant human IGF-1 resulted in decrease of the rate of proliferation of the cells. This suggests that some compound(s) in the non-transformed leaf extracts inhibits the activity of IGF-1 protein. These data suggest that human IGF-1 and IGF-1B produced and stored in plants were functional and maintained in an active conformation and can be used for therapeutic purposes. Such a cheap and abundant supply of the recombinant protein as well can now facilitate further research on the role of IGF-1B itself and its relationship to IGF-1.

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Chapter 1

Introduction

1.1. Production of recombinant proteins

Recent advances in biotechnology have made available many options for expressing and producing protein products. The potential of recombinant DNA technology lies in the effort of large-scale production, higher yields and greater purity of the product. To find the best expression system, one must ask which system offers the most significant advantages for the production of a particular recombinant product.

Recombinant protein production systems utilized today range from prokaryotic systems such as *Escherichia coli* (Demirhan *et al.*, 2000) and *Bacillus* (Ebisu *et al.*, 1996), to eukaryotic systems such as yeast (Hashimira, 1994; Gill *et al.*, 1999), *Aspergillus* (Archer, 1994), mammalian (Niu and Ashley, 2000) and insect cell cultures (Aztiria *et al.*, 2000; Hata *et al.*, 2000), transgenic animals (Velander *et al.*, 1997; Ueno *et al.*, 2000), urine (Kerr *et al.*, 1998) and plants (Frigerio *et al.*, 2000).

The expression of heterologous proteins in prokaryotic hosts has encountered a number of problems. The post-translational modifications such as glycosylation are not carried out. The protein processing occurs by a mechanism that differs significantly from that in eukaryotic cells. For example, in bacteria post-translational modifications required for bioactivity of many human proteins are not performed. This may be a reason why the high level protein expression in bacteria often leads to accumulation of insoluble protein aggregates or inclusion bodies (Cramer *et al.*, 1999). Also, another problem is incorrect folding which often results in biologically inactive molecules. Peptides whose biological activity depends on the correct formation of disulfide bridges are also frequently inactive: such bonds can not be formed in most bacteria

because of the reducing environment of the cytoplasm (Josephson and Bishop, 1988).

In yeast protein expression systems, the non-native forms of the proteins that are expressed often show different biological activities (Gellerfors *et al.*, 1989; Gill *et al.*, 1999). Also it was shown in yeast that the splicing mechanisms are not efficient for removing the introns of higher eukaryotes (Beggs *et al.*, 1980).

Insect cells may add only the high-mannose type of N-linked oligosaccharides. Studies of the mosquito cell lines from *Aedes aegypti* and *Aedes albopictus* indicate that the N-linked oligosaccharides produced in these species are deficient in sialic acid, galactose, and fucose (Butters and Hughes, 1981). Also, it seems that in insects, the correct post-translational modifications can not be performed which results in the expression of recombinant proteins which are not functional (Aztiria *et al.*, 2000).

Transgenic animals have been considered as suitable systems to express commercial proteins, especially in their milk since mammary glands of transgenic animals can perform correct post-translational modifications such as complex glycosylation (Lubon *et al.*, 1996). The idea that milk from transgenic animals could be a production medium for recombinant pharmaceutical proteins was suggested by the first experimental demonstration that sheep beta-lactoglobulin was produced in the milk of transgenic mice (Simons *et al.*, 1987). But, the recombinant proteins secreted in milk are not always in a proper biochemical form. Cleavage and glycosylation are not always carried out correctly. Also, it is noteworthy to mention that the generation of transgenic animals in general and transgenic livestock in particular is an extremely expensive and labor-intensive process. The cost of producing one transgenic sheep could be as high as US \$60,000 and as high as US \$500,000 for one transgenic cow (Janne *et al.*, 1998).

Mammalian cell culture also shows some limitations. For example, under optimal conditions, the expression level of proteins in mammalian cells like Chinese hamster ovary cells (CHO) is very low. To grow most mammalian cells usually fetal bovine serum (FBS) is required which is expensive and its composition may vary from batch to batch. Besides, the instability of

selected cell lines is often encountered (Sardana *et al.*, 1997).

Kerr and colleagues (1998) have used a bladder-specific promoter to direct expression of human growth hormone (hGH) into urine. The advantage is that urine can be collected throughout an animal's lifetime. But the collection of urine requires the use of catheters for constant collection and the standing position of animals is important. On the other hand the secretory pathway of the bladder is inefficient and limits the production system. Another important consideration is "leakiness" of promoter expression which was shown for some milk-specific promoters too. Leaky expression of recombinant proteins in animals can cause physiological problems including immunogenicity. For example, the promoter which was used for such a bladder 'bioreactor' was significantly leaky and hGH was observed in other tissues and made the animals obese because of the effects of GH (Meade and Ziomek, 1998).

In response to the fears or concerns with these commonly used expression systems, plant-based systems are now being more actively explored. Transgenic plants are potentially one of the most economical systems for large scale production of recombinant proteins for industrial and pharmaceutical uses. It is well known for example, that the most abundant protein synthesized throughout the biosphere is the photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase, "RubisCo" (Kamel, 1982). If cells like photosynthetic machinery cells or seed nitrogen-reserve cells (endosperm) have evolved to synthesize and accumulate large amounts of protein (sometimes upwards to 50% fresh weights), then perhaps plant cells can serve as an useful "platform technology" for manufacturing a recombinant protein of choice as well. Advantages of plant systems include the low cost of growing plants on large acreage, free inputs e.g. soil, CO₂, O₂, sun's energy and rain water. Plants being eukaryotic also, have post-translational modifications similar to mammalian systems. Carboxylation, hydroxylation, phosphorylation are some examples of having biological activity for some amino acids. For example, beta-hydroxylation of prolin in collagen is required for the formation of the heterotrimer. Theses requirements and the ability of plants to perform more exotic post-

translational modifications will determine what proteins can be expressed in plants in their active form. Finally, there is the possibility of administering transgenic plant foods by oral delivery in clinical settings.

Perhaps the most important advantage of plants involves product safety in face of "mad cow disease" and human pathogens such as HIV and other unknown agents. Such fears include the prions responsible for bovine spongiform encephalopathy and other related diseases that are involved in fermentation or mammalian cell production systems (Cramer *et al.*, 1999). The fact that some recombinant proteins from transgenic plants are now in clinical trials (Gastric lipase in transgenic tobacco and maize is in clinical trial by Meristem Company) and others are sold commercially (Avidin and beta-glucuronidase) demonstrates significant progress toward commercialization of this new drug technology.

1.2. Genetic engineering of plants

1.2.1. Methods for gene transfer

The developments of technology for foreign gene transfer into plant cells started with transformation of bacterial deoxyribonucleic acid (DNA) into plant cells. In 1973, Johnson *et al.* inoculated plant cells with a bacteriophage containing a bacterial beta-galactosidase gene. There are now several methods to transfer genes into plants and each method has its benefits and drawbacks. Introducing foreign DNA into a plant cell is the first step in successful transformation but the integration of foreign genes into plant chromosomes is a prerequisite to stable transformation. The two most commonly used methods of gene transfer into plants are *Agrobacterium*-mediated transformation and biolistic methods. *Agrobacterium*-mediated transformation is widely applicable in transforming dicotyledonous species. In this method of transformation a segment of DNA is first inserted into the binary vector (vectors based on plasmids that can replicate in both *E. coli* and *Agrobacterium*) and manipulated using *E. coli* as a host. The constructed plasmid molecules are transferred into *Agrobacterium* and finally the

desired segment of DNA can be integrated into a plant cell genome by the co-cultivation of plant cells and the transformed *Agrobacterium* cells (Komari *et al.*, 1998). Recently, there are reports of *Agrobacterium*-mediated transformation being efficient in monocotyledons as well, such as rice, maize and wheat (Heath *et al.*, 1997; Cheng *et al.*, 1997a; Cheng *et al.*, 1998). In the biolistic (from the words biological and ballistic) method, the DNA construct attached to small microparticles of some inert material e.g. gold, platinum or tungsten and the particles are bombarded through cell walls into the cells (Maenpaa *et al.*, 1999). This method has been used extensively for producing transgenic cereals. But multiple-copy insertion and gene silencing problems have been shown to be associated with various transgenes produced by the biolistic method (Kumpatla *et al.*, 1997; Palaugi and Balzergue, 2000). Therefore, *Agrobacterium* infection seems to be the best choice when it is possible.

1.2.2. Expression of heterologous peptides and proteins in plant cells

Plant genetic engineering has proved its potential in the production of valuable compounds in *planta* like herbicide resistance enzymes and entomocidal toxin proteins from *Bacillus thuringiensis* (*Bt*) (Ye *et al.*, 2001). The estimated global area of transgenic crops producing these recombinant proteins for the year 2000 was 44.2 million hectares which is almost twice the area of the United Kingdom. Transgenic traditional crops: soybean, cotton, canola and corn are globally planted. In case of soybean, in the year 2000, 36% of the soybean globally planted were transgenic (James, 2000). The crop-plant based production of proteins or “bio-farming” is one of the novel areas for production of industrial or therapeutic biomolecules. In this application the plant can be considered as a solar-powered bioreactor and an alternative to microbial, or animal cell expression systems. It has been proposed to use whole plant tissues for production of heterologous chemicals such as bioactive peptides (insulin, human growth factor, tissue plasminogen activator) or proteins (antibodies, human interferon, human serum albumin) and even commercially attractive bulk enzymes like alpha-amylase (Vandekerckhove *et al.*,

1989; deZoeten *et al.*, 1989; Sijmons *et al.*, 1990; Hiatt and Ma, 1992; Pen *et al.*, 1992).

Antibodies are one of the major proteins being studied. The first attempt to produce antibody in plants was done by Hiatt *et al.* in 1989. One application of antibodies expressed in plants is to combat plant disease, particularly viral diseases (Conrad and Fiedler, 1998). In addition, plant-produced antibodies are being used to combat infectious disease in animals and humans. EPIcyte Pharmaceutical, Inc. (San Diego, CA) a plantibody company is using corn plants to make monoclonal antibodies against the genital herpes virus [herpes simplex-2 virus (HSV-2)]. ProdiGene, Inc. (College Station, TX) is also using corn to produce monoclonal antibodies against sperm as a topical contraceptive (Erickson, 2000). Expression of an anti-cancer (colorectal cancer) monoclonal antibody in tobacco was reported by Verch *et al.* (1998). The mature IgG antibody was formed and accumulated in the endoplasmic reticulum (no mention of antibody yield from tobacco leaves was made).

Avidin and beta-glucuronidase are the first two proteins which were produced in transgenic maize and have entered the market (Sigma catalog # A 8706 for avidin and catalog # G 2035 for beta-glucuronidase) (Hood *et al.*, 1997; Witcher *et al.*, 1998). These proteins demonstrate the process of molecular farming of foreign proteins from transgenic plants. Zeigler *et al.* (1999) reported expression of another industrial enzyme in transgenic *Arabidopsis thaliana* (the complete genome sequence of *Arabidopsis* was determined by an international collaboration as the first complete genome sequence of a plant, Nature, Dec. 14, 2000). Endo-1,4-B-D-glucanase is a thermostable bacterial cellulase with cell-wall degrading activity. Because the enzyme is not active at normal temperatures, therefore no effects on the host plants themselves were detected. This enzyme was accumulated to levels as high as 25% of total soluble leaf protein. The market for this enzyme is in the areas of ethanol, textile, paper and detergent production from cellulose.

Another important plant-based production is that of vaccines. Selected subunits of the disease organism can be expressed in plants. If the selected antigen from the transgenic plant is

administered, an immune response in the host may be generated. In this way the risk of causing undesirable reactions is avoided which is possible with whole organism vaccines. One of the other advantages of plant-based vaccines is the ability of generating edible vaccines. In this way plant vaccines can be used directly as a feed or food (Hood and Jilka, 1999). One of the examples of plant-based vaccines is the production of Lt-B (heat-labile beta subunit) toxin in potato to prevent traveler's diarrhea caused by *E. coli* (Mason *et al.*, 1998).

All these considerations have led to an increased interest in expressing many other vaccine candidates and important proteins in plants over the past several years (Ganz *et al.*, 1996; Sardana *et al.*, 1997; Dalsgaard *et al.*, 1997; Kusnadi *et al.*, 1998; Arakawa *et al.*, 1998; Tackaberry *et al.*, 1999; Horuath *et al.*, 2000; Frigerio *et al.*, 2000; Wright *et al.*, 2001).

1.2.3. Secretion of proteins from plant cells and signal peptides

During the secretory pathway, proteins are synthesized on the rough endoplasmic reticulum (ER). The proteins are co-translationally translocated into the ER lumen. These proteins are transported through the intracellular compartments including ER and Golgi apparatus. The proteins pass through secretory vesicles, plasma membrane and hydrolytic compartments (vacuoles in plants and lysosomes in animals) (Okita and Rogers, 1996). The endomembrane system of plant cells has unique features which are not seen in yeast or animal cells. For example in plant cells there is the possibility of localization and assembly of large proteins in ER lumen or vacuole (Vitale and Chrispeels, 1992). Plant ER is able to synthesize heterologous proteins for industrial and pharmaceutical use (Vitale and Denecke, 1999). In plant cells like animal cells, secretion of the proteins needs the specific peptide sequence information within the protein. This peptide sorting determinant may be a signal peptide or an arrangement of the residues which forms the three-dimensional structure of the protein. In this way the destination of the proteins is determined within different compartments of the endomembrane system (Okita and Rogers, 1996). The so-called "signal hypothesis" which brought the 1999

Nobel Prize for Gunter Blobel was validated experimentally in 1975 (Blobel and Dobberstein, 1975). Blobel worked on transport through the ER. He proposed that the amino-terminal portion of a protein acts as a signal peptide.

This hypothesis has been used to explain the mechanisms of some disease. Also the hypothesis can assist to develop drugs that target specific components of the cell. Besides it is useful in the expression of proteins in different host systems (Birmingham, 1999). According to this hypothesis, one of the methods to achieve a high expression level for the foreign protein of interest in a host expression system, is to use signal peptide sequences in expression cassettes to direct the protein to specific subcellular compartments. For example, it was shown that the addition of the tetrapeptide KDEL (Lys, Asp, Glu, Leu) motif to the carboxyl terminal as an ER retention signal resulted in significantly improved expression levels of single-chain antibody fragments (scFvs) in transgenic potato (Schouten *et al.*, 1997). In another case, the highest accumulation of complete active recombinant scFv antibodies was obtained by retention of these proteins in the lumen of the ER using the same targeting signal peptide. This has been shown for leaves and seeds of transgenic tobacco as well as for potato tubers (Conrad and Fiedler, 1998). Fisher *et al.* (1999) targeted the scFv to the apoplast of transgenic tobacco cells using a murine leader peptide sequence and obtained more antibody compared to another construct in which the antibody was left alone to accumulate hopefully in the cytoplasm.

At the outset of this thesis research, I was not aware of any report on the expression of the human insulin-like growth factor 1 (IGF-1) in plants. Given the medical interest in the administration of IGF-1 to patients, I set out to study the possibility of expressing the human IGF-1 gene abundantly in transgenic plants.

1.3. Insulin-like growth factors

The insulin-like growth factors were discovered on the basis of their ability to stimulate cartilage sulfation and to replace the “sulfation factor activity” of growth hormone (GH)

(Salmon and Daughaday, 1957). This phenomenon led Salmon and Daughaday to propose that GH acted on skeletal tissues by inducing the formation of a direct acting intermediary growth factor. This hypothesis is commonly referred to as the somatomedin (mediator of somatotropin) hypothesis as growth hormone was originally called somatotropin. During the period in which the biological actions of sulfation factor were being characterized, parallel studies were initiated that attempted to define factors in serum that could stimulate insulin-like effects. It was shown that these factors were different from immunoreactive insulin. The insulin-like actions of these factors were not suppressed by addition of anti-insulin antibody. Therefore these factors were termed nonsuppressible insulin-like activity (NSILA) (Froesch *et al.*, 1966). It was found that highly purified NSILA and highly purified somatomedin each stimulated glucose incorporation into fat as well as sulfate incorporation into cartilage (Froesch *et al.*, 1976). Based on these findings it was concluded that these factors might be very similar or identical and that their structures were similar to insulin. Similarity to insulin was proven when the amino acid sequence of NSILA was obtained and was shown to be 48% homologous with human proinsulin (Rinderknecht and Humbel, 1978). Based on this, these factors were renamed insulin-like growth factor 1 (IGF-1). Sequencing of a second bioactive insulin-like molecule revealed its structure to be similar but not identical to IGF-1 and it was termed insulin-like growth factor 2 (IGF-2) (Rinderknecht and Humbel, 1978). Later sequencing of pure somatomedin-C showed its sequence to be identical to IGF-1 (Svoboda *et al.*, 1980).

Now it is known that these two proteins are pleiotropic. Both IGF-1 and IGF-2 are essential for normal fetal and postnatal mammalian growth. IGFs play a major role in cell growth and differentiation. IGF-2 is involved essentially in early fetal life, and IGF-1 in pre- and postnatal life. Circulating IGF-1 is mainly synthesized in the liver. IGF-1 circulates in blood at detectable concentrations (D'Ercole, 1996).

1.3.1. Structure

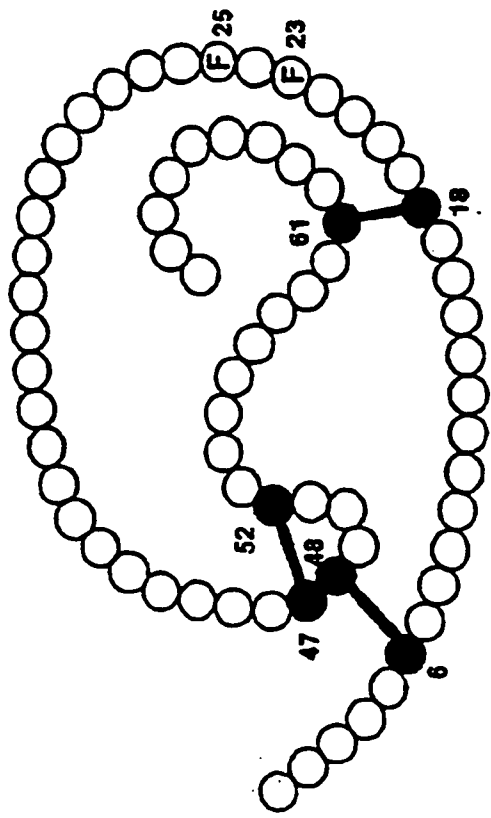
IGF-1 is a non glycosylated single chain polypeptide of 70 amino acid residues cross-linked by three disulfide bridges (Rinderknecht and Humbel, 1978). The expected molecular weight is 7,500 (Humbel, 1990). IGF-1 displays obvious homology to proinsulin and insulin (Rinderknecht and Humbel, 1978): positions 1 to 29 are homologous to insulin B chain and positions 42 to 62 (21 residues) to insulin A chain. A shortened “connecting” peptide (C-peptide domain) with 12 residues (positions 30 to 41) are comparable to positions 30 to 35 in proinsulin. An octapeptide sequence at the COOH-terminal end is also a feature not found in proinsulins (Fig. 1-3). The amino acid positions between IGF-1 and insulin suggest that there is a common ancestor for IGF-1 and insulin (Rinderknecht and Humbel, 1978). Insulin presumably evolved from IGF-1 to help the body to store energy during fasting (Van Wyk and Smith, 1999).

The A-domain of IGF-1 contains determinants for its growth-promoting activity. The B-domain is important for recognition of IGF-1 carrier proteins, which transport IGF-1 in the plasma and appear to modulate its delivery to target tissues (Magee *et al.*, 1999). The C-peptide domain enhances IGF-1 binding to IGF-1 receptors (Blundell *et al.*, 1978; Gill *et al.*, 1996). The D-domain of IGF-1 does not contribute directly to its growth promoting activity or to carrier protein recognition, but it is involved in binding of IGF-1 to receptors (Joshi *et al.* 1990) (Fig. 2). NMR (nuclear magnetic resonance) studies showed that stability of the protein is controlled by variability among side chains in the A or B domains (Hua *et al.*, 1998). In their study minimal models of proinsulin and IGF-1, designated mini-proinsulin and mini-IGF-1, were used. These models retained their native foldability although their biological activity was lost.

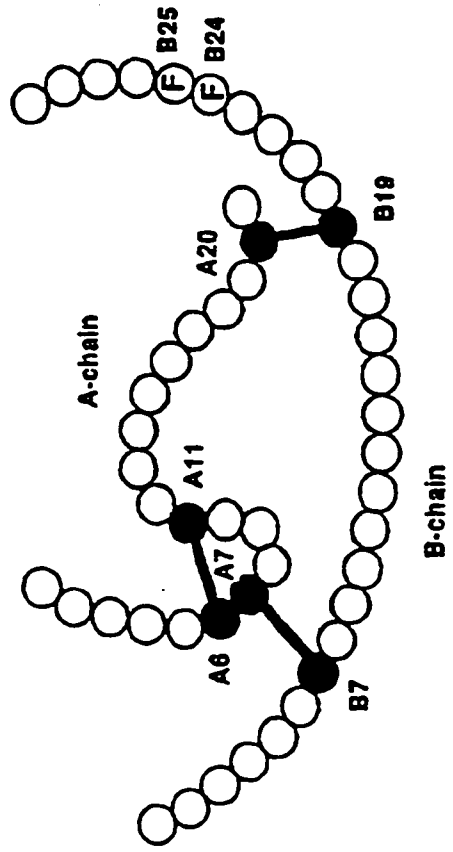
1.3.2. Molecular biology

Human IGF-1 is the product of a single, large gene, approximately 95 kb in length (Rotwein, 1991). The human IGF-1 gene is on the long arm of chromosome 12 (the human

Figure. 1. Primary structures of human IGF-1 and human insulin with disulfide bonds designated in black. IGF-1 is an intact 70-residue polypeptide chain. Human insulin consists of two polypeptide chains (A) and (B) linked by two disulfide bonds (Fransson *et al.*, 1997).

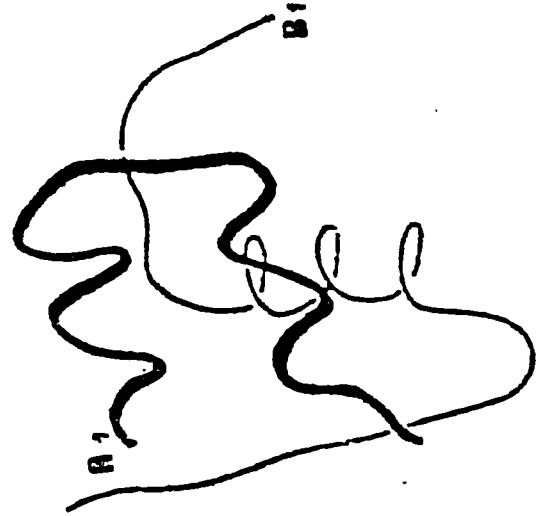


Human IGF-1

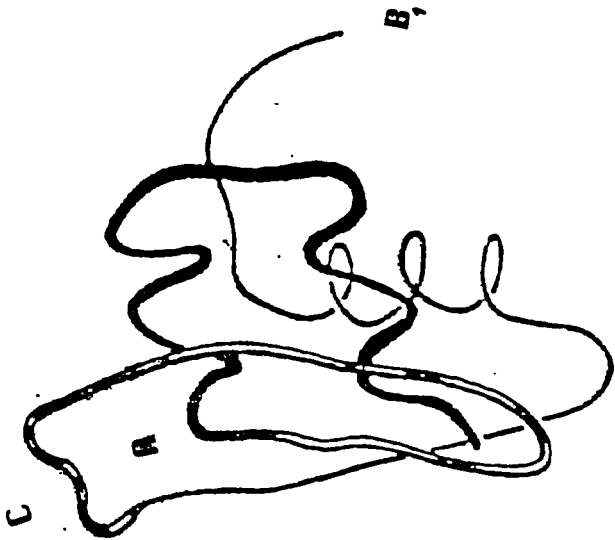


Human insulin

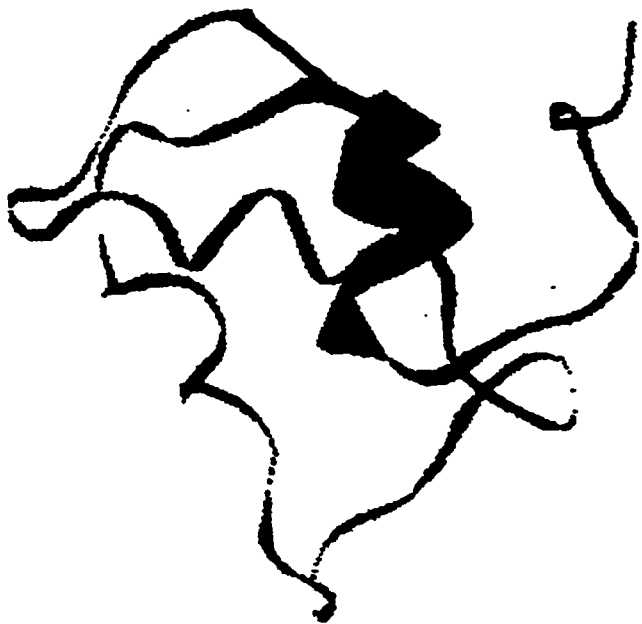
Figure. 2. 3-D (dimensional) structures of insulin, proinsulin and IGF-1 to demonstrate the family relationships. The structure of human insulin from PubMed (ID #. 1ZEH) is shown with blue color and the structure with red color is human IGF-1 from PubMed (ID #. 1BQT). 3-D structures of human insulin, proinsulin and IGF-1 from Narhi *et al.* (1993) also are presented to show the similarity in the structure.



Insulin



Proinsulin



IGF-1

Figure. 3. Comparative amino acid sequences of human insulin and IGF-1 (Rinderknecht and Humbel, 1978).

B-chain/domain

Insulin: FVNQHLCGSHLVEALYLVCGERGFFYTPKT

IGF-1: GPETLCGAELVDALQFVCGDRGFYFNKPT

C-domain

IGF-1: GYGSSRRAPQT

A-chain/domain

Insulin: GIVEQCCTSICSLYQLENYCN

IGF-1: GIVDECCFRSCDLRRLEMYCA

D-domain

IGF-1: PLKPAKSA

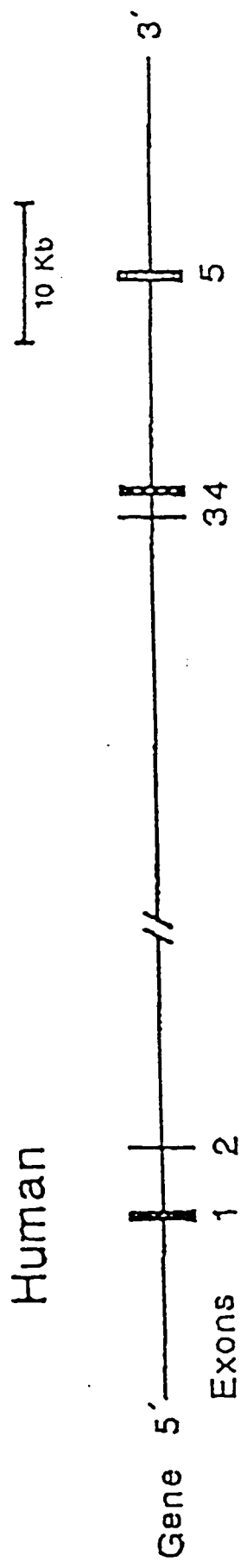
genome sequence was published in Nature on February 15, 2001 issue in which the high-resolution map of human chromosome 12 was included) and is composed of six exons, the last two of which are alternatively spliced to produce different IGF-1 (IGF-1A and IGF-1B) precursors. IGF-1A comprises exons 1, 2, 3, and 5 which encode 153 amino acids, and IGF-1B contains exons 1, 2, 3, and 4 which encode 195 residues (Fig. 4). Transcription of the IGF-1 gene results in multiple messenger RNAs ranging in size from 1 to 7.5 kb. Control of the processing of these mRNAs and their translation is a significant regulatory step in their expression (D'Ercole, 1996). The amino acid sequences of these two proteins are identical for the initial 134 residues which includes the co-translationally cleaved signal peptide (amino acids 1-48), the 70 residue IGF-1 molecule (amino acids 49-118), and 16 amino acids comprising the initial part of the carboxyl extension domain or E-peptide domain (Rotwein *et al.*, 1986, 1987). The IGF-1A precursor contains an additional 19 residues, and IGF-1B, 61 residues (Fig. 5).

The sequences of the primary IGF-1 translation products from different species are remarkably similar. Forty-three of the 48 amino-terminal amino acids comprising the signal peptide are identical between human and rat IGF-1. The carboxyl-terminal 35 amino acids of IGF-1A are also highly conserved with only five differences among human, rat, mouse, and pig precursors (Tavakkol *et al.*, 1988). Only the carboxyl terminus of the IGF-1B precursor is different among species. Fewer than 50% of residues are identical between human and rat or mouse, a probable result of the different mechanisms by which IGF-1B mRNAs are generated in these three species (Bell *et al.*, 1986; Roberts *et al.*, 1987; Shimatsu and Rotwein, 1987).

Despite the amino acid sequence similarity among IGF-1 precursors from different species, very little is known about biosynthesis of IGF-1 or about processing into the mature peptide. It seems that the entire 48 initial amino acids of human IGF-1A or IGF-1B can function as a co-translationally cleaved signal sequence (Rotwein *et al.*, 1987) targeting IGF-1 to the secretory pathway. In both precursors there is a single arginine residue at the first position after the carboxyl end of the mature protein, suggesting that a cleavage enzyme with a monobasic

Figure. 4. Structure and expression of the IGF-1 gene. Exons are numbered 1 through 5. Indicated below the gene is the pattern of alternative RNA splicing that generates the different mRNA transcripts. IGF-1A and IGF-1B mRNAs (1025, 1369 nucleotides respectively) are noted. Exons 1 and 2 encode portions of the signal peptide, exons 2 and 3 contain the mature IGF-1 molecule, exons 4 and 5 encode carboxy extension domain or E-peptide domain of IGF-1B and IGF-1A respectively (Daughaday and Rotwein, 1989).

Human



AUG
↓

UAG
↓



100 bp

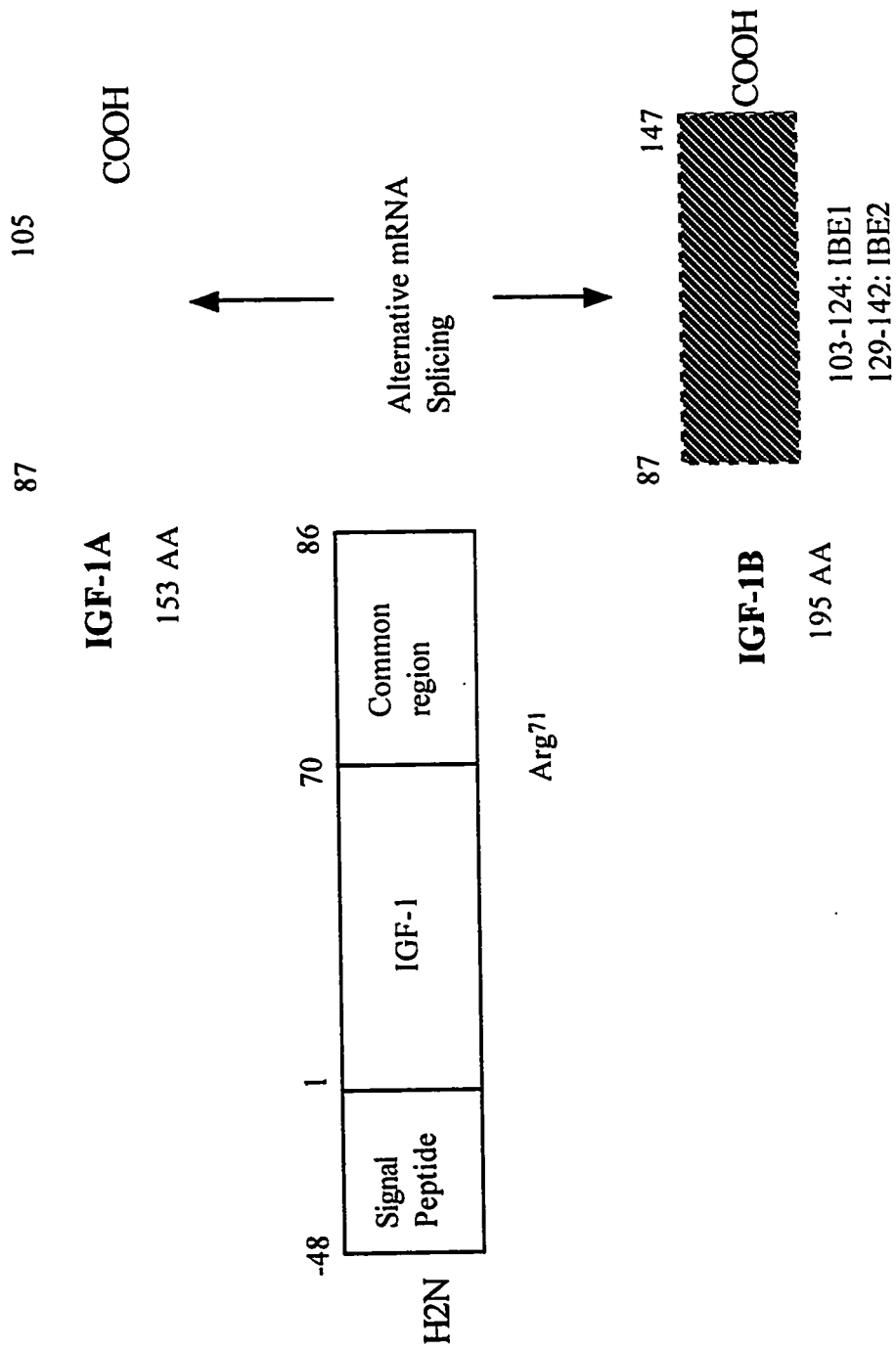
AUG
↓

UGA
↓



mRNAs

Figure. 5. Illustration of the human IGF-1A and IGF-1B proteins showing size and location of the signal peptide, the mature IGF-1, and the E- peptide domain. Residues 71-86 are common to both IGF-1A and IGF-1B. The position Arg⁷¹ is the recognition site for the cleavage enzyme during post-translational proteolytic processing (Daughaday and Rotwein, 1989).



recognition site is involved in post-translational proteolytic processing (Fig. 5) (Daughaday and Rotwein, 1989). Rotwein *et al.* (1986) suggested that tissue-specific factors may play a role in IGF-1 biosynthesis by influencing RNA splicing or protein processing and that the extension peptides themselves may show discrete biological functions.

Chew *et al.* (1995) suggested in hepatic tissue there is the splicing of exons 4-5-6 and production of a pro-IGF-1 molecule of 158 amino acids (24 amino acids in E-peptide domain). They suggested that the regulation of diverse activities of IGF-1 is complex and alternative splicing is a potential method of regulation of IGF-1 gene expression.

Investigation of the gene structure of IGF-1 among different species is essential to understand the evolutionary and functional significance of the expression of multiple IGF-1 mRNAs. The organization of the IGF-1 gene has been investigated in the human, rat, chicken, chum salmon and Japanese flounder (*Paralichthys olivaceus*) (Tanaka *et al.*, 1998). Studying the gene expression of IGF-1 in Japanese flounder suggested that some unknown transcription initiation factors are functioning in the promotion of IGF-1 expression. Nixon *et al.* (1999) studied IGF-1 gene expression in tissues and cartilage in horses. Alternative splicing of exon 4 of the equine IGF-1 gene resulted in the generation of two mRNA pools from a common nascent RNA structure. IGF-1A expression was evident in many tissues and IGF-1B was common in cartilage. Thus, mechanisms controlling gene expression of IGF-1 is complex and it remains one of the important unanswered questions in the field of growth hormone research. Another interesting phenomenon is the mitogenic activity of E-peptide domain of IGF-1 precursors especially IGF-1B (Siegfried *et al.*, 1992).

1.3.3. Function

There is considerable homology in structure between insulin and IGF-1 which results in similar functional behaviour of these compounds (Rinderknecht and Humbel, 1978). However,

the type of biological response is different with insulin or with IGF-1. For example, insulin is a potent activator of short-term metabolic processes, such as glucose transport, but it is a weak mitogen. IGF-1, on the other hand, is a potent activator of long-term growth effects, such as the stimulation of DNA synthesis (Zapf *et al.*, 1978) and therefore, is considered as a mitogenic agent. IGF-1 is involved in the regulation of somatic growth and cellular proliferation both *in vivo* and *in vitro* (Ferry *et al.*, 1999).

1.3.3.1. IGF-1 functions *in vitro*

In general, the effects of IGF-1 *in vitro* are on protein and carbohydrate metabolism, or longer term effects on cell replication and differentiation.

1.3.3.1a. Effects on cell cycle progression

The most widely studied effect of IGF-1 *in vitro* is the stimulation of DNA synthesis and cell replication. IGF-1 has been shown to function as a progression factor in the cell cycle (Adesanya *et al.*, 1999), and this function has been studied most extensively in BALB/c 3T3 (a cell type that does not produce IGF-1) fibroblast cells. Quiescent cells in G₀ can be induced to enter G₁ by platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). But these factors also have suppressive effects on the cells in G₁ which results in arrest of the cells in G₁. Treatment of the cells with IGF-1 in addition to one of these factors allows progression through G₁ and continuation through the cell cycle, resulting in DNA synthesis and cell proliferation (Jones and Clemmons, 1995). Overexpression of IGF-1 receptors in BALB/c 3T3 fibroblast cells diminished the need for the growth factors mentioned above, and cells proliferated in the presence of IGF-1 alone (Pietrzkowski *et al.*, 1992).

1.3.3.1b. Effects on cell proliferation

A wide variety of cells are stimulated by IGF-1 and show a mitogenic response. In

addition to fibroblasts, IGF-1 stimulates a mitogenic response in chondrocytes, osteoblasts, keratinocytes, thyroid follicular cells, smooth muscle cells, skeletal muscle cells, neuronal cells, mammary epithelial cells, mesangial cells, erythroid cells, thymic epithelium, oocytes, granulosa cells, and several cancer cell lines (for reviews see Sara and Hall, 1990; Bohme *et al.*, 1992; Barreca *et al.*, 1992; Werner and Le Roith, 2000; Miagawa *et al.*, 2000; Smith *et al.*, 2000; Maiorano *et al.*, 2000).

1.3.3.1c. Effects on cell death

One of the features of *in vitro* function of IGF-1 is its capacity in certain cells to inhibit cell death. This action has been best characterized in hematopoietic cells. In these cells, programmed cell death or apoptosis is believed to have an important role in the regulation of blood cell production by growth factors and IGF-1. IGF-1 along with its function as a stimulator of cell proliferation, acts also as an inhibitor of apoptosis (Williams *et al.*, 1990). In human erythroid progenitor cells, DNA breakdown which resulted from apoptosis was suppressed by IGF-1 (Muta and Krantz, 1993). IGF-1 has also been reported to prevent cell death in MCF-7 human breast carcinoma cells. In these cells apoptosis was induced by treatment of the cells with cyclohexamide (Geier *et al.*, 1993). Also it was shown that IGF-1 maintained chondrocyte survival and in this way the apoptosis was suppressed (Loeser and Shanker, 2000). IGF-1 also is considered as a factor which maintains the survival of human follicles in the early stage of their growth (Louhio *et al.*, 2000; Beg *et al.*, 2001).

1.3.3.1d. Effects on cell differentiation

IGF-1 was shown to be a potent inducer of differentiation in many different cell lines. IGF-1 can promote differentiation of chondrocytes (Geduspan and Solursh, 1993), neural cells (Pahlman *et al.*, 1991), adipocytes, osteoblasts (Sara and Hall, 1990) and a variety of muscle cell types (Kuemmerle, 2000).

1.3.3.1e. Effects on cell function

It is important to mention that hormone secretion from many cell types is regulated by IGF-1. For example, IGF-1 can stimulate hormone synthesis and secretion in ovarian cells, and also IGF-1 shows synergistic effects with FSH (Follicle Stimulating Hormone) and estrogen on these cells (Spicer *et al.*, 2000). In adrenal cells, IGF-1 increases ACTH (adrenocorticotrophic hormone) receptor number (Penhoat *et al.*, 1989; Le Roy *et al.*, 2000). It also has a regulatory effect on adrenal steroidogenic enzymes (Mesiano *et al.*, 1997). IGF-1 also effects a wide variety of other cell-specific functions. For example, IGF-1 induces release of histamine from basophils in response to immunoglobulin E (Hirai *et al.*, 1993).

In most cells with functional IGF-1 receptors, IGF-1 stimulates to some degree amino acid and glucose uptake and general protein synthesis (Bell and McDermott, 2000). In skeletal muscle *in vitro*, IGF-1 showed major insulin-like stimulatory affects on glucose uptake, glycolysis, and glycogen synthesis (Dimitriadis *et al.*, 1992).

1.3.3.2. IGF-1 functions *in vivo*

To study the functions of IGF-1 *in vivo*, the effects of IGF-1 infusion in experimental animals was investigated. In normal rats, IGF-1 infusion caused hypoglycemia, primarily by stimulating peripheral glucose uptake. But IGF-1 showed a minimal effect on suppressing hepatic glucose production (Jacob *et al.*, 1989). IGF-1 administration in other studies showed positive effects on wound healing (Suh *et al.*, 1992; Bitar, 2000), recovery of renal function (Miller *et al.*, 1992), total body protein increase (Tomas *et al.*, 1992) and had a role in control of muscle development (Reichel *et al.*, 2000). IGF-1 stimulated peripheral glucose uptake and glycogen synthesis normally in insulin-deficient diabetic rats (Rosetti *et al.*, 1991). In human, IGF-1 injection of 100 ug/kg (microgram per kilogram) rapidly caused hypoglycemia and was equipotent to 0.15 IU/kg (International Unit per kilogram) of insulin (Guler *et al.*, 1987). Also IGF-1 infusion significantly decreased the insulin-glucose ratio after caloric intake, suggesting

increased insulin sensitivity. The mechanism of this phenomenon is unknown but may be due to the IGF-1-mediated suppression of insulin. In this way insulin receptors are up regulated. Also, the suppression of GH is another outcome of IGF-1 infusion in which insulin resistance mediated by GH is removed (Boulware *et al.*, 1992). In the following section these insulin-like actions of IGF-1 are discussed in more detail.

1.3.3.3. The insulin-like actions of IGF-1

These are best appreciated in the *in vivo* studies of the effects of IGF-1 administration. As shown in Figures 1-3, IGF-1 is structurally related to insulin and mimics its effects. It is believed that IGF-1 has an important complementary role to insulin in the regulation of carbohydrate metabolism (Lewitt, 1994). Major differences between the effects of insulin and IGF-1 *in vivo* are related to the presence of the IGF-1 carrier proteins, which prolong IGF-1 actions. Administration of IGF-1 reduced hyperglycemia and improved insulin action in insulin resistance patients (Schoenle *et al.*, 1991). These glucose-lowering effects of IGF-1 raised interests in using IGF-1 in the treatment of different types of diabetes. In all of these cases, IGF-1 enhanced insulin action which led to improved glycemic control (Simpson *et al.*, 1998).

1.3.4. IGF-1 receptors

During the late 1970s biological studies moved away from tissue extracts, and studies utilizing cell culture systems were employed. A second major development in the early 1970s was the use of highly purified material to measure receptor binding. Since insulin receptor binding had been characterized, the next step was to determine if IGF-1 competed for binding to insulin receptors (Hintz *et al.*, 1972). There are two known receptors that specifically recognize IGF-1. The type 1 IGF-1 receptor has a high degree of homology with the insulin receptor and is the only IGF-1 receptor which is involved in IGF-1 mediated signalling functions (Steele Perkins *et al.*, 1988). It is a heterotetramer composed of paired, disulfide-linked alpha and beta subunits.

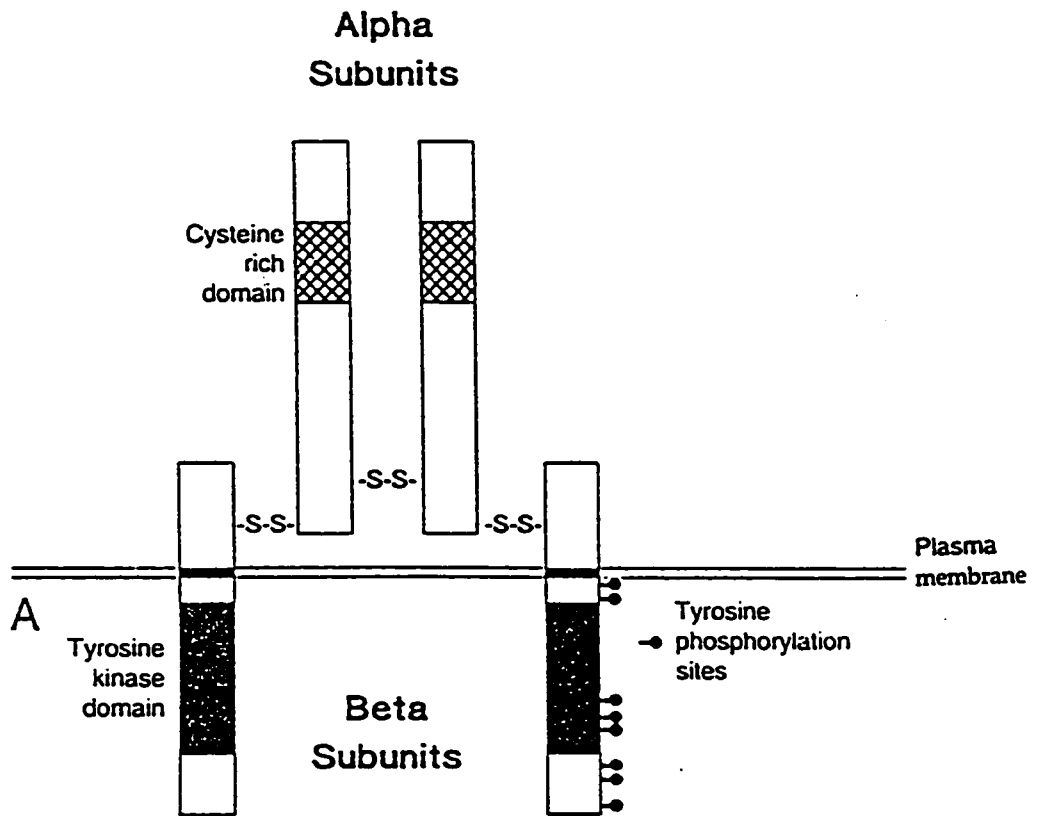
The alpha subunits are extracellular and bind ligand (IGF-1 more than insulin). The beta subunits span the cell membrane and include a tyrosine kinase and a carboxyl terminus containing three tyrosine residues that are important in signalling. The entire receptor is the product of a single gene (D'Ercole, 1996) (Fig. 6). The type 2 IGF-1 receptor is identical to the cation-independent mannose 6-phosphate receptor, which functions in the trafficking of lysosomal enzymes but has no known IGF signalling function (Nielsen, 1992). The insulin receptor can also bind IGF-1 with low affinity, and hybrid IGF/insulin receptors have been isolated that bind IGF-1 more than insulin. These receptors are formed in the cells that express both receptor genes related to insulin and IGF-1 (Soos *et al.*, 1993; Miyagawa *et al.*, 2000; Maggi and Cordera, 2001).

1.3.5. IGF-1 binding proteins (IGFBPs)

IGF-1 is present in the circulation and throughout the extracellular space and is in a form of binding to members of a family of high affinity IGFBPs. At least six major IGFBPs have been characterized. All share structural homology with each other and specifically bind IGF-1 (Baxter, 2000). The IGFBPs have been proposed to have four major functions that are essential to coordinate and regulate the biological activities of IGF-1. These proteins act as transport proteins in plasma and control the efflux of IGF-1 from vascular space. They also prolong the half-life of IGF-1 and regulate its metabolic clearance. They provide a means of tissue- and cell type-specific localization and directly modulate interaction of the IGF-1 with its receptors and thereby indirectly control biological actions. The molecular mechanism by which IGFBPs modulate IGF-1 actions is complex. Factors such as cell surface association, extracellular matrix association, phosphorylation, or proteolysis, which alter the affinity of IGFBPs for their respective ligands, can clearly alter target cell actions (Jones and Clemmons, 1995).

Another group of proteins which share structural similarities with the IGFBPs was discovered that has lower affinity for IGF-1. It was speculated that the IGFBP superfamily was

Figure. 6. Type 1 IGF-1 receptor structure (D'Ercole, 1996).



derived from an ancestral gene and during evolution, some members evolved into high-affinity IGF-1 binders and others into low-affinity IGF-1 binders (Hwa *et al.*, 1999).

1.3.6. Regulation of expression and function of IGF-1

Multiple factors have a role in regulating IGF-1 expression. GH and nutritional status are major regulators of IGF-1 (Rotwein, 1991; Guyda, 1997; Sirotkin *et al.*, 2000). Although the precise mechanisms are not defined, GH stimulates the transcription of IGF-1 in liver as well as in several other tissues (Rotwein *et al.*, 1993). As a result, hepatic IGF-1 mRNA and blood IGF-1 levels are elevated in states of GH excess. Nutritional deficiency, either protein or energy deprivation, results in depressed IGF-1 mRNA in liver. In other tissues also IGF-1 mRNA is depressed but often to a lesser extent. Also IGF-1 levels are reduced in blood in the case of nutritional deficiency (Ketelslegers *et al.*, 1995). Although transcription may be affected, low IGF-1 expression in nutritional deficiency seems to be resultant primarily from instability of IGF-1 mRNA and /or reduced translation. Furthermore, nutritional deficiency induces a state of relative GH resistance, which contributes to the reduced IGF-1 expression.

IGF-1 is regulated by many other factors too, generally those that are specific to the tissue of IGF-1 expression. For example, FSH stimulates IGF-1 mRNA in ovarian granulosa and testicular Sertoli cells, parathyroid hormone (PTH) does the same in cartilage, thyroid-stimulating hormone (TSH) stimulates IGF-1 in the thyroid, and estrogens in the uterus (D'Ercole, 1996; Klotz *et al.*, 2000; Spicer *et al.*, 2000).

Therefore, with these synergistic effects between IGF-1 and other hormones as well as similarity in function between IGF-1 and insulin, it is not surprising that IGF-1 has many clinical applications.

1.3.7. Clinical applications of IGF-1

IGF-1 is used as therapeutic agent in several clinical conditions. IGF-1 has been shown

to be the major mediator of the growth-promoting effects of GH. Treatment with IGF-1 is accompanied by increased insulin sensitivity, so IGF-1 may be a promising agent in the treatment of diabetes. In addition IGF-1 possesses hypoglycemic effects similar to insulin, therefore it is a promising therapeutic agent in subjects with certain defects in insulin receptor function. In the following situations IGF-1 is considered as a possible therapeutic agent:

1. Growth hormone-insensitive (Laron-type) syndrome (Carel *et al.*, 1996, Laron, 1999, Ranke *et al.*, 1999)
2. Type A insulin resistance syndrome (Schoenle *et al.*, 1991)
3. Catabolic states including acquired immunodeficiency syndrome (Hirschfeld, 1996)
4. Non insulin-dependent diabetes (Cusi *et al.*, 1995; Simpson *et al.*, 1998)
5. Insulin-dependent diabetes (Cusi *et al.*, 1995; Simpson *et al.*, 1998)
6. Osteoporosis (Hussain and Froesch, 1995)
7. Wound healing (Bitar, 2000).

The metabolic effects of IGF-1 appear to be beneficial in these conditions.

1.3.8. Production of recombinant human IGF-1

Because of the recent consideration of clinical uses of IGF-1 along with the demand of performing more research on IGF-1, recombinant IGF-1 has been expressed in several different host-vector systems, including yeast, *E. coli*, transgenic rabbits and mammalian cell cultures.

1.3.8.1. Expression of IGF-1 in *E. coli*

Early reports on IGF-1 expression in *E. coli* focused on expressing a cytoplasmic product (Buell *et al.*, 1984, Saito *et al.*, 1987). But to avoid the degradation of IGF-1 by cytoplasmic proteases, alternative approaches were used to target IGF-1 into the *E. coli* periplasmic space. Wong's group (1988) reported the expression of human IGF-1 into the periplasmic space. In this work, the synthetic IGF-1 was fused in frame to two of the signal sequences of *E. coli* Lam

B and Ompf sequences. A significant quantity of IGF-1 was processed and secreted into the periplasmic space while a small amount was found excreted into the medium. About 80% of expressed IGF-1 was accumulated as inclusion bodies which were insoluble and inactive. This is an example of the large number of problems observed when expressing heterologous proteins in bacteria (Joly *et al.*, 1998; Zhang *et al.*, 1998). Usually the precipitated material must be dissolved using strong chaotropic reagents, such as 8 M urea or 6 M guanidinium HCl, which are inconvenient to work with and expensive specifically in large scale applications (Samuelsson *et al.*, 1991). These chemicals can also cause irreversible modifications (e.g. carbamylation) to the protein structure, which affects the intended therapeutic uses of the product (Martson *et al.*, 1986). In addition, refolding is usually performed in very dilute solution to avoid reaggregation. These low concentrations lead to large working volumes, which again are not cost effective on a large scale (Weir and Sparks, 1987). After expression of IGF-1 in *E. coli* it was shown that in isolated material, IGF-1 was in misfolded or mismatched form (Samuelsson *et al.*, 1991). The mismatched form of IGF-1 has disulfide bonds at positions 6-47, 48-52 and biologically is inactive (Raschodrof *et al.*, 1988; Iwai *et al.*, 1989; Forsberg *et al.*, 1990). In native IGF-1 the disulfides are at positions 6-48, 18-61, 47-52 (Fig. 7). *In vitro* refolding of IGF-1 by oxygen has demonstrated that native, mismatched, and aggregated IGF-1 accumulated, even under dilute refolding conditions (Meng *et al.*, 1988; Milner *et al.*, 1999). Another important problem during refolding is the tendency to produce disulphide-linked dimers, trimers and multimers. The different misfolded forms of IGF-1 can rapidly shuffle between each other resulting in other misfolded forms of the protein even during the refolding process (Rosenfeld *et al.*, 1997). Thus, both the *in vivo* and the *in vitro* folding properties of IGF-1 raise concerns about the biological activity of the protein.

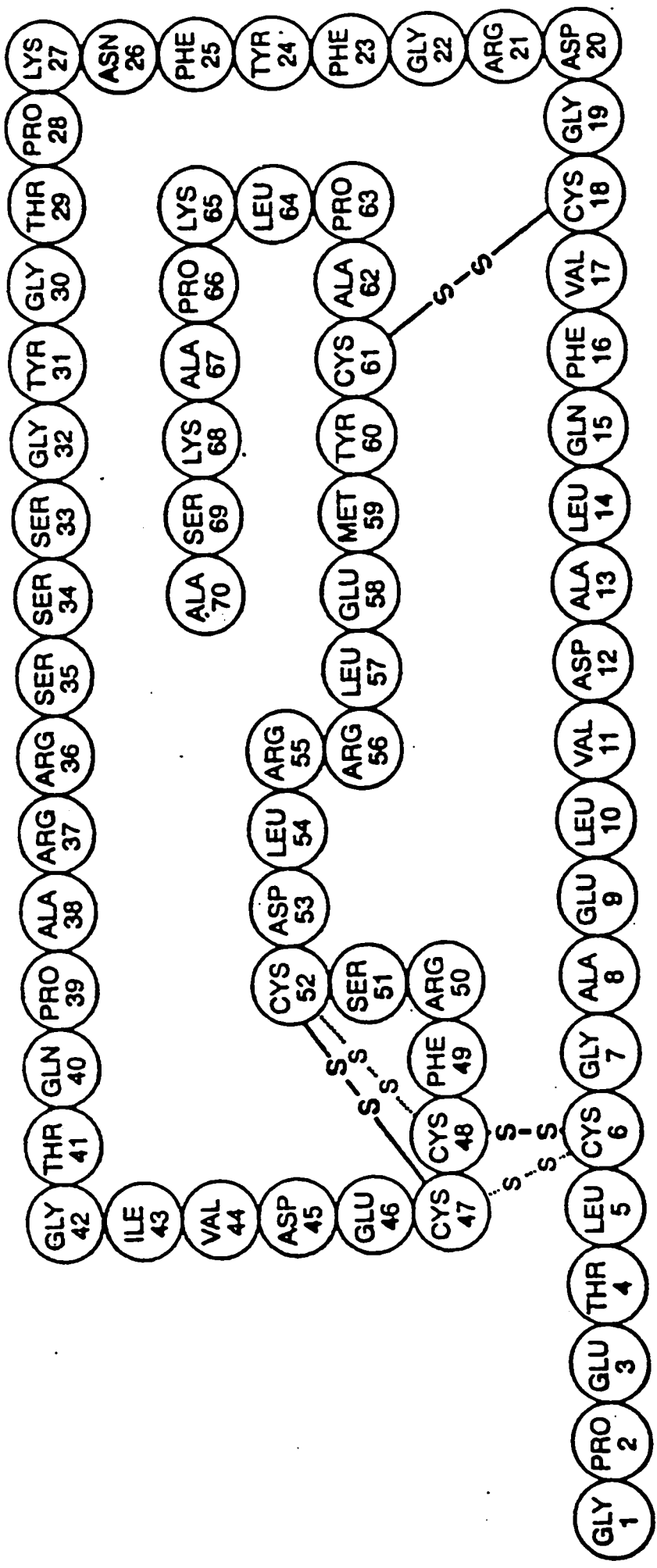
1.3.8.2. Expression of IGF-1 in yeast

The secretion process in yeast is similar to that of higher eukaryotic cells. It has been

possible to synthesize and secrete various foreign proteins of widely different origin in yeast, as for example, human haptoglobin (Van der Straten *et al.*, 1986). The secretory pathway in yeast involves several membrane structures that mediate the transfer of exported proteins from the site of synthesis at the endoplasmic reticulum via the Golgi apparatus to secretory vesicles (Schekman, 1992). But it seems that the possible post-translational modifications occurring when heterologous proteins are expressed and secreted in yeast are different from other eukaryotic systems. Most of the expressed proteins in yeast are glycosylated and this occurred with the expression of IGF-1 in yeast too. The observation that human IGF-1 is glycosylated in yeast but not in human demonstrates differences in substrate specificity between the yeast and mammalian mannosyltransferase enzymes (Gellerforst *et al.*, 1989). Also, insufficient secretion of IGF-1 into the medium in other cases of IGF-1 expression in yeast was reported. In addition, two disulfide bond isomers were found secreted into the medium (Bayne *et al.*, 1988). Elliot *et al.* (1990) also expressed IGF-1 in *Saccharomyces cerevisiae* using the yeast alpha-factor leader sequence (an effective secretion signal sequence in yeast). Approximately 10-20% of the IGF-1 was in a monomeric form, the remaining materials being disulfide-linked aggregates. Also the secreted IGF-1 was in glycosylated form. Sequence analysis suggested that Thr²⁹ was the site of glycosylation. Site-directed mutagenesis was used to convert Thr²⁹ to Asn²⁹. This substitution reduced, but did not eliminate IGF-1 glycosylation, suggesting additional glycosylation sites existed as well. In IGF-1 there are no potential sites for N-linked glycosylation (Asn-X-Ser/Thr), but there are three threonine and five serine residues. Serine and threonine residues may be the proper sites for O-glycosylation. On the other hand, it was reported that the production and secretion of human IGF-1 was deleterious to the growth of the yeast host strains. Therefore, Shuster *et al.* (1989) isolated the host strain mutant that relieved the toxicity of IGF-1 production.

Therefore it seems that expression of IGF-1 in yeast leads to the glycosylated form besides the misfolded form of the protein (Gill *et al.*, 1999). There is evidently need to find another expression system with better advantages especially regarding the expression of the

Figure. 7. The primary structure of human IGF-1. Native IGF-1 disulfide bonds (6-48, 18-61, 47-52) are indicated by solid lines and alternative disulfide bonds (6-47, 48-52) are indicated in dotted line (Miller *et al.*, 1993).



native form of IGF-1.

1.3.8.3. Expression of IGF-1 in the milk of transgenic rabbits

IGF-1 was expressed in the mammary gland of transgenic rabbits by Brem and colleagues in Germany (1994). They used a mammary-gland-specific expression system and IGF-1 occurred at levels up to 1 gram IGF-1 per liter rabbit milk. Another group in Austria (Zinovieva *et al.*, 1998) expressed IGF-1 in the milk of hemi- and homozygous transgenic rabbits for six generations. Homozygous females produced more recombinant protein than heterozygotes at levels up to an average of 543 microgram IGF-1 per ml rabbit milk. In these reports the native form of IGF-1 was obtained, but as mentioned before (section 1.1) the risk of pathogenic agents in the feed, the animals or their milk is a potential limiting factor if using this system to express IGF-1. Also, using this system is not economical in large scale production because of warren quarantine costs.

1.3.8.4. Expression of IGF-1 in mammalian cell cultures

Bayne *et al.* 1987 wanted to study the different binding domains of human IGF-1. Since the *E. coli* expression system failed to produce IGF-1 in native and active form, they assembled a synthetic gene (the nucleotide sequence for the gene was selected by optimizing the number of unique restriction endonuclease recognition sites) for human IGF-1 (encoding the seventy amino acids of human IGF-1 based on the known amino acid sequence) and expressed this gene in cultured mammalian cell lines. Their previous work showed that a chimeric plasmid containing the cytomegalovirus immediate early (CMV-IE) transcriptional regulatory region fused to the first 27 amino acids of bovine growth hormone (bGH) directed synthesis and secretion of bGH by transformed mouse fibroblasts. The synthetic human IGF-1 was inserted adjacent to the bGH signal peptide coding region to test whether the bGH signal peptide can direct secretion of a biologically active heterologous protein in transfected mouse fibroblast cells. IGF-1 was

secreted to the culture medium and biologically was active as monitored by the stimulation of DNA synthesis in vascular smooth muscle cells. They suggested that the plasmid containing the fusion of CMV-IE and bGH signal peptide to foreign proteins is a useful expression vector for the synthesis and secretion of heterologous proteins in cultured mammalian cells. In another study by Balloti *et al.* (1987) gene expression, receptor binding and growth promoting activity of IGF-1 was studied in cultured astrocytes from developing rat brain. They suggested that IGF-1 is synthesized in fetal rat astrocytes and acts as a growth promoter to stimulate astroglial cell growth during normal brain development. This system also should be considered with the limitations which were mentioned above, such as the risk of pathogenic agents and low level of expression.

1.3.8.5. General evaluation of existing expression systems

In the human health care market, there is a need to have at least 5000 tones production of therapeutic proteins. The capacity of current expression systems like stainless steel fermentors growing CHO cells to produce the world need of proteins is insufficient. For example, current recombinant factories cannot produce more than 500 kg of monoclonal antibodies as cancer therapeutics (L. Vezina, Medicago Inc., personal communication). Also, the risk of pathogenic agents is very high in these systems. In the case of using transgenic animals to express foreign proteins, the cost of production is high and this expression system is not able to provide the world demand of therapeutic proteins. In Table 1, costs, yields, limitations and the risk associated with various expression systems are summarized. Regarding the limiting factors of each expression system it is logical to study plants as an alternative system for expression of IGF-1 and IGF-1B.

1.4. Aim of the study

The production of human IGF-1 in plants may offer a cheap, safe and biologically active

Table 1. Commonly used expression systems

Characteristics	Host cell system						
	Bacteria	Yeast	Insect	Mammalian	Plant	Milk	Urine
Cell growth	rapid	rapid	slow	slow	moderate	slow	rapid
Cost	low	low	high	high	low	high	high
Expression levels	high	low-high	low-high	low	high	low	high
Protein folding required	refolding required	refolding required	proper	proper	proper	?	?
N-linked glycosylation	no	yes (mannose)	yes	yes	yes (sialic acid)	yes	?
O-linked glycosylation	no	no	no	yes	yes	yes	?
Pathogenic risk	high	high	high	high	low	high	high

source of human therapeutic proteins. Before this thesis project, no human IGF-1 was expressed in plants. The final goal of expressing human IGF-1 in recombinant expression systems is for human therapeutic application although the possibility of using this growth factor as a fish or animal growth factor supplement may also exist. The objective of this project was to express mature human IGF-1 in plants and determine its stability in the plant environment. A further objective of this project was to express a human IGF-1 precursor. Concerning the reports that IGF-1B (IGF-1 precursor) itself has mitogenic effects and may have some clinical applications led me to express this protein also in plants for the first time. The data of this project can be useful in investigating the function of IGF-1 and its precursor IGF-1B.

The transgenic nature, the translational process and biological activity of IGF-1 and IGF-1B in transgenic plants were characterized. A number of potential questions were assembled to address these goals.

1. Can one use experimental plants like the dicotyledenous *Nicotiana tabacum* or a commercially developed agricultural monocot like *Oryza sativa* (rice) for expression of human growth factors?

Our lab has expressed *E. coli* beta-glucuronidase (GUS) in tobacco (Robert *et al.*, 1989), human granulocyte macrophage colony stimulating factor (GM-CSF) in tobacco (Ganz *et al.*, 1995), *Bt* in rice (Cheng *et al.*, 1998). Thus, it was felt appropriate to see how well these two plants would handle the manufacture of these human growth factors.

2. To economize the time for construction of expression cassettes, can the original Lam B signal sequence from Genentech function in plants?

The Genentech company sent us the human IGF-1, IGF-1B coding sequences which were optimized for expression of IGF-1 and IGF-1B in *E. coli*. It was interesting to know, if these *E. coli*-optimized coding sequences could be expressed in transgenic plants. In this way, it might be possible to have the expression of even non-plant optimized coding sequences in a plant system.

3. Which plant host background would permit extracts of more active product?

4. What effect does a monocot promoter have in a dicot system on the expression level?

Historically, the Cauliflower mosaic virus (CaMV) 35S promoter has been widely used as a constitutive promoter for the expression of many proteins in plants. Since 1994, ubiquitin promoter has been used in monocot plants. By using ubiquitin promoter in a dicot plant like tobacco, one could get some idea of the effect of a monocot promoter in a dicot system. It is noteworthy to mention that 35S promoter has significant limitations when commercial bioproduction is the goal. Therefore, it was beneficial to investigate the role of ubiquitin promoter to drive constitutive transgene expression in tobacco.

5. How does plant-codon optimization affect the transgene expression level in plant systems?

When recombinant DNA technology was first applied to the overexpression of human proteins in *E. coli* (circa 1974-1977) the human somatostatin coding sequence was codon optimized for the *E. coli* t-RNA populations (R. L. Rodriguez, University of California, Davis, personal communication). When *Bt* toxin genes were first expressed in transgenic plants, codon optimizations were found to improve protein production by as much as 1000-fold (John Kemp, New Mexico State University, personal communication). Consequently, I too set out to improve this trick-of-the-trade or strategy to ensure maximal manufacture of IGFs in plants. So, one of my constructs contained plant-codon optimized IGF-1 sequence and the expression level of this construct was compared to the expression levels of *E. coli* –codon optimized sequence.

6. What is the role of IGF-1 precursor (IGF-1B) in human neuroblastoma cells?

Human neuroblastoma cells have receptors for IGF-1 and the response of these cells to IGF-1 is well documented and the effects well established. But it was interesting to investigate the response of these cells to IGF-1B, a longer polypeptide precursor chain.

7. Are plants feasible production systems to express human growth factors?

Chapter 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plasmids

The plasmids pBKIGF-2B and pJJIGF-1B were obtained from Genentech Inc. (South San Francisco, CA). The pGEM4Z plasmid was obtained from Promega. The pKHG4 (Le Gall *et al.*, 1994) binary vector for plant transformation vector has been made available to us by T. Candresse, INRA, France. This vector contained the neomycin phosphotransferase II gene for resistance to kanamycin and hygromycin phosphotransferase gene for resistance to hygromycin. For the maize ubiquitin promoter, pAHC25 plasmid was obtained from Dr. Peter Quail (US Department of Agriculture)

2.1.2. Rice prolamin signal sequence

The coding region (66 bp) of the rice prolamin signal sequence was synthesized by Dr. Xiongying Cheng in Dr. Altosaar lab based on a cDNA encoding the rice 13 kDa prolamin that was cloned by Masumura *et al.* (1990).

2.1.3. Nopaline synthase sequence

The nopaline synthase sequence (Nos-TER) for termination of transcription was obtained from the plasmid pAHC25 containing the maize ubiquitin promoter.

2.1.4. Chemicals and enzymes

Chemicals used for the preparation of the different solutions were from Sigma, Fisher Scientific, Promega and Gibco BRL. The restriction and modifying enzymes were from Gibco

BRL, Promega, Boehringer Mannheim, New England Biolabs and Pharmacia. The radionucleotide [³²P]dCTP) was from Amersham. The antibiotics (ampicillin, kanamycin, carbanicillin, ticarcillin) were either from Sigma or Gibco BRL. The oligonucleotide primers and oligos for gene assembly were synthesized by Dr. G. Alvarado from Synthaid Biotechnologies Inc. (Nepean, Ontario).

Preparations and compositions of the media are detailed in the appendix.

2.2. Methods

2.2.1. Plasmid constructs

2.2.1.1. The plasmid containing the bacterial signal sequence and the *Escherichia coli* (*E. coli*) codon-optimized IGF-1 sequence was designated as pULam B-IGF-1

It is noteworthy to mention that Genentech Inc. (South San Francisco, CA) is expressing IGF-1 in *E. coli*. Since IGF-1 expressed in *E. coli* has encountered many problems which were discussed in chapter 1 and IGF-1 was going to be used in patients, Genentech decided to collaborate with our lab to express IGF-1 in plants. Two plasmids were obtained from Genentech to start the expression of IGF-1 and IGF-1B in plants. pBKIGF-2B which was the Genentech expression vector for IGF-1 contained the Lam B signal sequence for controlled accumulation of IGF-1 in the periplasm of *E. coli*. The coding sequence of IGF-1 in this plasmid was originally from the human IGF-1 sequence which has been partially optimized for expression in *E. coli*. By using this plasmid in expression of IGF-1 in plants, one could see if the bacterial Lam B signal sequence was functional in plants. In our plant expression system we preferred to have IGF-1 coding sequence downstream of the maize ubiquitin promoter. This promoter is one of the strongest plant promoters known (Comejo *et al.*, 1993) so I chose it as the key promoter for expressing the IGF-1 protein, eventually in rice as the ultimate 'protein platform technology'. To facilitate construction of the IGF-1 vector, the IGF-1 coding sequence along with the LamB

signal sequence were subcloned into the pGEM4Z vector (Promega). The subcloning procedure is explained in more detail in Fig. 8. The Lam B-IGF-1 coding sequence was excised from the plasmid pBKIGF-2B (Genentech) using *Xba* I and *Hpa* I. The DNA was electrophoresed on a 0.8% agarose gel and the Lam B-IGF-1 fragment was excised and gene-cleaned (QIAGEN, Mississauga, Ontario). This fragment was subcloned into the multiple cloning site of the pGEM4Z plasmid (Promega) which was digested with *Sma* I and *Xba* I. The resulting plasmid was called pGIGF-1. The pGIGF-1 was cut with *Xba* I and *Sac* I to release the Lam B-IGF-1 fragment. To fuse Lam B-IGF-1 fragment downstream of the maize ubiquitin promoter in the pAHC25 (US Department of Agriculture) plasmid, the pAHC25 plasmid containing the maize ubiquitin promoter was digested with *Sma* I and *Sac* I. In this way the GUS (beta-glucuronidase) coding sequence was replaced with the Lam B-IGF-1 coding sequence insert by ligation. The final construct of the maize ubiquitin promoter, Lam B-IGF-1 and Nos-TER (the nopaline synthase sequence for termination of transcription) was designated pULam B-IGF-1 (Fig. 8).

2.2.1.2. Plasmid containing *E. coli* codon-optimized IGF-1 without bacterial signal sequence (pUIGF-1 construct)

As mentioned before, the plasmid pBKIGF-2B was from Genentech and contained the Lam B bacterial signal sequence + IGF-1. The goal of this experiment was to compare the expression level of *E. coli* partially optimized IGF-1 coding sequence with and without the bacterial signal sequence. For this purpose, the plasmid pBKIGF-2B (Genentech) was digested with *Xba* I and *Hpa* I to release the Lam B-IGF-1 fragment (Fig. 9). This fragment was ligated in the multiple cloning site of pGEM4Z (digested with *Xba* I and *Sma* I). The resulting plasmid pGIGF-1 was cut by *Nco* I and *Sac* I to release the IGF-1 fragment (without bacterial Lam B signal sequence). To put this IGF-1 fragment downstream of the maize ubiquitin promoter, the pAHC25 plasmid (US Department of Agriculture) was used. As mentioned before, this plasmid was digested with *Sma* I and *Sac* I to remove GUS (beta-glucuronidase) and to replace it with the

Figure 8. Construction of plasmid pULam B-IGF-1 (not to scale)

The Lam B-IGF-1 fragment from the plasmid pBKIGF-2B was subcloned into the pGEM4Z plasmid to facilitate vector construction. The resultant plasmid pGIGF-1 was digested with *Xba* I-*Sac* I to release the Lam B-IGF-1 fragment which was ligated to the plasmid pAHC25 (digested with *Sma* I-*Sac* I) to be downstream of the maize ubiquitin promoter. The Lam B-IGF-1 fragment is 344 bp. The maize ubiquitin promoter sequence is 2000 bp and Nos-TER sequence is 260 bp.

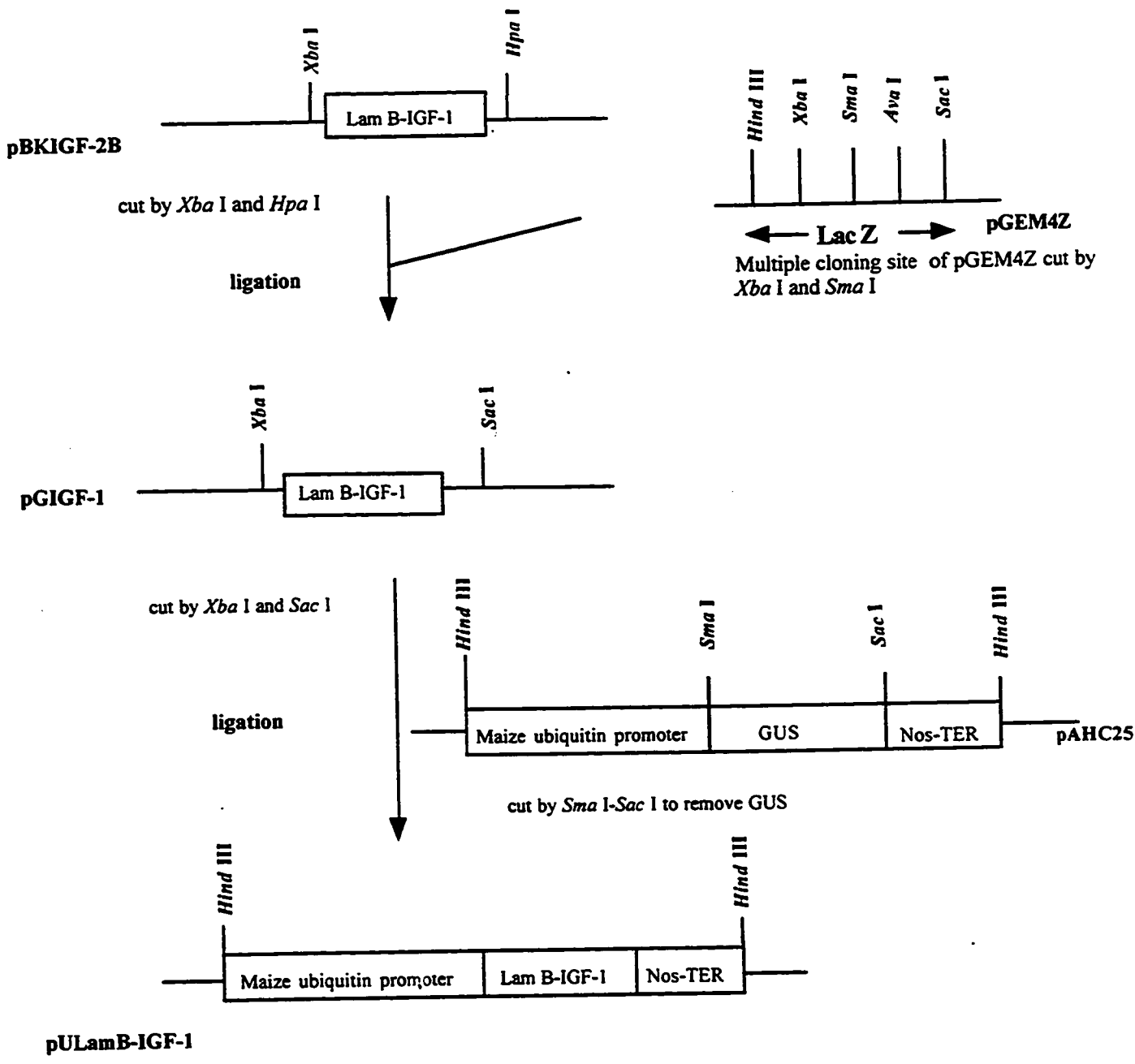
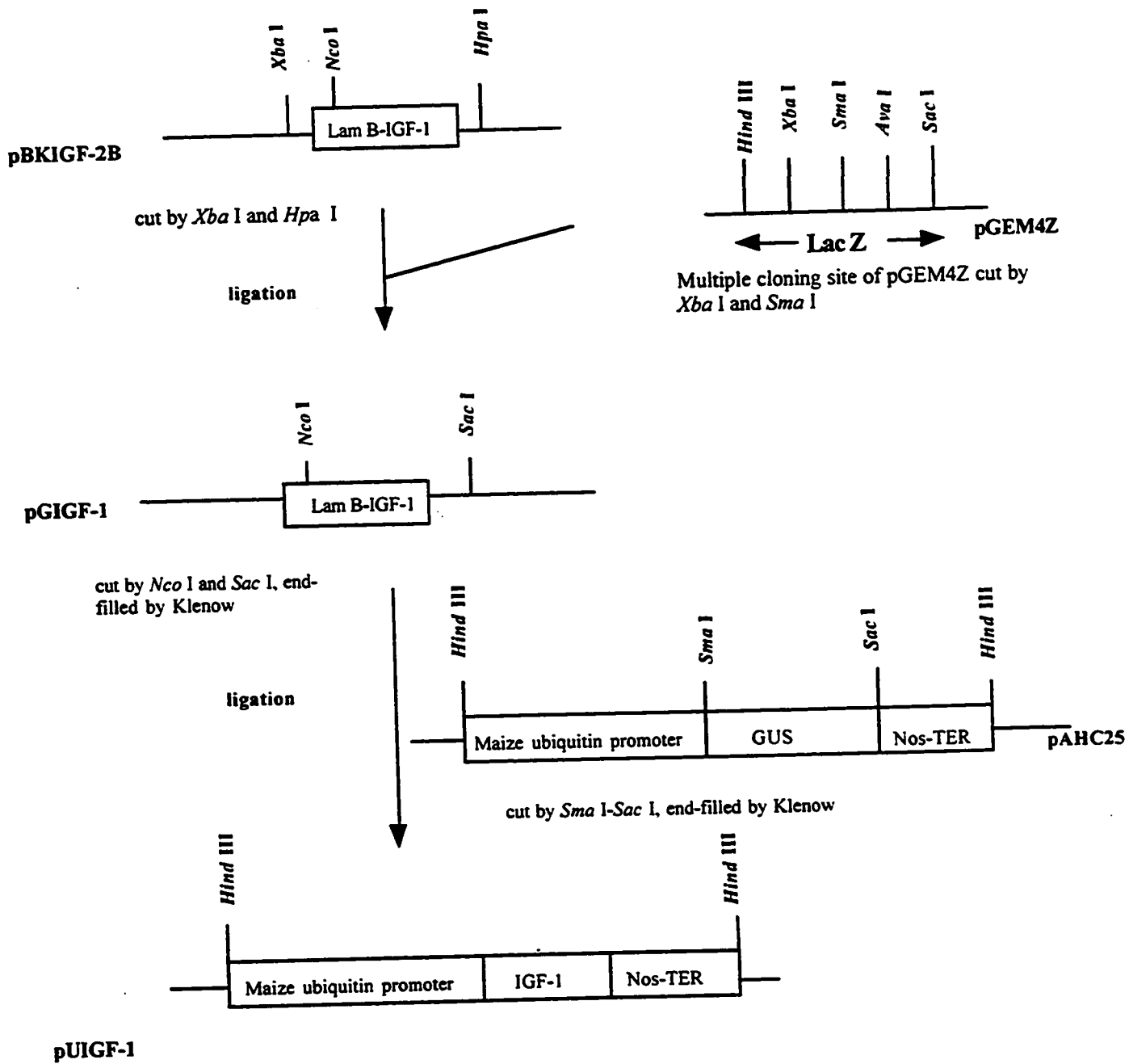


Figure 9. Construction of plasmid pUIGF-1(not to scale)

The Lam B-IGF-1 fragment from the plasmid pBKIGF-2B was subcloned into the pGEM4Z plasmid to facilitate vector construction. The resultant plasmid pGIGF-1 was digested with *Nco* I-*Sac* I to release the IGF-1 fragment. The IGF-1 fragment was ligated to the plasmid pAHC25 digested with *Sma* I-*Sac* I to be downstream of the maize ubiquitin promoter. The IGF-1 fragment is 268 bp. The maize ubiquitin promoter is 2000 bp and Nos-TER is 260 bp.



IGF-1 coding sequence insert. The final construct containing of the maize ubiquitin promoter, IGF-1 and Nos-TER (terminator sequence) was designated as pUIGF-1 (Fig. 9).

2.2.1.3. Plasmid containing bacterial Lam B signal sequence + *E. coli* codon-optimized IGF-1B (pULam B-IGF-1B construct)

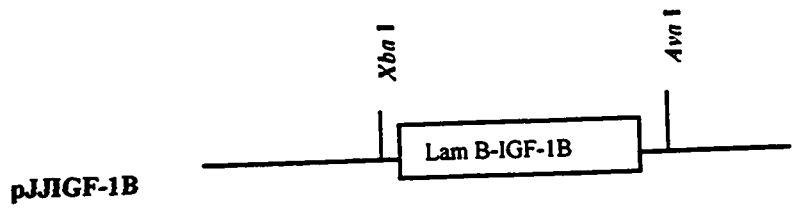
As mentioned the first Genentech plasmid (pBKIGF-2B) contained Lam B + *E. coli* optimized IGF-1 (70 residues). The second one (pJJIGF-1B) contained Lam B + *E. coli* optimized IGF-1B (the 147 residues prohormone). The IGF-1B codon optimized sequence also coded for the human precursor. Similar to IGF-1, I wanted to express IGF-1B in plants to compare the expression level of constructs with and without the bacterial Lam B signal sequence. For this purpose, pJJIGF-1B (Genentech) was digested by *Xba* I and *Ava* I to release the Lam B-IGF-1B (Fig. 10). The DNA was electrophoresed on a 0.8% agarose gel and the Lam B-IGF-1B fragment was excised and gene-cleaned. To facilitate the vector construction, the Lam B-IGF-1B coding sequence was subcloned into the multiple cloning site of pGEM4Z (Promega). The resulting plasmid was called pGIGF-1B. This plasmid was digested with *Xba* I and *Sac* I to release the Lam B-IGF-1B fragment. This recovered fragment was ligated into the pAHC25 plasmid (digested with *Sac* I and *Sma* I to remove GUS and to insert Lam B-IGF-1B fragment). The final construct containing the maize ubiquitin promoter, Lam B-IGF-1B coding sequence and Nos-TER was designated as pULam B-IGF-1B (Fig. 10).

2.2.1.4. Plasmid containing *E. coli* optimized IGF-1B without bacterial Lam B signal sequence (pUIGF-1B construct)

To compare the expression level of constructs containing IGF-1B with and without bacterial signal sequence in plant expression system, the construct containing IGF-1B without bacterial signal sequence was prepared. The plasmid pJJIGF-1B (Genentech) was cut by *Xba* I and *Ava* I to release the Lam B + IGF-1B (Fig. 11). To facilitate the vector construction, this

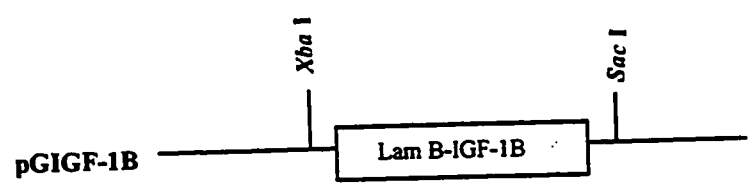
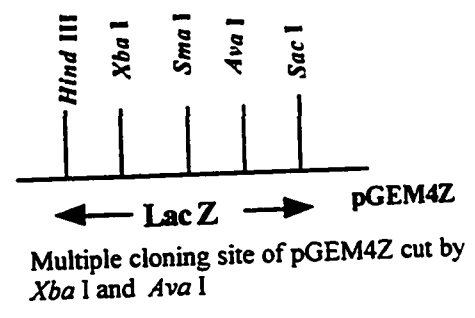
Figure 10. Construction of plasmid pULam B-IGF-1B (not to scale)

The Lam B-IGF-1B fragment was excised from plasmid pJJIGF-1B and subcloned into the pGEM4Z plasmid to facilitate vector construction. The resultant plasmid pGIGF-1B digested with *Xba* I-*Sac* I to release the Lam B-IGF-1B. This fragment was ligated to the plasmid pAHC25 digested with *Sma* I-*Sac* I to be downstream of the maize ubiquitin promoter. The Lam B-IGF-1B fragment is 542 bp. The maize ubiquitin promoter sequence is 2000 bp and Nos-TER sequence is 260 bp.



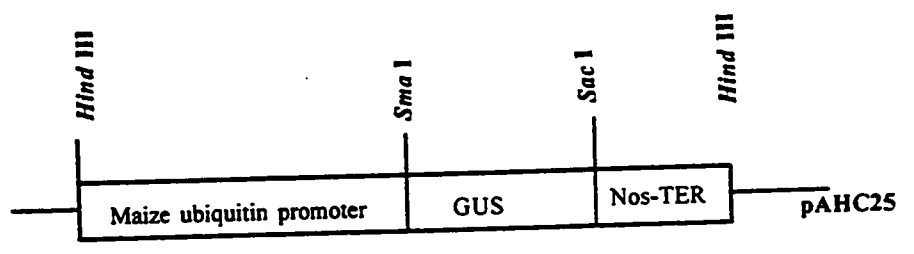
cut by *Xba* I and *Ava* I

ligation

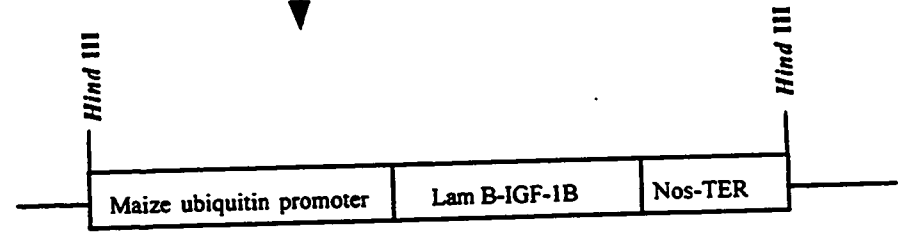


cut by *Xba* I-*Sac* I, end-filled by Klenow

ligation



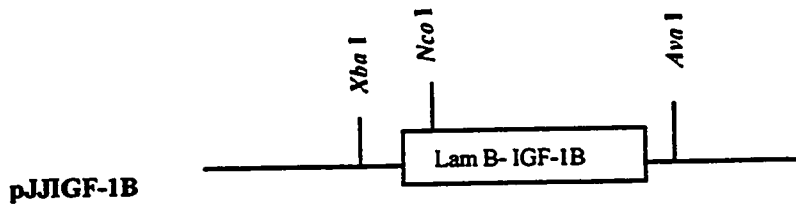
cut by *Sma* I-*Sac* I, end-filled by Klenow



pULamB-IGF-1B

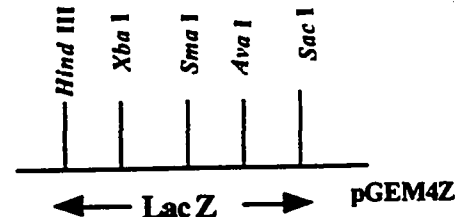
Figure 11. Construction of the plasmid pUIGF-1B (not to scale)

The Lam B-IGF-1B fragment was excised from plasmid pJJIGF-1B and subcloned into the pGEM4Z plasmid to facilitate vector construction. The resultant plasmid pGIGF-1B digested with *Nco* I-*Sac* I to release the IGF-1B fragment. This fragment was ligated to the plasmid pAHC25 digested with *Sma* I-*Sac* I to be downstream of the maize ubiquitin promoter. The IGF-1B fragment is 466 bp. The maize ubiquitin promoter sequence is 2000 bp and Nos-TER sequence is 260 bp.

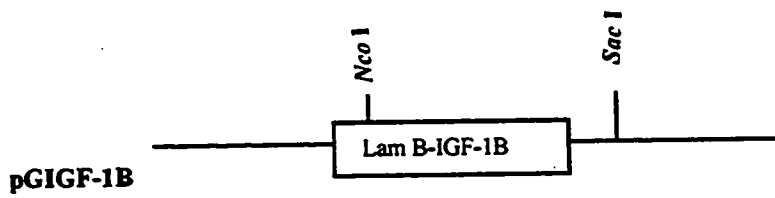


cut by *Xba* I and *Ava* I

ligation

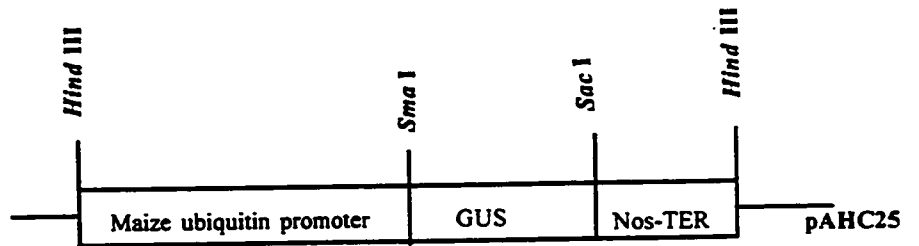


Multiple cloning site of pGEM4Z cut by *Xba* I and *Ava* I

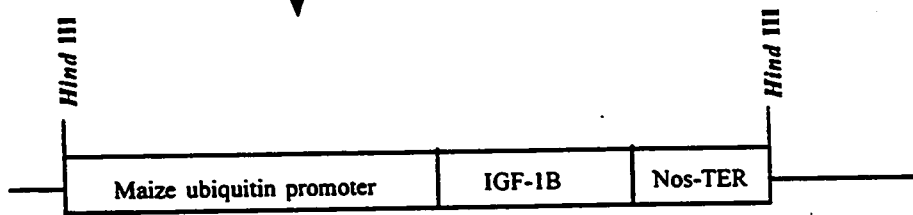


cut by *Nco* I-*Sac* I, end-filled by Klenow

ligation



cut by *Sma* I-*Sac* I, end-filled by Klenow



pUIGF-1B

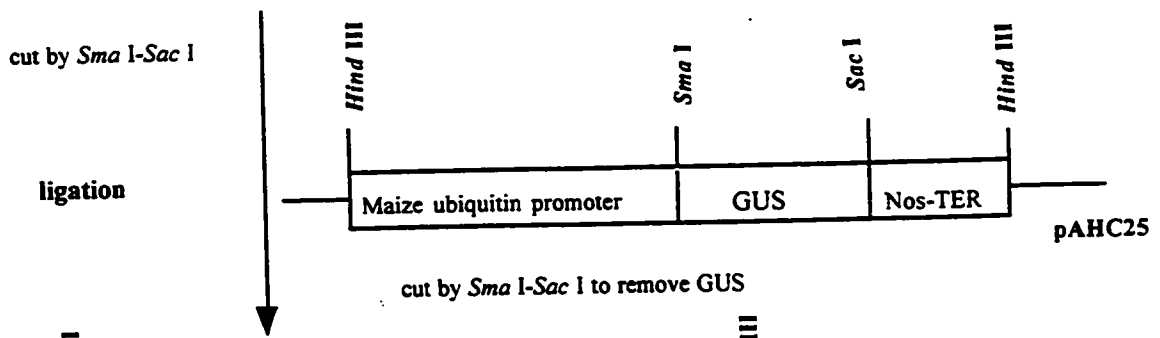
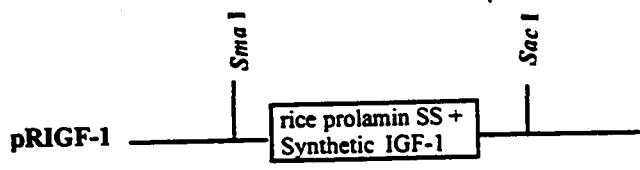
fragment was subcloned into the multiple cloning site of the pGEM4Z vector. The resulting plasmid pGIGF-1B was digested with *Nco* I and *Sac* I to release the IGF-1B fragment. The recovered fragment was ligated to the pAHC25 vector (digested with *Sac* I and *Sma* I to remove GUS and to insert IGF-1B fragment). The coding sequence was placed downstream of the maize ubiquitin promoter which is one of the strongest promoters in plants. The final construct containing maize ubiquitin promoter, IGF-1B, and Nos-TER (terminator sequence) was designated as pUIGF-1B (Fig. 11).

2.2.1.5. Plasmid containing plant codon-optimized IGF-1 (pUSYNTHETIC-IGF-1 construct)

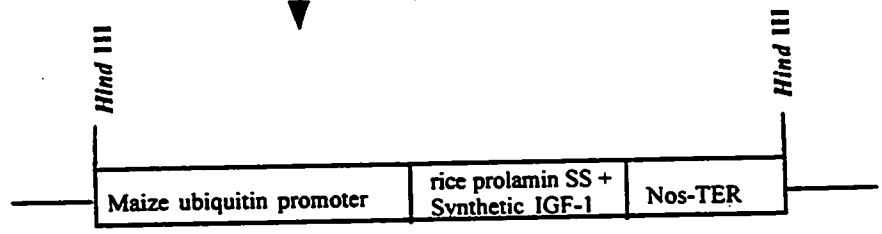
As mentioned before we made constructs based on the coding sequences which were obtained from Genentech and contained the partial *E. coli* optimized IGF-1 and IGF-1B coding sequences. These constructs were to test if they were functional in plant expression systems. To have a better expression level of IGF-1 in plant expression system, a construct containing a plant codon-optimized IGF-1 was made. To make this construct, the synthetic IGF-1 (plant codon-optimized IGF-1 coding sequence containing 70% GC along with rice prolamin signal sequence) was prepared by Dr. Xiongying Cheng in Dr. Altosaar lab. To reduce the potential of degradation of the recombinant proteins the synthetic IGF-1 was fused to the rice prolamin signal sequence which directs the recombinant protein to rice seed storage compartments in case of rice transformation. To make the construct containing synthetic IGF-1 along with rice prolamin signal sequence, 60 bp of rice prolamin signal sequence + IGF-1 coding sequence was ligated to the pAHC25 plasmid (digested with *Sac* I and *Sma* I to remove GUS and to insert the IGF-1 fragment). The construct containing the maize ubiquitin promoter, synthetic IGF-1 and Nos-TER was called pUSYNTHETIC-IGF-1 (Fig. 12).

These constructs were sequenced using the Cycle Sequencing PCR based method by the Cancer Research Institute (Ottawa). The DNA sequences of human IGF-1 and human IGF-1B genes with modifications made by Genentech and Dr. Cheng in our lab are shown in Figures 13-

Figure 12. Construction of the plasmid pUSYNTHETIC-IGF-1 (not to scale)
The plasmid pRIGF-1 was digested with *Sma* I-*Sac* I to release the rice prolamin signal sequence + synthetic human IGF-1. This fragment was inserted into the plasmid pAHC25 (digested with *Sma* I-*Sac* I) to be downstream of the maize ubiquitin promoter. The size of this fragment is 287 bp. The size of the maize ubiquitin promoter is 2000 bp and the Nos-TER sequence is 260 bp.



cut by *Sma* I-*Sac* I to remove GUS



pUSYNTHETIC-IGF-1

14. The Lam B bacterial signal sequence and rice prolamin signal sequence are presented in Figure 15. These constructs were used to transform *Agrobacterium tumefaciens* cells (summary of the constructs, Fig. 16).

2.2.1.6. Construction of binary vectors

Binary vectors are based on plasmids that can replicate in both *E. coli* and *Agrobacterium*. The binary vector pKHG4 was used as the plant transformation vector (Fig. 17). This binary vector contains a hygromycin phosphotransferase gene for resistance to hygromycin and a neomycin phosphotransferase gene for resistance to kanamycin. The pKHG4 binary vector also contains a GUS (beta-glucuronidase) gene as a reporter gene which is beneficial in detection of transgenic genomes in transgenic plants (section 2.2.6.1). The *E. coli* DH5 α strain was transformed with the binary vector pKHG4 (section 2.2.2.1). The DNA was extracted using QIAGEN miniprep kit (section 2.2.3) and was digested by *Hind* III to open the plasmid at its unique *Hind* III site. The digested plasmid was dephosphorylated using calf intestine alkaline phosphatase (CIAP). The dephosphorylation protocol supplied by Gibco BRL (Burlington, Ontario) was used. After the dephosphorylation, the DNA was collected for ligation to the expression cassettes (Fig. 8-12). The inserts were released by *Hind* III digestion and were recovered from 0.8% agarose gel by GeneClean kit from QIAGEN. The ligation reaction consisted of a 5:1 ratio of insert to vector. After an overnight incubation, 2 μ l of the ligation mixture was removed and used to transform competent *E. coli* cells (section 2.2.2.1). Colonies were screened on LB/kanamycin, hygromycin plates and transformed colonies were grown on LB (Luria-Bertani)/kanamycin, hygromycin medium (100 μ g kanamycin and 100 μ g hygromycin per medium). Colonies with the insert were identified by screening miniprep DNAs (1 μ g each) with *Hind* III digestion. The colonies containing the insert were used for *Agrobacterium* transformation (section 2.4.2).

Figure 13. The sequence of human IGF-1 (Rotwein *et al.*, 1986), *E. coli* codon-optimized IGF-1 and plant codon optimized IGF-1. I: IGF-1 amino acid residues; E: *E. coli* codon optimized IGF-1; Z: plant codon-optimized IGF-1 based on zeamatin sequence from *Zea mays*.

I H E Z
G P E T L C G A E L V D A L Q F V C G D
GGACCGGAGA CGCTCTGCGG GGCTGAGCTG GTGGATGCTC TTCAGTTCGT GTGTGGAGAC
T C A T G T A T C C C C G A C C C
C C C C C C C C C C C C C C C

I H E Z
R G F Y F N K P T G Y G S S S R R A P Q
AGGGCTTTT ATTTCAACAA GCCCACAGG TATGGCTCCA GCAGTCGGAG GCGCCCTCAG
C A C C A G T C A T C TC TC CC C C G
C C C C G G C C A G TCC CC C C G

I H E Z
T G I V D E C C F R S C D L R R L E M Y
ACAGGCATCG TGGATGAGTG CTGCTTCCGG AGCTGTGATC TAAGGAGGCT GGAGATGTAT
T T T T C A T T TCT C C C C C C C
C C C C A A C C C C C C C C C

I H E Z
C A P L K P A K S A
TGCGCACCCC TCAAGCCTGC CAAGTCAGCT
T G A G A G A G A G A G A G
C G G G G G G G G G G G G G G

Figure 14. The sequence of human IGF-1B (Rotwein *et al.*, 1986) and *E. coli* codon-optimized IGF-1B. I: IGF-1B amino acid residues; H: Human IGF-1B; E: *E. coli* codon optimized IGF-1B.

I H E
G P E T L C G A E L V D A L Q F V C G D
GGACCGGAGA CGCTCTGCGG GGCTGAGCTG GTGGATGCTC TTCAGTTCGT GTGTGGAGAC
T C A T G T A T C G A T T

I H E
R G F Y F N K P T G Y G S S S R R A P Q
AGGGCTTTT ATTCAACAA GCCCACAGGG TATGGCTCCA GCAGTCGGAG GCGCCCTCAG
C A C C A G T C A

I H E
T G I V D E C C F R S C D L R R L E M Y
ACAGGCATCG TGGATGAGTG CTGCTTCCGG AGCTGTGATC TAAGGAGGCT GGAGATGTAT

I H E
C A P L K P A K S A R S V R A Q R H T D
TGGCACCCC TCAAGCCTGC CAAGTCAGCT CGCTCTGTCC GTGCCAGCG CCACACCGAC

I H E
M P K T Q K Y Q P P S T N K N T K S Q R
ATGCCCAAGA CCCAGAAGTA TCAGCCCCCA TCACCAACA AGAACACGAA GTCTCAGAGA

I H E
R K G W P K T H P G G E Q K E G T E A S
AGGAAAGGTT GGCCAAAGAC ACATCCAGGA GGGAAACAGA AGGAGGGGAC AGAAGCAAGT

I H E
L Q I R G K K E Q R R E I G S R N A E
CTGCAGATCA GAGGAAAGAA GAAACAGCAG AGGAGGGAGA TTGGAAGTAG AAATGCTGAA

I H E
C R G K K G K
TGCAGAGGCA AAAAGGAAA A

Figure 15. (A). The sequence of bacterial Lam B signal sequence which was used in constructs containing *E. coli* codon-optimized IGF-1 and IGF-1B. (B). The sequence of rice prolamin signal sequence which was used in construct containing rice prolamin signal sequence + synthetic IGF-1.

A

M M I T L R K L P L A V A V
ATGATGATTA CTCTGCGCAA ACTTCCTCTG GCGGTTGCCG

A A G V M S A Q A M A
TCGCAGCGGG CGTAATGTCT GCTCAGGCCA TGGCC

B

M K I I F V F A L L A I V A
ATGAAGATCA TTTTCGTATT TGCTCTCCTT GCTATTGTTG

C N R S A R F D
CATGCAATCG CTCTGCGCGC GGTTTG

Figure 16. Maps of chimeric gene constructs (not to scale). The gene constructs containing the mature IGF-1 sequence under the control of maize ubiquitin promoter are shown here. The construct No. 1 contained the Lam B signal sequence (75 nucleotides encoding 25 amino acids) fused in-frame with the mature IGF-1 . Construct No. 3 contains rice prolamin signal sequence (66 nucleotides encoding 22 amino acids) fused in frame with the mature IGF-1 sequence. The size of constructs is 2604, 2528, 2547 bp respectively. The gene constructs containing the precursor IGF-1B sequence under the control of maize ubiquitin promoter also are shown. The size of constructs is 2802, 2726 bp respectively for constructs No. 4 and No. 5. The *Hind* III fragments from these constructs were individually cloned into a binary *Agrobacterium* vector pKHG4.

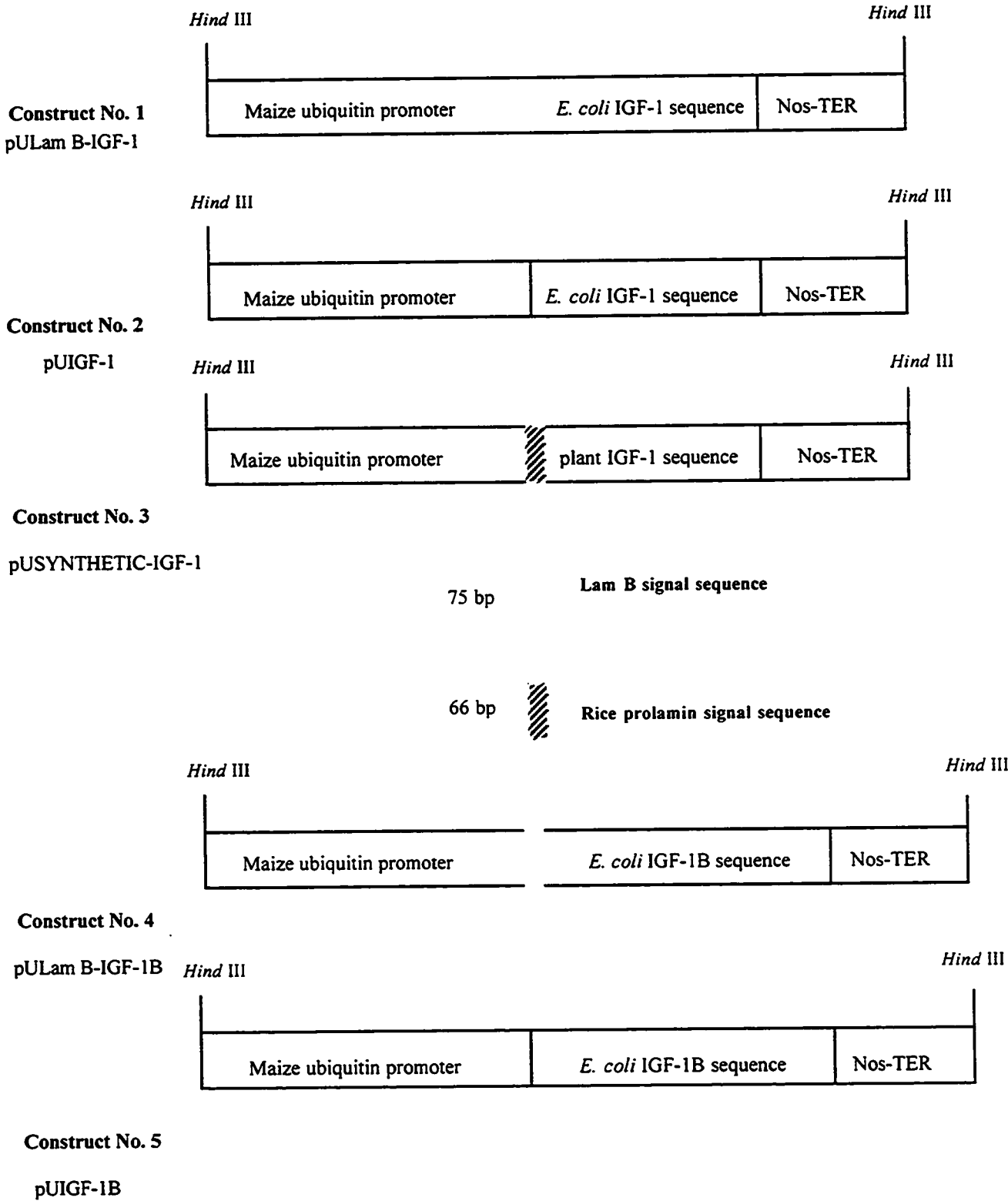
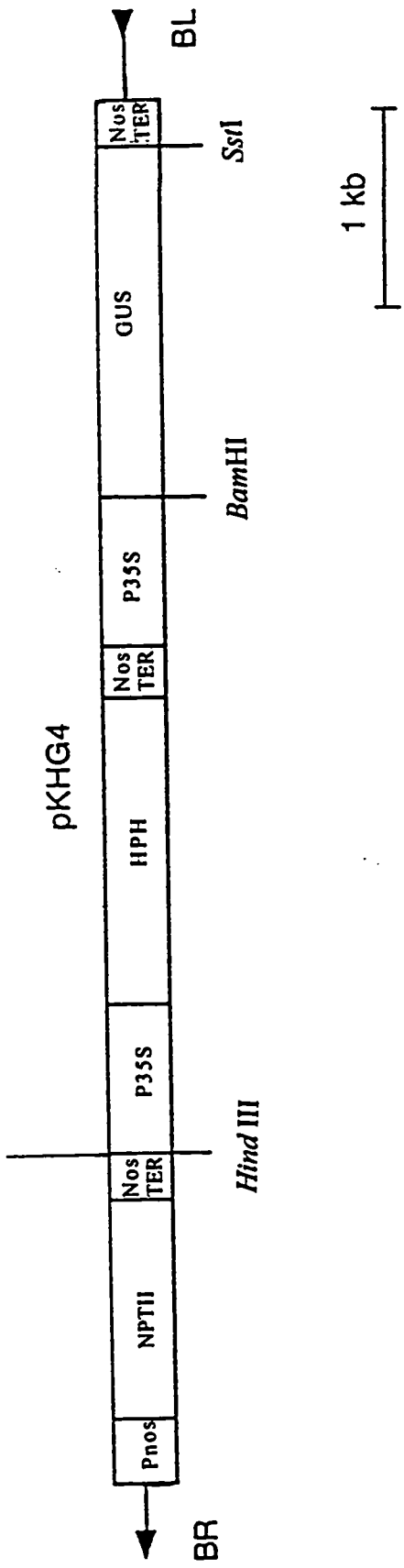


Figure 17. The map of binary vector pKHG4. BR, right border; BL, left border; HPH, hygromycin phosphotransferase; NPT II, neomycin phosphotransferase; P35S, CaMV 35S promoter; Pnos, nopaline synthase promoter; NT, 3' termination signal of nopaline synthase. The expression cassettes were introduced into the unique Hind III site.



2.2.2. Bacterial strains, growth conditions and transformation procedures

2.2.2.1. *Escherichia coli* (*E. coli*) transformation

Transformation of bacteria was performed using competent *E. coli* DH5 α cells using the supplier's protocol (Gibco BRL). The plasmids (the expression cassettes in the binary vector of pKHG4) (section 2.2.1.6) were mixed on ice with an aliquot of competent cells (50 μ l of competent cells). After 20 min a heat shock treatment was performed by incubation of the mixture at 42°C for 90 sec and then placing them on ice for 5 min. One ml of LB medium was added and the mixture was inverted several times and left at 37°C for 30-60 min. After incubation, the mixture was centrifuged (Micro-Centrifuge Model 235B, Fisher Scientific) at 10,000 rpm for 1 min. The pelleted cells were resuspended in approximately 100 μ l of LB medium and 50 μ l was spread on LB/agar plates containing 100 μ g/ml kanamycin and 100 μ g/ml hygromycin. The plates were incubated at 37°C overnight and antibiotic resistant colonies were analyzed by restriction analysis for the presence of the expression construct.

2.2.2.2. *Agrobacterium* transformation

Competent *Agrobacterium tumefaciens* cells LBA 4404 were prepared as described below for plasmid uptake followed by plant transformation. About 50 μ l of *A. tumefaciens* cells were put on YEP/agar (yeast extract, pepton and NaCl) plates without antibiotics. Plates were incubated at 28°C for 2 days to allow for colonies to appear. A single colony was grown overnight in 5 ml of YEP medium. The overnight culture was transferred to 50 ml of YEP liquid medium. After four hours of growth the culture was chilled, transferred to sterile centrifuge tubes and centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was discarded and one ml of 20 mM CaCl₂ was added. Aliquots of 100 μ l were dispensed in prechilled 1.5 ml sterile Eppendorf tubes. These competent cells were stored at -70°C.

The transformation of *A. tumefaciens* cells was performed using a protocol from CIONTECH. To an aliquot of 100 μ l of competent *A. tumefaciens* cells, one μ g of the plasmid

pKHG4 containing IGF-1 or IGF-1B fragments was added (section 2.2.2.1.6). After incubating on ice for 30 min and in liquid nitrogen for 5 min the cells were thawed at 42°C. Again the cells were put on ice for 5 min. The YEP or LB medium (500 μ l) was added and the mixture was incubated at 28°C for 2 hrs. The cells were pelleted and resuspended in 100 μ l of fresh YEP or LB medium. Then the cells were spread on YEP/agar or LB/agar plates containing 100 μ g/ml kanamycin and 100 μ g/ml hygromycin. These plates were incubated at 28°C for 2 days for antibiotic resistant colonies to appear. The colonies were put in 3 ml of YEP medium containing 100 μ g/ml of kanamycin and 100 μ g/ml of hygromycin and incubated at 28°C overnight with shaking. The culture was maintained in the form of a glycerol stock (700 μ l bacterial culture + 300 μ l sterile glycerol) and kept at -70°C. This stock culture was used to transform tobacco and rice.

2.2.3. Plasmid DNA miniprep (minipreps)

Miniprep kits were purchased from QIAGEN. The transformed bacterial DH5 α cells carrying the plasmid of interest were grown in 3.0 ml of LB or YEP medium containing the proper antibiotics (kanamycin 100 μ g/ml and hygromycin 100 μ g/ml) at 37°C overnight. Cells were centrifuged for 1 min to obtain the bacterial pellet. The supernatant was discarded and the pellet was resuspended in 250 μ l of buffer P1 (QIAGEN). Then 250 μ l lysis buffer (P2, QIAGEN) was added and the reaction was allowed to proceed for 5 min before being neutralized with 350 μ l of buffer N3 (QIAGEN). The mixture was cloudy and it was centrifuged for 10 min at 4°C. The supernatant was applied to a QIAprep spin column and centrifuged for 30 sec. The flow-through was discarded and the DNA collected on the column was washed with 750 μ l of PE wash buffer (QIAGEN) followed by 30 sec centrifugation. The flow-through was discarded and the spin column was centrifuged for 1 min to remove residual ethanol from the PE wash buffer. The DNA was eluted with 50 μ l of TE (Tris-EDTA) buffer.

2.2.4. Tobacco transformation (Horsch *et al.*, 1985)

Tobacco leaves (*Nicotiana tabacum* cv. Xanthi) were put in sterile petri dishes and sterilized for 30 sec in 70% ethanol followed by a 10 min soak in 1% Javex bleach (6% sodium hypochlorite). The leaves were washed several times with sterile dH₂O were cut into small disks. These leaf disks were put into MS (Murashige and Skoog, 1962) medium in petri dishes. About 10 leaf disks were put in each petri dish for one transformation.

Single transformed *A. tumefaciens* colony carrying the expression construct was used to inoculate a 5-ml YEP medium (50 mg/L kanamycin and 50 mg/L hygromycin) in a sterile 50 ml polypropylene centrifuge tube (CanLab, Mississauga, ON). After shaking overnight at 28°C using an orbital shaker with environmental control at a shake rate of about 200 rpm, the overnight culture was poured into 50 ml YEP medium containing the same selective antibiotics in a sterile 500 ml flask while shaking overnight under the same condition as the small culture above. The bacteria were collected by centrifugation in a 50 ml sterile polypropylene centrifuge tube in a Beckman TJ-6 centrifuge at 3000 rpm for 10 min at 4°C. The supernatant was poured off and the bacteria was resuspended in 10 ml of YEP medium without antibiotics. About 1 ml of this culture was poured into a sterile petri dish containing the tobacco leaf disks. About 10 leaf disks were placed into each sterile petri dish containing the bacterial culture for 10 min at RT (room temperature). The excess medium was removed by blotting the leaf disks onto sterile filter papers. These leaf disks were transferred to MS solid medium (appendix) plates. The plates were incubated in the dark at 20°C for 2 days. The co-cultivated leaf disks were transferred to selection and regeneration medium (appendix) at 20°C with 16 hours light and 8 hours dark. In this medium ticarcillin was used to kill off *A. tumefaciens* and hygromycin was used to select the transformed leaf disks. After one month calli developed and some shoots appeared (Fig. 18). The shoots were cut and transferred into a rooting medium in Phytakon™ vessels. After one month, plantlets developed roots and were placed into plant pots containing soil and placed in a greenhouse with 16 hours light at 26°C and 8 hours dark at 20°C. Plants were watered three

Figure 18. Regeneration and propagation of transformed tobacco plants. Leaf sections are on a regeneration medium containing 300 $\mu\text{g/ml}$ hygromycin and 500 $\mu\text{g/ml}$ ticarcillin. The leaf sections which are not transformed, are necrotized. Proliferation of resistant cells into calli and morphogenesis into plantlets is observed on transformed leaf sections.



times per week and fertilized with a water-soluble fertilizer (20:20:20) (Plant Products Co. Ltd. Brampton, ON) every two weeks. Plants were also sprayed with an insecticide (Safer Ltd. Scarborough, ON) two times per week. Plants grew normally (compared to controls) and flowered within nine months.

2.2.5. Rice transformation (Cheng *et al.*, 1997b)

2.2.5.1. Callus induction

In rice transformation usually embryogenic calli are induced from mature rice seed scutellum and are used as the target tissue for the gene transfer. Approximately, 100 dehusked mature rice seeds (93VA) were placed in 20 ml of 50% Javex solution with a drop of Tween 20 in a 100 ml sterile glass beaker covered with aluminum foil. The beaker was shaken on a shaker operated at 40 rpm for 30 min. Then, the seeds were rinsed in distilled water several times in a sterile hood. Sterilized seeds were put on the surface of MS Callus Induction Medium (appendix) in 9-cm petri dishes. The plates were wrapped with Parafilm and placed into an unlighted growth chamber at 26°C. Calli became visible in a week and were ready for transformation in three to four weeks after culture.

2.2.5.2. *Agrobacterium* Co-cultivation

Binary vectors (pKHG4) containing the expression cassettes (Fig. 8-12) were introduced into *A. tumefaciens* (LBA 4404) as mentioned before (section 2.2.2.2). The transformed *A. tumefaciens* cells with expression cassettes were plated on YEP medium with 50 mg/L kanamycin and 50 mg/L hygromycin and grown at 28°C for 48 to 72 hours to allow colonies to appear. A single colony from the plate was transferred to 5 ml AB medium (appendix), containing the same selective antibiotics under sterile conditions. The medium was shaken overnight at 28°C using an orbital shaker with environmental control at a rate of about 200 rpm. The overnight culture was poured into 50 ml AB medium containing the same selective

antibiotics and 100 μ M acetosyringone in a 500 ml flask and grown overnight under the same condition as the small culture above. The bacteria were collected by centrifugation in a 50 ml sterile polypropylene tube in a Beckman TJ-6 centrifuge at 3000 rpm for 10 min. The pelleted bacteria were washed once with MS Callus Induction Medium (appendix) containing 100 μ M acetosyringone and centrifuged again at 3000 rpm for 10 min. The pelleted bacteria were diluted with the same medium to a density of about 10^8 cells per ml. Three ml of diluted bacteria were poured into 6-cm sterile petri dishes for the callus immersion.

The embryogenic calli were selected from 3 to 4 week old callus culture and 200 to 400 mg of vigorously growing calli pieces (2-4 mm in diameter) were placed into the bacterial suspension and were immersed for 30 min with occasional shaking in a sterile laminar hood. The excess suspension from the calli was removed by placing them on a pad of dry sterile tissue paper. The inoculated calli were transferred to 2N6-AS (N6 vitamins-acetosyringone) medium in 9-cm petri dishes (50-100 mg/dish) and the plates were incubated at 28°C in the dark for two days. The plates were checked regularly for bacterial overgrowth.

2.2.5.3. Selection and regeneration of transformed rice calli

After the co-cultivation, the bacterial growth must be inhibited to allow for the preferential growth of the transformed plant cells and their subsequent selection. This was achieved by including in the culture media two types of antibiotics. Ticarcillin disodium was used for inhibiting the growth of bacteria and hygromycin was used for inhibiting the growth of untransformed plant cells, thus allowing transformed cells to grow into colonies. The selected cells then regenerated into plants in a regenerating medium (LHT medium, appendix). The 9-cm petri dishes of LHT containing 50-100 mg transformed calli were sealed by Parafilm and incubated at 26°C in the dark. The culture medium was renewed every two to three weeks. The visual hygromycin-resistant microcalli were usually visible one month after culture on the LHT medium. The calli that grew well were transferred into selection and regeneration medium

(LRHT, appendix) in 9- cm petri dishes. These plates were placed at 25°C under a photoperiod of 16 h light/8 h dark in a plant growth chamber. When the shoots from transformed calli were visible (Fig. 19), they were excised by surgical blade, and placed into the LRHT medium in the Phytakon™ vessels. These conditions were continued until the plantlets reached the top of the containers. The well developed plantlets (about 10 cm in height from the medium) were put in soil and were transferred to greenhouse. The analysis of rice plants was performed in the leaves of transgenic rice plants in a same way which was performed for transgenic tobacco leaves.

2.2.6. Characterization of transgenic genomes

2.2.6.1. Assay for GUS activity in transgenic plants (Jefferson, 1987)

The binary vector pKHG4 which was used for plant transformation contained a GUS (beta-glucuronidase) gene (Fig. 17). This experiment was performed as a preliminary tool to detect transgenic plants from non-transgenic ones. Small pieces of fresh young tobacco leaves and rice calli were incubated in phosphate buffer (50 mM NaPO₄ pH 7.0) containing 0.5 mg/ml of X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) which is the substrate of GUS. The incubation was carried out at 37°C from one hour to overnight. If a blue color was observed visually by eye, this was used as an indication of presence of T-DNA insert.

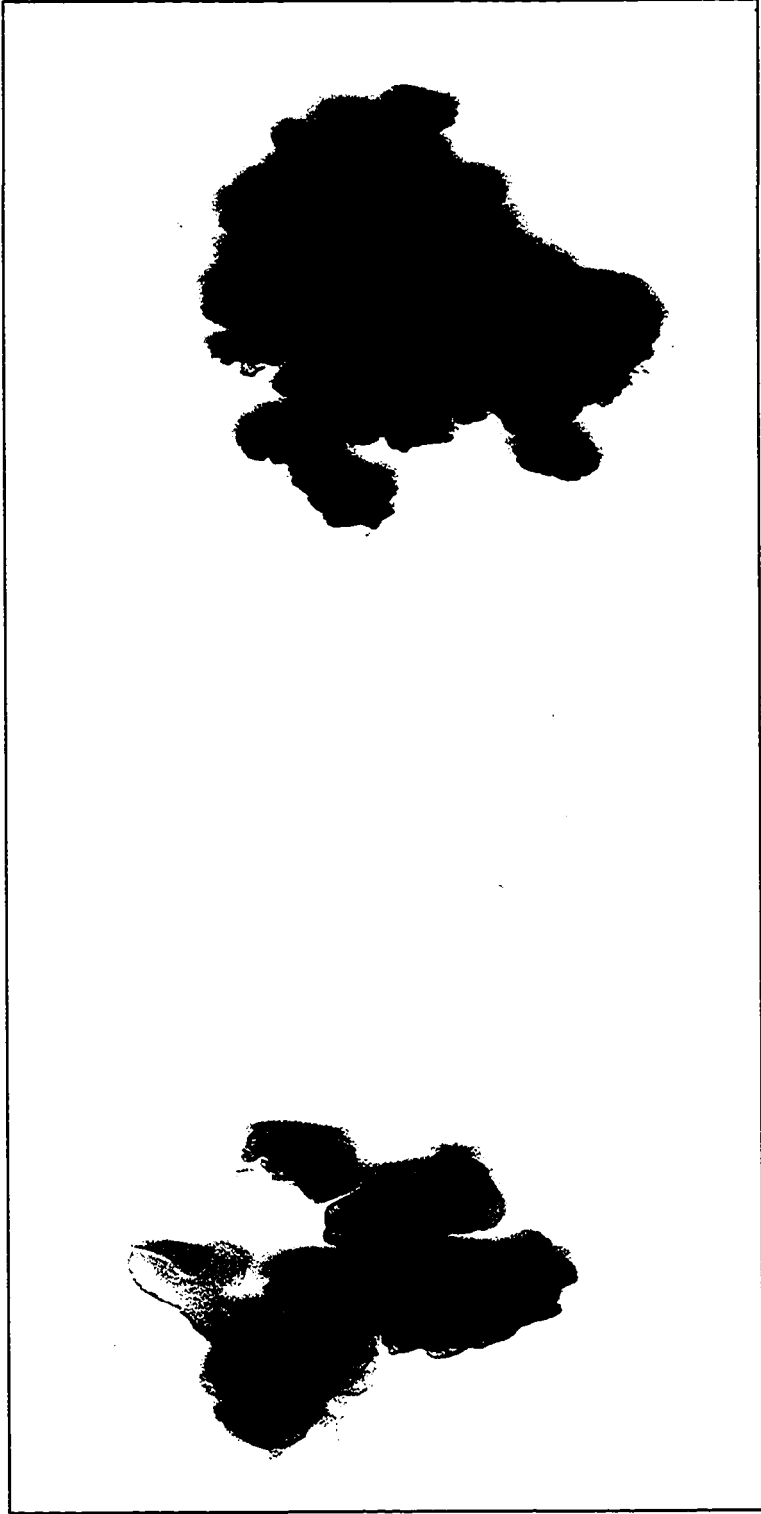
2.2.6.2. DNA extraction from tobacco leaves

Young leaves were used for DNA extraction. About three grams of leaves were placed into a sterile mortar that contained liquid nitrogen. The leaves were grounded to a fine powder using a pestle. The frozen powder was put in a 50 ml polypropylene centrifuge tube and 5 ml of DNA extraction buffer (appendix) was added. The centrifuge tube was inverted slowly several times and then incubated on ice for 15 min. The mixture was centrifuged (3000 rpm) at 4°C for 20 min. The supernatant was discarded and 2 ml of DNA lysis buffer was added to the pellet. The contents were mixed by gently vortexing. The suspension was incubated at 65°C for 30 min.

Figure 19. Continuous selection for transformed rice calli. Rice calli are on a selection and regeneration medium containing 50 mg/L hygromycin and 100 mg/L ticarcillin. A: The white, dry and rigid calli are transformed calli which are detectable from non-transformed calli (necrotized). B: The shoots from tranformed calli on the same medium as A.



A



B

After incubation, 2 ml of chloroform/isoamyl alcohol (24:1) was added and mixed thoroughly by inverting. The mixture was centrifuged (3000 rpm) at 4°C for 10 min. The top (aqueous) phase was carefully removed and placed into a fresh 50 ml polypropylene centrifuge tube. The chloroform/isoamyl alcohol extraction was repeated two more times. To the top phase 3 ml of iso-propanol were added and mixed by gentle inversion until a white-colored fiber (DNA precipitate) was formed. The mixture was centrifuged (3000 rpm) at 4°C for 5 min to precipitate DNA. The supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol by centrifugation (3000 rpm) at 4°C for 5 min. The ethanol was removed and again the centrifugation was performed to remove all traces of ethanol. The pellet was allowed to air dry and was resuspended in 200 μ l TE (Tris-EDTA) buffer. RNAase A (100 μ g) was added to the resuspended DNA followed by incubation at 37°C for two hours. The DNA concentration was measured at 260 nm using a spectrophotometer. The integrity of the DNA was verified on a 1% agarose gel.

2.2.6.3. Restriction analysis of total genomic DNA

Restriction analysis was performed by digestion of the genomic DNA with *Hind* III (NEB). Forty micrograms of genomic DNA was digested by 50 units of enzyme. The mixture was briefly centrifuged and incubated overnight at 37°C. The digested DNA was run on a 1% gel to confirm complete digestion and also the size of the released expression construct.

2.2.6.4. Agarose gels for DNA electrophoresis

Agarose gels for DNA separations were run in TAE (Tris-acetic acid-EDTA) buffer. The agarose (the concentration of agarose varied from 0.8%-1.2% depending on the size of the DNA to be separated) was dissolved in TAE buffer and 5 μ l/100 ml of 5 mg/ml ethidium bromide was added before casting the gel. Upon solidifying, the gel was run in 1 X TAE buffer.

2.2.6.5. Southern analysis of total genomic DNA

After overnight restriction digestion of forty micrograms of genomic DNA with *Hind* III, 6 X loading buffer (appendix) was added and after a brief centrifugation, the samples were loaded into a 0.8% agarose gel. Lambda marker (*Hind* III digested, Gibco BRL) and Phi X marker (*Hae* III digested, Gibco BRL) were used as markers along with the samples. After running the gel overnight, the gel was then UV illuminated and photographed using a Polaroid camera (Polaroid MP.4 Land Camera). A transparent ruler was placed along the marker lane to measure the distance of migration for the standards. The gel was placed in a DNA denaturing buffer (appendix) for 45 min. The gel was rinsed in distilled water and transferred to neutralization buffer (appendix) for 45 min. During this step, the transfer apparatus was set-up (Sambrook *et al.*, 1989). The DNA was transferred onto a nitrocellulose membrane (High Bond N, Amersham, Baie d'Urfe, Quebec). The transfer was allowed to proceed for 18-24 hours. The gel was photographed to confirm that complete transferring of DNA had occurred. After the transfer time was complete, the membrane was removed and soaked in 2 X SSC to remove any agarose residues. The membrane was placed on a paper towel to dry at least for 30 min at RT. Then the DNA was fixed to the membrane by baking the membrane for two hours at 80°C under the vacuum.

2.2.6.5.1. Radioactive DNA probes (random primer labeling)

The *Xba* I -*Hpa* I fragment from pBKIGF-2B (Genentech) was used as a probe to identify Lam B-IGF-1 transgenic plants (Fig. 8). The *Nco* I-*Hpa* I fragment from pGIGF-1 was used as a probe for identifying IGF-1 transgenic plants (Fig. 9). The *Xba* I-*Sac* I fragment from pGIGF-1B was used as a probe to identify Lam B-IGF-1B transgenic plants (Fig. 10). The *Nco* I-*Sac* I fragment from pGIGF-1B was used as the probe for identifying IGF-1B transgenic plants (Fig. 11). The PCR (polymerase chain reaction) amplified fragment was prepared to identify rice prolamin + synthetic IGF-1 transgenic plants. These fragments were excised for genecloning

after running on agarose gel. The QIAEX II gene-cleaning kit from QIAGEN was used. The concentration of the recovered fragments was estimated on a 1.2% agarose gel and used for the labeling reaction. The labeling reaction was performed by using the Ready To Go DNA Labeling Beads (-dCTP) kit from Pharmacia (Baie d'Urfe, Quebec). Fifty nanogram (45 μ l) of DNA was denatured by boiling for 3 min at 100°C then immediately placed on ice for 2 min. After a brief pulse centrifugation, 50 μ Ci of [³²P]dCTP was added and again a brief centrifugation was applied. This mixture was added to the supplied reaction mixture containing buffer, dATP, dGTP, dTTP, Klenow Fragment (7-12 units), and random oligodeoxyribonucleotide (mainly 9-mers) followed by vortexing and briefly pulsed centrifugation. The mixture was incubated at 37°C for 15 min. The unincorporated nucleotides were separated from the labeled DNA fragments using a MicroSpin S-300 HR column (Pharmacia).

2.2.6.5.2. Prehybridization and hybridization for Southern blots

The nitrocellulose membrane (baked) was placed in a tray containing 50 ml of prehybridization buffer (appendix) at 42°C under constant gentle shaking. After two hours, the buffer was replaced with fresh hybridization buffer (appendix). The purified radioactive probe was boiled for 3 min and rapidly cooled on ice for 5 min and then was added to the hybridization buffer. The hybridization was allowed to proceed for 16 hours at 42°C with gently shaking. The membrane was washed one time in 2 X SSC and 0.1% SDS (sodium dodecyl sulphate) solution at RT for 5-15 min, two with 1 X SSC and 0.1% SDS solution at 65°C for 15 min, one time with 0.4% X SSC and 0.1% SDS solution at 65°C for 15 min. Then the membrane was placed on a piece of filter paper to air dry and then was placed in a cassette on an intensifying screen and an X-ray film placed on top of the membrane. The film was developed after four hours to determine the exposure time need for the appearance of bands. Then the film was placed at -70°C until needed to be developed.

2.2.6.6. Polymerase chain reaction (PCR)

For PCR, 10 mg of young leaves were grounded and the DNA was extracted as described (section 2.2.6.2). The PCR reaction mixture consisted of 20 ng of plant genomic DNA, and amplifications were performed using IGF-1 and IGF-1B specific primers using the Ready To Go kit from Pharmacia Biotech in a Mastercycler gradient Eppendorf PCR machine. The PCR reactions were subjected to one cycle of 93°C for 3 min, 65°C for 45 sec, 72°C for 90 sec followed by 40 cycles of 94°C for 45 sec, 65°C for 45 sec, 72°C for 90 sec. Finally, an extension step at 72°C for 3 min was performed. Then 10 μ l aliquots were analyzed on 1% agarose gels. The oligonucleotide primers were synthesized by Dr. G. Alvarado (Synthaid Biotechnologies Inc.) as following:

Construct No. 1: 5' ATGATGATTACTCTGCGCAA3' forward primer
5'AGCAGATTTAGCGGGTTTCAG3' reverse primer
(Maize ubiquitin promoter + Lam B-IGF-1+ Nos-TER)

Construct No. 2: 5'GGTCCCGAAACTCTGTGC3' forward primer
5'AGCAGATTTAGCGGGTTTCAG3' reverse primer
(Maize ubiquitin promoter + IGF-1 + Nos-TER)

Construct No. 3: 5'ATGAAGATCATTTTCGTA3' forward primer
5'CGCGCTCTTGCCGGCTTGAG3' reverse primer
(Maize ubiquitin promoter + rice prolamin + synthetic IGF-1 + Nos-TER)

Construct No. 4: 5'GGTCCCGAAACTCTGTGC3' forward primer
5'TTTCCTTTTTGCCTCTGC3' reverse primer
(Maize ubiquitin promoter + Lam B-IGF-1B+ Nos-TER)

Construct No. 5: 5'GGTCCCGAAACTCTGTGC3' forward primer
 5'TTCCTTTTTTGCCTCTGC3' reverse primer

(Maize ubiquitin promoter + IGF-1B + Nos-TER)

2.2.7. Gene transcription

2.2.7.1. Extraction of total RNA from tobacco leaves

A maximum of 100 mg of young developing fresh leaves were collected and grounded to a fine powder using a pestle. The total RNA was extracted from the leaves using a RNeasy Mini kit from QIAGEN. Briefly, 450 μ l of buffer RLT (lysis buffer which contained guanidinium isothiocyanate) was added to leaf powder to disrupt the cells. The mixture was vortexed vigorously and was placed into spin column followed by centrifugation for two min at maximum speed. The flow-through was transferred to a new tube. In this way all the cell debris were removed and the lysate was homogenized. Then, 0.5 volumes ethanol (96%-100%) was added to the lysate and mixed well by pipetting. Ethanol provided appropriate binding conditions and then the sample was applied to an RNeasy mini spin column where the total RNA was bound to the membrane after centrifugation for 15 sec at 10,000 rpm. The RNA was treated with DNase I while bound to the silica-gel membrane by adding 10 μ l of DNase I in 70 μ l of a buffer from the kit followed by incubation at RT for 15 min. The column was washed by buffer RW1 (RNeasy Mini kit) and RNA was eluted in water (30-50 μ l RNase-free water). The RNA concentration and integrity was estimated on 1.0% RNA gel.

2.2.7.2. Preparation of RNA gels

RNA gel preparation and running conditions was based on the procedure of Fournery *et al.* (1992). Gels, buffers and samples were prepared in DEPC-treated (diethylpyrocarbonate) water. The gel, tray, gel comb, and running buffer tray were washed with distilled water and then presoaked overnight in a DEPC solution containing 1% SDS. The gel was made of 1.0% agarose

containing 1.9% formaldehyde). The 1 X running buffer (appendix) was prepared from a 10 X stock buffer. To 5 μ l of RNA 25 μ l of RNA loading buffer was added. Then RNA sample was heated at 65°C for 15 min. After cooling to 50°C, 1 μ l of ethidium bromide in DEPC-treated water (1 μ g/ μ l) was added and the sample was loaded onto the gel. The gel was run at 30V at RT. The bands were visualized and photographed under UV illumination.

2.2.7.3. Reverse-transcriptase PCR (RT-PCR)

To check if the transgene was expressed, the presence of IGF-1 and IGF-1B mRNA was verified by reverse transcription (RT) PCR amplification. The cDNA was synthesized using the Advantage™ RT-for-PCR kit from CLONTECH. Briefly, to 1 μ g (12.5 μ l) of RNA sample, 1.0 μ l of Oligo (dT)₁₈ primer was added. The RNA was heated at 70°C for two min, then was placed on ice and 6.5 μ l of master mix (5 X reaction buffer, dNTP mix, RNase inhibitor, MMLV: Moloney-Murine Leukemia Virus reverse transcriptase) were added. The mixture was heated at 94°C for 5 min followed by brief centrifugation. Then it was diluted to a final volume of 100 μ l (five fold) by DEPC-treated water. The cDNA was stored at -70°C. cDNAs were amplified as explained (2.2.6.6).

2.2.8. Detection of transgene protein product

2.2.8.1. Total protein extractions

Young leaves were used for total protein extraction. Approximately 500 mg of tobacco leaves were collected and placed in prechilled sterilized mortar. The leaves were grounded by the sterile pestle to a fine powder. The ground leaves were placed into Eppendorf tubes. Protein extraction buffer was immediately added in a one to one (w/v) ratio. The sample mixture was vortexed and then centrifuged for 10 min at 4°C (10,000 rpm). The supernatant was transferred to a new tube and centrifugation was repeated until clear supernatant was obtained. Total protein was determined with the Bio-Rad Protein Assay based on Bradford's method (1979). Bovine

serum albumin was used as a protein standard.

2.2.8.2. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay was performed by using Active™ IGF-1 ELISA kit from Diagnostic Systems Laboratories, Inc. (Montreal, QC). Briefly, the assay is an enzymatically amplified one-step sandwich-type immunoassay. In the assay, 20 μ l of standards, controls and extracted unknowns were incubated with anti-IGF-1 antibody labeled with the enzyme horseradish peroxidase (HRP) in microtitration wells coated with another anti-IGF-1 antibody. After incubation and washing, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by wavelength absorbance measurement at 450 nm. The absorbance measured was directly proportional to the concentration of IGF-1 present. A set of IGF-1 standards was used to plot a standard curve of absorbance versus IGF-1 concentration from which the IGF-1 concentrations in the unknowns could be calculated. Due to the cost of the ELISA kits only two measurements were performed for each plant extract.

2.2.8.3. Immunoprecipitation

Immunoprecipitation was conducted on total protein extracts from IGF-1B plants. To 10 μ l of total protein extract, 10 μ g of primary antibody was added and incubated at 4°C for 1 hour with end over end rotation. Then, 200 μ l of protein A agarose (Santa Cruz Biotechnology, Inc.) were added and incubated with mixing overnight at 4°C. The mixture was centrifuged and the pellet was washed four times with PBS (Phosphate-buffered saline). After the final wash, the protein concentration was measured by Bio-Rad Protein Assay. Then, 1 mg of total protein was loaded in the wells of a thick (1.5 mm) 20% SDS-PAGE gel. Western blotting continued as described in the following section.

2.2.8.4. Western blots

2.2.8.4.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Sambrook *et al.* (1989) was used for SDS-PAGE. The separating gel was a 20% Tris-glycine SDS. The stacking gel was a 5% Tris-glycine SDS-PAGE gel. For loading the samples, ammonium sulfate salt was added to the total extracted protein of each sample until the salt saturated the solution (35% of ammonium sulfate for IGF-1 and 50% for IGF-1B). The samples were incubated on ice for 30 min and centrifuged for 30 min at 10,000 rpm. The supernatant was discarded and each pellet was dissolved in one ml of PBS (Phosphate-buffered saline) pH 7.2. To remove the salt, each sample was dialyzed for 48 hours in 1 L PBS pH 7.2 at 4°C. The concentration of the protein samples was measured with the BIO-RAD Protein Assay and 1 mg of total protein was used for each well. SDS gel-loading buffer was added to each protein sample (1/4 of total protein volume) and then the samples were heated for 2 min at 100°C. The BIO-RAD electrophoresis apparatus model 422 ELECTRO-ELUTER was used to run the gel. The gel was run at 120 V until the dye passed the stacking gel and then was run at 180 V until the dye reached the bottom of the separating gel.

2.2.8.4.2. Protein transfer

The gel was transferred for 1 hour using the BIO-RAD SD, SEMI-DRY ELECTRO CELL. The nitrocellulose membrane 0.2 micron (BIO-RAD) was soaked in H₂O and then in transfer buffer for a few minutes. The nitrocellulose membrane was placed on the top of two Whatman papers presoaked in transfer buffer and the gel was put in direct contact of the membrane followed by two Whatman papers presoaked in transfer buffer. In this way the gel was on top of the assembly. Ten ml of transfer buffer was poured on the top and air bubbles were removed by rolling a glass pipette. The transfer was allowed to proceed at 10 V (350 milli amper) for 1 hour.

2.2.8.4.3. Probing and detection

The nitrocellulose membrane was put in blocking buffer for 1.5 hour. The gel was stained overnight with Coomassie Brilliant Blue R 250 (0.25%) solution (appendix). The gel was destained with the destaining solution. The prestained molecular weight markers from BIO-RAD were used to assess the efficiency of the transfer. Usually two identical gels were run and one was used for transferring and other was used for staining with Coomassie solution.

After incubating in blocking buffer, the nitrocellulose membrane was transferred to the primary antibody solution which contained 1/1000 dilution of polyclonal IGF-1 specific antibody (Genentech, South San Francisco) in blocking buffer for overnight at 4°C. Then the membrane was washed with wash buffer two times at RT with gentle rocking each time for 20 min. The membrane was then placed in secondary antibody solution containing 1/2000 in (blocking buffer) anti-rabbit antibody (alkaline phosphatase conjugated) for 1 hour at RT with gentle shaking. Two washes with wash buffer (appendix) as above were conducted.

The membrane was incubated in 10 ml of AP buffer (alkaline phosphatase detection buffer), 44 μ l of NBT (nitroblue tetrazolium chloride) and 33 μ l of BCIP (5-bromo-4-chloro-3-indolyphosphate). Once the bands developed, the membrane was washed with water and allowed to air dry.

2.2.9. Biological assay

To determine if the recombinant human IGF-1 produced in the transgenic plants was biologically active. The human cell line SH-SY5Y neuroblastoma cells was used (Bruke *et al.*, 1993; Zumkeller *et al.*, 1999; Zeidman *et al.*, 1999). These cells were transferred in triplicate into culture plates (6 wells) and incubated in the presence or absence of commercially available IGF-1 or aliquots of tobacco leaf extracts. The concentration of IGF-1 in different leaf extracts was determined and aliquots containing equal amounts of leaf-derived IGF-1 were used.

The SH-SY5Y cells obtained from Dr. Phipps' lab (National Research Council)) were

grown as suspension cultures in RPMI 1640 medium with fetal bovine serum (10%). The cells (1×10^4) were transferred in the wells of a 6-well culture plate. An aliquot of 0.5 ml RPMI medium 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 tobacco leaf extract containing 10 ng/ml of IGF-1, leaf extract from a non-transformed (NT) plant, leaf extract from NT plant + commercial IGF-1 (*E. coli*-derived), protein extraction buffer. The experiments were performed in triplicate and under sterile conditions. The cell growth was monitored at 24, 48, and 72 hours. The cells (live and clear cells) were counted using haemocytometry/trypan blue exclusion. The averages of two independent experiments were used in Microsoft Excel to generate the biological assay graphs. The standard deviations were also calculated. The same condition was used for IGF-1B plants.

2.2.10. Seed germination

Mature seeds from transgenic and non-transgenic tobacco plants were collected. The seeds were sterilized in 50% bleach for 30 min. Then they were rinsed several times with sterile distilled water. The sterilized seeds were put on half-MS medium containing hygromycin (100 mg/L). The seeds started to germinate after one week under light. The germinated seeds were transferred to rooting media and the plantlets were transferred to the soil and the second generation of transgenic plants (R_1) was obtained.

Chapter 3

Results

3.1. Expression of IGF-1 in transgenic tobacco plants

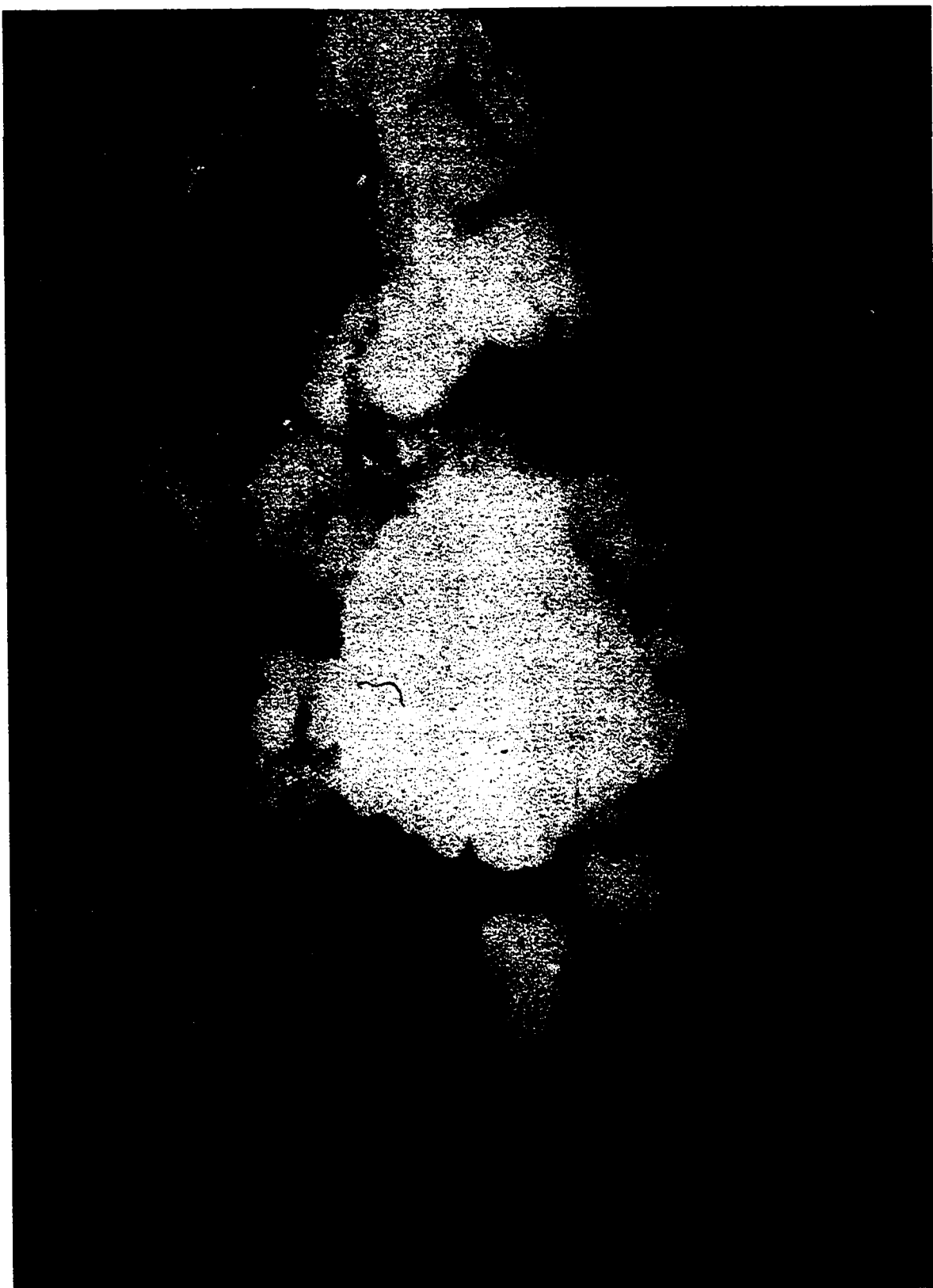
3.1.1. The recombinant human IGF-1 gene constructs

The DNA fragment encoding mature human IGF-1 was placed under the control of the maize ubiquitin promoter. For mature human IGF-1, two coding sequences were used, one with the original human sequence, which has been partially optimized for expression in *E. coli* and the other with a plant-codon optimized sequence (higher GC content coding sequence) which was expected to give a high level of expression in plant systems (Williams *et al.*, 1992). In some constructs, the Lam B signal sequence (an *E. coli* signal peptide) was cloned in front of the IGF-1 coding sequences to see if it is functional in plants (Fig. 16). The *Hind* III fragments containing the three IGF-1 constructs were cloned into a binary *Agrobacterium* vector pKHG4 (LeGall *et al.*, 1994).

3.1.2. Transgenic plants, integration of hIGF-1 DNA and analysis of expression

The *Agrobacterium* cells containing the chimeric pKHG4 were used to transform tobacco. Twenty independent transgenic plants were generated per construct. These plants were screened by ELISA and the transgenic plants that showed higher expression levels were chosen and regenerated for further studies. The transgenic nature of the plants was initially verified by a GUS activity assay (Fig. 20) and further was verified by performing Southern analyses. Purified tobacco genomic DNA from several transgenic plants was isolated and digested with *Hind* III, fractioned by agarose gel electrophoresis, transferred onto nylon membrane and probed with labeled fragments containing the IGF-1 coding sequence. Only the expected fragment was seen for IGF-1 plants (Fig. 21). This suggests an absence of any rearrangements in the IGF-1 coding

Figure 20. GUS (beta-glucoronidase) assay of transgenic plants. A: Tobacco leaf sections were incubated in phosphate buffer containing X-Gluc at 37°C as described in “ Methods” section. Blue spots indicate the GUS expression. B: Rice calli in phosphate buffer containing X-Gluc. Blue spots show the GUS activity.



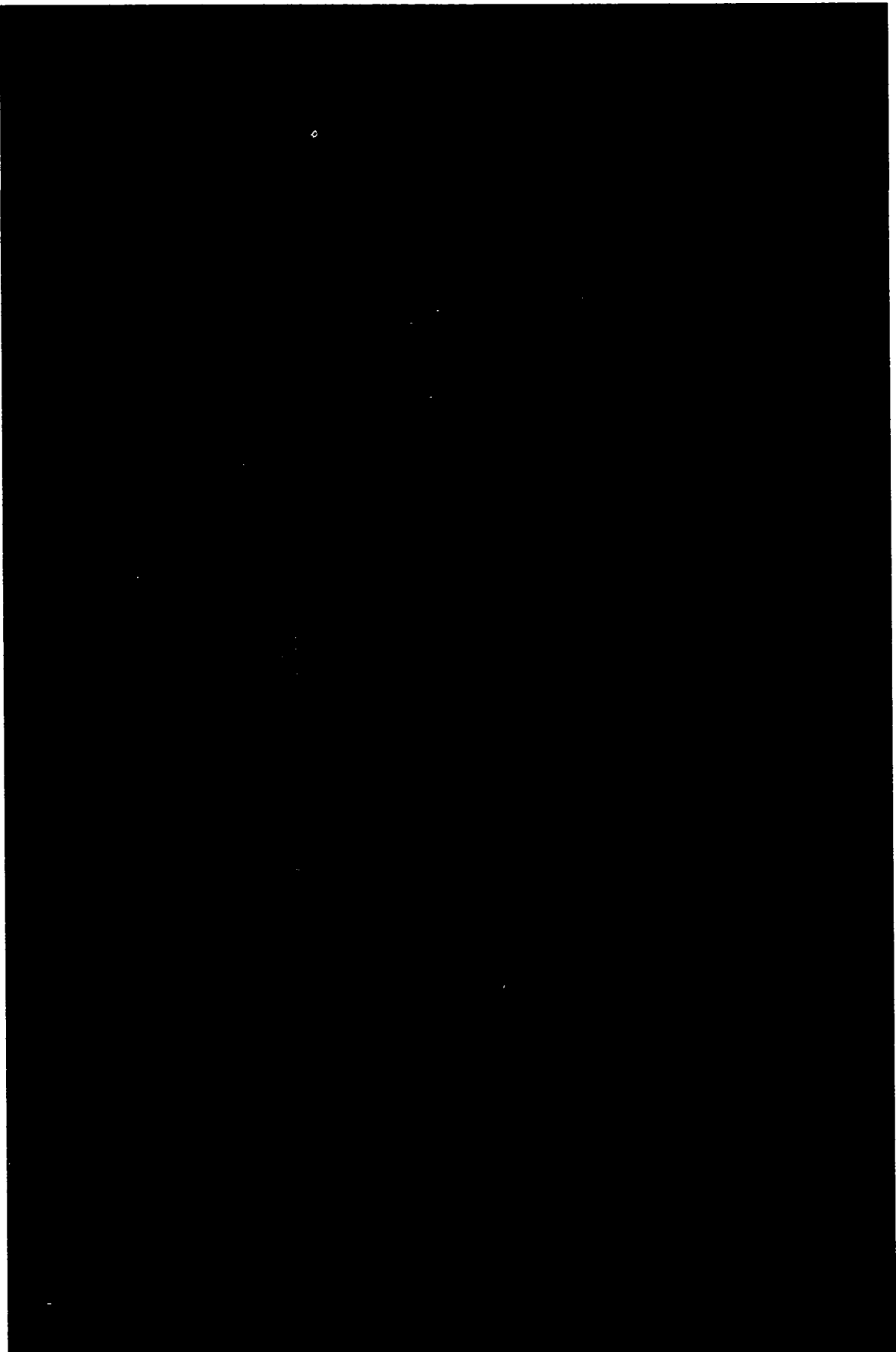
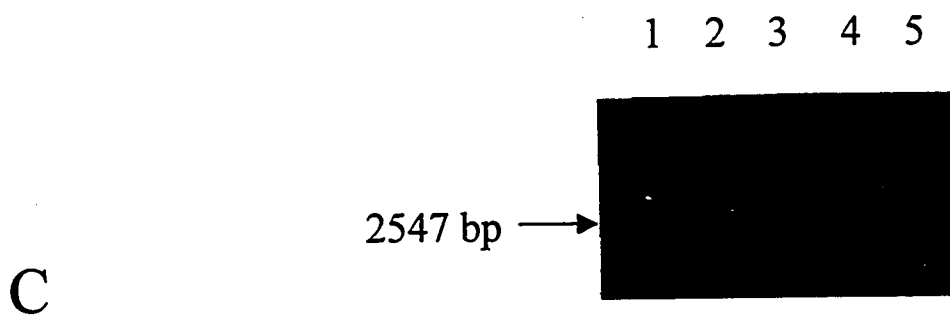
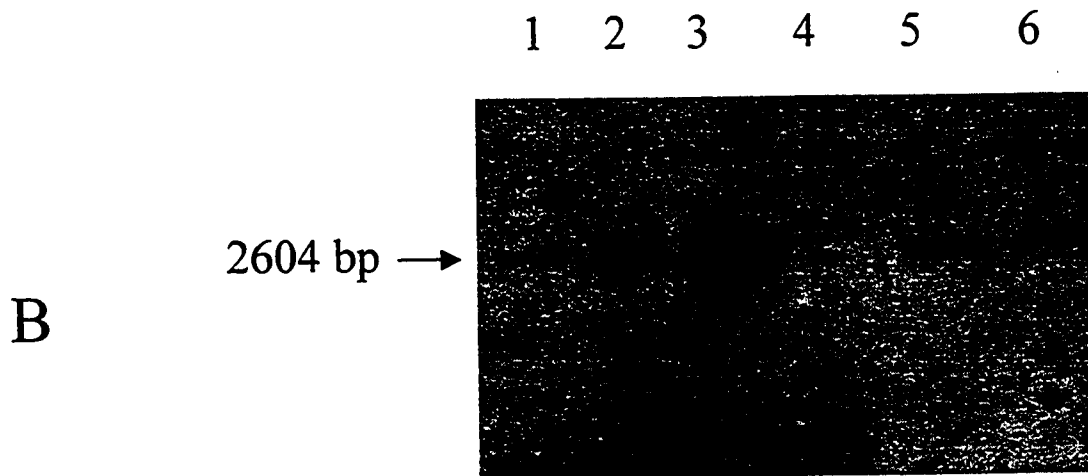
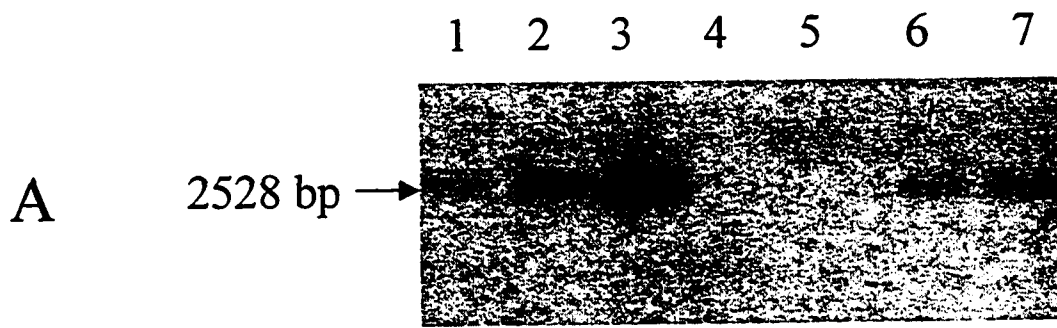


Figure 21. Screening Southern blots for IGF-1 sequence to identify transgenic tobacco plants.

A: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: empty; lane 5: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lanes 6, 7: DNA from two independent transgenic plants (#4, #11) cleaved with *Hind* III.

B: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lanes 5, 6: DNA from two independent transgenic plants (#34, #37) cleaved with *Hind* III.

C: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 3 (maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lane 5: DNA from transgenic plant (#7) cleaved with *Hind* III.



sequence and expression construct in these plants. No bands were observed for the non-transformed plant (NT). In order to obtain more information about copy number of transgene or sequence of interest, three “reconstruction” DNA samples (1 X, 5 X, 10 X) were included on each blot (Draper *et al.*, 1989).

These plants were verified for their transgenic nature using the PCR amplifications with primers specific to the mature IGF-1 (Fig. 22). These bands were identical in size to the ones obtained for the positive controls. No band of the expected size was obtained from the non-transformed tobacco plants.

To check if the transgene was expressed, total RNA was extracted from the leaves of transgenic plants. The presence of IGF-1 mRNA was verified by reverse transcription (RT) PCR amplification using the IGF-1 sequence-specific primers. The amplified fragments are shown for five IGF-1 plants (Fig. 23). These data suggest that the transgenes were being actively transcribed in the leaves of these transformed plants. Without the RT step, no fragment was amplified from total RNA confirming that the amplified fragments were not due to DNA contamination of the RNA preparation (panel A: lanes 12-14, panel B: lanes 10, 11). The negative controls (untransformed tobacco plant, water) did not show any amplified product (panel A, lanes 9, 10, panel B: lanes 7, 8).

3.1.3. IGF-1 ELISA and Western blot analysis

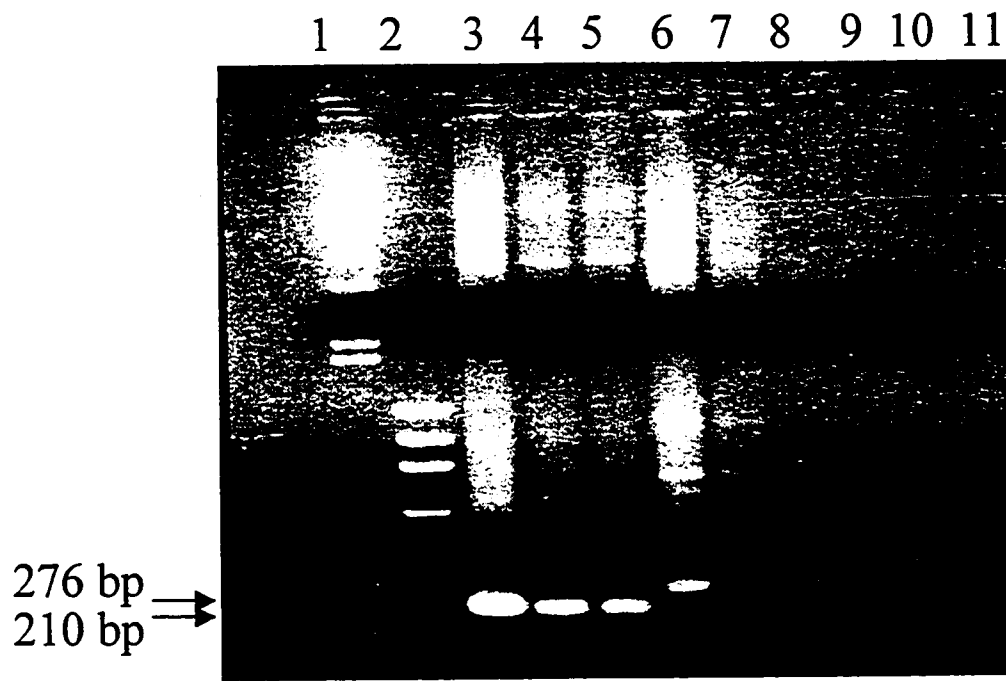
To test if the leaves of transgenic plants produced IGF-1, leaf protein extracts from transgenic and NT plants were assayed using a human IGF-1 immunoassay (Active™ IGF-1 ELISA, Diagnostic Systems Laboratories, Inc.). A standard curve was generated using *E. coli*-derived rthIGF-1. The optical density values obtained at 450 nm, for unknown samples, were used to estimate the rthIGF-1 concentrations from the standard curve. The results of the ELISA data on representative plants are shown in Table 2. According to ELISA results, two high

Figure 22. PCR detection of rthIGF-1 DNA in the leaves of transgenic tobacco plants.

A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: mature hIGF-1 coding sequence (210 bp) amplified from a plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER); lanes 4, 5: DNA from two independent transgenic plants, construct No. 2 (#4, #11); lane 6: positive control (276 bp) for plasmid containing construct No. 3 (maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER); lane 7: DNA from transgenic plant with construct No. 3 (#7); lane 8: empty; lane 9: DNA from a non-transformed plant; lane 10: H₂O as negative control; lane 11: empty.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: human IGF-1 coding sequence (285 bp) amplified from a plasmid containing construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER); lanes 4, 5: DNA from two independent transgenic plants containing construct No. 1 (#34, #37); lane 6: empty; lane 7: DNA from a non-transformed plant; lane 8: H₂O as negative control.

A



B

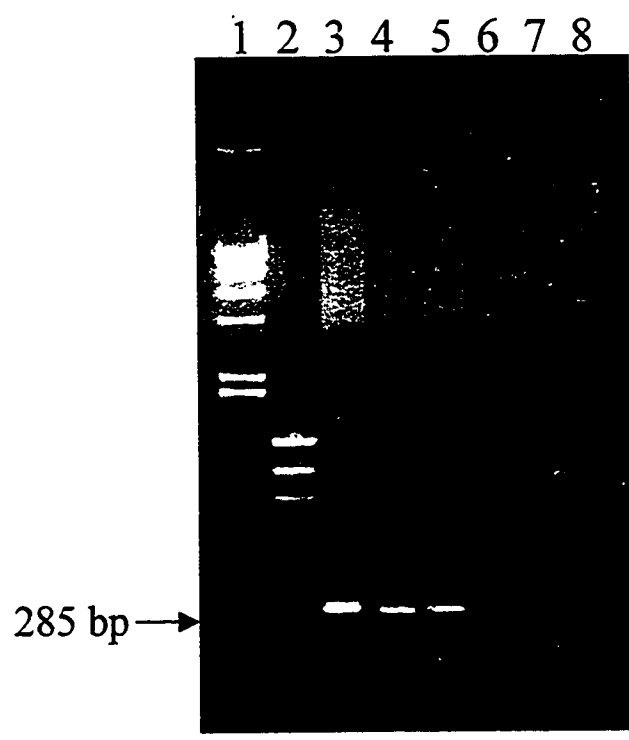


Figure 23. RT-PCR detection of the rthIGF-1 mRNA transcript in leaves of transgenic tobacco plants.

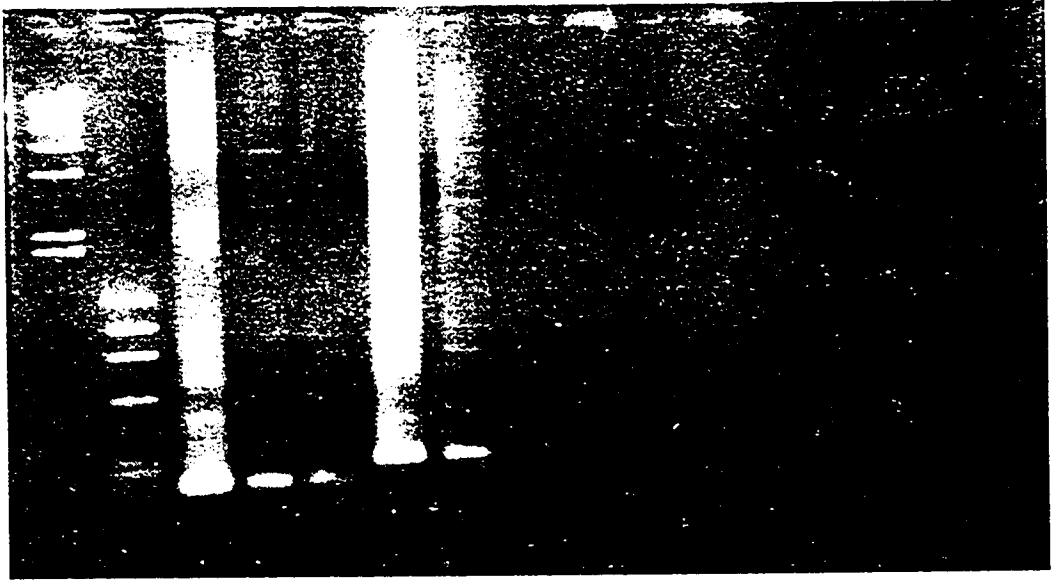
A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: mature hIGF-1 coding sequence (210 bp) amplified from a plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER); lanes 4, 5: total RNA from two independent transgenic plants, construct No. 2 (#4, #11); lane 6: positive control (276 bp) for plasmid containing construct No. 3 (maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER); lane 7: total RNA from one independent transgenic plant, construct No. 3 (#7); lane 8: empty; lane 9: total RNA from a non-transformed plant; lane 10: H₂O as negative control; lane 11: empty; lanes 12-14: total RNA from same transformed plants respectively without the reverse transcription step; lane 15: empty.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: positive control (285 bp) for construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER); lanes 4, 5: total RNA from two independent transgenic plants, construct No. 1 (#34, #37); lane 6: empty; lane 7: total RNA from a non-transformed plant; lane 8: H₂O as negative control; lane 9: empty; lanes 10,11: total RNA from same transgenic plants respectively without the reverse transcription step.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

A

276 bp →
210 bp →



1 2 3 4 5 6 7 8 9 10 11

B

285 bp →

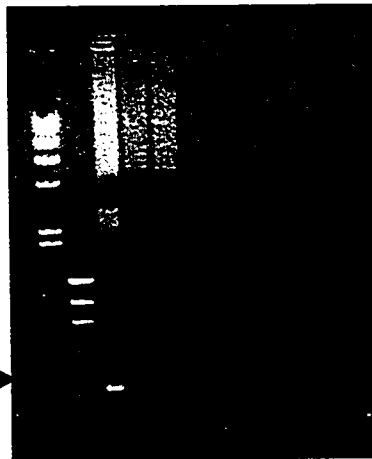


Table 2. Human IGF-1 immunoassay. The quantity of rthIGF-1 contained in the leaf extracts was estimated using the Active™ IGF-1 ELISA kit from Diagnostic Systems Laboratories, Inc. Diluted leaf extracts were added to pre-coated microtitre wells containing hIGF-1 specific monoclonal antibodies. The unbound substances were washed away with the wash buffer. The amount of rthIGF-1 was quantified using a horseradish peroxidase conjugated secondary antibody and a chromogen solution. A standard curve was generated using *E. coli* produced rthIGF-1 and the unknown concentrations from plant extracts were determined. The results were obtained as ng/ml of protein extract which converted to ng/mg of total protein by calculating the total protein in each plant extract (Bradford, 1976). NT, non-transformed tobacco plant as control.

Table 2. Human IGF-1 immunoassay on rthIGF-1 extracted from tobacco leaves (only the high expressers are shown).

Plant #	Construct #	Signal sequence	Codon bias	IGF-1 (ng/mg of total protein)
4	2	-	<i>E. coli</i>	129
11	2	-	<i>E. coli</i>	88
34	1	Lam B	<i>E. coli</i>	76
37	1	Lam B	<i>E. coli</i>	55
7	3	Rice prolamin	plant	241
NT	-	-	-	0

expressers of construct No. 1 which contained the Lam B signal sequence (#34, #37) and two of construct No. 2 which did not have the Lam B signal sequence (#4, #11) were obtained. Also, the construct which contained plant-codon optimized IGF-1 sequence (construct No. 3, plant # 7) showed higher expression level than the other constructs which contained sequences optimized for *E. coli* expression (Table 2).

The transgenic leaf extracts contained IGF-1 to a level of 129 ng/mg of total protein or 0.01% of total soluble proteins for construct No. 2 (maize ubiquitin + IGF-1 + Nos-TER) and 241 ng/mg of total protein or 0.02% of total soluble protein for construct No. 3 (maize ubiquitin + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER). The leaf extracts from a NT plant showed no immunoreactive material.

Further characterization utilized Western blotting experiments. The soluble protein extracts from leaves of two IGF-1 transgenic plants (#34 from construct No. 1, #4 from construct No. 2 and #7 from construct No. 3) which contained higher levels of IGF-1 protein (according to ELISA) were prepared and subjected to denaturing polyacrylamide gel electrophoresis. The proteins were transferred to membranes and probed with anti-IGF-1 antibody (Fig. 24). Polyclonal anti-IGF-1 antibody detected a protein of correct size (7.5 kDa) only in the extracts of transgenic plants. No band of this size was detected in NT samples. Also, the pattern of the *E. coli*-derived IGF-1 added to leaf extract of NT was the same as the pattern of *E. coli*-derived IGF-1 itself. This confirms that nothing from the leaf extracts is interfering with IGF-1. The position of *E. coli*-derived IGF-1 is indicated by two bands. The bands in addition in standard IGF-1 were reported by others also (Brem *et al.*, 1994).

3.1.4. Biological activity of the plant-expressed recombinant IGF-1

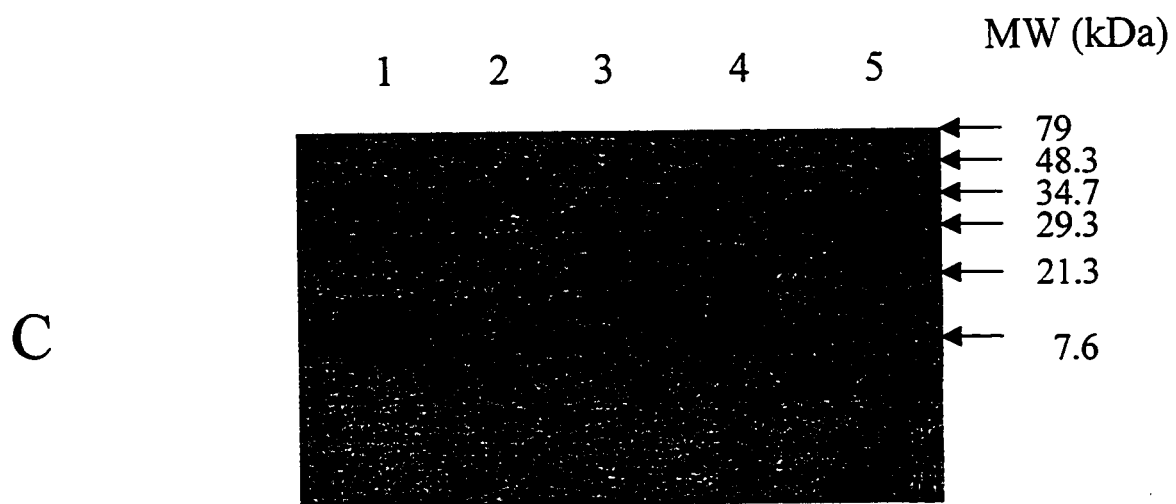
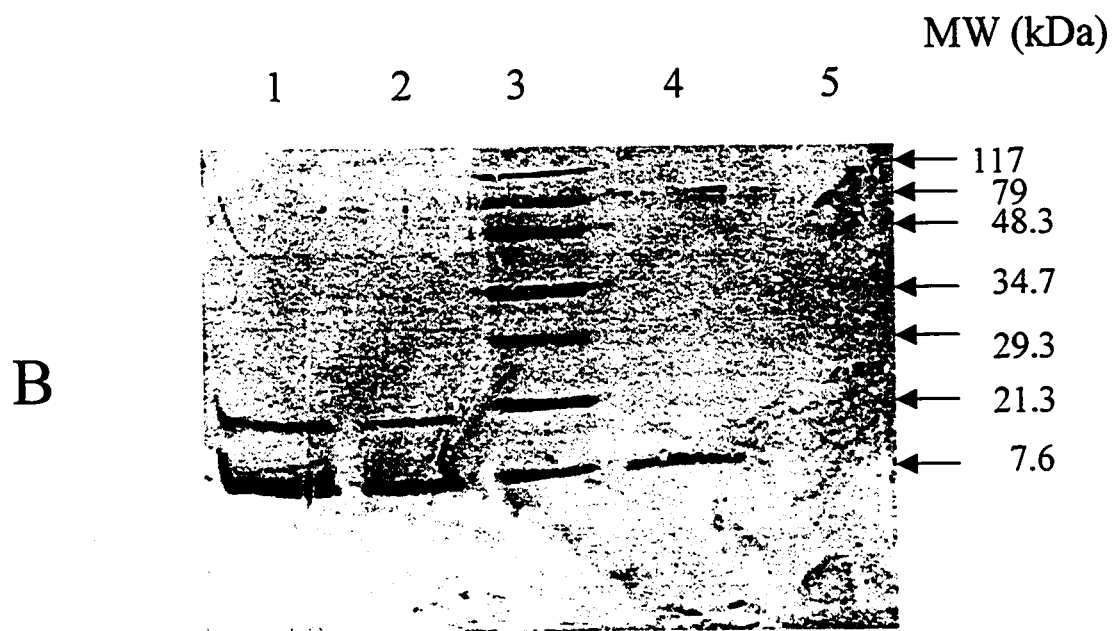
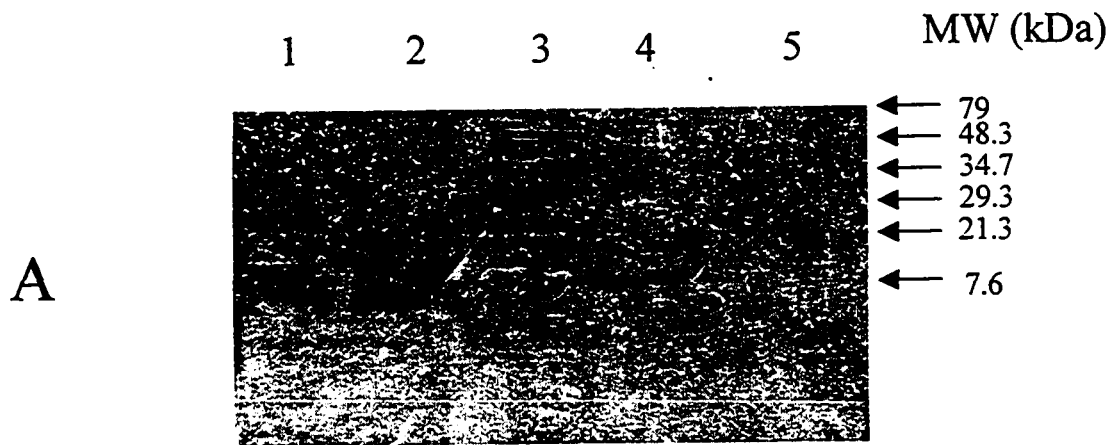
Further experiments were pursued to determine if the recombinant human IGF-1 produced in the transgenic plants was biologically active. For this purpose, human

Figure 24. Immunoblot detection of partially purified, ammonium sulphate precipitated rthIGF-1 protein in tobacco leaf extracts.

A: Lane 1: *E. coli*-derived rthIGF-1 (7.5 kDa) as standard; lane 2: *E. coli*-derived rthIGF-1 added to leaf extract from a non-transformed plant; lane 3: prestained SDS-PAGE standards (BIO-RAD); lane 4; leaf extract from an independent transgenic plant with construct No. 2 (maize ubiquitin promoter + IGF-1+ Nos-TER), plant #4; lane 5: leaf extract from a non-transformed plant.

B: Lane 1: *E. coli*-derived rthIGF-1 (7.5 kDa) as standard; lane 2: *E. coli*-derived rthIGF-1 added to leaf extract from a non-transformed plant; lane 3: prestained SDS-PAGE standards (BIO-RAD); lane 4; leaf extract from an independent transgenic plant construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1+ Nos-TER), plant #34; lane 5: leaf extract from a non-transformed plant.

C: Lane 1: *E. coli*-derived rthIGF-1 (7.5 kDa) as standard; lane 2: *E. coli*-derived rthIGF-1 added to leaf extract from a non-transformed plant; lane 3: prestained SDS-PAGE standards (BIO-RAD); lane 4; leaf extract from an independent transgenic plant construct No. 3 (maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1+ Nos-TER) #7; lane 5: leaf extract from a non-transformed plant.



neuroblastoma cell line SH-SY5Y was used (Zumkeller and Schwab, 1999; Zeidman *et al.*, 1999; Pahlman *et al.*, 1991). These cells were transferred in triplicate into a culture plate and incubated in the presence or absence of commercially available IGF-1 or aliquots of tobacco leaf extracts. Before addition to the medium, the concentrations of IGF-1 in different leaf extracts were determined and aliquots containing equal amounts of leaf-derived IGF-1 were used.

The results of these experiments are presented in Fig. 25-26. The bioassay medium alone (data not shown), leaf-extract from a non-transformed tobacco plant (NT) and protein extraction buffer (EB) added to assay medium did not support cell proliferation. The leaf extracts from the IGF-1 plants as well as the *E. coli*-derived IGF-1 when added individually to the medium (final concentration of IGF-1 in the medium: 10 ng/ml) supported proliferation of the cells as assessed at 24, 48 and 72 hours. As shown in Fig. 26, addition of transgenic tobacco leaf extracts to the growth medium resulted in proliferation of the cells. By increasing the final concentration of IGF-1 from 10 ng/ml to 50 ng/ml, cell proliferation was stopped and cell differentiation commenced as is expected from the role of IGF-1 in promotion of differentiation of SH-SY5Y cells (Pahlman *et al.*, 1991). These data suggest that the human IGF-1 produced and stored in plant cells was functional and was maintained in an active conformation. Also it was observed that leaf extracts from non-transformed tobacco inhibit the proliferation of SH-SY5Y cells. For example, with the simultaneous addition of both *E. coli*-derived IGF-1 and NT leaf extract to the same medium, a decrease in the number of cells was observed (Fig. 25). This decrease is in comparison to the number of cells when *E. coli*-derived IGF-1 alone is added to the medium. This suggests that some compound(s) in the non-transformed tobacco leaf extracts inhibits the activity of IGF-1 protein. This means that the biological activity of tobacco IGF-1 is likely similarly affected.

3.1.5. Second generation of transgenic plants (R₁)

To gain information on the stable Mendelian inheritance of the transgene and on the

Figure 25. Biological activity of tobacco expressed human IGF-1. The bioassays were performed on SH-SY5Y neuroblastoma cells. The results shown represent the proliferation of cells as measured at 24, 48, and 72 hours. The cells grown as suspension cultures were pipetted in triplicate into the wells of a culture plate. The cells were incubated in the presence or absence of aliquots of leaf extracts from transformed and non-transformed plants, and *E. coli*-derived IGF-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: leaf protein extraction buffer; NT: non-transformed leaf extract; #4, #11: leaf extracts from transgenic plants containing : maize ubiquitin promoter + IGF-1 + Nos-TER construct; #34, #37: leaf extracts from transgenic plants containing: maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER; #7: leaf extract from transgenic plant containing: maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER.

Biological Assay for R₀ IGF-1 Tobacco Plants

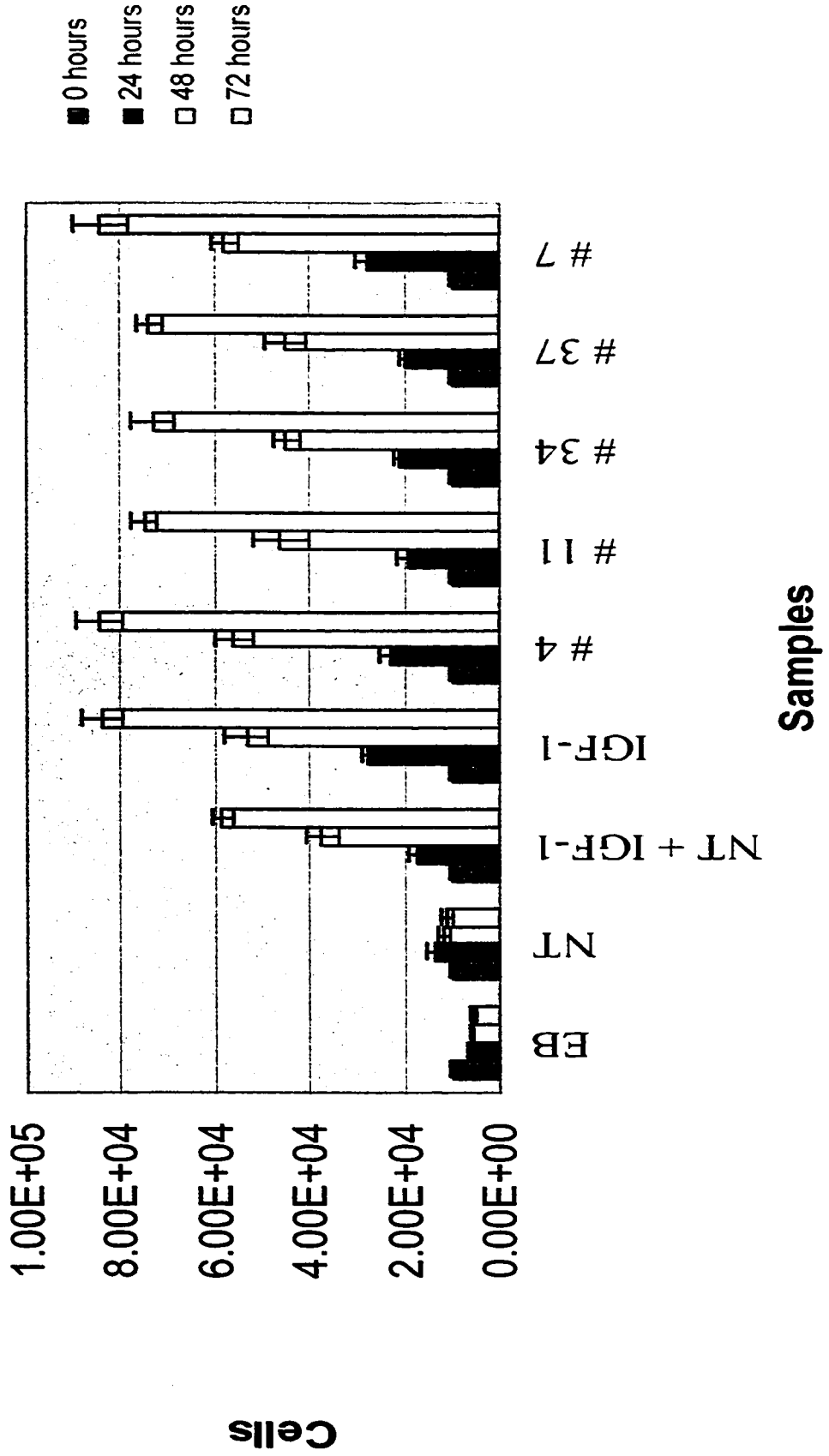
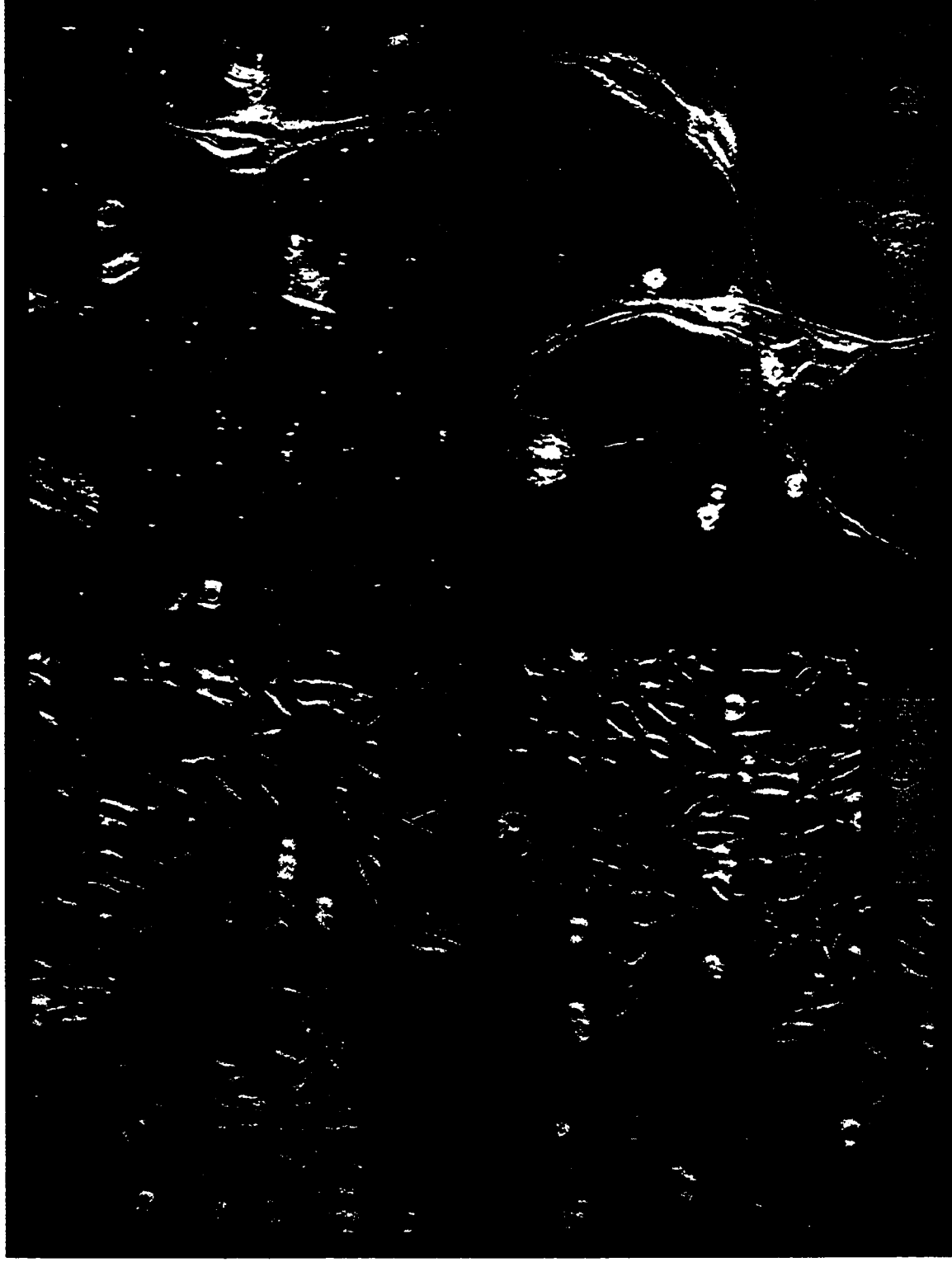


Figure 26. Effect of plant-derived IGF-1 on cell proliferation and cell differentiation. Protein extracted from the leaves of transgenic tobacco plants (#7) was tested for its ability to proliferate and differentiate SH-SY5Y neuroblastoma cells. Panel A: *E. coli*-derived IGF-1 standard, 10 ng/ml; Panel B: non-transformed plant extract at equivalent protein concentration; Panel C: independent transgenic tobacco plant extract (#7), 10 ng/ml, Panel D: independent transgenic tobacco plant extract (#7), 50 ng/ml (at this concentration, proliferation was stopped and differentiation was observed). The cells were monitored for one week with the magnification factor of 10X.

Biological Assay



activity of IGF-1 expression in the second generation of plants (R_1), the leaves from R_0 transgenic plants were assayed for Southern (Fig. 27), PCR (Fig. 28), RT-PCR (Fig. 29), Western blot (Fig. 30) and biological activity (Fig. 31). The obtained data confirm that there was stable production of human IGF-1 in the leaves of transgenic tobacco at least over one generation.

3.2. Expression of precursor hIGF-1B in transgenic tobacco plants

3.2.1. The recombinant human IGF-1B gene constructs

The DNA fragment encoding human IGF-1B (without 144 bp signal peptide) was placed under the control of the maize ubiquitin promoter. Two different constructs containing Lam B signal sequence from *E. coli* and without Lam B signal sequence were inserted along with IGF-1B coding sequence (Fig. 16). The *Hind* III fragments containing the two IGF-1B constructs were cloned into a binary *Agrobacterium* vector pKHG4.

3.2.2. Transgenic tobacco plants, integration of precursor hIGF-1B DNA and gene expression

The *Agrobacterium* cells containing the precursor coding sequence in chimeric pKHG4 were used to transform tobacco. Twenty independent transgenic plants were generated per construct. These plants were screened by ELISA and the transgenic plants that showed high expression levels were chosen and regenerated for further studies. The transgenic nature of the plants was verified by performing Southern analyses.

Purified tobacco genomic DNA from several transgenic plants was isolated and digested with *Hind* III, separated by agarose gel electrophoresis, transferred onto nylon membrane and probed with labeled *Xba* I-*Sac* I and *Nco* I-*Sac* I fragments containing the Lam B-IGF-1B and the IGF-1B sequences respectively. Only the expected fragment was seen for IGF-1B plants (Fig. 32). This suggests an absence of any rearrangements in the IGF-1B constructs in these plants.

Figure 27. Screening Southern blots of IGF-1 from transgenic tobacco plants (R₁).

A: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lanes 5, 6: DNA from two independent transgenic plants (#4, #11) cleaved with *Hind* III.

B: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lanes 5, 6: DNA from two independent transgenic plants (#34, #37) cleaved with *Hind* III.

C: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 3 (maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lane 5: DNA from transgenic plant (#7) cleaved with *Hind* III.

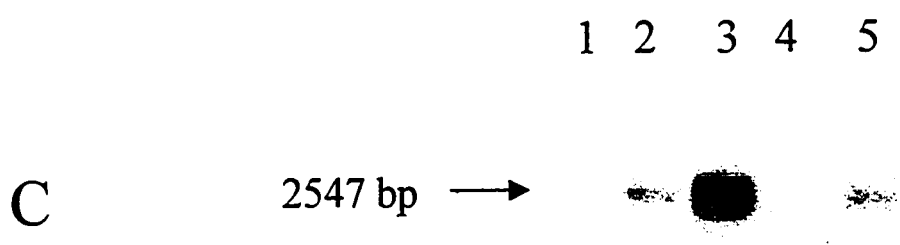
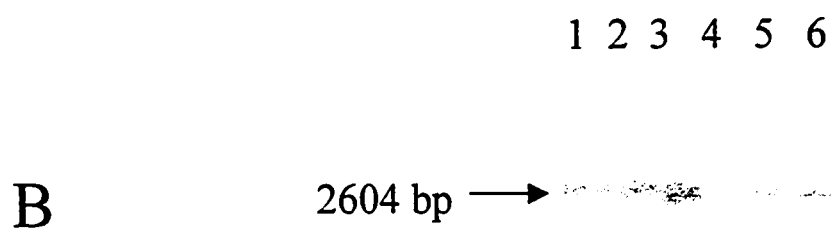
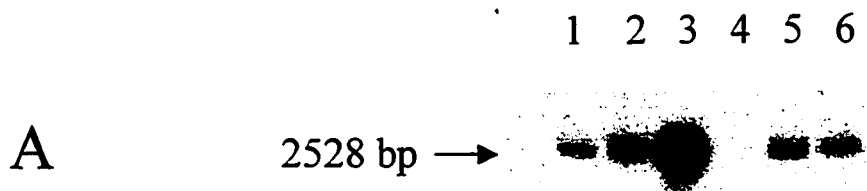


Figure 28. PCR detection of rthIGF-1 DNA in the leaves of transgenic tobacco plants (R_1).

A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: mature hIGF-1 coding sequence (210 bp) amplified from a plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER); lanes 4, 5: DNA from two independent transgenic plants, construct No. 2 (#4, #11); lane 6: positive control (276 bp) for plasmid containing construct No. 3 (maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER); lane 7: DNA from transgenic plant construct No. 3 (#7); lanes 8, 9: empty; lane 10: DNA from a non-transformed plant; lane 11: H₂O as negative control.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: human IGF-1 coding sequence (285 bp) amplified from a plasmid containing construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER); lanes 4, 5: DNA from two independent transgenic plants construct No. 1 (#34, #37); lane 6: empty; lane 7: DNA from a non-transformed plant; lane 8: H₂O as negative control.

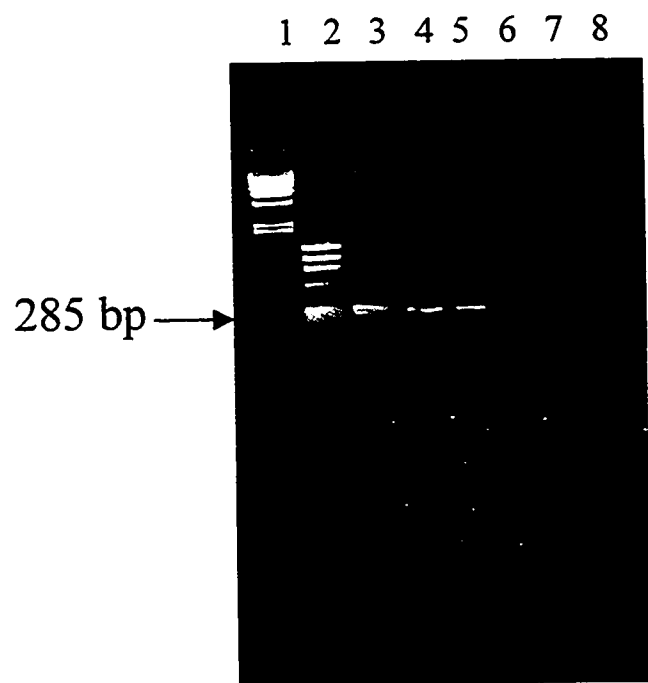
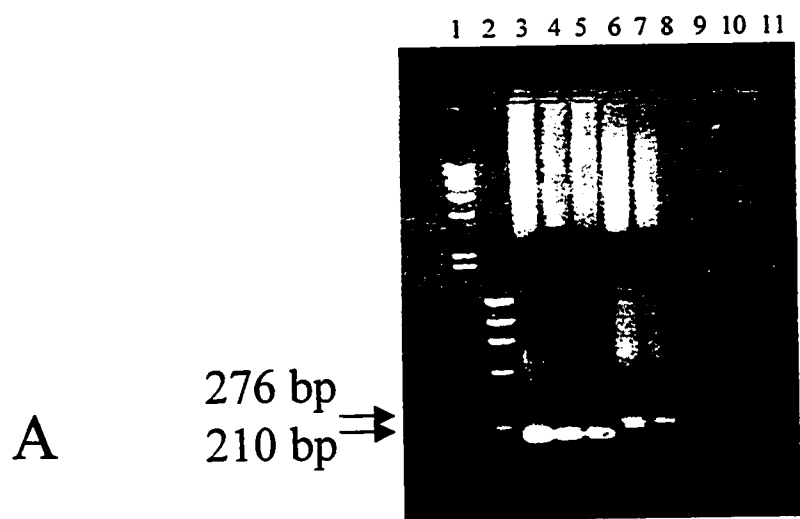
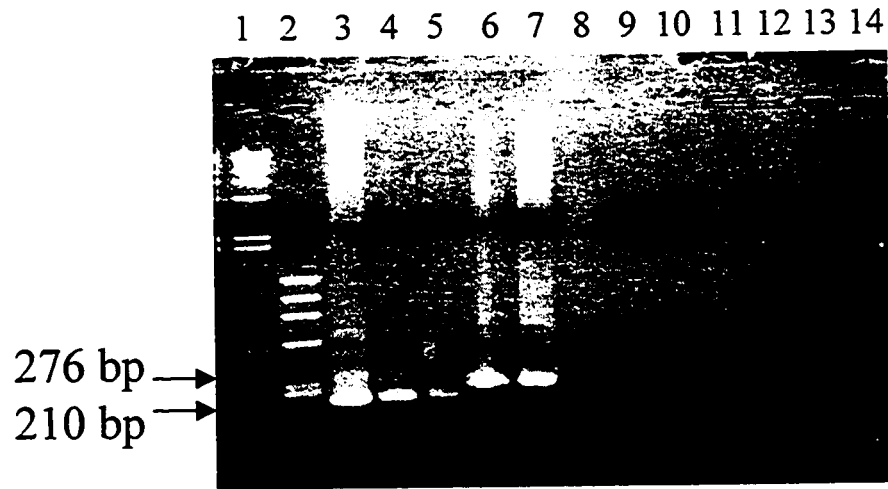


Figure 29. RT-PCR detection of the rthIGF-1 mRNA transcript in leaves of transgenic tobacco plants (R₁).

A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: mature hIGF-1 coding sequence (210 bp) amplified from a plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER); lanes 4, 5: total RNA from two independent transgenic plants, construct No. 2 (#4, #11); lane 6: positive control (276 bp) for plasmid containing construct No. 3 (rice prolamin signal sequence + synthetic IGF-1 + Nos-TER); lane 7: total RNA from one independent transgenic plant, construct No. 3 (#7); lane 8: empty; lane 9: total RNA from a non-transformed plant; lane 10: H₂O as negative control; lane 11: empty; lanes 12-14: total RNA from transformed plants respectively without the reverse transcription step.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: positive control (285 bp) for construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER); lanes 4, 5: total RNA from two independent transgenic plants, construct No. 1 (#34, #37); lane 6: empty; lane 7: total RNA from a non-transformed plant; lane 8: H₂O as negative control; lane 9: empty; lanes 10, 11: total RNA from transgenic plants respectively without the reverse transcription step.

A



B

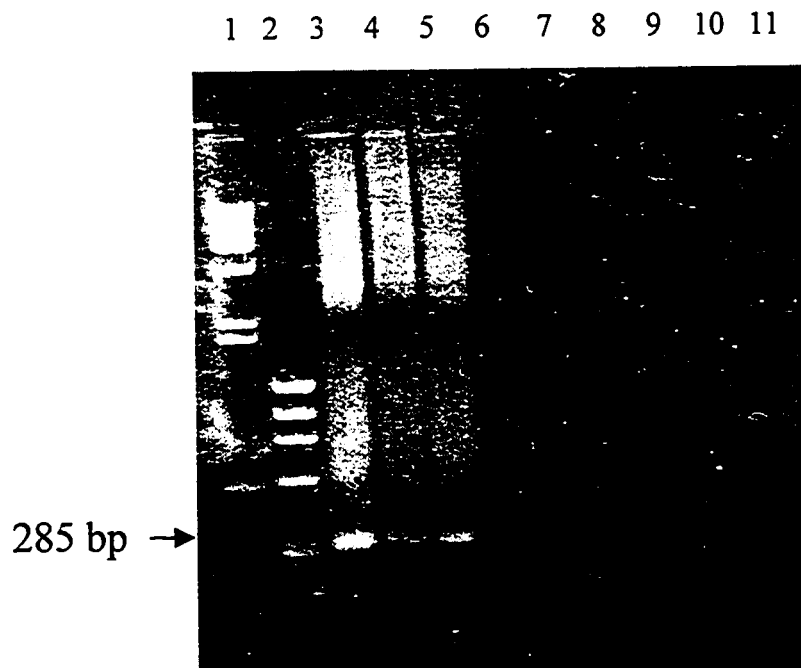


Figure 30. Immunoblot detection of partially purified, ammonium sulphate precipitated rthIGF-1 protein in tobacco leaf extracts (R₁).

A: Lane 1: leaf extract from a non-transformed plant; lane 2: leaf extract from an independent transgenic plant construct No. 2 (maize ubiquitin promoter + IGF-1+ Nos-TER) #4; lane 3: prestained SDS-PAGE standards (BIO-RAD); lane 4: *E. coli*-derived rthIGF-1 added to leaf extract from a non-transformed plant; lane 5: *E. coli*-derived rthIGF-1 (7.5 kDa).

B: Lane 1: *E. coli*-derived rthIGF-1 (7.5 kDa); lane 2: *E. coli*-derived rthIGF-1 added to leaf extract from a non-transformed plant; lane 3: prestained SDS-PAGE standards (BIO-RAD); lane 4; leaf extract from an independent transgenic plant construct No. 1 (maize ubiquitin promoter + Lam B signal sequence-IGF-1+ Nos-TER) #34; lane 5: leaf extract from a non-transformed plant.

C: Lane 1: *E. coli*-derived rthIGF-1 (7.5 kDa); lane 2: *E. coli*-derived rthIGF-1 added to leaf extract from a non-transformed plant; lane 3: prestained SDS-PAGE standards (BIO-RAD); lane 4; leaf extract from an independent transgenic plant construct No. 3 (maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1+ Nos-TER) #7; lane 5: leaf extract from a non-transformed plant.

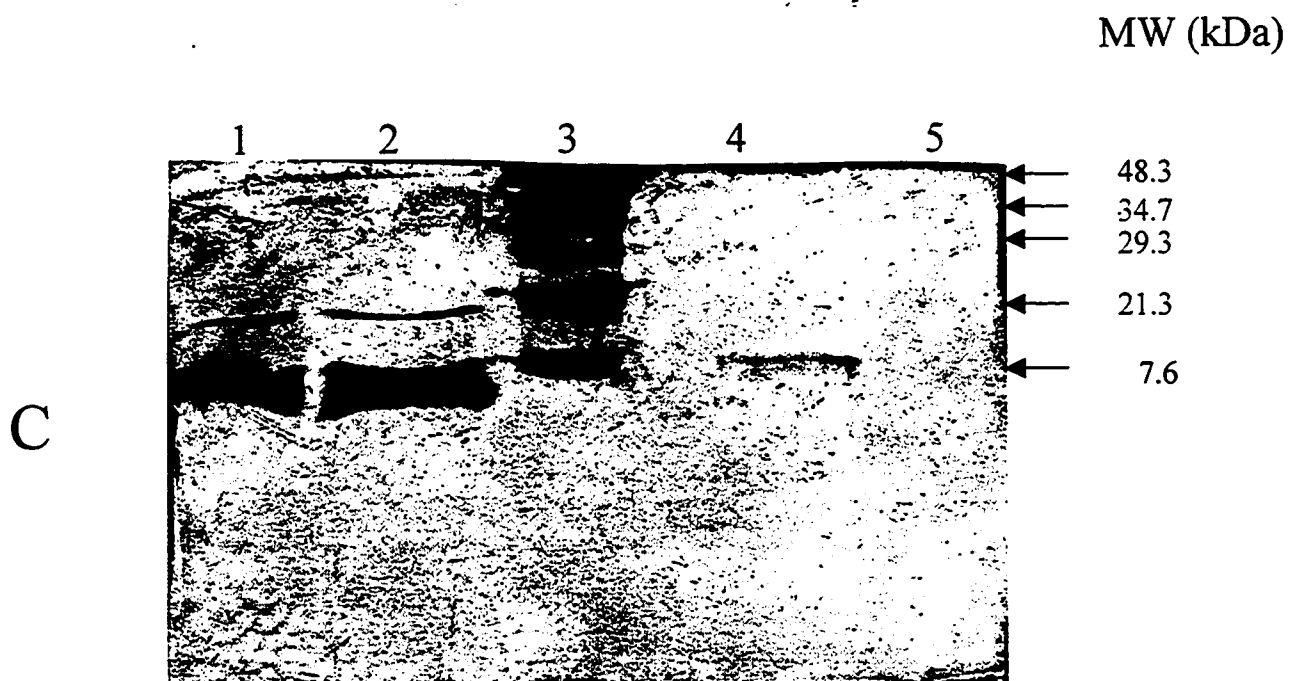
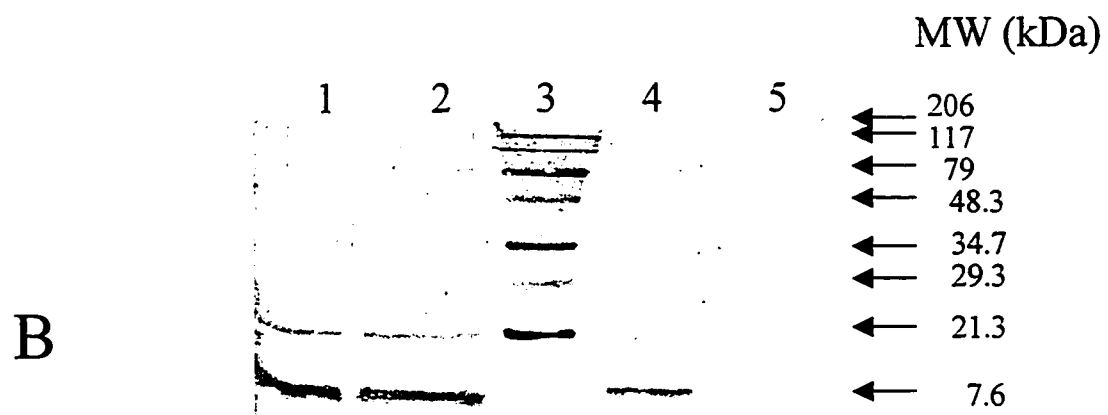
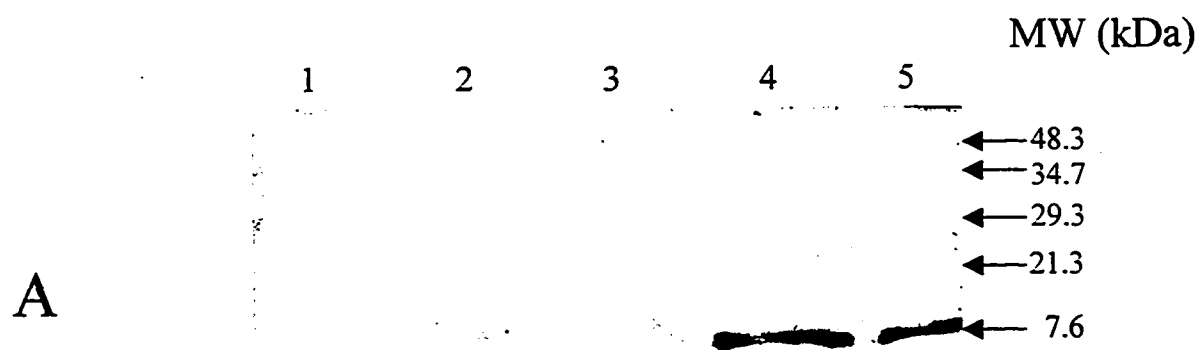


Fig. 3:

Figure 31. Biological activity of tobacco expressed human IGF-1 (R₁). The bioassays were performed on SH-SY5Y neuroblastoma cells. The results shown represent the proliferation of cells as measured at 24, 48, and 72 hours. The cells grown as suspension cultures were pipetted in triplicate into the wells of a culture plate. The cells were incubated in the presence or absence of aliquots of leaf extracts from transformed and non-transformed plants, and *E. coli*-derived IGF-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: leaf protein extraction buffer; NT: non-transformed leaf extract; #4, #11: leaf extracts from transgenic plants containing : maize ubiquitin promoter + IGF-1 + Nos-TER construct; #34, #37: leaf extracts from transgenic plants containing: maize ubiquitin promoter + Lam B signal sequence-IGF-1 + Nos-TER; #7: leaf extract from transgenic plant containing: maize ubiquitin promoter + rice prolamin signal sequence + synthetic IGF-1 + Nos-TER.

Biological Assay for R₁ IGF-1 Tobacco Plants

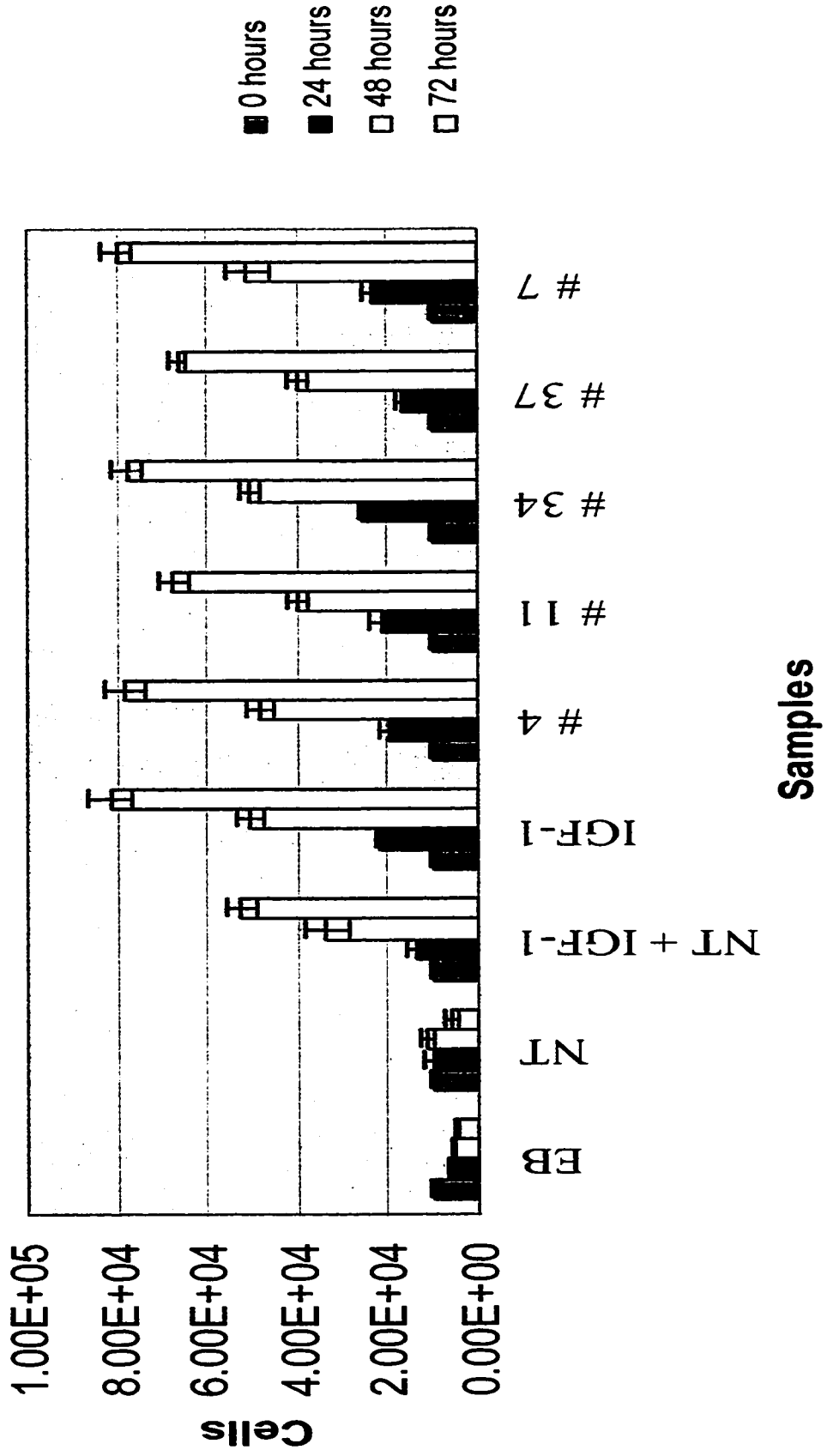
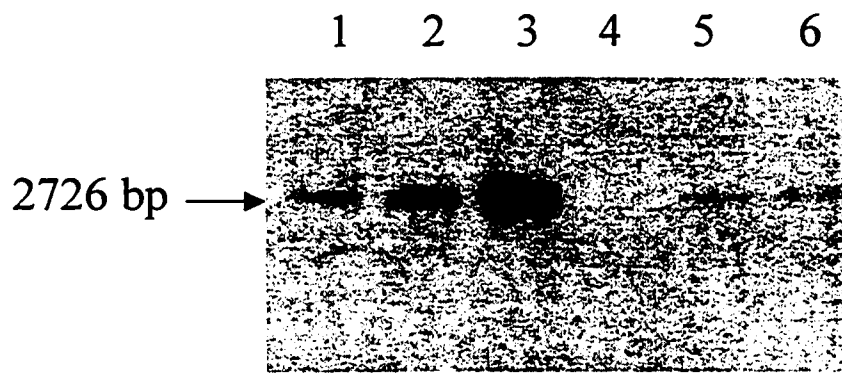


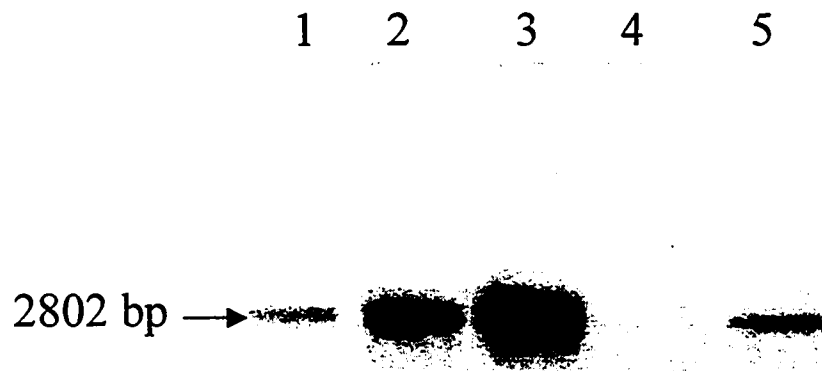
Figure 32. Screening Southern blots of IGF-1B to detect transgenic tobacco plants.

A: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 5 (maize ubiquitin promoter + IGF-1B + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lanes 5, 6: DNA from two independent transgenic plants (#5, #8) cleaved with *Hind* III.

B: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 4 (maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lane 5: DNA from transgenic plant (#6) cleaved with *Hind* III.



A



B

No bands were observed for the non-transformed plant (NT). The 1 X, 5 X, 10 X copy reconstructions were included on the blot.

These plants were verified for transgenic nature using the PCR amplifications with primers specific to the IGF-1B coding sequence (Fig. 33). These bands were identical in size to the ones obtained for the positive controls. No band of the expected size was obtained from the non-transformed tobacco plants (Panel A: lane 7; Panel B: lane 6).

To check if the transgene was expressed, total RNA was extracted from the leaves of transgenic plants. The presence of IGF-1B mRNA was verified by reverse transcription (RT) PCR amplification using the IGF-1B sequence-specific primers. The amplified fragments are shown for three IGF-1B plants (Fig. 34). These data suggest that the transgenes were being actively transcribed in the leaves of these transformed plants. Without the RT step, no fragment was amplified from total RNA confirming that the amplified fragments were not due to DNA contamination of the RNA preparation (Panel A: lanes 10, 11; Panel B: lane 8). The negative controls (untransformed tobacco plant, water) did not show any amplified product (Panel A: lanes 7, 8; Panel B: lanes 6, 7).

3.2.3. IGF-1B ELISA and Western blot analysis

ELISA was performed in the same way as was done for IGF-1 plants (Active™ IGF-1 ELISA, Diagnostic Systems Laboratories, Inc.). According to ELISA results, one high expresser which contained Lam B signal sequence (#6) contained rthIGF-1B to a level of 30 ng/mg of total protein. Two plants which did not have the Lam B signal sequence (#5, #8) contained 43 and 36 ng/mg of total protein (Table 3). All transgenic plants produced rthIGF-1B in leaves at levels that ranged from 5 ng/mg to 43 ng/mg of total protein of leaf extract (data not shown). The leaf extracts from a NT plant showed no immunoreactive material.

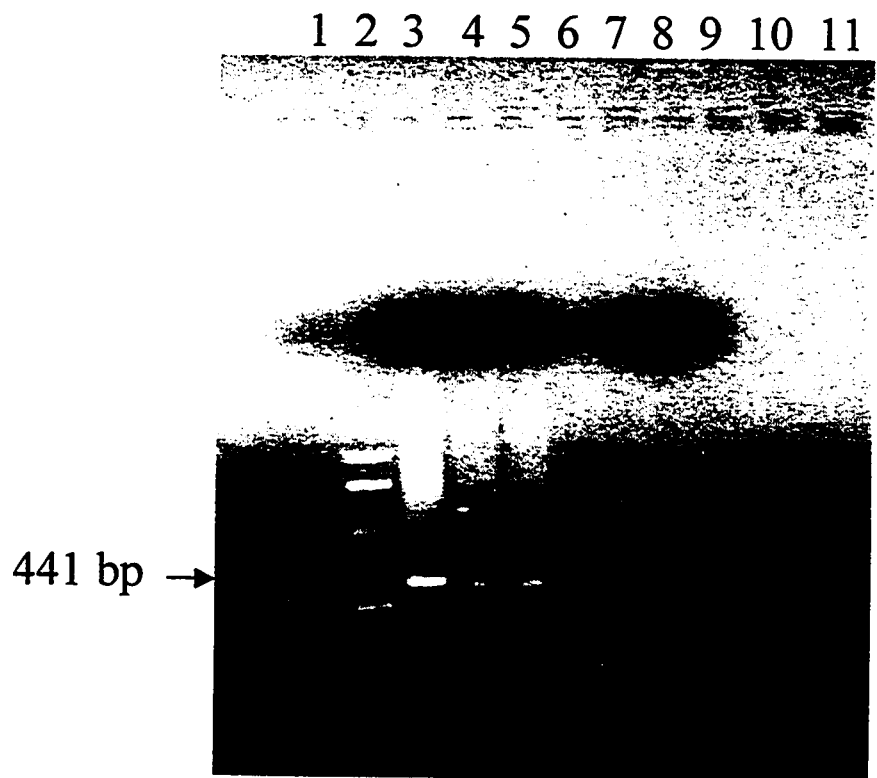
Further characterization of the IGF-1B protein utilized Western blotting experiments. The soluble protein extracts from leaves of three IGF-1B transgenic plants (#6 from construct No. 4,

Figure 33. PCR detection of rthIGF-1B DNA in the leaves of transgenic tobacco plants.

A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1B coding sequence (441 bp) amplified from a plasmid containing construct No. 5 (maize ubiquitin promoter + IGF-1B + Nos-TER); lanes 4, 5: DNA from two independent transgenic plants, construct No. 5 (#5, #8); lane 6: empty; lane 7: DNA from a non-transformed plant; lane 8: H₂O as negative control; lanes 9, 10, 11: empty.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1B coding sequence (516 bp) amplified from a plasmid containing construct No. 4 (maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER); lane 4: DNA from transgenic plant construct No. 5 (#6); lane 5: empty; lane 6: DNA from a non-transformed plant; lane 7: H₂O as negative control; lane 8: empty.

A



B

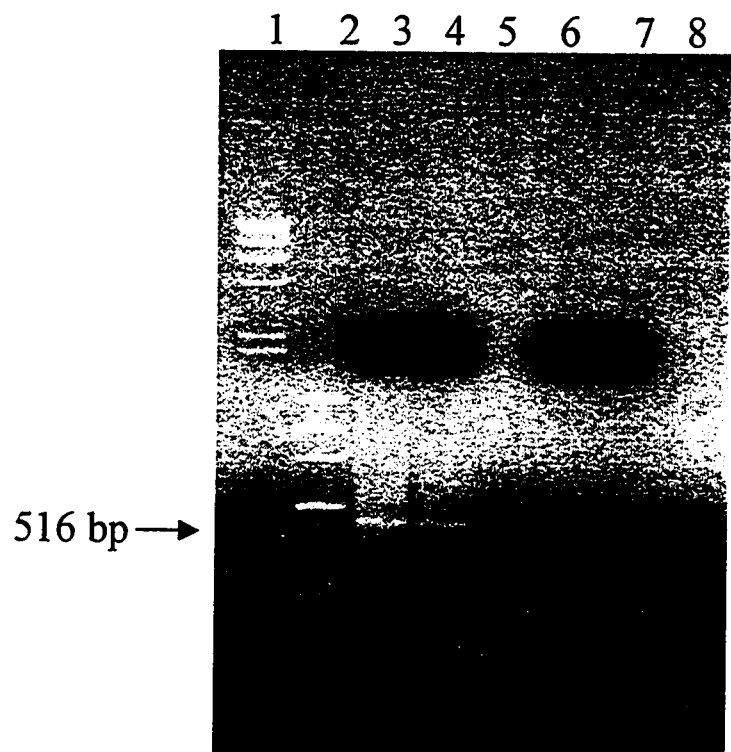


Figure 34. RT-PCR detection of the rthIGF-1B mRNA transcript in leaves of transgenic tobacco plants.

A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1B coding sequence (441 bp) amplified from a plasmid containing construct No. 5 (maize ubiquitin promoter + IGF-1B + Nos-TER); lanes 4, 5: total RNA from two independent transgenic plants, construct No. 5 (#5, #8); lane 6: empty; lane 7: total RNA from a non-transformed plant; lane 8: H₂O as negative control; lane 9: empty; lanes: 10, 11: total RNA from transformed plants respectively without the reverse transcription step.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: positive control (516 bp) for construct No. 5 (maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER); lane 4: total RNA from transgenic plant construct No. 5 (#6); lane 5: empty; lane 6: total RNA from a non-transformed plant; lane 7: H₂O as negative control; lane 8: total RNA from transgenic plant (#6) without the reverse transcription step.

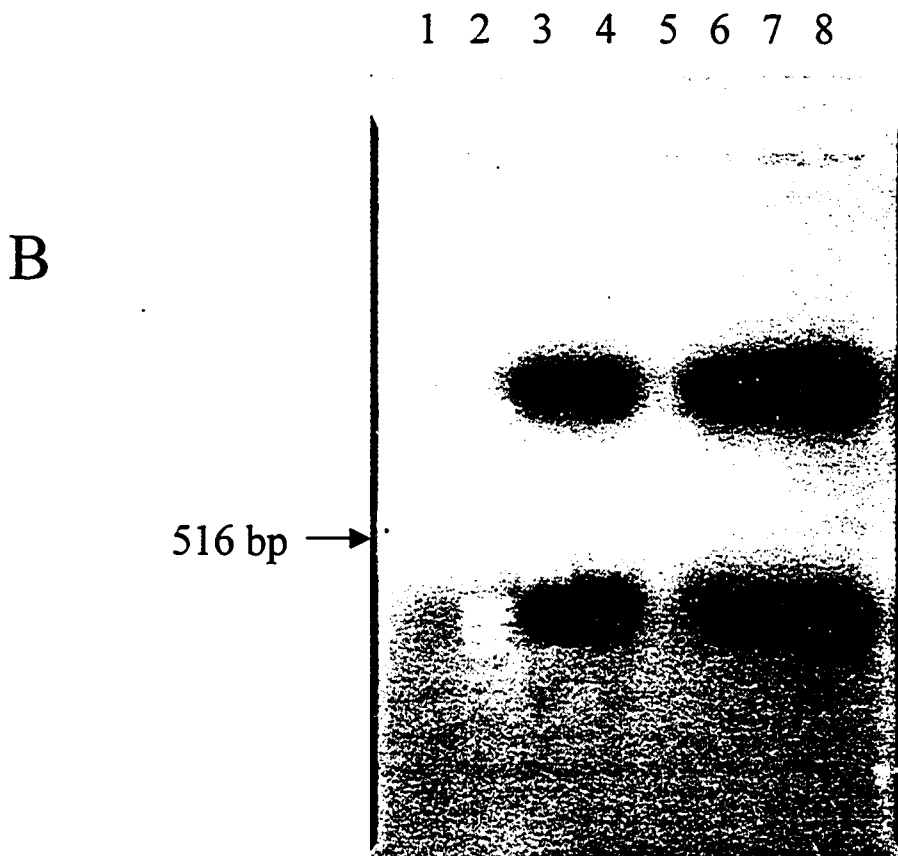
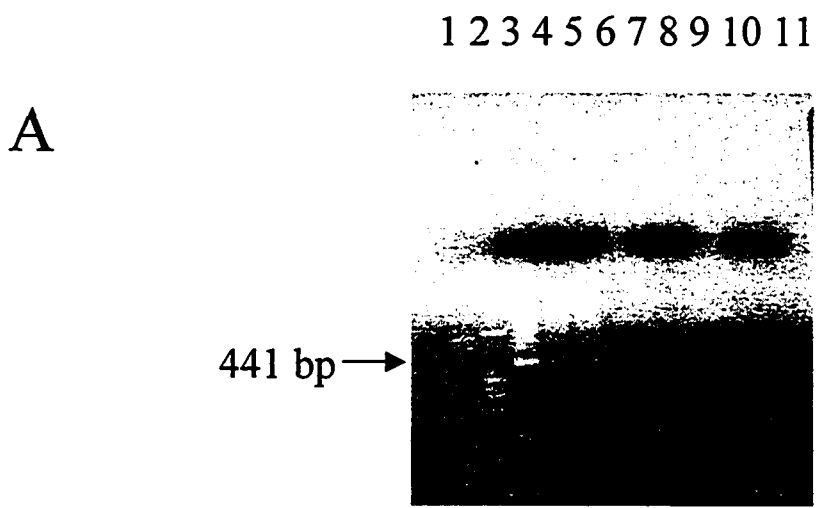


Table 3. Human IGF-1B immunoassay. The quantitative data obtained for representative plants is shown in this table. Leaf extracts were tested for the presence of human IGF-1B using the Active™ IGF-1 ELISA kit from Diagnostic Systems Laboratories Inc. Microplates pre-coated with monoclonal IGF-1 antibody were used. Diluted aliquots of leaf extracts were transferred into the wells. The unbound substrates were washed away. The amount of IGF-1 was quantitated using an horseradish peroxidase (HRP) conjugated secondary antibody and a substrate solution. A standard curve was calibrated and the results were derived using the standard curve. The results were obtained as ng/ml of protein extract which converted to ng/mg of total protein by calculating the total protein in each plant extract (Bradford, 1976). NT, non-transformed tobacco plant as control.

Table 3. Human IGF-1B immunoassay on rthIGF-1B extracted from tobacco leaves (only the high expressers are shown).

Plant #	Construct #	Signal sequence	Codon bias	IGF-1B (ng/mg of total protein)
5	5	-	<i>E. coli</i>	43
8	5	-	<i>E. coli</i>	36
6	4	Lam B	<i>E. coli</i>	30
NT	-	-	-	0

#5, #8 from construct No. 5) which contained higher levels of IGF-1B protein (according to ELISA) were prepared and subjected to denaturing polyacrylamide gel electrophoresis. The proteins were transferred to membranes and probed with anti-IGF-1 antibody (Fig. 35). Polyclonal anti-IGF-1 antibody detected a protein of 18 kDa only in the extracts of transgenic plants (the molecular weight of 147 residue signal-peptide-IGF-1B is 22 kDa, but IGF-1B in my constructs did not contain the 48 signal peptide residues which resulted in less molecular weight) No band of this size was detected in NT. No commercial IGF-1B standard was available to use as a molecular weight standard.

3.2.4. Biological activity of the plant-expressed recombinant IGF-1B

The recombinant human precursor IGF-1B produced in the transgenic plants was tested for biological activity. For this purpose, the same human cell line SH-SY5Y neuroblastoma cells were used in the same way as was performed for IGF-1 plants. Others have used the same cell lines to assay for IGF-1 and IGF-1B activities (Siegfried *et al.*, 1992). The results of these experiments are presented in Fig. 36. The bioassay medium alone (not shown), leaf-extract from a non-transformed tobacco plant (NT) and protein extraction buffer (EB) added to assay medium did not cause cell proliferation. The leaf-extracts from the IGF-1B plants as well as the *E. coli*-derived IGF-1 when added individually to the medium (final concentration of IGF-1B in the medium: 10 ng/ml) supported proliferation of the cells as assessed at 24, 48 and 72 hours. As shown in Figure 26, addition of transgenic tobacco leaf extracts to the growth medium resulted in proliferation of the cells. By increasing the final concentration of IGF-1B from 10 ng/ml to 50 ng/ml, cell proliferation ceased and cell differentiation began, as was shown for extracts from IGF-1 plants (Fig. 37). These data suggest that the human IGF-1B produced and stored in plant cells is functional and maintained in an active conformation. Also, the results suggest that some compound(s) in the non-transformed tobacco leaf extracts also inhibits the activity of IGF-1B protein as was shown for IGF-1 expressed in tobacco leaves.

Figure 35. Immunoblot detection of partially purified, ammonium sulphate precipitated rthIGF-1B protein in tobacco leaf extracts.

Lane 1: prestained SDS-PAGE standards (BIO-RAD); lane 2: leaf extract from a non-transformed plant; lanes 3, 4: leaf extracts from independent transgenic plants construct No. 4 (maize ubiquitin promoter + IGF-1B + Nos-TER) #5, #8; lane 5: leaf extract from transgenic plant containing construct No. 4 (maize ubiquitin promoter + Lam B signal sequence-IGF-1+ Nos-TER) plant #6.

MW (kDa)

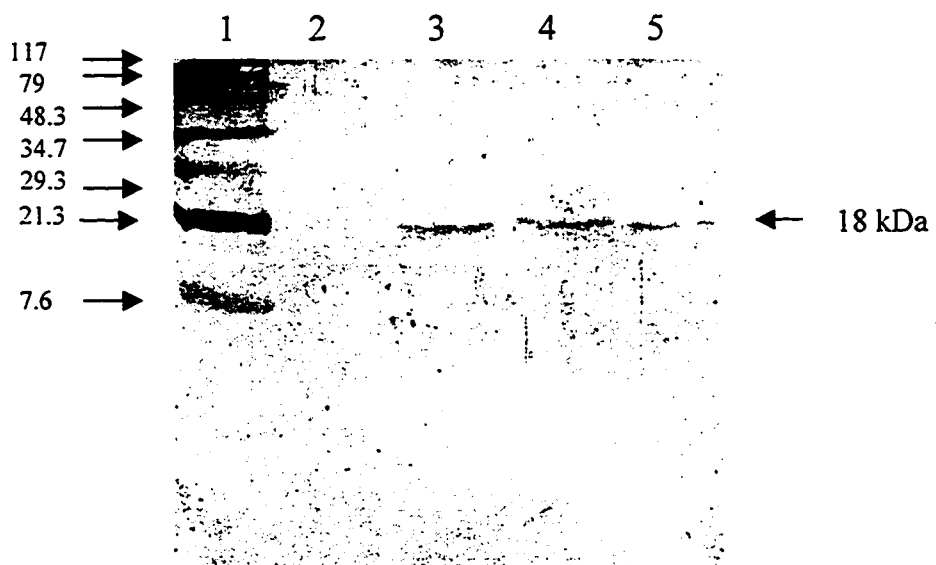
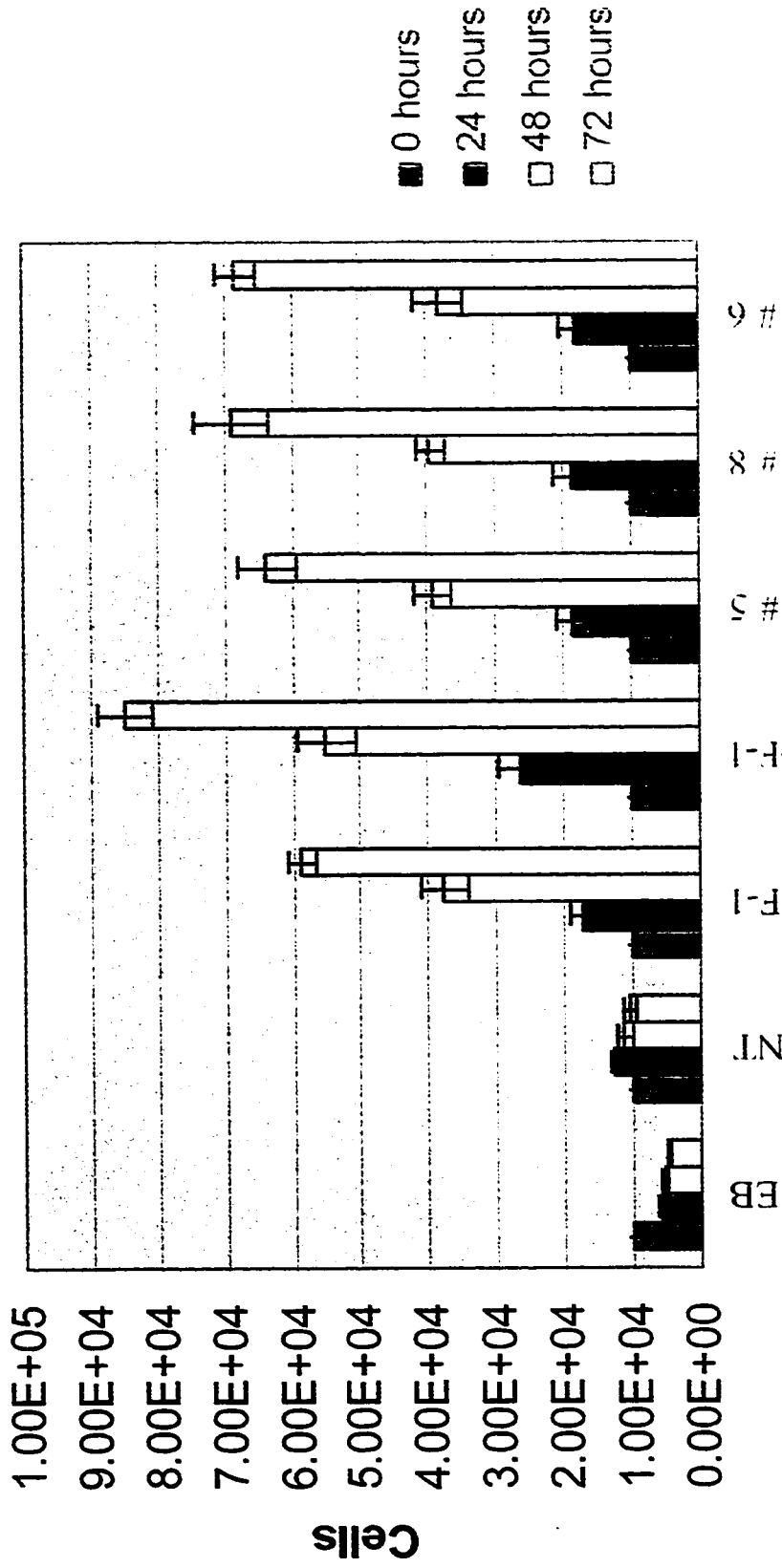


Figure 36. Biological activity of tobacco expressed human IGF-1B. The bioassays were performed on SH-SY5Y neuroblastoma cells. The results shown represent the proliferation of cells as measured at 24, 48, and 72 hours. The cells grown as suspension cultures were pipetted in triplicate into the wells of a culture plate. The cells were incubated in the presence or absence of aliquots of leaf extracts from transformed and non-transformed plants, and *E. coli*-derived IGF-1. All wells containing rthIGF-1 and rthIGF-1B had a final concentration of 10 ng/ml of rthIGF-1 and rthIGF-1B respectively. Cell number and viability were determined using haemocytometry/trypan blue exclusion. EB: leaf protein extraction buffer; NT: non-transformed leaf extract; #5, #8: leaf extracts from transgenic plants containing : maize ubiquitin promoter + IGF-1B + Nos-TER construct; #6: leaf extract from transgenic plant containing: maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER.

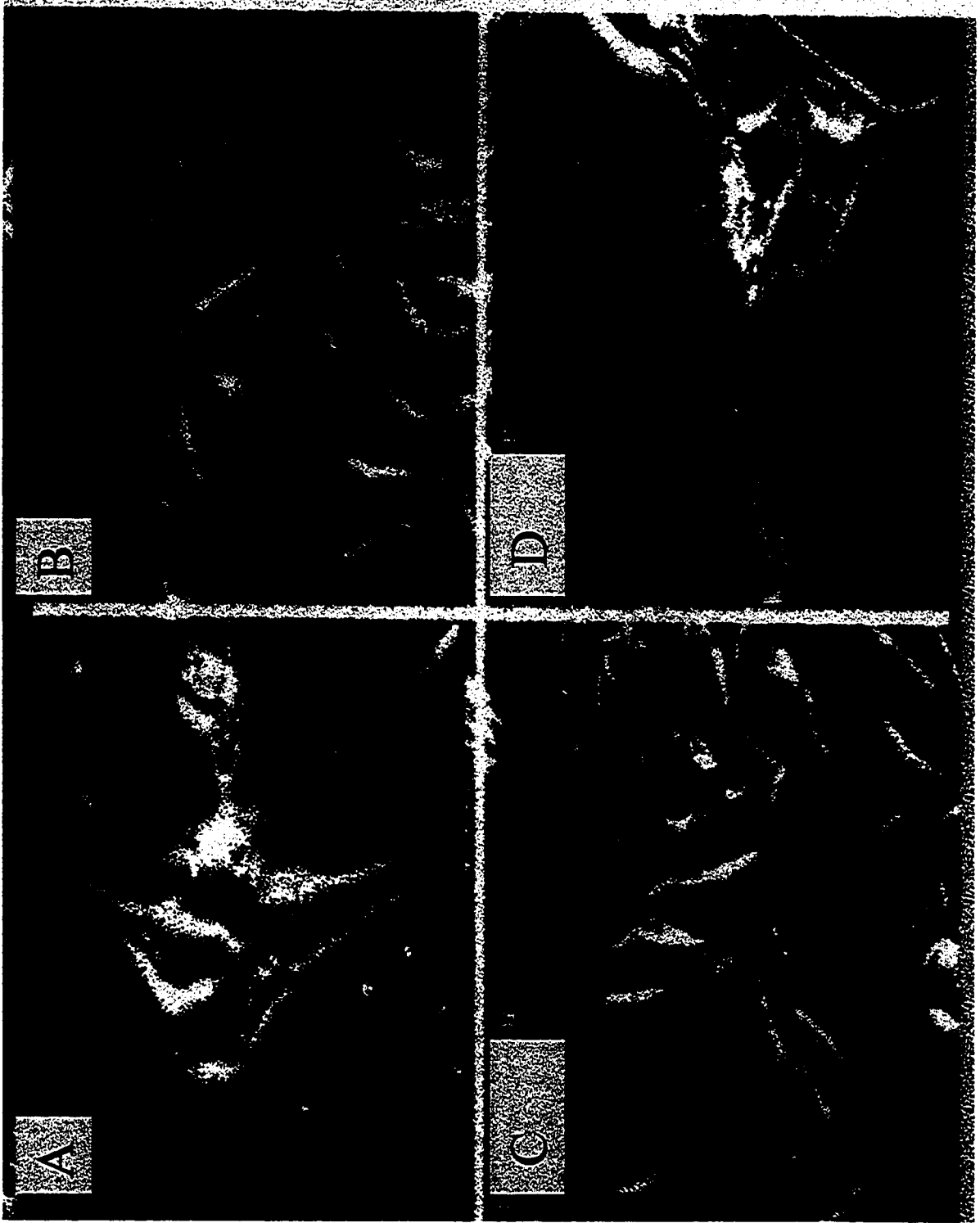
Biological Assay for R₀ IGF-1B Tobacco Plants



Samples

Figure 37. Effect of plant-derived IGF-1B on cell proliferation and cell differentiation . Protein extracted from the leaves of transgenic tobacco plant #5 was tested for its ability to cause proliferation and differentiation of SH-SY5Y neuroblastoma cells. Panel A: non-transformed plant extract at equivalent protein concentration as standard; Panel B: *E. coli*-derived IGF-1 standard, 10 ng/ml; Panel C: independent transgenic tobacco plant extract (#5), 10 ng/ml, Panel D: independent transgenic tobacco plant extract (#5), 50 ng/ml (at this concentration, proliferation was stopped and initiation of differentiation was observed). Magnification factor: 30X.

Biological Assay



3.2.5. Second generation of transgenic plants

To gain information on activity of IGF-1B expression in the first generation of plants, the leaves from the germinated seeds from regenerated transgenic plants were assayed for Southern (Fig. 38), PCR (Fig. 39), RT-PCR (Fig. 40), Western blot (Fig. 41) and biological activity (Fig. 42) which were shown as R₁ generation of the same transgenic plants. This confirms that there is stable production of human IGF-1B in the leaves of transgenic tobacco at least over one generation.

3.3. Expression of IGF-1 in transgenic rice plants

Construct No. 2 (Fig. 16) was expressed in transgenic rice plants. By screening with ELISA (same as tobacco plants), the one which showed higher expression levels (e.g. 371 ng/mg of total protein or 0.03% of total soluble leaf proteins for plant # 9) was chosen (GUS assay, Fig. 20, B) for further analyses like Southern, PCR, RT-PCR, Western and biological assay (Fig. 43-46). The details of these specific experiments and results are the same as those explained for transgenic tobacco plants made with construct No. 2. In this way, other constructs of IGF-1 could also be expressed in rice in the future, especially construct No. 3 which contains the rice 13 kDa prolamin signal sequence directing the expressed protein in seeds of transgenic rice plants is expected to yield even higher expression level.

Summary of the results for IGF-1 and IGF-1B is shown in Tables 4, 5.

Figure 38. Screening Southern blots of IGF-1B from transgenic tobacco plants (R₁).

A: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 5 (maize ubiquitin promoter + IGF-1B + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lanes 5, 6: DNA from two independent transgenic plants (#5, #8) cleaved with *Hind* III.

B: Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 4 (maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lane 5: DNA from transgenic plant (#6) cleaved with *Hind* III.

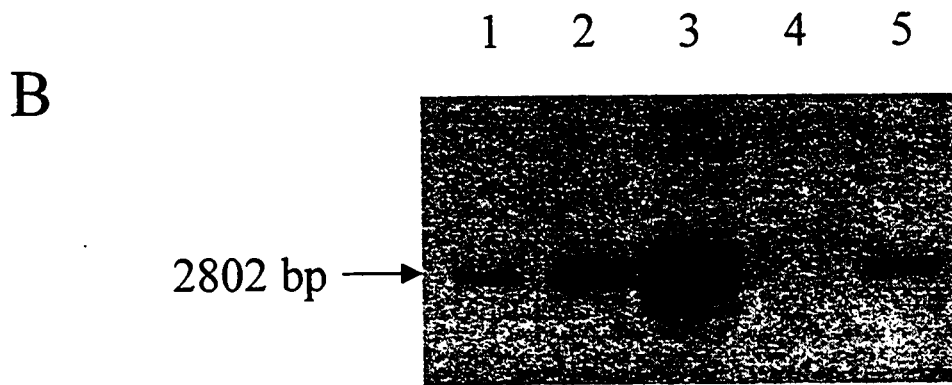
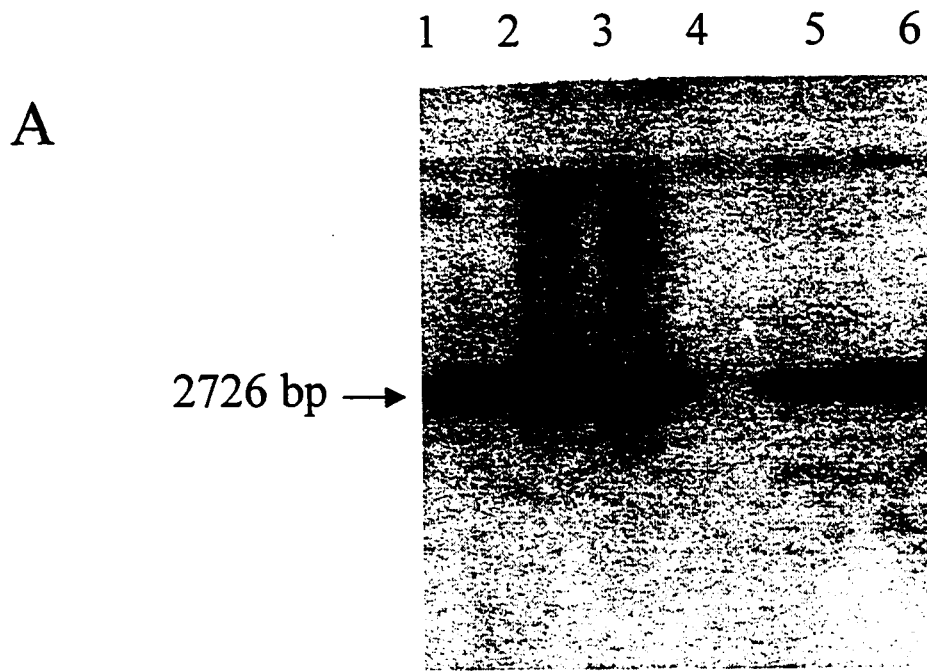
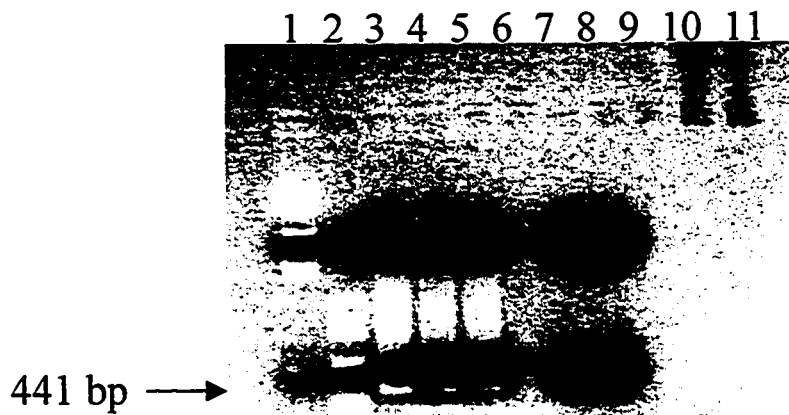


Figure 39. PCR detection of rthIGF-1B DNA in the leaves of transgenic tobacco plants (R₁).

A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1B coding sequence (441 bp) amplified from a plasmid containing construct No. 5 (maize ubiquitin promoter + IGF-1B + Nos-TER); lanes 4, 5: DNA from two independent transgenic plants, construct No. 5 (#5, #8); lane 6: empty; lane 7: DNA from a non-transformed plant; lane 8: H₂O as negative control; lanes 9, 10, 11: empty.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1B coding sequence (516 bp) amplified from a plasmid containing construct No. 4 (maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER); lane 4: DNA from transgenic plant construct No. 5 (#6); lane 5: empty; lane 6: DNA from a non-transformed plant; lane 7: H₂O as negative control; lane 8: empty.

A



B

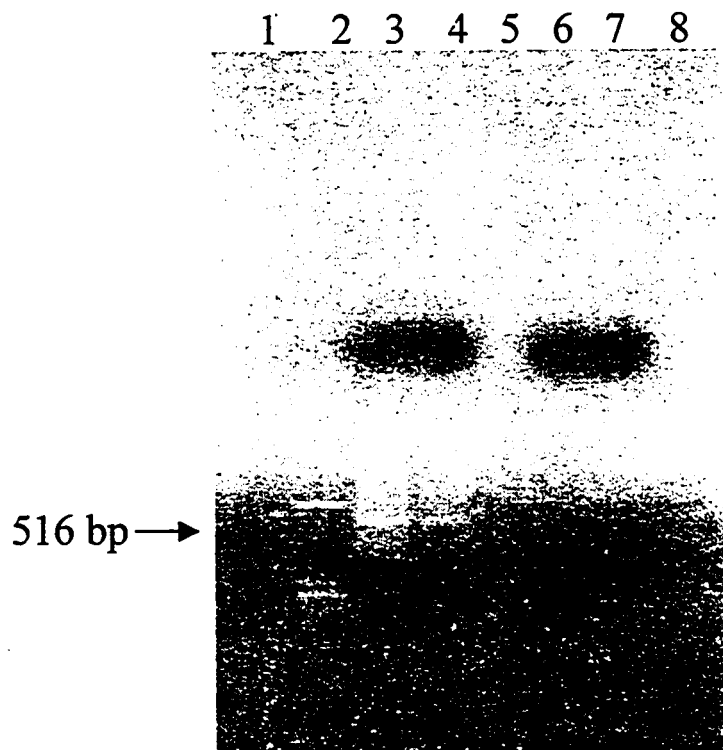
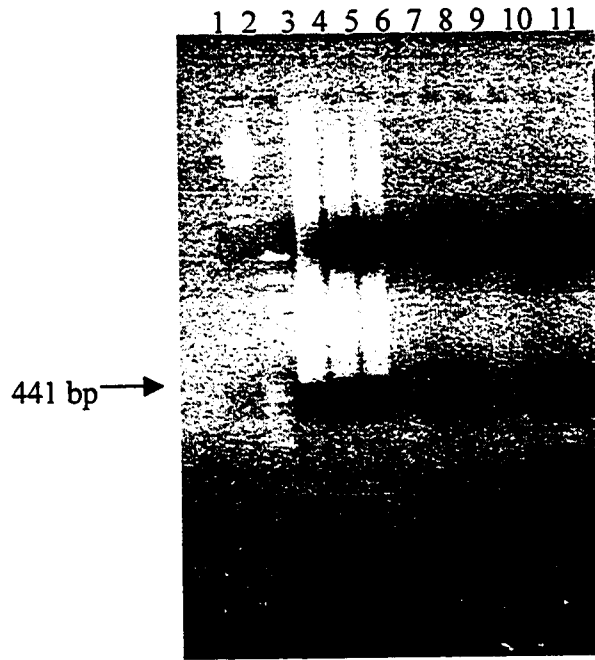


Figure 40. RT-PCR detection of the rthIGF-1B mRNA transcript in leaves of transgenic tobacco plants (R₁).

A: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1B coding sequence (441 bp) amplified from a plasmid containing construct No. 5 (maize ubiquitin promoter + IGF-1B + Nos-TER); lanes 4, 5: total RNA from two independent transgenic plants, construct No. 5 (#5, #8); lane 6: empty; lane 7: total RNA from a non-transformed plant; lane 8: H₂O as negative control; lane 9: empty; lanes: 10, 11: total RNA from transformed plants respectively without the reverse transcription step.

B: Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: positive control (516 bp) for construct No. 5 (maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER); lane 4: total RNA from transgenic plant construct No. 5 (#6); lane 5: empty; lane 6: total RNA from a non-transformed plant; lane 7: H₂O as negative control; lane 8: total RNA from transgenic plant (#6) without the reverse transcription step.

A



B

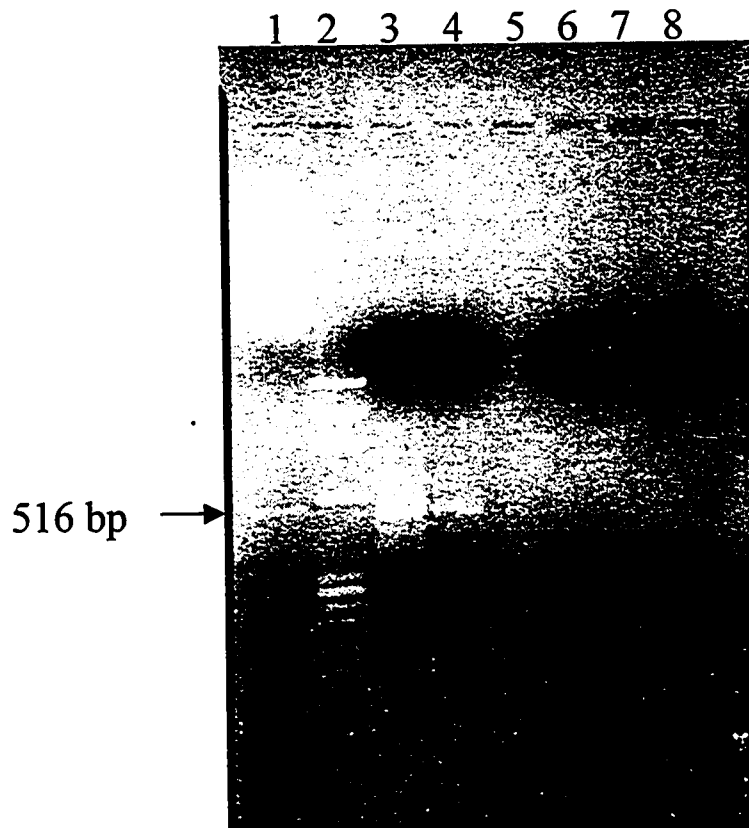


Figure 41. Immunoblot detection of partially purified, ammonium sulphate precipitated rthIGF-1B protein in tobacco leaf extracts (R₁).

Lane 1: leaf extract from a non-transformed plant; lanes 2, 3: leaf extracts from independent transgenic plants containing construct No. 5 (maize ubiquitin promoter + IGF-1B+ Nos-TER) #5, #8; lane 4: leaf extract from transgenic plant containing construct No. 4 (maize ubiquitin promoter + Lam B signal sequence-IGF-1+ Nos-TER) #6 lane 5: prestained SDS-PAGE standards (BIO-RAD).

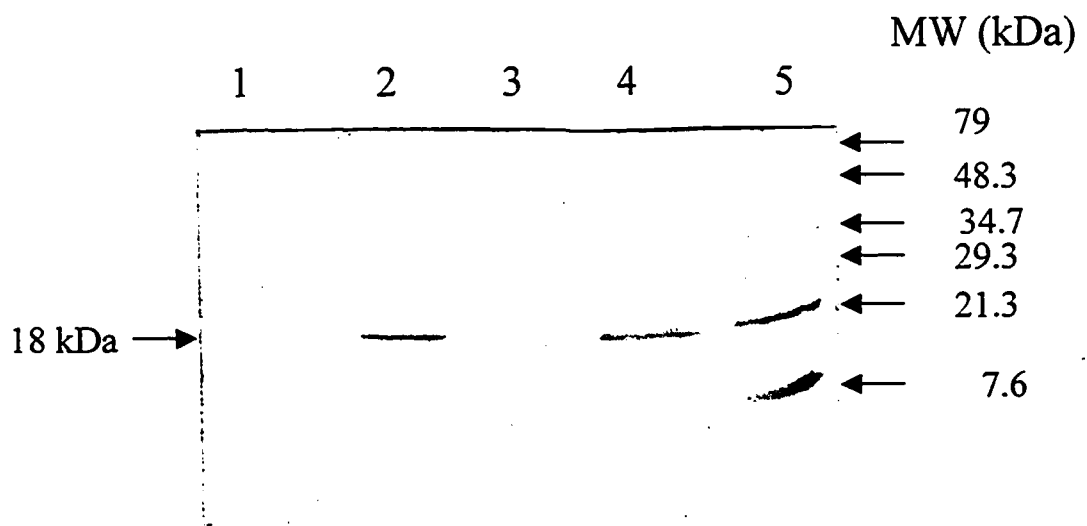
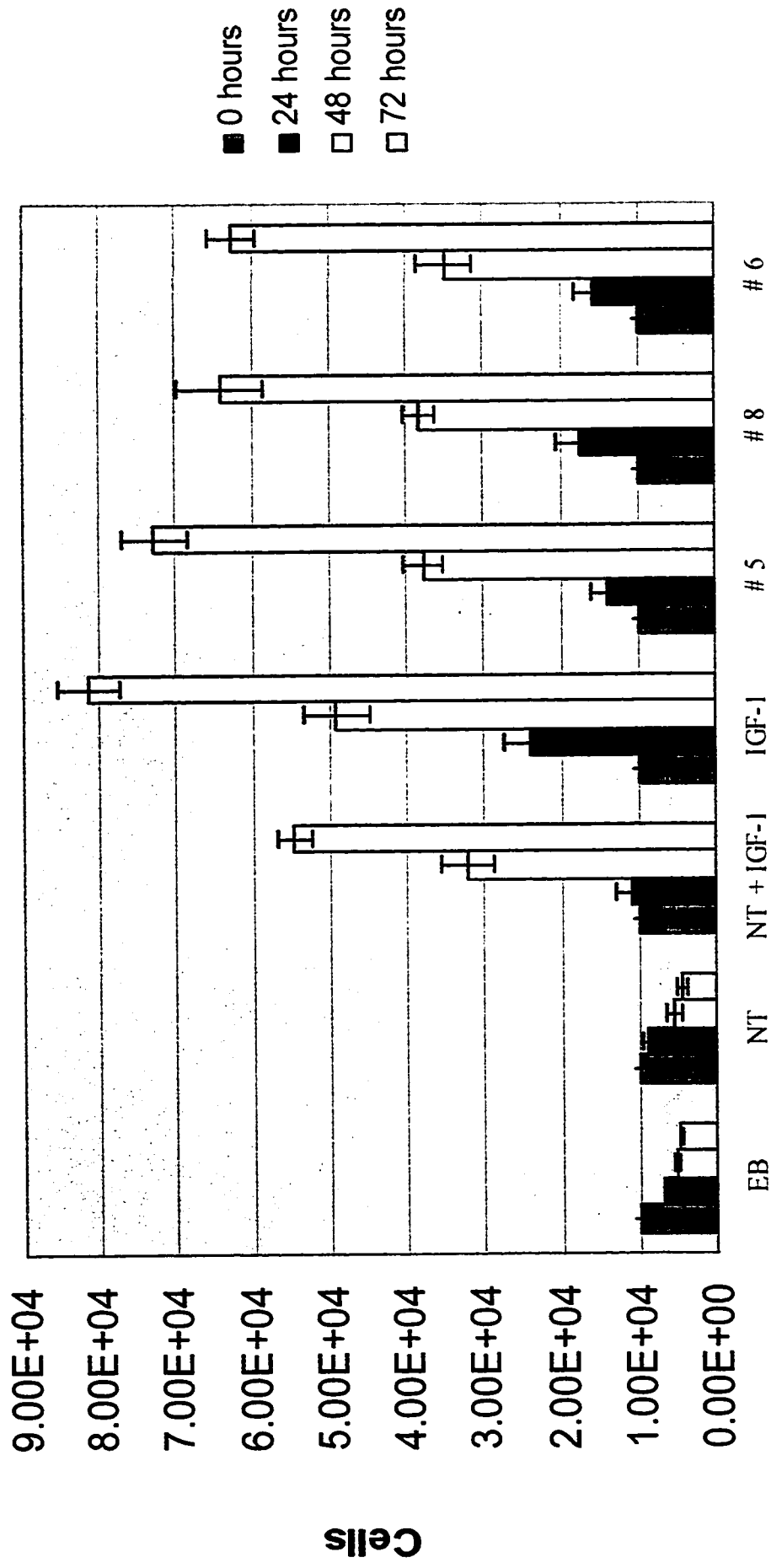


Figure 42. Biological activity of tobacco expressed human IGF-1B (R₁). The bioassays were performed on SH-SY5Y neuroblastoma cells. The results shown represent the proliferation of cells as measured at 24, 48, and 72 hours. The cells grown as suspension cultures were pipetted in triplicate into the wells of a culture plate. The cells were incubated in the presence or absence of aliquots of leaf extracts from transformed and non-transformed plants, and *E. coli*-derived IGF-1. All wells containing rthIGF-1 and rthIGF-1B had a final concentration of 10 ng/ml of rthIGF-1 and rthIGF-1B respectively. Cell number and viability were determined using haemocytometry/trypan blue exclusion. EB: leaf protein extraction buffer; NT: non-transformed leaf extract; #5, #8: leaf extracts from transgenic plants containing : maize ubiquitin promoter + IGF-1B + Nos-TER construct; #6: leaf extract from transgenic plant containing: maize ubiquitin promoter + Lam B signal sequence-IGF-1B + Nos-TER.

Biological Assay for R₁ IGF-1B Tobacco Plants



Samples

Figure 43. Analysis of transgenic rice plants.

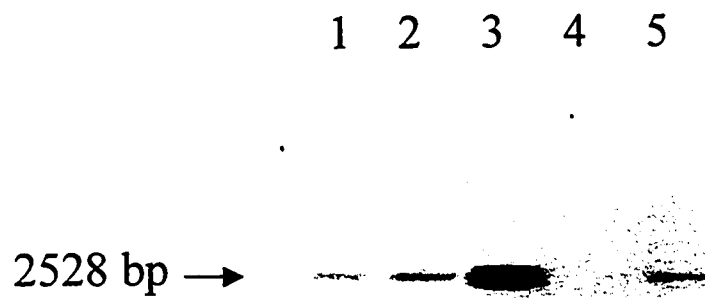
A: Southern blot analysis. Lanes 1, 2, 3: The *Hind* III insert released from the plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER) and reconstituted as 1, 5, 10 copy numbers respectively; lane 4: DNA from a non-transformed tobacco plant cleaved with *Hind* III; lane 5: DNA from transgenic plant (#9) cleaved with *Hind* III.

B: PCR detection of rthIGF-1 DNA in the leaves of transgenic rice plants.

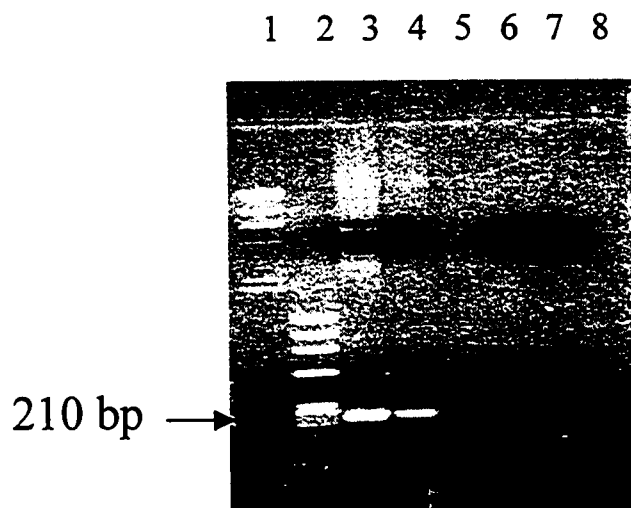
Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1 coding sequence (210 bp) amplified from a plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER); lane 4: DNA from transgenic plant, construct No. 2 (#9); lane 5: empty; lane 6: DNA from a non-transformed plant; lane 7: H₂O as negative control; lane 8: empty.

C: RT-PCR detection of the rthIGF-1 mRNA transcript in leaves of transgenic rice plants. Lane 1: Lambda DNA *Hind* III-digested marker; lane 2: ϕ X174 DNA *Hae* III-digested marker; lane 3: hIGF-1 coding sequence (210 bp) amplified from a plasmid containing construct No. 2 (maize ubiquitin promoter + IGF-1 + Nos-TER); lane 4: total RNA from transgenic plant construct No. 2 (#9); lane 5: empty; lane 6: total RNA from a non-transformed plant; lane 7: H₂O as negative control; lane 8: total RNA from transformed plant without the reverse transcription step.

A



B



C

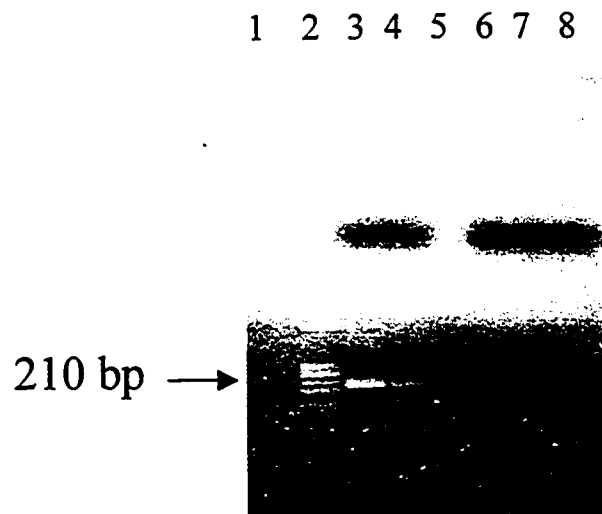


Figure 44. Immunoblot detection of partially purified, ammonium sulphate precipitated rthIGF-1 protein in rice leaf extracts.

Lane 1: leaf extract from a non-transformed plant; lane 2: leaf extract from an independent transgenic plant construct No. 2 (maize ubiquitin promoter + IGF-1+ Nos-TER) #9; lane 3: prestained SDS-PAGE standards (BIO-RAD); lane 4: *E. coli*-derived rthIGF-1 (7.5 kDa); lane 5: *E. coli*-derived rthIGF-1 added to leaf extract from a non-transformed plant.

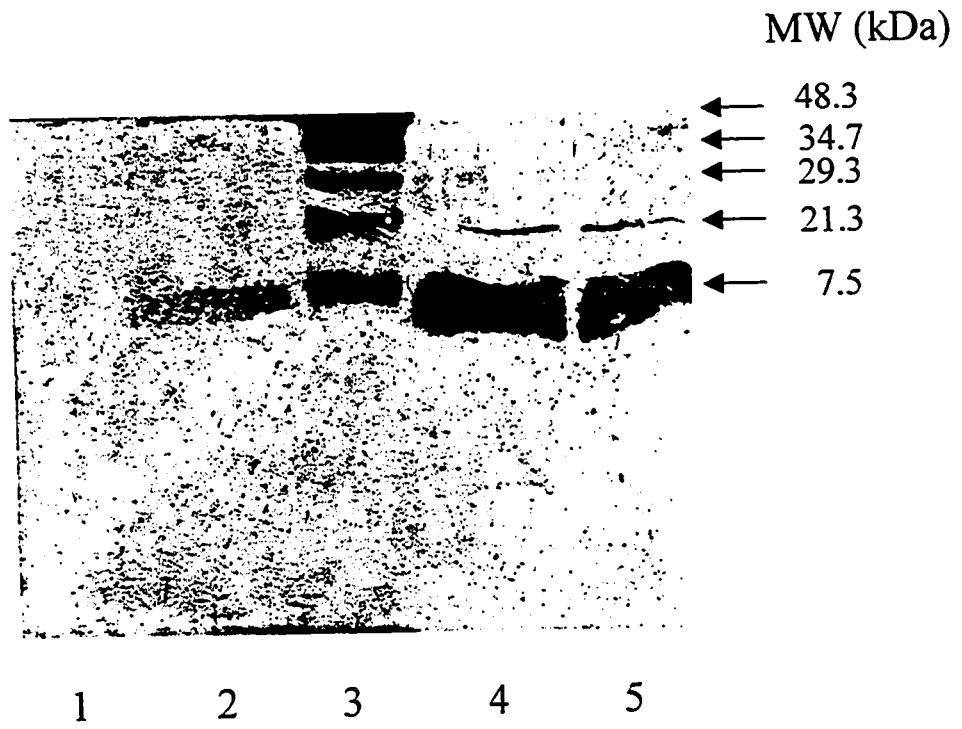
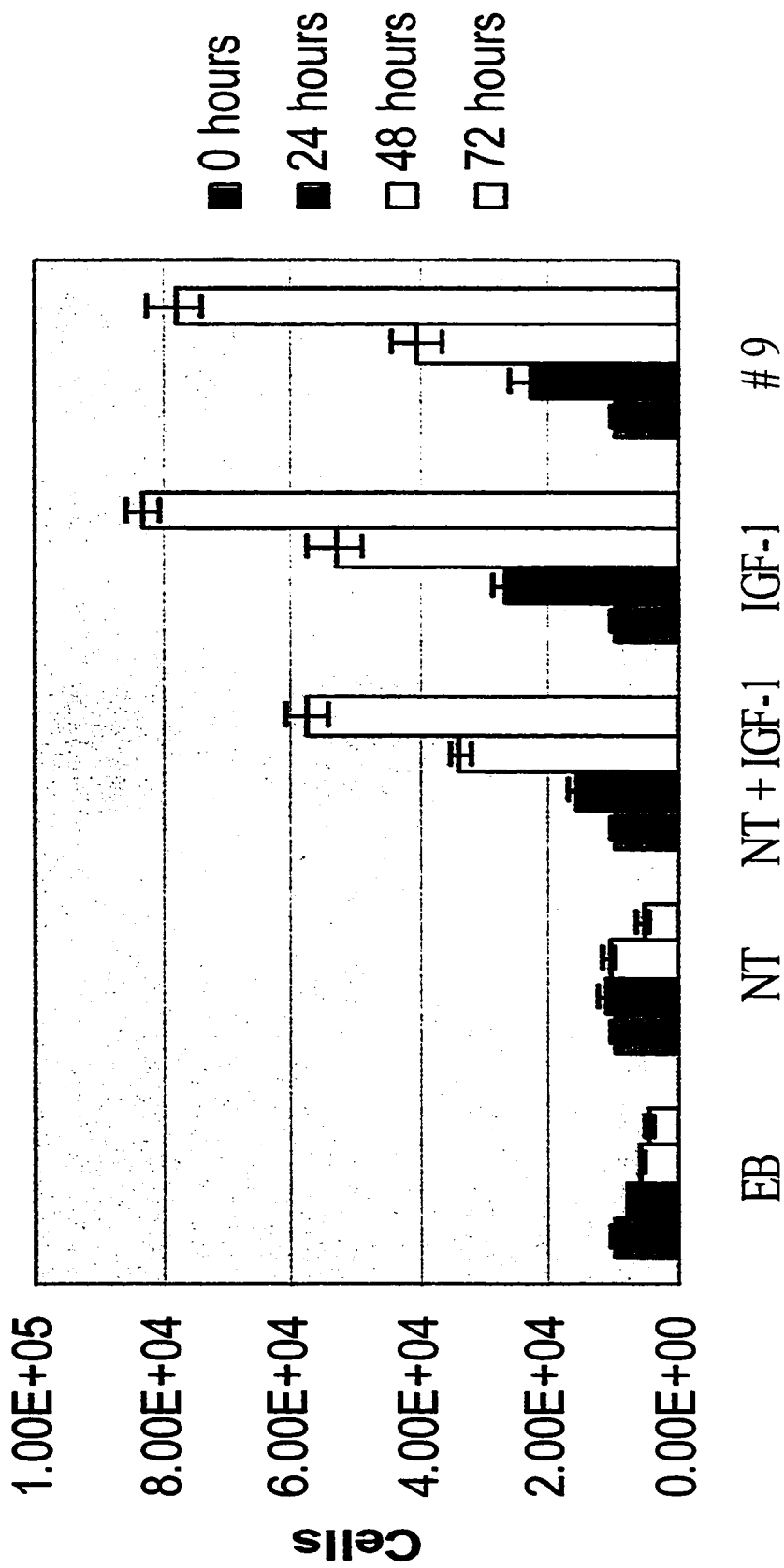


Figure 45. Biological activity of rice expressed human IGF-1. The bioassays were performed on SH-SY5Y neuroblastoma cells. The results shown represent the proliferation of cells as measured at 24, 48, and 72 hours. The cells grown as suspension cultures were pipetted in triplicate into the wells of a culture plate. The cells were incubated in the presence or absence of aliquots of leaf extracts from transformed and non-transformed plants, and *E. coli*-derived IGF-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: leaf protein extraction buffer; NT: non-transformed leaf extract; #9: leaf extract from transgenic plant containing: maize ubiquitin promoter + IGF-1 + Nos-TER construct.

Biological Assay for IGF-1 Rice Plant



Samples

Figure 46. Effect of plant-derived IGF-1 on cell proliferation and cell differentiation. Protein extracted from the leaves of transgenic rice plant (#9) was tested for its ability to proliferate and differentiate SH-SY5Y neuroblastoma cells. Panel A: *E. coli*-derived IGF-1 standard, 10 ng/ml; Panel B: non-transformed plant extract at equivalent protein concentration; Panel C: independent transgenic tobacco plant extract (#9), 10 ng/ml, Panel D: independent transgenic tobacco plant extract (#9), 50 ng/ml (at this concentration, proliferation was stopped and differentiation was observed). Magnification factor was 10 X.



Fig. 46

Table 4. Highest expressers of IGF-1

Plant #	Plant species	Promoter	Signal Sequence	Codon bias	IGF-1 (ng/mg Total Protein)	Southern PCR RT-PCR Western Biological assay
4	Tobacco	Ubi	-	<i>E. coli</i>	129	+
34	Tobacco	Ubi	Lam B	<i>E. coli</i>	76	+
7	Tobacco	Ubi	Rice prolamin	Plant	241	+
9	Rice	Ubi	-	<i>E. coli</i>	371	+

Table 5. Highest expressers of IGF-1B

Plant #	Plant species	Promoter	Signal Sequence	Codon bias	IGF-1B (ng/mg Total Protein)	Southern PCR RT-PCR Western Biological assay
5	Tobacco	Ubi	-	<i>E. coli</i>	43	+
6	Tobacco	Ubi	Lam B	<i>E. coli</i>	30	+

Chapter 4

Discussion

4.1. Exploring novel sources of human growth factor

A key role for molecular farming in modern biotechnology is its ability to increase the production levels of recombinant proteins to meet the market demand. Transgenic plants offer advantages for pharmaceutical biomolecule production, since plants can be grown economically on an agricultural scale and the recombinant macromolecules can be harvested from these crops. Plants can produce high levels of safe, functional, recombinant proteins and can be easily expanded to agricultural levels to satisfy medical and industrial applications and needs. Current applications of plants in biotechnology include the production of recombinant antibodies, enzymes, hormones, cytokines, plasma proteins and vaccines. Plant-based production of recombinant proteins requires only a virus-infected or transgenic plant, sunlight, mineral salts, air and water. Furthermore, modern agricultural practice enables easy scale-up, rapid harvesting and processing of large quantities of leaves or seeds (Pen, 1996).

This thesis is the first report of expression of recombinant human IGF-1 and its precursor IGF-1B in the leaves of transgenic plants.

4.2. High level expression of transgene proteins in plants

Expression of a particular candidate gene and subsequent translation of its mRNA in plant cells are affected by various factors. These include transgene copy number, transcriptional factors, promoter activity, untranslated regions (UTR), mRNA stability and translation, protein stability and modification. Promoters and enhancers play a crucial role in control of production of heterologous proteins at a particular plant growth and development stage or condition, or in a specific plant tissue (Dai *et al.*, 2000). Another strategy to improve expression in plants includes use of plant codon optimized gene sequences with increased GC content (Herbers and Sonnewald

1999). Some of these criteria are discussed in the following section.

4.2.1. Plant codon-optimized DNA sequences of the candidate genes

The ability to express foreign genes in plants depends on many factors. The successful expression of foreign genes in a variety of plants has been reported. However, this is not always the case. There is a growing list of foreign genes that are poorly expressed in plants. The genes best known for their low expression in plants are the *Bt* (*Bacillus thuringiensis*)-toxin genes, conveying insect resistance. Initial efforts to express *Bt*-toxin genes in plants yielded transgenic plants that produced little or no *Bt*-toxin mRNA or protein (Vaeck *et al.*, 1987). The problem appeared to be at the level of mRNA accumulation, because the plants that showed detectable *Bt*-toxin mRNA were also the most insect resistant (Vaeck *et al.*, 1987). Because of the potential agronomic importance of *Bt*-toxin genes, considerable effort has been made to increase the expression of these genes in plants. These efforts include expressing only the region of the gene encoding the insecticidal domain, modifying the 5' and 3' UTRs, generating protein fusions, and using a variety of strong promoters (Diehn *et al.*, 1998). The mRNA and protein levels were increased by resynthesizing the genes to be more "plant like". According to the codon usage database of a Japanese site (<http://www.kazusa.or.jp/en/database.html>), the GC content of the human genome is 53%, for the tobacco genome 44%, for rice 57% and for wild-type *Bt*-toxin genes 35%. Besides, tRNA (transfer RNA) varies from species to species. Therefore, changing the codon usage to a plant-preferred codon bias applied in most cases. This had the effect of raising the GC content of the wild-type gene. 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 (Diehn *et al.*, 1998). The transcripts of *cryIAb1* *Bt*-toxin gene was found to contain three cryptic introns. The inadvertent splicing of these transcripts was shown to be partly responsible for the low expression of this

gene in plants (Van Aarssen *et al.*, 1995). Therefore, by increasing the GC content to 49% for *cry1Ab1* and 47.7% for the *cry1Ac1* gene, a 100-fold increase was reported for expression in transgenic tobacco plants (Perlak *et al.*, 1990; Perlak *et al.*, 1991). The same codon modifications of these two toxin genes were successfully tested in maize suspension culture as a model monocot plant system (Sardana *et al.*, 1996). In this way it may be possible to design modified genes for each different plant species. However, the same modified (dicot codon optimized) versions of these toxin genes were examined in transformed rice genome. Accumulation of high levels (up to 3% of total soluble proteins) of Cry1Ab1 and Cry1Ac1 proteins was detected in R₀ plants (Cheng *et al.*, 1998). The relative half-lives of mRNA from a wild-type *cry1Ac1* and a highly modified synthetic *cry1Ac1* were determined by De Rocher *et al.* (1998). The wild-type *cry1Ac1* transcripts were degraded more rapidly than synthetic *cry1Ac1* transcripts in transformed BY-2 (Bright Yellow) tobacco cells, with a half-life comparable with that of transcripts known to be unstable in plants. Also, premature RNA synthesis resulted in termination of translation and subsequently in poor expression of wild-type *Bt*-toxin genes in plants. De Rocher *et al.* (1998) suggested that plant-codon optimization of transgenes can function in stabilizing their transcripts in plants. Therefore, all these factors are generally considered and used to determine a modified sequence.

In my thesis, chemical codon modification of the IGF-1 gene (the GC content of the hIGF-1 was increased from 58 to 70%) was modeled on a monocot codon bias to test for effect on expression. In one of the vectors the maize codon optimized IGF-1 coding sequence was preceded with a rice prolamin signal sequence. The long-term intent was to test it for protein accumulation in maize (both whole plant and cell culture) and rice seeds. Specifically, the codon usage of IGF-1 was redesigned according to the zeamatin cDNA by Dr. X. Cheng in Dr. Altosaar's lab.

Zeamatin is a major protein in corn that belongs to the group of pathogenesis-related (PR)

proteins (Batalia *et al.*, 1996). Pathogenesis-related (PR) proteins, are part of the plant's stress response and have been classified into five groups. Zeamatin is localized in the corn seed during maturation of the plant; there it probably protects the embryo from local fungal pathogens (Vigers *et al.*, 1992). Wilson *et al.* (2000) obtained 110 mg of purified zeamatin from 5 kg of corn seeds. This pilot-scale process for the purification of zeamatin was performed to obtain pharmaceutical grade zeamatin suitable for preclinical and clinical testing in humans. Zeamatin has the potential to be a novel antifungal compound that can be used either in single therapy or in combination with currently available antifungals. In the context of our protein farming project which might see one day bioreactor of maize endosperm cell culture, we found that zeamatin is one of the most abundant proteins expressed therein. On the basis of this fact alone, zeamatin was used to model the codon bias for the synthetic IGF-1 coding sequence. The zeamatin coding sequence has a GC content of about 70% which was attained in the synthetic IGF-1 construct.

When transgenic plants were analyzed by ELISA assay, the expression level of the IGF-1 construct which contained this plant-codon optimized IGF-1 coding sequence was almost two times higher than that of other constructs in transgenic tobacco plants where the codon usage had been left in the *E. coli* 'industrial' state (241 ng/mg of total protein vs. 129 ng/mg of total protein) (Table 2). The AT content of human IGF-1 is 42%, for *E. coli* optimized IGF-1 is 48%. The AT content of human IGF-1B is 45% and for *E. coli* optimized IGF-1B is 46%. Therefore, the high AT content of *E. coli* optimized IGF-1 and IGF-1B may be the reason for poor expression of these constructs in my plants. In the case of expression of *Bt*-toxin genes in plants, it was reported that these genes contained several runs of AATAAA. These sequences as poly (A) addition signals, are usually limited to the 3' untranslated region of eukaryotic genes. Besides, the AT sequences were found to be common in plant introns but rare in exons (Suton *et al.*, 1992).

These IGF-1 results are in agreement with the results of Sardana *et al.*, (1996) and Cheng

et al., (1998) where it has been shown that plant-codon optimized sequences supported higher expression levels.

4.2.2. Proper expression cassette

Several regulatory elements are critical to expression level in a transgenic system. These include: (a) constitutive or tissue specific promoter, (b) signal sequence, (c) enhancers, (d) advantageous position affects. In my thesis I explored two of these, (a) and (b).

(a). Choice of promoter, which mediates the timing, tissue-specificity, and level of transgene transcription, is a key determinant of transgene product yields and recovery strategies. The 35S promoter (from the cauliflower mosaic virus) was used to drive “constitutive” transgene expression in many proteins expressed in plants (Cramer *et al.*, 1999). The 35S promoter is quite active during seed development and has been used in production systems targeting recovery of recombinant proteins from seed. However, the 35S promoter has significant limitations when commercial bioproduction is the goal. Proteins that accumulate to high levels may negatively impact yield or overall health of the plant. Gene silencing phenomena resulting from the introduction of transgenes expressed under the control of 35S promoter have been discussed (Matzke and Matzke, 1995; Kumpatla *et al.*, 1997). Although the 35S promoter is active in monocot cells, its relative strength is substantially less than in dicot cells, and it is inactive in some cell types, e.g. pollen (McElroy and Brettel, 1994). The promoter from rice actin (*Act-1*) and maize ubiquitin (*Ubi-1*) have been shown to be significantly more active than the 35S promoter in monocot cells (McElroy and Brettel, 1994). Christensen and Quail (1996) made a variety of constructs with the *Ubi-1* promoter fused to a spectrum of selectable and scorable markers which have been used successfully in transforming a number of different monocot plants. For example, it was shown that this promoter was very active in rice by Cornejo *et al.* (1993). Also, expression of high levels of chicken egg white avidin and *E. coli* GUS (beta-glucuronidase) in maize embryos has been achieved using the maize ubiquitin promoter (Hood

et al., 1997; Witcher *et al.*, 1998).

In the present work, the maize ubiquitin promoter was used in both dicot transgenic tobacco and monocot transgenic rice plants. Use of a foreign monocot active promoter in a dicot host has been recently reported. For example, Leite *et al.*, (2000) expressed human growth hormone in transgenic tobacco seeds using the monocot tissue-specific promoter from sorghum gama-kafirin seed storage protein gene. The expression level for human growth factor was 0.16% of total soluble seed protein.

In this project, the ELISA data showed that the expression level of the construct which contains *E. coli*-optimized IGF-1 was higher in rice than the expression level of the same construct in tobacco (371 ng/mg of total protein vs. 129 ng/mg of total protein). This 3-fold difference may be due to better functioning of the maize ubiquitin promoter in rice as a homologous monocot plant than in tobacco which is a dicot plant.

(b). Signal sequences. Targeting may allow the protein of interest to be compartmentalized into specific organelles where it can avoid being rapidly degraded or interfering with cell metabolism. Target sequences can play a vital role in protein accumulation. Adding target sequences has led to increased levels of protein expression in some studies which were mentioned in Chapter 1.

For this thesis, a rice prolamin signal sequence was used as a signal peptide for construct No. 3 (Fig. 16). This signal sequence was based on a cDNA encoding the rice 13 kDa prolamin (Masumura *et al.*, 1990). Albumin (16 kDa), prolamin (13 kDa) and type II glutelin are the major storage proteins in rice. There are some conserved sequences in the 5'-flanking regions of these three rice seed storage protein genes (Nakase *et al.*, 1996). Use of such a monocot signal peptide should facilitate transport into the secretory pathway, thus enhancing protein deposition into the storage vesicles of endosperm cells within rice grains. Use of rice prolamin sequence as a signal sequence targeting a candidate protein into the protein bodies of transgenic seeds has been reported. Zhou and Fan (1993) fused the 5'-flanking region of the prolamin gene with the

beta-glucuronidase reporter gene. The resultant chimeric gene was used to transform tobacco. Histochemical analysis revealed the GUS activity in the endosperm tissues of tobacco seeds.

In the present work, the rice prolamin signal sequence was incorporated to direct the candidate protein into the reserve protein fraction of rice seeds. Such transgenic rice plants will eventually be harvested in order to test for the expression of IGF-1 in rice.

Also, the bacterial Lam B signal sequence was used in other constructs to see whether a prokaryote signal peptide could function directly in plants. There have been 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 (a) bacterial alpha-amylase using the the signal sequence of the same gene (Pen *et al.*, 1992), (b) human monoclonal antibody IgG using barley alpha-amylase signal peptide (During *et al.*, 1990) and (c) human serum albumin using tobacco PR-S (pathogenesis-related protein) signal peptide (Sijmons *et al.*, 1990). Mori and Cline (1998) used a signal peptide from *E. coli* that directed the thylakoid (chloroplast inner envelope membrane) targeting in plants. They suggested that the alternate targeting pathways in prokaryotes and plant thylakoids are analogous.

The present results showed that the DNA expression constructs containing a bacterial signal sequence also were successfully expressed in plants but the expression level of these constructs was lower than that of constructs without bacterial signal sequences (Tables 2, 3). It was shown by Bielefeld and Hollenberg (1992) that the bacterial signal sequence was not functional in yeast. When the bacterial signal sequence was replaced with the signal sequence of yeast, the secretion of the beta-lactamase protein increased by 75%.

In my work, the bacterial Lam B signal sequence was functional in tobacco plants, but the expression level was lower than the constructs without the bacterial signal sequence. For example, the expression level for plant #34 which contained the bacterial signal sequence was 76

ng/mg of total IGF-1 protein while plant #4 devoid of signal sequence made 129 ng/mg of total IGF-1 protein. According to these data there is almost a two-fold increase in the expression level of the proteins when the bacterial signal sequence is spliced out. Perhaps the presence of a bacterial signal sequence in the plant's protein formulation system is the reason for lower expression level of the constructs containing bacterial Lam B signal sequence for both IGF-1 and IGF-1B plants. Further analysis should provide a better idea of this phenomenon, for example, if the bacterial signal sequence was properly cleaved or incompletely processed, this will affect the level of protein accumulation. Since the constructs containing the bacterial signal sequence express the protein albeit at low levels, one can accept that the plant host system is a potential bioreactor in which to express any foreign protein without any prior codon modification is possible. Pre-commercial feasibility production, field trials could assess the logistical advantages of launching fermentor-type constructs in the field instead.

4.2.3. Transformation efficiency

Plant cell transformation technologies are important tools for the genetic manipulation and improvement of crop species. Various transformation methods allow the direct transfer of foreign genetic material into plant cells and are capable of giving rise to fertile plants. But, sometimes the transgene inactivation becomes a frequently observed phenomenon (Srivastava *et al.*, 1999). Though, not well understood, certain factors affecting transgene inactivation have been described that include multiple-copy integration. There is a much higher incidence of gene instability correlated with high transgene copy number, a common feature of biolistic-derived transgenics (Atkinson *et al.*, 1998).

In contrast, *Agrobacterium*-mediated transformation often results in single-copy or low-copy integration of full-length T-strands (transfer-DNA) carrying intact copies of the transgenes (Hansen *et al.*, 1997). Since *Agrobacterium* offers many such advantages for plant transformation, an *Agrobacteria*-mediated transformation system with these IGF-1 and IGF-1B

constructs was developed for tobacco and rice transformation in this project.

After co-cultivating leaf sections with *A. tumefaciens* harboring the IGF-1 and IGF-1B constructs, the selection process was initiated. From the growth of transgenic plants it appeared that the concentration of the two selection antibiotics was adequate for these experiments.

No phenotype abnormalities were observed for any of the regenerated plants. This may indicate that the insertion site(s) of IGF-1 and IGF-1B expression constructs did not disrupt any genes that are responsible for the normal phenotype characteristics. Besides, germination of the transgenic tobacco seeds indicated that the parental lines were fertile and viable. The second generation of transgenic tobacco plants also showed normal phenotypic characteristics.

4.3. Analysis of transgenic plants

In this present work, resultant transgenic plants were subjected to extensive molecular characterization regarding the integration of the introduced gene, copy number, transcription and translation process, protein synthesis and biological activity by using molecular biology methods such as Southern, PCR, RT-PCR, Western, ELISA and Biological Assay. These analyses were performed for both R_0 and R_1 generations of transgenic tobacco plants. The obvious problem of gene silencing for commercial production of proteins from transgenic plants suggests that, to obtain the commercial elite line of interest, one must select for not only high-expressing lines, but also for lines that are stable over many generations. For practical reasons, the stability of the transgenes was tested in transgenic tobacco at least over one generation.

4.3.1. Southern analysis

The transgenic nature of the plants was verified by performing Southern analysis. Southern analysis served several purposes. Southern blots confirmed that the transformed plants acquired the IGF-1 and IGF-1B constructs and the antibiotic selection procedure was effective. Further, the integrity of the expression constructs was deduced from Southern blot analysis and

this indicated the integration of the expected size. The data suggest the absence of any rearrangements in transgenic plants. It seems that in most cases there is one copy of the transgene which reflects the advantage of using *A. tumefaciens* transformation.

4.3.2. PCR analysis

The transgenic plants were verified for transgene integration using PCR amplifications with primers specific for each construct. The bands of expected sizes were obtained and no band was observed in non-transformed plants.

4.3.3. RT-PCR analysis

To verify the expression of transgenes, the presence of transgenic mRNA was shown by reverse transcriptase-PCR amplification. The amplified fragments of the expected sizes suggested that the transgenes were being actively transcribed in the leaves of transformed tobacco plants. The amplified fragments were not seen in non-transformed plants. To further confirm that the RT-PCR step was true and the mRNA was not contaminated with DNA, no product was PCR amplified when only mRNA was used as a template for the PCR reaction. The RT-PCR results indicated that the mRNA was intact and that the RT-PCR steps were authentic.

4.3.4. ELISA

By using an ELISA assay, the transgenic plants were analyzed to find the high expressers for each group of constructs. It is noteworthy that the ELISA kit, designed for IGF-1 and containing antibodies specific for IGF-1, was also used for the analysis of IGF-1B plants. It was shown that IGF-1B cross-reacts with antisera directed toward IGF-1 (Atkinson *et al.*, 1980; Clemmons *et al.*, 1981; Clemmons and Shaw, 1986). On the other hand, the suppliers of recently commercially available specific antibodies against IGF-1B mention the cross-reaction of these antibodies with IGF-1 (Research Diagnostic Inc. Catalog # RDI-ILGF1). Since IGF-1B as the

precursor of IGF-1 contains the IGF-1 itself, it is logical to accept that the antibodies against IGF-1 show cross-reaction with IGF-1B and vice versa. Also, it was reported that the high titer, specific antiserum, raised against a synthetic analog of a unique peptide region within the human IGF-1B, detected specific immunoreactivity in extracts of mouse, chicken, sheep, and human liver. This observation was used to make a cautionary note for using IGF-1B specific antibodies by Quinn *et al.* (1996).

The sensitivity of the assay was reported to be 0.03 ng/ml of rthIGF-1 by the supplier of the ELISA kit (Active™ IGF-1 ELISA, Diagnostic Systems Laboratories, Inc.). The leaves of all transformed tobacco plants produced recombinant proteins up to 241 ng/mg of total protein for IGF-1 and 43 ng/mg of total protein for IGF-1B. This indicated that every transgenic plant had the capacity to accumulate the human growth factors.

To roughly compare the level of expression of different proteins in different plants, the observed expression levels of some recombinant proteins is shown in Table 6. It is clear that some of the reasons for the differential performance of the different plant systems may include using different promoters, gene transfer methods, gene copy number, host genotype, numbers of transformants screened, and plant growth conditions.

4.3.5. Western blot

To test if the leaves of transgenic plants produced the transgene products, Western blot analysis was performed. By using ammonium sulphate precipitation, it was shown that hIGF-1 precipitated at 35% and hIGF-1B at 50% ammonium sulphate saturation. The ammonium sulphate precipitation experiments should result in the removal of some plant proteins and does not result in a homogeneous form of the plant synthesized rthIGF-1 and rthIGF-1B. Therefore, these experiments were conducted to obtain only partially purified recombinant proteins from the transgenic leaf extracts. In case of IGF-1B plants, immunoprecipitation was also applied. In these plants the levels of the expressed protein were lower than in IGF-1 plants. Therefore, the

Table 6. Transgene expression strategies and recombinant protein yield

Transgene product	Plant host	Promoter	Production levels (% of soluble protein)	Reference
Human Epidermal growth factor	Tobacco	35S	0.01%	Higo et al., 1993
Trout Growth hormone	Tobacco	35S	0.1%	Bosch et al., 1994
Hirudin	Canola	Oleosin	0.3%	Parmenter et al., 1995
Human Glucocerebrosidase	Tobacco	MeGA*	1%-10%	Cramer et al., 1996
Avidin	Maize	Maize ubiquitin	0.2%	Hood et al., 1997
Bt	Rice	Maize ubiquitin	3%	Cheng et al., 1998
GUS	Maize	Maize ubiquitin	0.7%	Witcher et al., 1998
Human α_1 -antitrypsin	Rice	Amy3D (rice α -amylase)	0.046-0.057%	Terashima et al., 1999
Human glutamic acid decarboxylase (GAD65)	Tobacco	35S	0.01%-0.04%	Porceddu et al., 1999
Human growth factor	Tobacco	gama-kafirin	0.16%	Leite at al., 2000

*This promoter is an inducible promoter that has been modified from a defense-related gene by CropTech (US. Patent 5670349).

recombinant protein could be distinguished on western blot by using immunoprecipitation. The size of the expressed protein was the same as the size of the standard for IGF-1 protein. For different constructs of IGF-1 and IGF-1B, the size of the expressed protein was similar, e.g. the size of the expressed protein from the construct containing Lam B signal sequence and from the construct without Lam B signal sequence or from the construct containing rice prolamin signal sequence was similar. This might be due to the inability of polyacrylamide gel to separate proteins with only small differences in size (2-3 kDa). Therefore, PAGE can not show the exact cleavage of the signal peptide in this case which should release the mature recombinant protein in plants. Ideally, N-terminal peptide sequencing can confirm the cleavage of the signal peptide. The proper cleavage of the signal peptide will result in the recombinant proteins being secreted into the lumen of the endoplasmic reticulum (ER). Through electron microscopy studies, it should be possible to find the exact subcellular locations of these recombinant proteins.

In case of the commercial standard protein, an extra band was observed that was reported by others (Brem *et al.*, 1994). However, for expressed protein in transgenic plants, just one single band was observed. In a personal communication with Dr. H. Kaplan (Dept. of Chemistry, University of Ottawa) it was mentioned that during the process of freeze-drying of some proteins, different isomers of proteins can be made and perhaps this extra band in the standard protein is a result of this process. In the case of IGF-1B expression, no standard protein was available and the size of the plant-expressed protein was 18 kDa (Fig. 35, Fig. 41). Clemmons and Shaw (1986) reported the molecular weight as 21.5 kDa for the human fibroblast-derived IGF-1B containing the signal peptide. Rotwein (1986) used a synthetic oligonucleotide probe and isolated the human IGF-1 cDNA clones from a liver library. Two IGF-1 precursors were obtained as IGF-1A and IGF-1B. The size of IGF-1B was 21.8 kDa.

4.3.6. Biological activity of recombinant protein from plants

Biological assays were performed to determine if the recombinant proteins produced in

transgenic plants were biologically active. In the case of IGF-1 expressed in transgenic plants, the data suggested that the human IGF-1 produced and stored in plant cells was functional and active. The SH-SY5Y neuroblastoma cell line is an IGF-1 dependent cell line that was previously used in biological assays (Lavie and Agranoff, 1996). The results in Figures 25 and 26 indicated proliferation of the SH-SY5Y cells was only possible when rthIGF-1 from *E. coli* or from transgenic plants was added to the medium. These experiments showed that SH-SY5Y cells required rthIGF-1 for their proliferation and that no SH-SY5Y cell proliferative factor(s) were present in the normal NT leaf extracts. In addition to the NT extracts not having proliferative capacity on SH-SY5Y cells, it appeared they diminished the biological activity of the *E. coli*-derived rthIGF-1 on SH-SY5Y cells (Fig. 25). This shows that compound(s) in the NT tobacco leaf extracts may reduce the biological activity of *E. coli*-derived rthIGF-1. These observations would suggest that biological activity of plant-derived rthIGF-1 is also similarly affected. This was also reported by Ganz *et al.*, (1996) in case of tobacco-derived GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor).

When the concentration of plant-derived rthIGF-1 was increased from 10 ng/ml to 50 ng/ml, the proliferation of the SH-SY5Y cells stopped and differentiation of the cells began. This mimicked exactly the role of IGF-1 in the cell cycle due to IGF-1's role in promotion of differentiation of SH-SY5Y cells (Pahlman *et al.*, 1991).

For biological assay of IGF-1B plants, the same SH-SY5Y cell lines were used. Others have also used the same cell lines for both IGF-1 and IGF-1B biological assays (Siegfried *et al.*, 1992). The biological assay for IGF-1B expressed in transgenic plants showed that the protein is biologically active and also mimics the pattern of IGF-1 in proliferation and differentiation of SH-SY5Y cell lines (Fig. 36, 37). This observation is important and brings to mind the possible clinical application of IGF-1B. It also raises more questions about the role of IGF-1B in different tissues and its relationship with IGF-1 regarding its mitogenic activity.

The biological assay of rthIGF-1 in rice was performed in the same way as was done for

rthIGF-1 in tobacco plants. The SH-SY5Y cell line was used and a similar pattern of rthIGF-1 activity was observed for rice. The effect of the NT leaf extract on *E. coli*-derived rthIGF-1 was significant in decreasing the proliferative effect. This again suggests that the leaf extract from NT plant contained substance(s) that diminished the activity of the *E. coli*-derived rthIGF-1. When the concentration of rthIGF-1 in transgenic rice was increased from 10 ng/ml to 50 ng/ml, the proliferation of the cells stopped and differentiation of the cells commenced (Fig. 46). The rthIGF-1 protein produced by mouse fibroblasts (Bayne *et al.*, 1987) and by transgenic rabbits (Brem *et al.*, 1994) was reported to be biologically active by using IGF-1 dependent cell lines.

4.4. Possible clinical application of IGF-1B

Rotwein *et al.*, (1986) suggested that tissue-specific factors may play a role in IGF-1 biosynthesis by influencing RNA splicing or protein processing and that the extension peptides themselves may show discrete biological functions. As mentioned before, the significance of the alternatively spliced forms of the IGF-1 gene are unclear. It is possible that they may differ in stability or rates of translation. These two IGF-1 precursors may have different functionally important roles. For example, they may interact differently with the IGF-1 receptor or binding proteins. It is also possible that the carboxyl terminal peptides or E-peptides generated by cleavage from the mature IGF-1 may have some biological actions (Glazebrook and Brindle, 1993). It was shown by Siegfried *et al.*, (1992) that a peptide based on part of the carboxyl terminal sequence of IGF-1B (103-124 aa) (Fig. 5) had potent mitogenic action on a lung cell line. They suggested that this part (IBE1) was a growth factor for human lung cells and also was active in liver extracts from nonhuman species. Therefore, IBE1 might be potentially a growth factor in other species. Another part of the carboxyl terminal sequence of IGF-1B designated as IBE2 (129-142 aa) (Fig. 5) was also biologically active. A synthetic analog of this peptide stimulated proliferation of human colon cancer cells. The IGF-1 gene, like the genes for glucagon and proopiomelanocortin (Miller *et al.*, 1983) produces multiple peptide products that

could have distinct physiological effects in different tissues. Other peptides are known that have multiple functions in different systems. For example, gastrin-releasing peptide is a fatal lung mitogen, a neuroendocrine hormone in the adult gut and an autocrine growth factor in small cell lung carcinoma (Cuttitta *et al.*, 1990). It is possible that IBE1 has functions distinct from IGF-1, with distinct organ or cell specificity. IGF-1B was expressed in transgenic mice to show the effect of overexpression of IGF-1 in myocytes which leads to an increased number of cells in heart (Reiss *et al.*, 1996) and therefore, this process may influence positively the performance of myocytes (Redaelli *et al.*, 1998).

Also, three recombinant E-peptides of proIGF-1A of rainbow trout, *Oncorhynchus mykiss*, were produced *in vitro* and showed mitogenic activity in a variety of cell lines. The 20 amino acid residues at the C-terminus of these peptides share 70% identity with their human counterparts (Tian *et al.*, 1999).

The biological assay of rthIGF-1B expressed in transgenic tobacco plants showed that expressed IGF-1B is biologically active. When administered in even higher concentrations, it caused the differentiation of the cell line, similar to the effect of IGF-1. Therefore, by expressing IGF-1B in transgenic plants further investigation can be done to verify the biological activity of this protein and its relationship to IGF-1. Such an abundant and economical supply may lead to possible clinical applications of this protein in future.

4.5. Conclusion and future applications

Human IGF-1 and its precursor IGF-1B were expressed in transgenic plants. The data showed that plants are a feasible alternative expression system to produce these human growth factors. The data also showed that IGF-1B expressed in plants has mitogenic activity similar to

IGF-1 expressed in plants, a phenomenon which raises more questions about IGF-1B and demands further investigation.

In my project the constructs were first put in tobacco plants. Tobacco is the standard host

that is widely used to test suitability of plant-based systems for bioproduction of recombinant proteins. At least three plant-based Biotech companies are transforming tobacco for biopharmaceutical production (CropTech Crop Inc., Large Scale Biology Inc. and Planet Biotechnology Inc.) (Cramer *et al.*, 1999).

Also, the expression of IGF-1 (Fig. 16, construct No. 2) was tested in rice. According to analysis of transgenic rice plants, the expression was successful. In future, the constructs can be modified in a way that IGF-1 will be targeted into the tobacco seeds or rice seeds (Fig. 16, construct No. 3). Several companies are developing production strategies involving transgene product accumulation in seeds. Companies targeting seed-based production using canola, corn or soybeans include SemBioSys Genetics, Agracetus (USA), Mogen International (The Netherlands), and Plantzyme (The Netherlands). Applied Phytologics (API, Davis, CA) is using transgenic rice and barley seed. Other crops being developed for biopharmaceutical protein or vaccine production include alfalfa, banana, potato, and tomato (Cramer *et al.*, 1999). Therefore, the construct containing the maize ubiquitin promoter, rice prolamin signal sequence + synthetic IGF-1 is a good choice for expression in a monocot plant. Higher accumulation may be obtained by expression of this cassette system in a monocot plant. As mentioned, in the work of Leite *et al.*, (2000), who expressed human growth hormone in transgenic tobacco seeds using the monocot tissue-specific promoter, the expression level was about 1/12 of the yield reported by Fiedler *et al.*, (1993) for the expression of an immunoglobulin in transgenic tobacco seeds. In the latter case, the promoter of the legumin gene, which encoded a dicot seed-storage protein, was used.

In summary the questions which were posed in the introduction section (page 32) as the goals of this thesis can now be addressed. It is possible to express human growth factors in both experimental plants like the dicot *Nicotiana tabacum* and commercially developed agricultural monocot like *Oryza sativa* (rice). To shorten the time for construction of plant expression

cassettes, the industrial coding sequences designed for *E. coli* can be used. Using a monocot promoter like the maize ubiquitin promoter in a dicot plant like tobacco is possible but the expression level is lower than when a monocot promoter is used in a monocot plant. And finally, plant-codon optimization affects the expression level dramatically. Table 7 summarizes the expression level of IGF-1 in different hosts.

Regarding the high expression level, 46.1%, of *Bt* gene in tobacco chloroplast by DeCosa *et al.* (2001), this type of transformation should be tried for human growth factor expression in transgenic plants. Also, if the IGF-1 transgenic plants could be grown in open field trials, the expression level might also be considerably higher than growing the plants in small green houses.

4.6. Prognosis

This thesis adapted the strategy of nuclear-controlled transformation because at the outset no sure-fire means of organellar transformation was available. It is important to note that recent advances in chloroplast transformation may have a strong bearing on the protein molecular pharming field.

Table 7. IGF-1 expression in various hosts

• <i>E. coli</i>	5.0 %	Joly <i>et al.</i> , 1998
• Yeast	1.0	Gill <i>et al.</i> , 1999
• Mammalian cell	0.007	Bayne <i>et al.</i> , 1987
• Milk	5-10	Brem <i>et al.</i> , 1994, Zinovieva <i>et al.</i> , 1998
• Plants	0.01-0.03	Panahi, Thesis 2001
• Chloroplast	46.1	De Cosa <i>et al.</i> , 2001

Appendix : Preparation and composition of the media

20 X AB buffer

60 g/L K_2HPO_4
20 g/L NaH_2PO_4

AB medium

5g/L glucose
50 ml/L AB buffer
50 ml/L AB salts

20 X AB salts

20 g/L NH_4Cl
6g/L $MgSO_4 \cdot 7 H_2O$
3 g/L KCl
0.2 g/L $CaCl_2$
50 mg/L $FeSO_4 \cdot 7H_2O$

AP buffer

100 mM Tris-Cl (pH 9.5)
100 mM $NaCl$
50 mM $MgCl_2$

blocking buffer

20 mM Tris (pH 7.4)
0.9% $NaCl$
0.25% gelatin
0.1% Triton-X-100 (v/v)
0.02% SDS

Coomassie blue staining solution

0.25% Coomassie Brilliant Blue R250 in:
10% acetic acid
45% methanol
45% H_2O

Coomassie blue destaining solution

10% acetic acid
45% methanol
45% H_2O

5 X Denhardt's solution

0.1% Ficoll
0.1% polyvinyl-polypyrrolidone (PVPP)
0.1% bovine serum albumin (BSA)

DNA extraction buffer

100mM Tris-Cl pH 8.0
5 mM EDTA (ethylene diamine tetraacetic acid)
2% PVPP
1% ascorbic acid
0.2% beta-mercaptoethanol

10 X DNA loading buffer

50% glycerol
0.1 M Tris pH 8.0
0.01 M EDTA
0.1% bromophenol blue
0.1% xylene cyanol

DNA lysis buffer

140 mM sorbitol
220 mM Tris-Cl pH 8.0
22 mM EDTA
800 mM NaCl
0.8% C-TAB (hexadecyltrimethyl-ammonium bromide)
1% Sarkosyl
50 ug/ml Proteinase K
1% PVPP

LB/agar plates

LB medium + 1% agar

LB medium

10 g/L bactotryptone
5 g/L yeast extract
10 g/L NaCl
pH 7.0

LHT medium

4.3 g/L MS (Murashige and Skoog) major and minor salts
1 mg/L thiamine.HCl
100 mg/L myo-inositol
30 g/L sucrose
2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid)
50 mg/L hygromycin

200 mg/L ticarcillin disodium
3 g/L Phytigel
pH 5.8

LRHT medium

4.3 g/L MS major and minor salts
1 mg/L thiamine.HCl
100 mg/L myoinositol
20 g/L sucrose
2 mg/L kinetin
0.5 mg/L IAA (indole-3-acetic acid)
50 mg/L hygromycin
100 mg/L ticarcillin disodium
3 g/L Phytigel
pH 5.8

MS Callus Induction Medium

4.3 g/L MS (Murashige and Skoog) basal medium
30 g/L sucrose
2 mg/L 2,4-D (2,4-Dichlorophenoxyacetic acid)
3 g/L Phytigel
pH 5.2

MS solid medium

4.3 g/L MS salt
3% sucrose
pH 5.8
agar 0.4%

2N6-AS medium

4.3 g/L N6 major salts
4.3 g/L N6 minor salts
0.01% N6 vitamins
1 g/L casamino acids
30 g/L glucose
100 uM acetosyringone
2 mg/L 2,4-D
3g/L Phytigel
pH 5.2

PBS

11.5 g/L Di-sodium hydrogen orthophosphate anhydrous
2.96 g/L sodium dihydrogen orthophosphate
5.84 g/L NaCl
pH 7.5

Protein extraction buffer

50 mM Tris pH 7.5
50 mM NaCl
1 mM PMSF
glycerol 100%
20 mM DTT
5 mM NH₄Cl

Protease Inhibitor Cocktail from Sigma containing AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride), E-64 [trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane], bestatin, leupeptin, aprotinin and sodium EDTA (Sigma, catalog # P2714).

RNA loading buffer

0.75 ml deionized formamide
0.15 ml 10 X MOPS [3-(N-morpholine) propanesulfonic acid] running buffer
0.24 ml formaldehyde
0.1 ml DEPC (diethylpyrocarbonate)
0.1 ml glycerol
0.08 ml of 10% bromophenol blue

RNA 10 X running buffer

0.2 M MOPS
10 mM EDTA
50 mM sodium acetate
pH 7.0

SDS gel-loading buffer

50 mM Tris-Cl (pH 6.8)
2% SDS
0.1% (w/v) bromophenol blue
10% glycerol
10% 2-beta-mercaptoethanol

20% separating SDS-PAGE gel

0.69 ml H₂O
6.66 ml of 30% acrylamide mix (29.0% acrylamide and 1.0% Bis-acrylamide, BIO-RAD)
1.3 ml of 1.5 M Tris-Cl (pH 8.8)
0.05 ml of 10% SDS
0.05ml of 10% ammonium persulphate
0.004 ml TEMED (N, N, N', N'-tetramethylethylenediamine)

Southern blot denaturing buffer

20 g/L NaOH
87.66 g/L NaCl

Southern blot neutralization buffer

60.5 g/L Tris pH 7.4
87.66 g/L NaCl

Southern blot hybridization buffer

50% deionized formamide
5 X SSC
0.1% SDS
100 pg/ml denatured salmon sperm DNA

Southern blot prehybridization buffer

50% deionized formamide
5 X Denhardt's solution
5 X SSC
0.1% SDS
100 pg/ml denatured salmon sperm DNA

5% stacking SDS PAGE gel

3.4 ml H₂O
0.83 ml of 30% acrylamide mix
0.63 ml of 1.0 M Tris-Cl (pH 6.8)
0.05 ml of 10% SDS
0.05 ml of 10% ammonium persulphate
0.005 ml TEMED

20 X SSC

0.3 M NaCl
300 mM sodium citrate
pH 7.0

TA buffer

0.33 M Tris pH 7.9
0.66 M potassium acetate
0.1 M magnesium acetate
0.04 M spermidine
5 mM DDT (dithiothreitol)

50 X TAE buffer

242 g Tris
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA pH 8.0

TE buffer

10 mM Tris-Cl

1mM EDTA
pH 8.5

Tobacco rooting medium

4.3 g/L MS salt
2% sucrose
pH 5.8
agar 0.8%
autoclave for 15 min at 121°C
cool to 65°C, then add
500 ug/ml ticarcillin disodium, and
300 ug/ml kanamycin

Tobacco selection and regeneration medium

4.3 g/L MS salts
3% sucrose
1.0 mg 6-Benzylaminopurine (BA)
0.1 mg/L alpha-naphthalene acetic acid (NAA)
pH 5.8
agar 0.5%
autoclave for 15 min at 121°C
cool to 65°C, then add
500 ug/ml ticarcillin disodium
300 ug/ml kanamycin

Transfer buffer for Western blot

39 mM glycine
48 mM Tris-Cl
0.037% SDS
20% methanol
pH 8.3

Wash buffer for Western blot

20 mM Tris (pH 7.4)
0.9% NaCl
50 ul Tween 20

YEP/agar plates

YEP medium + 1.5% agar

YEP medium

10 g/L yeast extract
10 g/L peptone
5 g/L NaCl
pH 7.0

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