

High-yield production of authentic human growth hormone using a plant virus-based expression system

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Summary

We describe here a high-yield transient expression system for the production of human growth hormone (hGH, or somatotropin) in transfected *Nicotiana benthamiana* leaves. The system is based on a recently described plant virus-based modular expression vector [Gleba, Y., Marillonnet, S. and Klimyuk, V. (2004) Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Curr. Opin. Plant Biol.* **7**, 182–188; Marillonnet, S., Giritch, A., Gils, M., Kandzia, R., Klimyuk, V. and Gleba, Y. (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. *Proc. Natl. Acad. Sci. USA*, **101**, 6852–6857], and represents a simple and fast alternative to stable transformation. By using various combinations of provector modules, hGH was produced in three compartments of the cell: the apoplast, the chloroplast and the cytosol. We found that targeting to the apoplast provided the highest amount of correctly processed and biologically active hGH, with a yield of up to 10% of total soluble protein or 1 mg per gram of fresh weight leaf biomass. These results indicate that the use of viral vectors for high-yield production of human therapeutic proteins in plants by transient expression provides an attractive alternative to production protocols using standard expression vectors in transgenic or transplastomic plants.

Keywords: human growth hormone, *Nicotiana benthamiana*, plant virus, protein processing, protein targeting, transient gene expression.

Introduction

Plants are becoming an attractive system for the production of recombinant proteins (Klimyuk *et al.*, 2005), including biopharmaceuticals (Ma *et al.*, 2003; Fischer *et al.*, 2004; Goldstein and Thomas, 2004; Schmidt, 2004), such as monoclonal antibodies (Ma and Hein, 1995; Valdes *et al.*, 2003), vaccines (Ma and Vine, 1999; Walmsley and Arntzen, 2003) and industrial proteins (Somerville and Bonetta, 2001). The production of recombinant therapeutic proteins has traditionally been performed in non-plant organisms, such as bacteria, fungi, cultured insect or mammalian cells (Benatti *et al.*, 1991). Bacterial and fungal expression systems are relatively cheap technologies, but are not suitable for all pharmaceutical proteins, as they are not always able to deliver the correct product, and either do not perform post-translational modifications or perform modifications different from those made in the cells of higher organisms (Becker and Hsiung, 1986; Schmidt, 2004). In contrast, recombinant proteins of human

origin are usually correctly synthesized and processed in mammalian expression systems (Denman *et al.*, 1991). However, the yield of proteins is often low and the production very costly. Mammalian cells are also very sensitive to environmental factors, complicating the production procedure significantly.

In comparison with these systems, molecular farming in plants offers several persuasive benefits. First, the existing infrastructure can be used for the production of a large amount of biomass in an open field or glasshouse environment, and for harvesting and storage, consequently keeping capital investments at a low level. Secondly, plant cells, like animal cells, are able to produce secreted proteins and to perform post-translational modifications. In addition, plant-derived products are less likely than animal cell lines to be contaminated with human pathogenic microorganisms, as plants are not hosts for most human infectious agents (Ma *et al.*, 2003; Goldstein and Thomas, 2004).

Plant-made recombinant proteins can be produced using either stably transformed plants or by transient expression.

The latter approach has several advantages over production from stably transformed plants; in particular, transient expression is very fast (Kapila *et al.*, 1997), whereas the production of stably transformed plants usually takes several months. In addition, stable transformation systems suffer from unpredictable levels of transgene expression as a result of gene silencing, because of varying transgene copy number and/or position effects, etc. (Kooter *et al.*, 1999).

In plants, processing steps in the secretory pathway, including protein folding, assembly, etc., are largely similar, if not identical, to those found in mammalian cells (Vitale and Denecke, 1999). Subcellular targeting of proteins to organelles or the intercellular space (apoplast) can increase the accumulation level and the stability of recombinant proteins, in some cases significantly, and has advantages for downstream processing of the protein (Di Fiore *et al.*, 2002; Fischer *et al.*, 2004).

Plant viral vectors are widely used for transient expression in plants (Scholthof *et al.*, 1996; Turpen, 1999; Yusibov *et al.*, 1999; Pogue *et al.*, 2002), with the genes of interest placed under control of the strong coat protein subgenomic promoter (CP-SgPr). An advantage of this system is that the desired protein can be produced at an extremely high level because of viral amplification. The viral vectors based on tobacco mosaic virus (TMV) are amongst the most efficient viral vectors developed for plants (Fitzmaurice *et al.*, 2002; Escobar *et al.*, 2003). We have recently described a transient expression system for protein production in plants that is based on the *in planta* assembly of complete viral vectors from separate modules (Gleba *et al.*, 2004; Marillonnet *et al.*, 2004). The system is extremely useful for fast optimization of the constructs, in order to provide the highest level of expression or for accurate protein processing. It allows for rapid expression of a coding sequence of interest under different controlling elements, such as different targeting/signal peptides. In this study, the human growth hormone (hGH, or somatotropin) was used as an example of a relevant biopharmaceutical protein that can be efficiently produced by this system in *Nicotiana benthamiana*. Somatotropin is an important drug that is used for the treatment of hypopituitary dwarfism in children and has potential for the treatment of Turner syndrome, chronic renal failure, human immunodeficiency (HIV) wasting syndrome and, possibly, treatment of the elderly (Tritos and Mantzoros, 1998). hGH is produced by the pituitary gland, which is located at the base of the brain. Originally, patients were treated with hGH extracted from glands taken from dead bodies. The process was limited by the supply of glands and carried a risk of transmitting slowly developing infections such as Creutzfeld–Jakob disease. Consequently, hGH was one of the first recombinant proteins produced in

bacteria (Becker and Hsiung, 1986). Expression of hGH in plants has been reported in two publications (Leite *et al.*, 2000; Staub *et al.*, 2000). Leite *et al.* (2000) succeeded in expressing hGH with the correct N-terminal amino acid sequence in tobacco seeds, but the yield was very low (maximum of 0.16% of total soluble seed protein). In contrast, in the second study (Staub *et al.*, 2000), the expression of hGH in transplastomic tobacco provided for a high yield [over 7% of total soluble protein (TSP)], but accurate processing of the N-terminus remained a problem. Here, we show that high-yield production of correctly processed and biologically active recombinant hGH identical to its human counterpart is possible in plants.

Results

Description of the viral modules prepared for transient expression of hGH

The viral provector system is designed for the *in planta* assembly of functional replicons from non-functional provector modules delivered by *Agrobacterium*. Functional replicons are formed by recombination between 5' and 3' modules as a result of the action of a site-specific recombinase, which can be expressed transiently from a third co-delivered construct or stably from a transgenic host. In this work, a single 5' provector module (pICH10570; Figure 1) was used in combination with several 3' modules. The 5' module contained the TMV RNA-dependent RNA polymerase, the movement protein (MP) and the CP-SgPr, which drives expression of the gene of interest cloned in the 3' module after recombination.

hGH is a secreted protein, and the first 26 amino acids of the protein consist of a signal peptide that directs the expressed protein into the secretory pathway. This signal peptide is cleaved on secretion into the endoplasmic reticulum (ER), resulting in a secreted protein starting with phenylalanine. Four 3' modules were prepared in order to produce the protein in different cell compartments. In the first construct, pICH11901, the coding sequence of the mature protein was cloned without a signal peptide sequence for expression in the cytosol. As the mature growth hormone starts with a phenylalanine at the N-terminal end, a methionine start codon had to be added for expression in the cytosol (a second amino acid, an alanine, was also present after the methionine start codon due to cloning of the coding sequence using a *NcoI* restriction site). A second construct, pICH11731, contained the full hGH precursor sequence with the native human signal peptide sequence. If the signal peptide is functional in plants, as in human cells, it should target the expressed protein to the ER, and a proteolytic cleavage would

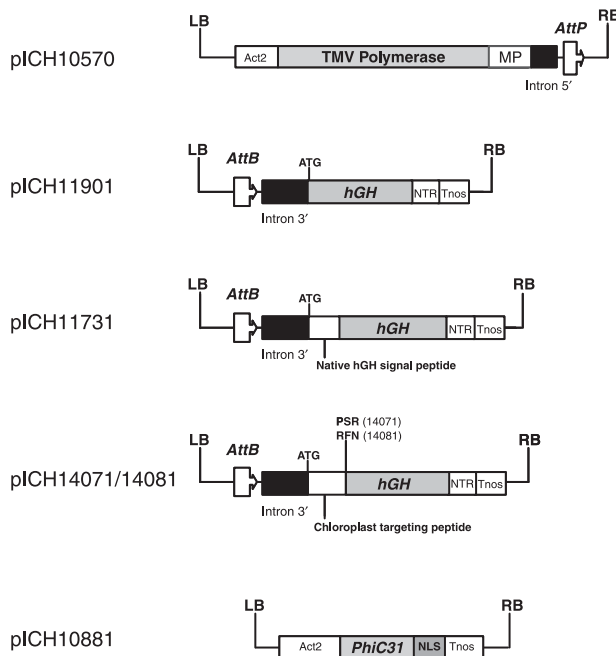


Figure 1 Modules of the provector system. The provector system consists of a 5' module (pICH10570) that can be combined with different 3' modules (pICH11731, pICH11901, pICH14071 and pICH14081). Both modules are assembled *in planta* by expression of a recombinase from construct pICH10881. All constructs are cloned in pBIN19-based binary vectors between the T-DNA left and right borders (LB and RB). Act2, *Arabidopsis* actin2 promoter; AttP, AttB, *Streptomyces* phage PhiC31 recombinase attachment sites; MP, viral movement protein; NLS, nuclear localization signal; NTR, cr-TMV 3' non-translated region; Tnos, nopaline synthase terminator; TVCV-polymerase, turnip vein clearing virus RNA-dependent RNA polymerase.

result in a processed molecule starting with a phenylalanine. Finally, two modules, pICH14071 and pICH14081, were prepared for targeting the protein into the chloroplast. In these constructs, the hGH coding sequence was fused to a consensus amino acid sequence of chloroplast targeting signals from the ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) small subunit precursor proteins from different plant species. The three C-terminal amino acids of the signal peptide were changed from VCR to either PSR (pICH14071) or RFN (pICH14081) (Figure 1 and 'Experimental procedures') to create a cleavage sequence expected to lead to a phenylalanine at the N-terminus for the processed protein (sequence determined by comparative analysis of natural chloroplast-targeted proteins that are cleaved upstream of a phenylalanine). This artificial amino acid sequence was subsequently converted into a nucleotide sequence chosen to conform to the codon usage of dicot plants.

Construct pICH10881 contained a gene encoding the PhiC31 integrase protein, which catalyses site-specific recombination between the AttB and AttP sites present in 5' and 3'

proectors. The assembly of the modules at the DNA level is followed by transcription in the nucleus and by a splicing reaction that leads to the removal of the recombination site and of flanking intron sequences that are present between the CP-SgPr and the gene of interest. Construction of the plasmids is described in 'Experimental procedures'.

Transient expression of hGH in *N. benthamiana* leaves

N. benthamiana leaves were infiltrated with mixes of *Agrobacterium* cultures containing the modules pICH10570 and pICH10881 in all cases and, in addition, pICH11731 for targeting hGH to the apoplast (sector AP; Figure 2), pICH11901 for expression in the cytoplasm (sector CY; Figure 2), or pICH14071 or pICH14081 for targeting hGH to the chloroplast (sector CL; Figure 2). Six to eight days after infiltration with an *Agrobacterium* mix containing pICH11731 for targeting to the apoplast, necrosis appeared in the infiltrated area (sector AP; Figure 2). Twelve days after treatment, the tissue was almost completely dried. Necrosis also appeared after infiltration of *Agrobacterium* cultures carrying the construct pICH11901 (sector CY; Figure 2), but was delayed and less strong than with the apoplast targeting construct. In contrast, no necrosis could be seen in areas infiltrated with modules for targeting the protein to the chloroplast (sector CL; Figure 2).

The expression of hGH in infiltrated areas was analysed by Western blot (Figure 2) and quantified using hGH enzyme-linked immunosorbent assay (ELISA). For targeting hGH in the apoplast with pICH11731, first hGH was detected by Western blot analyses 3–4 days after infiltration, and the concentration reached a maximum after about 12 days. Interestingly, intact hGH was still present in leaf tissue samples that were necrotic and even dried.

The protein that accumulated 12 days after infiltration could be clearly visualized on Coomassie-stained sodium dodecylsulphate-polyacrylamide gels (Figure 3). The absolute concentration of the recombinant protein reached a maximal value of 1 mg/g fresh tissue or approximately 10% of TSP. This amount of protein could be reliably achieved in independent experiments and was not significantly influenced by experimental variability, the age of the leaf material, etc.

In the cytosol, the amount of hGH expressed was so low that detection was possible only in immunoblot analyses. Nevertheless, a protein of the predicted molecular weight could be detected. ELISA revealed a maximal expression level of 0.01% TSP.

As measured by ELISA, targeting hGH to the chloroplast with construct pICH14081 led to the expression of 0.075%–0.1% TSP. In the case of pICH14071, the expression was

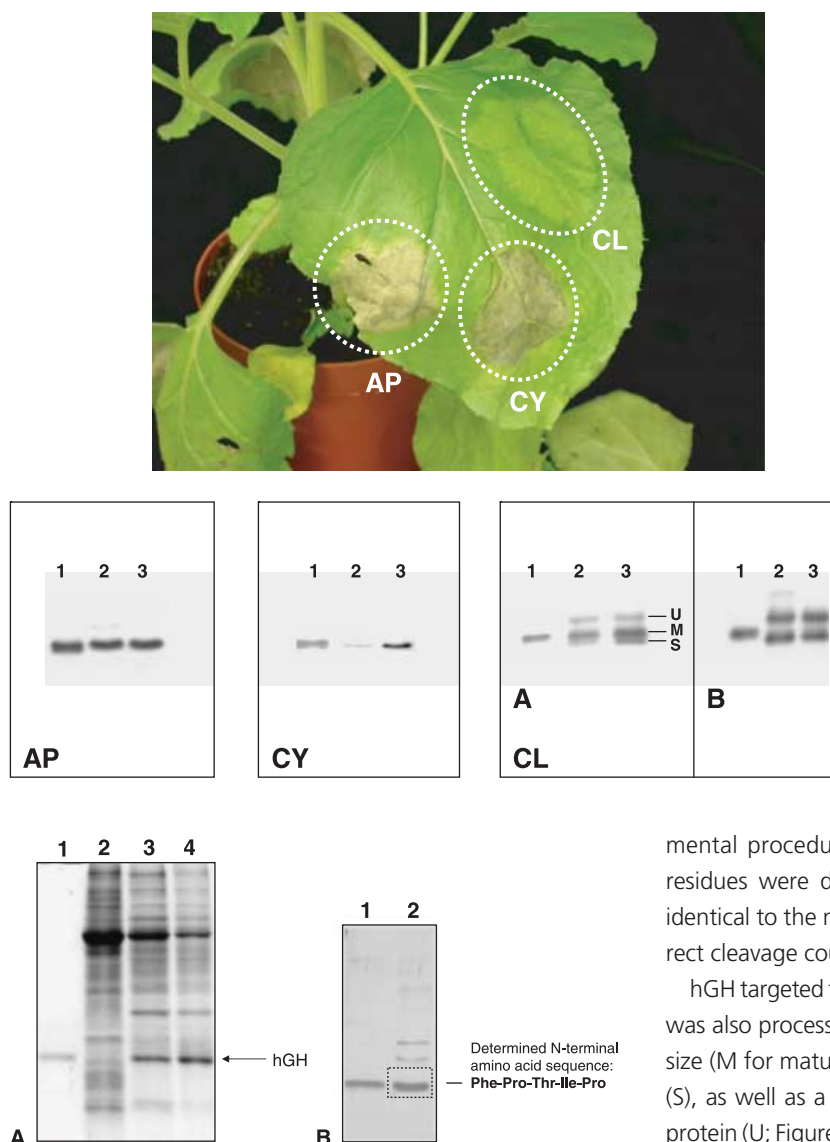


Figure 2 Analysis of human growth hormone (hGH) accumulation in *Nicotiana benthamiana*. 5' modules, 3' modules and integrase-containing plasmids were transformed into *Agrobacterium tumefaciens* and infiltrated together in equal amounts into *N. benthamiana* leaves. Leaf sectors were infiltrated with *Agrobacterium* mixes containing pICH10570, pICH10881 and pICH11731 for targeting the protein into the apoplast (sector AP), pICH11901 for targeting in the cytoplasm (CY), or pICH14081 (A) or pICH14071 (B) for targeting to the chloroplast (CL). The presence of the hGH protein was shown by Western blot analysis. Total soluble protein extracts of two leaves transfected independently (lanes 2 and 3) are compared with a 22-kDa hGH control protein from Sigma (lane 1). Other abbreviations are explained in the text.

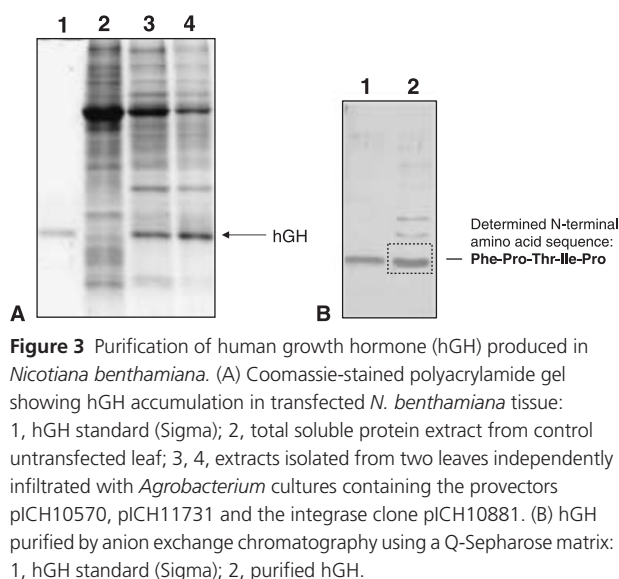


Figure 3 Purification of human growth hormone (hGH) produced in *Nicotiana benthamiana*. (A) Coomassie-stained polyacrylamide gel showing hGH accumulation in transfected *N. benthamiana* tissue: 1, hGH standard (Sigma); 2, total soluble protein extract from control untransfected leaf; 3, 4, extracts isolated from two leaves independently infiltrated with *Agrobacterium* cultures containing the provectors pICH10570, pICH11731 and the integrase clone pICH10881. (B) hGH purified by anion exchange chromatography using a Q-Sepharose matrix: 1, hGH standard (Sigma); 2, purified hGH.

higher, but the amount of hGH processed to the expected size was very low.

The native signal of hGH is accurately processed in *N. benthamiana*

hGH protein targeted to the apoplast using vector pICH11731 was purified by anion exchange chromatography and sequenced by automatic Edman degradation (see Figure 3B and 'Experi-

mental procedures'). The first five N-terminal amino acid residues were determined as Phe-Pro-Thr-Ile-Pro, which is identical to the native mature hGH protein. No sign of incorrect cleavage could be detected with this analysis.

hGH targeted to the chloroplast using provector pICH14081 was also processed, but in addition to a protein of the exact size (M for mature protein), a smaller band was also present (S), as well as a larger band of the size of the unprocessed protein (U; Figure 2). The ratio of correctly processed hGH was approximately 75% and varied slightly in different samples. To determine whether cleavage of the chloroplast signal peptide led to an N-terminus identical to the native mature protein, we attempted to purify the 22-kDa protein fraction produced with pICH14081, but were unsuccessful as a result of the small amount of hGH present in the tissue. Targeting to the chloroplast using provector pICH14071 led to a higher hGH concentration than with pICH14081, but most of the protein was not processed to the correct size.

hGH expressed with viral vectors is bioactive

Bioactivity of the growth hormone produced in the apoplast and the chloroplast was tested using an Nb2-11 cell bioactivity assay. This assay uses quiescent Nb2-11 lymphoma cancer cells that are unable to proliferate unless growth hormones are provided (see 'Experimental procedures').

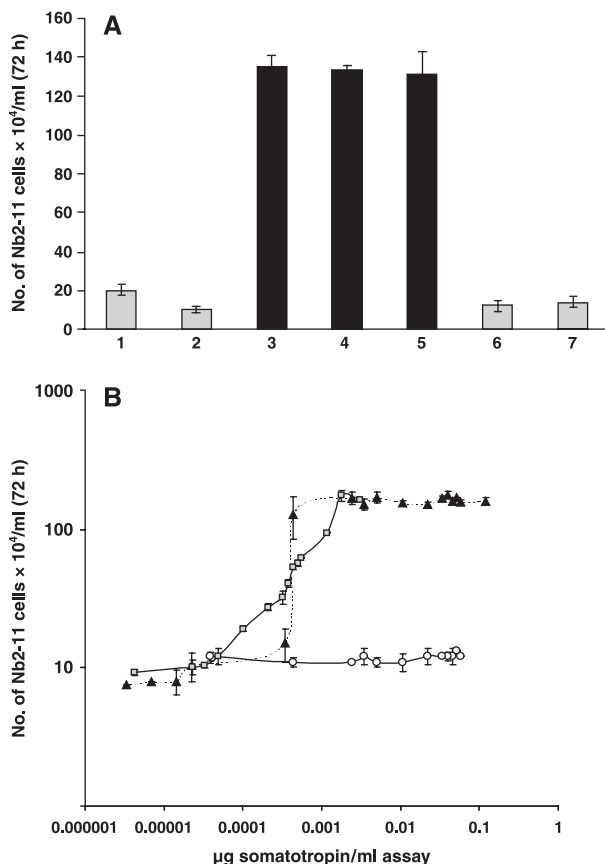


Figure 4 Bioactivity analyses of recombinant human growth hormone (hGH). (A) Stimulation of Nb2-11 cells: 1, starting cell culture; 2, cell culture incubated for 72 h without any treatment; 3, 4, cell cultures incubated for 72 h after addition of crude protein extracts prepared from leaves infiltrated with pICH11731 (3) or pICH14081 (4) (both extracts contained approximately 20 ng hGH); 5, cell cultures spiked with 20 ng hGH purified from *Nicotiana benthamiana* leaf tissue (see Figure 3B); 6, addition of a crude extract from leaves infiltrated with viral provectors expressing green fluorescent protein (GFP) instead of hGH (construct pICH7410; Marillonnet *et al.*, 2004); 7, addition of a crude extract from an untreated *N. benthamiana* plant. (B) Positive growth response of Nb2-11 cells after addition of increasing concentrations of crude extract into the cell medium. Extracts were prepared from tissue transfected with pICH10570, pICH10881 and either pICH11731 (▲) or pICH14081 (□). Control plants were infected with a comparable vector expressing GFP (○).

hGH protein accumulated in the apoplast (pICH11731) or in the chloroplast (pICH14081) induced proliferation of the Nb2-11 cells (Figure 4A). The effect increased proportionally to the concentration of added growth hormone until saturation was reached (Figure 4B). The same effects were achieved by spiking crude extracts of untreated *N. benthamiana* plants with purified protein (data not shown), or by adding crude extracts from tissue expressing hGH. As a negative control, extracts from *N. benthamiana* plants infiltrated with vectors expressing green fluorescent protein (GFP) (pICH7410; Marillonnet *et al.*, 2004) or from plants that were not treated

at all did not induce proliferation of Nb2-11 cells. Therefore, it can be concluded that no endogenous molecules from the plant or the cell extracts were capable of stimulating or inhibiting the proliferation rate of Nb2-11 cells.

Protein extracts obtained from leaf sectors containing hGH in the cytosol (pICH11901) were not included in the analysis, as the low yield of hGH protein that could be detected did not allow us to measure the activity of a defined proportion of hGH accurately. When larger volumes of crude extract were added to Nb2-11 cells, no effects, or only very slight effects, on cell proliferation could be observed.

Discussion

We have shown here that the viral provector system reported earlier (Marillonnet *et al.*, 2004) can be used to express pharmaceutical recombinant proteins in plants, hGH in the present example. In addition to providing a high level of heterologous protein expression, the provector system represents a versatile system that allows rapid testing of various combinations of genetic elements by simply mixing *Agrobacterium* cultures containing different viral modules. In the present example, all modifications were made in the 3' provector module as a library of 5' modules was not available at the time at which this study was performed. However, a number of 5' provector modules have now been constructed that contain various signal peptides in different preconstructed 5' modules. Such modules allow the testing of various signal peptides with a unique 3' module that contains the coding sequence of the mature protein without any signal peptide sequence. A convenient feature of the provector system is that cloning does not have to be carried out on full-length clones, and can therefore be performed easily.

Biopharmaceuticals produced in plants are subjected to the same standards of performance and safety as those derived from other production systems. One important aspect is correct processing of the protein to obtain a product that is as close as possible to proteins that are already in therapeutic use. In a previous study, Staub *et al.* (2000) reported the development of a transgenic line of *N. tabacum* that produced hGH in chloroplasts at a level of up to 7% of TSP. With the goal of obtaining a processed protein with an N-terminus identical to the human mature protein, the authors produced a ubiquitin-hGH fusion protein which, after proteolytic processing, would be expected to produce the exact mature protein. However, only small amounts of correctly processed hGH were present in extracts, and most of the produced hGH had an extra amino acid, a proline, at the amino-terminal end. Although the hGH produced in this work was bioactive, it was

not identical to other recombinant proteins characterized in human clinical trials. In contrast with this work, the N-terminal end of the apoplast-targeted hGH produced using the provector system is exactly the same as the mature human protein. Accurate processing appeared to take place for all of the expressed protein, as no unprocessed protein could be detected by immunoblot analyses, and no sequence other than the exact mature protein could be detected from N-terminal sequence analysis. Thus, it can be concluded that all the expressed hGH represents an active form identical to hGH that is already in therapeutic use. Hence, potential immunogenic effects due to an altered amino acid sequence of the protein, as was the case for met-hGH produced in *Escherichia coli* (Becker and Hsiung, 1986), are excluded.

From the results described here, it can be concluded that targeting into subcellular compartments stabilizes the recombinant protein. High levels of hGH were found to be toxic to plant tissues. Toxicity does not simply result from a high level of recombinant protein expression, as tissue expressing even higher levels of another protein (GFP) displayed no toxicity at all (Marillonnet *et al.*, 2004). Toxicity was not strictly correlated with the amount of bioactive protein produced, because no necrosis of the tissue was observed by targeting hGH to the chloroplast, whereas toxicity was induced by targeting of the protein in the cytosol. However, more bioactive protein was obtained in the chloroplast than in the cytosol. The highest level of bioactive protein was produced in the apoplast, followed by the chloroplast. Native hGH expressed in human cells contains two disulphide bonds. The lower level of bioactive protein produced in the chloroplast might result from the fact that conditions outside of the ER are generally less favourable for the formation of disulphide bonds than conditions inside the ER (Hwang *et al.*, 1992). However, the high level of bioactive hGH produced in the chloroplast by Staub *et al.* (2000) argues against this hypothesis. Rather, modification of the cleavage site sequence probably led to inefficient N-terminal processing, as evidenced by the variety of hGH proteins of different sizes detected by Western blot. The low level of expressed protein detected in the cytoplasm might result from the degradation of improperly folded protein. This assumption was confirmed by the fact that, in some Western blot analyses, traces of degraded hGH could be obtained (data not shown).

In addition to hGH, we have also expressed several other biopharmaceutical proteins using the provector system, including single chain antibodies, human cytokines and interferon- α 2B. The latter protein accumulates to a concentration that is even higher than that of hGH described here. High expression could be achieved only by fusing the interferon coding

sequence to a plant apoplast-targeting signal peptide (the *Nicotiana plumbaginifolia* calreticulin signal peptide; Borisjuk *et al.*, 1998). Again, cytosolic expression was detected, but at a much lower level, similar to the situation observed for hGH.

In summary, to our knowledge, the amount of correctly processed and active hGH produced with the provector system exceeds the yields obtained with other plant expression systems reported so far (Leite *et al.*, 2000; Staub *et al.*, 2000). Assuming a leaf mass of about 20 g per *N. benthamiana* plant, our system allows for the production of up to 20 mg of hGH from a single plant in less than 12 days.

The infiltrations described in this work were performed on a small scale. Two alternatives could be used for industrial scale-up. The first strategy involves the use of systemic viral vectors, as described in Marillonnet *et al.* (2004). With this approach, a 3' provector carries, in addition to the gene of interest, a coat protein gene (the CP gene of the U5 strain of TMGMV) under control of an extra subgenomic promoter. The expression of CP leads to the systemic movement of viral RNA. We tested this approach and found that it worked: 10–14 days after infiltration of a leaf of an *N. benthamiana* plant, a significant amount of hGH was isolated from non-inoculated systemic leaves. The protein was tested for bioactivity as described above and found to be active (data not shown). A second and more efficient strategy for scale-up involves the infiltration of entire plants (Magniffection; Gleba *et al.*, 2005) using constructs similar to those described here (lacking CP), either in the form of a provector or of pre-assembled complete viral vectors (Marillonnet *et al.*, 2005). This approach offers many advantages over the use of viral vectors that express CP, including higher yields, faster expression and increased level of biosafety. When GFP was expressed using such an approach, the absolute protein yield reached 4 g of recombinant protein per kilogram of fresh leaf biomass in *N. benthamiana* and up to 2.5 g/kg in tobacco (*N. tabacum*), which is 25–40% of TSP. Therefore, the provector system has great potential for the profitable production of costly biopharmaceutical proteins in plant bioreactors.

Experimental procedures

Construction of the viral vectors

5' Provector constructs were cloned in pBIN19-based binary vectors. For DNA recombination, the integrase system from *Streptomyces* phage PhiC31 (Thorpe and Smith, 1998) was used. Additionally, the third intron from the *Petunia hybrida* PSK7 gene (Accession no. AJ224165) was split and introduced into the provectors according to Marillonnet *et al.*

(2004). The construction of pICH10881 is described in the same publication.

An hGH cDNA with a sequence identical to the native sequence (GENBANK accession no. NM000515) was chemically synthesized (by ATG:biosynthetics GmbH, Merzhausen, Germany) and cloned in the 3' provector construct, resulting in pICH11731. The 26-amino-acid native signal peptide was replaced in constructs pICH14071 and pICH14081 by two 59-amino-acid synthetic chloroplast targeting sequences that differ in the three C-terminal amino acids at the cleavage site (sequence MASSM LSSAA VVATR ASAAQ ASMVA PFTGL KSAAS FVTR KQNNL DITSI ASNGG R, followed by amino acids PSR in pICH14071 and RFN in pICH14081). In pICH11901, the native signal peptide was deleted and replaced by amino acids MA.

Delivery of *Agrobacterium* into plants

The infiltration of *Agrobacterium* into *N. benthamiana* plants was performed according to a modified protocol described previously (Yang *et al.*, 2000). *Agrobacterium tumefaciens* strain GV3101 transformed with individual constructs was grown in Luria–Bertani (LB) medium supplemented with rifampicin (50 mg/L) and carbenicillin (50 mg/L) at 28 °C. The cells of an overnight culture (5 mL) were sedimented by centrifugation (10 min, 4500 *g*) and resuspended in 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (pH 5.5) supplemented with 10 mM MgSO₄. The bacterial suspensions were adjusted to a final OD₆₀₀ (OD, optical density) of 0.8 and equal volumes were mixed before infiltration. Infiltration of fully expanded leaves was performed using a syringe without a needle. After infiltration, the plants were further grown under glasshouse conditions.

Isolation and analysis of the hGH protein

The infiltrated leaves were harvested and frozen for storage at –80 °C. Crude extracts were made by grinding 100 mg of frozen leaf tissue and extracting the proteins with 300–1000 µL of buffer containing 0.15 M Tris-HCl and 5 mM β-mercaptoethanol (pH 8.0).

hGH purification was performed by anion exchange chromatography using a Q-Sepharose Fast Flow column (Amersham, Buckinghamshire, UK). The buffer for equilibration, binding and washing contained 0.05 M Tris-HCl (pH 8.0), 2 mM ethylenediaminetetraacetic acid (EDTA) and 15 mM NaCl. The protein was eluted by NaCl gradient of the elution buffer (0.15 M Tris, pH 8.0, 2 mM EDTA), reaching a protein elution peak in the region of 125 mM NaCl.

Protein purification and Western blot analyses were performed according to Staub *et al.* (2000). The antibodies included mouse anti-hGH IgG (Research Diagnostics Inc., Concord, MA) and goat anti-mouse IgG (Sigma Aldrich, Munich, Germany).

ELISAs were carried out using the hGH ELISA-Kit from Roche (Penzberg, Germany).

N-terminal microsequencing was performed by TopLab GmbH (Martinsried, Germany) using automatic Edman degradation [sequencer *Procise* 492 (Applied Biosystems, Foster City, CA) combined with On-Line PTH Analyser 140C, UV-detector 756A (Applied Biosystems)].

Activity analysis of hGH by Nb2-11 bioassay

The bioactivity of hGH was assayed by testing the proliferation dependence of the lymphoma rat cell line Nb2-11 in the presence of hGH (Gout *et al.*, 1980; Dattani *et al.*, 1995). The Nb2-11 cell line was obtained from the European Collection of Cell Cultures (ECACC). Cultures were routinely grown at 37 °C, 5% CO₂ for 72 h in Fischer's medium supplemented with 1.0% fetal bovine serum (FBS), 10% horse serum (HS), 0.075% bicarbonate, 0.05 mM 2-mercaptoethanol and 2 mM glutamine before transferring the cells to Fischer's SM medium (medium above without FBS). The Nb2-11 cells were suspended in this assay medium at a density of 200 000 cells/mL and incubated overnight. The assay was performed in microtitre plates containing 0.5 mL of cell suspension per well. Five hundred microlitres of assay medium containing different concentrations of either purified hGH or crude extracts of *N. benthamiana* plants were then added to the wells of the microtitre plates. The cells were counted after 72 h of incubation under the conditions described above.

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