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GENETIC TRANSFORMATION AND HYBRIDIZATION

Expression of human growth hormone in transgenic rice cell suspension culture

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Abstract Human growth hormone (hGH), a pituitary-derived polypeptide, evidences a wide range of biological functions, including protein synthesis, cell proliferation, and metabolism. A synthetic hGH gene (shGH) has been synthesized on the basis of plant-optimized codon usage via an overlap PCR strategy and located in a plant expression vector under the control of the rice amylase 3D (Ramy3D) promoter, which is induced by sugar starvation. The plant expression vector was introduced into rice calli (*Oryza sativa* L. cv. Donjin) via particle bombardment transformation methods. The integration of the shGH gene into the chromosome of the transgenic rice callus was verified via genomic DNA PCR amplification and shGH expression in transgenic rice suspension cells was confirmed via Northern blot analysis. The shGH protein was detected in the transgenic rice cell suspension culture medium following induction with sugar starvation, using Western blot analysis. The quantity of shGH that accumulated in the transgenic rice cell suspension medium was 57 mg/l. The shGH accumulated in the transgenic rice cell suspension culture medium evidenced a biological activity similar to that of *Escherichia coli*-derived recombinant hGH. These results indicate that the shGH was generated and accumulated in the transgenic rice cell suspension culture medium, and manifested biological activity.

Keywords Human growth hormone · Rice cell suspension culture · Rice amylase 3D promoter

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Introduction

Human growth hormone (hGH) plays central roles in growth, development and sexual maturation, and has been studied extensively since 1944, when the secreted peptide hormone was first isolated (Li and Evans 1944). hGH is an anterior pituitary-derived polypeptide hormone harboring 192 amino acid residues, and folds into a four-helix bundle structure containing two disulfide bridges (De Vos et al. 1992). The predominant form has a molecular weight of 22 kDa. Endocrine hGH exerts a direct effect on somatic growth regulation, mediated via interaction with a membrane-bound receptor, the growth hormone receptor (GHR) (Zhu et al. 2001; Roith et al. 2001). hGH has therapeutic applications in the treatment of dwarfism, bone fractures, skin burns, and bleeding ulcers (Raschke et al. 2007; Losada et al. 2002; Mattes et al. 1975). The hGH is a principal participant in the control of several complex physiological processes, including growth and metabolism. Growth hormone is also of considerable interest as a drug applicable to both humans and animals.

Transgenic plant cell suspension culture systems have been previously utilized in the production of recombinant pharmaceutical proteins (Lee et al. 1997; Fischer and Emans 2000; Shin et al. 2003). The plant cell culture system is associated with several advantages, including the reduction of the risk of mammalian viral and bacterial toxin contamination, ease of purification, low cost of plant

culture media, and the capacity to produce biologically active proteins and assemble multimeric proteins, such as antibodies (Fischer et al. 1999). Plant cell suspension cultures offer greater control over the production environment, and simpler and cheaper downstream processing and purification for proteins secreted from the cells. Therefore, plant cell culture systems may constitute an optimal means for the production of small-to-medium quantities of high-priced, high-purity, specialty recombinant proteins.

Although plant cell suspension cultures have many advantages, the low expression level possible with this technique has been considered to be one of the limiting factors in the production of foreign proteins. Several reports have addressed the improvement of protein expression in transgenic plants via the modification of the gene with plant optimized codon usage, the chloroplast targeting system, the viral expression system, and rice amylase promoter expression systems (Shin et al. 2003; Daniell et al. 2002; Warzecha et al. 2003; Canizares et al. 2005).

In this study, a synthesized human growth hormone gene (shGH) was modified on the basis of rice-optimized codon usage, and introduced subsequently into a transgenic rice suspension culture under the control of the rice amylase 3D promoter. The feasibility of the production and biological activity of shGH accumulated in the medium of transgenic rice cell suspension culture system was tested in this study.

Materials and methods

Construction of plant expression vector

The synthetic human growth hormone gene (shGH) (GenBank accession No. NM 000515) was modified on the basis of rice optimized codon usage (http://www.kazusa.or.jp/codon/) and synthesized via an overlap PCR strategy (Fig. 1). The PCR product of the shGH gene was cloned into pGEM T-Easy vector (Promega, Madison, WI), and its DNA sequence was confirmed via DNA sequence analysis. The shGH gene, which was fused with the signal sequence of rice amylase 3D (Ramy3D), was introduced into the plant expression vector containing HTP (Hygromycin phosphotransferase) as a selection marker for hygromycin B and the rice Ramy 3D promoter expression system. This plant expression vector was designated pMYN449 (Fig. 2).

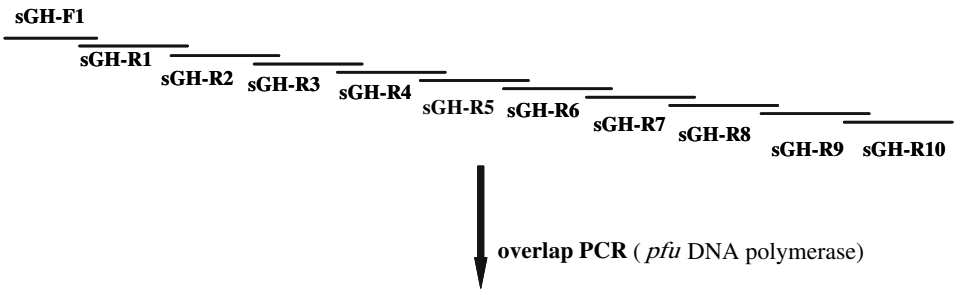

Fig. 1 Overlap PCR strategy for synthesis of shGH

synthetic hGH (mature peptide region, 654 bp)

sGH-F1 5'-TTCCCTACCATTCCGTTGTCCAGGCTCTTCGACAACGCCATGCTGCGCGCCCATCGCCTGCACCAGCTGGCC-3'
sGH-R1 5'-GAATGAGTACTTCTGCTCCTTTGGGATGTAGGGCTCCTCGAACTCCTGGTAGGTGTCGAAGGCCAGCTGGTGCAGGCGATGGGCGCGCAG-3'
sGH-R2 5'-CCGGTTGGATGGTGTCGGAATAGACTCTGAGAAGCACAGAGAGGTCTGTGGGTTCTGGAGGAATGACTTCTGCTCCTTTGGGATGTA-3'
sGH-R3 5'-CTGGATGAGGAGCAGGGAGATGCGGAGCAGCTCCAGGTTGGACTTCTGCTGTGTCTCCTCCCGGTTGGATGGTGTCGGAATAGACTCTGA-3'
sGH-R4 5'-GCCGTACACCAGGCTGTTGGCGAAGACGCTCCTGAGGAACTGCACTGGCTCCAGCCAAGACTGGATGAGGAGCAGGGAGATGCGGAGCAG-3'
sGH-R5 5'-CAGCGTCTGGATGCCCTCCTCGAGGTCCTTGAGGAGGTCGTAGACGTTGCTGTCAGAGGCGCCGTACACCAGGCTGTTGGCGAAGACGCT-3'
sGH-R6 5'-GCTGTAGGTCTGCTTGAAGATCTGGCCAGTCCGTGGGCTGCCATCCTCCAGTCTGCCCATCAGCGTCTGGATGCCCTCCTCGAGGTCCTT-3'
sGH-R7 5'-GTACAGCAGGCCGTAGTTCTTGAGGAGGGCGTCATCGTTGTGTGAGTTGGTGTCGAACTTGCTGTAGGTCTGCTTGAAGATCTGGCCAGT-3'
sGH-R8 5'-AGAGCGGCACTGCACGATGCGCAGGAATGTCTCGACCTTGTCCATGTCCTTCCTGAAGCAGTAGAGCAGGCCGTAGTTCTTGAGGAGGGC-3'
sGH-R9 5'-ACTCACTCACTCACTCACTCATCAGAAGCCGCAGCTGCCCTCCACAGAGCGGCACTGCACGATGCGCAGGAATGT-3'
sGH-R10 5'-AAGAGCTCACTCACTCACTCACTCACTCATCAG-3'

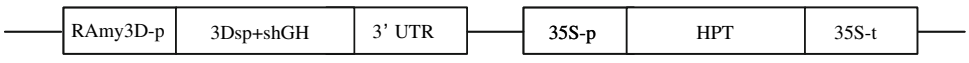

Fig. 2 Plant expression vector. The synthetic human growth hormone gene (shGH), fused with the Ramy3D signal sequence (3Dsp), is located between the rice amylase 3D promoter (Ramy3D-p) and the 3′ untranslated region (3′UTR). The plant expression vector contains the hygromycin phosphotransferase (HPT) expression cassette for the hygromycin B selection of transgenic callus under the control of the 35S Cauliflower Mosaic Virus promoter (35S-p) and terminator (35S-t)

Rice transformation

Rice calli (*Oryza sativa* L. cv. Dongin) were prepared and transformed via a particle bombardment-mediated transformation technique (Chen et al. 1998). After bombardment, the explants were transferred to N6 selection medium (N6S) (Chu et al. 1975) supplemented with 2,4-dichlorophenoxyacetic acid (2 mg/l), sucrose (30 g/l), proline (0.5 g/l), glutamine (0.5 g/l), casein enzymatic hydrolysate (0.3 g/l), gelite (2 g/l) and hygromycin B (35 mg/l) as selection markers at 2–3-week intervals. The putative transgenic calli resistant to the selection marker were selected in the N6S medium.

Detection of the shGH gene in the transgenic calli

Genomic DNA was isolated from the putative transgenic calli with a DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The genomic DNA concentration was determined at a wavelength of 260 m in a UV spectrophotometer. The presence of the shGH gene in the genomic DNA (400 ng) of the putative transgenic calli was detected via PCR amplification using the specific primer set for the shGH gene; the forward primer used was 5′-TTCCCTACCATTCCGT TGTCCAG-3′ and the reverse primer used was sGH-R10. The PCR amplification program proceeded as follows; 10 min of denaturation at 94°C followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final 10-min extension step at 72°C. The PCR products were then separated via electrophoresis on 1.0% agarose gel.

Establishment of cell suspension cultures

Transgenic calli harboring the shGH gene were used to establish the cell suspension culture. These cultures were grown in darkness in a shaking incubator at 110 rpm at 25°C in a 50 ml volume of N6S (N6SS) to which kinetin (0.2 mg/l), but not gelite, had been added. Ten ml of inocula were transferred to new media every 9 days. In order to induce the expression of the shGH gene, the media were removed from the suspension culture via aspiration and N6SS medium (without sucrose) was added to a density of 10% (weight of wet cells/volume of N6SS medium).

Northern blot analysis

Total RNA was isolated from transgenic rice suspension cells grown in liquid N6SS media (without sucrose) using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) then electrophoretically separated on formaldehyde-containing agarose gel (Sambrook et al. 1989). The separated RNA was transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech RPN82B, Piscataway, NJ). This membrane was then hybridized with a ^{32}P-labeled shGH probe using the Prime-a-Gene labeling system (Promega U1100, Madison, WI) at 65°C in Hybridization Incubator (FINEPCR Combi-H, Seoul, Korea). The membrane was washed twice in 2x SSC and 0.1% SDS, and then washed twice more with 2x SSC and 1% SDS for 15 min each at 65°C. The hybridized bands were detected via autoradiography on X-ray film (Fuji Photo Film Co. HR-G30, Tokyo, Japan).

Western blot analysis

Transgenic rice suspension cells were harvested and homogenized by grinding with a mortar and pestle at 4°C in extraction buffer (1:1 W/V) (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM ethylenediaminetetraacetic acid, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20). The homogenate was centrifuged at 17,000 x g in a Beckman GS-15R centrifuge for 15 min at 4°C in order to remove insoluble cell debris. The sample aliquots were separated via 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated bands were blotted onto nitrocellulose filters. After blocking with 5% skim milk, the filter was incubated with mouse monoclonal anti-human growth hormone antibody (R&D Systems Inc., LG Life Sciences Biotech, Seoul, Korea), followed by binding to an anti-mouse IgG conjugated to horseradish peroxidase as a secondary antibody. A 4-chloro-1-naphthol reagent was applied as a colorimetric detection substrate by horseradish peroxidase, as described by Sambrook *et al.* (Sambrook et al. 1989).

Quantification of shGH protein in suspension culture

After recombinant shGH production was induced via the incubation of transgenic rice cells in N6SS without sucrose, the suspension culture medium was subjected to 3 min of centrifugation at 200x*g*. One ml sample of the resulting culture supernatant was then dialyzed against phosphate-buffered saline (PBS) overnight at 4°C and utilized for quantitative ELISA. The concentration of recombinant shGH was determined using an rhGH-specific ELISA kit (Endogene, Woburn, MA), in accordance with the manufacturer's instructions.

Biological activity of shGH accumulated in transgenic suspension culture medium.

The biological activity of shGH accumulated in the transgenic cell suspension medium was determined by

measuring the induced proliferation of hGH-required Nb2 node lymphoma cells (Tanaka et al. 1980). In brief, growth factor-starved 1 x 10^5 Nb2-11 cells were suspended in 100 µl of RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 5% FBS (HyClone Laboratories Inc., Logan, UT). Serial dilution of hGH accumulated in the transgenic cell suspension medium was added into each of the wells and the *E. coli*-derived hGH was used as a positive control for the analyses of biological activity. After 27 h of incubation, 1 Ci of [methyl-H^3] thymidine (Amersham Lifescience, Piscataway, NJ) was added into each well and incubated for an additional 32 h. The cells were then harvested with a cell harvester (Inotech, Switzerland) and the tritium content was determined using a Liquid Scintillation Counter (Packard, USA).

Results

Vector construction and plant transformation.

In order to synthesize the human growth hormone (shGH) gene, primers were synthesized on the basis of the DNA sequences modified based on rice optimized codon usage, and were used subsequently for overlap PCR amplification (Fig. 1). The shGH gene, which was fused with the Ramy3D signal sequence, was introduced into plant expression vector under the control of the Ramy3D promoter, which is induced via the removal of sucrose from the medium (Fig. 2). The rice calli were transformed with pMYN449 and transferred onto hygromycin B-containing medium. The putative transgenic calli were then subjected to PCR amplification in order to determine the presence of the shGH gene. All putative transgenic calli evidenced a size consistent with that of the shGH gene (654bp). No PCR products were detected in the wild-type calli (Fig. 3).

Northern and Western blot analyses

Northern blot analysis was conducted in order to confirm shGH gene expression in transgenic cell suspension cultures after the induction of gene expression under sucrose starvation conditions. Positive shGH signals were detected in 19 of 20 transgenic suspension cell lines (Fig. 4). No signals were detected in one transgenic and one non-transgenic calli (Fig. 4). As is evidenced by Northern blot analysis, the transgenic callus evidencing high levels of mRNA expression (line 2, 3, 6, 8, 12, and 17) were selected for further experiments.

The production of the shGH protein in transgenic cell suspension culture media was assessed using immunoblot analysis with anti-human growth hormone antibody. The shGH proteins were detected in the transgenic plant cell suspension culture medium and not in the suspension culture medium of the non-transgenic calli (Fig. 5). The

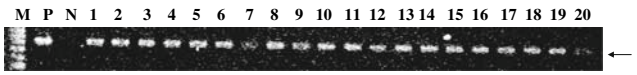

Fig. 3 Detection of the shGH gene in transgenic callus. Genomic DNA (400 ng) isolated from transgenic calli was used to demonstrate the presence of the shGH gene via PCR amplification. Lane M is a 100 bp DNA Ladder (New England Biolabs, Beverly, MA); lane P is the pMYN449 plasmid used as a positive PCR control; lane N is the non-transgenic callus genomic DNA used as a negative control; lanes 1–20 show the PCR products from transgenic callus genomic DNA

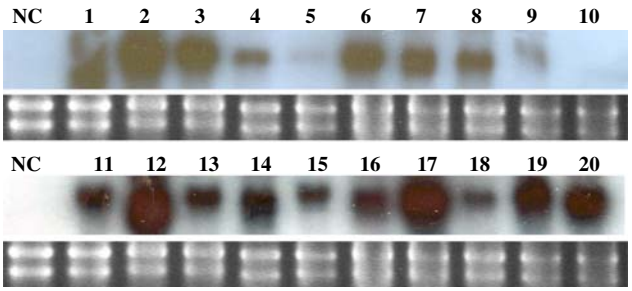

Fig. 4 Northern blot analysis to determine shGH expression. The total RNA extracts from transgenic rice suspension cells after induction via sugar starvation were subjected to Northern blot analysis in order to detect the shGH gene transcripts. Lane NC is the total RNA extract of non-transgenic rice suspension cells used as a negative control; lanes 1–20 are the total RNA extracts of transgenic rice suspension cells

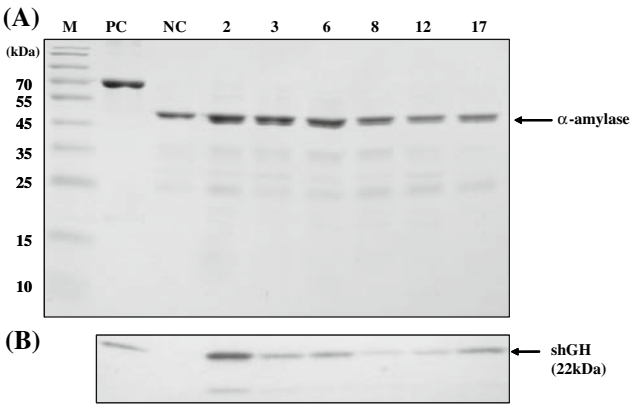

Fig. 5 Western blot analysis of shGH in transgenic rice cell suspension medium. The shGH proteins accumulated in the suspension medium of transgenic rice cells after induction with sucrose starvation were separated via SDS-PAGE (**a**) and subjected to Western blot analysis (**b**). Lane M is the molecular weight markers (Bio-Rad, Hercules, CA); lane PC is *E. coli*-derived hGH recombinant protein; lane NC is the non-transgenic rice suspension medium used as a negative control; lanes 2, 3, 6, 8, 12, and 17 are transgenic rice suspension culture media. *The arrow* indicates the bands of shGH protein accumulated in the suspension medium

full-length (approximately 22 kDa) and smaller size variants of shGH were detected in Western blot analysis (Fig. 5).

Measurement of shGH accumulation in suspension culture medium

The quantities of shGH accumulated in the suspension culture medium were determined using an rhGH-specific ELISA kit. The shGH accumulated in the transgenic cell suspension medium was obtained on day 7 after induction under sugar starvation conditions. The accumulation of shGH in the suspension culture medium of transgenic rice callus line 2 reached maximum levels at 57 mg/L (Fig. 6). The time course production of shGH proteins on every other day from day 3 to day 21 showed the maximum production on day 7 and decreased quickly thereafter (data not shown).

Biological activity of shGH accumulated in suspension culture medium

The proliferation of Nb2 node lymphoma cells, which require hGH for proliferation, were assessed in order to determine the biological activity of shGH accumulated in the suspension culture medium of transgenic rice cells evidencing the highest levels of shGH protein expression. The biological activity of shGH accumulated in the transgenic rice cell suspension culture was similar to that of the *E. coli*-derived recombinant hGH. No biological activity was detected in the medium from the non-transgenic cell suspension culture (Fig. 7).

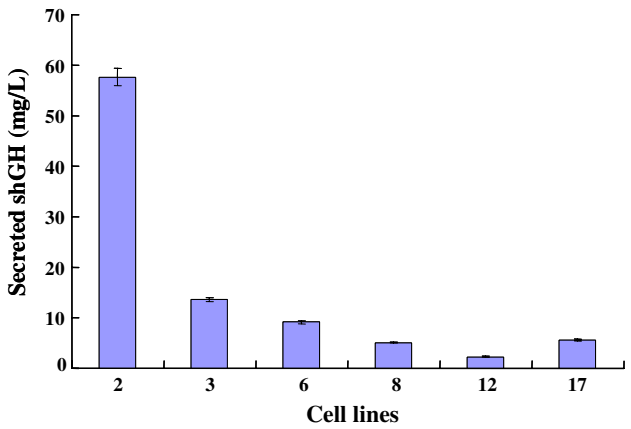

Fig. 6 Measurement of shGH accumulation in suspension culture medium. The concentration of accumulated shGH protein in the medium of the suspension cultures at day 7 after induction with sugar starvation were measured with an rhGH-specific ELISA kit

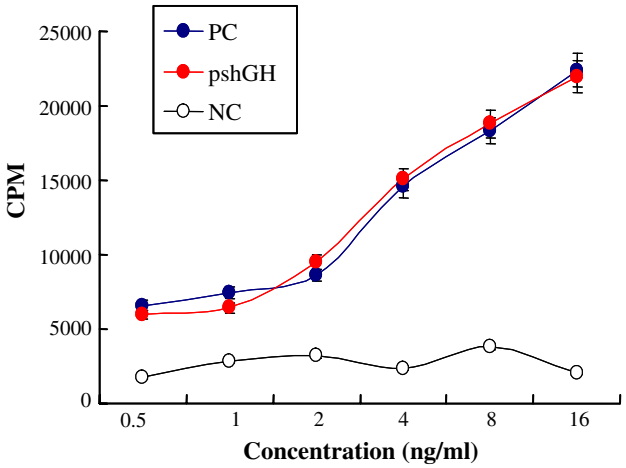

Fig. 7 Biological activity of shGH accumulated in transgenic cell suspension medium. The biological activity of accumulated shGH (pshGH) in the transgenic rice suspension culture medium was measured depending on the proliferation of Nb2 node lymphoma cells. *E. coli*-derived recombinant hGH was used as a positive control (*PC*) and the medium derived from non-transgenic rice callus was used as a negative control (*NC*)

Discussion

Recently, transgenic plant cell suspension cultures have been employed in the production of pharmaceutical proteins. However, the relatively low productivity of foreign proteins has been considered to be one of the limiting factors in this process. The strong promoter, the rice amylase 3D promoter (Ramy3D) that is induced by sugar starvation in transgenic rice cell suspension culture, improved the GM-CSF protein expression level by 1,000-fold as compared to the 35S CaMV promoter expression system in a transgenic tobacco cell suspension culture system (Shin et al. 2003). The modified LTB gene based on plant optimized codon usage effected a 200-fold increase in the level of LTB protein expression as compared to that of the unmodified LTB gene in transgenic tobacco plants (Kang et al. 2004). Although hGH has been shown to be expressed in transgenic tobacco nuclear and plastid systems, tobacco cell cultures, and soy and maize seed systems (Leite et al. 2000; Staub et al. 2000; Russell et al. 2005), high levels of hGH expression have been observed in tobacco cell cultures at 0.7 mg/L, an amount $>$ 35-fold lower than those observed in microbial secretory systems, including *E. coli* ($<$ 25 mg/L) (Chang et al. 1987) and *Pichia pastoris* (49 mg/L) (Ecamilla-Trevino et al. 2000). In this study, shGH, which was modified on the basis of rice optimized codon usage and under the control of the Ramy3D promoter expression system, evidenced the highest levels of accumulated shGH expression in a rice cell suspension medium at 57 mg/l. This enhancement in expression levels occurred as the consequence of the strength of the Ramy3D promoter

and the reduction of protease activity secreted into suspension media derived from transgenic rice cells via sucrose starvation (Shin et al. 2003).

The shGH proteins accumulated in the transgenic rice cell suspension culture medium were detected in two bands by Western blot analysis. This result has also been observed in other expression systems; transgenic tobacco cell cultures, soy, maize (Staub et al. 2000) and *E. coli* (Canova-Davis et al. 1990). In maize, the lower hGH protein band might be a product of additional exoproteolytic activity. Protein degradation is considered to be a principal limiting factor for the high-level production of foreign proteins within plant cell suspension cultures. The reduction of heterologous protein levels in plant cell cultures has been previously described (Terashima et al. 1999; Wongsamuth and Doran 1997), and truncated protein fragments have been detected in the Western blot analyses of plant protein extracts (Russel 1999; Ma et al. 1994). The inhibition of protease activity is crucial for protecting secreted proteins against degradation as the result of extracellular protease attack. The expression and secretion of proteinase inhibitors in transgenic rice cell suspension media reduced the relative protease activity to 23% (Kim et al. 2007). Co-expression with protease inhibitors may prove to be a potential strategy for protecting the cleavage and increasing the production of hGH in transgenic rice cell suspension cultures.

In this study, we constructed a plant expression vector containing the synthetic human growth hormone gene modified on the basis of the rice optimized codon usage under the control of the Ramy3D promoter, and introduced this vector into a transgenic rice suspension culture. It was verified that the recombinant human growth hormone with biological activity was generated and accumulated in the transgenic rice suspension culture medium.

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