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Production and Secretion of Human Interleukin-18 in Transgenic Tobacco Cell Suspension Culture

Niti Sharma[1], Tae-Geum Kim[2], and Moon-Sik Yang[2]*

[1] Basic Science Research Institute, Chonbuk National University, Jeonju 561-756, Korea
[2] Division of Biological Sciences, Research Center for Bioactive Materials, Chonbuk National University, Jeonju 561-756, Korea

Abstract Interleukin-18 (IL-18), otherwise known as interferon-gamma-inducing factor (IGIF), is one of several well characterized and important cytokines that contribute to host defenses. The complementary DNA (cDNA) of mature human interleukin-18 gene (hIL-18) was fused with the signal peptide of the rice amylase 1A gene (Ramy1A) and introduced into the plant expression vector under the control of a duplicated CaMV 35S promoter. The recombinant plasmid was transformed into tobacco (*Nicotiana tabacum* L. cv Havana) using the *Agrobacterium*-mediated transformation method. The integration of the hIL-18 gene into the genome of transgenic tobacco plants was confirmed by polymerase chain reaction (PCR) amplification and its expression was observed in the suspension cells that were derived from the transgenic plant callus by using Northern blot analysis. The hIL-18 protein was detected in the extracts of the transgenic callus and in the medium of the transgenic tobacco suspension culture by using immunoblot analysis. Based upon enzyme-linked immunosorbant assay (ELISA) results, the expression level of the hIL-18 protein approximated 166 μg/L in the suspension culture medium. Bioassay results from the induction of interferon-γ from a KG-1 cell line indicated that the hIL-18 secreted into the suspension culture medium was bioactive.

Keywords: hIL-18, cell suspension culture, tobacco, 35S CaMV promoter

INTRODUCTION

Transgenic plant cell suspension culture systems have been used to produce heterologous proteins [1-5]. The plant cell culture systems have several advantages: reduction of risk by mammalian viral contamination, ease of purification, low cost of plant culture media, and the production of biologically active proteins including the assembly of complex multimeric proteins such as antibodies [6]. Therefore, plant culture systems may prove to be a highly favorable means of producing small-to-medium quantities of high priced, high purity, specialty recombinant proteins.

Interleukin-18 (IL-18), formally known as interferon-γ-inducing factor (IGIF), is a member of the IL-1 cytokine superfamily [7]. The first identification of IL-18 was in mice with endotoxin shock induced by the sequential injection of *Propionibacterium acnes* and bacterial lipopolysaccharides (LPS) [7]. Soon thereafter, Ushio, *et al.* [8] characterized the human form of IL-18 (hIL-18). IL-18 is expressed in a wide range of cells including Kupffer cells, macrophage, T cells, B cells, osteoblasts, keratinocytes, dendritic cells, astrocytes, and microglia cells [9]. IL-18 is a potent proinflammatory cytokine capable of inducing IFN-γ, GM-CSF, TNF-α, and IL-1 in immunocompetent cells and to up-regulate the expression of certain chemokine receptors. The combination of IL-18 with IL-12 induces the production of IFN-γ in T cells, B cells, and NK cells [7,10,11]. IL-18 is also involved in inflammatory diseases by direct induction of TNF-α production in human monocytes and, together with INF-γ, indirectly stimulating IL-1β [12]. IL-18 has an important role in protecting the host against severe infections involving intracellular bacteria, viruses, fungi, and protozoa. IL-18 also enhances tumor rejection by its potent capacity to up-regulate the cytotoxic actions of NK and $CD8^+$ T cells *in vivo* [10]. In contrast, IL-18 induces naïve T cells to develop into Th2 cells. IL-18 in the absence of IL-12 induces the production of IL-4 and IL-13 by NK cells, master cells and basophils [13,14]. Therefore, IL-18 is an important and critical regulator of both innate and adaptive immune responses and influences nonimmune mechanisms of a hosts defense and inflammation responses.

The expression and production of bioactive hIL-18 in transgenic tobacco plants has previously been reported [15]. In this study, a transgenic tobacco suspension culture system was developed and used to produce and secrete the hIL-18 protein into suspension culture medium. The hIL-18 was easily purified and shown to have bio-

***Corresponding author**
Tel: +82-63-270-3339 Fax: +82-63-270-4334
e-mail: mskyang@chonbuk.ac.kr

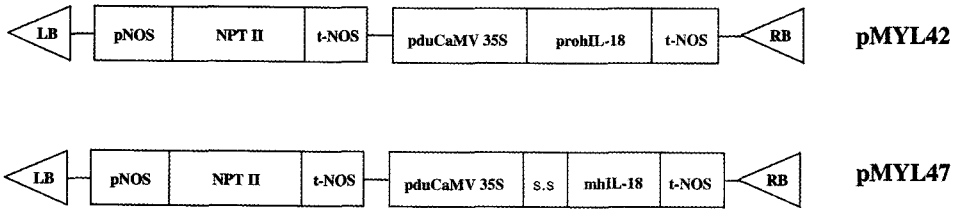

Fig. 1. Plant expression vector of pMYL42 and pMYL47. Genes located within the T-DNA sequence flanked by the right and left borders (RB and LB) include the mature hIL-18 (with signal sequence (s.s) of Ramy 1A) or pro hIL-18 (with pro signal peptide) gene (mhIL-18 and prohIL-18, respectively), an NPT II (neomycin phosphotransferase II) expression cassette for kanamycin selection of transformed plants, t-NOS which is the polyadenylation signal from the nopaline synthase gene in *A. tumefaciens* and Pnos which is the promoter from the *A. tumefaciens* nopaline synthase gene.

logical activity.

MATERIALS AND METHODS

Construction of the Plant Expression Vector and Plant Transformation

The complementary DNA (cDNA) of hIL-18 was synthesized from the total RNA preparation of LPS-stimulated peripheral blood mononuclear cells (PBMC) by the reverse transcription-polymerase chain reaction (RT-PCR) and fused with the signal peptide of Ramy1A for expression in yeast [16]. The pro hIL-18 (prohIL-18) with the pro signal, which is not fused with Ramy1A signal peptide, and the mature hIL-18 (mhIL-18) without the pro signal, which is fused with Ramy1A signal peptide were introduced into plant expression vectors under the control of a duplicated cauliflower mosaic virus 35S promoter (duCaMV 35S) and the resulting plasmids were denoted pMYL42 and pMYL47, respectively (Fig. 1). The recombinant plasmids were transformed into *Agrobacterium tumefaciens* LBA4404 by the tri-parental mating transformation method [17].

Tobacco (*Nicotiana tabacum* L. cv Havana) leaf discs were transformed using *A. tumefaciens* harboring the pMYL42 or pMYL47 plasmids. Following co-incubation with *A. tumefaciens* in solution, the explants were transferred to MS medium [18] supplemented with 0.1 mg/L of naphthaleneacetic acid (NAA), 1 mg/L of 6-benzylaminopurine (BAP), 30 g/L of sucrose, 8 g/L of agar, 100 mg/L of kanamycin and 300 mg/L of cefotaxime. The explants were transferred on the fresh medium every 3 weeks. The developing plant shoots were transferred to the MS medium containing kanamycin and cefotaxime, without phytoregulators, to induce root formation.

Detection of the hIL-18 Gene in Transgenic Plants

Genomic DNA was isolated from transgenic plants using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The concentration of genomic DNA was measured at 260 nm in a UV spectrophotometer. The presence of hIL-18 gene in transgenic plant genomic DNA was determined by PCR amplification analysis using the primer set specific for the hIL-18 gene; the forward primer for the proIL-18 gene is 5′-CGGGATCCATGGCTGCTGAACCAGTAGAAGAC-3′, the forward primer for the mIL-18 gene is 5′-AACTTGACAGCCGGGTACTTTGGCAAGCTT-3′ and the reverse primer for both genes is 5′-CGGAATTCCTAGTCTTCGTTTTGAACAGTGAA-3′. Amplification used a program of denaturing involving 94°C for 10 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and a final extension step of 72°C for 10 min. The PCR products were separated by electrophoresis in a 1.0% agarose gel.

Establishment of Cell Suspension Cultures

After confirmation of the presence of the hIL-18 gene in transgenic plants, transgenic calli were induced from transgenic plant leaf tissue using the same conditions as for the tobacco transformation. These transgenic calli were transferred into 50 mL (in 300 mL flask) of MS medium containing 1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.05 mg/L of kinetin, 100 mg/L of kanamycin and 3% sucrose at 25°C in a shaking incubator at 110 rpm. A 10 mL inoculum was transferred to fresh medium every nine days. The fresh weight of suspension cells was measured to compare the cell growth between transformed suspension cells with pMYL42 and pMYL47.

Northern and Western Blot Analyses

The total RNA was isolated from transgenic suspension cells after 5 days culture using the RNeasy plant total RNA extraction kit (Qiagen) and separated by electrophoresis through agarose gel containing formaldehyde (30 μg per lane) [19]. The separated RNA was then transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech RPN82B, Piscataway, NJ, USA). The membrane was hybridized with a ^{32}P-labeled hIL-18 probe using the Prime-a-Gene labeling system (Promega U1100, Madison, WI, USA) at 65°C in a Hybridization Incubator (FINEPCR Combi-H, Seoul, Korea). The membrane was washed twice with 2 × SSC and 0.1% SDS, and the wash procedure repeated twice more with 2 × SSC and 1.0% SDS for 15 min at 65°C. The hybridized bands were detected by autoradiography using X-ray film (Fuji Photo Film co. HR-G30, Tokyo, Japan).

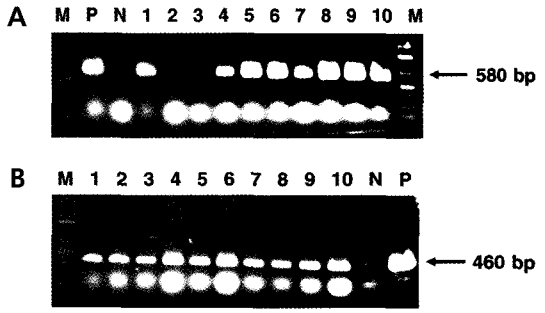

Fig. 2. Detection of the IL-18 gene in transgenic plants. Genomic DNA (400 ng) isolated from transgenic tobacco leaf tissues was used to demonstrate the presence of (A) the prohIL-18 gene or (B) the mhIL-18 gene using PCR amplification. (A), lane M is a 100 bp DNA Ladder (New England Biolabs, Beverly, MA, USA); lane P is a pMYL42 plasmid used as a control for PCR; lane N is non-transgenic plant genomic DNA used as a negative control; and lanes 1-10 are PCR products from transgenic plants (1~10). (B), lane M is a 100 bp DNA ladder (New England Biolabs); lane P is a pMYL47 plasmid used as a control for PCR; lane N is non-transgenic plant genomic DNA used as a negative control; and lanes 1-10 showed PCR products from transgenic plants (1~10).

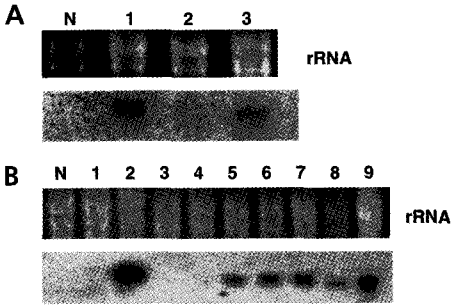

Fig. 3. Expression of hIL-18 in transgenic cell suspension cultures. The total RNA from suspension culture cells transformed with (A) pMYL42 or (B) pMYL47 was isolated and separated on the agarose gel containing formaldehyde. The separated RNA was hybridized with a ^{32}P-labelled hIL-18 probe. (A), lane N contains the total RNA of non-transgenic plant used as a negative control; lanes 1-3 contain that of transgenic plants (1~3). (B), lane N contains the total RNA of non-transgenic plant; lanes 1-9 contain that of transgenic plants (1~9). Before blotting, the gel used for the blot was stained with ethidium bromide to indicate that a similar amount of RNA has been loaded for each sample.

Transgenic plant leaf tissues were homogenized by grinding in 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 tissue homogenate was centrifuged at 17,000 g in a Beckman GS-15R centrifuge for 15 min at 4°C to remove insoluble cell debris. Sample aliquots were separated by SDS-polyacryla-mide gel electrophoresis (SDS-PAGE) and the bands blotted onto a nitrocellulose filter. After blocking with 5% skim milk, the filter was incubated with anti-hIL-18 antiserum (Koma Biotech Inc., 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 substrate for colorimetric detection by horseradish peroxidase according to Sambrook *et al.* [19].

Quantification of IL-18 in Suspension Culture

Medium containing recombinant hIL-18 in the transgenic suspension culture was centrifuged at 200 × g for 3 min. A one mL sample of the resulting culture supernatant was dialyzed against phosphate-buffered saline (PBS) overnight at 4°C and then used for quantitative ELISA. The concentration of recombinant hIL-18 was determined using a Human IL-18 colorimetric sandwich ELISA kit (R&D systems 7620, Minneapolis, MN, USA) according to the manufacturer's instructions.

Biological Activity of hIL-18

The suspension culture medium was centrifuged at 200 × g for 3 min, dialyzed against PBS buffer overnight at 4°C and sterilized by passage through a 0.2 μm syringe filter. The protein concentration was measured by the Bradford assay using the Bio-Rad Protein Assay Kit [20] and adjusted to 20 μg/mL for further analyses. An aliquot of 100 μL of sterile culture filtrate was serially diluted with PBS and used for the bioassays. To measure the biological activity, 100 μL of a human myelomonocytic KG-1 (ATCC CCL246) cell suspension (3 × 10^6 cells/mL) was mixed in the wells of a 96 well microtiter plate (Nunc, Roskilde, Denmark) with 100 μL of either hIL-18 standards or the medium samples. The microtiter plate was incubated for 24 h at 37°C in a 5% (v/v) CO_2/air humidified atmosphere and the production of IFN-γ by hIL-18 treated human myelomonocytic KG-1 cells measured using an ELISA [21].

RESULTS AND DISCUSSION

Vector Construction and Plant Transformation

The hIL-18 gene fused with the Ramy1A signal sequence was introduced into a plant expression vector under the control of duplicated CaMV 35S promoter to secrete hIL-18 protein into a suspension culture medium for easy purification (Fig. 1). The leaf tissues of tobacco were used for transformation with either pMYL42 or pMYL47 plasmids, harboring pro- or mature hIL-18 genes, by an *Agrobacterium*-mediated transformation. The putative transgenic plants were selected in the medium containing kanamycin (100 mg/L) and subjected to PCR amplification to detect the presence of the hIL-18 gene. The results demonstrated a corresponding size to the pro- and mature hIL-18 genes (580 and 460 bp, re-

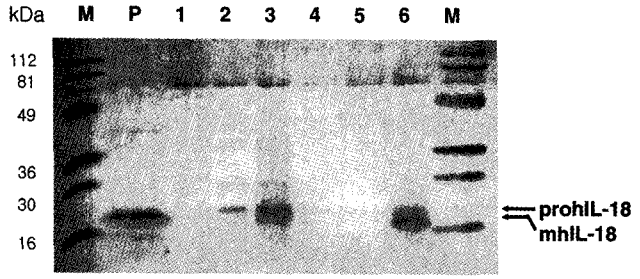

Fig. 4. Immunoblot detection of hIL-18 protein in transgenic plants. The transgenic plant cell protein extracts were analyzed for expression of the hIL-18 protein using anti-hIL-18 antibody as the primary antibody. Lane M contains molecular weight markers (Bio-Rad, Hercules, CA, USA); lane P is a human IL-18 protein produced in *E. coli*; lane 1 is a protein extract of non-transgenic callus (100 μg protein per lane) as a negative control; lane 2 is a protein extract of transformed callus with pMYL42-1: lane 3 is protein extract of transformed callus with pMYL47-2; lane 4 is a medium sample of non-transgenic suspension culture; lane 5 is a medium sample of transformed plant suspension culture with pMYL42; lane 6 is a medium sample of transformed plant suspension culture with pMYL47. The arrows indicate the bands of pro hIL-18 and mature hIL-18 proteins.

spectively). No band could be identified in the non-transgenic tobacco plants (Fig. 2).

Northern and Western Blot Analyses

Northern blot analysis was conducted to confirm expression of hIL-18 gene in the transgenic suspension cells that are transformed with pMYL42 or pMYL47 plasmids. Positive signals for hIL-18 gene expression were detected in both transgenic cells. There was no signal detected in non-transgenic tobacco cells (Fig. 3). As shown in Northern blot analysis, pMYL42-1 and pMYL47-2 transgenic cell suspension cultures showing the highest expression level of mRNA were selected for further experiments. Although a hIL-18 DNA band was detected by PCR analysis in transgenic plants, some transgenic plants showed no or only minor mRNA bands for hIL-18 by Northern blot analysis. These different expression levels of the hIL-18 gene likely depend on different incorporation sites of the target gene in the chromosome of different plants, often termed "position effect" [22,23].

The production of the hIL-18 protein in transgenic plant and suspension culture medium were examined using immunoblot analysis with anti-hIL-18 antibody. The transgenic plant transformed with pMYL42 showed pro hIL-18 (24 kDa) only in the plant protein extracts and not in the suspension culture medium because there is no signal peptide in the pMYL42 plasmid. The transgenic plant transformed with pMYL47 demonstrated the mature hIL-18 (18 kDa) in the transgenic suspension culture medium and a slightly higher band in the plant protein extract. The slightly higher band in the plant protein extract is due to no processing of signal peptide (Fig. 4).

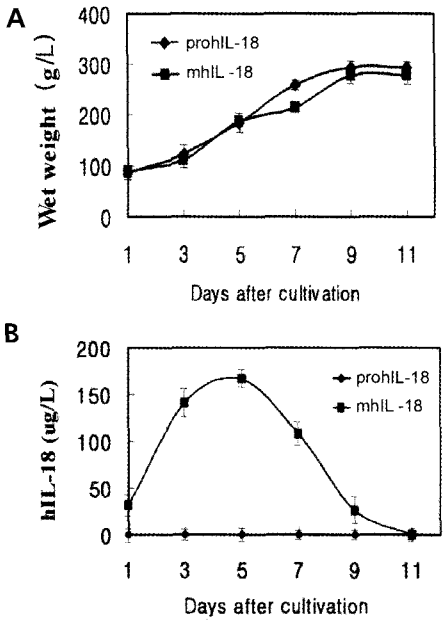

Fig. 5. Time courses of (A) cell growth and (B) expression level of hIL-18 in transgenic cell suspension culture. The expression level of hIL-18 in the medium of a transgenic suspension culture was examined. The prohIL-18 curve is from the medium of transgenic suspension culture with pMYL42-1 while the mhIL-18 curve is from the medium of transgenic suspension culture with pMYL47-2.

Production, Secretion, and Biological Activity of the hIL-18 Protein

The amounts of hIL-18 secreted into suspension culture medium were determined by quantitative ELISA (Fig. 5). The maximum expression level of hIL-18 in transgenic plants transformed with pMYL47 was found in the exponential phase at day 5 after transfer to fresh medium with a yield approximating 166 μg/L. However, the accumulation of IL-18 secreted into suspension culture medium decreased after 5 days culture. The reduction of accumulation of IL-18 may be due to protease activity secreted into suspension culture medium. The protease activity secreted into suspension culture increased during suspension culture [24].

The biological activity of hIL-18 secreted from the transgenic cells was analyzed by measuring IFN-γ production in KG-1 cells. The medium of non-transgenic and transgenic tobacco suspension cells with pMYL42 did not elicit the production of IFN-γ in the KG-1 cells. In contrast, the positive control of *E. coli* recombinant IL-18 and the suspension culture medium of transformed plants with pMYL47 indicated the production of IFN-γ from KT-1 cells (Fig. 6).

CONCLUSION

In conclusion, we have demonstrated that the cDNA of

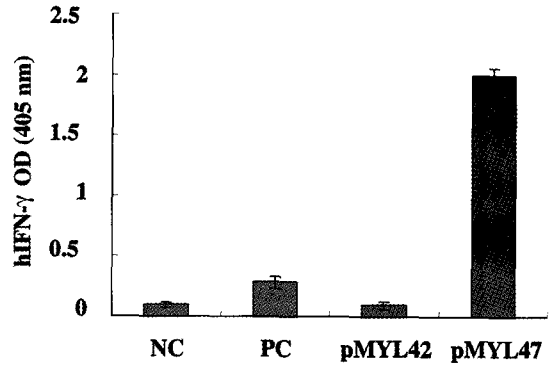

Fig. 6. Measurement of the biological activity of transgenic suspension cultures. The medium of transgenic suspension cultures was examined to detect IFN-γ secretion from KG-1 cells. Human IL-18 produced in *E. coli* was used as a positive control and compared to the hIL-18 secreted from transgenic plants. NC is a medium of non-transgenic suspension culture and PC is recombinant hIL-18 purified from *E. coli*.

human IL-18 gene was introduced into transgenic tobacco and that a transgenic plant suspension culture could be established to produce and to secrete hIL-18 into cell suspension culture medium leading to protein purification. The highest expression level of hIL-18 observed was during the exponential growth phase at day 5 after transfer to fresh medium for a yield of 166 μg/L. This transgenic suspension culture system could be adapted to the production and purification of pharmaceutical grade proteins. However, the production of hIL-18 has been decreased in stationary stage (day 5). It is speculated that the expression level of hIL-18 could be improved by the addition of a protein stabilizing agent [25] or the use of a Ramy3D system in a different transgenic rice suspension culture.

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