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Expression of Human Interleukin-11 and Granulocyte-Macrophage Colony-Stimulating Factor in Transgenic Plants

LEE, BO-YE[1], JEONG-HYUN LEE[1], HOON-SEOK YOON[1], KYUNG HO KANG[2], KYUNG-NAM KIM[3], JAE-HONG KIM[1], JU-KON KIM[4], AND JEONG-KOOK KIM[1]*

[1]*School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea*
[2]*National Institute of Crop Science, Rural Development Administration, Suwon 441-857, Korea*
[3]*Department of Molecular Biology, Sejong University, Seoul 143-747, Korea*
[4]*Division of Bioscience and Bioinformatics, Myongji University, Yongin 449-728, Korea*

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Abstract The production of therapeutic proteins for human diseases in plants results in many economic benefits, including reduced risk of animal virus contamination, high yields, and reduced production and storage costs. Human cytokines, interleukin-11 (hIL-11) and granulocyte-macrophage colony-stimulating factor (hGM-CSF), cDNAs were introduced into rice or tobacco, using either the maize ubiquitin promoter or the 35S promoter. The primary hIL-11 transgenic rice plants exhibited stunted growth and a sterile phenotype, whereas the hIL-11 transgenic tobacco plants did not. This suggests that hIL-11 expression in rice disrupts the normal growth and development of the plant. The regeneration efficiency of rice calli transformed with hGM-CSF was found to be approximately a quarter of that seen with the hIL-11, suggesting that hGM-CSF expression is more deleterious to the regeneration of rice calli than is hIL-11. However, the surviving hGM-CSF transgenic rice plants exhibited a normal phenotype of growth. Therefore, it appears that only those transgenic rice lines that expressed the human cytokines in small quantities were able to survive the selection process.

Key words: Human cytokines, interleukin-11, GM-CSF, transgenic plant, rice, tobacco

Many different expression systems, including those involving microbial and mammalian cells, have been used in the production of heterologous enzymes, cytokines, antibodies, and other recombinant proteins [15–17, 19, 21]. Each of these systems carries both advantages and disadvantages with regard to the production of recombinant proteins. Nevertheless, the production of such human proteins in plants may have many benefits [5, 8, 9, 26]. First, recombinant proteins produced in transformed plant cells are more likely to be safe for human consumption, since plant pathogens such as fungi and viruses can be more easily monitored, and may not be pathogenic to humans. Second, growing plants is much less expensive than raising animals for the production of proteins. Third, there are less ethical problems involved in the use of plants than the use of animal systems. However, the use of plants also carries a distinct disadvantage in that the glycosylation patterns of plants are different from those of humans [8].

In the early stages of molecular farming, recombinant proteins were produced using transgenic tobacco plants. Tobacco is a good system in that it can generate high biomass yields. However, tobacco and other plant species tend to produce noxious chemicals, such as alkaloids that often cannot be removed from the generated recombinant proteins [28]. Cereals usually do not produce such toxic compounds and have therefore come to be regarded as an alternative. In particular, rice and wheat, the world's principal cereals, potentially constitute economical and convenient production systems [28].

Rice (*Oryza sativa* L.) is the most important crop in Asia. It is self-fertilized under natural conditions, and it is unlikely that its pollen would fertilize other plants. In addition, the expression in rice seeds introduces the possibility of oral administration of some therapeutic proteins without the necessity of expensive purification processes.

Human IL-11 is a cytokine that is biologically related to IL-6, leukemia inhibitory factor (LIF), oncostatinM (OSM), and ciliary neurotrophic factor (CNTF) [7, 10]. Human IL-11 exerts a preventative effect against apoptotic cell death and inhibits preadipocyte differentiation. Human IL-11 cDNA encodes for a 199-amino acid precursor with a 21-amino acid hydrophobic signal peptide, which is

*Corresponding author
Phone: 82-2-3290-3157; Fax: 82-2-929-9028;
E-mail: jkkim@korea.ac.kr

cleaved to generate a 23 kDa biologically active form. Human IL-11 contains no cysteine residues or potential glycosylation sites [1, 24].

Human GM-CSF (granulocyte-macrophage colony-stimulating factor) is a hematopoietic growth factor and an activator of mature myeloid cells. Recombinant GM-CSF variants have been clinically studed for the treatment of a variety of diseases, including cancer, several infectious diseases, and some hematopoietic diseases. Mature GM-CSF, which consists of 127 amino acids, is generated by the cleavage of a hydrophobic 25-amino acid leader sequence residue [2, 18, 29]. Human GM-CSF has been previously produced in transgenic tobacco plants, as well as in rice cell suspension cultures [25, 27]. The primary objective of this study was to evaluate the possibility of obtaining transgenic rice plants that generate large quantities of hIL-11 and hGM-CSF.

MATERIALS AND METHODS

Plasmid Construction

The hIL-11 and hGM-CSF cDNAs were cloned into pMJU vector containing the maize ubiquitin promoter and the phosphinothricin resistance gene for rice (*Oryza sativa L.* cv. Dongjin) transformation experiments [14]. The hIL-11 and hGM-CSF cDNA clones were provided by Dr. Jae-Hong Kim of Korea University. hIL-11 was also cloned into pCAMBIA2300M2 containing the Cauliflower Mosaic Virus (CaMV) 35S promoter, neomycin phosphotransferase for tobacco (*Nicotiana tabacum cv. samsunNN*) transformation. The pCAMBIA2300M2 was kindly provided by Dr. Kyung-Hee Paek of Korea University. The constructs were mobilized into *Agrobacterium tumefaciens* strain LBA4404, using either the tri-parental mating or freeze-thaw methods [6, 12, 13].

Rice Transformation

Dehusked seeds were washed in 70% ethanol for 5 min and then rinsed with sterilized water in order to remove any traces of ethanol. Seed sterilization was conducted on a shaker, using a 50% sodium hypochloride solution. After 15 min, the solution was removed, and the seeds were washed 5–6 times thoroughly with sterilized water. The sterilized seeds were transferred into Petri dishes containing callus induction medium. After 4 weeks of incubation in darkness at 280°C, approximately 1–2 mm long calli initiated from the scutella were subcultured on fresh callus induction medium for 3 additional days.

Agrobacterium strains containing the constructs were then allowed to grow on AB media [20] supplemented with 50 mg/l carbenicillin and 10 mg/l tetracycline for 3 days at 28°C in darkness. The *Agrobacterium* cells were scraped from the AB media plates and resuspended for 60 min in liquid co-cultivation medium supplemented with 100 μM acetosyringone. Embryogenic calli were immersed in *Agrobacterium* suspension for 15 min–20 min. Agroinfected calli were blotted dry on sterile 3MM paper, transferred onto the co-cultivation medium, and then incubated for an additional 3 days at 28°C in darkness.

After co-cultivation, *Agrobacterium*-infected calli were washed in order to kill the *Agrobacterium*. These washed calli were then transferred to selection medium supplemented with 250 mg/l cefotaxime and 4 mg/l phophinotricin. After 2 weeks, the calli were subcultured onto fresh selection medium. The cultures were then incubated at 28°C in darkness. After 2 additional weeks on the selection medium, the calli exhibited sustained proliferation at one or more regions. Continuous selection on MS medium supplemented with 250 mg/l cefotaxime and 4 mg/l phophinotricin resulted in the appearance of apparently resistant and proliferating embryogenic calli. The cultures were then incubated at 28°C in light. Calli-producing roots and shoots in regeneration medium were transferred into nursery soil for 1 week and then finally transferred into soil.

Tobacco Transformation

Tobacco seeds were sterilized in 100% alcohol for 1 min and in 50% hypochloride for 1 min. The seeds were then rinsed 5 times with sterilized water, and the sterile seeds were positioned on Murashige and Skoog (MS) agar media [23]. Leaf discs, approx. 1 cm^2, were infected with *A. tumefaciens* harboring the hIL-11 construct in MS broth medium, supplemented with 2 mg/l benzylaminopurine (BAP) and 0.1 mg/l naphthaleneacetic acid (NAA) for 2 days at 28°C. After 2 days of co-cultivation, the leaf disks were washed in sterilized water and then transferred to shooting medium (MS agar media containing 2 mg/l BAP, 0.1 mg/l NAA, 200 mg/l carbenicillin, and 250 mg/l kanamycin). The leaves of the shoot formation were transferred onto rooting medium (MS agar media containing 1 mg/l indole-3-butyric acid). Roots, regenerated from these shoots after 2–3 weeks, were further incubated for growth into small plantlets. Putative transgenic plants were multiplied *in vitro*, transplanted into soil, and maintained under standard greenhouse conditions, eventually yielding whole plants.

RT-PCR Analysis and Northern Analysis

Total RNAs were prepared with TRI-reagent (MRC Inc., Cincinnati, OH, U.S.A.) according to the manufacturer's instructions. For cDNA synthesis, 4 μg of total RNA was primed with random hexamers. RT-PCR was conducted via 94°C incubation for 5 min, followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 60 sec, with a 5-min extension at 72°C. The primers used in the detection of the hIL-11 transcript were 5'-ACA GCT GAG GGA CAA ATT CC-3' (forward) and 5'-GAG TCT TCA GCA GCA GCA GT-3' (reverse). The primers used in the

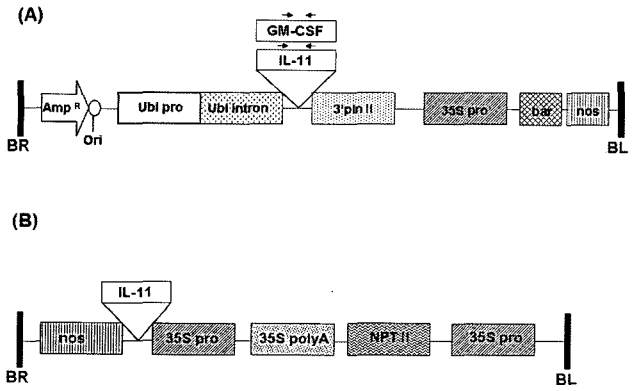

Fig. 1. Plasmid constructs utilized for the expression of hIL-11 and hGM-CSF in rice or tobacco.
(A) In pMJU, a rice transformation vector, the maize ubiquitin promoter (Ubi pro) was used to overexpress hIL-11 or hGM-CSF. The small arrows indicate the approximate location of primers used for detection of hIL-11 or hGM-CSF cDNAs. (B) In pCAMBIA 2300M2, a tobacco transformation vector, the CaMV 35S promoter (35S pro) was used to overexpress hIL-11. The bar indicates the phosphinothricin resistance gene and NPTII stands for neomycin phosphotransferase.

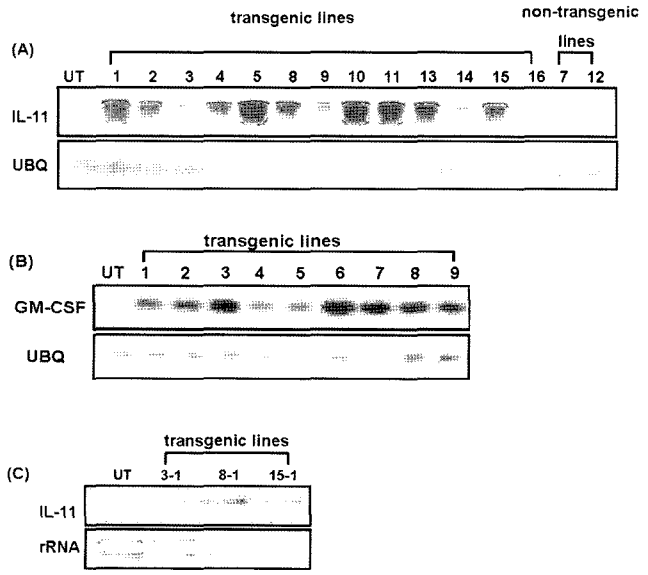

Fig. 2. Expression of human cytokines, hIL-11 and hGM-CSF, in transgenic plants.
(A) Expression of hIL-11 in transgenic rice plants. The hIL-11 mRNAs in the transgenic plants were analyzed via RT-PCR. Lines 7 and 12 are non-transgenic. Line 6 did not survive long enough to obtain an RNA sample from it. (B) Expression of hGM-CSF in transgenic rice plants. The hGM-CSF mRNAs in the transgenic plants were analyzed via RT-PCR. Ubiquitin mRNA (UBQ) was analyzed as a control. (C) Expression of hIL-11 in transgenic tobacco plants. The hIL-11 mRNAs in transgenic plants were analyzed by Northern hybridization. Total RNAs were extracted from the leaves of the T1 transgenic plants. Negative photography of the EtBr-stained rRNA bands for equal loading controls.

detection of the hGM-CSF transcript were 5'-CTG CTG CTG AGA TGA ATG AAA-3' (forward) and 5'-CTT CTG CCA TGC CTG TAT CA-3' (reverse).

Thirty μg of total RNA was then separated on 1% agarose gel containing 17.5% formaldehyde, and transferred to a Hybond N nylon membrane (Amersham, Buckinghamshire, U.K.). A [α-^{32}P] dCTP-labeled hybridization probe, corresponding to the hIL-11 coding region, was prepared with a random prime labeling kit (Amersham). The membranes were washed with 2×SSC and 1×SSC containing 0.1% SDS at 65°C for 10 min. The membranes were directly visualized with a BAS-2500 image analyzer (Fuji, Tokyo, Japan).

RESULTS

Generation and Characterization of the hIL-11 or hGM-CSF Transgenic Plants

Full-length hIL-11 or hGM-CSF cDNAs, carrying their own signal peptides, were cloned into a vector for the preparation of transgenic rice or tobacco plants. For the transgenic rice plants, the maize ubiquitin promoter was utilized, whereas the CaMV 35S promoter was utilized for the transgenic tobacco plants. However, hGM-CSF was cloned only into rice transformation vector, since its use in transgenic tobacco plants had already been reported [25].

In order to validate the integration of the hIL-11 and hGM-CSF cDNAs into the rice genomes, the genomic DNAs from the putative transgenic rice plants were assessed by PCR analysis. The putative transgenic plants were also evaluated for the presence of the hIL-11 and hGM-CSF transcripts via RT-PCR (Fig. 2). Among 16 putative hIL-11 transgenic lines, lines 7 and 12 were not found to harbor the cDNA in their genomes. However, all 9 hGM-CSF lines contained the hGM-CSF cDNA in their genomes. The abundance of the hIL-11 and hGM-CSF transcripts in the transgenic rice lines appeared to be quite low since these transcripts were undetectable by Northern analysis.

In the case of the transgenic tobacco plants, 15 out of 19 putative transgenic lines turned out to contain hIL-11 cDNA-specific fragments. In order to confirm hIL-11 expression, the total RNAs were extracted from transgenic tobacco plants, and RT-PCR was conducted. Transgenic tobacco plants with one transgene insert per genome were selected, based on a 3:1 ratio of kanamycin-resistant to susceptible phenotypes. The hIL-11 expression in these selected T1 transgenic plants was re-examined using Northern analysis (Fig. 2C). The hIL-11 specific transcripts were detected, thereby indicating that the hIL-11 gene could be overexpressed in the tobacco plants.

Constitutive Expression of hIL-11 in Rice Plants Causes a Dwarf and Early Flowering Phenotype

The primary hIL-11 transgenic rice plants exhibited a stunted growth phenotype (Fig. 3A): They were shorter

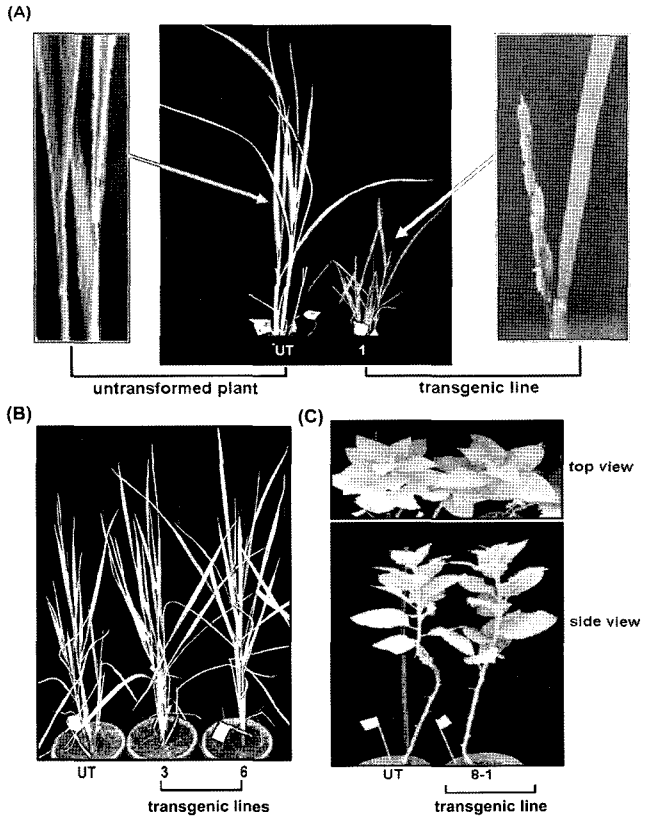

Fig. 3. Phenotypes of transgenic plants expressing hIL-11 and hGM-CSF.

(A) The primary transgenic rice lines expressing hIL-11. All transgenic lines exhibited phenotypes characterized by dwarfism and early flowering. A representative line (1) was shown with the untransformed plant (UT). The enlarged picture on the right shows the panicle, which appeared above the flag leaf of the transgenic line 1. On the other hand, the enlarged picture on the left shows no panicle above the vegetative leaves of the untranformed plant. (B) The primary transgenic rice lines expressing hGM-CSF. No primary transgenic lines exhibited abnormal phenotypes. (C) T_1 transgenic tobacco lines expressing hIL-11. The T_1 transgenic tobacco expressing hIL-11 exhibited no abnormal phenotypes.

(29.82±0.372 cm on average) and the leaves were smaller than those of the untransformed and non-transgenic plants. These hIL-11 transgenic lines flowered 35 to 65 days after they had been transferred into soil, which is much earlier than that observed in the untransformed plants and non-transgenic lines 7 and 12. The hIL-11 transgenic plants produced less numbers of tillers, and all tillers exhibited empty heads of grain: That is, all of the flowers remained unfertilized, although they produced tillers. Flowers of the hIL-11 transgenic rice plants also exhibited infertility. However, we detected no abnormalities in the external morphology of their flowers. The levels of the RT-PCR products from the hIL-11 primary transgenic lines appeared to generally correlate with the extent of severity of phenotypes. However, it was impossible to confirm that the correlation was inheritable, because the transgenic lines were sterile. Unlike in the hIL-11 transgenic rice plants, hIL-11 expression in the transgenic tobacco plants caused no phenotypic alterations in the plants. The plants appeared normal and flowered at a time similar to the untransformed plants (Fig. 3C).

The hGM-CSF transgenic rice plants, however, exhibited no abnormal phenotypes (Fig. 3B). These hGM-CSF transgenic lines flowered at a similar time as did the untransformed plants. Upon selection on regeneration media, the calli that had been transformed with the hGM-CSF construct exhibited much lower regeneration efficiency than those with the hIL-11 construct. The calli harboring the hIL-11 construct exhibited a 1.3% survival rate, whereas the calli with the hGM-CSF construct showed only a 0.36% survival rate, suggesting that the expression of hGM-CSF is more deleterious to regeneration of the transformed calli.

DISCUSSION

Molecular farming in plants offers the pharmaceutical industry a host of exciting new opportunities for the production of large quantities of proteins for use in both disease diagnosis and therapy. Recombinant human cytokines are expected to be of great market value [4]. The production of human cytokines, including interleukin-12 and interleukin-18, in transgenic plants has already been reported [10, 31].

In this work, transgenic rice plants that expressed hIL-11 or hGM-CSF have been constructed. However, these hIL-11 transgenic rice plants exhibited a phenotype characterized by early flowering and dwarfism. This indicates that hIL-11 expression in rice disrupts the normal growth and development of the transgenic plants. All of the transgenic rice plants appeared to express hIL-11 or hGM-CSF genes at quite low levels, since their mRNAs were detectable with RT-PCR analysis, but not with Northern analysis. This suggests that the overexpression of these two human cytokines may be toxic to regeneration of transformed calli on selective media, so that only transformed calli that generate these cytokines at low levels are able to survive the selection process. In fact, the regeneration efficiency was also determined to be quite low, especially in the case of the hGM-CSF transformed calli. hGM-CSF had already been successfully produced in transgenic tobacco seeds [25]. In this work, transgenic tobacco plants that overexpressed hIL-11 also exhibited no abnormalities. Therefore, the effects of expression of these cytokines differ from plant to plant. Considering its low level of expression in the transgenic rice plants, the effects of hIL-11 expression on plant development were unexpected. However, the expression of human therapeutic proteins in plants has sometimes proven previously to be detrimental to plant development. For example, the constitutive expression of human erythropoietin protein was found to induce

stunted growth, late flowering, and male infertility in transgenic tobacco [3]. Our speculation on the effect of hIL-11 and hGM-CSF expression in rice and tobacco transgenic plants may be incorrect, because we did not prove whether the hIL-11 or hGM-CSF proteins purified from the transgenic plants had their activity. In fact, we tried to detect the protein level in the transgenic rice lines using Western blotting, but it was unsuccessful, and this failure was probably due to very a low level of the protein. Since we did not detect the hIL-11 protein in the transgenic tobacco plants, we could not exclude the possibility that the lack of any phenotype in the transgenic tobacco might have been due to inactive hIL-11 protein produced through misfolding and/or glycosylation at an unexpected amino acid residue.

In order to avoid the problems with the transgenic rice plants that we encountered in this study, a number of alternatives could be considered. First, an inducible promoter might be used. Although it was the rice cell suspension culture system, an inducible promoter was found to be more effective than the constitutive promoters in increasing the yield of hGM-CSF [27]. The second approach might involve the targeting of cytokines specific to compartments, such as chloroplast, endosperm, or apoplast: Human interleukin-10 was also successfully targeted to chloroplasts [22], and human lysozymes have also been expressed in the endosperms of transgenic rice [30].

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