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Transcription of Human Interleukin-2 Gene in *Nicotiana tabacum* Driven by CaMV35S Promoter

Park, Soo Min and Choo Bong Hong*

Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea

Human interleukin-2 (IL-2) gene was inserted next of the 35S-transcript promoter of cauliflower mosaic virus in a binary vector and introduced into tobacco genome by *Agrobacterium*-mediated transformation. Genomic DNA blot analysis of the *in vitro* regenerated transgenic tobacco confirmed that the introduced human IL-2 gene was successfully integrated into tobacco genome. RNA blot analyses were carried out for the IL-2 transcript to examine expression of the introduced IL-2 gene. Specific signals only from the transgenic tobacco plants indicated that the introduced human IL-2 gene was properly transcribed. The transgenic tobacco plants were self-pollinated and the seeds were harvested. Differences in the level of the transcript were more apparent during the germination of the seeds and between the T_2 generation tobacco plants.

Key words: CaMV35S promoter, human IL-2 transcript, *Nicotiana tabacum*

Agrobacterium tumefaciens in conjunction with a binary vector is able to transfer a foreign gene into plant chromosome, and the transferred gene has been expressed in plants according to the function of the promoter placed in front of the gene. The genes transferred and expressed in plants are those of prokaryotes, plants and animals, and thus there seems to be no limitation of genes that can be transformed into and expressed in plants (Goy and Duesing, 1995). For a strong and constitutive expression of a foreign gene, the 35S-transcript promoter of cauliflower mosaic virus (CaMV35S promoter) has been widely used. With the promoter, it is common to be able to detect the transcript of the target gene in the transgenic plant in major organs including leaf, stem, petal and root (Kay *et al.*, 1987; Prat *et al.*, 1989; Flavell, 1994).

We introduced the human interleukin-2 (IL-2) gene into tobacco chromosome using *A. tumefaciens* and a binary vector. Human interleukin-2 is a protein which allows the long-term proliferation of T-cells following interaction with antigen, and the immunotherapeutic effect of IL-2 was reported in advanced renal cell carcinoma, malignant melanoma, colorectal cancer, B-cell lymphoma and Hodgkin's disease (Hannien *et al.*, 1993). Binary vector, pBKS1, is a derivative of pGA472 (An, 1987) and has a CaMV 35S promoter to lead the expression of a foreign gene (Suh *et al.*, 1994).

Human IL-2 cDNA was cloned by Taniguchi *et al.* (1983), and for a mass production of the protein, IL-2 cDNA was inserted and expressed in *Escherichia coli, Saccharomyces cerevisiae,* mammalian cell and insect cell (Taniguchi *et al.*, 1983; Devos *et al.*, 1983; Smith *et al.*, 1985; Kang *et al.*, 1989). In line of the effort to develop transgenic plants for the production of IL-2, we analyzed the expression pattern of the IL-2 gene driven by CaMV35S promoter in transgenic tobacco plants at the transcript level.

MATERIALS AND METHODS

Plants and bacteria

Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) seeds were germinated on vermiculite and sterilized soil mixture (1:1). The plants were maintained at the temperature of 25±2°C and photoperiod of 16 h light. *Escherichia coli* strain HB101 was used for the gene manipulation process. *Agrobacterium tumefaciens* strain LBA4404 was used for the transformation of tobacco plant.

Transformation of *E. coli* and *A. tumefaciens*

Introduction of plasmid DNA into *E. coli* was car-

*Corresponding author: Fax +82-2-874-1206

ried out as described by Sambrook *et al.* (1989). Introduction of plasmid DNA into *A. tumefaciens* was carried out as described by An (1987) with slight modifications. *A. tumefaciens* growing in YEP medium (10 g yeast extract, 10 g peptone, 5 g NaCl per liter, pH 7.0) was harvested at log phase by centrifugation at 3000×g for 5 min at 4°C. Bacterial pellet was resuspended in 20 mM ice-cold $CaCl_2$ solution. Plasmid DNA was added to the bacterial preparation, quickly frozen in liquid nitrogen and thawed at 37°C for 5 min. YEP medium was added and incubated at 28°C for 2-4 h with shaking at 150 rpm. Bacteria carrying the IL-2 gene in pBKS1 were selected on a kanamycin (50 μg/ml) containing YEP-agar plate.

DNA isolation and manipulation

Plasmid DNA was isolated from *E. coli* and *A. tumefaciens* by alkaline lysis method as described by Sambrook *et al.* (1989). Restriction digestion, agarose gel electrophoresis, DNA extraction and purification from agarose gel and DNA ligation were performed by standard procedures (Sambrook *et al.*, 1989; the manufacturers' suggestions). Restriction and modifying enzymes were purchased from KOSCO (Korea) and Promega (U.S.A.)

Transformation of tobacco and maintenance of transgenic tobacco

Tobacco was transformed according to Horsh *et al.* (1985). Young and healthy leaves were collected from two months old plants. Leaves were cut to squares and cocultured with *A. tumefaciens* carrying the human IL-2 gene in pBKS1 for two days in the dark at 25°C in callus induction medium (MS medium supplemented with 2 mg/ L of NAA and 0.5 mg/ L of BAP). After cocultivation, leaf discs were washed with MS medium and cultured on a MS solid medium supplemented with cefotaxim (100 mg/L) and kanamycin (200 mg/L). Regeneration of tobacco plants was carried out on MS medium supplemented with cefotaxim (100 mg/L) and kanamycin (200 mg/ L), and fully regenerated plants were moved to soil. Plants were maintained in a green house with double doors. Plants were self-fertilized, and seeds harvested were kept at 4°C. Seeds were placed on MS solid medium supplemented with kanamycin (300 mg/L) to induce imbibition and germination.

Genomic DNA blot analysis

Chromosomal DNA was isolated from the young leaves of tobacco as described by Junghans and Metzlaff (1990). Extracted genomic DNA was digested with *Sal*I, electrophoresed on a 0.6% agarose gel in the presence of ethidium bromide and blotted onto Hybond-N membrane (Amersham, U.K.). The membrane was prehybridized and hybridized in 50% formamide and 5 × SSPE at 37°C and washed in 0.2× SSPE and 0.1× SDS at 37°C. The membrane was exposed to an X-ray film (Kodak, X-OMAT, U.S.A.) with two intensifying screens (DuPont, U.S.A.) at -70 °C. Other conditions were as described by Sambrook *et al.* (1989).

RNA blot analysis

Total RNA was extracted from the transgenic and nontransgenic tobacco plants as described by Hong and Jeon (1987). Ten g of total RNA was electrophoresed on a 0.8% agarose gel containing 17.5% formaldehyde and blotted onto Hybond-N membrane. Prehybridization and hybridization were carried out in 50% formamide and 5× SSPE at 42°C and washed in 0.2× SSPE and 0.1× SDS at 42°C. The membrane was exposed to an X-ray film with two intensifying screens at -70°C. RNA loaded on each lane

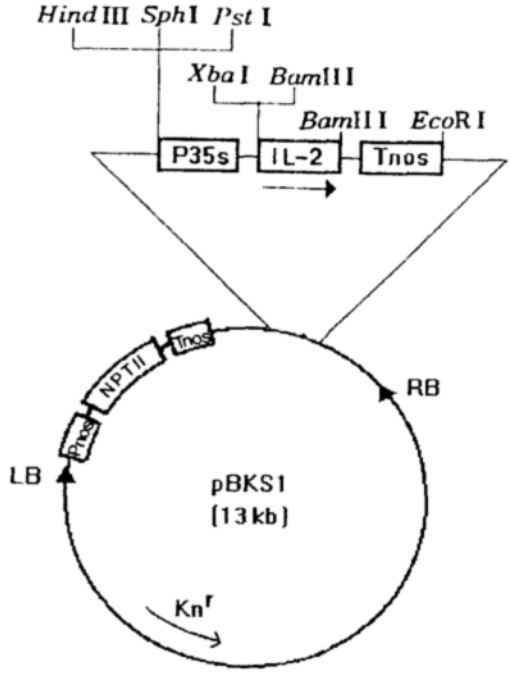

Fig. 1. A binary vector pBKS1 carrying human interleukin-2 gene. The IL-2 gene was inserted into *Bam*HI site between P35s and Tnos. Kn^r, kanamycin resistance gene; LB, left border sequence of T-DNA; NPTII, neomycin phosphotransferase II gene; P35s, promoter of cauliflower mosaic virus 35S transcript; RB, right border sequence of T-DNA; Tnos, terminator of nopaline synthase gene.

was normalized spectrophotometrically and by methylene blue staining of ribosomal RNA. Other conditions were as described by Sambrook *et al.* (1989).

RESULTS AND DISCUSSION

Human IL-2 cDNA clone, pKS2, was cut with *Eco*RI and subcloned into pGEM4. After amplification in *E. coli*, the plasmid DNA was isolated and cut with *Bam*HI. The *Bam*HI fragment was eluted from an agarose gel and put into the *Bam*HI-cut and dephosphorylated pBKS1, a transformation vector for higher plants (Fig. 1). To identify the direction of IL-2 gene, nucleotide sequence at the joining region of pBKS1 and the IL-2 gene was determined which confirmed that the 'ATG' translation initiation codon of IL-2 gene and the 3' terminus of CaMV35S promoter were properly connected in sense orientation (data not shown). The recombinant plasmid was moved into *A. tumefaciens* and used for the transformation of tobacco.

Genomic DNA blot hybridization for the putative *in vitro* regenerated transgenic tobacco plant with the ^{32}P-labelled IL-2 gene as a probe identified one or two strong bands for each transgenic line. From the nontransgenic tobacco, there was no apparent band observed (Fig. 2).

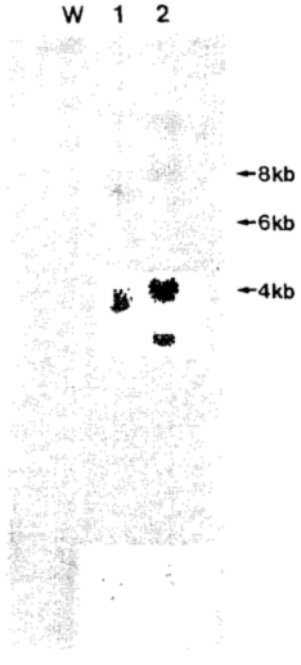

Fig. 2. Genomic DNA blot hybridization pattern for the introduced IL-2 gene in transgenic tobacco plants. W, nontransgenic W38 tobacco plant; 1 and 2, *Sal*I digestions of two different transgenic tobacco lines. The sizes indicated are from 1 kb DNA ladder.

RNA blot hybridization with the ^{32}P-labelled IL-2 gene for the RNA extracted from the kanamycin-resistant transgenic plants showed single major band at about 900 nucleotides from 5 different transgenic lines. Intensity of the bands was alike among the transgenic lines. From one transgenic line and nontransgenic plant, the hybridization band was not observed (Fig. 3).

Transgenic plants producing IL-2 transcripts were self-fertilized, and seeds were harvested. RNA blot hybridization for the transgenic tobacco seeds detected minimal levels of IL-2 transcript. After imbibition of the seeds, the level of IL-2 transcript increas-

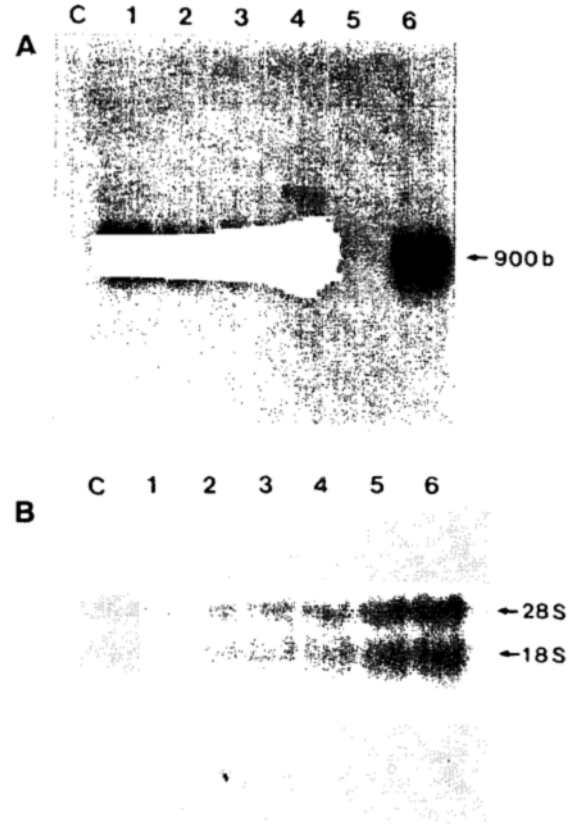

Fig. 3. RNA blot hybridizations for the IL-2 transcript in transgenic tobacco plants. A. RNA from T_1 transgenic tobacco plants was separated on a formaldehyde agarose gel and hybridized to the ^{32}P-labelled IL-2 cDNA. B. To normalize RNA on each lane of the gel, RNA was stained with methylene blue to compare the amount of ribosomal RNA. C, nontransgenic tobacco plant; 1, 2, 3, 4, 5 and 6, T_1 generation transgenic tobacco plant lines. 28S and 18S represent the positions for the large and small ribosomal RNAs.

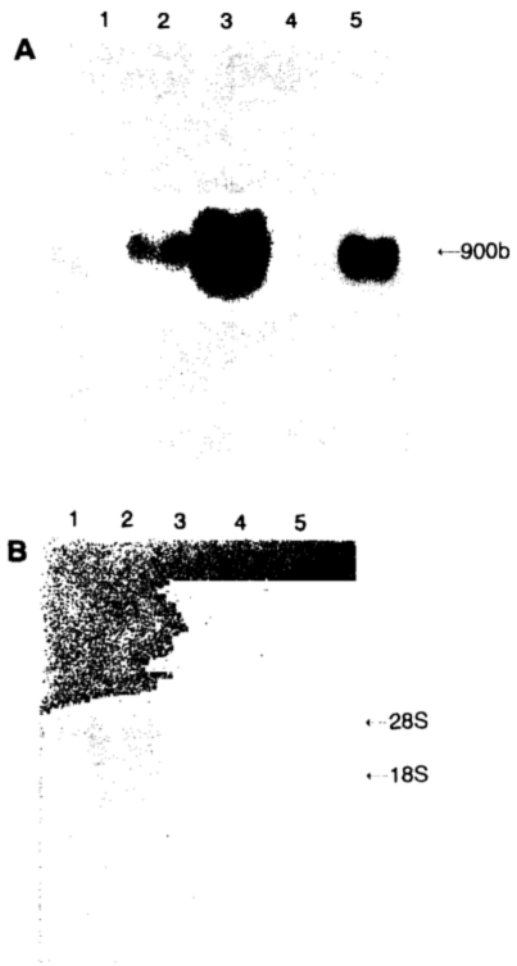

Fig. 4. RNA blot hybridization for the IL-2 transcript in germinating transgenic tobacco plants. A. RNA was isolated from dry seeds (1), seeds imbibed for 2 days (2) or 4 days (3), a nontransgenic plant as a negative control (4), and a T_1 generation transgenic tobacco plant as a positive control (5). RNA was separated on a formaldehyde agarose gel and hybridized to the ^{32}P-labelled IL-2 cDNA. B. Methylene blue stained ribosomal RNA from the samples as in A. 28S and 18S represent the positions for the large and small ribosomal RNAs.

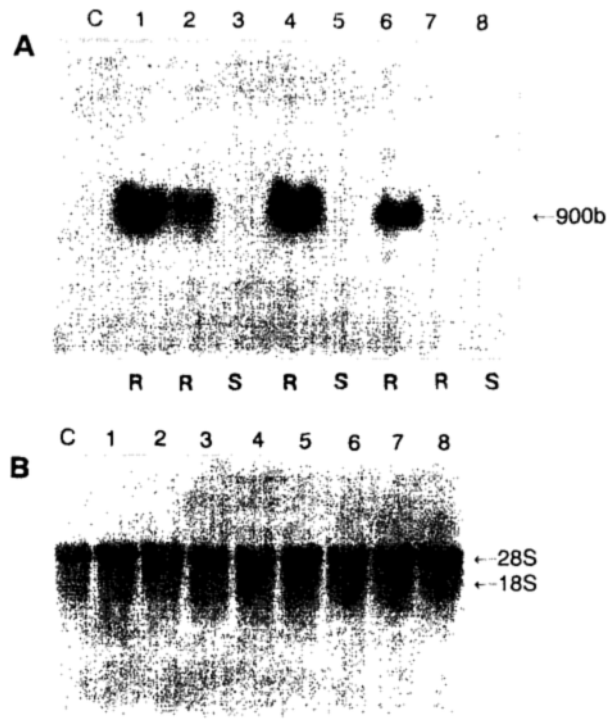

Fig. 5. RNA blot hybridization for the IL-2 transcript in T_2 transgenic tobacco plants. A. RNA from T_2 transgenic tobacco plants was separated on a formaldehyde agarose gel and hybridized to the ^{32}P-labelled IL-2 cDNA. B. To normalize RNA on each lane of the gel, RNA was stained with methylene blue to compare the amount of ribosomal RNA. C, nontransgenic tobacco plant; 1, 2, 3, 4, 5, 6, 7 and 8, T_2 generation transgenic tobacco plant lines; R, plant showing kanamycin-resistance; S, plant showing kanamycin-sensitivity. 28S and 18S represent the positions for the large and small ribosomal RNAs.

ed dramatically and after 4 days of imbibition, the level of transcript detected was more than 4 times higher than the transcript level of the fully grown leaves of T_1 and T_2 generation tobacco plants (Fig. 4). The IL-2 transcript level among the T_2 tobacco plants was largely variable. One order of difference in the band intensity was often observed between T_2 tobacco plants (Fig. 5).

The 35S promoter of the cauliflower mosaic virus is one of the most often used promoters in plants. It drives the transcription of an 8.2 kb polycistronic mRNA of its own, and is widely active in different unrelated species, both monocots and dicots (Prat *et al.*, 1989). It carries expression of a gene constitutively and strongly (Kay *et al.*, 1987). The result presented here (Fig. 5) suggests that the activity of CaMV35S promoter can be dependent on the developmental status of a plant. Marginal level of the IL-2 transcript detected from the dry seeds was probably from the stored IL-2 mRNA in the seeds rather than the *de novo* synthesized. The IL-2 transcript level went up very quickly upon imbibition which indicated a favorable environment for the function of the CaMV35S promoter quickly established in the seeds. The level of IL-2 transcript detected at the fourth day of imbibition was much higher than that of fully grown leaves which probably indicated a stronger CaMV35S promoter activity at this developmental stage.

The difference in the transcript level of a foreign gene among the transgenic plants was attributed to the position effect and gene silencing effect of the gene inserted into chromosome (Culver *et al.*, 1993; Matzke *et al.*, 1994; Neuhuber *et al.*, 1994). In this study from some of the transgenic tobacco plants which showed kanamycin-resistance, we could not observe the transcript of the human IL-2 gene. Especially, this variation was significantly increased in T_2 generation. This often observed aspect of loosing expression after the sexual transmission of the gene to the next generation suggests that the position effect and the gene silencing effect could be increased upon the rearrangement of genes during the crossing over of DNA in meiosis for the preparation of sexual cells and the fertilization process of the sexual cells.

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