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The Effect of the Recombinant Human Interleukin-2 Gene in Potato (*Solanum tuberosum* cv. Superior)

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To examine the effect of the T-cell growth factor (human interleukin-2), we constructed a binary vector, pSSK-1, carrying the recombinant human interleukin-2 (rhIL-2) gene, and transferred it into *Agrobacterium tumefaciens*. Using this construct, we then transformed potato explants (*Solanum tuberosum* cv. Superior), achieving 100% regeneration of shoots on a modified MS medium. Of the putative transformed shoots, 81% rooted and were selected on 200 mg/L kanamycin. Both Southern and northern analyses verified the transformation events. An ELISA test also indicated that the rhIL-2 protein was produced from rhIL-2-transformed potatoes. To determine whether this protein was biologically active in the potato cells, we performed a biological assay using the IL-2 dependent cell line, CTLL-2. The suspension containing extract from the transformants showed significant proliferation of the IL-2 dependent CTLL-2 cells, whereas cells did not proliferate in the nontransformed potato. We then grew the verified rhIL-2 transgenic potatoes in soil, and compared their performance with that of nontransgenic potatoes as well as those that had been transformed with GUS. Growth rates, as calculated from plant heights, were up to 50% higher than for either the nontransgenic or the GUS-transformed potatoes. Similar patterns were found with *Arabidopsis thaliana* plants treated in the same manner. All of these results suggest that rhIL-2 may function as a growth factor in potato.

Keywords: *Arabidopsis thaliana*, potato (*Solanum tuberosum* cv. Superior), recombinant human interleukin-2 (rhIL-2), T-cell growth factor

Recent advances in gene transfer technology have made it possible to engineer plants that produce proteins of pharmaceutical importance (Hiatt et al., 1989; Ma et al., 1995; Hong and Park, 1997; Zeitlin et al., 1998). Because of these technical advances, plant systems provide a viable alternative to microbial or animal cell expression systems for mass production of valuable proteins. In addition, these proteins may be more correctly modified with plant cells rather than through reliance on microbial expression systems (Hiatt, 1990).

Interleukin-2 (IL-2) is a pharmaceutically important cytokine secreted by helper T lymphocytes. Recombinant IL-2 (rIL-2) has been clinically applied in many human diseases, including cancers. The major function of IL-2 in humans is to activate various cells in the immune system, including helper T cells, B cells, macrophages, natural killer (NK) cells, and lymphokine-activated killer (LAK) precursors (Jeffrey et al., 1992). Human IL-2 (hIL-2) is composed of 133 amino acid residues, with a molecular weight of 15 kDa. In humans, IL-2 is synthesized as a prepro-protein, which is processed to the mature product by sequential proteolytic cleavage as it passes through the endoplasmic reticulum and Golgi complex. In contrast, the mature protein in microbial expression systems may be incompletely processed, secreted inefficiently, or be difficult to purify from the host cells (Taniguchi et al., 1983; Smith, 1988).

Recombinant human IL-2 (rhIL-2) has recently been produced through baculovirus-infected *T Trichoplusia. ni* insect cells. However, extremely high, subtoxic doses of rIL-2 are required to maintain a therapeutic concentration in the circulatory system (Rosenberg et al., 1985). Furthermore, a single injection of rIL-2 does not sufficiently sustain that therapeutic effect because of its short half-life and rapid clearance (Bubenik et al., 1985). Therefore, a large quantity must be available, but the current production system for rIL-2 is very expensive.

Potato (*Solanum tuberosum* L.) is an important crop species that is amenable to basic cell and tissue culture techniques, while being sensitive to *Agrobacterium* infection (Sree-Ramulu, 1987). Moreover, it can be produced in high quantities across many different agroecologies (e.g., subtropical, temperate, semi-arid, or humid mediterranean areas), and it possesses exceptional qualities of carbohydrates, proteins, and vitamins. In this study, we investigated rhIL-

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2 expression in the potato by constructing the plasmid pSSK-1 to replace the *udi* A gene of pBI121 with the rhIL-2 gene. Then, to determine whether rhIL-2 expressed by plant cells was biologically active, we performed a biological assay using the IL-2 dependent cell line, CTLL-2. Finally, to elucidate the relationship between rhIL-2 transgenic and nontransgenic potatoes, we used GUS-transgenic potatoes as the positive control to study expression of the rhIL-2 gene and its effect on plant growth.

MATERIALS AND METHODS

Construction of Plasmid for Plant Transformation

Nucleotide sequences were determined using a sequence kit (version 2.0; United States Biochemical Corp., Cleveland, OH, USA), according to manufacturer's instructions. Plasmid pBI121 (Jefferson et al., 1987), from Clontech, comprises pBIN19, which contains neomycin phosphotransferase II (NPT II) for selection; an 0.8-kb fragment containing the promoter for the 35S CaMV gene; and a 1.9-kb PstI fragment from pRJ225 that contains *udi* A. Plasmid pSSK-1, a derivative of pBI121, replaced the *udi* A gene (GUS gene) with the rhIL-2 gene (obtained from Dr. Hong, Seoul National Univ.) (Fig. 1). *Agrobacterium tumefaciens* strain *AGL1* was used for the transformations, and culturing was done at 30°C on a YEP medium supplemented with 50 mg/L kanamycin.

Potato Transformation

Stem explants prepared from in vitro-grown potato plants (*S. tuberosum* cv. Superior) were precultured for 2 d in a Stage I medium (MS salts, 0.5 g/L MES, 20 g/L sucrose, 0.4 mg/L IAA, and 2.24 mg/L BAP; Murashige and Skoog, 1962; Horsch, 1985). The precultured explants were soaked in overnight cultures of *A. tumefaciens,* which had been diluted (1:20) with the Stage I medium (minus the growth regulators) for 10 min. These explants were then transferred onto fresh Stage I media and cultured in the dark. After 3 d, they were moved to a Stage II medium (Stage I medium plus 50 mg/L kanamycin and 100 mg/L timentin), and cultured for 10 d under a 16-h photoperiod. Afterward, the samples were transferredI onto a Stage III medium (MS salts, 0.5 g/L MES, 20 g/L sucrose, 2.24 mg/L BAP, 0.1 mg/L GA, 50 mg/L kanamycin, and 100 mg/L timentin) to regenerate shoots.

Characterization of Transgenic Shoots

Potato stem segments were cocultivated with *A. tumefaciens* that harbored disarmed Ti-plasmids and bore specific gene constructs. The segments were placed on a selection medium containing 50 mg/L kanamycin and 100 mg/L timentin. Selection was then carried out in two steps. First, the regenerated shoots were selected in the presence of kanamycin (50 mg/L). Second, those shoots were tested on an MS medium containing 200 mg/L kanamycin to check for rooting.

Root Transformation of *Arabidopsis thaliana*

Using methods described by Valvekens et al. (1988), we cut roots from *A. thaliana* explants into 0.5-cm sections, then combined them with 0.5 to 1.0 mL of an *Agrobacterium* culture. This culture had been grown overnight at 30°C in YEP at 200 rpm. The root sections were co-cultivated for 2 min, then each piece was placed in a Petri dish containing solidified CIM media (0.5 mg/L 2,4-D and 0.05 mg/L kinetin). After 2 d, the lumps were re-divided into small explants and transferred to solidified SIM media (5 mg/L 2IP, 0.15 mg/L IAA, 50 mg/L kanamycin, and 750 mg/L vancomycin). Shoots emerged intermittently from the green calli over the next several weeks.

Genomic DNA Isolation and Southern Hybridization

We isolated genomic DNA from the in-vitro shoot

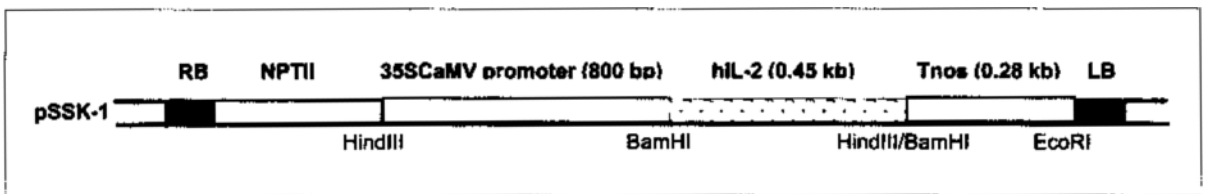

Figure 1. Scheme for the construction of pSSK-1. RB, Right border sequence; LB, left border sequence; NPTII, neomycin phosphotransferase II; Tnos, nos terminator.

cultures according to the method of Cone (1989). Ten μg of genomic DNA was digested with BamHI/HindIII, separated on a 0.8% agarose gel, then transferred onto a nylon membrane in 0.4 M NaOH. The membrane was prehybridized at 65°C in Church buffer (7% SDS, 1% BSA, 1 mM EDTA, and 250 mM Na_3PO_4; pH 7.2) for 20 min, and hybridized in the same solution now containing a radio-labeled rhIL-2 probe (Church and Gilbert, 1984). This membrane was washed twice in 2X SSC and 0.5% SDS for 30 min at 65°C, then twice in 0.2X SSC and 0.5% SDS for 30 min at room temperature. The membrane filter was exposed to X-ray film with an intensifying screen at −70°C. For the probe, the 450-bp fragment encoding the rhIL-2 gene was separated from the pSSK-1 plasmid and radio-labeled with a random priming method (Feinberg and Vogelstein, 1983).

RNA Extraction and Northern Hybridization

Total RNA was extracted from the potato leavesaccording to the method of Hong and Jeon (1987). Northern blot analysis was performed as described by Sambrook et al. (1989). RNA samples (20 μg) were electrophoresed on a 1.0% agarose gel containing 0.67 M formaldehyde, then transferred to a nylon membrane. Conditions for hybridization were the same as for Southern hybridization, except that a temperature of 42°C was used.

Quantification of rhIL-2 by ELISA

ELISA was performed using the method of Jeyaseelan et al. (1987). First, we coated the microtiter with crude protein extracts. We then applied an antibody (anti-mouse-anti-interleukin-2-IgG diluted with PBST to 1:1000) to each well. Afterward, a second antibody (AP-conjugated anti-rabbit IgG diluted with PBST to 1:25,000) was added. When color appeared, the reaction was stopped by adding 3 M NaOH to each well. Absorbance of the well contents was read at 405 nm.

Cell Line for Bioassay

IL-2 dependent CTLL-2 cells (ATCC, Rockville, MD, USA) were maintained in an RPMI-1640 medium (10% FBS, 2 mM glutamine, 0.5 mM sodium pyruvate, 5×10^2 mM 2-mercaptoethanol, and 50 U/mL rhIL-2; Genzyme, Cambridge, MA, USA) at 37°C in a humidified 5% CO_2/air atmosphere.

RhIL-2 Bioassay

Following methods described by Gillis et al. (1978), we performed a biological assay for rhIL-2, using the IL-2 dependent cell line, CTLL-2. The proteins were passed through 0.2-μm filters. Serial dilutions of the sample solutions were then distributed to 96-well microplates that contained 5×10^3 CTLL-2 cells per well in a final volume of 200 μL. After 24 h of culture in a CO_2 incubator, the cells were pulsed with 0.5 μCi ^3H thymidine per well, and incorporation was measured after 6 h. Because one unit/ml of IL-2 yields a half-maximal proliferation of CTLL-2 cells under these conditions, we calculated the concentration of rhIL-2 in our samples as the reciprocal of the dilution giving half-maximal proliferation.

Growth of Transgenic Plants

To quantify growth rates, we cut transgenic potato plants into 0.5-cm-long pieces, and cultured them in bottles containing MS media. After one month, the rooted sections were transplanted into soil. For the sake of comparison, we also grew nontransgenic potato plants as well as those that had been transformed with GUS. Heights from each line were measured at 10-d intervals for 3 months. In addition, the heights of transplanted *A. thaliana* transformants were measured over a 45-d period.

RESULTS AND DISCUSSION

Southern and Northern Analysis

To confirm the incorporation of the rhIL-2 gene into the potato genome, we extracted genomic DNA from transgenic potato leaves and hybridized it with a radio-labeled probe of rhIL-2. Figure 2 shows that a 450-bp fragment was indeed transformed in the transgenic plants. Northern hybridizations revealed expression of the rhIL-2 transcript in some of the transgenic plants, but not in the controls that were transformed only with the pBI121 vector (Fig. 3). The intensity of the northern signal differed among the transgenic plants, probably due to the "positional effect"of the transgene in the chromosome (Culver et al., 1993). Line A showed the strongest signal among the rhIL-2 transgenic potatoes. Because it is controlled at a variety of levels, including the initiation of transcription and processing, transgene expression may

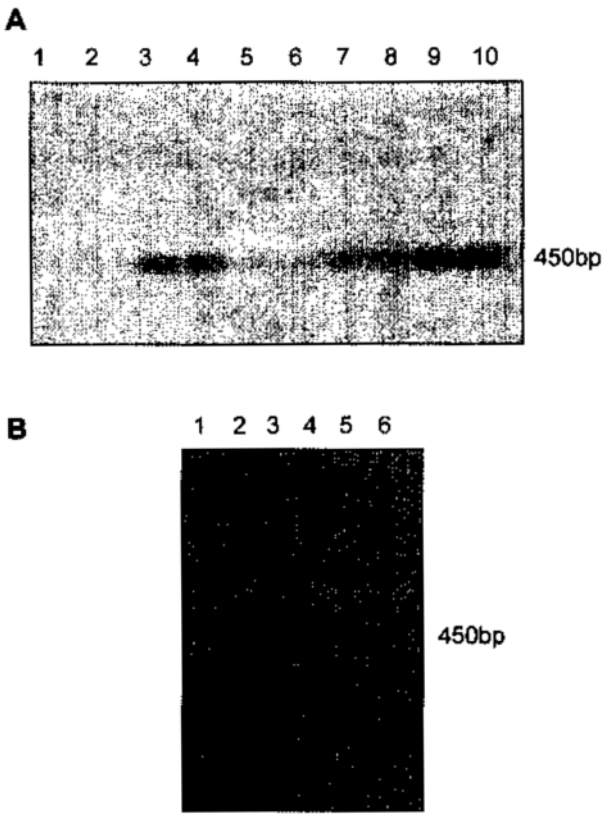

Figure 2. Southern blot hybridization of genomic DNA isolated from the transgenic potatoes. Genomic DNA (10 μg) was digested with BamHI. The digested DNA segments were separated on a 0.8% agarose gel and then transferred onto a nylon membrane. The blot was hybridized with the ^{32}P-labeled rhIL-2 fragment. **A.** Lane 1, 1-kb DNA ladder; Lane 2, untransformed potatoes; Lanes 3 - 10, transformed potatoes, **B.** Lane 1, 1-kb DNA ladder; Lane 2, untransformed *A. thaliana* plant; Lanes 3 - 6, transformed *A. thaliana* plants.

be modulated at one or more of these levels (Jefferson et al., 1987).

Quantification and Bioassay of rhIL-2

ELISA (enzyme-linked immunosorbent assay) is a highly versatile and sensitive technique for qualitative or quantitative determinations of practically any antigen or antibody (Engvall and Perlmann, 1971). Here, we used ELISA to quantify the amount of rhIL-2 produced from transgenic potatoes. All five tested lines that expressed the rhIL-2 transcript also accumulated the rhIL-2 protein. However, their optical densities differed at 405 nm (Fig. 4), with Line A having both the highest optical density as well as the strongest signal in our northern analysis. These results suggest that, although the rhIL-2 gene is successfully expressed in

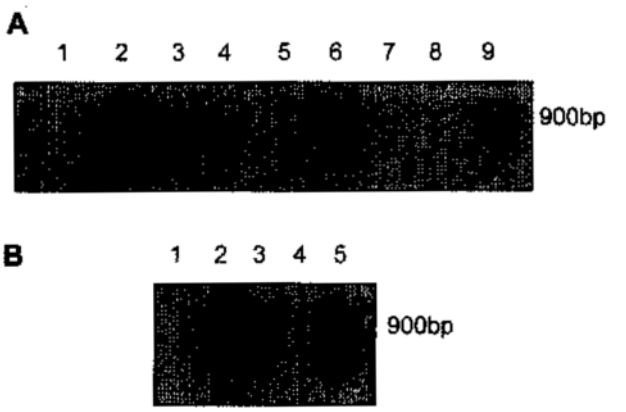

Figure 3. Expression of the rhIL-2 gene in transgenic potatoes. Total RNA was fractionated in a 1% agarose gel containing 0.67 M formaldehyde, and transferred onto a nylon membrane. The RNA blot was hybridized with the ^{32}P-labeled rhIL-2 probe. **A.** Lane 1, untransformed potatoes; Lanes 2 - 9, transformed potatoes: Lane 2, line A; Lane 3, line E; Lane 4, line F; Lane 6, line M; Lane 9, line T. (Lanes 5, 7, and 8 were not detected). **B.** Lane 1, untransformed *A. thaliana* plant; Lanes 2 - 5, transformed *A. thaliana* plants.

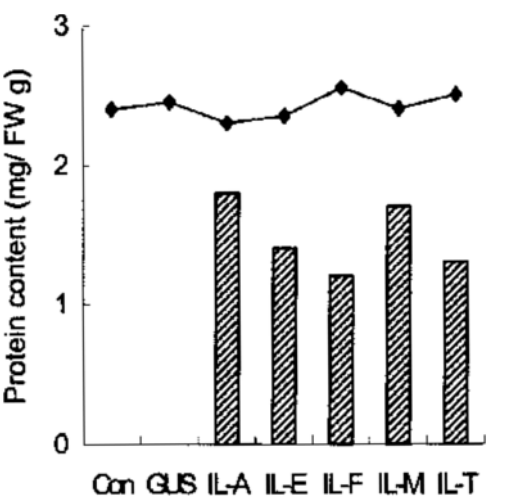

Figure 4. Quantification of rhIL-2 protein. Total protein content was measured by a Lowry assay; for rhIL-2, optical density was measured at 405 nm after ELISA test. ◆, total protein content; ▨, rhIL-2 content.

transgenic potatoes, its expression in each line may be differentially controlled at various levels, e.g., translation, processing of mRNA, and protein degradation (Hong and Park, 1997). The level of expression for rhIL-2 in this study was low, but it could be increased significantly through methods proposed by Stiekema et al. (1988) and Bernd et al. (1990).

To determine whether the rhIL-2 expressed in the transformants was biologically active, we conducted a

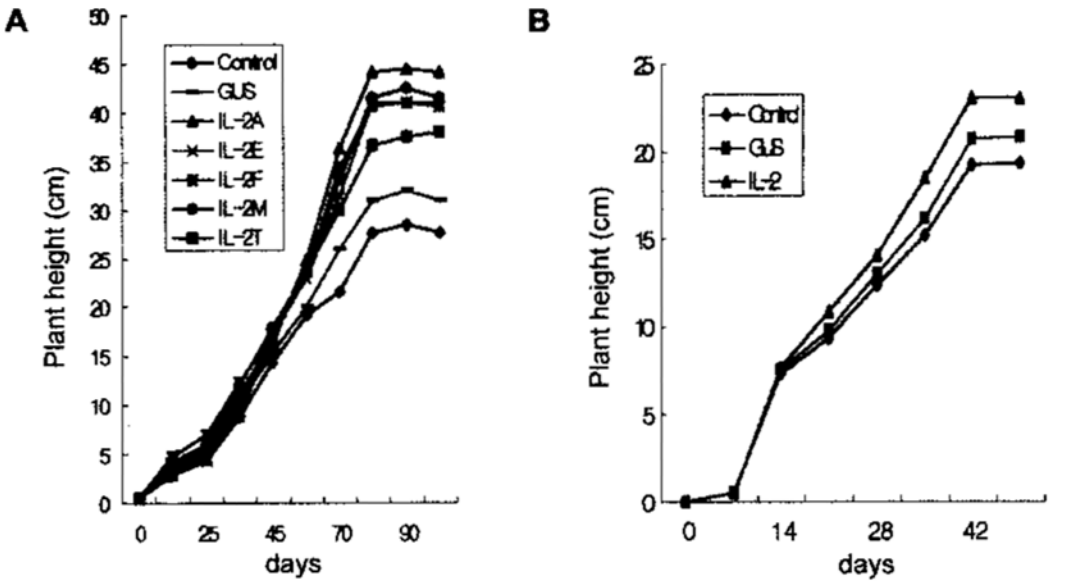

Figure 5. Height comparisons among transgenic potatoes (A) and among *A. thaliana* plants (B).

Table 1. Biological activity of rhIL-2 expressed by the pSSK-1. Extracts of transformed potato tissues were prepared as described in the Materials and Methods section.

Samples	3H-thymidine incorporated (cpm)	
Recombinant hIL-2 Protein	8.00 U/mL	8.270
	4.00	8.732
	2.00	6.762
	1.00	4.824
	0.50	3.081
	0.25	2.086
	0.13	1.638
	0.07	1.041
Nontransformed Potato	1:90	0.000
	1:27	0.000
	1:81	0.000
RhIL-2 Transformed Potato	1:90	7.739
	1:27	5.147
	1:81	2.840

^{3}H-thymidine incorporation assay using the IL-2 dependent CTLL-2 cell line, and examined its ability to support proliferation of CTLL-2 cells. As shown in Table 1, the suspension from our transformants demonstrated significant proliferation of those cells, while that from nontransformed potatoes did not.

Growth of Transgenic Plants

During the transformation process, we perceived that the rhIL-2 transgenic potato plants were growing rapidly. To verify this, we produced hundreds of rhIL-2 transgenic potato plants and randomly chose five lines among them. As expected, the growth rate for the rhIL-2 transgenic potato plants was high, with the heights of plants from transformant Line A, in particular, being 50% taller (42 cm) than the controls after 80 d. Likewise, the heights of transplanted rhIL-2 transgenic *A. thaliana* plants after 45 d also increased by about 20% compared with the growth rate of untransformed plants (Fig. 5). [In this evaluation, we took into account the difference in the growth cycle between potato and *A. thaliana*.] In addition, the rhIL-2 transgenic potatoes produced many more tubers than did the GUS transgenic potatoes (data not shown). These results suggest that rhIL-2 may play an important role in both the growth and differentiation of the potato.

In summary, we have shown that the gene responsible for animal bioactive proteins also can be expressed in plants, although more detailed study is required for increasing that expression (Stiekema et al., 1988; Bernd et al., 1990). Further research is also necessary for stabilizing these synthesized proteins, and for describing the growth stimulation mechanism in plants.

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