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Review

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A Novel Oxidative Stress-inducible Peroxidase Promoter and Its Applications to Production of Pharmaceutical Proteins in Transgenic Cell Cultures

Ok-Sun Lee[1], Sun-Mi Choi[2], Suk-Yoon Kwon[2], Haeng-Soon Lee[1], Kee-Yeun Kim[2], Jae-Whune Kim[3], Sang-Soo Kwak[2*]

[1]Lab. of Plant Cell Biotechnology; [2]Lab. of Environmental Biotechnology, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Oun-dong 52, Yusong-gu, Daejeon 305-806, Korea; [3]R&D Center, Microplants Co., LTD, Shinsung-dong 6, Yusong, Daejeon, 305-345, Korea

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Abstract

A strong oxidative stress-inducible peroxidase promoter (referred to as *SWPA2* promoter) was cloned from cell cultures of sweetpotato (*Ipomoea batatas*) and characterized in transgenic tobacco cultured cells in terms of biotechnological applications. Employing a transient expression assay in tobacco protoplasts, with five different 5'-deletion mutants of the *SWPA2* promoter fused to the β-glucuronidase (GUS) reporter gene, the 1314 bp deletion mutant showed approximately 30 times higher GUS expression than the CaMV 35S promoter. The expression of GUS activity in suspension cultures of transgenic cells derived from transgenic tobacco leaves containing the -1314 bp *SWPA2* promoter-GUS fusion was strongly expressed following 15 days of subculture compared to other deletion mutants, suggesting that the 1314 bp *SWPA2* promoter will be biotechnologically useful for the development of transgenic cell lines engineered to produce key pharmaceutical proteins. In this respect, we developed transgenic cell lines such as tobacco (*Nicotiana tabacum* L. BY-2), ginseng (*Panax ginseng*) and Siberian ginseng (*Acanthopanax senticosus*) using a *SWPA2* promoter to produce a human lactoferrin (hLf) and characterized the hLf production in cultured cells. The hLf production monitored by ELISA analysis in transgenic BY-2 cells was directly increased proportional to cell growth and reached a maximal level (up to 4.3% of total soluble protein) at the stationary phase in suspension cultures. The *SWPA2* promoter should result in higher productivity and increased applications of plant cultured cells for the production of high-value recombinant proteins.

Introduction

Plant cell suspension cultures are very important in the field of plant biotechnology for development of transgenic plants, for mass propagation and for the biosynthesis of key pharmaceutical proteins. Cell aggregates, so-called calli, formed from the wounded parts of explants might have a high potential to produce various antioxidant components to overcome oxidative stress derived by wounding. However, there are no detailed reports on the biochemical studies of cultured cells in terms of antioxidants. Cultured plant cells might be a good source of material for studies of antioxidant mechanisms and mass production of antioxidants, because they are considered to be grown under high oxidative stress conditions.

In previous reports, we have investigated the levels of

* Corresponding author, E-mail; sskwak@kribb.re.kr
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antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD), and low molecular weight antioxidants such as ascorbate and glutathione in 100 cell lines derived from different plant species (Kim et al., 1994; You et al., 1996; Ahn et al., 1999; Lee et al., 2000). Among the antioxidant activities investigated, plant cultured cells were found to have much higher levels of antioxidant enzymes such as SOD and POD than differentiated plant tissues. Particularly, a sweetpotato (*Ipomoea batatas*) cell line produced a very high level of POD activity (Kwak et al., 1995). Thus, we established an efficient production system of POD in suspension cultures of sweetpotato, showing that the A2 POD isoenzyme composes approximately 7.5% of total soluble cell protein. We isolated four POD cDNAs, three anionic *swpa1, swpa2, swpa3* and one neutral *swpn1*, from suspension cultures of sweetpotato. Moreover, we have characterized their expressions in terms of oxidative stresses, all four genes were predominantly expressed in cultured cells of sweetpotato (Huh et al., 1997; Kim et al., 1999). Interestingly, *swpa2* was particularly strongly expressed under these conditions. In contrast, it was not expressed in differentiated plant tissues. Furthermore, *swpa2* was strongly induced by environmental stresses such as wounding, chilling and ozone in leaves of sweetpotato, suggesting that this gene was regulated by a stress-inducible promoter.

Production of pharmaceutical proteins in cultured plant cells has been actively studied due to its potential commercial utility. Only a small number of low molecular compounds derived from plants such as shikonin and taxol have been successfully produced in plant suspension cultures (Fujita et al., 1984; Yukimune et al., 1996; Choi et al., 2000). However, with recent advance in plant metabolic engineering, protein expression in plant cell suspension cultures has significant potential (Yun et al., 1995; Shinmyo et al., 1998; Herbers and Sonnewald, 1999; Doran, 2000; Giddings et al., 2000). A powerful expression system using an appropriate promoter is key requisite for expression of foreign genes efficiently in cultured plant cells.

Recently we isolated a novel oxidative stress-inducible POD promoter cloned from cell cultures of sweetpotato (*I. batatas*) and its subsequent characterization using an *in vitro* transient assay, transgenic tobacco plants and cultured cells (Kim et al., 2003). The results highlighted the potential utility of the *SWPA2* promoter for the development of stress-tolerant transgenic plants and import-antly for the generation of transgenic cell lines to synthesize key biological materials.

Lactoferrin is an iron-binding glycoprotein with an approxi-mately 80 kDa which was originally found in milk. High levels of lactoferrin expression have been identified in neutrophils (Masson et al., 1969) and in lactating mammary glands (Green and Pastewka, 1978). Lactoferrin plays a significant protective role in human milk. Human lactoferrin (hLf) contains a specific antimicrobial domain consisting of a loop of 18 amino acid residues (Bellamy et al., 1992). Moreover, lactoferrin also has antibacterial (Nibbering et al., 2001), antifungal (Soukka et al., 1992), anti-endotoxin (Zhang et al., 1999), and antiviral activities (Hasegawa et al., 1994). Recombinant hLf has been produced in fungi (Ward et al., 1992), yeast (Liang and Richardson, 1993), and mammalian systems, including cows (Nuijens et al., 1997; van Berkel et al., 2002). However, animal and fungi production systems require expensive purification processes and harbor harmful mammalian disease-causing viruses, microbes, fungi and prions of animal cell origin (Arakawa et al., 1999).

A great advantage of plant cell suspension cultures is that recombinant proteins can be produced under certified conditions with large scale-up at low cost, but yields are low compared with stably transformed plants and yeast (Fishcher et al., 1999). Thus, a strong inducible promoter with a high expression in plant cell cultures will be required to overcome the low yield of target proteins including hLf. In this paper, we decribe a strong oxidative-stress inducible POD (*SWPA2*) promoter isolated from cell cultures of sweetpotato and its application to development of transgenic tobacco BY-2 cell lines with a fast cell growth and transgenic cell lines of medicinal plants such as ginseng (*Panax ginseng*) and Siberian ginseng (*Acanthopanax senticosus*) to produce a hLf protein in cell cultures.

Materials and Methods

Molecular cloning and characterization of a genomic *SWPA2*

Isolation of *SWPA2* genomic clone (Figure 1) and characteriza tion of its promoter were described in previous report in detail (Kim et al., 2003).

Fluorometric GUS activity and staining

Extraction of proteins and GUS activity in crude extracts was determined florometrically as described (Jefferson, 1987). Histochemical staining for GUS activity was performed essentially as described by Jefferson (1987) with X-gluc as a substrate. Protein content of sample extracts was determined according to the method of Bradford (1976) by use a Bio-Rad Protein Assay Kit with BSA as a standard. Data are presented as the mean of GUS activity from at least three independent determinations.

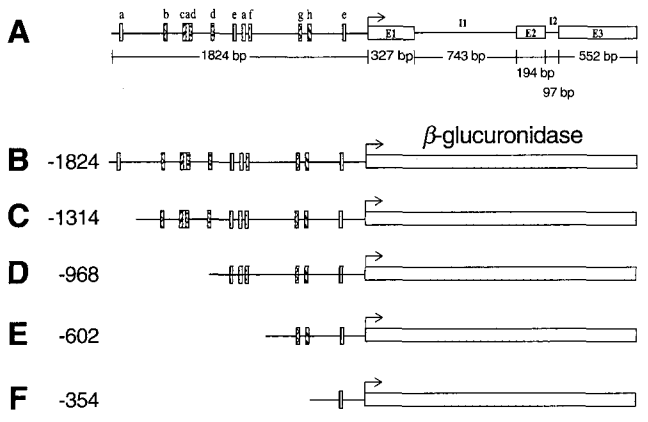

Figure 1. Functional architecture of sweetpotato (*Ipomoea batatas*) (Kim et al., 2003). Possible *cis*-elements involved in oxidative stress include a, GCN-4; b, Oct-1; c, HSE; d, AP-1; e, CATT box; f, TATA box; g, G-box; h, Sp-1. Introns 1 and 2 denoted by I1 and I2, respectively. Exons 1, 2 and 3 denoted by E1, E2 and E3, respectively. The Genbank Accession No for *SWPA2* genomic clone is AF453791.

Preparation of transgenic tobacco plants and cultured cells to express GUS protein

Agrobacterium tumefaciens strain LBA4404 harboring each pBS1314, pBS 1824 or pBI121 was used to transform tobacco plants (*Nicotiana tabacum* cv. Xanthi) by the leaf disc method (Horsch et al., 1985). Transformants were selected on MS (Murashige and Skoog, 1962) medium supplemented with 200 mg/L kanamycin and 300 mg/L claforan. Kanamycin resistant shoots were directly formed on cut edges of leaf discs. Rooting of kanamycin resistant plantlets was carried out in the selectable MS medium without growth regulators. After acclimation, the plants were transplanted to potting soil and maintained in a greenhouse. For callus induction, leaf segments from *in vitro* transgenic plants were incubated for 17 days on medium, containing MS salts (Duchefa Biochemical Co., Netherlands), 3% sucrose, 2 mg/L 1-naphthaleneacetic acid (NAA), 0.25 mg/L 6-benzyl-aminopurine (BA) and 1 mg/L thiamine-HCl.

Preparation of transgenic tobacco cell lines to produce hLf protein

The construction of a plant expression vector using *SWPA2* promoter and hLf cDNA (U0764) and transformation of tobacco cells were described in previous report in detail (Choi et al., 2003). Northern and Western blot analysis were also described in previous report in detail (Choi et al., 2003).

Protein extraction and determination of hLf content

Transformed tobacco cells (1 g of fresh weight) were homogenized on ice with a mortar in an equal volume of ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0) and centrifuged at 12000 g for 15 min at 4°C. The soluble protein concentration in the homogenate supernatant was determined (Bradford, 1976). An enzyme-linked immunosorbent assay (ELISA) was conducted on protein extracts from transformed and non-transformed (control) cells. The presence of recombinant hLf was assessed by ELISA according to the BIOXYTECH® Lactof-EIATMTMT™ (OxiResearchTM™).

Results and Discussion

Analysis of *SWPA2* promoter deletion mutants using tobacco protoplasts

To investigate the promoter activity of the *SWPA2* gene (Figure 1), five deletion mutants of this region (Figure 1B-F) were studied in a transient-expression assay employing tobacco (BY-2) protoplasts (Figure 2). The activity of *SWPA2* promoter mutants was examined by measuring the activity of the β-glucuronidase (GUS) reporter enzyme. The -1314 bp fragment supported the highest levels of GUS activity, which was approximately 30 times higher than that supported by the 35S promoter of CaMV. A deletion from -1314 to -968 bp slightly reduced GUS activity compared to the CaMV 35S promoter. Further deletion to -354 bp gradually reduced *SWPA2* expression to two-thirds those of the CaMV 35S promoter. The 1824 bp fragment showed a lower level of expression than the CaMV 35S promoter. Taken together, these results suggested that

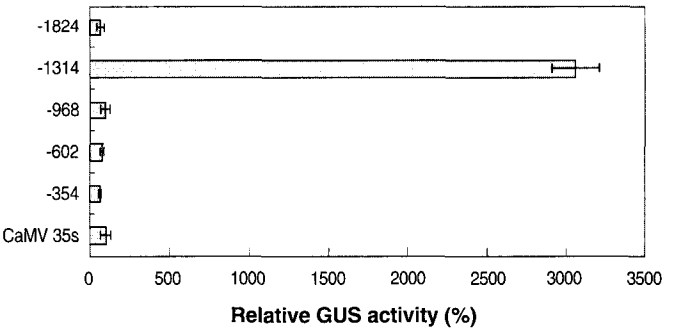

Figure 2. Functional analysis of *SWPA2* promoter deletion mutants in tobacco (BY-2) protoplasts (Kim et al., 2003). Deletion mutants defined as numbers of base pairs from the site of translation. GUS activity is expressed relative to that supported by CaMV 35S promoter, which was 5176±96 pmol/min/mg protein. Data are mean±SE of three replicates.

positive *cis*-elements may be located in the regions between -1314 and 968 bp, whereas negative *cis*-elements may exist in the regions between -1824 and 1314 bp. The *cis*-elements such as GCN-4, AP1 and HSE in the region from -1164 to -1188 bp may therefore be important for gene expression. The characterization of exact positive and negative *cis*-elements remains to be determined.

The *SWPA2* promoter directs high GUS expression in transgenic tobacco cell lines

Transgenic calli were successfully developed from the leaves of transgenic plants containing *SWPA2* promoter deletion mutants. Transgenic calli containing 1314 and 1824 bp *SWPA2* promoters were strongly stained with X-gluc, whereas those containing the CaMV 35S promoter showed a weak GUS staining, reflecting the GUS activity of each deletion promoter (Figure 3A). Transgenic calli containing -1314 and -1824 bp *SWPA2* promoters showed approximately 3.3 and 1.9 times higher GUS activity than those containing the CaMV 35S promoter after 30 days of subculture (Figure 3B).

After establishment of suspension cultures from these calli, their respective GUS activities were investigated (Figure 4). The cell growth of -1314 bp, -1824 bp and CaMV 35S cell lines was similar in pattern, showing a typical sigmoidal growth curve, with maximum growth at 15 days after subculture (DAS) (Figure 4A). Hence, there is no significant difference in cell growth among these three cell lines. The GUS activity established in CaMV 35S:: GUS containing cells showed a constitutive low-level of GUS expression regardless of the cell growth phase. However, the GUS activity in the -1314:: GUS cell line showed a minimum level at 5 and 7 DAS, but markedly increased

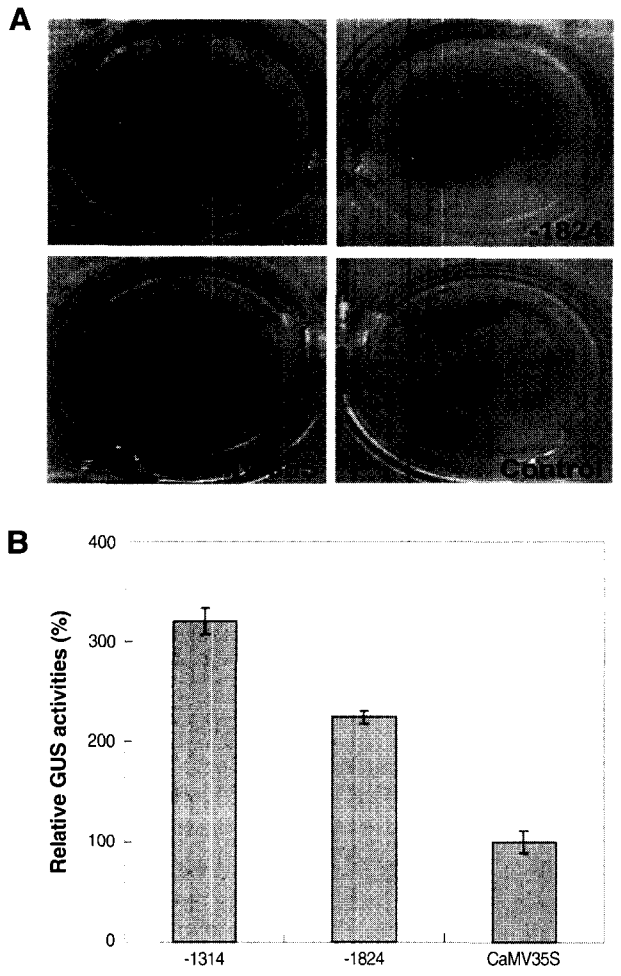

Figure 3. Expression of GUS protein in calli derived from leaves of transgenic and non-transgenic tobacco plants (Kim et al., 2003). GUS staining (A) and GUS specific activity (pmol/min/mg protein) after 30 days of subculture (B). The calli was cultured on MS medium supplemented with 2 mg/L NAA, 0.25 mg/L BA and 3% sucrose. GUS activity is expressed relative to that supported by CaMV 35S promoter, which was 4707±522 pmol/min/mg protein. Data are mean ±SE of three independent replicates.

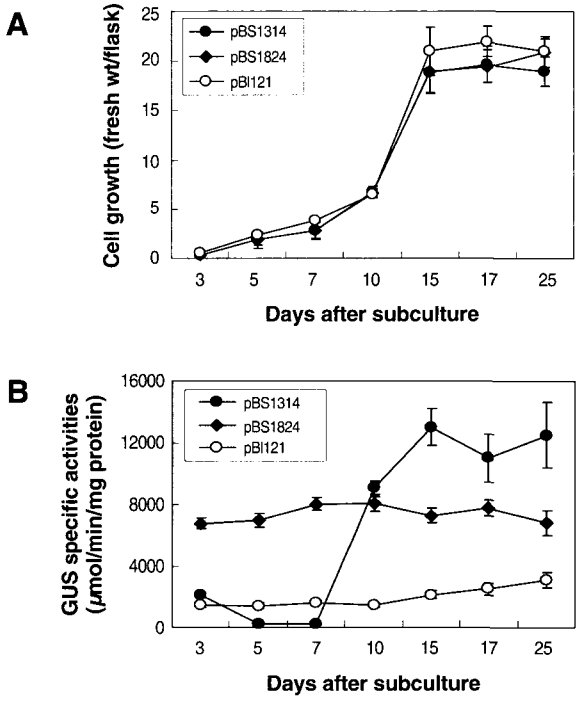

Figure 4. Expression of GUS activity following subculture of cells derived from leaves of transgenic and non-transgenic tobacco plants (Kim et al., 2003). Cell growth (A) and GUS specific activity (pmol/min/mg protein) (B) in three cell lines. pBS1314 and pBS1824 transformants indicate the transgenic cells containing the 1314 bp and 1824 bp promoter/ GUS fusion gene in pBI101.1 vector, respectively. Data are mean±SE of three replicates.

GUS activity from 7 DAS, which reached a maximum level at 15 DAS and sustained a high level of GUS activity with further cultures (Figure 4B). In the case of the -1824 :: GUS cell line, a high level of constitute GUS was observed during every growth phase. As expected, the GUS activity established by the 1314 bp *SWPA2* promoter showed high levels at stationary growth phase (15 DAS), reflecting the accumulation of *SWPA2* transcripts during cell growth (Kim et al., 1999). The GUS activities of -1314 :: GUS and -1824 :: GUS cell lines were approximately 6.2 and 1.8 times higher than that recorded in CaMV 35S :: GUS cell line.

In a previous study, we discovered that *SWPA2* was not expressed in any tissues of differentiated plants, whereas this gene was strongly expressed in cultured cells, and induced by wounding, chilling and ozone in intact leaves (Kim et al., 1999). High GUS activity in transgenic tobacco cell lines containing -1314 and -1824 bp *SWPA2* promoters well reflected the POD expression in cell cultures of sweetpotato (Kim et al., 1999).

There are some differences of GUS expression between experiment systems such as protoplasts, leaves and cultured cells and deletion mutants of the *SWPA2* promoter, suggesting the existence of complicated factors among experiments in terms of stress. The difference among experiment systems remains to be determined.

The -1314 *SWPA2* promoter can be considered as an oxidative stress-inducible promoter, since it has several *cis*-elements involved in oxidative stress in animal cells (Figure 1), and it may regulate GUS expression in transgenic plants and cultured cells by oxidative stress (Figures 3 and 4). In conclusion, the results indicate that an oxidative stress-inducible *SWPA2* promoter, particularly -1314 bp promoter, from sweetpotato, will be biotechnologically useful for the development of stress-tolerant plants and for the development of transgenic cell lines to produce useful biological materials. In addition, the *SWPA2* promoter may have high utility for studies investigating oxidative stress signal transduction. The transcription factors in relation to positive and negative *cis*-elements are currently under investigation.

Transformation and cell line selection to produce hLf

In order to produce a human lactoferrin protein in plant cultured cells, we developed a transgenic tobacco (*Nicotiana tabacum* L. BY-2) cell line using an oxidative stress-inducible peroxidase (*SWPA2*) promoter and characterized the production of human lactoferrin in cultured cells (Choi et al., 2003). A construct containing a targeting signal peptide for tobacco endoplasmic reticulum fused to human lactoferrin cDNA under the control of *SWPA2* promoter was engineered.

Tobacco cultured cells was transformed by *Agrobacterium tumefaciens* carrying a SWPA2pro :: ER-hLf/pCGN1578 plasmid. Transformed tobacco calli were selected on MS medium containing 150 mg/L kanamycin. Kanamycin-resistant calli were formed after 3-4 weeks of cultures on selection medium. Thirteen independent kanamycin-resistant cell lines were obtained and the presence of hLf and nptII gene in tobacco cells was detected by PCR (data not shown here). The transformed tobacco cells had a normal appearance and had similar growth characteristics as the non-transformed control cells.

The levels of hLf protein of thirteen PCR positive cell lines were analyzed to select high-yielding cell lines by ELISA analysis. Transgenic calli expressed the hLf contents ranging from 0.7% to 2.7% of total soluble protein (data not shown here). Six transgenic cell lines (T1-T6) producing a high level of hLf protein were selected for further study in suspension cultures.

Molecular analysis of hLf high-yielding cell lines

To confirm the stable integration of the hLf gene into the nuclear genome of host cells, Southern blot analysis was performed with the tobacco cell lines (T1-T6) with high levels of hLf protein, using the ^{32}P-labeled hLf cDNA as a probe (data not shown). After *Eco*RI digestion, six cell lines produced a single band, indicating that the hLf gene was properly incorporated into the genomic DNA of tobacco cells.

Total RNA isolated from the same cell lines as Southern blot analysis was analyzed by northern hybridization with an hLf-specific probe to examine the expression of hLf gene in transgenic tobacco cells. Northern blot analysis indicated the presence of 2.3 kb transcripts (Figure 5A). The hLf gene was highly expressed in four transgenic tobacco cell lines such as T1, T2, T4, and T5, whereas was detected at a very low level in other two transgenic cell lines, T3 and T6. The expression of hLf gene was not detectable in non-transformed control cells. The transgenic tobacco cell lines were tested for the expression of the lactoferrin protein by Western blot analysis. Recombinant hLf protein synthesized in six transgenic cell lines (T1-T6) is shown in Figure 5B. The immunoreactive hLf levels differed among the transgenic cell lines tested. The transgenic tobacco cells except for T3 produced hLf protein with 80 kDa and 40 kDa, but extracts of non-transformed (C) callus did not react with the anti-hLf antibody. T3 cell line produced hLf protein with 80 kDa only.

In previous other studies, Mitra and Zhang (1994) reported expression of hLf in tobacco calli, which pro-

duced only truncated hLf protein with molecular weight of 48 kDa. A full length and lower molecular masses of recombinant hLf protein were detected in transgenic tobacco plants (Salmon et al., 1998). Recently a full-length hLf was isolated from transgenic tobacco and potato plants (Chong and Langridge, 2000). However, it is not known why partial-length lactoferrin is produced in tobacco cells. It is possible that the plant-produced lactoferrin protein does not undergo proper folding and the unfolded part is degraded (Mitra and Zhang, 1994).

High expression of hLf in suspension cultures

To investigate the production levels of hLf in suspension cultures, three cell lines (T1, T2, and T5) showing a high hLf were cultured in the same liquid medium. After establishment of suspension cultures, the hLf levels of three cell lines were investigated during cell growth. Cell growth in suspension cultures follows a typical sigmoidal growth curve (Figure 6A). After an initial lag period of up to 3 or 5 days after subculture (DAS) cell mass increased exponentially, reaching a maximum at 7 or 11 DAS. The growth pattern of the three cell lines was almost similar, except that T5 cell line showed a faster growth than other cell lines.

The hLf contents in three cell lines (T1, T2, and T5) were linearly increased during the cell growth in a culture-dependent manner, showing a maximum level at stationary growth phase. The hLf content in T5 line was markedly increased from 3 DAS, which reached a maximum level at 9 DAS and sustained a high level of hLf contents to the end of the cultures (Figure 6B). In the case of the cell lines T1 and T2, a gradual increase of hLf level was observed during cell growth. The maximal levels of hLf in three lines of T1, T2, and T5 reached 3.6%, 3.8%, and 4.3% of total soluble protein, respectively. As expected, the production of hLf increased according to the cell growth and then showed the maximal level after the stationary phase in suspension cultures in reflection the characteristics of *SWPA2* promoter (Kim et al., 2003). The *SWPA2* promoter is highly expressed following the stationary growth phase. The GUS activities of a *SWPA2* :: GUS cell line were approximately 6.2 times higher than that recorded in CaMV 35S :: GUS cell line (Figure 4)(Kim et al., 2003).

We anticipate that the tobacco cell lines in this study will be good materials for the production of human lacto-

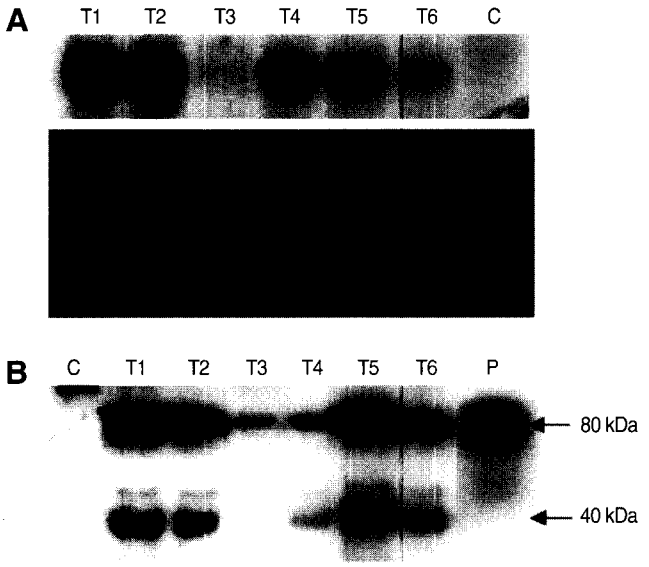

Figure 5. Transformation of tobacco cells with the gene encoding a human lactoferrin (Choi et al., 2003). A, northern blot analysis of transgenic cell lines. Total RNA (15 μg) of each sample was fractionated on 1% agarose gel, transferred to a membrane, and hybridized with the same probe as Southern analysis. Etidium bromide staining of the gel was shown as a loading control. B, Western blot analysis of transgenic cell lines. Lane M: marker, Lane C: protein extract from non-transformed control tobacco callus, Lanes T1-T6: protein extract from transgenic tobacco cell lines, Lane P: commercially available lactoferrin (100 ng).

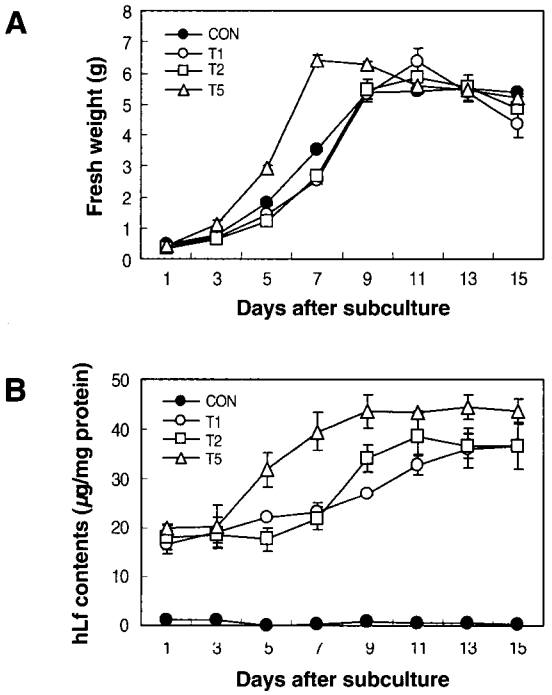

Figure 6. Changes in cell growth (A) and contents of human lactoferrin (B) in suspension cultures of three transgenic tobacco cell lines (Choi et al., 2003). Cells were grown in the 100 mL Erlenmeyer flask. CON represents non-transformed control cells. T1, T2, and T5 represent transgenic cell lines.

ferrin by large-scale cultures. Recently we successfully developed transgenic cell lines of medicinal plants such as ginseng (*Panax ginseng*) and Siberian ginseng (*Acanthopanax senticosus*) using *SWPA2* promoter to produce high level of a hLf protein (unpublished data).

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