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High expression of a human lactoferrin in transgenic tobacco cell cultures

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Abstract

Transgenic *Nicotiana tabacum* cell lines were developed expressing the human lactoferrin gene driven by the oxidative stress-inducible peroxidase (*SWPA2*) promoter. Western blot analysis showed the accumulation of both the full-length human lactoferrin protein as well as a immuno-reactive truncated fragment. Accumulation of human lactoferrin as monitored by ELISA increased proportionally to cell growth and reached a maximal (up to 4.3% of total soluble proteins) at the stationary phase of growth. Protein extracts from transgenic tobacco cells exhibited antibacterial activity.

Introduction

Lactoferrin is an iron-binding glycoprotein with an approximate mass of 80 kDa, which was originally found in milk. High levels of lactoferrin expression have been identified in neutrophils and in lactating mammary glands. Lactoferrin plays a significant protective role in human milk. Moreover, lactoferrin also has antibacterial, antifungal, anti-endotoxin, and antiviral activities (Arakawa *et al.* 1999).

Recombinant human lactoferrin (hLf) has been produced in fungi, yeast, and mammalian systems, including cows (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).

Plant cultured cells have become attractive systems for production of secondary metabolites and recombinant proteins post transformation. Most applications of plant cell suspension cultures in biotechnology are aimed at the production of naturally occurring secondary metabolites such as shikonin (Fukui *et al.* 1990), taxol (Seki *et al.* 1997). 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.

Mitra & Zhang (1994) first reported expression of the hLf gene under control of the CaMV 35S promoter in tobacco cultured cells, which contained approx. 1.8% of total cellular protein. Recently, transgenic plants expressing hLf were developed and produced at a maximal level of 0.3% of total cellular protein (Salmon *et al.* 1998, Anzai *et al.* 2000, Chong & Langridge 2000). Transgenic potatoes were developed that expressed lactoferrin at a level of 0.1% of total soluble protein.

Thus, while the expression of hLf in transgenic plant has been reported previously, only expression and extraction levels of 0.3% have so far been obtained. Recently, increased expression levels of several proteins have been attained by expressing foreign proteins in chloroplasts of higher plants (Heifetz & Tuttle 2001). However, transgenic plants produced via plastid transformation have so far only been generated in tobacco.

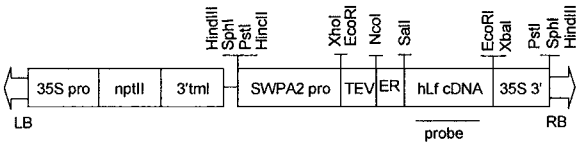

Fig. 1. Structure of plant expression vector SWPA2pro::ER-hLf/pCGN1578 for tobacco transformation. *SWPA2* pro: sweetpotato peroxidase (*SWPA2*) promoter, TEV: tobacco etch virus leader sequence, ER: signal peptide of calreticulin; 35S 3′: CaMV 35S transcription terminator, 35S pro: CaMV 35S promoter, nptII: neomycin phosphotransferase gene, 3′tml: tml terminator. LB and RB: T-DNA left and right border sequences, respectively. Bar represents probe from 1 kb fragment of hLf cDNA for Southern and Northern blot analysis.

The fast-growing cultured cells of tobacco (*Nicotiana tabacum* cv. BY-2) have doubling times of 11 h and are suitable hosts for recombinant-enzyme production (Shinmyo *et al.* 1998). To express a foreign gene efficiently in cultured cells, a good expression system using an appropriate promoter and optimized conditions for high expression is essential. Recent improvements in the design of novel promoters and other elements together with gene targeting will lead to significant improvements in product yields.

The *SWPA2* promoter is a strong oxidative stress-inducible peroxidase (POD) promoter, which was cloned from sweetpotato (*Ipomoea batatas*) (Kim *et al.* 2002). The *SWPA2* promoter was strongly expressed in cultured cells, and highly induced in response to environmental stresses in intact plants. Furthermore, GUS protein in suspension cultures of transgenic tobacco cells using a *SWPA2* promoter was strongly expressed following the stationary stage of cell growth (Kim *et al.* 2002). Therefore, we anticipate that the *SWPA2* promoter will be biotechnologically useful for the development of particular transgenic cell lines engineered to produce key pharmaceutical proteins. Here, we report that transgenic tobacco cells expressing a hLf gene under control of the *SWPA2* promoter results in high levels of hLf production.

Materials and methods

Construction of plant expression vector

The human lactoferrin (hLf) cDNA (U07643) was modified at the 5′ end to introduce restriction sites facilitating the replacement of the hLf signal peptide sequence with the tobacco endoplasmic reticulum calreticulin signal peptide sequence. A cDNA clone encoding hLf was amplified by PCR using the 5′ primer with *Sal*I site, 5′-GTCGACGGCCGTAGGAGAAGGAG-3′ and 3′ primer with *Xba*I site, 5′-GGCCATCTAGATCGGTT TTACTTCCTGA-3′. 5′ Primer was designed to contain a hLf without the sequence encoding the signal peptide (position 149 bp). Calreticulin signal peptide fragment (ER) was achieved by PCR and ligated to replace the hLf signal peptide sequence and confirmed by DNA sequencing. The ER-hLf fragment was ligated into the corresponding site of pRTL2 vector. Finally *Hin*dIII fragment (*SWPA2* promoter-ER-hLf) containing the chimeric gene under the control of the *SWPA2* promoter was inserted into the *Hin*dIII site of pCGN1578 with the neomycin phosphotransferse (*nptII*) as a selectable marker. The resultant binary plasmid was named SWPA2pro::ER-hLf/pCGN1578 (Figure 1) and transformed into *Agrobacterium tumefaciens* EHA101 for plant transformation.

Transformation of tobacco cells

Suspension cultured cells of tobacco BY-2 were transformed by the *Agrobacterium* co-cultivation method. Transformed tobacco cells were selected on Murashige & Skoog (MS 1962) medium containing 0.18 mg 2,4-D l^{-1}, 150 mg kanamycin l^{-1}, and 300 mg Claforan l^{-1}. Individual kanamycin-resistant clusters were transferred to fresh selection medium every 3 weeks.

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) and centrifuged at 12 000 *g* for 15 min at 4 °C. The soluble protein concentration in the homogenate supernatant was determined using the Bradford method. The presence of recombinant hLf from transformed and non-transformed (control) cells was assessed by enzyme-linked immunosorbent assay (ELISA) according to the BioxyTech *Lactof*-EIA protocol (*Oxi*Research).

Southern and Northern blot analysis

Genomic DNA was extracted from tobacco cell lines with high level of hLf protein, and digested with *Eco*RI. It was electrophoresed on 0.8% agarose gel, blotted onto Zeta-Probe GT membrane (Bio-Rad), and hybridized to the 1 kb of hLf cDNA-specific probe. Probe was labeled with [α-^{32}P]dCTP using a

*redi*prime II kit (Amersham Pharmacia Biotech UK Ltd.). Hybridization was carried out in 0.25 M sodium phosphate (pH 7.2) containing 7% (v/v) SDS at 60 °C. After hybridization the blot was washed once with 20 mm sodium phosphate (pH 7.2), 1% SDS at room temperature for 10 min, then twice with the same solution at 60 °C. For Northern analysis, total RNA was extracted with a TRIzol (Gibco/BRL), following the manufacturer's instructions. Approx. 15 μg total RNA was electrophoresed on a 1% agarose gel containing 0.67 M formaldehyde and blotted onto a Zeta membrane (Bio-Rad). Radiolabelling probe and hybridization were performed as above.

Western blot analysis

Total soluble proteins were extracted from transformed tobacco cells as described above. Protein extracts (50 μg) were separated on a 10% (w/v) acrylamide gel along with 100 ng of commercially available lactoferrin (Sigma) as a standard. The resolved proteins were transferred to a PVDF nylon membrane (Millipore Co.). The membrane was immersed in a blocking solution (1% BSA and 10 mM Tris/HCl, pH 7.4, 150 mM NaCl) for 1 h at 4 °C. The membrane was incubated for overnight in a 1:20 000 dilution of a commercially available polyclonal antibody conjugated with peroxidase (rabbit anti-human lactoferrin, Biodesign International). After washing five times with TBST buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), the blot was detected with ECL plus Western Blotting Detection System (Amersham Pharmacia Biotech UK Ltd.), following the manufacturer's instructions.

Assay of antibacterial activity

To determine the effect of antibacterial activity, total soluble proteins were extracted from transgenic tobacco cells. Three different bacterial strains, *Salmonella typhymirium* (KCTC1925), *Staphylococcus aureus* (KCTC1916) and *Escherichia coli* DH5α, were selected for the growth inhibition assay and grown to an A_{600} of 0.4, and aliquots of 1 ml were transferred to culture tubes. Total soluble proteins (500 μg) were added to each culture tube and incubated at 37 °C on a rotary shaker until the A_{600} of the control cell suspension reached to 1. Commercially available lactoferrin (500 μg) (Sigma) and extract from non-transformed control tobacco cells were used as positive and negative controls, respectively. After incubation, the total number of colony-forming units was determined by serial dilution on LB plates and counting the number of colonies (Chong & Langridge 2000).

Results and discussion

Transformation and cell line selection

Tobacco cells were transformed by *Agrobacterium tumefaciens* carrying a SWPA2pro::ER-hLf/pCGN1578 plasmid. Transformed tobacco cells were selected on MS medium containing 150 mg kanamycin l^{-1}. Kanamycin-resistant cells 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 expression of hLf in thirteen PCR positive cell lines were analyzed to select high-yielding cell lines by ELISA analysis. Transgenic cell lines expressed the hLf contents ranging from 0.7% to 2.7% of total soluble proteins (data not shown here). Six transgenic cell lines (T1 to 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 to T6) with high levels of hLf protein, using the ^{32}P-labeled hLf cDNA as a probe (Figure 2A). After *Eco*RI digestion, six cell lines showed 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 (T1 to T6) 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 2B). 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.

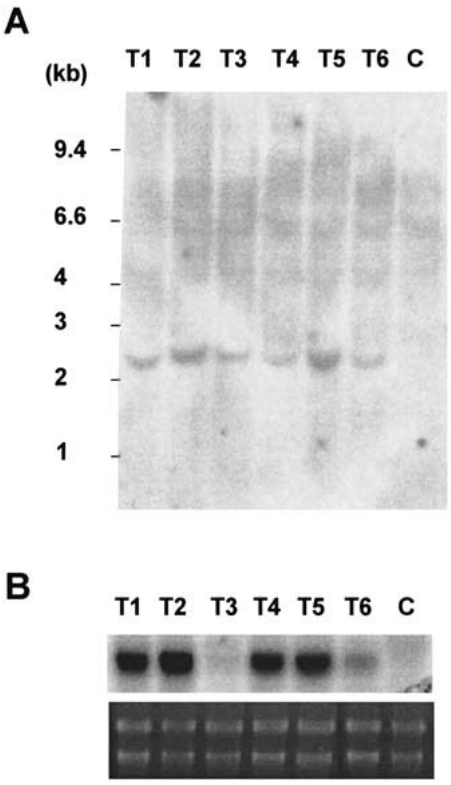

Fig. 2. Transformation of tobacco cells with the gene encoding a human lactoferrin. (A) Southern blot of transgenic (T1–T6) and non-transformed control (C) cell lines. Total genomic DNA (20 μg) was digested with *Eco*RI, transferred to a membrane, and hybridized with ^{32}P-labeled human lactoferrin cDNA probe as marked in Figure 1. Numbers at left are size marker. (B) 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. Ethidium bromide staining of the gel was shown as a loading control.

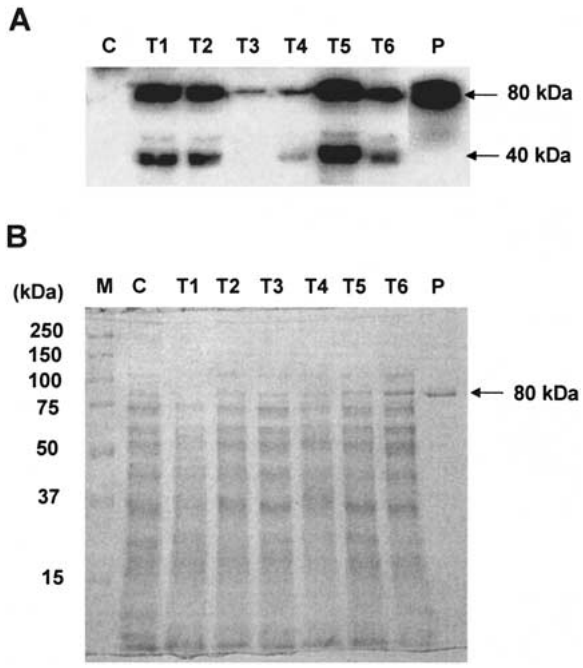

Fig. 3. Immunodetection of human lactoferrin protein in transgenic tobacco cell lines. Total soluble proteins from tobacco cell lines were fractionated by SDS-PAGE and blotted onto a nylon membrane and probed with a commercially available anti-lactoferrin antibody. (A) Western blot analysis. Lane M: marker, lane C: protein extract from non-transformed control tobacco cells, lanes T1–T6: protein extract from transgenic tobacco cell lines, lane P: commercially available lactoferrin (100 ng). (B) Coomassie staining of an SDS-PAGE with the same loading order as in panel A. Lane P: commercially available lactoferrin (1 μg).

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 to T6) is shown in Figure 3. 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 & Zhang (1994) reported expression of hLf in tobacco calli, which produced 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 & Langridge 2000). However, it is not known why partial-length lactoferrin is produced in tobacco cells. It was hypothesized that the plant-produced lactoferrin protein does not undergo proper folding and the unfolded part is degraded (Mitra & 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 4A). After an initial lag period of up to 3 or 5 d after subculture (DAS), the cell mass increased exponentially, reaching a maximum at 7 or 11 DAS. The growth pattern of the

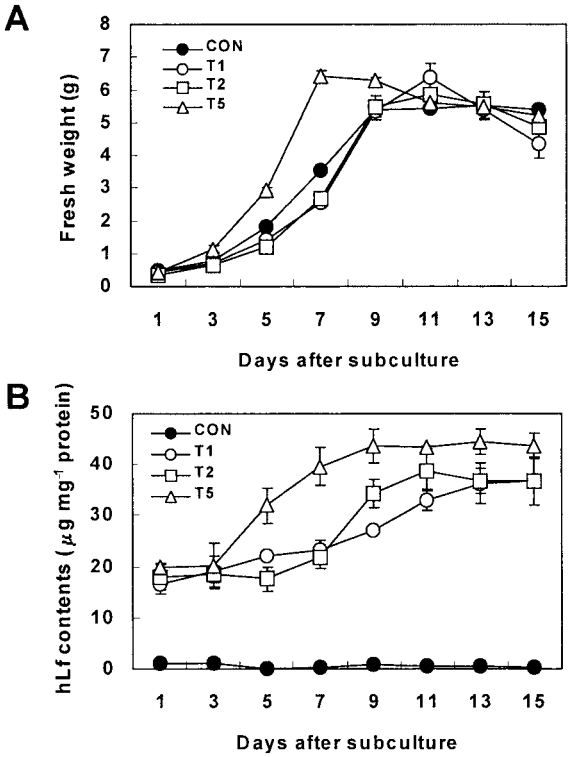

Fig. 4. Changes in cell growth (A) and contents of human lactoferrin (B) in suspension cultures of three transgenic tobacco cell lines. Cells were grown in the 100 ml Erlenmeyer flask. CON represents non-transformed control cells. T1, T2, and T5 represent transgenic cell lines.

three cell lines was almost similar, except that T5 cell line showed a faster growth than other cell lines.

The hLf content in the three cell lines (T1, T2, and T5) 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 4B). 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.* 2002). The *SWPA2* promoter is highly expressed following the stationary growth phase. The GUS activities of a SWPA2::GUS cell line

Table 1. Antibacterial activity of total protein extract from transgenic tobacco cells expressing a human lactoferrin.

Strains	Cell growth (%)		
	Control cell extract	Lactoferrin (commercial) (500 μg ml^{-1})	Transgenic cell extract
Salmonella typhymirium	100 ± 2.3	22.3 ± 1.2	87.7 ± 1.6
Staphylococcus aureus	100 ± 1.7	45.8 ± 1.5	79.9 ± 2.6
Escherichia coli (DH5α)	100 ± 0.9	1.5 ± 0.5	51.8 ± 1.9

Data represent the average of three experiments.

were approx. 6.2 times higher than that recorded in CaMV 35S::GUS cell line (Kim *et al.* 2002).

Antibacterial activity of hLf producing cell lines

To test antibacterial activities of the recombinant hLf protein, total protein extracts from transgenic cell line (T5) with the highest level of hLf was prepared. As shown in Table 1, recombinant hLf from transgenic tobacco cells slightly inhibited the growth of three bacterial strains tested. Since protein extracts prepared from transgenic tobacco cells had more than 4% of the total soluble proteins, the concentration of hLf was estimated to be over 20 μg (in 500 μg total soluble proteins) for each antibacterial assay. Antibacterial effects from extracts of T5 cell line showed a reduction in colony formation in three bacterial strains tested. However, incubation of the bacteria with a purified commercial hLf (500 μg) generated a significant antibacterial effect.

These results are consistent with previous other reports (Mitra & Zhang 1994, Chong & Langridge 2000). The transgenic tobacco cells-synthesized 48 kDa hLf-derived peptide and transgenic potato-synthesized full-length hLf possessed substantially higher antibacterial activity than commercially available purified lactoferrin. In addition, purified recombinant hLf from transgenic rice also showed the antimicrobial activity (Anzai *et al.* 2000).

This manuscript only demonstrates the high-yield production of a hLf using a high-expression promoter in plant cultured cells. The optimization culture conditions for cell growth and hLf production remains to be determined in the future. The high expression system developed in this study produced over two-fold

more hLf than the amount generated by the CaMV 35S promoter (Mitra & Zhang 1994).

We anticipate that hLf levels could be further increased by optimized cultivation conditions and controlled elicitation. The *SWPA2* promoter should result in higher productivity and increased applications of plant cultured cells for the production of high-value recombinant proteins. Improvements in plant gene expression together with increased choice of expression hosts may lead to significant advances in the large-scale production of pharmaceutical proteins.

Acknowledgements

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