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BRIEF COMMUNICATION

# Production of human lactoferrin in transgenic cell suspension cultures of sweet potato

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## Abstract

Shoot apical meristem-derived calli were transformed with a hLF cDNA in an attempt to produce human lactoferrin (hLF) in transgenic cell suspension cultures of sweet potato [*Ipomoea batatas* (L.) Lam.]. Calli were bombarded with tungsten particles coated with the binary vector pLSM1 containing a hLF cDNA under the control of the 35S promoter and the neomycin phosphotransferase gene as a selection marker. Calli were then transferred to Murashige and Skoog (MS) medium supplemented with 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 mg $dm^{-3}$ kanamycin. Kanamycin-resistant calli were selected at four-week intervals and subcultured. Cell suspension cultures were established in liquid MS medium with 4.52 μM 2,4-D. Southern and Northern blot analyses confirmed that hLF cDNA was incorporated into the plant genome and was properly expressed in the cells. ELISA analysis showed that transgenic cells produced hLF up to 3.2 μg $mg^{-1}$(total protein).

*Additional key words*: genetic transformation, *Ipomoea batatas*, particle bombardment.

Human lactoferrin (hLF) is an iron-binding protein found in milk that plays an important role in the immune system response. hLF also prevents the growth of pathogens, exerts antibacterial and antiviral properties, controls cell and tissue damage caused by oxidation, and facilitates iron transport (Arakawa *et al.* 1999). In an attempt to produce hLF on a large scale, recombinant hLF has been used in various systems, including transgenic yeast (Liang and Richardson 1993) and cows (Van Berkel *et al.* 2002). Transgenic tobacco, rice, and tomato plants producing hLF exhibited antibacterial activity against pathogens (Zhang *et al.* 1998, Humphrey *et al.* 2002, Lee *et al.* 2002). Transgenic tobacco plants also exerted antiviral activity against CMV-Y (Liu *et al.* 1999). Transgenic potato tubers producing hLF have been proposed as a component of infant formulas and baby foods in order to exempt these products from expensive purification processes (Chong and Langridge 2000). We transformed sweet potato cells with a hLF cDNA to produce hLF in plant cell cultures as a precursor to production of the human therapeutic protein on a large scale.

Embryogenic calli derived from the shoot apical meristem of sweet potato [*Ipomoea batatas* (L.) Lam., cv. Yulmi] (Liu *et al.* 1989, Min *et al.* 1994) were maintained by subculturing on Murashige and Skoog (1962; MS) medium supplemented with 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (MS1D) at four-week intervals. The culture medium used throughout the experiments consisted of MS inorganic salts, 100 mg $dm^{-3}$ myo-inositol, 0.4 mg $dm^{-3}$ thiamine·HCl, 3 % (m/v) sucrose, and 0.4 % (m/v) Phytagel (*Sigma*, St. Louis, MO, USA). The pH of the medium was adjusted to 5.8 before autoclaving. Medium was dispensed into plastic Petri dishes. Unless mentioned otherwise, all cultures were incubated at 25 ºC in the dark.

A hLF cDNA containing the 3' untranslated region

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(UTR) with the 5` UTR region isolated from a human mammary gland tissue cDNA library (*Clontech*, Palo Alto, CA, USA) was inserted into the *Hin*dIII/*Bal*II restriction site of the binary vector pGA748 under the control of the CaMV35S promoter. PGA748 carries the neomycin phosphotransferase gene as a selectable marker. The resulting vector named pLSMI (Fig. 1*A*) was then introduced into *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method.

Embryogenic cell clumps were placed on filter paper discs (70 mm in diameter), then placed on MS1D in Petri dishes. Cell clumps were subjected to bombardment with tungsten particles (1.0 μm) coated with the pLSM1 vector using a *Bio-Rad* particle delivery system (PDS 1000/He) with a constant 9 cm target distance in a 71 cm Hg vacuum using 7 600 000 Pa rupture disks. Bombarded cell clumps were placed on MS1D for seven days before transfer to MS1D with 100 mg $dm^{-3}$ of kanamycin (MS1Dkm). Bombarded cell clumps were subcultured at four-week intervals.

To establish cell suspension cultures, approximately 1 g (fresh mass) of kanamycin-resistant cell lines (fast growing, off-white, friable, non-embryogenic calli) was transferred to a 250 $cm^3$ Erlenmeyer flask containing 10 $cm^3$ of liquid MS1Dkm. The flask was maintained on a gyratory shaker at 100 rpm. After two weeks, 20 $cm^3$ of liquid MS1Dkm was added to the flask. After an additional two weeks, 5 $cm^3$ of suspension culture was transferred to 250 $cm^3$ Erlenmeyer flasks containing 50 $cm^3$ of liquid MS1Dkm. Suspension cultures were maintained using liquid MS medium supplemented with 4.52 μM 2,4-D by subculture at two-week intervals.

Total genomic DNA was extracted from kanamycin-resistant cell clumps using a *Qiagen DNeasy*$^R$ *Plant Maxi Kit* following the manufacturer's instructions. After digestion with *Hin*dIII, 50 μg of digested DNA was electrophoresed on 0.8 % agarose gel and blotted onto *Zeta-Probe*$^R$ *GT* blotting membrane (*Bio-Rad*, Hercules, CA, USA). The 1.0 kb *Eco*RI-*Bgl*II DNA fragment containing the hLF cDNA was labeled with [α-$^{32}$P] dCTP and was used as a probe to detect transgenic cell lines. Pre-hybridization and hybridization were performed in 0.25 M sodium phosphate (pH 7.2) buffer and 7 % (m/v) sodium dodecyl sulphate (SDS) at 58 ºC overnight. The membranes were washed twice in 20 mM sodium phosphate (pH 7.2) buffer and 1 % (m/v) SDS at 58 ºC for 10 min.

Total RNA was extracted from kanamycin-resistant cell clumps using *TRIzol*$^{TM}$ reagent (*GIBCO/BRL*, Grand Island, NY, USA) following the manufacturer's instructions. Approximately 40 μg of total RNA was subjected to electrophoresis using an agarose gel containing 5.1 % (v/v) formaldehyde followed by blotting onto *Zeta-Probe*$^R$ *GT* blotting membrane. The probe used for Southern blot analysis was also used for northern blot analysis. Prehybridization, hybridization, and washing conditions were the same as for Southern blot analysis.

Suspension-cultured transgenic cells and wild-type cells were harvested after 10 d of subculture, frozen in liquid nitrogen with 50 mM Tris·HCl buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (protein inhibitor) and 8 mM $MgCl_2 . 6 H_2O$, then ground using a mortar and pestle. After centrifuging at 4 ºC at 13 000 *g* for 10 min, the supernatant was collected and the protein concentration was determined by the Bradford method (Bradford 1976). An amount of 20 μg of protein was subjected to ELISA analysis using an ELISA kit (*Oxis International*, Portland, OR, USA).

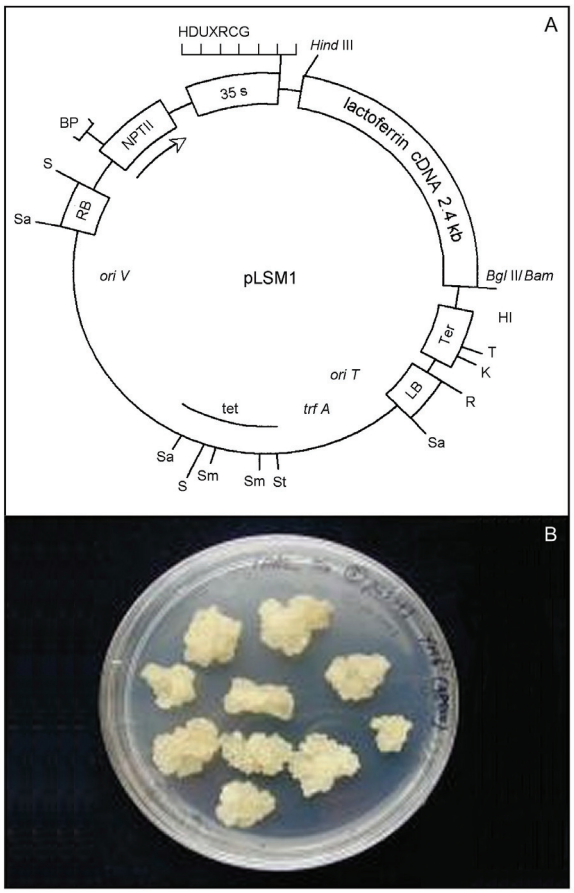


Fig. 1. The binary vector pLSM1 and a transgenic sweet potato cell line. *A*: The pLSM1 binary vector containing hLF cDNA; 35S - the cauliflower mosaic virus 35S promoter, NPT II - neomycin phosphotransferase II; LB and RB - T-DNA left and right borders, respectively. *B*: A transgenic cell line on the selection medium.

Three kanamycin-resistant cell lines were selected from bombarded cell clumps after four months of subculture (Fig. 1*B*). Southern blot analysis confirmed that the hLF cDNA was incorporated into the plant genome (Fig. 2). The copy number of the incorporated gene was one in cell line 3, but greater than three in cell lines 1 and 2. Northern blot analysis detected a 2.3 kb band from all the

transgenic cell lines (Fig. 3), indicating that the transgene was properly expressed at the transcriptional level. However, the expression level for cell lines 1 and 2 was many times higher than for cell line 3. Differences in hLf expression at the transcriptional level in different cell lines were reflected at the translational level, as determined by ELISA analysis. A transgenic plant containing multiple copies of a transgene expresses the transgene at a lower level than a plant containing a single copy of the transgene, possibly due to co-suppression (see Vaucheret *et al*. 1998).

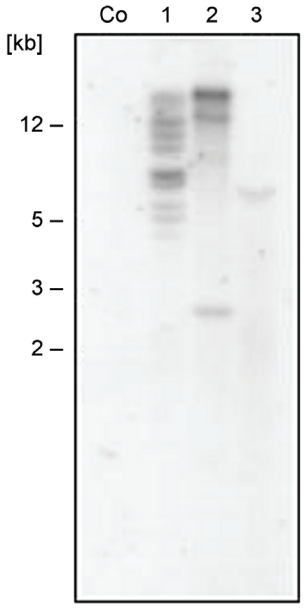


Fig. 2. Southern analysis of genomic DNA from transgenic sweet potato cells with hLF cDNA. Equal amounts (50 μg) of genomic DNA were digested with the restriction enzyme (*Hin*dIII), electrophoresed in 0.8 % agarose gel, and blotted onto a membrane. The blot was hybridized with the 1.0 kb *Eco*RI-*Bgl*II DNA fragment of hLF cDNA as a probe. Co - a wild-type cell line; 1, 2, and 3 - transgenic cell lines. The positions of molecular mass markers are indicated on the left.

However, cell lines with multiple copies of the transgene expressed the transgene many more times than the cell line with a single copy at the transcriptional and translational levels in this study. Cell line 5 produced hLF at a level of 3.2 μg $mg^{-1}$(total protein) [0.32 % (m/m) of total extracted protein]. This amount was the highest among the three cell lines. hLF has been produced in transgenic tobacco leaves (Salmon *et al.* 1998), potato tubers (Chong *et al*. 2000), and rice grains (Nandi *et al*. 2002) at levels of up to 0.3, 0.1, and 0.5 %, respectively. Transgenic cell lines were readily dispersed in liquid medium and proliferated as subcultures proceeded. However, the expression of hLF apparently did not inhibit cell growth (data not shown).

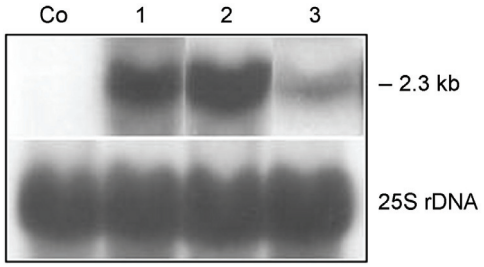


Fig. 3. Northern blot analysis of total RNA isolated from transgenic sweet potato cells. Forty μg of total RNA was loaded in each lane. The blot was hybridized with the 1.0 kb *Eco*RI-*Bgl*II DNA fragment of hLF cDNA as a probe. Co - a wild-type cell line; 1, 2, and 3: transgenic cell lines.

Human proteins are in great demand for the treatment of a variety of diseases. Some proteins can be purified from blood, however this procedure is expensive and runs the risk of contamination with the AIDS or hepatitis C viruses. Proteins can be produced in human cell culture, but costs are high and the output is small. Much larger quantities can be produced in bacteria and yeast, but the proteins produced can be difficult to purify and they lack the appropriate post-translational modifications that are needed for efficacy *in vivo*.

Production of foreign proteins in plant cell cultures has advantages over production in whole plants in certain situations. Less time is required to develop a plant cell culture system than to develop a whole plant system. A few weeks are required to produce foreign proteins in bioreactors compared with a few months in whole plants grown in open fields. Bioreactors provide more controlled and reproducible conditions than open field growth. Therefore, regulatory requirements for therapeutic proteins can be met more easily using cells produced *in vitro* (Doran 2000). We expressed hLF in sweet potato cells in an attempt to produce the protein on a large scale. Cultured cells may be used as a nutritional supplement or as a health food after dehydration. The overall results of this study indicate that plant cell cultures can be used for production of a human therapeutic protein. The possibility of viral infection causing human diseases is thus avoided.

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