

Development of transgenic rice lines expressing the human lactoferrin gene

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Abstract Lactoferrin is an 80-kDa iron-binding glycoprotein that is found in high concentrations in human milk. Human lactoferrin (*hLF*) has several beneficial biological activities including immune system modulation and antimicrobial activity. In the present study, we developed a method of *hLF* expression through introducing the *hLF* gene construct into *Oriza sativa* cv. Nakdong using the *Agrobacterium*-mediated transformation system. The expression of the *hLF* gene under the control of the rice glutelin promoter was detected in the seeds of transgenic rice plants. Transformed rice plants were selected on media containing herbicide (DL-phosphinothricin) and the integration of *hLF* cDNA was confirmed by Southern blot analysis. The expression of the full length hLF protein from the grains of transgenic rice plants was verified by Western blot analysis. The lactoferrin expression levels in the transformed rice grains determined by enzyme-linked immunosorbent assay accounted for approximately 1.5% of total soluble protein. Taken together, these data indicate that rice grains expressing hLF can be directly incorporated into infant formula and baby food.

Keywords human lactoferrin gene, rice, transgenic plants

Introduction

Lactoferrin is an iron-binding, multifunctional glycoprotein that is found in high concentrations in human milk. Human

lactoferrin (*hLF*) has several biological activities including protection from pathogens, immune system modulation, regulation of iron absorption, antimicrobial activity, and promotion of cellular growth (Arakawa et al. 1999; Lönn-erdal 1996).

Human milk-fed infants are healthier than formula-fed infants (Hanson and Korotkova 2002), which may in part be due to the lack of important components including HLF in infant formula. The potential of lactoferrin to act both as an antimicrobial and an immune regulatory agent in addition to its nutritional and pharmaceutical value has stimulated considerable interest in the development of its expression, which may provide the beneficial factors present in human milk when added to infant formula. Recombinant (rhLF) has been produced in various systems, including fungi (Ward et al. 1992), transgenic yeast (Liang and Richardson 1993), mice (Platenburg et al. 1994), and cows (Van Berkel et al. 2002). However, both animal and fungal production systems require a high degree of purification as well as safety and toxicity testing prior to use as a food additive or in infant formula (Chong and Langridge 2000; Nandi et al. 2002). In contrast, when expressed in transgenic plants, lactoferrin can be directly incorporated into food additives without the purification of rhLF, providing a convenient advantage over these other expression systems. The expression of rhLF in plants has been reported in tobacco (Salmon et al. 1998), potato (Chong et al. 1997), tomato (Lee et al. 2002), rice (Nandi et al. 2002), and sweet potato cell culture systems (Min et al. 2006).

There are several distinct advantages of expressing rhLF in rice (Nandi et al. 2005). Rice is generally regarded as safe (GRAS) for consumption and rice-based foods are considered hypoallergenic. Thus, rice grains expressing rhLF may be processed for use in infant formula without extensive purification steps. In the present study, we expressed the full length rhLF gene under the control of a rice glutelin

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promoter in the japonica type rice cultivar, *Oryza sativa* L. cv. NakDong.

Materials and methods

Plant material, regeneration, and culture conditions

Mature seeds of rice (*Oryza sativa* L. cv. Nakdong) were surface-sterilized in 70% ethanol for 5 min, then in 2% sodium hypochlorite for 15 min, and washed five times in sterile distilled water. The seeds were then cultured on N6 callus induction medium (Chu et al. 1975) containing 2 mg/L 2,4-D and incubated at 28°C in darkness for three weeks. The calli derived from scutella of seeds were subcultured in fresh N6 medium at 28°C in darkness for three days. Actively growing pieces of calli were used for rice transformation.

Construction of *hLF* rice expression vector

The plasmid *pHLF* containing the 2.1 kb full length *hLF* cDNA fragment was kindly provided by Prof. Pauline Ward (Baylor College of Medicine, Houston, Texas, USA). The *hLF* cDNA was digested with *Hind*II-*Hind*III and the fragment cloned into the pBluescript II KS plasmid (pBKS-*hLF*). The *Xho*I-*Sma*I fragment containing the rice glutelin promoter was isolated from pBKS-GB and inserted into the *Xho*I-*Hind*II sites of pBKS-*hLF* (pBKS-Glu/*hLF*). The *Xho*I-*Not*I fragment containing the *hLF* cDNA with the glutelin promoter was isolated from pBKS-Glu/*hLF* and inserted into the pSB binary vector (pSBG-*hLF*) such that the vector contained the mature human lactoferrin gene under the control of the rice glutelin promoter, the PinII terminator, and the herbicide resistant bar gene as a selectable marker. The resulting plasmid (pSBG-*hLF*) was introduced into *Agrobacterium tumefaciens* strain LBA4404 using the tri-parental mating method (Simon 1984).

Generation of transgenic rice plants

Agrobacterium tumefaciens strain LBA4404 harboring the pSBG-*hLF* plasmid was used for transformation. The *Agrobacterium* with pSBG-*hLF* was grown overnight at 28°C in Luria-Bertani (LB) medium containing 10 mg/L tetracycline and 50 mg/L spectinomycin. The culture was centrifuged at 2500 rpm for 15 min, and the cell pellet re-suspended in 25 ml of AAM medium (Philip and Bowler 2002) containing 200 μM acetosyringone. The calli were then immersed in the bacterial suspension for 1 h at room temperature. Following incubation, the *Agrobacterium* cells were removed by blotting on sterile filter paper. The infected calli were transferred to N6 medium containing 200 μM acetosyringone and incubated in the dark for three days at 25°C. After co-cultivation, the infected calli were washed five times with sterile water containing 250 mg/L carbenicillin, placed on MS selection medium (MS salts and vitamins, 100 mg/L myo-inositol, 1 mg/L L-proline, 500 mg/L L-alanine, 300 mg/L casein enzymatic hydrolysate, 2 mg/L 2,4-D, 30 g/L sucrose, 3 g/L phytigel, 250 mg/L carbenicillin, and 6 mg/L DL-phosphinothricin), and cultured at 28°C in darkness for two to three weeks. Following the cultivation, healthy green shoots were selected and transferred to MS regeneration medium (MS salts and vitamins, 1 mg/L NAA, 5 mg/L kinetin, 30 g/L D-sorbitol, 15 g/L maltose, 4 g/L phytigel, and 3 mg/L DL-phosphinothricin) for full plant formation with an extensive root system. Following rooting, the transgenic plants were transferred to a greenhouse and grown to maturity.

Detection of the lactoferrin gene in transgenic rice plants

Total genomic DNA was extracted from the young leaves of the transgenic rice using CTAB extraction methods (Murray and Thompson 1980). For Southern blot analysis, 5 μg of genomic DNA were digested with the *Bam*HI restriction enzyme. The digested DNAs were electrophoresed on 1%

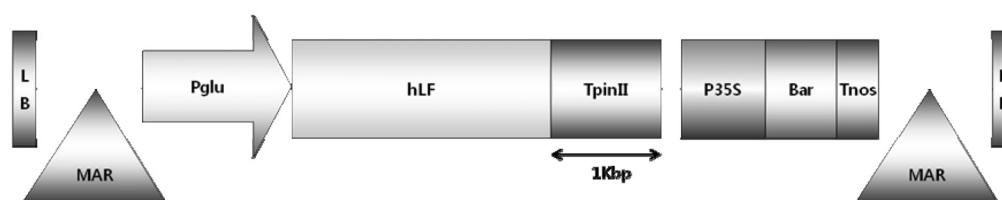


Fig. 1 Partial map of the pSBG-*hLF* binary vector harboring the human lactoferrin gene.

Pglu: rice glutelin promoter; *hLF*: human lactoferrin cDNA; TpinII: proteinase inhibitor II terminator; P35S: CaMV 35S promoter; *Bar*: phosphinothricin acetyltransferase gene; Tnos: nopaline synthase terminator; MAR: matrix attachment region; LB and RB: T-DNA left and right borders, respectively

agarose gels, blotted onto nylon membranes (Hybond-N+, GE Healthcare), and hybridized to 1 kb of the *hLF* cDNA-specific probe. The probe was labeled with [α - 32 P]dCTP using Ready-to-go DNA labeling beads (GE Healthcare). Hybridization was performed in hybridization solution (1% BSA, 1 mM EDTA, 0.5 M NaPO₄, pH 7.2, 7% SDS, 1 mg/ml salmon sperm DNA) at 65°C overnight. Following hybridization, the blot was washed with washing solutions (1st solution, 2× SSC, 0.1% SDS; 2nd solution, 1× SSC, 0.1% SDS; 3rd solution, 0.2× SSC, 0.1% SDS) at 65°C for 15 min per solution. The membranes were then exposed to X-ray film (Fuji Photo Film).

Immunoblot detection of lactoferrin proteins in transgenic rice plants

Total soluble proteins were extracted from the seeds of transgenic rice plants by grinding in the presence of the protein extraction buffer (50 mM Tris-HCl pH 7.5, 1% 2-mercaptoethanol, 10% glycerol, 1 mM PMSF, and 2% PVPP). The homogenate was centrifuged at 14,000 rpm for 20 min at 4°C. The soluble protein concentration in the extracts was then determined (Bradford 1976). Approximately 20 μ g total protein from each sample were loaded onto 8% (v/v) SDS-polyacrylamide gels along with 100 ng of commercially available lactoferrin (Sigma) as a standard. The protein bands were separated by polyacrylamide gel electrophoresis, and transferred onto PVDF membranes (GE Healthcare) through electroblotting with a Mini Trans-Blot[®] (Bio-Rad). The membranes were blocked in 5% skim milk in TBST buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. The blocked membrane was probed with rabbit anti-*hLF* antibodies (Sigma) followed by detection using a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma). The recombinant protein was visualized by colorimetric reaction in which the membrane was incubated in a BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) substrate solution until the positive control standard was clearly evident.

Determination of lactoferrin content in transgenic rice grains

The transgenic rice grains (1 g fresh weight) were homogenized on ice with a mortar in a double volume (w/v) of cold extraction buffer (50 mM Tris-HCl, pH 7.5) and centrifuged at 14,000 × g for 15 min at 4°C. The total soluble protein concentration in the homogenate supernatant was determined using the Bradford method. The content of recombinant hLF from transformed and non-transformed rice plants was

assessed by enzyme-linked immunosorbent assay (ELISA) according to the BioxyTech Lactof-EIA protocol (OxiResearch).

Results and discussion

Generation of transgenic rice plants

Rice calli were transformed using *A. tumefaciens* carrying the pSBG-*hLF* binary vector (Fig. 1). The transformation vector contains the mature human lactoferrin gene under the control of the rice glutelin promoter, a PinII terminator, and the herbicide resistant *bar* gene as a selectable marker. The infected calli were initially cultured in MS selection medium containing 250 mg/L carbenicillin and 3 mg/L DL-phosphinothricin and the shoots differentiated from transformed calli (Fig. 2A). Resistant shoots were transferred onto MS regeneration medium containing 250 mg/L carbenicillin and 3 mg/L PPT and cultured for an additional three weeks. All the shoots grew well and rooted in the medium (Fig. 2B). Thirty-four independent PPT-resistant rice plants were transferred to pots and grown in a greenhouse (Fig. 2C). The plants regenerated and grew in a comparable fashion to the non-transgenic wild-type plant.

Basta spray assays were conducted to evaluate the functional

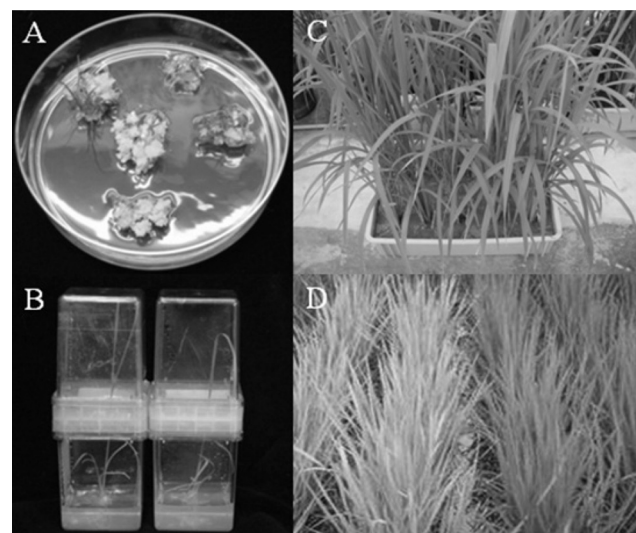


Fig. 2 Formation and expression of the *bar* gene in transgenic rice plants. A: Shoot induction from the DL-phosphinothricin-resistant embryogenic calli; B: Development of independent transformants in the regeneration medium; C: Transgenic rice plants growing in the green house; D: Comparison of transgenic rice showing Basta resistance with wild-type plants. Transgenic plants grown for 60 days in the field were sprayed (on the leaves) with 3 g/L of the herbicide (Basta). The photograph shows the plants one week after the spraying

bar gene expression in putative T₁ transgenic rice plants in a GMO field. When sprayed with Basta (3 g/L), the transformants showed complete resistance to the herbicide, while the non-transformed wild-type plants became withered and eventually died within two weeks of treatment (Fig. 2D). These results confirmed the successful insertion of the *Bar* gene into the genome of the transgenic rice plants.

Detection of the *hLF* gene in transgenic rice plants

To confirm the stable integration of the *hLF* gene into the nuclear genome of host cells, Southern blot analysis was performed with 25 independent T₀ transgenic lines that produced T₁ seeds, using the ³²P-labeled *hLF* cDNA fragment as a probe. As shown in Fig. 3, 22 transgenic rice plants showed a clear band of the expected 2.1 kb length, indicating that the *hLF* gene was correctly incorporated into the genomic DNA of the plant. No hybridization signal was observed in genomic DNA from control plants or the remaining three lines (Fig. 3, lanes 1, 7, and 26).

Expression of the lactoferrin protein in transgenic rice plants

Four representative T₃ transgenic rice lines were selected by southern blot analysis and agronomic characteristics. These lines were analyzed for the expression of the rhLF protein through Western blot analysis. The rhLF protein synthesized in the grains of transgenic plants is shown in Fig. 4. The rhLF proteins (Fig. 4, lanes 2–5) migrated as a single band at the expected molecular weight of approximately 80 kDa,

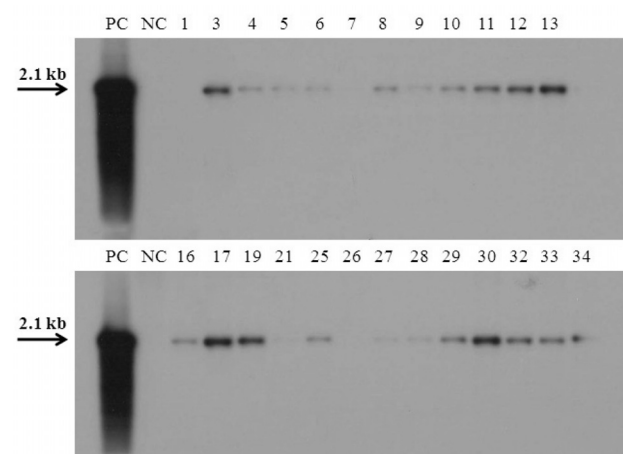


Fig. 3 Southern blot analysis of transgenic rice plants carrying the *hLF* gene. Total genomic DNA (5 µg) from 25 representative transgenic rice plants was digested with BamHI. The blot was probed with a 1 kb *hLF* gene fragment. Lane PC: plasmid DNA digested with the same enzymes; NC: non-transformed negative control; lanes 1–34: transgenic rice plants

indicating that full length lactoferrin was synthesized in the transgenic rice plants. Extracts from the untransformed plants did not react with the anti-lactoferrin antibody (Fig. 4, lane 1).

Recombinant hLF contents in transgenic rice grains

Transgenic rice plants containing *hLF* cDNA were screened for the expression levels of recombinant lactoferrin using ELISA. The results of indirect ELISA experiments demonstrated that all transgenic plants expressed variable levels of the recombinant rhLF protein in grains. The levels of rhLF detected varied from 0.63% of soluble protein to 1.51% (Fig. 5). The wild-type rice plant did not express the rhLF protein.

A previous study reported that rhLF was expressed in rice at significantly higher levels than in other transgenic plant systems, reaching 0.5% of the grain weight (Nandi et al. 2002). Furthermore, the expression of rhLF in *Nicotiana benthamiana* and tobacco cell cultures reportedly achieved

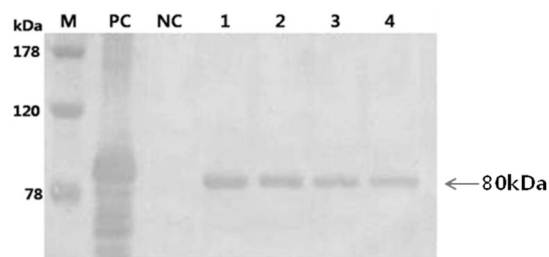


Fig. 4 Immunodetection of human lactoferrin protein synthesized in the grains of transgenic rice plants. Twenty micrograms of total soluble protein from transgenic rice grains were resolved by SDS-PAGE and membranes were probed with anti-lactoferrin antibodies. The arrow indicates the hLF protein synthesized in the transgenic rice plants. Lane M: molecular weight marker; lane PC: commercially available hLF as standard; lane NC: non-transformed negative control; lanes 1–4: transgenic rice lines (pGF 5-1-1, 11-1-1, 13-1-1, 29-1-1, and 32-2-1)

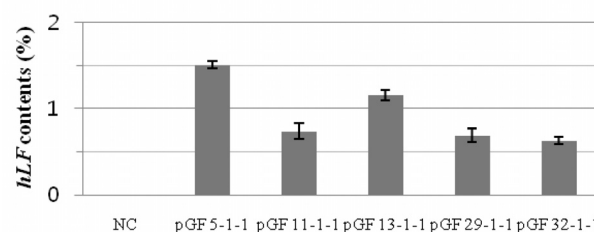


Fig. 5 Levels of recombinant hLF protein in the grains of transgenic rice plants. Total soluble proteins were extracted from the grains of transgenic rice plants. Approximately 1 µg protein was analyzed by ELISA. Experiments were repeated three times. NC: non-transformed rice plant; pGF5-1-1, 11-1-1, 13-1-1, 29-1-1, and 32-2-1: transgenic rice plants

levels of 0.6 and 4.3% of the total soluble protein, respectively (Choi et al. 2003; Li et al. 2004). However, the rhLF protein from tobacco or non-edible plants requires extensive purification before it can be considered for any food application, making it an unlikely commercially viable product (Lönnerdal 2002). In contrast, as rice is safe (GRAS) for consumption and rice-based foods including rice-based infant formulas are considered to be hypoallergenic, the expression of rhLF in rice seeds could contribute to the production of a nutritional hLF supplement for non-breast fed infants as well as children and adults. In addition, the expression of recombinant hLF in seeds enables long-term storage at ambient temperature as the protein accumulates in a stable form.

In conclusion, we confirmed the expression of the *hLF* gene in transgenic japonica rice cv. Nakdong obtained through *Agrobacterium*-mediated transformation. The *hLF* gene was integrated into the genomic DNA of transgenic rice plants and expressed in the grains of these plants. The expression levels of recombinant hLF represented up to 1.5% of the total soluble protein in transgenic rice grains. Based on these results, four T₃ lines were advanced to T₄ generations in a GMO field. At present, we are conducting molecular analysis to confirm the gene copy number for the selection of homozygous lines with agronomic characteristics similar to those of non-transformants. This study demonstrates the use of transgenic japonica rice cv. Nakdong in applications for human biological functions, efficacy, and safety.

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