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Expression of Human Lactoferrin Gene in Transgenic Rice (*Oryza sativa* L.)

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ABSTRACT Lactoferrin is an 80-kDa iron-binding glycoprotein known to exert many biological activities, such as facilitating iron absorption and having antimicrobial and anti-inflammatory effects. Rice can be a useful target for edible food plants to introduce human lactoferrin, because it has lower allergenicity and is likely to be safer than microorganisms or transgenic animals. A cDNA fragment encoding human lactoferrin (*HLF*) driven by the maize polyubiquitin promoter, along with herbicide resistance gene (*bar*) driven by CaMV 35S promoter, was introduced into rice (*Oryza sativa* L. cv. Dong Jin) using the *Agrobacterium* -mediated transformation system. Putative transformants were initially selected on the medium containing bialaphos. The stable integration of the *bar* and *HLF* genes into transgenic rice plants was further confirmed through polymerase chain reaction (PCR) and Southern blot analyses. The expression of the full length HLF protein from various tissues such as grains and young leaves of transgenic rice was verified by Western blot analysis. Analysis of progeny also demonstrated that introduced genes were stably inherited to the next generation at the Mendelian fashion.

Introduction

Lactoferrin is an 80-kDa iron-binding glycoprotein of the transferrin family (Bowman et al. 1988). It is present in milk as well as other exocrine secretions such as tears, saliva, and the cervical mucus. It is also found in granules of neutrophils during inflammatory responses (Masson et al. 1969). Lactoferrin exhibits bacteriostatic activity against a wide range of gram-negative and gram-positive bacteria due to its ability to chelate iron, which is essential for microbial growth (Arnold et al. 1980). It has also other biological activities including antiviral activity, antioxidant activity and immunomodulatory activity (Lönnerdal and Iyer 1995, Sanchez et al. 1992). In addition, lactoferrin has a bactericidal activity. It destabilizes the outer membrane of gram-negative bacteria through the liberation of lipopolysaccharides from their cell walls (Ellison et al. 1992). Pepsin cleavage of human lactoferrin (HLF) yields a peptide fragment called lactoferricin H, which has enhanced antibacterial activity compared to intact lactoferrin (Bellamy et al. 1992).

The potential of lactoferrin as an antimicrobial and immune regulatory agent in addition to its nutritional and pharmaceutical value has stimulated considerable interest in development of an expression system which can provide large amounts of biologically active recombinant lactoferrin

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(Chong et al. 2000). Recombinant human lactoferrin (rHLF) has been produced in lower eukaryotes such as *Saccharomyces cerevisiae* (Liang and Richardson 1993) and *Aspergillus* (Ward et al. 1992), and in a variety of mammalian systems, including baby hamster kidney cells (Stowell et al. 1991), transgenic cows (van Berkel et al. 2002) and human 293 cells (van Berkel et al. 1995). Lactoferrin has also been expressed in edible crop plants such as tobacco plants (Mitra and Zhang 1994, Salmon et al. 1998), potato plants (Chong and Langridge 2000) and rice plants (Nandi et al. 2002, Suzuki et al. 2003).

There are several distinct advantages for expressing rHLF in rice (Nandi et al. 2005). Rice is generally regarded as safe (GRAS) for consumption and rice-based foods are considered as hypoallergenic. Thus, rice grains expressing rHLF may be processed for use in infant formula without extensive purification steps. To date, expression of human lactoferrin gene has been successfully achieved in the indica type rice cultivar only. In this study, we expressed the full length *rHLF* gene under the control of the maize ubiquitin-1 promoter in the japonica type rice cultivar, *Oryza sativa* L. cv. Dong Jin.

Materials and Methods

Plant Material, Regeneration and Culture Conditions

Mature seeds of rice (*Oryza sativa* L. cv. Dong Jin) were surface-sterilized in 70% ethanol for 1 min, then in 4% sodium hypochlorite containing 200 μl Tween-20 for 15 min, and washed five times in sterile distilled water. These seeds were cultured on N6 callus induction medium (Chu et al. 1975) containing 2 mg/L 2,4-D and incubated under the continuous irradiation of fluorescent light (2,000 lux) at 25℃ for three weeks. The calli were aseptically excised from the cotyledons and mature seeds and then used for transformation.

Plasmid Construction of Plant Transformation Vectors Containing HLF

The binary vector pCUMB-gfp (V-E9) contains a maize polyubiquitin promoter, *Arabidopsis thaliana* small subunit of ribulose bisphosphate carboxylase (*ArbcS*) gene, and the herbicide resistant *bar* gene as a selectable marker. The plasmid pHLF containing a full-length HLF cDNA was kindly provided by Prof. Pauline Ward (Baylor College of Medicine, Texas, USA). Four oligonucleotides for a HIV-1 tat protein transduction domain (PTD; amino acids 47-59, RKKRRQRRR) (Wender et al., 2000) were synthesized. Each primer was annealed to generate a double-stranded oligonucleotide, and inserted into the *Eco*RV·*Hind*III and *Eco*RV·*Eco*RI sites of pBluescript II KS (+) vector, respectively, which was named as pBlue-tat. The sequences of tat PTD for fusion to N- and C-terminals of the *HLF* gene were (tat-1) 5'-AGCTTATGAGGAAGAAGCGGAGACAGCGACGAAGAGAT-3', (tat-2) 5'-ATCTCTTCGTCGCTGTCTCCGCTTCTTCCTCATA-3', (tat-3) 5'-ATCAGGAAGAAGCGGAGACAGCGACGAAGATAGG-3', and (tat-4) 5'-AATTCCTATCTTCGTCGCTGTCTCCGCTTCTTCCTGAT-3'. The *HLF* gene was amplified using the PCR method in a volume of 50 μl with 100 ng of pHLF, 100 pmol of each primer (cHLF-M, 5'-AGGTCGACATGAAACTTGTCTTCCTC-3'; cHLF-R, 5'-AGGTCGACCTTCCTGAGGAATTCACA-3'; the *Hinc*II sites are underlined), 200 μM dNTPs, and 2.5 U of *Pwo* DNA polymerase (Roche, Mannheim, Germany). The reaction mixture was denatured at 95℃ for 5 min and subjected to 30 cycles of 93℃ for 30 s, 52℃ for 45 s, and 72℃ for 2 min in a thermal cycler (Applied Biosystems GeneAmp PCR System 2400). The PCR fragment was purified with a gel purification kit (Qiaquick; Qiagen, Hilden, Germany), digested with HincII, and subcloned into the *Eco*RV site of pBlue-tat. The tat-fused *HLF* cDNA gene was confirmed by sequence analysis. The plasmid pBR-βgal (Park et al. 2001) containing the *Bifidobacterioum adolescentis* β-galactosidase (β-gal) gene was used as a template. The primers were 5'-AGGTTAACCATGACTCAACGTAGAGCCTATCGTTGGCCC-3' (GAL-1) and 5'-AGCTACAGTCAAAAGATAGCAAGCTTTTGTTCGGGCGA-3' (GAL-2). Total 30 cycles were performed with denaturation at 95℃ for 30 s, annealing at 52℃ for 45 s, synthesis at 72℃ for 2 min, and extension at 72℃ for 10 min. The amplified β-gal gene fragment was located between CaMV35S promoter and the termination sequence of the octopin synthase gene. Finally, the tat-fused *HLF* cDNA gene and β-gal cassette were inserted into the *Bam*HI and *Hind*III sites of

pCUMB-gfp (V-E9) vector, respectively. The resulting plasmid pCUMB-gfp::HLF (V-E9TFTβ) (Figure 1) was introduced into *Agrobacterium tumefaciens* strain EHA105 by electorporation. All DNA manipulations were performed according to standard procedures (Sambrook et al. 1989).

Selection and Regeneration of Putative Transgenic Plants

Agrobacterium tumefaciens strain EHA105 (Hood et al. 1993) harboring a plasmid pCUMB-gfp::HLF (V-E9TFTβ) was used for transformation. The *Agrobacterium* with pCUMB-gfp::HLF (V-E9TFTβ) was grown overnight at 28℃ in Luria-Bertani (LB) medium containing 100 mg/L kanamycin and 10 mg/L rifampicin. The culture was centrifuged at 2,200 rpm for 20 min, and the cell pellet was resuspended in equal volume of N6 medium. The calli that were freshly cultured for three days were transferred into the *Agrobacterium* suspension. After incubating for 10 min at room temperature, the excess *Agrobacterium* cells were removed by blotting on sterile filter paper. Infected calli were transferred onto the filter papers which were placed on N6 medium supplemented with 100 μM acetosyringone. Co-cultivation was carried out at 28℃ for 5-7 days in the dark. After co-cultivation, the infected calli were washed more than three times with sterile water containing 250 mg/L carbenicillin and transferred to MS medium (Murashige and Skoog 1962) containing 250 mg/L carbenicillin and 2.5 mg/L bialaphos. After cultivation in the selection medium, healthy green shoots over 3 mm were selected and transferred to MS regeneration medium for full plant formation with extensive root system. After rooting, the transgenic plants were transferred to a greenhouse and grown to maturity.

Polymerase Chain Reaction (PCR) and DNA Gel Blot Analysis

Total genomic DNA was isolated from young leaves of the transgenic rice and wild-type plants according to the CTAB method (Murray and Thompson 1980). In order to verify the presence of the *HLF* gene in transgenic plants, a set of primers designed to amplify the *HLF* gene was as follows: forward primer: 5'-AGGTCGACATGAAACTTGTCTTCCTC-3', corresponding to the proximity of the 5'-end of the *HLF* gene, and reverse primer: 5'-AGGTCGACCTTCCTGAGGAATTCACA -3', corresponding to the 3' end of the *HLF* gene. The amplified fragment using this pair of primers should result in a product of 2.1 kb. The PCR was carried out as follows; a first denaturation step at 95℃ for 5 min, 30 cycles at 94℃ for 30 s, at 52℃ for 45 s, at 72℃ for 2 min, and a final

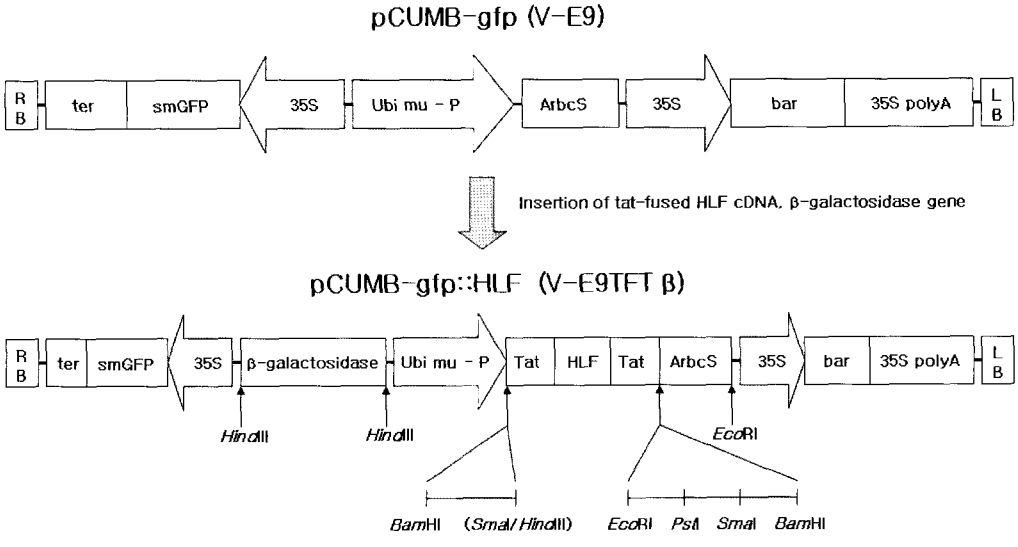

Figure 1. Schematic summary of the construction of plasmid pCUMB-gfp::HLF (V-E9TFTβ) used for transformation. RB, right border; LB, left border; bar, phosphinothricin acetyl transferase gene; ArbcS, Arabidopsis Rubisco small subunit; HLF, human lactoferrin gene; Tat, transduction domain of HIV; Ubi mu-P, polyubiquitin mutant promoter of maize; ter, terminator; 35S, cauliflower mosaic virus (CaMV) 35S RNA promoter.

extension step at 72℃ for 10 min. The amplified products were analyzed by electrophoresis in 1.0% agarose gels.

For genomic DNA blot, 10 μg of genomic DNA was digested with *Bam*H I or *Eco*R I and electrophoresed on 0.8% agarose gel. DNA bands were transferred onto a nylon membrane (Hybond™, Amersham Biosciences) as described by Sambrook et al. (1989). The blot was probed with [α-^{32}P] dCTP-labeled *HLF* cDNA fragment. Hybridization, cleavage of plant and plasmid DNA, and autoradiography were carried out according to the laboratory manual of Sambrook et al. (1989).

Segregation of T_1 Progeny for Bialaphos Resistance

Transgenic plants and their progeny were evaluated for tolerance to bialaphos that would indicate functional expression of the *bar* gene. Seeds of self-pollinated progeny were aseptically sown on a 1/2 MS medium containing 2.5 mg/L bialaphos. Each experiment was carried out in triplicate (20 seeds per dish). Bialaphos-resistant and sensitive seedlings were scored 4 weeks after planting. The data were obtained from three independent experiments and a Chi-square test (χ^2 test at $p = 0.05$) was used to determine whether the segregation ratios observes among T_1 progeny for the inheritance of the bar gene fit Mendelian segregation pattern.

Protein Isolation and Western Blotting

Total soluble proteins were extracted from young leaves and seeds of glasshouse-grown transgenic rice plants by grinding in the presence of protein extraction buffer (50 mM Tris-Cl pH 8.0, 0.3 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride). The homogenate was centrifuged at 4℃ for 40 min at 11,000 xg. Protein concentration in the extracts was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Fifteen μg of protein from each extract was loaded and electrophoresed under denaturing conditions (SDS-PAGE) in a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membrane by electroblotting. Membrane was blocked by incubation in 0.2% non-fat milk in TBS (20 mM Tris pH 7.5, 500 mM NaCl), then rinsed in TTBS (20 mM Tris pH 7.5, 500 mM NaCl, 0.1% Tween-20) before incubation with the rabbit anti-HLF antiserum (Sigma) at a dilution of 1:3,000 in TTBS. After rinsing in TTBS, the membrane was cross- reacted with a second antibody consisting of alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad) at the recommended dilution. Bands corresponding to HLF were detected using Immuno-Star™ Chemiluminescent Protein Detection Systems (Bio-Rad).

Results and Discussion

Generation of Transgenic Rice Plants

Infected calli, which were co-cultivated with the *Agrobacterium* containing pCUMB - gfp::HLF (V-E9TFTβ), were initially cultured on the shoot induction medium composed of MS medium containing carbenicillin only. Shoots (>3 mm) derived from calli were then transferred to MS regeneration medium containing 250 mg/L carbenicillin and 2.5 mg/L bialaphos. Shoots differentiated from transformed calli were survived and grew well, whereas shoots of untransformed control callus were changed to brown color and stopped growing (Figure 2A). Resistant shoots were then transferred onto the MS medium supplemented with bialaphos and cultured for additional three weeks. Nearly all the shoots grew well and rooted in the medium (Figure 2B). The stable transformation efficiency, estimated by the number of bialaphos- resistant shoots recovered per 100 calli infected was 11% (data not shown). Bialaphos-resistant plants were transferred to pot and grown in a greenhouse. The plants regenerated were observed to grow normally compared to non-transgenic wild type plants.

Basta spray assay was conducted to evaluate the functional *bar* gene expression in putative T_0 transgenic plants. When sprayed with Basta (150 mg/L), the transformants showed complete resistance to the herbicide but the untransformed wild type control plants became withered and eventually died within 1 week following the treatment (Figure 2C). These results indicate that the *bar* gene was

successfully inserted into the genome of transgenic plant and normally expressed.

Molecular Analysis of Transgenic Plants

Twenty independent bialaphos-resistance rice plants were obtained after transformation with pCUMB-gfp::HLF (V- E9TFTβ). Among them, four transgenic plants were selected randomly and further analyzed for the presence of the *HLF* gene in the genome through PCR amplification and Southern hybridization. PCR amplification of genomic DNAs isolated from these transgenic lines was first undertaken using primers specific to *bar* and *HLF* cDNA. All the T_0 shoots tested showed expected PCR products, which are 550 bp and 2.1 kb DNA fragments of the *bar* and *HLF*, respectively. No amplified product was detected from genomic DNA of untransformed plants (data not shown).

Southern blot analysis of genomic DNAs strengthened the results of PCR analysis, showing that the T-DNA region was integrated into the genome of the transformed plants. As shown in figure 3A, presence of a 2.1 kb fragment hybridizing to the *HLF* gene probe in all putative transgenic

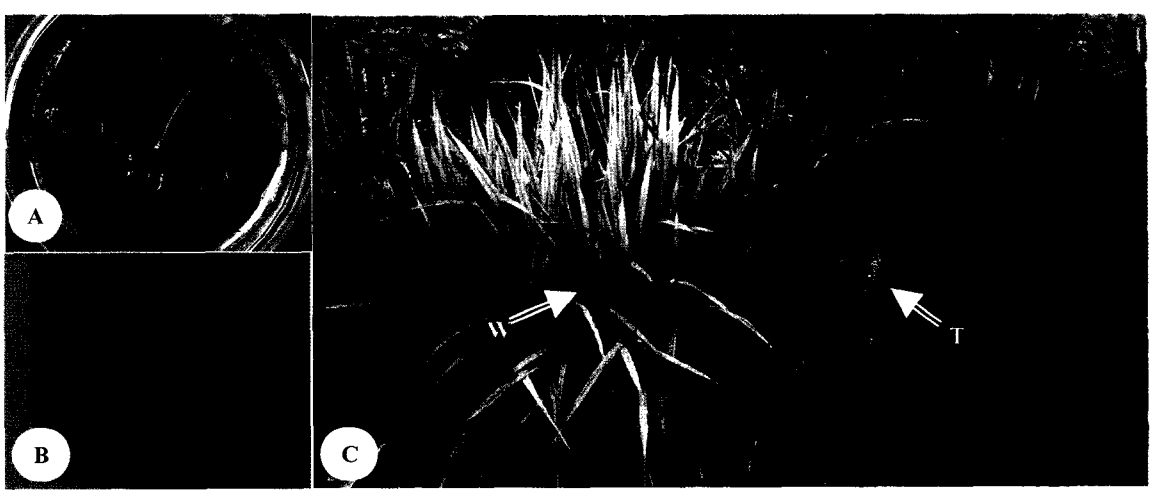

Figure 2. Expression of *bar* gene in transgenic rice (cv. Dong Jin). (A) Shoot regeneration on MS medium supplemented with 2.5 mg/L bialaphos. (B) Confirmation of transformation with root induction medium containing 5 mg/L bialaphos. (C) Comparison of transgenic plant showing Basta resistance with wild-type counterpart. Photographic observations were recorded one week after the Basta (150 mg/L) treatment. T, transgenic plant; W, wild-type control plant.

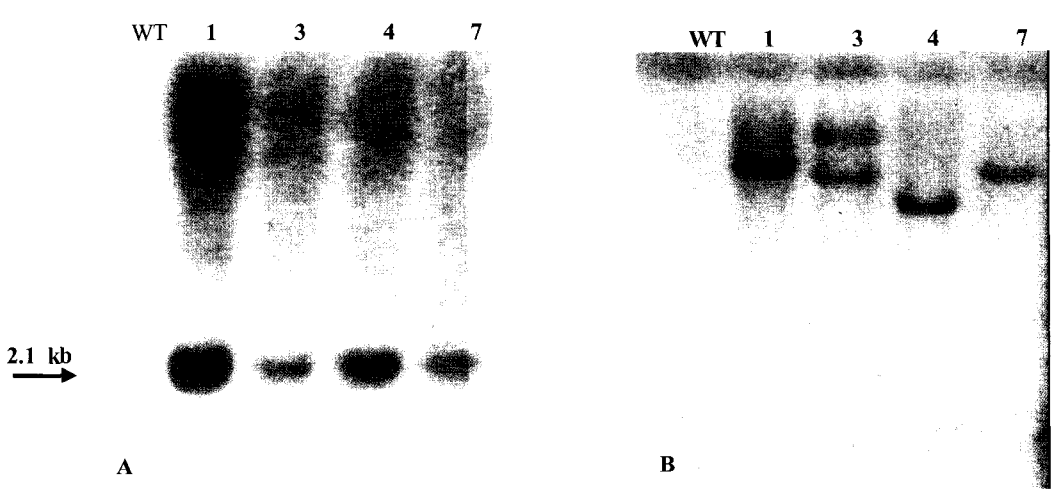

Figure 3. Southern blot analyses of four representative transgenic plants (line 1, 3, 4 ,7) and wild-type (WT) control plant. (A) Total genomic DNA was digested with *Bam*H I hybridized with 2.1 kb *HLF* probe. (B) Total genomic DNA was digested with *Eco*R I hybridized with 550 bp *bar* probe.

plants verified the introduction of the full length coding sequence of the *HLF* gene. No hybridization signal was obtained from the untransformed wild type plant.

We also assessed the stable integration of the *bar* gene in the T_0 generation of the transgenic lines. Total DNAs of the plants were digested with *Eco*RI. As illustrated in figure 1, *Eco*RI restriction digests of genomic DNAs would yield various bands larger than 1.9 kb that hybridize to the *bar* gene probe. The number of bands hybridizing with the *bar* probe would, in turn, reflect the number of sites into which T-DNA has been integrated. The different size of hybridizing bands also indicates integration of T-DNA into the different sites in rice genome. When probed for the *bar* gene integration, transgenic plants with varying copy numbers were observed, whereas no hybridization signal was detected in the non-transformed plants. The copy number of the integrated genes was one (lane 1, 4, 7) or two (lane 3), as shown in Figure 3B. The sizes of hybridizing bands were also varied in each transgenic line, indicating that these are independent transgenic lines.

Taken together, the pattern of bands hybridized with the *HLF* or *bar* probe clearly showed integration of at least one copy of each gene (Figure 3).

Inheritance of the *bar* Gene to T_1 Progeny

In order to assess whether the *bar* gene was transmitted to the next generation in a normal Mendelian fashion, genetic segregation analysis of Basta resistance and sensitivity was performed in T_1 progenies derived from self-pollinated transgenic lines. The T_1 progenies of 10 independent lines were disinfected and allowed to germinate in a 1/2 MS medium. After 7 days, seedlings were transferred to the same medium containing 50 mg/L bialaphos. The number of plants resistant or sensitive to the bialaphos was scored after 2 weeks. The χ^2 analysis of the T_1 progenies showed that seven lines among ten tested were segregated with 3:1 ratio, whereas two lines (line 4 and 5) showed 1:1 segregation ratio and one line (line 3) segregated with 15:1 ratio (Table 1). The fact that line 3 showed 15:1 segregation ratio was consistent with the data obtained from genomic Southern blot analysis. In case of the line 4, we expected a segregation ratio of 3:1 for inheritance of the bar gene, because the genomic DNA gel blot analysis indicated that this line carried a single copy of transgene insertion. However, an aberrant 1:1 segregation ratio was observed. This phenomenon might be associated with lower transgene expression levels, gene silencing in homozygous plants of T_1 progenies. It has been reported that change of gene dosage is one of the key factors influencing transgene expression levels and stability in transgenic rice (James et al. 2002)

Table 1. Segregation of bialaphos resistance in T_1 progeny of transgenic rice.

Lines	No. of seed tested	NO. of Bar^R	Ratio (R:S)	Expected ratio	χ^2-value	p-value
1	20	14	2.3:1	3:1	0.26	0.61
2	20	16	4.0:1	3:1	0.26	0.61
3	20	19	19:1	15:1	0.05	0.82
4	20	11	1.2:1	1:1	0.20	0.65
5	20	12	1.5:1	1:1	0.80	0.37
6	20	16	4.0:1	3:1	0.26	0.61
7	20	15	3.0:1	3:1	0.00	1.00
8	20	14	2.3:1	3:1	0.26	0.61
9	20	13	1.9:1	3:1	1.06	0.30
10	20	14	2.3:1	3:1	0.26	0.61

Seeds were cultured on 1/2 MS medium containing 5 mg/L bialaphos for 2 weeks. Seedlings longer than 5cm were classified as resistant (Bar^R). Lines 1, 2 and 6-10, with one expressed copy of the transgene; line 3, with two copies of the transgene; lines 4 and 5, semidominant. χ^2-values indicate significant fit to the expected ratio.

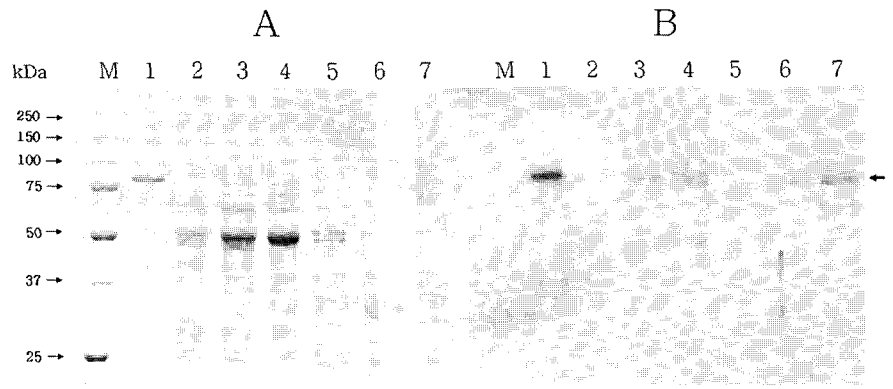

Figure 4. Immuno-detection of human lactoferrin protein expressed in young leaves and grains of transgenic rice plants. Total soluble proteins from young leaves and grains were fractionated by SDS-PAGE (A), blotted onto a nitrocellulose membrane, and probed with a rabbit anti-human lactoferrin primary antibody and detected with alkaline phosphatase conjugated goat anti-rabbit secondary antibody (B). Plant-expressed lactoferrin migrates as a single band with an identical mobility to human lactoferrin with an estimated molecular weight of approximately 80 kDa. Lane M, molecular weight marker; lane 1, native HLF; lane 2, leaf extract from wild-type plant; lane 3-4, leaf extracts from transgenic plant line 1 and 7; lane 5, seed soluble protein from wild-type plant; lane 6-7, seed soluble proteins from transgenic plant line 1 and 7.

Expression of Human Lactoferrin Protein in Transgenic Rice

Two representative transgenic rice cell lines (line 1 and 7) were selected and analyzed for expression of rHLF. Total soluble proteins extracted from leaves and grains of these transgenic rices were loaded on SDS-PAGE gel (Figure 4A) and electrophoretically transferred to a nitrocellulose membrane for immunoblotting. The lactoferrin protein expressed in transgenic plants is shown in Figure 4B. The plant-expressed lactoferrin proteins (lane 3, 4, 6, and 7) migrated as a single band in the SDS-PAGE gel with molecular weight of approximately 80 kDa, which was equivalent to the full length commercial HLF protein (lane1). Similarities in the molecular mobility pattern indicate that full length lactoferrin protein synthesized in rices may be correctly processed, although the expression level of HLF was not high. Low level of rHLF expression was observed in potato plants also (Chong and Langridge 2000), in which HLF was only 0.01% of total soluble protein. However, very high level of expression in the rice grain was obtained by using a strong and tissue- specific promoter (Nandi et al. 2002). In contrast to transgenic plants, untransformed plant protein extract did not react with the anti-lactoferrin antibody (lane 2 and 5). The data also indicated that the *HLF* gene is expressed in leaf tissues and grains at similar level. These transgenic lines should be further examined for bacteriocidal effects against a variety of human pathogenic bacteria.

Acknowledgements

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