DOI:10.1021/jf903904s

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Porcine Lactoferrin Expression in Transgenic Rice and Its Effects as a Feed Additive on Early Weaned Piglets

Tzu-Tai Lee,[†,‡] Chi-Chung Chang,[‡] Rung-Shian Juang,[‡] Ray-Bin Chen,[‡] Hsiu-Ya Yang,[‡] Li-Wei Chu,[‡] Shih-Rong Wang,[§] Tung-Hai Tseng,[∥] Chang-Sheng Wang,[‡] Liang-Jwu Chen,*[,‡] and Bi Yu*[,‡]

[†]Department of Biotechnology, Ming-Dao University, Changhau, 523, Taiwan, [‡]Institute of Molecular Biology, Department of Animal Science, and Department of Agronomy, National Chung Hsing University, Taichung 402, Taiwan, [§]Division of Biotechnology, Animal Technology Institute Taiwan, Miaoli 350, Taiwan, and [∥]Division of Biotechnology, Taiwan Agricultural Research Institute, Wufeng, Taichung 413, Taiwan

The purpose of this study is to determine the growth performance and immune characteristics of early weaned piglets receiving rice bran expressing porcine lactoferrin as a feed additive. Full-length cDNA encoding porcine lactoferrin (LF) driven by a rice actin promoter was transformed into rice plants, and its integration into the rice genome was verified by Southern blot analysis. The expression of recombinant LF (rLF) in whole grains and rice bran was also confirmed, and the amount of rLF accumulated in rice bran was estimated by immunoblot assay to be approximately 0.1% of rice bran weight. An iron-binding assay showed that the rLF retained iron-binding activity and the binding capacity of 1 mg/mL rLF would be saturated by 100 μM of $FeCl_3$. Thirty-six early weaned piglets at 21 days old were randomly selected into two groups and fed a diet containing 5% transgenic rice bran containing 50 mg/kg rLF (rLF group) and 5% rice bran (control group) to investigate the piglets' growth performance and immune characteristics. The results showed no significant difference in growth performance between the groups during the feeding period. However, the aerobic bacteria, anaerobic bacteria, and coliform counts in the cecal contents of the rLF-fed group were significantly lower than those of the control group. Additional immune characteristics such as the IgG concentration in the rLF group was higher than the control group at the 28th day, but leukocyte counts and the peripheral lymphocyte ratio remained similar. In summary, porcine LF expressed in rice bran, a byproduct of rice, can be used as a functional additive to improve antimicrobial capabilities and IgG concentration of early weaned piglets.

KEYWORDS: Recombinant porcine lactoferrin; transgenic rice; animal feed; growth performance; immunity characteristics

INTRODUCTION

There are many stress conditions that affect growth during piglets' early weaning period when their feed is changed from liquid to solid form. Bacterial and viral infections, as well as environmental changes, can result in retarded growth during this stressful period, and outbreaks of diarrhea and even mortality of the piglets may occur (*1*). Incorporating additives to the solid feed, such as antibiotics, can help lower infection rates, but widespread application is restricted due to possible antibiotic residues and the development of bacterial resistance (*2*). An alternative animal feed additive, lactoferrin, has been used to reduce or eliminate antibiotic consumption in animal husbandry (*3*) and to strengthen the piglets' natural immune systems (*4*).

*Corresponding author. (L.-J.C.) Tel: +886-4-22851885. Fax: +886-4-22850347. E-mail: ljchen@nchu.edu.tw. (B.Y.) Tel: +886-4-22860799. Fax: +886-4-22860265. E-mail: byu@dragon.nchu.edu.tw.

Lactoferrin (LF) is an iron binding glycoprotein belonging to the transferrin family. Its molecular mass ranges from 72 to 85 kDa depending on the mammalian species. It is commonly found in physiological fluids such as gland secretions and neutrophilic leukocytes, and is most abundant in colostrums (*5*). LF consists of a single polypeptide chain folded into two structurally homologous lobes which are linked by a short hinge-like peptide, and each lobe has an iron-binding site to bind one molecule of ferric ion Fe^{3+} (*6*). The antimicrobial characteristic of LF is due to its high affinity for Fe^{3+} ion, which competes with the free iron needs of bacteria, and the LF proteolysis process generates lactoferricin (1−47 amino acids) and kaliocin-1 (152−182 amino acids), both of which are small peptides with antimicrobial activity (*7*, *8*).

LF has been used for protection against microbial infection (*9*), modulation of inflammatory responses (*10*), regulation of immune functions (*11*), and cellular growth promotion (*12*) as well as transcriptional regulation and intestinal iron absorption (*13*).

 Published on Web 03/26/2010

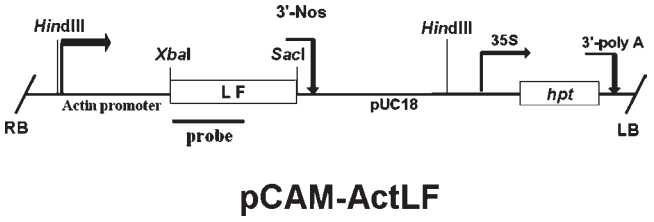

Figure 1. Schematic diagram of plasmid construct pCAM-ActLF. A plasmid construct in vector pCAMBIA1300 (8958 bp, accession number AF234296) with a porcine lactoferrin (LF) cDNA fragment (2061 bp, accession number NM 214362), driven by a rice actin promoter (1255 bp) (*22*) and an NOS terminator (300 bp, 3′-Nos) was created for rice transformation. Restriction enzyme sites of *Hin*dIII, *Xba*I, and *Sac*I are indicated. LB, RB, and *hpt* represent the left border, right border, and the hygromycin phosphotransferase gene, respectively.

The combination of iron and lactoferrin in mucosal secretions affects the aggregation of pathogenic bacteria and inhibits the attachment of both bacteria and viruses to the cell membrane by binding to host cells and viral particles (*14*).

Further immunomodulatory studies, such as those on mice which received oral administrations of LF, showed increased IgG and IgA concentrations in small intestinal mucous and stimulated growth in both Peyer's patches and spleen cells (*15*). Additional studies indicated that, when directly injected with LF, mice would have strengthened small intestine immune systems due to increased activation of natural killer cells, such as macrophages and T-cells (*16*). In weaned piglets, LF used as a feed additive improved the animals' immune functions and serum iron level as well (*4*). These studies demonstrate the positive effects of LF on animal immunomodulatory regulation.

Given the limited amounts of LF available in colostrum as well as human and cow's milk, there have been numerous attempts to obtain large amounts of LF by using transgenic rice (*17*−*19*) and transgenic potatoes (*20*), which can yield relatively larger amounts of LF. These plant-expressed rLFs have also been shown to retain the biochemical and physiological properties of their native counterparts (*17*−*21*). As such, application of these purified plant-based rLFs in infant formula or animal feed have been suggested; however, studies related to feeding plant materials containing LF directly to early weaned animals, such as piglets, and evaluating its antimicrobial and immunomodulatory activity have not been performed. As there has been no study to date on the biofunctionality of rice-expressed recombinant LF (rLF) used as an animal feed additive for early weaned piglets, and in an attempt to provide a cost-effective solution, we have recently engineered a porcine LF gene to be expressed in rice grains to enable large scale production of LF and to allow the rice bran to be added directly to the feed. The present study utilizes transgenic rice bran expressing LF as a feed additive and evaluates its effects on the growth performance and immune characteristics of early weaned piglets. Positive results from this study will demonstrate the economic value of LF transgenic rice and its application benefits to the feed industry.

MATERIALS AND METHODS

Plasmid Construction. A full-length cDNA fragment (2061 bp) of porcine lactoferrin (accession number NM 214362) driven by a rice actin promoter (1255 bp) (22) was first constructed in pUC18, then inserted into the *Hin*dIII site of vector pCAMBIA1300 (accession number AF234296) to form pCAM-ActLF (**Figure 1**). This plasmid vector was then transferred to *Agrobacterium tumefaciens* strain EHA105 for rice transformation. The hygromycin phosphotransferase (hpt) gene driven by the 35S promoter was used as a selection marker.

***Agrobacterium*-Mediated Transformation and Selection for Transgenic Rice.** *Oryza sativa japonica* cv. TNG67, a major rice variety cultured in Taiwan, was selected as the target plant for transformation. Rice calli, derived from immature embryos, were used as the material for *Agrobacterium*-mediated transformation, according to the report of Lee et al. (*23*, *24*). Rice calli were incubated in a suspension of *Agrobacterium tumefaciens* containing pCAM-ActLF for 30 min, then removed from the suspension, and transferred to a solid 2N6-AS medium (2-fold N6 salts, 2 mg/L 2,4-dichlorophenoxyacetic acid, 1 g/L casamino acid, and 30 g/L sucrose, pH 5.2) for another 72 h of cocultivation at 28 °C in the dark. After cocultivation, rice calli were washed in a liquid medium with cefotaxime (250 mg/L) and plated on a solid 2N6-CH medium (2-fold N6 salts, 2 mg/L 2,4-dichlorophenoxyacetic acid, and 30 g/L sucrose, pH 5.7) for 3 weeks. These rice calli were then transferred to RS-selection medium (MS salts, 1 g/L casamino acid, 0.02 mg/L naphthalene acetic acid, 2 mg/L kinetin, 30 g/L sorbitol 30 g/L, sucrose 50 mg/L, hygromycin, pH 5.7) and subcultured every 2 weeks until the shoot regenerated. Regenerated plantlets (T0 plants) were transplanted to soil in pots in a greenhouse at the Taiwan Agricultural Research Institute, Taichung. Putative transgenic rice plants were selected by resistance to hygromycin, and the existence of the LF gene was detected by PCR using primers LF27A: 5′-CCAACATGTTCATGAAGCTCTTCATCC-3′ and LF25B: 5′- CTCGAGCTCCGGTTTTACCTCATCA-3′.

Rice Genomic DNA Isolation and Southern Blot Analysis. Genomic DNA was isolated from 3-week-old seedlings of transgenic rice lines according to Dellaporta et al. (*25*). Aliquots of DNA (20 μg) were digested with *Hin*dIII or *Xba*I, fractionated on a 1% agarose gel, transferred onto the Zeta-probe GT membrane (BioRad, USA), and hybridized with a ^{32}P-labeled LF probe. Hybridization and detection were carried out according to the manufacturer's instructions.

Selection of Homozygous Transgenic Lines. T1 seeds from four representative transgenic lines were germinated in media containing hygromycin, and 40 seedlings chosen from each transgenic line were grown in the greenhouse. To screen homozygous transgenic rice lines, 30 T2 seeds harvested from each T1 transgenic plant were cultured in the presence of hygromycin. Lines with all 30 seeds germinated in the hygromycin-containing media were picked up as putative homozygous transgenic lines as described by Lee et al. (*22*). The same screening methods were applied to the progeny of T3 through T6 generations to obtain homozygous lines, and the seeds from these T6 homozygous lines were used for further assays. The existence of the LF gene for each line in the seedling stages was also confirmed by PCR and Southern blot analysis as described in the previous section.

Protein Extraction and Immunoblot Assay. Antibody to be used against LF was raised in a rabbit, and an anti-LF antiserum was prepared and purified as described by Wu et al. (*26*). Proteins from whole grains of TNG67 (WT) and rice bran (RB) as well as the whole grains (WG) and leaves (L) of transgenic lines were extracted as described by Lee et al. (*27*). The protein samples were then fractionated on a 12.5% SDS−PAGE gel and transferred onto nitrocellulose membrane with a BioRad Trans-Blot system according to the manufacturer's instructions. The membrane was first probed with primary anti-LF antibody and then treated with secondary antirabbit antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA). After antibody recognition, the membrane was incubated with NBT (nitro blue tetrazolium chloride, 50 mg/mL) and BCIP (5-bromo-4-chloro-3-indolyphosphate, 50 mg/mL) in a 10 mL alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.5) for color development as described by Lee et al. (*27*).

Preparation of Porcine rLF from Rice Grains and Iron-Binding Assay. Fifty grams of matured rice grains from transgenic rice line A14 were ground in liquid nitrogen with an extraction buffer (50 mM Tris/HCl, 1 mM EDTA, 100 mM NaCl, and 0.1%(v/v) Triton X-100, pH 6.5). The extracted proteins were partially purified by 40−55% of ammonium sulfate precipitation, and the precipitated proteins were collected by centrifugation and then resuspended and dialyzed against the initial buffer to remove excess salts. These proteins were further purified through a heparin-Sepharose column with an ÄKTA purifier 10 system (Amersham Pharmarcia Biotech. Inc.). The amounts of purified protein were detected by the Bradford method using a protein assay kit (Bio-Rad, USA).

Determination of the iron-binding assay was performed according to the methods described by Sorrentino et al. (*28*). The partially purified

Table 1. Composition of the Experimental Diet (g/kg)

ingredients	treatments	
	control	rLF
yellow corn	534.9	534.9
soybean meal (CP, 445 g/kg)	150.0	150.0
full fat soybean meal (CP, 380 g/kg)	100.0	100.0
fish meal (CP, 650 g/kg)	82.5	82.5
rice bran	50.0	0.0
transgenic rice bran[a]	0.0	50.0
dried whey	50.0	50.0
soybean oil	11.8	11.8
limestone	9.0	9.0
dicalcium phosphate	7.3	7.3
sodium chloride	2.5	2.5
vitamin premix[b]	1.0	1.0
mineral premix[c]	1.0	1.0
Calculated Values		
metabolizable energy (kcal/kg)	3349.1	3349.1
crude protein (%)	21.0	21.0
calcium (%)	0.78	0.78
total phosphorus (%)	0.72	0.72
lysine (%)	1.27	1.27
lactoferrin (mg/kg)	0.00	50.00

[a] One kilograms of transgenic rice bran expressed approximately 1 g of lactoferrin. The 50 g of transgenic rice bran in the rLF-diet contains 50 mg of lactoferrin. [b] Supplied per kg of diet: vit. A, 15000 IU; vit. D3, 3000 IU; vit. E, 30 mg; vit. K3, 4 mg; riboflavin, 8 mg; pyridoxine, 5 mg; vit. B12, 4 mg; Ca-pantothenate, 19 mg; niacin, 50 mg; folic acid, 1.5 mg; biotin, 60 mcg. [c] Supplied per kg of diet: Co ($CoCO_3$), 0.255 mg; Cu ($CuSO_4 \cdot 5H_2O$), 10.8 mg; Fe ($FeSO_4 \cdot H_2O$), 90 mg; Zn (ZnO), 68.4 mg; Mn ($MnSO_4 \cdot H_2O$), 90 mg; Se (Na_2SeO_3), 0.18 mg.

porcine rLF was treated with iron-removing buffer (0.1 M acetic acid, 0.1 M sodium acetate, 40 mM EDTA, and 0.2 M sodium phosphate, pH 4.0) to remove ferric ions from the rLF, then transferred to the initial reaction buffer (5 mM Tris-HCl, pH 7.4, and 50 mM NaCl), and then subjected to dialysis to remove all of the released ions in the rLF preparation. The same amount (1 mg) of original rLF (orLF) or iron-removed rLF (irLF) dissolved in 1 mL of reaction buffer was subjected to absorption analysis with an absorbance reading of 465 nm for 20 min in the presence of different concentrations (0, 10, 100, and 1000 μM) of $FeCl_3$. Data are presented as the mean values of three independent assays.

Proteolytic Digestion of Lactoferrin. The purified porcine rLF and transgenic rice bran were dissolved in the HCl solution with pepsin (Sigma P-7000, 4 g/L). The hydrolysis reaction was performed at 39 °C for 2 h. The reaction mixtures were then neutralized to pH 6.6 by adding 1 N $NaHCO_3$. The reaction mixtures were removed by centrifugation at 3,000*g* for 10 min. The supernatants were retained and freeze-dried. The resulting lyophilized powders were used in further antimicrobial experiments (*29*).

Antimicrobial Activity Assay. For the determination of inhibitory activity, strains of *Salmonella typhimurium* E29, *E. coli* DH5α, and *Lactobacillus reuteri* Pg4 (*30*) were cultured for 16 to 20 h with the XLD (Xylose Lysine Desoxycholate agar, Difco 27885), Coliform (Merck 110426, Darmstadt, Germany) and MRS (de Man Rogosa and Sharpe agar, Difco 288130) media, respectively. The treatments were (1) PBS buffer (pH 7.2), (2) purified rLF hydrolysate (62.5 μg LF/plate), and (3) transgenic rice bran hydrolysate (including 25 μg LF/plate). A standard inoculum of logarithmic phase cells at a final concentration of approximately 10^8 or 10^9 cfu/mL (*Salmonella* 10^9 and *E. coli* and *Lactobacillus* 10^8) was diluted and cultured on agar plates mixed with 500 μL of PBS dissolved hydrolysates (lyophilized powder for transgenic rice bran hydrolysate), and colony-forming units were then determined.

Animals Feeding and Diet. Thirty-six cross-bred 21-day-old weaning piglets were randomly allotted into two dietary treatments of 5% rice bran (control group) and 5% rLF transgenic rice bran (rLF group). Each treatment had three replicates of 6 piglets for a 28-day feeding period. The piglets were housed in traditional weaning pens. Fresh feed and water were provided ad libitum during the experimental period. The average temperature during the experiment was 26.3 °C. Diets were formulated to meet the NRC (*31*) recommendations and formulated to be isonitrogenous and isocaloric with 21% CP and 3350 kcal of ME/kg as shown in **Table 1**. The body weight and feed intake of each pen was determined on the 28th day. On the 14th and 28th days of the feeding period, cecal contents were collected to analyze the total aerobic bacteria, total anaerobic bacteria, and coliform counts, and blood was collected to measure the IgG, T-cell, and B-cell counts in plasma. We did not analyze the values of the different parameters prior to the dietary treatments to reduce the number of piglets slaughtered. In addition, all weaning piglets of this study were in excellent physical condition; we therefore assumed that all of the parameters were at similar levels in the beginning and that the difference resulted from the treatments. The experiment was carried out at National Chung Hsing University, and the experimental protocol for animal use was approved by the Animal Care and Use Committee.

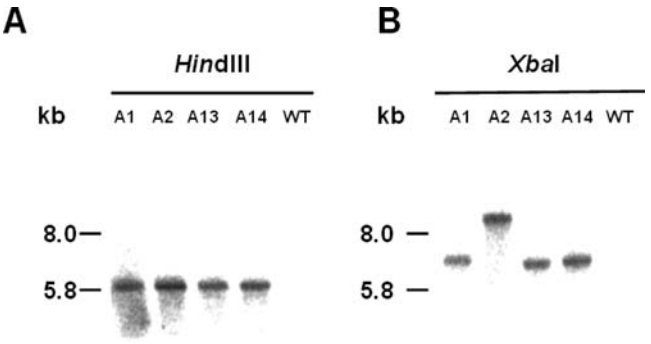

Figure 2. The insertion of the porcine rLF gene in the genome of transgenic lines. Twenty micrograms of genomic DNA extracted from seedlings of the TNG 67 (WT) and four transgenic rice lines (A1, A2, A13, and A14) were digested with *Hin*d III (**A**) or *Xba* I (**B**) and loaded on an agarose gel for separation, then transferred to membranes, and probed with a ^{32}P-labeled LF cDNA fragment as indicated in **Figure 1**. The molecular size markers in kb are shown on the left side of each gel.

Bacterial Counts in Cecal Contents. The cecal contents of weaning piglets measuring approximately 1 g were collected and then diluted with 9 mL of sterile PBS. The plate media used were Plate Count Agar (PCA, Difico) for the total aerobic bacterial count, Reinforced Clostridial Agar (RCA, Oxide) for the total anaerobes count, and Coliform Agar (Difico, USA) for the coliform count. The plates were incubated in an incubator at 37 °C for 1 day (PCA and RCA) or 2 days (Coliform). The bacterial colonies were counted at the end of each incubation period.

IgG Content. IgG concentration was determined by using a double antibody technique ELISA kit (Bethyl Laboratories Inc., Montgomery, TX). Absorbance was measured at 450 nm as described by Hankins et al. (*32*).

White Blood Cells Counts. Blood collected from the piglets was diluted by an autodilutor, and white blood cell counts were measured using a microcell counter (Sysmex F-800, Japan) as described by Lien et al. (*33*).

T-Cell and B-Cell Contents. Fluid cell fluorescence detection was used to measure the percentages of T-cells and B-cells, and MSA4 and 76-7-4 antibodies were used to evaluate these two cells. The ratio of both cells was then analyzed by WinMDI 2.8 software as described by Hankins et al. (*32*).

Statistical Analysis. Data was analyzed as a randomized complete block design using the general linear model procedure (SAS Institute, Inc., Cary, NC). A difference between the two means was tested using Duncan's multiple range tests (*34*). A significant level of 0.05 was used.

RESULTS

Expression and Accumulation of Porcine rLF in Transgenic Rice Plants. More than 20 transgenic rice lines containing recombinant porcine lactoferrin gene were obtained through *Agrobacterium*-mediated transformation, and the existence of the rLF gene in the rice genome was confirmed by PCR and Southern blot analysis. Four homozygous lines (A1, A2, A13, and A14) stably expressing rLF for more than 6 generations (T6) were selected for further

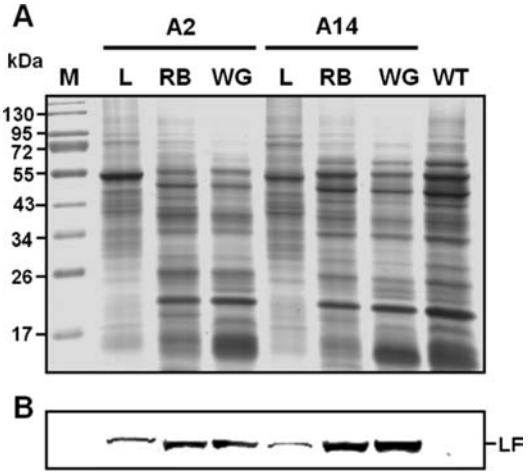

Figure 3. The protein levels of rLF in transgenic rice. Proteins, 30 μg each, isolated from leaves (L), rice bran (RB), and whole grains (WG) of transgenic rice lines A2 and A14, and the whole grains of TNG 67 (WT) were resolved by 12.5% SDS−PAGE gel and stained with Coomassie blue (**A**). A duplicated gel was transferred onto a nitrocellulose membrane and then subjected to immunoblotting with antibodies against rLF (**B**). Numbers on the left (M) indicate the molecular weight markers.

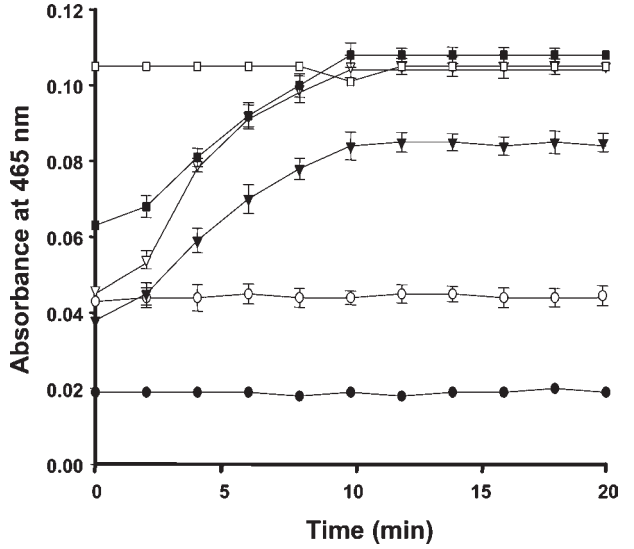

Figure 4. Iron binding activity of rice-expressed rLF. The same amount (1 mg) of rice-expressed rLF originally purified from transgenic rice (orLF, □) and iron-removed rLF (irLF) from the original rLF were dissolved in 1 mL of reaction buffer, then subjected to absorption reading analysis at 465 nm for 20 min in the presence of $FeCl_3$ in 0 μM (○), 10 μM (▼), 100 μM (▽), and 1000 μM (■). Protein extracted from TNG 67 (WT, ●) incubated with 100 μM $FeCl_3$ was used as a control. Data presented are the mean values of three independent assays.

analysis. The insertion of the porcine rLF gene in the genome of these 4 transgenic lines was detected by Southern blot (**Figure 2**). As expected, all four transgenic lines possessed an approximately 5.8 kb *Hind*III fragment (**Figure 2A**) when hybridized with the ^{32}P-labeled LF probe (**Figure 1**). Hybridization with *Xba*I digested fragments revealed only one copy of the porcine LF gene in each of the selected transgenic rice lines (**Figure 2B**).

The accumulation of rLF in leaves and mature rice grains of A2 and A14 homozygous lines was detected by immunoblot analysis (**Figure 3**). An equal amount (30 μg) of protein extracted from each sample was subjected to SDS−PAGE and stained with

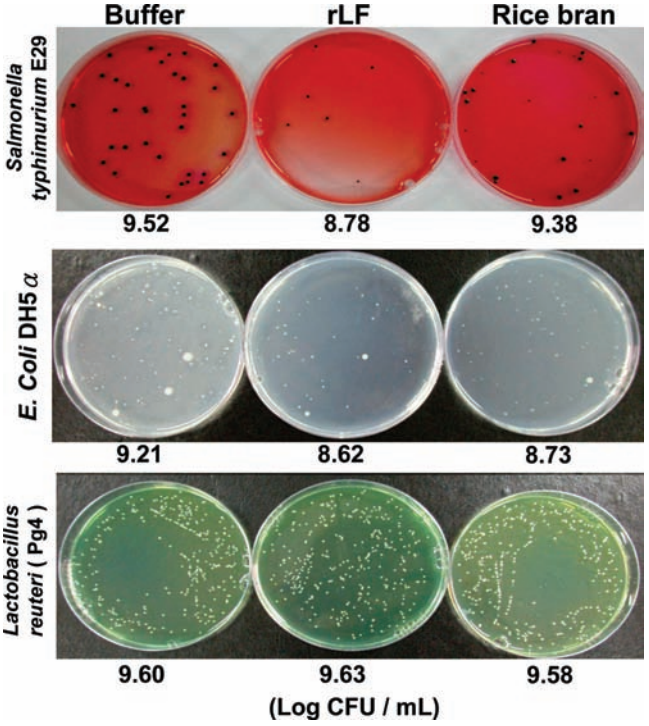

Figure 5. Antimicrobial activity of pepsin-treated porcine rLF and transgenic rice bran. The hydrolysates of pepsin-digested porcine rLF (62.5 μg LF/plate) and transgenic rice bran (approximately 25 μg LF/plate) were incubated with *Salmonella typhimurium* E29, *E. coli* DH5α, and *Lactobacillus reuteri* Pg4 grown in the XLD, Coliform, and MRS medium, respectively, for the determination of inhibitory activity. The CFU/mL (in logarithmic values) were determined for each treatment.

Coomassie blue R-250 (**Figure 3A**). The rLFs were recognized in all samples of transgenic lines, and accumulation was higher in rice bran (RB) and whole grain (WG) than in leaves (**Figure 3B**). The transgenic rice expressed rLF was further subjected to MS/MS sequencing, and the sequences of digested peptide fragments confirmed the success of transgenic porcine rLF expression (Supporting Information, Figure 1). The expression level of porcine rLF in rice bran was estimated to be approximately 1% of the total extractable protein by comparing it to the known amount of purified porcine lactoferrin in the immunoblot assay (**Figure 3** and data not shown).

Iron Binding Activity of Rice-Expressed Porcine rLF. The iron-binding activity of rLF can be measured by its characteristic absorbance at 465 nm (*17*). The original purified porcine rLF (orLF) revealed a reading of 0.10 throughout the 20 min reaction period with the same amount of protein extracted from TNG67 (WT) background reading at 0.02 (**Figure 4**). This result suggested that the porcine rLF expressed in transgenic rice was already saturated with ferric ions.

The iron bound to the original purified porcine rLF was then removed with an acidic pH (pH 4.0) iron-removing buffer. These iron-removed rLF (irLF) were incubated with various concentrations of $FeCl_3$ to measure their iron-binding activity, and results showed that after application of the iron-removing buffer, the irLF has a reading of 0.04, suggesting that most ferric ions have been removed but that some residual amounts remain. When adding 10 μM $FeCl_3$ to the irLF, the absorbance characteristic at 465 nm increases until the 10 min mark and then levels off at 0.08. The reactions with 100 μM and 1000 μM $FeCl_3$ added reached the same levels of absorbance, of approximately 0.10. These results demonstrated that the rice-expressed porcine rLF retained certain

Table 2. Effect of Transgenic Rice Bran on the Growth Performance of Weaning Piglets

	treatments[a]		
items	control	rLF	SEM
initial weight (kg)	5.9	5.9	0.13
end weight (kg)	10.9	10.8	0.65
average daily gain (g)	193.4	189.3	13.2
average daily feed intake (g)	323.6	306.1	22.4
feed conversion (feed/gain)	1.85	1.75	0.05

[a] The treatments were the same as those described in **Table 1**. The rLF-diet contains 50 mg of lactoferrin.

Table 3. Effect of Transgenic Rice Bran on the Bacterial Population of the Cecal Contents of Weaning Piglets[a]

	treatments[b]		
	log CFU/g		
days/items	control	rLF	SEM
At the 14th Day			
aerobic bacteria	8.54	8.30	0.345
anaerobic bacteria	10.00	10.18	0.521
coliform	8.03	7.98	0.325
At the 28th Day			
aerobic bacteria	8.40 a	7.51 b	0.575
anaerobic bacteria	10.29 a	9.48 b	0.416
coliform	9.05 a	7.00 b	0.440

[a] Means in the same row without the same letters are significantly different ($p < 0.05$). [b] The treatments were the same as those described in **Table 1**. The rLF-diet contains 50 mg of lactoferrin. Each value represents the mean of 3 pens (2 pigs per pen).

levels of iron-binding activity and that the binding capacity of rLF will reach saturation at 100 μM of $FeCl_3$.

Antimicrobial Activity Assay in Vitro. An assay against a laboratory *E. coli* strain was first performed to test antimicrobial activity using original purified porcine rLF (orLF), iron-removed rLF (irLF), and bovine LF. The results showed that both forms of rLF inhibited the growth of *E. coli*, and a better antimicrobial activity was observed when iron was partially removed (Supporting Information, Figure 2). In order to mimic the effect of transgenic rice bran as a feed additive on early weaned piglets, purified porcine rLF (62.5 μg) and transgenic rice bran, containing approximately 25 μg of rLF, were digested in vitro by pepsin before adding to the cultures of a pathogenic bacterium *S. typhimurium* E29, a beneficial bacterium *L. reuterin* Pg4, and a laboratory *E. coli* strain DH5α. The results from the rLF and transgenic rice bran treatments showed an antimicrobial effect on *S. typhimurium* E29 and *E. coli* DH5α, but not on the beneficial bacterium *L. reuterin* Pg4 (**Figure 5**).

Growth Performance and Intestinal Morphology. Thirty-six cross-bred 21-day-old weaning piglets were randomly allotted into two dietary treatments of 5% rice bran and 5% rLF transgenic rice bran. The body weight and feed intake of each pen were determined on the 28th day. **Table 2** shows the growth disparity in weaning piglets when fed rLF transgenic rice bran feed. The initial body weights of the weaned piglets in both groups were 5.9 kg. During the 28 day experiment period, the end weights, average daily gain, and feed intake of both groups were similar, but the feed conversion ratio (feed/gain) of the rLF fed group was lower than that of the control group (1.75 vs 1.85), indicating a better feed-to-gain ratio. In addition, other parameters, such as xylose absobance capacity (Supporting Information, Table 1), jejunum villus height, and crypt depth (Supporting Information, Table 2) were analyzed. However, these parameters of rLF group did not reveal a significant difference from those of the control group (Supporting Information, Table 2).

Table 4. Effect of Transgenic Rice Bran on the IgG Content of Weaning Piglets[a]

	treatments[b]		
	(mg/mL)		
days/items	control	rLF	SEM
at the 14th day	30.3	35.0	3.98
at the 28th day	14.0 a	24.2 b	2.15

[a] Means in the same row without the same letters are significantly different ($p < 0.05$). [b] The treatments were the same as those described in **Table 1**. The rLF-diet contains 50 mg of lactoferrin. Each value represents the mean of 3 pens (2 pigs per pen).

Table 5. Effect of Transgenic Rice Bran on Leukocyte Counts and Peripheral Lymphocyte Ratios of Weaning Piglets at the 28th Day

	treatments[a]		
items	control	rLF	SEM
leukocyte counts ($(\times 10^6)/\mu$L)	6.70	6.83	0.69
lymphocytes in leukocyte (%)	30.51	22.81	2.99
T-cells in lymphocyte (%)	79.34	81.57	4.33
B-cells in lymphocyte (%)	6.21	4.96	1.22

[a] The treatments were the same as those described in **Table 1**. The rLF-diet contains 50 mg of lactoferrin. Each value represents the mean of 3 pens (2 pigs per pen).

Bacterial Population in Cecal Contents. Table 3 lists the effects on aerobic, anaerobic, and coliform bacterial populations in cecal contents when weaning piglets were fed rLF transgenic rice bran. Although bacterial populations in the cecal contents of both groups showed no difference on the 14th day after treatment, all bacterial populations in the cecal contents of the rLF group were significantly reduced and were smaller than that of the control group on the 28th day, and in particular, the coliform count was lower by 2 log CFU/g.

IgG in the Plasma. Table 4 shows the effect of transgenic rice bran on IgG content of weaning piglets. The IgG concentration detected in weaning piglets fed with rLF transgenic rice bran showed no statistical difference from those of the control group at the 14th day. On the 28th day, the IgG concentration was significantly higher than that of the control group (24.2 mg/mL vs 14.0 mg/mL, $p < 0.05$).

Leukocyte Counts and Peripheral Lymphocyte Ratio. Table 5 indicates that after a 28 day feeding period of rLF transgenic rice bran, the leukocyte count difference in weaning piglets was not statistically significant (control vs rLF is 6.70 vs 6.83). As for the percentage of lymphocytes in leukocytes, the percentage of T-cells in lymphocytes increased, and the percentage of B-cells in lymphocytes decreased, resulting in an overall decrease in the percentage of lymphocytes in leukocytes (30.51% vs 22.81%).

DISCUSSION

The present study demonstrated that porcine LF can be successfully expressed in transgenic rice grains through a plasmid construct containing the coding region of the porcine LF gene driven by a rice actin promoter. The expression level of recombinant porcine LF in rice bran was estimated to be approximately 1% of the total extractable protein, a figure obtained by comparing it to a known amount of porcine lactoferrin in an immunoblot assay. This level of rLF is equivalent to approximately 0.1% of

the rice bran weight, which is lower than the expression level (up to 0.5% of grain weight) of recombinant human lactoferrin (rHLF) in transgenic rice driven by an endosperm-specific glutelin promoter (*17*).

The physiological characteristics of rHLF, such as its iron-binding capacity and antimicrobial activity, have been demonstrated previously (*17*), and its potential economic application was evaluated as well (*35*). Although the glycosylation pattern of rHLF is different from that of the native human LF, a range of biochemical and biophysical analyses indicates that the purified rHLF is identical to its native human counterpart (*35*). The study of van Berkel et al. (*36*) revealed that different glycosylations of human rLF from a human derived 293(S) cell line did not affect the properties of iron binding and affinity toward bacterial lipopolysaccharide. It has also been illustrated that rLF still retained its biochemical properties and structure even when introduced into different species such as animals, plants, or bacteria (*21*). The iron-binding assay in the present study demonstrated that the porcine rLF, isolated originally from rice grains, had been saturated with ferric ions and that the iron-removed porcine rLF could still resume its iron-binding activity. These iron-binding properties are similar to those of rHLF expressed in rice (*17*, *18*).

The in vitro antimicrobial activity of rice-based rLF, similar to that of rice-expressed rHLF (*17*, *18*, *37*), was first demonstrated using laboratory *E. coli* strain (Supporting Information, Figure 2). In addition, an in vitro pepsin-mediated digestion of rLF and transgenic rice bran to mimic the possible proteolyzed rLF in the gastrointestinal tract revealed positive effects on the suppression of *S. typhimurium* E29 and no inhibition on the beneficial bacterium *L. reuteri* Pg4 (**Figure 5**). It has been demonstrated that lactoferrin has crucial antimicrobial activities against many Gram-positive and Gram-negative pathogens including *E. coli* spp., *Salmonella typhimurium*, *Streptococcus* spp., *Vibrio cholerae*, *Enterococcus* spp., and *Bacillus subtilis* (*38*). Furthermore, rLF seems to promote the growth of beneficial bacteria, such as *Lactobacillus* and *bifidobacteria* (*39*). On the basis of the similar results obtained by porcine rLF and rHLF (*17*, *18*, *37*), we proposed that rice-expressed porcine rLF would retain the same antimicrobial properties as its native porcine counterpart. To test this notion and to analyze the effects of porcine rLF used as an animal feed additive, we added transgenic rice bran directly into the feed as a formula supplement to analyze its antimicrobial activity and to evaluate its modulation of the immune system in weaned piglets.

The antimicrobial activity of lactoferrin is due to its high affinity for ferric ions, which exploit the free iron that microorganisms require and inhibit the attachment of both bacteria and viruses to the cell membrane, by binding to host cells and viral particles (*9*, *14*). In addition, lactoferricin, a LF proteolyzed polypeptide found in the gastrointestinal tract generated by the pepsin-mediated digestion of lactoferrin, has an even stronger antimicrobial activity than lactoferrin (*40*). It has been suggested that lactoferricin releases lipopolysaccharide (LPS) intrinsically from the cell membrane of Gram-negative bacteria and damages the outer membrane of bacteria (*41*). In the present study, we demonstrated antimicrobial activity in the cecum of weaning piglets by measuring the population change of aerobic/anaerobic and coliform bacteria on the 14th and 28th day of transgenic rice bran feeding. Although the bacterial populations showed no differences on the 14th day of feeding, significant reduction of the three types of bacterial populations have been observed on the 28th day after treatment. These results not only confirm the antimicrobial activity of porcine LF but also provide evidence that the application of LF in the form of transgenic rice bran is an effective method. Furthermore, a similar approach constitutively expressed a human lactoferrin and its N-lobe in rice plants which could confer disease resistance (*42*), further extending the potential application of our transgenic rice plants.

The effects of transgenic rice bran on the growth performance of weaning piglets were also evaluated, and the results showed no significant difference between treatments in ADG, ADFI (**Table 3**), xylose absobance capacity (Supporting Information, Table 1), jejunum villus height, and crypt depth (Supporting Information, Table 2) except where the rLF group showed slightly better performance in feed conversion than the control group. However, previous studies demonstrated that Holstein calves fed with supplemental LF showed a significant increase in daily weight gain as well as feed efficiency and reduced use of daily medication during the preweaning phase (*43*, *44*). Recently, a study showed that adding 0.1% of pure human LF in feed could improve growth performance as well as affect the intestinal microflora and intestinal morphology of weaning piglets (*45*). Another study showed that when animal feed was supplemented with 0.1% of pure human LF, although the ADG, ADFI, and feed conversion showed no differences compared to the control group, there was still a decrease in the diarrhea ratio of weanling piglets (*4*). The lack of difference in growth performance levels could be attributed to the weaning age and body weight of the piglets (*18*). The similar growth performance results of the present study could be attributed to the weaning age and body weight of the piglets as well as the 20 times lower amount of rLF that was provided compared to that of Shan et al. (*4*)

Three main populations of the peripheral blood leukocytes of mammals include lymphocytes, monocytes, and granulocytes (*46*). T-cells and B-cells are known to play major roles in host immune response, in cell-mediated immunity and antibody-mediated immunity, respectively. In the present study, porcine rLF did not enhance the ratio of peripheral lymphocytes and those of the T-cells and B-cells in the blood of weaning piglets. Furthermore, in the present study the levels of lymphocytes and T-cells were not increased by rLF treatment, and such results could be due to the exclusion of external interference, such as the presence of tumor cells in groups of weaning piglets, which would induce strong immune responses. There are two different arguments about the effects of LF on lymphocyte development. One is the positive effect, that LF could promote the formation of lymphocytes (*47*, *48*); the other is that it would suppress the formation of lymphocytes (*49*). The function of LF as a promoter or suppressor of lymphocyte formation has not been completely confirmed, but it is certain that LF increases the growth of natural killer cells and killer T cells (*50*). Kuhara et al. (*51*) and Wang et al. (*50*) also presented data showing that when mice are implanted with cancer cells of the large intestine (Co26Lu), orally administered LF was able to activate peripheral leukocytes, including spleen lymphoid T-cells of $CD4^+$, $CD8^+$, and asoalo-$GM1^+$ cells, which increased the killing abilities of the T-cells. In addition, Micallef et al. (*52*) showed that LF could stimulate intestinal epithelium cells to secrete IL-18, which could promote the activation of native killer cells and $CD4^+$ T cells to improve immune function. Therefore, it has been suggested that LF could activate T-cells and suppress the activities of tumor cells despite the results of the present study, which do not necessarily demonstrate that capability.

There have been numerous studies demonstrating the beneficial application of LF in the diet of infant animals during the postbirth or weaning period due to its advantageous effects in boosting the specific or nonspecific immune systems and the attribution of many other physiological functions (*4*, *21*, *45*).

Furthermore, studies show that oral administration of bovine LF extracted from cow milk could raise the immunity levels of small intestinal mucous and increase IgG counts in mucous and plasma in mice. Although the increase of IgG contents in weaned piglets at the 28th day after treatment cannot directly conclude a biological significance, Debbabi et al. detected anti-LF IgG in the intestinal fluid and serum, and the IgG secretion was enhanced in Peyer's patches and spleen from LF-fed mice. Their findings suggested that LF could act as an immunostimulating factor on the mucosal immune system (*15*). Therefore, the increase of IgG content in the results of this study could be attributed to the accumulation and stimulation effects of the rLF provided in the diet during the piglets' late weaned period. This suggests that using transgenic rice bran containing procine LF can be used as a feed additive with positive immunostimulating effects.

In the present study, although the concentration of plasma IgG was higher on the 14th day after feeding than on the 28th day, there is no significant difference between the rLF treatment group and the control group. This result could be partially attributed to the possibility that a piglet may receive the influence of antigens from components of feed such as soybean meal and that this may lead to a higher IgG concentration at an earlier stage, such as at the 14th day. However, at the 28th day after being fed with rLF, higher IgG concentrations in the rLF group than those in the control group were observed ($p < 0.05$, 24.2 mg/mL vs 14.0 mg/mL). This illustrates that even with lower amounts of rLF, nonspecific immune reactions in piglets can still be improved. Taken as a whole, this study concludes that porcine LF can be successfully expressed in transgenic rice bran driven by a rice actin promoter and that the direct application of transgenic rice bran in piglet feed can enhance the antimicrobial capabilities and IgG concentration of early weaned piglets.

ABBREVIATIONS USED

ADFI, average daily feed intake; ADG, average daily gain; Anti-rLF, antibody against porcine lactoferrin; BW, body weight; IgG, immunoglobulin G; LF, lactoferrin; pAct, actin promoter construct with pCAMBIA plasmid; rLF, recombinant lactoferrin.

ACKNOWLEDGMENT

We thank Jia-Sue Lin, I-Wen Wang, Kuang-Chih Lee, and Wen-Bin Tseng for their technical assistance with the transgenic plants and protein preparations.

Supporting Information Available: Effect of transgenic rice bran on xylose concentration of plasma after oral xylose IgG of weaning piglets for 14th day; effect of transgenic rice bran on jejunum villus height and crypt depth of weaning piglets; sequence analysis of rice-expressed porcine rLF by MS/MS; and antimicrobial activity of porcine rLF and bovine LF against *E. coli*. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Pluske, J. R.; Hampson, D. J.; Williams, I. H. Factors influencing the structure and function of the small intestine in weaned pig: A review. *Livest. Prod. Sci.* **1997**, *51*, 215–236.

(2) Frydendahl, K. Prevalence of serogroups and virulence genes in Escherichia coli associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches. *Vet. Microbiol.* **2002**, *85*, 169–182.

(3) Humphrey, B. D.; Huang, N.; Klasing, K. C. Rice expressing lactoferrin and lysozyme has antibiotic-like properties when fed to chicks. *J. Nutr.* **2002**, *132*, 1214–1218.

(4) Shan, T.; Wang, Y.; Wang, Y.; Liu, J.; Xu, Z. Effect of dietary lactoferrin on the immune functions and serum iron level of weaning piglets. *J. Anim. Sci.* **2007**, *85*, 2140–2146.

(5) Masson, P. L.; Heremans, J. F.; Schonne, E. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* **1969**, *130*, 643–658.

(6) Moore, S. A.; Anderson, B. F.; Groom, C. R.; Haridas, M.; Baker, E. N. Three-dimensional structure of diferric bovine lactoferrin at 2.8Å resolution. *J. Mol. Biol.* **1997**, *209*, 711–734.

(7) Sallmann, F. R.; Baveye-Descamps, S.; Pattus, F.; Salmon, V.; Branza, N.; Spik, G..; Legrand, D. Porins OmpC and PhoE of *Escherichia coli* as specific cell-surface targets of human lactoferrin. Binding characteristics and biological effects. *J. Biol. Chem.* **1999**, *274*, 16107–16114.

(8) Viejo-Díaz, M.; Andrés, M. T.; Pérez-Gil, J.; Sánchez, M.; Fierro, J. F. Potassium efflux induced by a new lactoferrin-derived peptide mimicking the effect of native human lactoferrin on the bacterial cytoplasmic membrane. *Biochemistry* (*Moscow*) **2003**, *68*, 217–227.

(9) Arnold, R. R.; Brewer, M.; Gauthier, J. J. Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. *Infect. Immun.* **1980**, *28*, 893–898.

(10) Hayashida, K.; Kaneko, T.; Takeuchi, T.; Shimizu, H.; Ando, K.; Harada, E. Oral administration of lactoferrin inhibits inflammation and nociception in rat adjuvant-induced arthritis. *J. Vet. Med. Sci.* **2004**, *66*, 149–154.

(11) Mattsby-Baltzer, I.; Roseanu, A.; Motas, C.; Elverfors, J.; Engberg, I.; Hanson, L. A. Lactoferrin or a fragment thereof inhibits the endotoxin-induced interleukin-6 response in human monocytic cells. *Pediatr. Res.* **1996**, *40*, 257–262.

(12) Bezault, J.; Bhimani, R.; Wiprovnick, J.; Furmanski, P. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. *Cancer Res.* **1994**, *54*, 2310–2312.

(13) Ward, P. P.; Chu, H.; Zhou, X.; Cunneely, O. M. Expression and characterization of recombinant murine lactoferrin. *Gene* **1997**, *204*, 171–176.

(14) Thomassen, E. A.; van Veen, H. A.; van Berkel, P. H.; Nuijens, J. H.; Abrahams, J. P. The protein structure of recombinant human lactoferrin produced in the milk of transgenic cows closely matches the structure of human milk-derived lactoferrin. *Transgenic Res.* **2005**, *14*, 397–405.

(15) Debbabi, H.; Dubarry, M.; Rautureau, M.; Tomé, D. Bovine lactoferrin induces both mucosal and systemic immune response in mice. *J. Dairy Res.* **1998**, *65*, 283–293.

(16) Yajima, M.; Yajima, T.; Kuwata, T. Intraperitoneal injection of lactoferrin ameliorates severe albumin extravasation and neutrophilia in LPS-induced inflammation in neonatal rats. *Biomed. Res.* **2005**, *266*, 249–255.

(17) Nandi, S.; Suzuki, Y. A.; Huang, J.; Yalda, D.; Pham, P.; Wu, L.; Bartley, G.; Huang, N.; Lönnerdal, B. Expression of human lactoferrin in transgenic rice grains for the application in infant formula. *Plant Sci.* **2002**, *163*, 713–722.

(18) Suzuki, Y. A.; Kelleher, S. L.; Yalda, D.; Wu, L.; Huang, J.; Huang, N.; Lonnerdal, B. Expression, characterization, and biology activity of recombinant human lactoferrin in rice. *J. Pediatr. Gastroenterol. Nutr.* **2003**, *36*, 190–199.

(19) Rachmawati, D.; Mori, T.; Hosaka, T.; Takaiwa, F.; Inoue, E.; Anzai, H. Production and characterization of recombinant human lactoferrin transgenic Javanica rice. *Breed Sci.* **2005**, *55*, 213–222.

(20) Chong, D. K.; Langridge, W. H. Expression of full-length bioactive antimicrobial human lactoferrin in potato plants. *Transgenic Res.* **2000**, *9*, 71–78.

(21) Gonzalez-Chavez, S. A.; Arevalo-Gallegos, S.; Rascon-Cruz, Q. Lactoferrin: structure, function and applications. *Int. J. Antimicrob. Agents* **2009**, *33*, 301–308.

(22) Zhang, W.; McElroy, D.; Wu, R. Analysis of rice Act1 5′ region activity in transgenic rice plants. *Plant Cell* **1991**, *3*, 1155–1165.

(23) Lee, T. T.; Chung, M. C.; Kao, Y. W.; Wang, C. S.; Chen, L. J.; Tzen, J. T. C. Specific expression of a sesame storage protein in transgenic rice bran. *J. Cereal Sci.* **2005**, *41*, 23–29.

(24) Lee, T. T.; Wang, M. M. C.; Hou, R. C. W.; Chen, L. J.; Su, R. C.; Wang, C. S.; Tzen, J. T. C. Accumulation of a sesame 2S albumin

enhances methionine and cysteine levels of transgenic rice seeds. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1699–1705.
(25) Dellaporta, S. L.; Wood, J.; Hicks, J. B. Maize DNA Miniprep. In *Molecular Biology of Plants*; Malmberg, R., Messing, J., Sussex, I., Eds.; Cold Spring Harbor Laboratory: Plainview, NY, 1984; pp 36−37.
(26) Wu, S. C.; Chen, H. L.; Yen, C. C.; Kuo, M. F.; Yang, T. S.; Wang, S. R.; Weng, C. N.; Chen, C. M.; Cheng, W. T. K. Recombinant porcine lactoferrin expressed in the milk of transgenic mice enhances offspring growth performance. *J. Agric. Food Chem.* **2007**, *55*, 4670–4677.
(27) Lee, T. T.; Leu, W. M.; Yang, H. H.; Chen, B. C. M.; Tzen, J. T. C. Sesame oleosin and prepro-2S albumin expressed as a fusion polypeptide in transgenic rice were split, processed and separately assembled into oil bodies and protein bodies. *J. Cereal Sci.* **2006**, *44*, 333–341.
(28) Sorrentino, S.; D'Alessandro, A. M.; Maras, B.; Di Ciccio, L.; D'Andrea, G.; De Prisco, R.; Bossa, F.; Libonati, M.; Oratore, A. Purification of a 76-kDa iron-binding protein from human seminal plasma by affinity chromatography specific for ribonuclease: structural and functional identity with milk lactoferrin. *Biochim. Biophys. Acta* **1999**, *1430*, 103–110.
(29) Babinszky, L.; van der Mear, J. M.; Bear, H.; der Hartog, L. A. An in vitro method for prediction of the digestible crude protein content in pigfeeds. *J. Sci. Food Agric.* **1990**, *50*, 173–178.
(30) Yu., B.; Liu, J. R.; Hsiao, F. S.; Lee, T. T.; Chiou, P. W. S. The probiotic and adherence properties of *Lactobacillus reuteri* Pg4 expressing the rumen microbial β-glucanase. *Asian-Aust. J Anim. Sci.* **2008**, *21*, 1324–1329.
(31) NRC. *Nutrient Requirements of Swine*; National Academy Press: Washington D.C., 1998.
(32) Hankins, C. C.; Noland, P. R.; Burks, A. W., Jr.; Connaughton, C.; Cockrell, G.; Metz, C. L. Effect of soy protein ingestion on total and specific immunoglobulin G concentrations in neonatal porcine serum measured by enzyme-linked immunosorbent assay. *J. Anim. Sci.* **1992**, *70*, 3096–3101.
(33) Lien, T. F.; Horng, Y. M.; Wu, C. P. Feasibility of replacing antibiotic feed promoters with the Chinese traditional herbal medicine Bazhen in weaned piglets. *Livest. Prod. Sci.* **2007**, *107*, 97–102.
(34) Duncan, D. B. Multiple range and multiple F test. *Biometrics* **1955**, *11*, 1–42.
(35) Nandi, S.; Yalda, D.; Lu, S.; Nikolov, Z.; Misaki, R.; Fujiyama, K.; Huang, N. Process development and economic evaluation of recombinant human lactoferrin expressed in rice grain. *Transgenic Res.* **2005**, *14*, 237–249.
(36) van Berkel, P. H.; Geerts, M. E.; van Veen, H. A.; Kooiman, P. M.; Pieper, F. R.; de Boer, H. A.; Nuijens, J. H. Glycosylated and unglycosylated human lactoferrin both bind iron and show identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibilities towards tryptic proteolysis. *Biochem. J.* **1995**, *312*, 107–114.
(37) Conesa, C.; Rota, C.; Castillo, E.; Perez, M.; Calvo, M.; Sanchez, L. Antibacterial activity of recombinant human lactoferrin from rice: effect of heat treatment. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 1301–1307.
(38) Valenti, P.; Antonini, G. Lactoferrin: an important host defense against microbial and viral attack. *Cell. Mol. Life Sci.* **2005**, *62*, 2576–2587.
(39) Sherman, M. P.; Bennett, S. H.; Hwang, F. F.; Yu, C. Neonatal small bowel epithelia: enhancing anti-bacterial defense with lactoferrin and *Lactobacillus* GG. *BioMetals* **2004**, *17*, 285–289.
(40) Ballamy, W.; Takase, M.; Yamauchi, K.; Wakabayashi, H.; Kawase, K.; Tomita, M. Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta* **1992**, *112*, 130–136.
(41) Yamauchi, K.; Tomita, M.; Giehl, T. J.; Ellison, R. T. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect. Immun.* **1993**, *61*, 719–728.
(42) Takase, K.; Hagiwara, K.; Onodera, H.; Nishizawa, Y.; Ugaki, M.; Omura, T.; Numata, S.; Akutsu, K.; Kumura, H.; Shimazaki, K. Constitutive expression of human lactoferrin and its N-lobe in rice plants to confer disease resistance. *Biochem. Cell Biol.* **2005**, *83*, 239–249.
(43) Joslin, R. S.; Erickson, P. S.; Santoro, H. M.; Whitehouse, N. L.; Schwab, C. G.; Rejman, J. J. Lactoferrin supplementation to dairy calves. *J. Dairy Sci.* **2002**, *85*, 1237–1242.
(44) Robblee, E. D.; Erickson, P. S.; Whitehouse, N. L.; McLaughlin, A. M.; Schwab, C. G.; Rejman, J. J.; Rompala, R. E. Supplemental lactoferrin improves health and growth of Holstein calves during the preweaning phase. *J. Dairy Sci.* **2003**, *86*, 1458–1464.
(45) Wang, Y. Z.; Shan, T. Z.; Xu, Z. R.; Feng, J.; Wang, Z. Q. Effects of the lactoferrin (LF) on the growth performance, intestinal microflora and morphology of weanling pigs. *Anim. Feed Sci. Technol.* **2007**, *135*, 263–272.
(46) Wang, F.; Williams, T. J.; El-Awar, F. Y.; Pang, V. F.; Hahn, E. C. Characterization of porcine peripheral blood leukocytes by light-scattering flow cytometry. *Can. J. Vet. Res.* **1987**, *51*, 421–427.
(47) Bi, B. Y.; Lefebvre, A. M.; Dus, D.; Spik, G.; Mazurier, J. Effect of lactoferrin on proliferation and differentiation of the Jurkat human lymphoblastic T cell line. *Arch. Immunol. Ther. Exp.* **1997**, *45*, 315–320.
(48) Esaguy, N.; Freire, O.; Van Embden, J. D.; Aguas, A. P. Lactoferrin triggers in vitro proliferation of T cells of Lewis rats submitted to mycobacteria-induced adjuvant arthritis. *Scand. J. Immunol.* **1993**, *38*, 147–152.
(49) Richie, E. R.; Hilliard, J. K.; Gilmore, R.; Gillespie, D. J. Human milk-derived lactoferrin inhibits mitogen and alloantigen induced human lymphocyte proliferation. *J. Reprod. Immunol.* **1987**, *12*, 137–148.
(50) Wang, W. P.; Iigo, M.; Sato, J.; Sekine, K.; Adachi, I.; Tsuda, H. Activation of mucosal intestinal immunity in tumor-bearing mice by lactoferrin. *Jpn. J. Cancer Res.* **2000**, *91*, 1022–1027.
(51) Kuhara, T.; Iigo, M.; Ito, T.; Ushida, Y.; Sekine, K.; Terada, N.; Okamura, H.; Tsuda, H. Orally administered lactoferrin exerts an antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. *Nutr. Cancer* **2000**, *38*, 192–199.
(52) Micallef, M. J.; Yoshida, K.; Kawai, S.; Hanayama, T.; Kohno, K. In vivo antitumor effects of murine interferon-gamma-inducing factor/interlekin-18 in mice bearing syngeneic Meth A sarcoma malignant ascites. *Cancer Immunol. Immunother.* **1997**, *43*, 361–367.

Received for review November 9, 2009. Revised manuscript received March 11, 2010. Accepted March 16, 2010. This work was supported by grants from the National Science Council (NSC 89-2311-B-005-015) and Council of Agriculture (93AS-4.1.2- FD-Z1) of Taiwan to L.-J.C.