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Expression, purification, and characterization of recombinant human transferrin from rice (*Oryza sativa* L.)

Deshui Zhang^{a,*}, Somen Nandi^{a,1}, Paula Bryan^{a,2}, Steve Pettit^b, Diane Nguyen^a, Mary Ann Santos^b, and Ning Huang^{a,b,*}

^a Ventria Bioscience, 2860 W Covell Blvd., Suite 1, Davis, CA 95616

^b InVitria, 2120 Milestone Dr., Suite 102, Fort Collins, CO 80525

Abstract

Transferrin is an essential ingredient used in cell culture media due to its crucial role in regulating cellular iron uptake, transport, and utilization. It is also a promising drug carrier used to increase a drug's therapeutic index via the unique transferrin receptor-mediated endocytosis pathway. Due to the high risk of contamination with blood-borne pathogens from the use of human- or animal plasma-derived transferrin, recombinant transferrin is preferred for use as a replacement for native transferrin. We expressed recombinant human transferrin in rice (*Oryza sativa* L.) at a high level of 1% seed dry weight (10 g/kg). The recombinant human transferrin was able to be extracted with saline buffers and then purified by a one step anion exchange chromatographic process to greater than 95% purity. The rice-derived recombinant human transferrin was shown to be not only structurally similar to the native human transferrin, but also functionally the same as native transferrin in terms of reversible iron binding and promoting cell growth and productivity. These results indicate that rice-derived recombinant human transferrin should be a safe and low cost alternative to human or animal plasma-derived transferrin for use in cell culture-based biopharmaceutical production of protein therapeutics and vaccines.

Keywords

Human serum transferrin; Recombinant protein; Rice (*Oryza sativa* L.); Serum-free cell culture medium

Iron is an essential element used by all eukaryotic organisms and most microorganisms as a cofactor for numerous proteins or enzymes involved in respiration, DNA synthesis, and many other critical metabolic processes [1]. Cellular iron deficiency can arrest cell proliferation and even cause cell death, whereas the excessive iron will be toxic to cells by reacting with oxygen via the Fenton reaction to produce highly reactive hydroxyl radicals that cause oxidative damage to cells [1,2].

*Corresponding Authors Ventria Bioscience, 2860 W Covell Blvd., Suite 1, Davis, CA 95616. Tel., 5307921419. Fax, 5307921427. DZhang@ventria.com; NHuang@ventria.com.

¹Present address: Department of Molecular & Cellular Biology, University of California, Davis, CA 95616

²Present address: Life Technologies, 6055 Sunol Blvd., Pleasanton, CA 94566

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To overcome the dual challenges of iron deficiency and overload, a family of iron carrier glycoproteins collectively called transferrins has evolved in nearly all organisms to tightly control cellular iron uptake, storage, and transport to maintain cellular iron homeostasis [3]. The transferrin protein family includes serum transferrin (TF); lactoferrin (LF) found in mammalian extracellular secretions such as milk, tears, and pancreatic fluid; melanotransferrin (mTF) which is present on the surface of melanocytes and in liver and intestinal epithelium; and ovotransferrin (oTF) found in bird and reptile oviduct secretions and egg white. While all members of the transferrin protein family can bind iron to control free iron level, TF is currently the only protein that has been proven to be able to transport iron into cells [1].

TF is a single-chain glycoprotein of 679 amino acid residues including 38 cysteine residues which are all disulfide bonded. TF consists of two homologous halves, each comprising about 340 amino acid residues and sharing about 40% sequence identity [1,4,5]. The two homologous halves are shown by X-ray crystallography to fold into two distinct globular lobes called N- and C-terminal lobes [1,4]. Each lobe comprises two dissimilar domains (N1 and N2 in the N- lobe; C1 and C2 in the C- lobe) separated by a deep cleft, where the iron binding site is located. The iron-binding ligands in each lobe are identical, which involves the side chains of an aspartic acid, two tyrosines, a histidine and two oxygen molecules from a synergistic carbonate anion [1,2,4–6].

The cellular iron uptake and transport is normally driven by a TF/TF receptor (TFR)-mediated endocytotic process [1]. When TF is free of iron (apo-TF), the two domains of each TF lobe (N1, N2 and C1, C2) remain apart from each other, forming a large water-filled cleft for easy access by the ferric iron. The apo-TF can then binds one (monoferric TF) or two iron molecules (diferric TF or holo-TF) by the coordination of iron-binding ligands at the extracellular pH of 7.4. The diferric TF then binds to TFR on the cell surface in a way that the TF C- lobe binds laterally to the helical domain of the dimeric TFR ectodomain while the TF N-lobe is sandwiched between the bulk of the dimeric TFR ectodomain and the cell membrane [7,8]. This TF-TFR complex is then endocytosed into the early endosome, where the acidic environment (pH 5.5) triggers the conformational change of TF-TFR and the subsequent release of iron from TF by first protonating and dissociating the synergistic anion followed by protonating iron binding-related His and/or Tyr ligands [1,6]. Finally, the apo-TF-TFR complex is recycled to the cell surface, where the neutral extracellular pH will dissociate the complex and release the TF for re-use.

The TF-TFR complex-mediated endocytosis pathway of iron transport is not only biologically significant for maintaining cellular iron homeostasis, but also has important pharmaceutical applications. TF is a requisite component of almost all serum-free cell culture media to ensure iron delivery for propagating cells and maintaining sustained growth in mammalian culture for the production of therapeutic proteins and vaccines [9–12]. In addition, TF has also been actively pursued as a drug-delivery vehicle due to its unique receptor-mediated endocytosis pathway as well as its added advantages of being biodegradable, nontoxic, and nonimmunogenic [13–15]. TF not only can deliver anti-cancer drugs into primary proliferating malignant cells where the TFR is abundantly expressed [14], but also can deliver drugs to the brain by crossing the blood-brain barrier (BBB), which is a major barrier for administrated drugs to reach the central nervous system (CNS) [13,15,16]. TF can also be exploited for the oral delivery of protein-based therapeutics [17,18], as TF is resistant to proteolytic degradation and TFR is abundantly expressed in human gastrointestinal (GI) epithelium [17,19].

With the increasing concerns over the risk of transmission of infectious pathogenic agents from the use of human or animal plasma-derived TFs in both cell culture and drug delivery

applications, recombinant transferrin (rTF) is preferred to native TF [20]. Recombinant human TF (rhTF) has long been pursued in a variety of expression systems [21], but proves to be challenging largely due to hTF's complicated structural characteristics as described above. The commonly used *E. coli* system for production of recombinant proteins has proved to be impractical for producing rhTF, as the expressed rhTF protein remains in insoluble inclusion bodies and the yield of functionally active rhTF after renaturation is very limited [22]. Although both the insect cell (baculovirus) [23] and mammalian cell [21] expression systems have been shown to be able to express the bioactive rhTF, neither of them can provide enough quantity to be commercially available because of the low expression level and the high cost of production. Recently, bioactive rhTF has been expressed in *Saccharomyces Cerevisiae* [24] and become commercially available. This yeast-derived rhTF, however, still remains very expensive (Millipore, Billerica, MA). To address the problems of both the shortage and the high cost of rhTF, alternative expression systems need to be explored.

With the advancement of plant molecular biology in general and the improvement of plant transformation techniques in particular, plant hosts have become a powerful system to produce recombinant proteins cost-effectively and on a large scale [25–28]. In this paper, we report the high level expression of rhTF in rice grains, and the purification as well as the biochemical and functional characterization of rhTF. The expression level of rhTF is estimated at 1% seed dry weight. The rhTF was able to be extracted with saline buffer and purified by a one step anion exchange chromatographic process to greater than 95% purity. The rice-derived rhTF was shown to display similar structural characteristics and biological functionalities to that of native hTF.

Materials and Methods

Materials

All DNA restriction enzymes and modified enzymes used for developing the expression vector were purchased from New England Biolabs (Beverly, MA). All the plant cell culture medium components, antibiotics, and the Extract-N-Amp Plant PCR kit used in this study were purchased from Sigma (St. Louis, MO). The 4–20% Tris-Glycine gels, 6% TBE urea gels, and pH 3–10 IEF gels were purchased from Invitrogen (Carlsbad, CA). The rabbit anti-hTF antibody used for both western and dot immuoblot detections was from (Abcam, Cambridge, MA). The hTF ELISA assay kit was purchased from Bethyl Labs (Montgomery, TX). Chromatographic media Phenyl Sepharose 6 FF, Q (quaternary amine) Sepharose FF and DEAE (diethyl amino ethane) Sepharose FF were obtained from GE (Piscataway, NJ). The native hTF and the yeast-derived aglycosylated rhTF were from Sigma (St. Louis, MO) and Millipore (Billerica, MA), respectively. The peptide-N-glycosidase F was from Sigma. DMEM/F12 medium was obtained from SAFC Bioscience (Lenexa, KS). FBS and ITSE cocktail were purchased from Invitrogen (Carlsbad, CA). All other chemicals and reagents were analytic grade or purer.

Development of hTF expression vector and plant transformation

To obtain high level expression of rhTF in rice seeds, the mature hTF protein amino acid sequence (under Swiss-Prot accession number **P02787**) was back translated to a nucleotide sequence with the codons optimized towards the codon-usage preference of rice genes (<http://www.kazusa.or.jp/codon>). At the same time, internal repeats and other features that might affect mRNA stability or translation efficiency were avoided. Compared to the native gene sequence for mature hTF, nucleotides in 339 out of a total of 679 codons were modified in the codon-optimized nucleotide sequence encoding hTF without altering the amino acid sequence, and the G+C content was increased to 65% from 50% in the native

hTF gene sequence. To facilitate the subcloning of hTF gene into an expression vector, the *MlyI* blunt-cutting restriction site that allows a cut right before the first nucleotide of the hTF gene was engineered, while two consecutive stop codons followed by an *XhoI* restriction site were engineered after the last genetic codon of hTF gene. The entire gene sequence was synthesized by the company DNA2.0 (Menlo Park, CA), and subcloned into a pUC-derived plasmid, creating an intermediate plasmid designated as pVB23.

The expression plasmid pAPI405 comprising rice seed storage protein glutelin 1 gene promoter (Gt1) and its signal peptide encoding sequence (GenBank accession no. [Y00687](#)), the GUS (beta-glucuronidase) reporter gene, and the nopaline synthase (*nos*) gene terminator of *Agrobacterium tumefaciens* was used to prepare a vector for the expression of rhTF. To replace the GUS reporter gene with the synthesized hTF gene, the plasmid pAPI405 was digested with *NaeI* and *XhoI* to remove the GUS reporter gene while the plasmid pVB23 was digested with *MlyI* and *XhoI* to recover the hTF gene. Then, these two restriction fragments were gel purified and fused together by ligation with T4 DNA ligase, creating the hTF expression vector pVB24 (Figure 1).

The plasmid pAPI146 was used to provide a selection marker in plant transformation. The pAPI146 consists of the *hpt* (hygromycin B phosphor-transferase) gene encoding the hygromycin B-resistant protein under the control of rice beta-glucanase 9 gene promoter, which restricts the expression of *hpt* gene only in rice calli [29].

The linear expression cassette DNA fragments comprising the region from promoter to terminator (without the superfluous backbone plasmid sequence) in both pVB24 and pAPI146 plasmids were prepared by double digestion with *EcoRI* and *HindIII*, and used for transformation. Microprojectile bombardment-mediated transformation of embryonic calli induced from the mature seeds of two cultivars, Tapei309 and Bengal (*Oryza sativa* L. subsp. *Japonica*), was performed as described before [29]. Before the regenerated transgenic seedlings were transferred to soil, PCR analysis of the plants were conducted with primers specific to the hTF gene using the Extract-N-Amp Plant PCR kit (Sigma, St. Louis, MO), and plants shown as negative were discarded. The regenerated transgenic plants are referred to as R₀ plants or transgenic events, and their progeny in successive generations are designated as R₁, R₂, etc.

Expression analysis of recombinant hTF from transgenic rice

To identify transgenic events expressing rhTF, pooled R₁ seeds from each transgenic event (R₀) were analyzed because of the genetic segregation of hemizygous hTF gene in the selfed R₁ seeds. Eight R₁ seeds from each transgenic event were randomly picked, dehusked, and placed into eight wells within the same column of a 96 deep-well plate. Five hundred microliters of PBS buffer (pH 7.4) and two 2 mm diameter steel beads were dispensed into each well. Then, a homogeneous extract was produced by agitating the plate with a Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ) for 20 min at 1300 strokes/min followed by centrifugation with a microplate centrifuge at $1,800 \times g$ for 20 min at 4 °C. Equal amounts of supernatant extract of each seed from the same transgenic event were pooled. Two microliters of the pooled protein extracts from each transgenic event were spotted onto a nitrocellulose membrane. The blot was blocked in 5% non-fat milk in Tris buffered saline tween-20 (TBST) buffer for 1 h, and then incubated with rabbit anti-hTF antibody (Abcam, Cambridge, MA) in TBST buffer at a concentration of 1 µg/ml for 1 h followed by washing 4 times (5 min each) with TBST buffer. Then, the blots were incubated with 1:20,000 diluted anti-rabbit HRP (horseradish peroxidase)-conjugated antibody (BioRad, Hercules, CA) in TBST buffer for 1 h followed by 3 washes, 5 min each in TBST buffer, and one wash in TBS buffer for 5 min. The dot blots were then incubated with the enhanced

chemiluminescence (ECL) reagent (Perice Biotechnology, Rockford, IL) for 5 min, and then exposed to X-ray film for signal detection.

The seed protein extracts from positive transgenic plants identified by immuno-dot blot were resolved on a 4–20% Tris-glycine SDS-PAGE gel (Invitrogen, Carlsbad, CA), electro-blotted onto a 0.45 μ m nitrocellulose membrane for 1 h at 100V in a Bio-Rad Protean System (BioRad, Hercules, CA). The subsequent western blot detection procedure was the same as described for dot-immunoblot except that the secondary antibody was the anti-rabbit alkaline phosphatase-conjugated antibody (BioRad, Hercules, CA) at a 1:4000 dilution and that the blot was developed with BCIP/NBT substrate (Sigma, St. Louis, MO).

To investigate the tissue-specificity of rhTF expression, proteins were extracted from roots, stems, leaves, leaf sheaths, anthers with pollens, grain husks, pistils, immature seeds, and mature seeds, respectively, with PBS buffer (pH 7.4), resolved on a 4–20% Tris-glycine SDS-PAGE gel, and immuno-detected by anti-hTF antibody using the method as described above.

Quantification of rhTF was performed by ELISA (enzyme-linked immunosorbent assay) with a hTF ELISA assay kit (Bethyl Labs, Montgomery, TX) by following the manufacturer's instructions, except that the purified hTF from Sigma was used to produce the standard curve.

Extraction and purification of rhTF

To find the optimal extraction condition for rhTF, the effect of temperature, buffer pH, ionic strength, and mixing time on protein extraction was investigated using 100 mg of rice seed flour in each treatment. The temperature effect on rhTF extraction was examined by extracting 100 mg of rice seed flour in 1 ml of PBS buffer, pH 7.4 at room temperature (RT), 37 °C, 40 °C, and 60 °C, respectively, for 1 h. The effect of buffer pH on rhTF extraction was determined by extracting 100 mg of rice seed flour in each Eppendorf tube with 1 ml of 25 mM sodium acetate at pH 4.5, 5.0, 6.0; 25 mM Tris-HCl at pH 7.0, 7.5, 8.0, 9.0; and 25 mM CAPS, pH 10.0 for 1 h at RT. The ionic strength effect on rhTF extraction was determined by extracting 100 mg of rice flour in each of 1 ml 25 mM Tris-HCl, pH 8.0 with 100, 200, and 500 mM sodium chloride for 1 h at RT. The time effect on rhTF extraction was determined by extracting 100 mg of rice flour in 1 ml of 25 mM Tris-HCl, pH 8.0 for 10, 30, 60, and 120 min, respectively. After extraction, all samples were centrifuged at $13,000 \times g$ for 20 min at RT, and the supernatants were assayed to estimate the total soluble protein (TSP) and rhTF protein content.

The purification of rhTF protein was tested with hydrophobic interaction chromatography (HIC) medium Phenyl Sepharose 6 FF, anion exchange chromatography media Q (quaternary amine) and DEAE (diethyl amino ethane) Sepharose FF (GE, Piscataway, NJ), respectively, using the Biologic LP chromatography system (Bio-Rad, Hercules, CA). Each type of chromatography media was packed to 5 cm high in a 1×10 cm Bio-Rad Econo column. The purification of rhTF protein using Phenyl Sepharose resin was carried out essentially as described in [23]. For the purification of rhTF protein with anion exchange chromatography, the seed crude total proteins were extracted with 25 mM Tris-HCl buffer, pH 7.5 at a ratio of 1 to 10 of flour to buffer (g/ml) for 30 min at RT followed by centrifugation at $15,000 \times g$ for 30 min. The supernatant was filtered through a 0.2 μ m filter, and then loaded onto a DEAE or Q Sepharose column pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.5. After the column was washed with 25 mM Tris-HCl buffer, pH 7.5 to the UV and conductivity baseline, the rhTF protein was eluted either by linear gradient from 0 to 100 mM NaCl in 25 mM Tris-HCl buffer, pH 7.5 or by a step elution with 40 mM NaCl in 25 mM Tris-HCl buffer, pH 7.5.

Amino-terminal sequence analysis

The purified rhTF was resolved on a 4–20% Tris-glycine SDS-PAGE gel (Invitrogen, Carlsbad, CA) and electroblotted onto a PVDF membrane (Bio-Rad, Hercules, CA) in 50 mM CAPS buffer, pH 10.0. The blot was stained with 0.1% Ponceau S in 0.1% acetic acid for 5 min, and destained with 0.1% acetic acid and ddH₂O. The protein band corresponding to rhTF was excised and sequenced using an ABI 494-HT Procise Edman Sequencer at the Molecular Structure Facility at the University of California, Davis, CA, US.

MALDI (Matrix-Assisted Laser Desorption Ionization) analysis of rhTF

Three sources of TFs, rice-derived rhTF, yeast-derived aglycosylated rhTF (Millipore, Billerica, MA), and native hTF (Sigma, St. Louis, MO), were all dialyzed against 50 mM sodium acetate, 5 mM EDTA, pH 4.9 overnight followed by dialyses in ddH₂O to deplete iron that was bound to TFs. These iron-free or apo-TFs were further desalted using ZipTipTM μ -C18 pipette tips (Millipore, Billerica, MA), eluted with a solution of 70% acetonitrile (ACN), 0.2% formic acid, and 5 mg/ml MALDI matrix (α -cyano-4-hydroxycinnamic acid), and spotted onto the MALDI target and analyzed with an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems Inc., Foster City, CA) at the Molecular Structure Facility at the University of California, Davis, CA, US.

PNGase F digestion of rhTF

To evaluate the glycosylation status of rice-derived rhTF, the purified rhTF protein was subjected to digestion with peptide-N-glycosidase F (PNGase F) (Sigma, St. Louis, MO). The yeast-derived aglycosylated rhTF (Millipore, Billerica, MA) and native hTF (Sigma, St. Louis, MO) were also included for comparison. All TFs were desalted and buffer exchanged for 20 mM ammonium bicarbonate, pH 8.6 using 10 KDa MWCO Microcon spin columns (Millipore, Billerica, MA) to a final TF concentration of 0.5 mg/ml. Then, 45 μ l of each type of TF was transferred into an Eppendorf tube followed by adding 5 μ l of 10 X denaturant (0.2% SDS, 10 mM 2-mercaptoethanol, 20 mM ammonium bicarbonate, pH 8.6) and boiling for 10 min. After the samples were cooled to RT, 5 μ l of 15% Triton X-100 was added followed by the addition of 5 μ l (2.5 units) PNGase F to remove the glycans from TFs. The reaction was carried out at 37°C overnight (16 h) and analyzed by resolving 15 μ l of each reaction on 4–20% Tris-glycine SDS-PAGE gel (Invitrogen, Carlsbad, CA) and staining with LabSafe Gel Blue (G Biosciences, St. Louise, MO).

Analysis of the isoelectric point of rhTF

The isoelectric point of rice-derived apo-rhTF was determined using a pre-cast Novex IEF (isoelectric focusing) gel, pH 3–10 (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. The native apo-hTF (Sigma, St. Louis, MO) and the yeast-derived aglycosylated apo-rhTF (Millipore, Billerica, MA) were also loaded on the gel for comparison. The running condition was 100 V for 1 h, 200 V for 1 h, and 300 V for 30 min. The gel was then fixed in 136 mM sulphosalicylic acid and 11.5% trichloroacetic acid (TCA) for 30 min and then stained with 0.1% Coomassie Brilliant Blue R-250 followed by destaining.

RP-HPLC analysis of rhTF

Both native apo-hTF (Sigma, St. Louis, MO) and rice-derived apo-rhTF were prepared in buffer A containing 0.1% trifluoroacetic acid (TFA) and 5% ACN at a concentration of 50 μ g/ml and filtered through a 0.2 μ m syringe filter (PALL, Port Washington, NY). Then 2.5 μ g of each protein sample was injected into a pre-equilibrated Zorbax 3000SB-C8 column (Agilent, Santa Clara, CA) with buffer A using a Beckman Coulter System Gold 126 solvent module (Beckman, Fullerton, CA). The column was washed with three column volume of

buffer A, and then run with a gradient from buffer A to 100% buffer B containing 0.04% TFA and 95% ACN in 12 column volume.

Iron-binding assay of rhTF

To test the reversible iron binding capacity of rice-derived rhTF, the purified rhTF was first dialyzed against 50 mM sodium acetate, 5 mM EDTA, pH 4.9 overnight followed by sequential dialysis in ddH₂O two times for 2 h each and 25 mM Tris-HCl, pH 7.5 three times for 2 h each to remove the iron that was bound to rhTF. Then, the apo-rhTF at a concentration of 5 mg/ml in 25 mM Tris-HCl buffer, pH 7.4 + 10 mM NaHCO₃ was titrated with increasing amount of iron (III)-nitrilotriacetate (Fe³⁺-NTA). The spectra were scanned from 700 to 380 nm after each addition of Fe³⁺-NTA, and the reading was corrected for dilution. The iron-saturated rhTF was dialyzed in 25 mM Tris-HCl buffer, pH 7.5 overnight with three buffer changes to remove the unbound iron, resulting in the holo-rhTF. The iron-binding status of rhTF with different iron saturation levels was assayed by examining the mobility of rhTF on the Urea-PAGE gel with the method as described in [30,31,48]. Approximately 2 µg of each TF sample was mixed with equal volume of 2 × sample buffer (89 mM Tris-borate, pH 8.4, 7 M urea, 50% sucrose, 0.01% bromophenol blue), loaded onto a Novex precast 6% TBE-Urea PAGE gel (7M urea) (Invitrogen, Carlsbad, CA), and electrophoresed in a buffer containing 89 mM Tris-borate, 20 mM EDTA, pH 8.4 for 2 h at 170 V. The gel was stained with Coomassie blue.

Cell growth and antibody productivity assay of rhTF

The rice-derived rhTF was compared to the native holo-hTF (Sigma, St. Louis, MO) to test its effect on proliferation and productivity of hybridoma cells under serum-free conditions. The log phase Sp2/0-derived hybridoma cells AE1 (ATCC HB-72) were prepared by growing in DMEM/F12 medium + 1% FBS + ITSE supplement (insulin 10 µg/ml, TF 5.5 µg/ml, Sodium selenite 0.0067 µg/ml, ethanolamine 2.0 µg/ml (Invitrogen, Carlsbad, CA). The cells were then washed three times with DMEM/F12 without supplements to remove FBS and TF, and seeded in serum-free assay medium (DMEM/F12 supplemented with ISE (no TF) and 1g/L CellastimTM (recombinant human albumin) (Invitria, Fort Collins, CO) at 0.8×10^5 viable cells/ml. A dose response study was carried out by adding rhTF or its native counterpart hTF (Sigma, St. Louis, MO) into assay medium at concentrations of 0.03, 0.1, 0.3, 1.0, 5.0, and 30 µg/ml and examining their cell proliferation effect after three days of growth in a humidified incubator, 37°C, 6% CO₂. The negative control was the same assay medium without any added TF, while 10% FBS and ITSE cocktail (Invitrogen, Carlsbad, CA) in assay medium were positive controls. The assay was carried out in duplicate 1 ml stationary cultures for each condition. The concentration of viable cells was determined by a Guava PCA cell counter. The cell proliferation effect of rhTF was further evaluated by using cell growth curve. The AE1 cells were grown in assay medium with the addition of rhTF or native hTF at 10 µg/ml, and the concentration of viable cells was determined every day for six days.

The cell productivity of rhTF was assayed by quantifying the amount of antibody produced in hybridoma cells at day 6 through ELISA. After cells and debris were removed from the media by centrifugation, the antibody quantity was measured using by ELISA as instructed by the manufacturer (Bethyl Labs, Montgomery, TX).

Results and Discussion

Expression analysis of rhTF

From the particle bombardment transformation of two rice cultivars, Bengal and Taipei 309, by using linear rhTF gene expression cassette DNA (Fig. 1), we obtained 568 regenerated

plants (R_0), of which 555 plants were confirmed to contain the rhTF gene by PCR analysis (data not shown). In total, 195 independent transgenic rice plants were fertile and produced seeds (R_1).

The expression screening analysis of R_1 seeds by immuno dot-blot assay of protein extracts showed that 54 plants exhibited detectable expression of rhTF (Fig. 2). The SDS-PAGE analysis revealed a predominant protein band corresponding to the molecular weight of native hTF in positive transgenic seeds but not in the wild-type rice seeds (Fig. 3a), and the band was shown to specifically cross-react with anti-hTF antibody (Fig. 3b).

An analysis of the tissue specificity of rhTF expression demonstrated that the rhTF was expressed only in the maturing and mature seeds, but not in the root, stem, leaf, leaf sheath, grain husk, anther including pollen, and the pistils (Fig. 4). This is consistent with previous finding that the Gt1 gene promoter is developmentally regulated and active only in maturing rice seeds [32,33].

The transgenic events with high level expression of rhTF were identified by the densitometric analysis of the immuno dot signals followed by ELISA quantification. The expression level of rhTF in R_1 seeds was shown to be about 40% of total soluble protein (TSP). However, the measurement of rhTF expression level as a percent of TSP varied significantly depending on different extraction buffers and conditions used because the extracted amount of native rice seed proteins was significantly impacted by pH, ionic strength, and temperature (data not shown). Therefore, the percent of biomass dry weight represented by rhTF is a more reliable estimate of rhTF expression level. The expression level of rhTF in some selected transgenic events was up to 8.8 mg per gram (0.088%) of dry R_1 seed; and reached over 10 mg per gram (1%) of seed dry weight at R_2 generation and remained stable in subsequent generation (Table 1). The relatively lower expression level of rhTF in R_1 seeds compared to that in subsequent generation seeds is likely because of the poor plant growth performance and seed development of R_0 plants. Similar observations have been reported by others [34,35].

Low expression yield of recombinant proteins has been identified as one of the major limitations of plant expression systems [26,36], and Farran et al. (2002) suggested that the critical limit of plant-derived recombinant protein expression level for commercial viability is 0.01% mass weight [37]. The rice-derived rhTF expression level is 100 fold higher than this suggested critical limit. This extremely high expression level will contribute to significantly reduce the production cost, and will also benefit the downstream purification.

Extraction and Purification of rhTF

Identification of the optimal extraction conditions for rhTF is important for developing a purification procedure that allows to increase protein purity and to reduce purification costs. The effect of buffer pH on rhTF extractability was tested in a range from 4.5 to 10.0. It was shown that while the amount of TSP increased with the increase in pH, the extracted rhTF protein was shown to increase with increase in pH from 4.5 to 7.0 but no substantial difference in the pH range from 7.0 to 10.0 (data not presented). Comparison of the effect of extraction time showed that 30 min extraction was already able to exact the maximum amount of rhTF. Neither the salt concentration nor the extraction temperature showed a significant effect on the rhTF extractability (data not shown). These results indicated that extraction of rhTF from rice flour with 25 mM Tris-HCl, pH 7.5 for 30 min at RT was the optimal condition to maximize the extraction of rhTF while minimizing the extraction of rice native proteins.

To develop a cost-effective procedure for purification of rhTF, different chromatography media and conditions were tested. The HIC column with a Phenyl Sepharose was shown to be able to purify rhTF at a purity of 90%. However, the requirement of a step of precipitating rice native proteins with ammonium sulphate before loading the protein extracts onto the Phenyl Sepharose column could reduce the yield of rhTF and also add the purification cost. The weak anion exchange chromatography DEAE showed that the rhTF bound to the DEAE resin in the extraction buffer 25 mM Tris-HCl, pH 7.5 without the need of buffer exchange, while some rice proteins leaked out of the resin into the flow-through fractions during loading and washing. The rhTF could then be eluted from the DEAE resin with 40 mM NaCl in 25 mM Tris-HCl, pH 7.5, and was at a purity of greater than 95% based on the SDS-PAGE (Fig. 5). The purification of rhTF by the strong anion exchange chromatography Q Sepharose resin showed a very similar chromatographic profile to that of DEAE Sepharose column. However, the Q Sepharose resin bound rhTF protein more strongly than DEAE Sepharose resin, and the rhTF protein needed to be eluted with higher concentration of salts, resulting in coeluting more rice proteins. With the DEAE chromatography, we purified rhTF with four batches of 100 g seed flour and each batch consistently yielded the recovery rate of rhTF to 60% calculated on the basis of protein mass as determined by ELISA. These results showed that a one-column DEAE chromatography method can effectively purify rhTF from rice grain protein extracts. The ease of purifying rhTF with a single purification step is presumably enabled by both the high expression level of rhTF and the relatively simple protein composition of the rice grain [38], because either of them will lead to a higher enrichment of target protein in the starting material for purification, which can help simplify the purification process and reduce the cost. The ease and low cost of purification of recombinant proteins from rice grains have also been shown in our prior work on recombinant lactoferrin [39] and lysozyme [40,41].

Biochemical Characterization of rhTF

Amino (N)-terminal sequence analysis—Since a rice seed storage protein signal sequence targeting to the protein body in endosperm was fused to the N-terminus of the rhTF, N-terminal sequencing of rhTF was carried out to examine whether the rice signal sequence was cleaved correctly. Eleven sequencer cycles were analyzed, and the N-terminal sequence of rhTF was revealed as V-P-D-K-T-V-R-W-X^c-A-V, which is identical to nhTF except that the expected cysteine amino acid residue at cycle 9 was not determined. The undetected cysteine is expected because cysteine, without special modification, cannot be detected by N-terminal sequencing. This result indicates that the rice signal sequence before the mature rhTF protein was correctly removed at the expected position.

Molecular weight of rhTF—The MALDI analysis was carried out to estimate the molecular weight of rice-derived rhTF. A close-up view of the MALDI spectrum of rhTF revealed a peak comprising two small split peaks on top with molecular weights of 75,255.6 and 76,573.8 Da, respectively (Fig 6). This MALDI spectrum is similar to that of the yeast-derived aglycosylated rhTF but different from the N-glycosylated nhTF spectrum, which showed a single peak of 80,000 Da mass (Data not shown). The mass for the first split small peak of the rice-derived rhTF is close to the calculated mass of non-N-glycosylated nhTF (75,181.4 Da) with a mass shift of just 74.2 Da, and the mass for the second split small peak showed a mass increase of 1,392.4 Da. The size discrepancy between rhTF and N-glycosylated nhTF as revealed by MALDI is consistent with the finding as shown in the SDS-PAGE gel analysis of rhTF (Fig. 5). Furthermore, the rice-derived rhTF molecular weight as revealed by MALDI is similar to that of the yeast-derived aglycosylated rhTF, suggesting that the rice-derived rhTF may not be N-glycosylated.

Glycosylation modifications—To further verify if the rice-derived rhTF is non-N-glycosylated, rhTF was subjected to PNGase F digestion with native hTF and yeast-derived aglycosylated rhTF as controls (Fig. 7). The native hTF contains two N-glycosylation sites (N413 and N611) [42], whereas the yeast-derived aglycosylated rhTF has two mutations of its N-glycosylation sites (N413Q and N611Q), rendering the protein without N-glycosylation [24]. As expected, the N-glycosylated nhTF showed a clear downward shift in electrophoretic mobility after PNGase F treatment, and the yeast-derived aglycosylated rhTF showed no change before and after the PNGase F treatment. Surprisingly, the electrophoretic mobility of rice-derived rhTF also remained unchanged before and after the PNGase F treatment, and its molecular size was the same as that of deglycosylated native hTF by PNGase F and yeast-derived aglycosylated rhTF. This result is consistent with the data revealed by MALDI analysis, and they all suggest that rice-derived rhTF is not N-glycosylated. The absence of N-glycosylation in rice-derived rhTF is, however, inconsistent with our prior finding on recombinant human lactoferrin (a close relative to hTF), which is expressed in rice grain using the same expression vector for rhTF and shown to be N-glycosylated [39,43]. The mechanism of the formation of non-N-glycosylated rhTF warrants further investigation.

Isoelectric focusing point of rhTF—The isoelectric point (pI) of rice-derived rhTF was shown to be 6.3, which is same as the pI of yeast-derived aglycosylated rhTF but one unit higher than the pI of the native hTF (5.3) (Fig. 8). The pI discrepancy between rhTF and native hTF is due to the negatively charged sialic acid residues present in the native hTF but absent in both rice-derived and yeast-derived rhTFs. The native hTF has two N-linked oligosaccharide chains, and each chain terminates in two or three antennae, each with terminal sialic acid residues [42,44]. It has been reported that loss of the sialic acid residues leads to a cathodic shift of the pI of TF molecules [45]. The yeast-derived aglycosylated rhTF has no N-linked glycans and sialic acid residues. The rhTF expressed in rice grain is not expected to have sialic acids either, as plants are presumably not capable of synthesizing sialic acids or at best just contain negligible amounts [46,47].

Conformation of rhTF—The conformation and integrity of rice-derived apo-rhTF was assessed by comparing with the apo-nhTF using reverse phase liquid chromatography (RP-HPLC). RP-HPLC resolved both the rhTF and nhTF into a major peak corresponding to their respective monomer form of the molecule, and the two peaks were shown to have the same retention time (Fig. 9), suggesting that rice-derived rhTF has similar conformational structure as nhTF.

Biological function assay of rhTF

Iron binding assay—The biological function of TF can be measured by assessing its ability to bind and release iron reversibly. The purified partially iron saturated (pis) rhTF from rice grains showed a salmon-pink color, a characteristic color of iron-bound TF, suggesting that rhTF has already bound iron in rice grains. After being dialyzed against 50 mM sodium acetate, 5 mM EDTA, pH 4.9 overnight followed by sequential dialysis in ddH₂O and 25 mM Tris-HCl, pH 7.5, the pinkish rhTF became colorless (Fig. 10A), an indication of iron release from the pis-rhTF, resulting in the conversion of pis-rhTF into apo-rhTF. Spectrophotometric titration of this apo-rhTF with iron (Fe³⁺-NTA) showed a broad peak in the region from 465 to 470 nm, and the peak grew in size as the rhTF was gradually saturated with the increasing concentration of iron (Fig. 10D). At the same time, the pink color also gradually showed up in the titrated rhTF solution and became darker when rhTF was saturated with iron (Fig. 10A). The saturation of apo-rhTF with iron resulted in the production of holo-rhTF.

To evaluate the iron binding status of purified pis-rhTF and its derived apo- and holo- isoforms after iron depletion and saturation, these rhTF samples were subjected to a urea-PAGE gel electrophoresis analysis. The apo- and holo-rhTF both showed a single band but with slower and faster electrophoretic mobility, respectively, in the urea-PAGE gel (Fig. 10B). The slower and faster migrating forms of rhTF reflected the conformational change of rhTF without or with bound iron [24,30]. The pis-rhTF showed three bands in the urea-PAGE gel; the slowest and the fastest bands corresponded to the apo- and holo- forms of rhTF, respectively, whereas the middle band represented the monoferric form of rhTF. The coexistence of apo-, holo- and monoferric-rhTF in the purified rhTF indicated that rhTF had been indeed partially saturated with iron in the rice grain. The monoferric form of rhTF was further inferred to have an iron bound in C-lobe of rhTF because the band was shown to be closer to the apo-rhTF, which is a characteristic of C-terminal monoferric TF [30,48]. In normal serum with an iron concentration insufficient to saturate TF, the two monoferric forms of hTF (C- and N- terminal) can be revealed in the urea-PAGE gel because both N- and C-terminal iron-binding sites are occupied with iron although the N-terminal site is normally preferentially occupied [49,50]. However, when the serum is dialyzed against a buffer at pH 7.4, iron is found to preferentially bind to the C-terminal site so that the N-terminal monoferric TF is undetectable in the urea-PAGE gel [50]. Similarly, the rice-derived rhTF was extracted and purified at pH 7.5 followed by a step of diafiltration at pH 7.5 to concentrate and buffer exchange, and thus these conditions could cause the C-terminal iron-binding site of rhTF to be predominantly occupied with iron, resulting in the absence of the band corresponding to N-terminal monoferric rhTF.

The electrophoretic mobility of rice-derived apo- and holo-rhTF in urea-PAGE gel was compared with that of native hTF and the yeast-derived aglycosylated rhTF (Fig. 10. C). It was shown that the rice-derived apo- or holo- rhTF migrated with the same mobility exhibited by their corresponding form of yeast-derived aglycosylated rhTF. These results showed that rice-derived rhTF was able to bind and release iron reversibly. However, both apo- and holo- native hTF exhibited faster mobility compared to their respective counterpart of recombinant hTF. The faster electrophoretic mobility of native hTF is associated with its possession of negatively charged sialic acid residues that are absent in both rice- and yeast-derived rhTFs.

Effect of rhTF on cell growth and antibody production—Rice-derived pis-rhTF was shown to have an equivalent dose response as native holo-hTF for the proliferation of hybridoma cells (Fig. 11. A). Less than saturating levels of activity were observed at concentrations from 0.03 to 1 $\mu\text{g/ml}$ with similar EC_{50} value of about 0.3 $\mu\text{g/ml}$. Likewise, a similar maximum effect was observed at 5 and 30 $\mu\text{g/ml}$ that supported cell proliferation to 12.0×10^5 cells/ml. The maximum effect was similar to the ITSE cocktail control containing 5.5 $\mu\text{g/ml}$ native hTF. In addition, hybridoma cells grown in medium with either rice-derived rhTF or native hTF showed similar growth curves (Fig. 11. B), supporting that rhTF has the same proliferation effect as native hTF. Similar effects of rhTF and native hTF on production of antibody were also seen (Fig. 11. C). These data show that pis- rhTF is equivalent to the native holo-form of hTF in stimulating cell growth and antibody production. Likely, the partially iron-saturated rhTF quickly becomes iron saturated due to the presence of iron in the medium.

In summary, we have expressed hTF in rice grain at a high level of 10 grams per kilogram of seed dry weight (1%). The recombinant hTF protein can be extracted with salt-water buffer and purified using a single chromatographic step on DEAE Sepharose column. The rice-derived rhTF was shown to be non-N-glycosylated by MALDI and PNGase F enzyme digestion analyses although the entire amino acid sequence of rhTF including its N-glycosylation sites were not genetically modified. The rice-derived rhTF was proved to be

not only structurally similar to the native hTF, but also functionally the same as native hTF in terms of reversibly binding iron and promoting cell growth and productivity. The rice-derived rhTF will be a safe and low cost alternative to human or animal plasma-derived TF for use in biopharmaceutical cell culture, and it is available under trade name, Optiferrin from InVitria.

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Abbreviations used

TF	transferrin
hTF	human transferrin
TFR	transferrin receptor
LF	lactoferrin
HRP	horseradish peroxidase
IEF	isoelectric focusing
PCR	polymerase chain reaction
MALDI	matrix-assisted laser desorption ionization
ELISA	enzyme-linked immunosorbent assay
TFA	trifluoroacetic acid
ACN	acetonitrile
SDS	sodium dodecyl sulfate
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
BCIP/NBT	5-bromo, 4-chloro, 3-indolylphosphate (BCIP)/nitro blue tetrazolium chloride (NBT)
PAGE	polyacrylamide gel electrophoresis
TBST	Tris buffered saline tween-20
PBS	phosphate-buffered saline
DEAE resin	diethyl amino ethane
Q resin	quaternary amine
PVDF	polyvinylidene difluoride
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
ITSE	a mixture of insulin, transferrin, selenite, and ethanolamine
DMEM	Dulbecco's modified Eagle medium
PNGase F	peptide-N-glycosidase F
RP-HPLC	reversed-phase high-performance liquid chromatography

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Figure 1.

Schematic diagram of the gene construct for the expression of hTF protein. Gt1 promoter, rice seed storage protein glutelin gene promoter; SP, Gt1 signal peptide; hTF, human transferrin; T-Nos, nopaline synthase gene terminator of *Agrobacterium tumefaciens*.

Figure 2.

Expression screening analysis of transgenic rice seeds expressing hTF using immuno dot-blot. Rice seed total soluble proteins were extracted with 0.5 ml of PBS buffer, pH 7.5 per seed at room temperature for 1 h followed by centrifugation. Two microliters each of pooled protein extract from each transgenic event were spotted onto a nitrocellulose membrane. The spots A to F, 1–12, contained seed protein extracts from 72 transgenic rice events. The spots G, 1–6 and G, 7–12 contained seed protein extracts from non-transgenic rice cultivar Bengal and Tapei309, respectively. The spots H, 1–6 contained 10, 20, 50, 100, 200, and 500 ng of native hTF (Sigma) spiked into 2 μ l of Bengal seed protein extract, respectively. The spots H, 7–12 contained 10, 20, 50, 100, 200, and 500 ng of native hTF (Sigma) spiked into 2 μ l of Tapei309 seed protein extract, respectively.

Figure 3.

SDS- polyacrylamide gel electrophoresis (SDS_PAGE) and immunoblot analysis of rhTF expressed in rice grain. Total soluble proteins were extracted from rice flour of transgenic lines expressing rhTF and non-transgenic line Bengal with 25mM Tris-HCl, pH 7.5 at a 1:10 ratio (g/ml) of buffer to rice flour. Total soluble proteins were directly loaded and resolved on two 4–20% Tris-glycine SDS-PAGE gels (Invitrogen) without any protein concentration, enrichment or purification. One gel was stained with Coomassie blue (A), and the other was used for western blot immuno-detection by anti-hTF antibody (B). The native hTF was from Sigma; the arrow head indicates the protein bands corresponding to rhTF. M, Molecular weight standard; 1, 20 μ g of native hTF (Sigma); 2, wild-type rice (Bengal) seed protein extract; 3–8, represent transgenic events VB24-17, 54, 57, 401, 77, and 136, respectively.

Figure 4.

Tissue specificity of rhTF expression in rice. Two 4–20% Tris-glycine SDS-PAGE gels (Invitrogen) were run simultaneously, one gel was stained with LabSafe Gel Blue (G Biosciences) (A), and the other was used for western blot immuno-detection with anti-hTF antibody (B). Lanes 1–9, represent crude protein extracts, 10 µg each, from roots, stems, leaves, leaf sheaths, anthers with pollens, grain husks, pistils, immature seeds, and mature seeds, respectively, and lane 10 is 4 µg of commercial native hTF (Sigma). Arrow indicates hTF protein bands.

Figure 5. SDS- polyacrylamide gel electrophoresis (SDS_PAGE) of protein extracts, different fraction pools from the purification of rice-derived rhTF. CK1 and CK2 represent native hTF (Sigma) and yeast-derived aglycosylated rhTF (Millipore), respectively; M, molecular weight marker; 1, Crude grain extract containing rhTF; 2, flow-through from DEAE-Sepharose column; 3, eluate from DEAE-Sepharose column; 4, fractions from high salt cleaning of DEAE-Sepharose column after the elution of rhTF; Arrow, protein bands corresponding to hTF

Figure 6.

MALDI mass spectra of purified rice-derived rhTF (a close-up view). The apo- rhTFs was desalted using ZipTipTM μ -C18 pipette tips (Millipore, Billerica, MA), eluted with a solution of 70% acetonitrile (ACN), 0.2% formic acid, and 5 mg/ml MALDI matrix (α -cyano-4-hydroxycinnamic acid), and analyzed with an Applied Biosystems 4700 Proteomics Analyzer.

Figure 7. PNGase F treatment of rice-derived rhTF. M, molecular weight marker; 1, 2, and 3, represent native hTF (Sigma), rice-derived rhTF, and yeast-derived aglycosylated rhTF (Millipore), respectively. + and -, represent with and without PNGase F treatment, respectively.

Figure 8.

Isoelectric focusing gel analysis of rice-derived rhTF. Four micrograms of TF in dH₂O was resolved on a Novex vertical slab IEF gels (pH 3–10, Invitrogen) at 100 V for 1 h, 200 V for 1 h, and 300 V for 30 min. 1, native hTF (Sigma); 2, Yeast-derived aglycosylated rhF (Millipore); 3, rice-derived rhTF.

Figure 9.

RP-HPLC comparison of rice-derived rhTF and native hTF. Two and half μg each of rice-derived apo-rhTF and native apo-hTF (Sigma) in buffer A containing 0.1% trifluoroacetic acid (TFA) and 5% ACN were injected to a pre-equilibrated Zorbax 3000SB-C8 column (Agilent). The column was washed with three column volume of buffer A, and then run with a gradient from buffer A to 100% buffer B containing 0.04% TFA and 95% ACN in 12 column volume.

Figure 10.

Iron-binding characteristics of rice-derived rhTF. (A), Color appearance of rhTF (5mg/ml) with different iron saturation levels. 1. partially-iron-saturated (pis) rhTF; 2. apo-rhTF made from purified pis- rhTF; 3. holo-rhTF made from apo-rhTF. (B), urea-PAGE gel (Invitrogen) analysis of rhTF with different iron saturation levels. The TF samples from left to right on the gel are the same as in Panel A. (C), urea-PAGE gel analysis of rice-derived rhTF and the comparison with commercial sources of hTF. Two micrograms of each protein sample were resolved on a precast 6%TBE-urea gel (Invitrogen) under 170 V for 2 h. 1, native apo-hTF (Sigma); 2, Yeast-derived aglycosylated apo-rhf (Millipore); 3, rice-derived apo-rhTF; 4, native holo-hTF (Sigma); 5, Yeast-derived aglycosylated holo-rhf (Millipore); 6, rice-derived holo-rhTF. (D), visible spectra of rhTF in response to different concentrations of ferric iron. Apo-rhTF (5 mg/ml in 25 mM Tris-HCl buffer, pH 7.5 + 10 mM NaHCO₃) was titrated with increasing amounts of iron (III)-nitrilotriacetate (Fe³⁺-NTA). The visible spectra were scanned from 700 to 380 nm after each addition of Fe³⁺-NTA, and the reading was corrected for dilution.

Figure 11.

Effect of rhTF on cell growth and antibody production. A. Growth of hybridoma cells in serum-free media supplemented with no hTF, 0.03, 0.1, 0.3, 1, 5 or 30 $\mu\text{g/ml}$ native hTF (holo form, from Sigma), rice-derived rhTF, ITSE or 10% FBS (Invitrogen). Shown is viable cell concentration after three days. B. Growth of Sp2/0 hybridoma in serum-free medium. The 6 day growth curve of Sp2/0 hybridoma in serum-free medium supplemented with either 10 $\mu\text{g/ml}$ native hTF or rice-derived rhTF, or unsupplemented medium. C. Increase in antibody production by hybridoma cells in serum-free medium supplied with TF. The concentration of antibody after six days of culture is shown. The medium was supplemented with either 10 $\mu\text{g/ml}$ of rice-derived rhTF or native hTF.

Table 1

Quantification of rhTF expression levels over three generations in rice grains

Generation	VB24-17		VB24-54		VB24-57	
	n	Mean±Std	n	Mean±Std	n	Mean±Std
R ₁ ^a	8	8.8 ± 0.9	8	8.0 ± 0.8	8	7.7 ± 0.3
R ₂ ^b	59	10.2 ± 1.7	64	10.0 ± 1.7	76	10.1 ± 2.1
R ₃ ^c	10	10.5 ± 1.8	10	10.5 ± 1.4	15	10.1 ± 1.6

^aEight R₁ positive seeds from each transgenic event were assayed

^bOne gram of pooled R₂ seeds from a single TF-positive R₁ plant was assayed

^cOne gram of pooled R₃ seeds from each single homozygous R₂ plant was assayed