

MCDB 201L: Molecular Biology Laboratory
Professor: Maria Moreno

By submitting this essay, I attest that it is my own work, completed in accordance with University regulations.—Sabrina Gill

Cloning of the Yellow Stripe 1 gene and of the promoter of a
Tapetal Development and Function gene in *Oryza sativa japonica*

by Sabrina Gill, Elaine Lee, Michael Turner,
Ashley Viehmann, Elena Poiata, and Maria Moreno

Abstract

Micronutrient malnutrition plagues over half the world's population. Our goal is to help provide people with necessary nutrients by enriching Japanese rice (a major food staple) with iron (one of the most important micronutrients). *Ys1a* is a gene in *Oryza sativa japonica* and is in a family of genes involved in iron uptake from the rhizosphere and in the phloem transport of iron. TDF1 is a gene in *Oryza sativa japonica* that plays an important role in developing seeds. We hypothesized that by using TDF1's promoter to drive the expression of *Ys1a*, we can increase the iron content in the seeds (the edible portion) of the plant. We successfully cloned the TDF1 promoter into a TOPO vector and the *Ys1a* gene into a pGEX vector. These constructs can be used to create a plant transformation vector, consisting of the *Ys1a* gene flanked by the promoter and terminator of TDF1.

Introduction

Micronutrient malnutrition (MNM), also known as hidden hunger, afflicts over half the world's population.¹ Biofortification, which is the use of micronutrient dense crops to deliver nutrients, offers the most sustainable, cost effective, and far-reaching approach to providing necessary micronutrients to the over three billion people in need.² Iron deficiency, which is the main cause of anemia, is the most widespread micronutrient deficiency, and around 40% of infants in developing countries are iron deficient. There is evidence that rice can be biofortified by the insertion of genes that assist in the transportation and absorption of iron.³ The purpose of our work is to enrich Japanese rice, a major food staple, with iron, one of the most important micronutrients.

There were two parts to this project. The first part involved cloning the TDF1 promoter into a TOPO vector. This construct can be used to create a plant transformation vector using the promoter and terminator from the TDF1 (Tapetal Development and Function) gene from gDNA from *Oryza sativa japonica*.⁴ We choose TDF1 because of its role in controlling callose dissolution and in tapetal differentiation and function.⁴ The tapetum is a highly specialized, transient tissue surrounding microspores and grains during their development in all land plants.⁴ The importance of TDF1 proteins in developing seeds suggests that a plant transformation vector constructed from TDF1's promoters and terminators would help the expressed protein accumulate in edible portions of the plant. If the gene inserted into the TDF1 plant transformation vector increases iron content, then the seeds of the plant would contain more iron, and people eating the rice would intake more iron.

The second goal was to create a protein expression vector using Ys1a from cDNA. Ys1 (yellow stripe 1) and Ys1-like (YSL) transporters are used in iron-chelating compounds known as mugineic family phytosiderophores.⁵ We chose Ys1a because the role of YsL homologs suggests that Ys1a genes are also involved in iron uptake from the rhizosphere and in the phloem transport of iron.⁶ We hypothesize that if the Ys1a gene was incorporated into an expression vector constructed using TDF1's promoters and terminators, Ys1a would be expressed where the TDF1 gene would normally be expressed, which should increase the iron content in edible portions of the rice plant.

We successfully cloned the TDF1 promoter into a TOPO vector and the Ys1a gene into a pGEX vector. These constructs can be used to create a plant transformation vector, consisting of

the Ys1a gene flanked by the TDF1 promoter and the TDF1 terminator on either side. Using the TDF1 promoter to direct transcription of Ys1a will, hopefully, result in Ys1a production around the seeds of the plants. This will increase the iron content of the edible portion of Japanese rice, thereby biofortifying the plant.

Materials and Methods

Amplification and Modification of a DNA Sequence

The promoter of TDF1 was amplified from *Orzya sativa* genomic DNA (*O. sativya gDNA*) by polymerase chain reaction (PCR) and modified using mutagenic forward (5'-tacttccaatccATCAACCCGTTTTGTTTTGG-3') and reverse (5'-tccccgggCTCCCCCTCTCAG CTCCTACCA-3') primers. Mutagenic sequences are shown in blue, and the mutagenic sequence flanking the reverse primer contains an SmaI enzyme restriction site. PCR samples with a final reaction volume of 25 ul were prepared with .52 μM forward primer, .52 μM reverse primer, either Phusion PCR Master Mix with High-Fidelity buffer (NEB) or Phusion PCR Master Mix with GC buffer (NEB), with or without dimethyl sulfoxide (DMSO), and 50 ng/μL *O. sativa* gDNA. Touch-down thermal cycling was performing as follows: denaturation at 98°C for 2 min; 5 cycles of 15 s at 98°C, 30s at 62°C, 1 min at 72°C; 5 cycles of 15 s at 98°C, 30s at 61°C, 1 min at 72°C; 5 cycles of 15 s at 98°C, 30s at 60°C, 1 min at 72°C; one cycle of 15 s at 72°C.

PCR amplification was used to amplify the Ys1a gene from a complementary DNA construct of Ys1a in a PMB plasmid (*O. sativa* cDNA) by the conditions described above. The forward primer was 5'-tacttccaattccATGGAGCACGCCGACGCGGAC -3', and the reverse primer was 5'tatccacctttactTTAGCTTCCAGGCGTAAACT TC-3'. Mutagenic sequences, shown in blue, were extension sequences for ligation independent cloning. Touch-down thermal cycling was performed as follows: denaturation at 98°C for 2 min; 5 cycles of 15 s at 98°C, 30s at 67.5°C, 1 min at 72°C; 5 cycles of 15 s at 98°C, 30s at 66.5°C, 1 min at 72°C; 5 cycles of 15 s at 98°C, 30s at 65.5°C; 1 min at 72°C; one cycle of 15 s at 72°C.

The PCR products were visualized through agarose gel electrophoresis (AGE) on agarose gels (0.8%) using SYBR Safe DNA Gel Stain (Invitrogen). Then, PCR products were purified using the Qiaquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions.

Cloning and Bacterial Transformation of DNA Sequences

Purified PCR product of TDF1 promoter was ligated into the pCR-BluntII-TOPO vector (Invitrogen) (hereby referred to as pCR-BluntII-TOPO::TDF1-PRO) according to the manufacturer's instructions.

The pCR-Blunt-TOPO::TDF1-PRO vector was cloned into One Shot TOP 10 Competent

Cells (Invitrogen) using the pCR-BluntII-TOPO vector from the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to the manufacturer's instructions. 50 μ l and 150 μ l of pCR-BluntII-TOPO::TDF1-PRO transformed bacteria were plated onto luria broth agar with 50 μ g/ml kanamycin.

Ligation independent cloning (LIC) was used to generate a pGEX vector that contained Ys1a. 5 ng/ μ L of the pGEX-6-P2-LIC plasmid vector was digested with 0.3 U/ μ L of *Bsal* restriction enzyme (NEB), NEB3 Buffer (NEB), Bovine Serum Albumun (NEB) (final reaction volume 100 μ L) and was incubated for 2 hours at 50°C and 15 min at 65 °C. Digested vectors were treated with T4 DNA polymerase by incubating final reaction volumes of 100 μ L, consisting of 50ng/ μ L purified vectors, T4 Buffer (NEB), 2.5mM dGTP (Invitrogen), Bovine Serum Albumin (NEB), 5mM DDT (Molecular Probes, Inc.), and .15 U/ μ L T4 DDNA polymerase (NEB) for 30 min at 22°C and at 70 °C for 20 min. Purified Ys1a was treated with T4 DNA polymerase under the same conditions, with the exception that the final reaction volume was 10 μ L and 2.5 mM dCTP (Invitrogen) replaced dGTP. Annealing occurred when the *Bsal*-digested-T4-treated vectors and the T4-treated Ys1a were mixed in a 2:1 ratio and incubated for 10 min at 22 °C (hereby referred to as pGEX-6-p2-LIC::Ys1a). 50 μ l and 150 μ l of pGEX-6-p2-LIC::Ys1a transformed bacteria were plated onto luria broth agar containing 100 μ g/ml carbenicillin and 5% sucrose.

Screening Bacterial Colonies

PCR amplification was used to screen for recombinant plasmids containing the desired pCR-Blunt-TOPO::TDF1-PRO insert. The samples contained the following: .52 μ M forward primer (5'-GCTATGACCATGATTACGCCAAG-3'), .52 μ M reverse primer (5'-ACGTTGTAACGACGGCCAG-3'), Phusion PCR Master Mix with High-Fidelity Buffer (NEB), and 2% DMSO. Touch-down thermal cycling was performed as follows: one cycle of 10 min at 95°C and 30 s at 98°C; 30 cycles of 10 s at 98°C, 30s at 65°C, 45s at 72°C; one cycle of 2 min at 72°C.

To screen for recombinant plasmids containing the desired pGEX-6-p2-LIC::Ys1a insert, samples were prepared as described above, and the same thermal cycle was performed. The forward primer was 5'-ATGCGTTCCTCCAAAATTAGTTTGT-3', and the reverse primer was 5'-CTTACAGACAAGCTGTGACCGTCT-3'.

Isolation of Plasmid DNA

Plasmid DNA was isolated by alkaline lysis from a liquid culture of bacterial cells containing pGEX-6-p2-LIC::Ys1a or pCR-BluntII-TOPO::TDF1-PRO from the QIAGEN Plasmid Midi Kit (Qiagen, Inc) according to the manufacturer's instructions.

DNA Sequencing and Analysis

Reactions containing .444 uM forward primer, .444 uM reverse primer, and 27.8 ng/ul plasmid DNA template were sequenced at the W.M. Keck DNA Sequencing Facility. BLAST was used to analyze the sequences.

Results

The purpose of this experiment was to insert a gene involved in the transportation and absorption of iron (we examined the Ys1a gene) into a promoter-terminator cassette made using the TDF1 gene. After Polymerase Chain Reaction (PCR) amplification of *Oryza sativa* DNA and PCR purification of the Ys1a and TDF1 promoter, we successfully cloned the TDF1 promoter into a TOPO vector and the Ys1a gene into a pGEX vector. The alignment of the virtual TDF1 promoter to the sequenced regions of the pCR-BluntII-TOPO::TDF1-PRO (Fig. 9) and the alignment of the virtual Ys1a gene to the sequenced regions of pGEX-6-p2-LIC::Ys1a (Fig. 10) indicated that the TDF1 promoter and Ys1a were present in the plasmids sent for sequencing. BLAST analysis of the amino acid sequence generated from the sequenced regions of pGEX-6-p2-LIC::Ys1a vector revealed that the theoretical protein expressed by this construct matches the protein produced by the Ys1a gene (Fig. 11).

Amplification and Purification of TDF1 Promoter and Ys1a

To obtain the TDF1 promoter, *Oryza sativa* gDNA was amplified using mutagenic forward and reverse primers for the TDF1 promoter under various conditions (with HF polymerase or with GC polymerase, and with or without DMSO.) Comparison against 1kb ladder revealed sharp bands of 1.0 kb fragments in lanes three and nine. (Fig 1) Those two samples were purified and run in an AGE against 1kb ladder. This AGE also revealed sharp bands of about 1.0 kb fragments, and indicated that PCR with HF Polymerase and DMSO amplifies the TDF1 promoter effectively. A virtual map of the TDF1 promoter shows that it is 1035 bp (Fig. 2), which matches the length of the 1.0 kb DNA fragments found in the AGE.

To obtain the Ys1a gene, a cDNA construct of Ys1a in a plasmid was amplified with mutagenic forward and reverse primers for Ys1a, with or without DMSO, and with HF polymerase or GC polymerase. AGE against 1kb ladder indicated about 2.0 kb fragments lanes three and five, which contain GC polymerase (Fig. 3). An AGE of purified DNA against 1kb ladder also showed DNA fragments 2.0 kb long, indicating that the PCR described above with GC Polymerase and DMSO will amplify Ys1a effectively. A virtual map of Ys1a shows that it is 2023 bp (Fig. 4), which matches the 2.0 kb DNA fragments found in the AGE.

Transformation of TDF1-PRO and Ys1a into Bacterial Plasmids

For the purpose of transforming the TDF1 promoter into bacterial plasmids, the TDF1 promoter was ligated to a pCR-BluntII-TOPO vector and then transformed into competent *E. coli* host cells (Fig. 5). After bacterial colony PCR was performed, recombinant colonies were identified by AGE. Compared to the negative control in lane one, lanes three through eight indicate that bacterial colonies pSG-2 through pSG-8 with pCR-BluntII-TOPO::TDF1-PRO contain TDF1's promoter (Fig. 6).

For the purpose of transforming Ys1a into bacterial plasmids, ligation independent cloning was performed using the prepared pGEX-6-p2 vector, and then pGEX-6-p2-LIC::Ys1a was transformed into competent *E. coli* host cells (Fig. 7). After bacterial colony PCR, recombinant colonies were identified by AGE. Compared to the negative control (lane nine), lanes three, four, six, and eight indicated that that bacterial colonies pSG-3, pSG-4, pSG 6, and pSG-8 were transformed with pGEX-6-p2::Ys1a (Fig. 8).

Sequencing DNA (both Ys1a and TDF1-PRO) and Preparing DNA Sequencing Reactions

Plasmids containing pCR-Blunt-TOPO::TDF1-PRO and plasmids containing pGEX-6-p2::Ys1a were sent to be sequenced to establish if the plasmids contained the TDF1 promoter or Ys1a, respectively. Alkine lysis was used to isolate pCR-Blunt-TOPO::TDF1-PRO and pGEX-6-p2::Ys1a plasmids, and a UV spectrophotometer was used to determine that the concentrations of plasmid DNA templates were 0.5 μ g/ μ l and 0.35 μ g/ μ l respectively. Sequencing reactions were prepared, and DNA sequencing was performed. The alignment of the virtual construct of the TDF1 Promoter (Fig.1) to the sequenced regions of the pCR-BluntII-TOPO::TDF1-PRO and the alignment of the virtual Ys1a construct (Fig. 3) to the sequenced regions of pGEX-6-p2::Ys1a indicated that the TDF1 promoter and Ys1a were present in the plasmids of bacterial cells sent for sequencing (Fig. 9, Fig. 10).

BLAST Analysis of Ys1a

The predicted amino acid sequence was generated from the DNA sequencing results (Fig. 11). The molecular weight of this predicted protein was 72149.94 Daltons.

BLAST analysis, performed on the predicted protein generated from the DNA sequencing results, revealed that the DNA sequence inserted in the pGEX vector codes for the

Ys1a protein. COG1297, a predicted membrane protein with an unknown function, was a conserved domain (Fig. 12). The evolutionary relationship between Ys1a's predicted protein and all other proteins in GenBank revealed a high degree of similarity to the Yellow Stripe 1 (Ys1) transporter in maize and barley, and to a vacuolar iron transporter (TgVit1) in *Tulipa gesneriana* (Fig. 13).

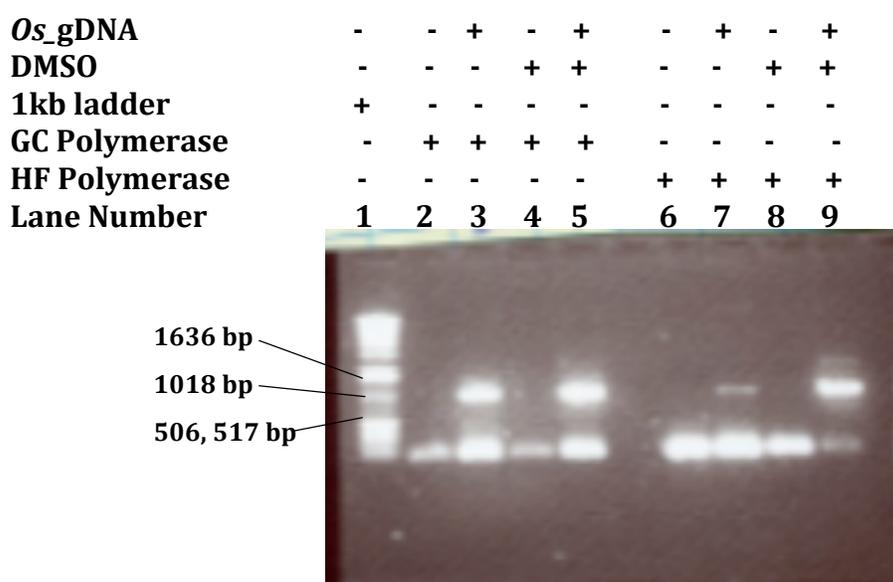


FIG. 1 Agarose gel electrophoresis of TDF1-PRO. AGE of PCR amplification of *Orza Sativya* gDNA, using mutagenic forward and reverse primers for the TDF1 promoter, was performed using DMSO or no DMSO, and GC polymerase or HF polymerase. Lane one contained the 1kb ladder. Lanes two through five contained GC polymerase. Lanes six through nine contained HF polymerase. Lanes four, five, eight, and nine contained DMSO. Lanes three, five, seven, and nine contained *Os_gDNA*, and lanes two, four, six, and eight were negative controls. Comparison to the 1kb ladder revealed 1.0 kb fragments were present in lanes three, five, seven, and nine. The nonspecific band in lane nine and the TDF1 promoter band in lane seven were very faint. The small fragments at the bottom of every lane were most likely residue from primers. PCR with HF Polymerase and DMSO amplifies the TDF1 promoter most effectively.

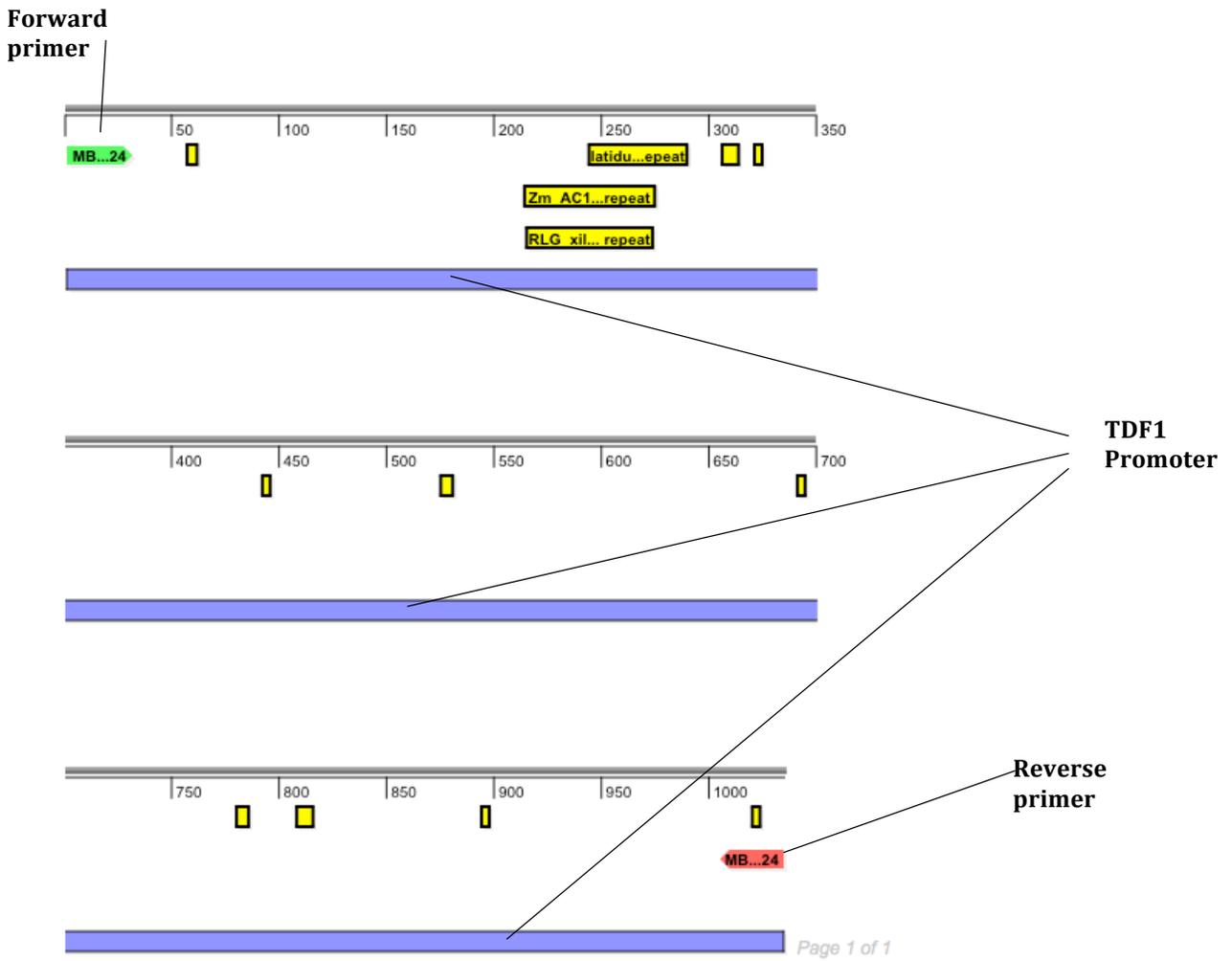


FIG. 2 Virtual Linear Map of TDF1 promoter. The TDF1 promoter is 1035 bp, including its mutagenic forward and reverse primer sequences.

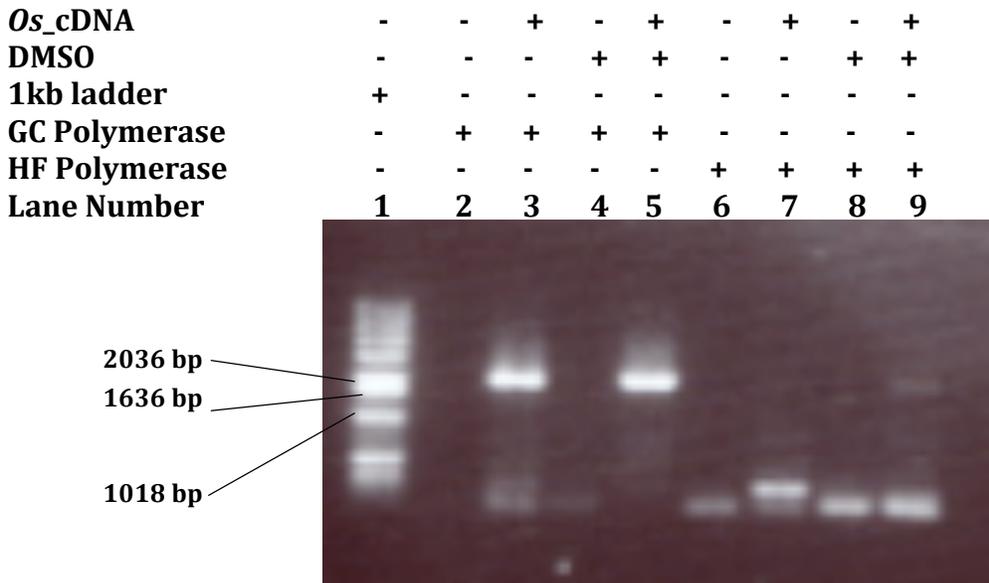


FIG. 3 Agarose gel electrophoresis of Ys1a. PCR amplification of a cDNA construct of Ys1a in a plasmid, using mutagenic forward and reverse primers, was performed with GC Polymerase or HF Polymerase, with DMSO or without DMSO, and with negative controls. Lane one contained the 1kb ladder. Lanes two through five contained GC polymerase. Lanes six through nine contained HF polymerase. Lanes four, five, eight, and nine contained DMSO. Lanes three, five, seven, and nine contained *Os_cDNA*, and lanes two, four, six, and eight were negative controls. Comparison to the 1kb ladder indicated 2.0 kb fragments in lanes three and five. The residue above these fragments may be from smearing of the high quantity of DNA fragments. Lanes two, four, six, and eight were negative controls that show no bands, except for very small fragments, which are probably residue from the primers. Lane nine contained a very faint about 1.8 kb fragment. PCR with GC polymerase and DMSO will amplify Ys1a effectively.

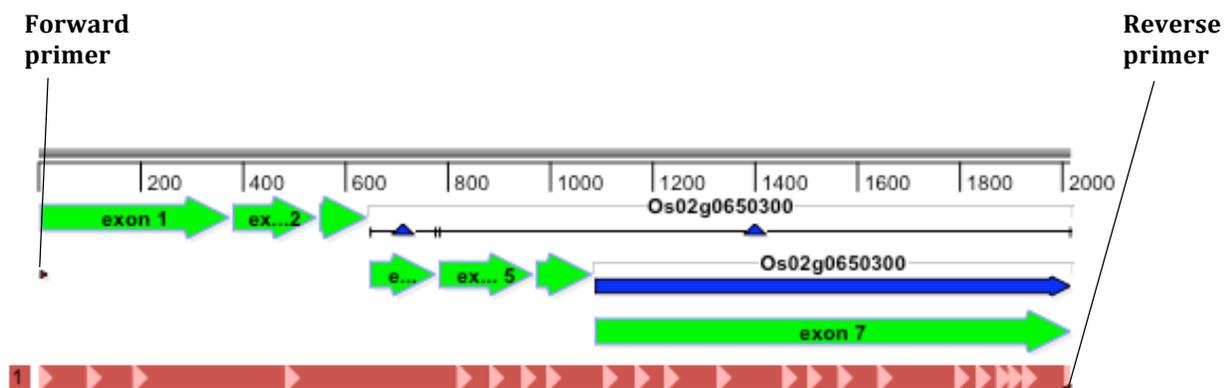


FIG. 4 Virtual Linear Map of Ys1a. The Ys1a coding region in *Os_cDNA* is 2023 bp. The open reading frame (the uninterrupted red bar) between the mutagenic forward and reverse primers for Ys1a is continuous.

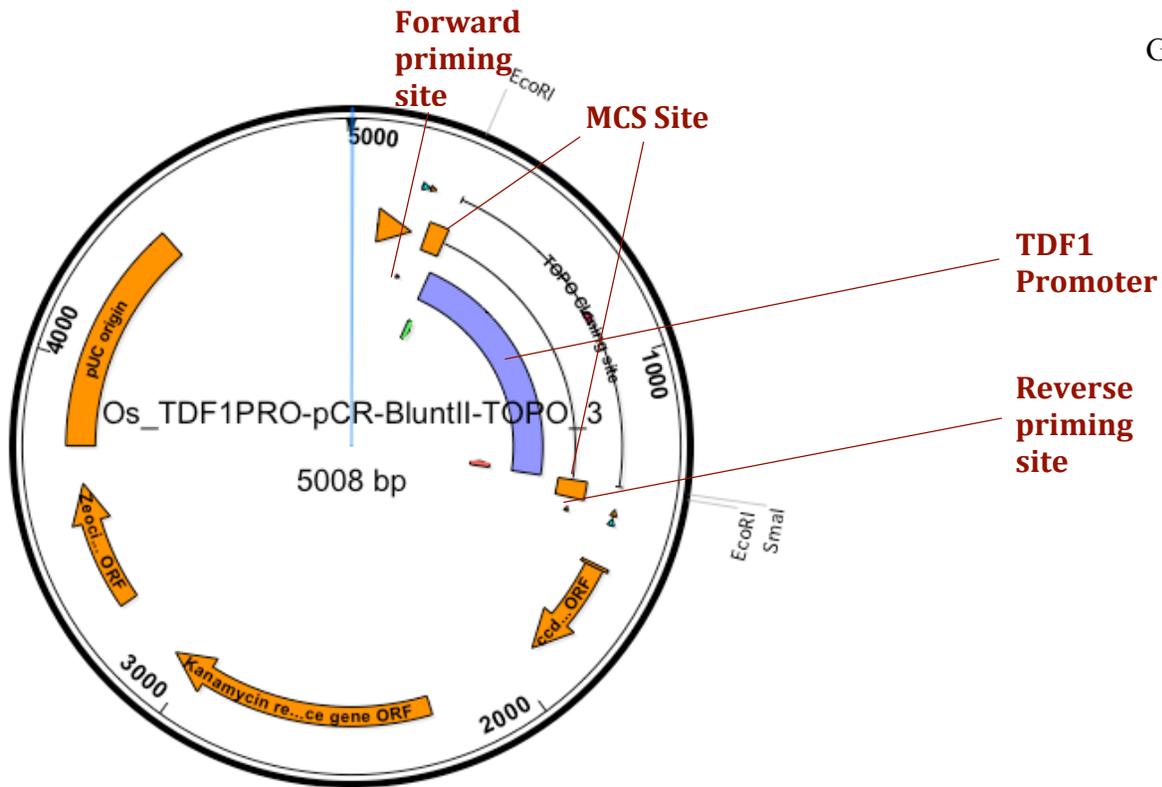


FIG. 5 pCR-Blunt-TOPO::TDF1-PRO virtual clone map. Virtual clone map of recombinant *E. coli* plasmid that contains the pCR-BluntII-TOPO::TDF1-PRO vector, showing the multiple cloning site, the TDF1 promoter gene, the SmaI and EcoRI restriction enzyme sites, and the forward and reverse priming sites for this TOPO vector.

Bacterial Colony	-	-	1	2	3	4	5	6	7
1kb ladder	+	-	-	-	-	-	-	-	-
Lane number	1	2	3	4	5	6	7	8	9

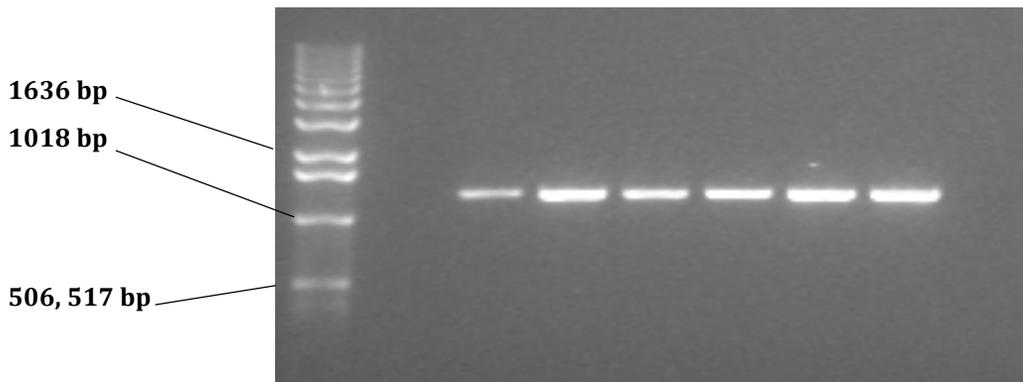


FIG. 6 Identification of positive recombinant bacterial colonies with pCR-BluntII-TOPO::Ys1a. Compared to the 1kb ladder (lane one) and the negative control (lane two), the 1.4 kb bands in lanes three through eight indicated that bacterial colonies pSG-2 through pSG-8 were transformed with pCR-BluntII-TOPO::TDF1-PRO .

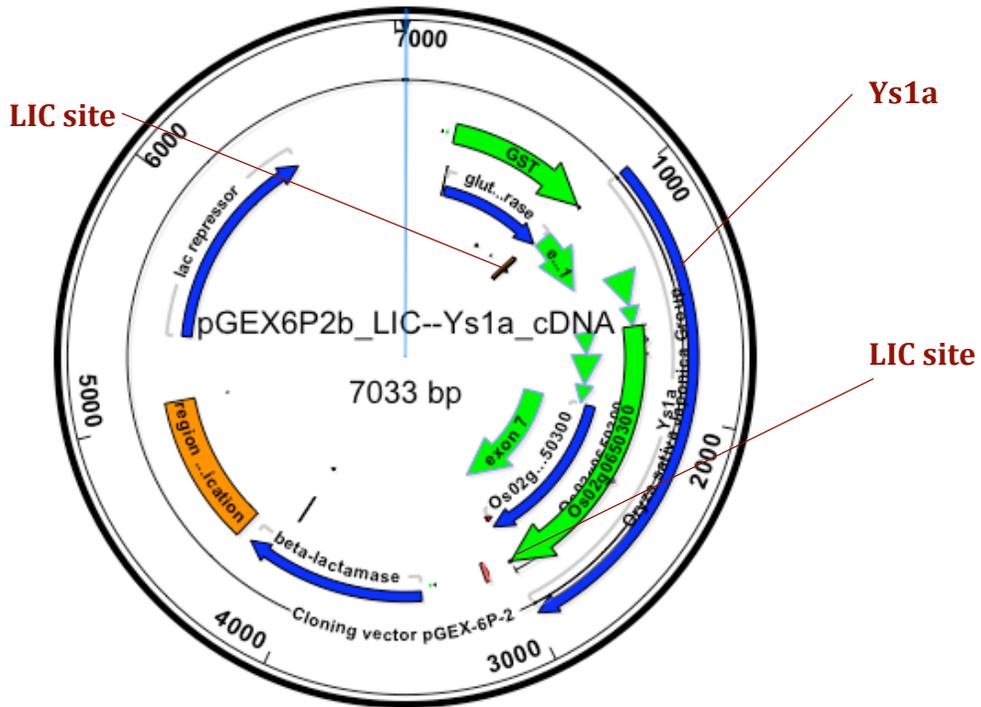


FIG 7 pGEX-6-p2-LIC::Ys1a virtual clone map Virtual clone map of recombinant *E. coli* plasmid that contains the pGEX-6-p2-LIC::Ys1a vector. The Ys1a gene, and the two ligation independent cloning (LIC) sites for this pGEX vector are shown.

Bacterial Colony	-	1	2	3	4	5	6	7	8
1kb ladder	+	-	-	-	-	-	-	-	-
Lane number	1	2	3	4	5	6	7	8	9

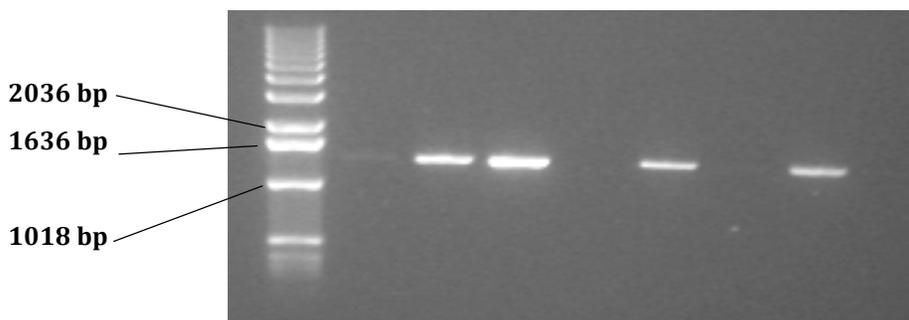


FIG. 8 Identification of positive recombinant bacterial colonies pGEX-6-p2-LIC::Ys1a. Compared to the negative control (lane nine) and the 1kb ladder (lane one), the about 1.5 kb bands in lanes three, four, six, and eight indicated that that bacterial colonies pSG-3, pSG-4, pSG-6, and pSG-8 contain the pGEX-6-p2-LIC::Ys1a vector. The bands in lane three and four were brighter than the bands in lanes six and eight. There was a very faint band about 1.5 kb band in lane two.

tacttccaatccATCAACCCGTTTTGTTTTGGGGAAAATCAAAAGACTTGTCTCCCAAAAAA
 GTCAGTATGGCGGATACATCATATCTATATACGTATTCCATCTAGCAAGTGCGACTG
 TCGGAGTAGGATCGCTGATGTATATTCACGCCTGATGTTTGAAACTTGTGTGAAGCG
 CACTACCAACAGTAAATAAATCTGCCATATCTCTATGGACAAAACCATCAGAGTGGC
 GCAGCGGAAGCGTGGTGGGCCCATAACCCACAGGTCCCAGGATCGAAACCTGGCTC
 TGATAAAATTTGTTGATTTTTTTTTTCTCTCATTTTTTGTGCAACTGATCCTCATTTTCAG
 TTCTGGACAGATGCTTACAACCTTTATTAATTCTCCAACCTTTTAGCATCATTTTCAGTTC
 TGGACAGATGCTTACAATTTGATTAATCCTCCAACCTTTTTATCGTAACGACATGACC
 AGATGTCCAGATCGCATCTTCAGAAAGCTCGTAGCAGCGCTCGAGACCTGATAAGT
 GATCACCCCCCAACACATCTCCGAGCCTAAACTAAAACCGATTCCGCGGCGTCGA
 AGTCGTGATAAATCATCACGTCGAACCCGTGAAAATTCTTAAACACCATCATCAAAT
 CCCATCATTAGATTATAAGCCTTACCAACCTCCATCAGCCAGCTAAATTAACCTCGAC
 GCCCCCTGGTAAAACCTGTTGATTTGCCGGAAGGAAGCAGTTGCAGGCGCTGGTCATT
 ATAAACTTAACCAGCGCCATAAACTAATCCTGCCCCCCTGCATGCGTCGCTGCTG
 ATCTAAAAAAAAGAACCATTTGTGCGTGATTATTGTTACCGGGCTCACGCCTCCGT
 CGCATGGCTTCGCTTCGCCTGCCTCCTGCCATGTTTTTACCATGCCTTCCTCGTCAGT
 GAGATCCTGTCTATATGTGCCGTTGCTTGCTCCGGCCTTTTGCAGTGATCTCGTCAGA
 GTTGGTGTGTTGTTGTTGAGCTCACCGTGGTAGGAGCTGAGAGGGGGAGccccggggga

FIG. 9 Virtual DNA sequence of TDF1 Promoter. The sequenced regions of pCR-BluntII-TOPO::TDF1-PRO using the forward primer and the reverse complement of the reverse primer for the TOPO vector aligned perfectly with the virtual TDF1 promoter.

TACTTCCAATCCatggagcacgccgacgcggaccgcacgcgggtggcgccccgagatcgggagcctgcacgacgaggacg
 cggaggcggaccggcgcgggcgaggagatggagcggctgcagccgtggcgggagcaggtgacggcgcgcggggtggggcg
 cggcgctgacgggttcgtgttcagcgtcatcgtgatgaagatcgcgctcaccacggggctggtgccacgctcaacatcctccgccgct
 gctcgccttctcgcgctccgcggctggacgcgcgctggagcgcctcggttctcgcgcggcccttcacggcgaggagaacacc
 gtcgtccagacctgcgccgtcgcctgctacacatagccttcggcggtgggttcggatcgacattgctgggctgaacaaggagacgtac
 gagctggccgggaactcaccaggcaatgtgccaggagctataaggagcctgggattggctggatggtagggctcctcctgctattagc
 ttcgcggtaacctcagcttgattcccctaagaaaggctttggtcgtagactacaaattaactatccaagtggaactgcaacagctgttctat
 caacgggtccacacagcccaaggagataagaatgcaaaattgcaactccatgggttctaaaatattttgggcttagctattttggagcttctt
 ccaatggttctacacgggaggcaatgcctgtggattgtcagttccctacttttggctgaaagcctggaacaatcattcttctttgatttagc
 ctacatatgttggtgcgggatgattgtcacaccttgaacctctctacctccttggctgcggctattcatggggaataatgtggccactt
 atcagcaaacataagggggactggtaccctgcaaacatacctgaaagcagcatgacaagttgtatggttacaagtcgttttatgcattgctc
 tgattatgggggatggcctgtaccatttftaaagtaccggtgctactgtaagagttacataatagatttaaccgaaaaagttagcaaca
 cagcatcagaggagggtgatgtttcattagatgatctac-aacgtgatgaggtattcaagaggggtactgtccctt-
 ctggatggcatacagtggttacttctgtaagcatcattcggtaatcaccataaccgataatgttcgacaagtgaagtgtactacgtgatc
 atagcctacgcgctcggcccgggtgctaggattcgcgaattcctacggagcagggctcacggacatcaacatggggtacaactacggcaag
 atcgtctcttcgtctttgcagctgggctggcaaggacaatggcgtcatagccggccttgggtggcaccctggtgaagcagctggtgctc
 gtctctgccgatctgatgcagatcgaagacgggacatcaccctgacatcgccgaggtccatgctcgtgggagagctcatcgggacag
 gattggctgctcatcgcgccactgacgttcatgctgttctacagggcgttgacatcggggaacccgatggctactggaaggcgccatac
 gcgctgatctaccgcaacatggcgatcctcgggatcaggggatcctcggcgctgccaagcactgcctctcgtctccgtcgggttctcgc
 gttcgcctgctcacgaacgtggcgaggagcgcctcggcgaggtacaagaagctcgtgccgctgccgacggcgatggccgtgccg
 ttctcgtagggcgagcttcgccatgacatgtgcgtcgggagcctggtgctgtttgcttggacaagatgaacaagaaggagctgcctt
 catggtgcctgctgttgcgtctgggctcatgtgtggcgtatggcatttgaccctccttctctatccttgcctcgtcctaagattaagccaccgat
 ctgcatgaagtttacgcctggaagctaaCAGTAAAGGTGGATA

FIG. 10 Virtual DNA sequence of Ys1a. The sequenced regions of pGEX-6-p2-LIC::Ys1a using the forward primer and the reverse complement of the reverse primer for this pGEX vector aligned perfectly with the virtual Ys1a promoter.

MEHADADRTRVAPEIGSLHDEDAEADPARAREMERLQPWREQVTARGVVAAALIGFVF
SVIVMKIALTTGLVPTLNISAALLAFLALRGWTRALERLGFSPRPFTRQENTVVQTCAVA
CYTIAFGGGFGSTLLGLNKRTYELAGNSPGNVPGSYKEPGIGWMVGLLLAISFAGNLSLI
PLRKALVVDYKLTYPSTGATAVFLINGFHTAQGDKNAKLQLHGFLKYFGLSLFWSFFQW
FYTGGNACGFVQFPTFGLKAWKQSFFDFSLTYVGAGMICSHLVNLSTLLGAVISWGIM
WPLISKHKGDWYPANIPESMSTLYGYKSFLCIALIMGDGLYHFVKVTGVTAKSLHNR
NRKSVSNTASEEGDMVSLDDLQRDEVFKRGTVPSWMAYSYFLLSIIAVITIPIMFRQVK
WYYVIIAYALGPVLGFANSYGAGLTDINMGYNYGKIALFVFAAWAGKDNGVIAGLVV
GTLVKQLVLVSADLMHDLKTGHLTLTSPRMLVGEIGTGIGCFIAPLTFMLFYRAFDIG
NPDGYWKAPYALIYRNMAILGIEGIALPKHCLSLSVGFFAFAVLTNVARDALPARYKK
LVPLPTAMAVPFLVGASFAIDMCVGSLLVFAWNKMNKKEAAFMPAVASGLMCGDGI
WTFPSSILALAKIKPPICMKFTPGS

FIG. 11 Predicted Protein Sequence of Ys1a. The virtual DNA sequence of Ys1a was used to generate the predicted protein sequence. The theoretical Ys1a protein is 672 amino acids and weighs 72149.94 Daltons. 9.114 is the isoelectric point, and the charge is 16.440 at a pH of 7.0. There are 56 strongly basic amino acids (K,R), 41 strongly acidic amino acids (D,E), 295 hydrophobic amino acids (A,I,F,W,V), and 150 polar amino acids (N,C,Q,S,T,Y).

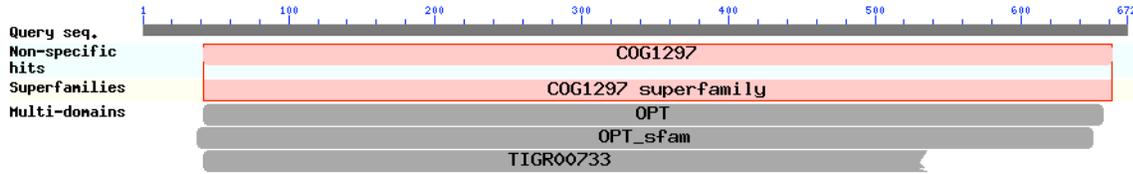


Fig. 12

Conserved domains revealed by BLAST analysis. A comparison of the predicted protein sequence to other proteins in GenBank shows a conserved domain of COG1297, which is a predicted membrane protein with an unknown function.

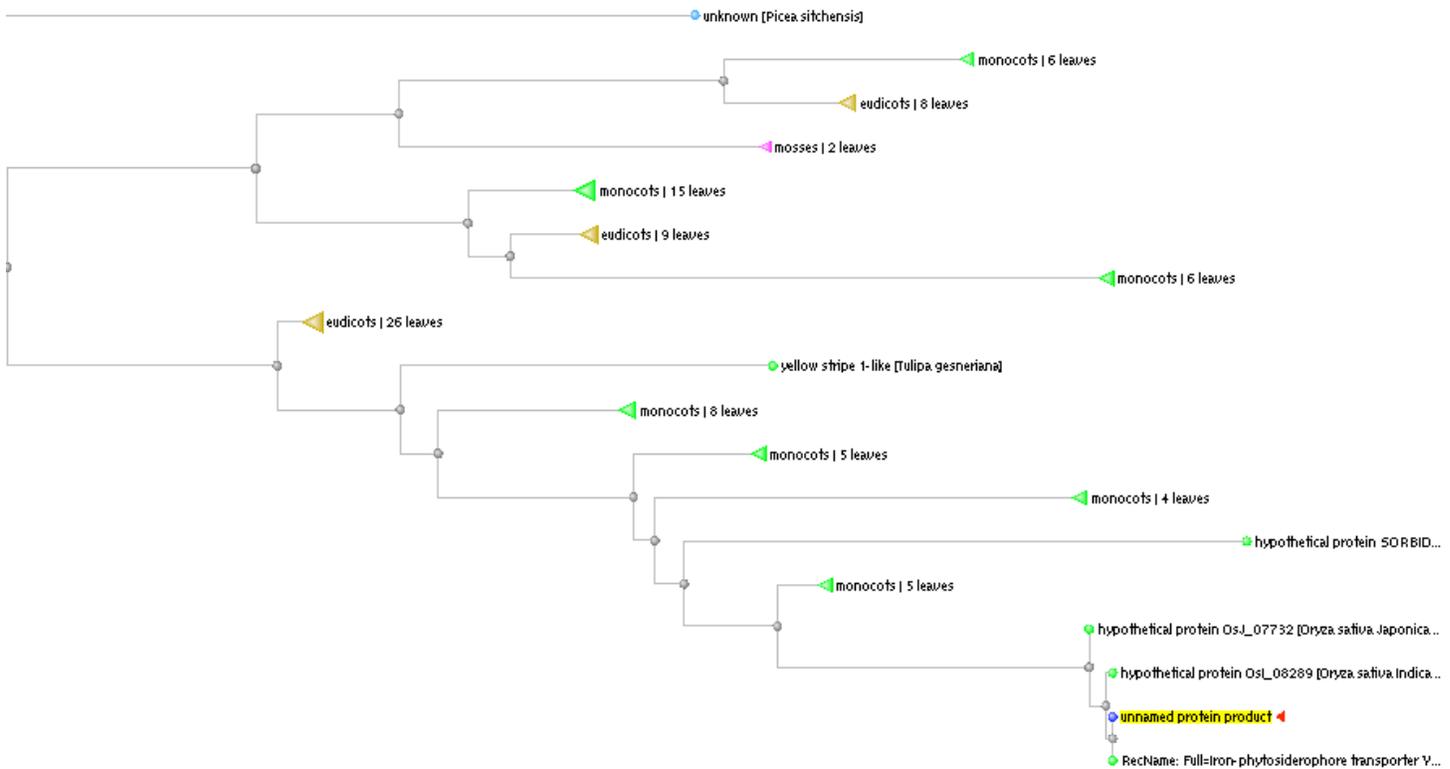


Fig. 13 Distance Tree of Ys1a.

The evolutionary relationship between the predicted Ys1a protein sequence all other proteins in the GenBank, according to BLAST analysis, revealed a high degree of similarity to the Yellow Stripe 1 (Ys1) transporter in maize and barley, and to a vacuolar iron transporter (TgVit1) in *Tulipa gesneriana*.

Discussion

Our first goal was to create a promoter-terminator cassette using the promoter and terminator of the TDF1 gene in *Oryza sativa*. We plan to use the TDF1 promoter to drive expression of a gene involved in iron uptake or storage, and we hypothesize that this could increase iron content in the edible portion of Japanese rice by producing the protein of interest around the seeds of the plants, where TDF1 is usually produced. Our second goal was to create an expression vector using Ys1a, a gene involved in the iron uptake and phloem transport of iron. We successfully cloned the TDF1 promoter into a TOPO vector and the Ys1a gene into a pGEX vector. The sequenced DNA from these vectors matched the virtual TDF1 promoter and Ys1a gene perfectly (Fig. 9,10). BLAST analysis of the amino acid sequence generated from the sequenced regions of the pGEX vector revealed that the theoretical protein generated by this construct would have the conserved domain of COG1297, a predicted membrane protein with an unknown function (Fig. 12), and would match the protein produced by the Ys1a gene (Fig. 11).

The BLAST analysis of amino acid sequence of Ys1a also revealed a high degree of similarity to a vacuolar iron transporter (TgVit1) in *Tulipa gesneriana* and to Yellow Stripe 1 (Ys1) transporters in maize and barley (Fig. 13). TgVit1 contributes to the blue coloration and is expressed exclusively in blue-colored epidermal cells.⁷ In some gramineous plants, roots secrete phytosiderophores in response to iron deficiency and take up iron through Ys1 transporters.⁸ This transporter in maize (ZmYS1) is expressed in leaf blades, sheaths, and seminal roots, while the transporter in barley (HvYS1) is only present in roots.⁸ Differences in the gene expression pattern also suggest that ZmYS1 is involved in both iron acquisition and intracellular transport metals in maize, but HvYS1 is only involved in iron acquisition by barley roots.⁸ Ys1a may only be involved in iron acquisition like HvYS1, but it may be involved in intracellular iron storage and transport like TgVit1 and ZmYS1, and therefore capable of increasing the iron content in edible portions of *Oryza sativa*.

We have shown that the vectors containing the Ys1a gene and the promoter of the TDF1 gene can be used to transform bacteria. Further studies may include using affinity chromatography analysis to determine if Ys1a can be expressed in rosetta cells, creating a plant transformation vector of a gene involved in the iron uptake or storage of iron flanked by the promoter and terminator of TDF1, using that plant transformation vector to transform Japanese rice plants, and testing the seeds (the edible portion) of the genetically engineered plants to

measure the effect of increased Ys1a expression on the plant's iron content.

Literature cited

1. Mayer J.E., Pfeiffer W.H., Beyer P. *Biofortified crops to alleviate micronutrient malnutrition*. **2008**. *Curr Opin Plant Biol.* 11(2):166-170.
2. Gilani G.S., Nasim A. *Impact of foods nutritionally enhanced through biotechnology in alleviating malnutrition in developing countries*. **2007**. *J AOAC Int.* 90(5):1440-4.
3. Sautter C., Poletti S., Zhang P., Gruissem, W. *Biofortification of essential nutritional compounds and trace elements in rice and cassava*. **2006**. *Proc Nutr Soc.* 65(2):153-9.
4. Zhu J., Chen H., Li H., Gao J.F., Jiang H., Wang C., Guan Y.F., Yang Z.N. *Defective in Tapetal development and function 1 is essential for anther development and tapetal function for microspore maturation in Arabidopsis*. **2008**. *Plant J.* 55(2):266-77.
5. Inoue H., Kobayashi T., Nozoye T., Takahashi M., Kakei Y., Suzuki K., Nakazono M., Nakanishi H., Mori S., Nishizawa N.K. *Rice OsYSL15 is an iron-regulated iron (III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings*. **2009**. *J. Biol. Chem.* 284(6):3470-3479.
6. Jean M.L., Schikora A., Mari S., Briat J.F., Curie C. *A loss of function mutation in AtYSL1 reveals its role in iron and nicotianamine seed loading*. **2005**. *Plant J.* 44(5):769-82.
7. Momonoi K., Yoshida K., Mano S., Takahashi H., Nakamori C., Shoji K., Nitta A., Nishimura M. *A vacuolar iron transporter in tulip, TgVit1, is responsible for blue coloration in petal cells through iron accumulation*. **2009**. *Plant J.* 59(3):437-47.
8. Ueno D., Yamaji N., Ma J.F. *Further characterization of ferric-phytosiderophore transporters ZmYS1 and HvYS1 in maize and barley*. **2009**. *J Exp Bot.* 60(12):3513-20.