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Professor: Maria Moreno

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

Cloning of the *Oryza sativa* ferric chelate reductase promoter-terminator fusion into a pYU2735 plasmid: generation of a universal construct toward rice biofortification

by Micah Johnson, Michael Turner, Elena Poiata, Stephanie Vadasz, Nathan Yardley, and Maria Moreno

Abstract

Iron deficiency is the most common nutritional disorder in the world. Our goal is to alleviate this problem by enriching the grain of the *Oryza sativa* staple crop (rice) with iron. Ferric chelate reductase is a gene involved in the uptake and transport of iron and is expressed ubiquitously in the plant. We successfully generated an *O. sativa* ferric chelate reductase promoter-terminator fusion and cloned this insert into a pYU2735 plasmid. This universal plasmid can be used to carry out translational fusions to insert genes of interest between the *O. sativa* ferric chelate reductase promoter and terminator regions. We propose that this strategy will allow a cell-type-specific determination of the gene expression pattern, and hypothesize that when used along with similar constructs generated from other genes, this construct will ultimately help achieve enrichment of rice grain with bioavailable iron.

Introduction

Iron deficiency is the most common nutritional disorder in the world [1]. More than one billion people suffer from iron deficient anemia, which causes permanent cognitive impairment and results in an estimated 800,000 deaths annually [2]. But for various reasons, conventional alleviation strategies such as pharmaceutical supplementation have had limited success in reducing the prevalence of iron deficient anemia in developing countries [3]. It has thus been suggested that biofortified crops may provide a more sustainable and cost-effective solution [2]. Because rice is a

primary food source for more than half of the world's population, biofortification of rice holds promise for alleviating iron deficiencies—and in countries where the staple food is rice, the per capita consumption is so high (ranging from 62 to 190 kg/year) that even a small increase in its nutritive value is highly significant [4, 5].

Biofortification efforts depend not only on the total amount of iron in the plant, but on the *location* of the iron within the plant—iron must be in the edible portion to have human benefit. For instance, iron content is quite high in the rice leaf (100-200 ppm) but very low in the rice grain (3 ppm) [2]. So while Ishimaru et al. were able to double iron uptake and thus cause an 8-fold increase in grain yield, they did not increase the iron content of the rice grain [2]. It has thus been proposed that the challenge is to create an enhanced "sink" for iron in the seed [6]. Previous studies have successfully overexpressed ferritin in the seed by introducing the soybean ferritin gene into rice, resulting in a three-fold increase of iron content in seeds. However, a further rise in the iron concentration cannot be achieved by enhancing ferritin expression [4]. Instead, it has been suggested that the limiting factor for iron accumulation may be iron uptake and/or transportation—indeed, rice plants transport only 4% of iron in the shoot to the seeds [7, 8].

The present study thus focuses on the promoter region of the gene for ferric chelate reductase, which reduces Fe^{3+} to the more soluble Fe^{2+} for transport—in fact, much evidence suggests that iron cannot be transported without being reduced [9]. Previous research has focused on the role of the gene in iron uptake (through reduction and transport at the root-soil interface), but several studies indicate that the gene is also expressed in the stem, leaf, flower, and panicle, suggesting it may also play an important role in the transport of iron throughout the plant [9, 10, 11]. But this work has only grossly characterized the locations of gene expression. We aim to characterize ferric chelate reductase gene expression at the cell-type-specific level, allowing a more detailed understanding of iron transport in the plant and thus facilitating efforts to genetically

modify rice so as to ultimately enrich iron accumulation in the grain. As an important step toward that goal, we carried out a genetic transformation by generating an *O. sativa* ferric chelate reductase promoter-terminator fusion and cloning this insert into a pYU2735 plasmid. This paper describes the successful generation of this recombinant vector and discusses its potential as a universal plasmid to be used in the biofortification of rice.

Materials and Methods

Amplification of DNA Sequences

Target DNA sequences were amplified by polymerase chain reaction (PCR) in a final reaction volume of 25 µl consisting of Phusion High-Fidelity PCR Master Mix (New England BioLabs or NEB), 0.52 µM forward and reverse primers, and template DNA (0.2 ng/µl plasmid DNA or 2ng/µl genomic DNA), with or without 2% DMSO.

The Ligation Independent Cloning (LIC) Cassette was amplified from pNIC28-Bsa4 plasmid DNA (Opher Gileadi) using forward primer (5'-GTACTTCCAATCCATGGAGACC-3') and reverse primer (5'-GTATCCACCTTTACTGGAGACCG-3'). Thermal cycling conditions were as follows: initial denaturation at 98°C for 30 seconds; 35 cycles of denaturation at 98°C for 10 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute; then a final extension at 72°C for 5 minutes followed by a hold at 4°C.

The *O. sativa* ferric chelate reductase terminator region was amplified from genomic DNA using mutagenic forward primer (5'-tcgccccgggTCTGTAGACCAGAGCTAGTACGACAC-3') and mutagenic reverse primer (5'-tatccacctttactgTAATAGTTAGTTGGAAAATCCTC-3'). Lowercase letters denote extensions: 3'-promoter sequence extension in forward primer, LIC extension in reverse primer. Blue letters denote *Sma*I digestion site. Thermal cycling conditions were as follows: initial denaturation at 98°C for 2 minutes; 35 cycles of denaturation at 98°C for 10

seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 1 minute 30 seconds; then a final extension at 72°C for 5 minutes followed by a hold at 4°C.

The *O. sativa* ferric chelate reductase promoter-terminator fusion was amplified from a purified mixture of *O. sativa* ferric chelate reductase promoter and terminator DNA using mutagenic forward primer (5'-tacttccaatccatgGCCACGGGAAGTCTATC-3') and mutagenic reverse primer (5'-tatccacctttactgTAATAGTTAGTTGGAAAATCCTC-3'). Lowercase letters denote LIC extensions. Thermal cycling conditions were identical to the conditions described above, except immediately following initial denaturation there were an additional 10 cycles of denaturation at 98°C for 10 seconds and annealing/extension at 72°C for 2 minutes.

PCR reactions were resolved on 0.8% agarose gels stained with SYBR Safe dye (Invitrogen). Samples were prepared with RapidRun Loading Dye (Affymetrix) and loaded alongside 1 Kb DNA Ladder (Invitrogen). The gels were run at 110-130V for 20-30 minutes.

LIC Cassette and terminator PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Corporation) according to manufacturer's specifications, except sodium acetate (pH 5.0) was added to each mixture to a final concentration of 0.10 M prior to centrifugation. The promoter-terminator fusion PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN Corporation) according to manufacturer's specifications, with the exception described above.

Ligation Independent Cloning (LIC)

The pYU2735 vector (50 ng/µl) was digested with 0.3 U/µl *Bsa*I restriction enzyme (NEB) in a final reaction volume of 100 µl consisting of NEB4 Buffer (NEB) and Bovine Serum Albumin (BSA, NEB), and was incubated for 2 hours at 50°C and 15 minutes at 65°C. The digested plasmid vector DNA was purified using the QIAquick PCR Purification Kit (QIAGEN Corporation) according to manufacturer's specifications and treated with T4 DNA polymerase in a final reaction

volume of 100µl consisting of T4 Buffer (NEB 2), 2.5 mM dGTP (Invitrogen), 0.1 µg/µl BSA (NEB), 5 mM DTT (Molecular Probes, Inc.), and 0.15 U/µl T4 DNA Polymerase (NEB) by incubating for 30 minutes at 22°C and 20 minutes at 70°C.

The *O. sativa* ferric chelate reductase promoter-terminator PCR product was treated with T4 DNA polymerase in a final reaction volume of 10 µl consisting of T4 Buffer (NEB 2), 2.5 mM dCTP (Invitrogen), 0.5 µg/µl BSA (NEB), 50 mM DTT (Molecular Probes, Inc.), and 0.15 U/µl T4 DNA Polymerase (NEB) by incubating at the same conditions described above.

The *Bsa*I-T4 treated pYU2735 vector and T4 treated promoter-terminator PCR insert were combined in a 1:2 ratio and incubated for 10 minutes at 22°C to generate the annealed pYU2735::Os_FeCheRed-PRO-*Sma*I-TER complex.

Bacterial Transformation and Screening

The pYU2735::Os_FeCheRed-PRO-*Sma*I-TER annealed complex was transformed into One Shot® TOP10 chemically competent cells (Invitrogen) according to manufacturer's specifications. Cells were plated on LB agar with 100 µg/ml ampicillin and 5% sucrose.

Bacterial colony PCR was used to screen for colonies containing the desired insert using Phusion High-Fidelity PCR Master Mix (NEB), 0.52µM forward primer (5'-ACGTTGTAAAACG ACGGCCAG-3'), and 0.52µM reverse primer (5'-GCTATGACCATGATTACGCCAAG-3') in a final reaction volume of 25µl. Thermal cycling conditions were as follows: one cycle at 95°C for 8 minutes and 98°C for 2 minutes; 35 cycles of denaturation at 98°C for 20 seconds, annealing at 65°C for 15 seconds, and extension at 72°C for 2 minutes; then a final extension at 72°C for 5 minutes followed by a hold at 4°C.

Isolation of Plasmid DNA

Plasmid DNA was isolated from identified recombinant clones by using the QIAfilter Plasmid Midi Kit (QIAGEN) according to manufacturer's specifications.

DNA Sequencing and Analysis

DNA sequencing was performed at the W. M. Keck Foundation Biotechnology Resource Center, Yale University. *In silico* DNA sequence analysis was performed with DNASTAR Lasergene 8 software and BLAST (http://www.ncbi.nlm.nih.gov/guide).

Results

Analysis of ferric chelate reductase gene and protein structure

In order to create a construct toward use in the biofortification of rice, we designed a DNA construct comprised of the *O. sativa* ferric chelate reductase promoter and terminator regions. Coding sequences for genes of interest can be cloned into this region; the *O. sativa* ferric chelate reductase CDS is shown here (Fig. 1). BLAST analysis revealed that the predicted protein contains two conserved domains: a ferric reductase-like transmembrane component and a FAD/NADP-like binding domain (Fig. 1C) [12, 13, 14].

Figure 1. Ferric Chelate Reductase: Gene and Protein Structure
Panel A shows the 2277 bp *O. sativa* ferric chelate reductase gene coding sequence between the *O. sativa* ferric chelate reductase promoter and terminator regions. Panel B shows the CDS splicing pattern, with exons labeled 1 through 9. Panel C shows conserved protein domains in the predicted

protein (758 amino acids), with transmembrane domain at residues 191-331 and FAD/NADP-like binding domain at residues 343-537.

Generation of ferric chelate reductase promoter-terminator fusion

In order to generate the promoter-terminator construct, the *O. sativa* ferric chelate reductase promoter region was amplified by PCR from genomic DNA resulting in a fragment of 1109 bp (Fig. 2A). The *O. sativa* ferric chelate reductase terminator region was also amplified by PCR from genomic DNA resulting in a fragment of 387 bp (Fig. 2B). These two pieces were used to generate a promoter-terminator construct by fusion PCR resulting in an amplified fragment of 1480 bp (Fig. 2C). After recombination with the pYU2735 plasmid, the promoter-terminator fusion and its flanking sequences in the plasmid were amplified resulting in a fragment of 1712 bp (Fig. 2D).

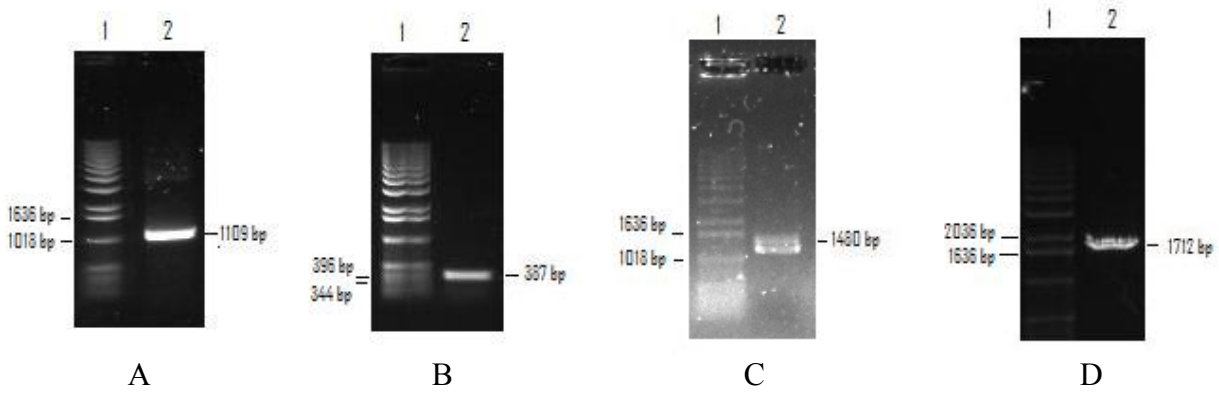

Figure 2. Generating the Promoter-Terminator Fusion
Lane 1 in each panel shows 1kb ladder. Panel A shows purified promoter fragment, which is 1109 bp. Panel B shows purified terminator fragment, which is 387 bp. Panel C shows purified promoter-terminator fusion, which is 1480 bp. Panel D shows purified promoter-terminator fusion amplified from transformed plasmid, which is 1712 bp.

Generation of recombinant pYU2735 plasmid and bacterial transformation

To replicate and store the plasmid of interest, ligation independent cloning was performed to transform the bacteria with the annealed pYU2735::Os_FeCheRed-PRO-*Sma*I-TER complex. Bacterial colonies were present after growth on media containing ampicillin and sucrose, having

been transformed with the plasmid containing the promoter region, the *SmaI* restriction site, the terminator region, the origin of replication, and the ampicillin resistance gene (Fig. 3).

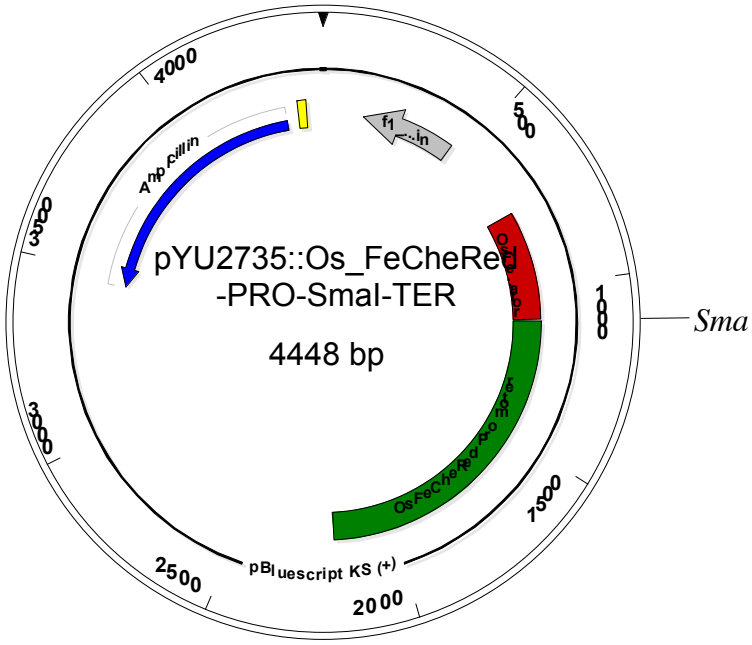

Figure 3. Vector Map of Recombinant pYU2735 Plasmid
Map of recombinant pYU2735 plasmid containing origin of replication (gray), ampicillin resistance gene (promoter in yellow, coding sequence in blue), *O. sativa* ferric chelate reductase promoter (green), *Sma*I restriction site (in promoter-terminator junction), and *O. sativa* ferric chelate reductase terminator (red).

DNA sequencing and alignment

To confirm that the pYU2735::Os_FeCheRed-PRO-*Sma*I-TER construct was successfully generated, the recombinant plasmid was sequenced and found to align 100% with the Os04g0444800 reference sequence obtained from GenBank (Fig. 4).

ttccaatccatgGCCACGGGAAGTCTATCGATGTTTCATTTTTTTCAACTCACACGTCATTTTTC
TTTTACCTCATATATAGCTCCAGTTTATCTTCATATCCAACCCTGCCACGTGGTCCAATC
CAAATCCGGCAAAACGGCCGGCTTGAGATTAGTACTTCGCTAATGATTATCATCCCCTC
CTACCTCCCGAACACTCCTGTGGGCCACTGGGCCCCACGCGGGAGCTGTGTGCCGGGC
CCACCGCCCTCGTCTCCCTCTCATCCGCGGGTGGTAACCCCTCTCCACACTCCTGTCGCT
GACGCTGCTGCTCCCGTCGGAGGAGACGTCGGAGTCGCGGTTCCTTCTTTCCCCGTCGA
TCGCCGAGGACCACGAGCACGCCCACGAACTGGCAAATCCTTCGCTACGAGCGGATAA
GAGGCCCCGCCACCGCGCGGCGCTGTCCCACCCCGCCAGCCACCGGAAATTCCCTTGG
CTGGGGAAAATCTCCGCGCAACAACTACGGTTAATTTTGGAGAATATCCTTGGCGTTTA
CGCCTTCGCAGGAGATGAAAACCGCAGCCATTCGTCTGAATTTCGATTTGACAGTTCGT
GCGGTGCGGCTCCCAATAATTTCTCTCTTTTTTCCCCCTTAATCACATACCCTCCCCCTC
CCGTTCCCCAACGTGTGGTACTGTAATTTCCCCCCTGATAATTCAGGCGATTTCCGTGG
CAGATTTGTCCGGTTTTTTCACGAACTAAATCATCTGTAAAGATTTGTGAAAAAAAATA
TATTACCCGCTGAAAAATCAATCGAGGCATACGGAGATTTGGCTGGGGCCTTCACGAA
TCAGGAGCACACGTTGGATGTGTGGCTCTCGCCAAGCTCGCTCGGCCACTGTCCCCAAC
TGCAACCACCTGGCGATGACGCCTCCCGTACACTCTCCTCACCGATGATCCATCGCCGT
CGCCATTGCCAGCGACCGGCGCCCGCGAGGACCTCGCGTCTTGGCCGCATAAAGCGGC
GGCAGCAGCGCCCCCTGGCCCTGGCTGGTCGAGGGGAGGGCGCGAGTTTCCGTGGATT
CCGCGGCTCTACCGTTCACAGCCGCTCGCCcccgggTCTGTAGACCAGAGCTAGTACGAC
ACTCTGCGCAGGTGGCGAACATATTAATTTTTAATTGATAATAGTAGTTAATATGTACT
GTATAAAATAACTAAAGATTAAAATTATAGCCGAGGCCCTGCACCAATAGTAAATCCA
TCCATCCATCCTGTTTCCTGTACAGTACACTACACATACGACAAGCTCGCCGTCGTCGG
CGCGTGCATGTATATATTCTGCATGATCGGAAACGTGAGCATACGTGAAGGCATTACG
AATTCACATACGTCATATGAAGTTTCATGTCGGATGCGTGGTTGCATTCCCTTTTTCCTT
TTGTTTTTCTTTTCTTCTTTGAGGATTTTCCAACTAACTATTAcagtaaaggtgg

Figure 4. DNA Sequence Alignment for pYU2735::Os_FeCheRed-PRO-*Sma*I-TER
The sequenced region of the pYU2735::Os_FeCheRed-PRO-*Sma*I-TER construct aligned 100% with the reference sequence obtained from GenBank. *O. sativa* ferric chelate reductase promoter and terminator regions are shown here; lowercase letters denote flanking plasmid sequences, blue letters denote *Sma*I digestion site.

Discussion

Our goal was to create a universal plasmid that can be used to carry out translational fusions to insert genes of interest between the promoter and terminator regions of the *O. sativa* ferric chelate reductase gene. We accomplished this by creating a promoter-terminator construct by fusion PCR and then inserting it into a pYU2735 plasmid by LIC cloning (Fig. 3) [15]. The sizes of the bands visualized by AGE suggest that the promoter and terminator regions were successfully

amplified by PCR (Fig. 2A, 2B), that fusion PCR successfully generated the promoter-terminator construct (Fig. 2C), and that the fusion construct was successfully recovered in bacteria (Fig. 2D). DNA sequencing verified that the construct was generated successfully, as the sequence aligned 100% with the reference sequence for the pYU2735::Os_FeCheRed-PRO-*Sma*I-TER construct (Fig. 4). We plan to insert the LIC cassette into the promoter-terminator fusion, which will then allow us to clone genes of interest into the vector between the *O. sativa* ferric chelate reductase promoter and terminator regions.

A promising future direction is the translational fusion of a reporter gene (such as green fluorescent protein) into the *O. sativa* ferric chelate reductase promoter-terminator region. Introducing this construct into rice and employing *in vivo* localization should allow us to determine—at the cell-type-specific level—when and where in the plant the gene is expressed. This enables the general determination of where genes inserted into our universal plasmid are expressed, facilitating efforts to find the combinations of promoters and genes that will optimize the uptake, transport, and accumulation of iron. Using this same strategy with promoters of other iron genes will allow a comprehensive analysis of iron in the plant, making possible the identification of any "weak links" that limit the ultimate accumulation of iron in the edible portion of the plant. By pairing an appropriate iron uptake, transport, or accumulation gene with a promoter that has an expression pattern that includes the region of the plant identified as limiting, we hypothesize that iron content can be increased in the edible portion of rice. We also suggest that a similar approach might be successful in the biofortification of other plants that are staple crops in the developing world, such as members of the bean family.

Furthermore, results from our BLAST analysis suggest protein function and thus allow us to hypothesize a possible role for *O. sativa* ferric chelate reductase in this biofortification effort. The conserved FAD/NADP-like binding domain suggests that the mechanism of action for the reduction

of Fe^{3+} to Fe^{2+} may involve the binding of FAD or NADP to the conserved pocket and the subsequent transfer of an electron to iron. The conserved transmembrane domain also supports the finding that ferric chelate reductase plays a role in transporting reduced iron ions across membranes in the cell. Additionally, the high similarity of *O. sativa* ferric chelate reductase to ferric chelate reductase genes in other species suggests that the gene's function in rice is very similar to that of homologous genes in other species. Of particular interest is the high similarity to the Fe(III)-chelate reductase in the fruit *Citrus junos.* Researchers' evidence that this protein may play a crucial role in long-distance transport between organs and tissues suggests that our protein likely plays a similar role in iron transport throughout the rice plant [16]. This leads us to hypothesize that *O. sativa* ferric chelate reductase could be a promising candidate gene to pair with a promoter that drives expression in a region of the plant with limited iron transport. Finally, the fact that *O. sativa* ferric chelate reductase has been located ubiquitously throughout the plant also allows us to make the preliminary hypothesis that its promoter could be useful to pair with either an uptake gene (because it is found in the root) or an accumulation gene (because it is found in a variety of locations within the plant) [9, 10, 11].

In this study, we successfully inserted a promoter-terminator fusion of the *O. sativa* ferric chelate reductase gene into a pYU2735 plasmid. Cloning a reporter gene into this plasmid will enable an unprecedented investigation of gene expression patterns at the cell-type-specific level. We hypothesize that the comprehensive use of this strategy with iron uptake, transport, and accumulation genes will allow us to increase the level of bioavailable iron in the edible portion of the rice crop. This will be a crucial step in alleviating iron deficiencies that affect over one billion people worldwide [2].

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