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# Iron Bioavailability of Maize Hemoglobin in a Caco-2 Cell Culture Model

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**ABSTRACT:** Maize (*Zea mays*) is an important staple crop in many parts of the world but has low iron bioavailability, in part due to its high phytate content. Hemoglobin is a form of iron that is highly bioavailable, and its bioavailability is not inhibited by phytate. It was hypothesized that maize hemoglobin is a highly bioavailable iron source and that biofortification of maize with iron can be accomplished by overexpression of maize globin in the endosperm. Maize was transformed with a gene construct encoding a translational fusion of maize globin and green fluorescent protein under transcriptional control of the maize 27 kDa γ-zein promoter. Iron bioavailability of maize hemoglobin produced in *Escherichia coli* and of stably transformed seeds expressing the maize globin−GFP fusion was determined using an in vitro Caco-2 cell culture model. Maize flour fortified with maize hemoglobin was found to have iron bioavailability that is not significantly different from that of flour fortified with ferrous sulfate or bovine hemoglobin but is significantly higher than unfortified flour. Transformed maize grain expressing maize globin was found to have iron bioavailability similar to that of untransformed seeds. These results suggest that maize globin produced in *E. coli* may be an effective iron fortificant, but overexpressing maize globin in maize endosperm may require a different strategy to increase bioavailable iron content in maize.

**KEYWORDS:** *biofortification, hemoglobin, iron bioavailability, transgenic maize*

## INTRODUCTION

Iron deficiency is the most prevalent nutrient deficiency, affecting an estimated 2 billion people worldwide,[1] and even mild deficiency has a wide range of negative health effects.[2] Despite a variety of efforts and international mandates to reduce iron deficiency, minimal progress has been made, particularly in some of the most isolated and resource-poor regions of the world where processing-based fortification strategies have not been effective. Iron deficiency is a particular challenge in areas where maize is a staple crop because its high phytate content causes low iron bioavailability.[3] Novel strategies are necessary to reduce iron deficiency in populations consuming maize, and biofortification may provide this novel approach.

Biofortification is the development of staple crop genotypes that have improved nutritional qualities and is considered a cost efficient and self-perpetuating means of providing nutrients in staple crops.[4,5] There are significant challenges to overcome prior to the development of iron-biofortified maize. Some variation of total iron in maize grain has been found through evaluation of diverse maize genotypes,[6,7] but improvement in total iron content may not increase bioavailable iron unless phytate is significantly reduced.[8] Low-phytate maize mutants[9] can improve iron bioavailability,[10,11] but have low yields and other negative effects.[12,13] Although natural variability in maize iron bioavailability[14] may provide a path to breeding improved varieties, transgenic biofortification could provide another route. Grains have been engineered for improved iron in a variety of ways.[7] Expression of soybean ferritin in rice resulted in bioavailability similar to that of ferrous sulfate fortified rice when evaluated in a rat hemoglobin repletion model,[15] and human lactoferrin produced in rice had bioavailability similar to that of ferrous sulfate in young women.[16] Reduction of phytate has been achieved in maize through expression of *Aspergillus niger* phytase, without the undesired agronomic effects seen in low-phytate mutants.[17] Improved iron bioavailability was also achieved with coexpression of fungal phytase and soybean ferritin[18] and by expression of soybean ferritin in low-phytate maize.[19]

Transgenic biofortification faces its own challenges. Biotechnology that makes use of genes from different species may not be well accepted. A cisgenic approach, in which genes and genetic elements from the species of interest are used to produce a desired phenotype,[20] may be more acceptable,[21] particularly if there is a nutritional benefit.[22] Maize contains multiple genes that have the potential to increase total iron or iron bioavailability when overexpressed. One such gene encodes maize globin. Absorption of heme iron from animal sources is not affected by dietary factors such as phytate.[23] Symbiotic soybean leghemoglobin was found to be as bioavailable as bovine hemoglobin,[24] indicating there is potential for other plant hemoglobins to be used as iron biofortificants. Additionally, iron in soybean leghemoglobin did not decrease in bioavailability after cooking, whereas the iron bioavailability of ferrous sulfate was significantly reduced by

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cooking,[24] further indicating that plant hemoglobins may be useful as iron fortificants.

The function of symbiotic plant globins is to produce an anoxic environment for nitrogen-fixing bacteria by reversibly binding oxygen. Whereas symbiotic plant globins are found only in legumes, all plants examined to date have genes coding for nonsymbiotic globin proteins,[25] and these globin genes in maize have been characterized.[26] Nonsymbiotic globins seem to play a role in regulating hormone signal transduction.[27] They are able to reversibly bind oxygen and they seem to be involved with stress response.[28] In maize seedlings, maize globin mRNA levels increased in root tips under stress due to high salt or low oxygen, and expression of maize globin in tobacco allowed greater tolerance to submergence or salt.[29] When barley globin was overexpressed in maize tissue culture cells under low oxygen, nitrous oxide levels were reduced.[30] To our knowledge, the iron bioavailability of nonsymbiotic plant globins has not yet been determined.

Overexpression of maize hemoglobin in maize endosperm is a potential avenue for the development of maize with highly bioavailable iron that could circumvent the inhibitory effect of phytate. Endosperm is the starchy part of the maize kernel, containing carbohydrates and proteins that are used during germination. The endosperm is a desirable target for overexpression of proteins for human nutrition as it is the part of the kernel that is most often consumed. The objective of this study is to evaluate the iron bioavailability of nonsymbiotic maize globin expressed in maize endosperm and of maize hemoglobin expressed in *Escherichia coli*.

## METHODS

**Expression Vector Construction.** A construct containing the *Zea mays* endosperm specific 27 kDa γ-zein promoter, a translational fusion of *Z. mays* globin 1 (ZmG) and green fluorescent protein (GFP) coding sequences, and the nos terminator, as shown in Figure 1, was used to transform maize. To create this construct, the

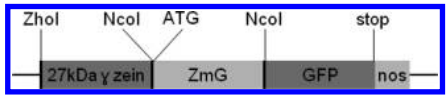


**Figure 1.** Gene construct used to create maize overexpressing maize globin. The construct contains the endosperm specific 27 kDa γ-zein promoter, a fusion of maize globin (ZmG) and green fluorescent protein (GFP) coding sequences, and the nos terminator. The position of the start codon ATG and the translational stop codon are located as indicated here. This construct is designed to result in a translational fusion product with GFP fused to the C-terminus of ZmG.

pAct1IsGFP-1 plasmid,[31] containing the modified GFP gene sGFPs65T[32] (Genbank accession ABB59985) and nos terminator sequence (modified from Genbank accession V00087), was modified by the addition of the 27 kDa γ-zein promoter (Genbank accession EF061093). This promoter was previously cloned from maize inbred Va26 and chosen because it was shown to produce high expression of GFP in maize endosperm tissue.[33] GFP was included as a visual marker. The nos terminator was chosen because it has been shown to be an effective transcriptional terminator in many biotechnology applications. The predicted maize globin protein was not found to contain signal peptides with SignalP,[34] and no signal peptides or targeting sequences were added.

The cDNA for maize globin 1 was obtained from the Iowa State University Expressed Sequence Tag Library (Genbank accession BM333948). A PCR product containing the coding sequence for the mature ZmG protein was amplified using Pfu polymerase (Stratagene, La Jolla, CA, USA) from the ZmG cDNA. The primers were designed such that the PCR product included an *Nco*I site in conjunction with the start codon and completes an additional *Nco*I site at the end of the PCR product, just before the natural stop codon of ZmG, such that there are no changes in the amino acid sequence of ZmG itself but two additional amino acids are added at the C-terminal end of ZmG, proline and tryptophan (forward primer, CGCCCTT**CCATGG**CACTCGCGGAGGCC; reverse, **CCATGG**CATCGGGCTTCATCTCCC; bold nucleotides are the *Nco*I sites and underlined nucleotides are not in the coding sequence of ZmG).

The PCR product was subcloned to pCR 2.1 Topo Vector (Invitrogen Corp., Carlsbad, CA, USA) for amplification in *E. coli* XL1 Blue (Stratagene, La Jolla, CA, USA). The ZmG was restriction digested from pCR 2.1 by *Nco*I and was inserted into the vector containing the 27 kDa γ-zein promoter and GFP at the *Nco*I restriction site such that ZmG and GFP formed a translational fusion product (ZmG−GFP) with GFP fused to the C-terminus of ZmG. The sequence of the construct was verified by DNA sequencing at the Iowa State University DNA facility prior to transformation.

**Plant Transformation, Tissue Culture, and Seed Production.** Stable transformation by gold particle bombardment of HiII Type II callus[35] was accomplished at the Plant Transformation Facility at Iowa State University. Callus was cobombarded with the globin−GFP expression vector and with a plasmid containing the *bar* gene for bialaphos resistance.[36] T0 callus was raised on bialaphos selective media, and resistant callus was screened with PCR with two primer sets for the presence of the ZmG−GFP construct using GoTaq (Promega, Madison, WI, USA) (forward primer 1, CCGATCGACACCATGGCACTCGCGGAG; reverse 1, CTTGCTCACCATGGCATCGGGCTTCATC; forward primer 2, GATGAAGCCCGATGCCATGGTGAGCAAG; reverse 2, CTGCAGCCGGGCGGCCGCTTTACTTG). The first primer set covered the fusion region between the 27 kDa γ-zein promoter and globin coding sequence to the fusion between globin and GFP. The second primer set covered the globin and GFP fusion region to the end of the GFP coding region. This approach eliminated any ambiguity that may have arisen from amplification of the native globin gene or 27 kDa γ-zein promoter.

Transformed calli that were PCR positive for the gene construct were regenerated to plants in the Plant Transformation Facility Greenhouse and crossed to the inbred B73 to create F1 kernels. All subsequent plants were grown at the Iowa State University Transgenic Nursery in Ames, IA, USA. Kernels from F1 events found to be positive for the transgenic protein were advanced to the BC2F1 generation in 2007 by crossing to B73. In 2009 plants grown from seeds with visible GFP expression were crossed to three nontransgenic inbred lines: B110, B73, and Mo17. The ZmG−GFP kernels with the highest fluorescence were chosen for iron bioavailability analysis. This ear was produced by crossing the inbred B110 with a heterozygous BC2F1 plant containing event 73. As a control, we included an ear that expressed GFP that was not fused to hemoglobin. This line was developed by Shepherd[33] and was grown in the same nursery as the ZmG−GFP. This allows us to rule out the influence of GFP on iron bioavailability. The ears chosen for analysis were segregating for their respective transgenes, as was expected on the basis of their pedigrees, and kernels expressing the transgene were compared to kernels from the same ear that did not express the transgene. In this way, environmental and genetic effects were minimized. Segregation ratios and 50-kernel mass were determined in duplicate for three randomly selected ears that were also from crosses of BC2F1 event 73 with B110. A chi square test was used to determine whether the actual segregation ratios were significantly different from the expected 50% transgene positive and 50% transgene negative ratio. A Student's *t* test was used to determine whether masses of positive and negative kernels were significantly different.

**Maize Hemoglobin and Antibody Production.** Maize globin was expressed in *E. coli*, purified, and characterized as described by Smagghe et al.[37] The protein was purified using metal affinity chromatography, resulting in a single band on an SDS-PAGE gel, and had a Soret/280 ratio that was characteristic of pure hemoglobin.[37] For

this study, heme iron in the purified protein was confirmed with FTIR spectroscopy as described by Kundu et al.[38] Oxygenated hemoglobin was prepared by dissolving in 20 mM Tris, pH 4.8, and 10 mM dithiothreitol, and the FTIR spectrum was determined. Carbon monoxide was blown over the sample for about 10 s, and the sample was scanned again to obtain the deoxygenated hemoglobin spectrum. The purified maize hemoglobin (ZmHb) was used as an antigen for polyclonal antibody production in rabbits (ProSci Incorporated, Poway, CA, USA).

**Transgenic Kernel Screening.** Screening for transgene positive kernels consisted of visual screening (Figure 2) for GFP fluorescence

**Figure 2.** Kernels from a representative ear segregating for ZmG-GFP in white light (top) and in blue light (485 nm) with an orange filter (535 nm) (bottom). Fluorescing kernels appear bright yellow-green, whereas nonfluorescing kernels appear dark or orange.

using a Dark Reader hand lamp (Clare Chemical, Dolores, CO, USA) followed by measuring fluorescence of individual visually positive kernels in a 24-well plate with a spectrofluorometer (Tecan, Mannedorf-Zurich, Switzerland) at 485 nm excitation and 535 nm emission wavelengths. Fluorescence of flour for the iron bioavailability study was determined by placing equivalent amounts of flour from each sample into a 6-well Costar plate (Corning, Lowell, MA, USA) and measuring four points within each well. Comparisons between samples were made with Student's *t* test.

In the F1 generation, the presence of the ZmG−GFP gene fusion product in fluorescent kernels was confirmed by immunoassay. Flour (50 mg) ground from positive kernels and the two untransformed parents (B73 and A188) was suspended in 500 $\mu$L of Lammeli buffer, boiled, and centrifuged, and 15 $\mu$L of the supernatant was loaded on 15% SDS-PAGE gels. Separated proteins were then transferred to a nitrocellulose membrane and blocked overnight with 5% milk in Tris-buffered saline with 0.05% Tween 20 (TBST). Membranes were probed for 3 h either with anti-GFP monoclonal antibody (Living Colors, Clontech, Mountain View, CA, USA) or with anti-ZmHb polyclonal antibody (described above) with 1:25000 dilution in 2% milk in TBST. Membranes were then probed for 1 h with horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse antibody, respectively, both diluted 1:50000 in 2% milk in TBST. Immunoreactive bands were visualized by chemiluminescence.

**Iron Bioavailability of Maize Hemoglobin Produced in *E. coli*.** Whole seeds of the inbred maize line B73, grown at the ISU Agronomy Farm in 2009, were ground into fine flour with a Sorvall grinder (Thermo Fisher Scientific Inc., Waltham, MA, USA). The flour was fortified with one of the following fortificants: ZmHb produced in *E. coli*, bovine hemoglobin (Sigma-Aldrich, St. Louis, MO, USA), or ferrous sulfate (Sigma-Aldrich). For each fortificant, 30 ppm iron by weight was added to the flour. No fortificant was added to the negative control flour sample. Because bioavailability of an iron fortificant can be affected by cooking[39] and because virtually all maize for human consumption is cooked, the fortified corn flour was made into simple porridge by heating 3.5 g of flour with 10 mL of deionized water and 1 mM ascorbic acid in a glass pot until stiff (about 5 min). The porridge was then freeze-dried.

Each sample was digested in duplicate as described by Glahn[40] and modified by Proulx.[24,41] The freeze-dried samples were mixed with 15 mL of 0.1 M HCl and adjusted to pH 2.0 with 1 M HCl. Then, 1 mL of pepsin solution (1.2 g 1:10000 porcine pepsin A in 10 mL 0.1 mol/L HCl) was added, and the sample was incubated for 1 h at 37 °C with shaking at 500 rpm to mimic gastric digestion. The pH was then adjusted to 6 with 1 mol/L $NaHCO_3$, and 5 mL of pancreatin and bile solution (0.05 g of 4× USP porcine pancreatin and 0.3 g of bile extract in 35 mL of 0.1 mol/L $NaHCO_3$) was added. To mimic duodenal digestion, the samples were incubated at 37 °C with shaking for 15 min. The digests were then heat treated for 4 min at 100 °C to inactivate proteases, as suggested previously,[42] centrifuged at 5000*g* for 10 min, and frozen prior to use.

The iron uptake experiment was conducted as described by Proulx and Reddy.[41] All reagents for cell culture work were from Sigma-Aldrich or Gibco BRL (Grand Island, NY, USA) unless otherwise mentioned. Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) at passage 18 were grown in a culture flask containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% v/v nonessential amino acids, and 1% v/v antibiotic−antimycotic solution. Cells were maintained at 37 °C in an incubator with 5% $CO_2$. Medium was changed three times weekly. At 7 days, the cells were rinsed with Earle's balanced salt solution (EBSS), trypsinized to dissociate the cells, and centrifuged at 22.6*g*. The cells were seeded at passage 29 at a density of $5 \times 10^4$ cells/cm$^2$ on collagenized (type 1 rat tail collagen) 12-well Costar cell culture plates (Corning).

At 15 days post seeding, the cell monolayer was rinsed with EBSS. For cell uptake, 0.5 mL of serum-free medium and 0.5 mL of the supernatant of each digest were added to each cell culture well in a randomized complete block design and incubated for 2 h. A subsequent 0.5 mL of serum-free media was then added, followed by further incubation for 22 h. Serum-free medium consisted of DMEM with 1% v/v nonessential amino acids, 1% v/v antibiotic−antimycotic solution, 0.4 mg/mL hydrocortisone, 0.5 $\mu$g/mL selenium, 3.4 $\mu$g/mL triiodothyronine (T3), 1 mmol/mL piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), 2 $\mu$g/mL EGF (epidermal growth factor), and 1 mg/mL insulin.[39] After 24 h of total incubation, digests and media were removed by aspiration, and cells were rinsed with 1.5 mL of EBSS. The cells were then lysed by the addition of 0.5 mL of deionized water to each well and sonicated with a probe-type sonic dismembranator at lowest setting (<1 W output) for 15 s.

Protein in cell lysates was determined in duplicate with the Bradford Coomassie assay (Pierce Laboratories, Rockford IL, USA), and ferritin was determined by radioimmunoassay (Fer-Iron II, Ramco Laboratories, Stafford, TX, USA) and measured with a Cobra-II gamma counter with SpectraWorks software (Packard BioSciences, Meriden, CT, USA). Iron bioavailability was expressed as relative bioavailability (RBA) to the positive control on a plate-by-plate basis. Comparisons between respective transformed and untransformed maize were made with Student's *t* test.

**Iron Bioavailability of Transformed Maize Kernels.** Kernels were visually separated into GFP positive and negative kernels for both ZmHb−GFP and GFP transformations. A ground sample (0.25g) of

each transgenic (+GFP) or nontransgenic (−GFP) grain was digested with 1.7 μmol of Fe with or without 34 μmol of ascorbic acid (1:20 Fe/ascorbic acid), applied to cells, and iron bioavailability was assessed as described above. On the basis of our initial results with low response without added iron, we added iron to all samples. To further increase the ferritin response, ascorbic acid was also added to some samples. A positive control with ferrous sulfate plus ascorbic acid alone and a negative control without added iron or ascorbic acid were treated identically to the flour samples. RBA was calculated as described above using the positive control as a reference. The total iron content of each sample was measured using the nonheme iron assay described by Proux et al.[41] The FTIR spectrum was used to determine heme iron content of ZmHb−GFP as described above for maize hemoglobin produced in *E. coli*.

## RESULTS

**Bioavailability of Iron in Maize Flour Fortified with Maize Hemoglobin Produced in *E. coli*.** To determine the usefulness of maize nonsymbiotic hemoglobin as an iron biofortificant, we examined the bioavailability of maize hemoglobin produced in *E. coli* (ZmHb) using a Caco-2 cell culture model to compare ZmHb to other fortificants. The presence of heme iron in ZmHb was confirmed by FTIR spectroscopy by observing the shift of the oxygenated to the deoxygenated hemoglobin peak (Figure 3). The size of the

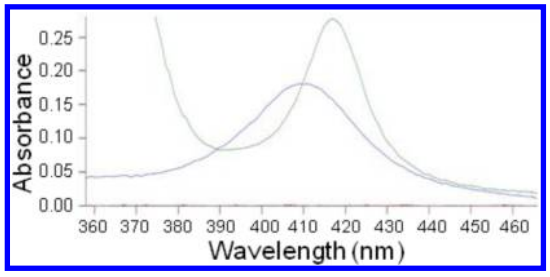


**Figure 3.** Heme iron in ZmHb produced in *E. coli* as detected with FTIR. Oxygenated ferric maize hemoglobin has a peak at 412 nm (abs 0.155), and deoxygenated ferric maize hemoglobin has a peak at 418 nm (abs 0.255).

ZmHb protein was also confirmed as 18 kDa by Western blot (Figure 4), similar to the 18.3 kDa reported by Arechaga-Ocampo.[26] The iron bioavailability of maize flour fortified with ZmHb was not significantly different from that of flour fortified with bovine hemoglobin (BHb) or ferrous sulfate ($FeSO_4$), but all three were significantly different from an unfortified maize flour sample ($p = 0.0005$), as shown in Figure 5. Whereas these data do not support the hypothesis that hemoglobin iron is more bioavailable than nonchelated forms of iron such as ferrous sulfate, the observation that the iron bioavailability maize hemoglobin is similar to that of bovine hemoglobin and ferrous sulfate suggests that maize hemoglobin may be an effective plant-based source of highly bioavailable iron.

**Production of the ZmG−GFP Fusion Protein in Transgenic Maize Kernels.** We next set out to produce transgenic maize plants expressing maize globin. To facilitate detection of transgenic kernels, the globin coding sequence was translationally fused to GFP by elimination of the stop codon within the globin gene. Of 80 calli screened, 20 were PCR positive for both regions of the construct. In the F1, kernels from 15 events were visually positive for GFP, and the other 5 events were discarded. There are a number of possible reasons why 6 events were PCR positive but did not have visible GFP fluorescence in the F1 generation, including transgene silencing

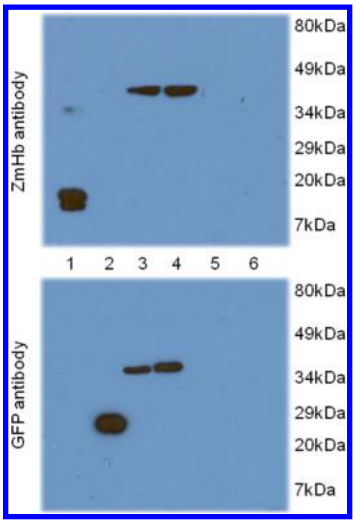


**Figure 4.** Western blots of maize endosperm using antibodies to GFP or maize hemoglobin (ZmHb). Lanes on both blots: 1, maize hemoglobin produced in *E. coli* (30 ng); 2, GFP standard (Clontech, Mountain View, CA, USA) (20 ng); 3, transgenic event 26 F1; 4, transgenic event 65 F1; 5, untransformed A188; 6, untransformed B73. Maize hemoglobin is expected to be approximately 18 kDa, and GFP is expected to be approximately 25 kDa, so the fusion protein is expected to be approximately 43 kDa.

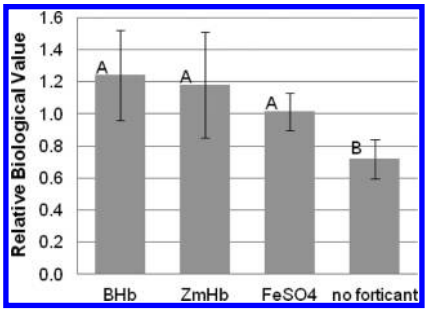


**Figure 5.** Iron bioavailability of fortified maize flour expressed as ferritin synthesis (ng ferritin/mg cell protein) relative to ferrous sulfate. Iron bioavailabilities for bovine hemoglobin (BHb), maize hemoglobin (ZmHb), and ferrous sulfate ($FeSO_4$) are not significantly different, but all three are significantly different from that of flour with no added fortificant ($p = 0.0005$, $n = 8$).

and rearrangement of the transgene during bombardment or integration into the genome. Of these 15 events, 7 were selected for further experiments on the basis of the number of available kernels.

Segregation ratios were determined visually for three randomly selected ears from crosses of event 73 in BC2F1 with the inbred line B110. Two of the three ears did not have segregation ratios that were significantly different from the expected 50% transgene positive and 50% transgene negative ratio ($p = 0.2373$ and 0.5859, $n = 232$ and 273 kernels, respectively). The third ear did have significant variation from the expected ratio ($p = 0.0007$, $n = 186$), with 62.4% positive kernels. We conclude that the transgene was successfully incorporated into the maize genome and transmitted through meiosis. The masses of visually GFP positive and negative kernels from the same ear were not significantly different ($p = 0.9348$, $n = 100$ kernels each from three ears, data not shown), indicating that the transgene does not cause a change in mass. There were significant differences in kernel mass between ears

**Table 1. Relative Iron Bioavailability (Rba), Expressed As Ferritin Synthesis (ng ferritin/mg Cell Protein) in Relation to a Positive Control (Ferrous Sulfate Plus Ascorbic Acid), And Total Iron (mg/g) of Maize Flour of Visually Identified Gfp Positive and Negative Maize Kernels Expressing Transcriptional Fusion of Maize Globin and Gfp (Zmg−gfp) and Gfp Alone[a]**

| | | RBA (%) | | | | total iron (μg/g) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *n* | mean | SEM | *p* | *n* | mean | SEM | *p* |
| ZmG−GFP with AA | positive | 4 | 12.59 | 1.71 | 0.29 | 2 | 18.71 | 0.71 | 0.06 |
| | negative | 4 | 14.83 | 0.91 | | | 16.25 | 0.80 | - |
| ZmG−GFP without AA | positive | 4 | 5.00 | 0.39 | 0.44 | 2 | - | - | - |
| | negative | 4 | 5.51 | 0.50 | | | - | - | - |
| GFP with AA | positive | 4 | 8.92 | 1.70 | 0.32 | 2 | 15.49 | 0.36 | 0.24 |
| | negative | 4 | 6.67 | 1.16 | | | 14.09 | 0.68 | - |
| GFP without AA | positive | 4 | 3.70 | 0.43 | 0.23 | 2 | - | - | - |
| | negative | 4 | 3.05 | 0.23 | | | - | - | - |

[a]Both transformed and untransformed ZmG-GFP and GFP samples were tested with and without ascorbic acid. Statistical comparison was conducted with Student's *t* test.

($p < 0.0001$) that can be attributed to genetic and environmental differences between the plants that produced them.

The observation of GFP in kernels suggested that the transgene was successfully transcribed and translated. We next characterized the ZmG−GFP polypeptide. The predicted fusion protein has a molecular weight of approximately 43 kDa, with 18 kDa contributed from maize globin and 25 kDa contributed by GFP, and this molecular weight was confirmed by SDS-PAGE with immunoblot detection (Figure 4) in the seven events that were tested. No proteins in either of the untransformed parental lines reacted with either the ZmG or GFP antibodies, suggesting that concentrations of native globin protein in maize endosperm are too low to detect and that there are no maize proteins similar to GFP. Representative blots including two of the events expressing the ZmG−GFP fusion protein are shown in Figure 4.

Measured fluorescence levels of ZmG−GFP and GFP alone were significantly higher between visually identified GFP positive and negative kernels ($p < 0.0001$). However, total iron in ZmG−GFP positive flours was slightly higher than in their nontransformed negative controls (18.71 vs 16.25 μg/g), but the difference was not significant ($p = 0.06$, Table 1) when tested in the pooled maize flours. Although total iron content was 7% higher in the ZmG−GFP maize, heme iron was undetectable. As expected, no difference in total iron content was found between GFP alone transformed and non-transformed seeds.

**Iron Bioavailability of Transformed Maize Flours.** The mean ferritin values, expressed in ng ferritin/mg protein, were 242 and 287 with added ascorbic acid and 97 and 106 without ascorbic acid for ZmG−GFP positive and ZmG−GFP negative samples, respectively. Average ferritin response ($n = 4$) with the positive control was 1935.1, and the negative control was 38.5 ng/mg protein. The RBA (compared to a ferrous sulfate plus ascorbic acid) and iron content of the transformed and untransformed flours is shown in Table 1. When tested with added ascorbic acid, the RBA was 15% lower with ZmG−GFP positive compared to negative control; however, the difference was not significant ($p = 0.29$). A similar 9% nonsignificant ($p = 0.44$) difference was found when ascorbic was not added to the samples. Overall, adding ascorbic acid improved iron bioavailability by 2.5- and 2.7-fold in ZmG−GFP positive and negative samples, respectively, but the relative differences were the same between positive and negative maize.

Similar results were found when we tested the maize expressing GFP alone. There was no significant difference in RBA between GFP positive and negative seeds when tested with (8.92 vs 6.67%) and without ascorbic acid (3.7 vs 3.05%). The ferritin responses with the positive control and the negative controls were similar to those of a previous experiment, 2018 and 35.6 ng/mg protein.

## DISCUSSION

The objective of this study was to determine the potential of maize hemoglobin as iron fortificant by evaluating the iron bioavailability of maize hemoglobin expressed in *E. coli* and of nonsymbiotic maize globin expressed in maize endosperm. Our results show that maize hemoglobin is as bioavailable as ferrous sulfate when added to flour at equal concentrations of iron. However, transgenic maize kernels expressing maize globin fused to GFP were found to have similar bioavailability as kernels from the same ear that were not expressing ZmG−GFP. Further studies are needed to confirm these effects across different transformation events and environments.

Maize has a significant amount of phytate, which is a potent inhibitor of iron absorption. Increasing total iron content may not be an effective method for improving iron nutrition, which may require strategies that improve iron bioavailability. The strategy of overexpressing ferritin received attention because plant ferritins have shown absorption as high as ferrous sulfate in rat models[16] and in humans.[17] It is now understood that ferritin is degraded during digestion and iron associated with the protein will enter the common nonheme iron pool, making it susceptible to phytate inhibition.[43] Unlike ferritin, hemoglobin has a unique uptake pathway and transport mechanisms,[44] and the heme pyrrole is protective against the inhibitory effects of phytate and other nonheme iron inhibitors.[23]

Because globin is produced as a translational fusion on the N-terminus of GFP, the presence of GFP in transformed kernels suggests that globin is also present. Western blots confirm the presence of a fusion protein of the expected size that reacts with both GFP and ZmHb antibodies. GFP greatly facilitated kernel screening, and the results with ZmG−GFP expressing kernels could be useful for developing future plant


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transformations overexpressing globin without GFP. Having GFP in the seeds may not be of concern because Richards et al., in a rodent feeding study, showed no risk with regard to overall health or allergenicity with GFP.[45] Therefore, ZmG−GFP lines could be used to evaluate iron bioavailability in future animal feeding studies with little concern with GFP. However, on the basis of not seeing positive results of RBA and not detecting heme iron with ZmG−GFP positive kernels, we speculate that GFP could have inhibited folding of globin or iron incorporation iron with globin. No increase found in iron bioavailability in ZmG−GFP positive kernels compared to negative may be due to no increase in iron content, especially heme iron. No significant difference in iron bioavailability between GFP positive and negative seeds suggests that the presence of GFP is not affecting bioavailability but does not rule out the negative effect of GFP on iron incorporation in the globin.

Because of the ubiquitous nature of heme compounds within cells, it was assumed that the ferrochelatase activity required for heme pyrrole insertion into the expressed ZmHb would be accomplished without need of further genetic modification. Previous transgenic expression studies found that heme incorporation into the globin protein did not require overexpression of enzymes responsible for heme synthesis.[36] The three-dimensional structures of multiple plant globins have been determined to have a heme binding pocket,[27,46] and maize globin expressed in *E. coli* for this study was found to contain heme iron, indicating that plant globin could contain heme iron in vivo. However, only low concentrations of the native globin protein have been found in plant tissue,[28] and incorporation of heme iron into plant globin in planta has not been detected.

Even though expression of ZmG−GFP was detected with fluorescence and confirmed with Western blot, we were unable to reliably detect heme, possibly due to low concentration of the protein in endosperm tissue. The fusion protein could not be identified on SDS-PAGE (not shown), suggesting that expression is low. In maize flour, chemiluminescence assay and FTIR spectroscopy for heme iron, and immunoprecipitation with the ZmHb antibody followed by measurement of total iron all had inconclusive results. Attempts to find native globin protein in untransformed seed and seedling tissues found no reactivity with the ZmHb antibody, suggesting that the native globin is not present in detectable concentrations. The maize globin presumed expressed in plant tissues was not detected by Western blot in roots, stems, or leaves of 14-day seedlings when exposed to 48 h of oxygen-reduced environment or 250 mM NaCl (data not shown). These results were not entirely unexpected, as Aréchaga-Ocampo et al. detected maize globin with Western blot in 5−7-day seedlings but found that levels of globin were greatly decreased in 14-day seedlings.[26]

Drakakaki et al. showed that localization of recombinant proteins contributes to protein functionality.[47] Whereas subcellular localization of the ZmG−GFP protein within the endosperm was not investigated in this study, it was expected to accumulate in the cytoplasm because signal sequences were not used. It is known that the presence of transgenic hemoglobin can have significant effects on plant growth and can alter concentrations of a wide variety of metabolites.[48,49] However, compartmentalization may compromise heme pyrrole incorporation. It is suggested that the enzymes in plants responsible for heme synthesis and the insertion of iron into heme pyrroles are associated with the mitochondria and chloroplasts,[50−52] but it is not currently clear where heme pyrrole insertion into globin takes place. In *Arabidopsis*, alfalfa, and cotton, hemoglobin is found in the cytosol and in the nucleus,[53] indicating that cytoplasmic accumulation of the protein would be sufficient for heme pyrrole insertion. Maize globin sequence is not predicted to have a signal sequence.[34] Still, appropriate targeting could result in higher protein expression levels or greater heme pyrrole incorporation.

This study establishes the possibility of altering iron bioavailability in maize carrying a transgene encoding maize hemoglobin. Important questions about the feasibility of this approach remain. It will be important to examine the stability of transgene expression in different environments and in particular to understand the impact of environmental iron levels on transgene expression. Furthermore, it will be important to understand the impact of accumulation of the transgene-derived globin protein on iron homeostasis in the plant. Large disruptions in iron homeostasis could interfere with iron-dependent metabolic processes such as photosynthesis and could lead to reductions in grain yield.

Hemoglobin expressed in *E. coli* has bioavailability similar to that of bovine hemoglobin but not in transformed seed, suggesting iron is not incorporated in the pyrrole ring or the pyrrole ring is not incorporated into the globin protein. It is possible that endosperm cells do not make sufficient amounts of pyrrole to meet the requirements of the overexpressed globin protein. A second consideration is that the pyrrole may not be produced in the same subcellular compartment as the globin protein. In conclusion, this study shows that expressing globin alone may not necessarily be useful unless we include strategies for iron incorporation. Future studies are needed to perform transformations without GFP or express enzymes that involved iron incorporation to increase heme iron content.

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