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**Abstract**
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**Keywords**
Department of Genetics, Development, and Cell Biology, Biofortification; iron bioavailability; transgenic maize; low phytic acid 1-1

**Disciplines**
Food Science | Human and Clinical Nutrition | Other Plant Sciences

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# Genetic Modification of *Low Phytic Acid 1-1* Maize to Enhance Iron Content and Bioavailability

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Supporting Information

**ABSTRACT:** High phytate content in staple food crops is a major barrier to successful iron biofortification. We have exploited the *low phytic acid 1-1* (*lpa1-1*) mutant of maize to generate transgenic plants with up-to 70 $\mu$g/g seed iron through the endosperm-specific overexpression of soybean ferritin, resulting in more than 2-fold improvement in iron bioavailability. The levels of bioavailable seed iron achieved in this study greatly exceed any achieved thus far and closely approach values estimated to have a nutritional impact on target populations. Gene expression studies reveal a large induction of the *YS1* transporter in leaves and severe repression of an iron acquisition gene *DMAS1* in roots, suggesting significant alterations in the iron homeostatic mechanisms in transgenic *lpa1-1*. Furthermore, preliminary tests show that the high-iron *lpa1-1* seeds have higher germination rates and seedling vigor when compared to those of the nontransgenic seeds, which may help improve their value to plant breeders.

**KEYWORDS:** biofortification, iron bioavailability, transgenic maize, *low phytic acid 1-1*

## INTRODUCTION

Iron (Fe) deficiency anemia (IDA) afflicts an estimated 2 billion people worldwide and accounts for over 20% of maternal mortality and 30% of childhood morbidity rates (WHO 2010). Much of these child and maternal iron deficiency cases are in developing countries such as Asia and Africa, whose populations are sustained on a few staple food crops (for e.g., rice, wheat, and maize), resulting in insufficient dietary iron intakes. Biofortification of staple food crops, therefore, provides a cost-effective and sustainable alternative over traditional methods such as food fortification for improving human iron nutrition.[1−4] However, iron biofortification is a difficult problem due to the high amounts of antinutrients (for example, phytate) in cereal and legume-based foods.[1−3] Phytate (*myo*-inositol 1,2,3,4,5,6,-hexakisphosphate) acts as an antinutrient by strongly chelating multivalent metal ions such as iron, zinc, and calcium, and forming insoluble salts with poor bioavailability of these minerals.[5] Moreover, Fe accumulation in seeds of food crops is a complex polygenic phenomenon involving a number of tightly integrated homeostatic mechanisms (iron uptake from soil by the roots, transport and distribution within the aerial parts of the plant, and import and storage in seeds) to avoid toxic effects of iron overload and thus forms an effective physiological barrier for genetic modification of plants.[6−8]

Biofortification programs based on conventional breeding have taken advantage of existing genotypic differences in seed Fe concentrations to identify varieties with improved Fe content.[9−14] Assuming a daily consumption of approximately 300 g of grains per day and a daily reference intake (DRI) of 18 mg of Fe (FAO/WHO 2005), it is estimated that an optimum Fe level of ~60 $\mu$g/g in grains is necessary to meet the daily nutritional requirement of iron. However, the best varieties identified via conventional breeding show only a modest increase in Fe content (~25 $\mu$g/g for maize).[11−14] Furthermore, the seed Fe concentrations are greatly influenced by soil conditions and environmental effects, and show no significant correlation with Fe bioavailability.[12−15]

Considerable progress has been made in recent years to modify seed iron content via genetic engineering by overexpressing different Fe-regulated proteins in target food crops.[16−22] Ferritin, in particular, has been widely used to enhance Fe content of staple food crops, due to its high-Fe binding capacity of up to 4,500 atoms of Fe per molecule. While these prior biotechnological efforts have been successful in enhancing Fe content of staple food crops, efforts to improve Fe bioavailability from food crops have met with only marginal success.

Fe bioavailability from plant foods is influenced by a variety of factors. Phytate[23−25] and polyphenols[23,26] inhibit Fe absorption, while ascorbic acid[27−29] and meat [30,31] enhance Fe absorption. Fe bioavailability also depends on the chemical form of Fe, such as ferrous sulfate ($FeSO_4$) and Fe bound to ferritin. Studies with purified soybean ferritin suggested that ferritin-Fe bioavailability may be similar to $FeSO_4$, a highly bioavailable form of Fe.[32] However, factors (for example, phytate) that affect iron absorption of $FeSO_4$ similarly affect the absorption of ferritin-Fe.[33] To circumvent the inhibitory effect of phytate on Fe absorption, transgenic rice and maize varieties overexpressing both ferritin and phytase (an enzyme that hydrolyzes phytate) were generated.[19,20] Although studies with transgenic maize clearly demonstrated a negative correlation between phytate content and Fe bioavailability, it is difficult to interpret the usefulness of heterologous phytase expression in planta, as staple foods are generally subjected to high heat during cooking, and even heat stable phytases such as

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those expressed in rice and wheat showed a significant loss of activity after 20 min of boiling.[19,34]

Another practical approach to addressing the nutritional problem of low Fe bioavailability and heterologous phytase expression in plants is to utilize the low phytic acid mutant(s) of crop plants. Several mutant lines of maize such as *lpa1-1* and *lpa2-1* with 50−66% reduction in phytate[35] and the *lpa241* mutant with 90% reduction in phytate[36,37] have been studied in some detail. Mutations such as those in *lpa241* have been reported to have severe negative effects on seed viability, germination, and plant growth, resulting in various degrees of yield penalty[36,37] and therefore are not attractive to plant breeders. However, first field trails conducted with *lpa1-1* mutant indicated little or no effect on seed germination and plant growth.[35,38] In addition, nutritional studies showed that *lpa1-1* maize improved Fe absorption by approximately 50% in humans,[39] thus making it an attractive target for iron biofortification of maize.

## MATERIALS AND METHODS

**Plasmid Construct.** The parent plasmid is pRBS from Aluru et al.[40] The ferritin gene (GenBank accession no. M64337) was isolated from soybeans by RT-PCR using gene-specific primers (given below) and was then cloned into the NcoI/SacI site of pRBS to generate the plasmid pMSF (Figure 1).

FORWARD - 5' TCTAGAATGGCTCTTGCTCCATCCAAAGTT 3'

REVERSE – 5' CTCGAGTAATCAAGAAGTCTTTGATCAAAG 3'

**Maize Transformation.** The homozygous *lpa1-1* mutant seeds (low phytate) of maize backcrossed to inbred maize line A188 were kindly provided by Dr. Victor Raboy, USDA-ARS, Idaho (vraboy@uidaho.edu), and the A188 seeds (normal phytate) were obtained from the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS), Ames, IA. Maize transformations were performed using a standard protocol for biolistic transformation at the Iowa State University Plant Transformation Facility.[41] Immature zygotic embryos of the A188*lpa1-1* (hereafter referred to as *lpa1-1*) and the A188 germplasm were cotransformed with plasmid pMSF and plasmid pBAR184, which carries the *Streptomyces hygroscopicus* phosphinothricin acetyltransferase gene (*bar*) under the control of the maize ubiquitin promoter. Transformed calli resistant to the herbicide bialaphos and positive for the presence of soybean ferritin gene were regenerated into plants ($T_0$) in the greenhouse under controlled conditions.[42] Because of poor synchronization of male and female flowers from transformed $T_0$ plants, $T_1$ kernels/seeds were obtained by pollinating the $T_0$ plants (as females) with pollen from nontransgenic *lpa1-1* or A188, respectively. To generate $T_2$ and $T_3$ seeds, the $T_1$ and $T_2$ seeds, respectively, were planted in the greenhouse, and the resulting plants were screened for the presence of soybean ferritin gene via genomic PCR. Approximately, 9−10 $T_1$ seeds and 5−6 $T_2$ seeds were planted for each transformation event and/or maize line. Mature plants positive for the transgene were then self-pollinated.

**Protein Immunoblot Analysis.** Total protein was extracted from transgenic $T_1$ maize seeds with acetone precipitation. Seeds (~1 g) were soaked in water for 24−36 h at room temperature and ground to a fine paste in ice cold 10 mM sodium phosphate buffer at pH 7.2. The slurry was centrifuged at 8,000*g* for 10 min at 4 °C. Cold acetone (4× vol) was added to the resulting supernatant, and samples were incubated at −20 °C for 16 h to precipitate soluble proteins. The protein pellet was collected after centrifugation at 15,000*g* for 15 min at 4 °C, air-dried for 30−45 min, and resuspended in 10 mM sodium phosphate buffer at pH 7.2. Protein concentration was measured using the Bradford protein assay

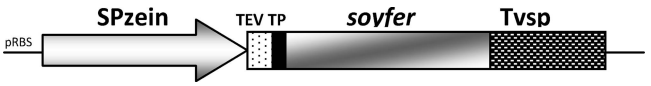



**Figure 1.** Plasmid construct. The parent plasmid was pRBS.[40] SPzein, "super 27 kD γ-zein promoter" obtained by repeating the −444/−174 region of the endosperm-specific γ-zein promoter; TEV, tobacco etch virus 5′ untranslated region; TP, transit peptide from pea Rubisco small subunit (*rbcS*); Soyfer, soybean ferritin cDNA; and Tvsp, soybean vegetative storage protein terminator.

(Bio-Rad Hercules, CA). For immunoblot analyses, approximately 50 μg of protein was electrophoresed through 12% SDS−polyacrylamide gels and transferred onto nitrocellulose membranes (Whatman, Maidstone, Kent, UK) according to the method described by Towbin et al.[43] The membranes were probed with a polyclonal antibody specific for soybean ferritin protein (a gift from Dr. Paul Scott, Iowa State University), and proteins were visualized using the ECL immunodetection assay method (Pierce, Erembodegum, Belgium). Ferritins have a molecular mass of 540 kDa with a subunit size of approximately 28 kDa.[44]

**Ferritin Quantification.** Total protein was isolated from individual $T_2$ seeds of transgenic maize lines using the acetone method described under immunoblot analysis. Protein from nontransgenic *lpa1-1* and A188 seeds served as respective controls. The ELISA protocol previously described in Lukac et al.[45] was used to measure ferritin concentrations. Briefly, 5 μg protein samples and standards made in bicarbonate buffer were coated onto Nunc Immuno Plates (Sigma-Aldrich, MO). After washing the wells twice with PBST buffer, the proteins were probed with an antiferritin polyclonal antibody (1:500 dilution) followed by HRP-conjugated goat anti rabbit antibody. The specificity of the antigen−antibody reaction was read using a chemiluminescent substrate with a Microplate reader. Ferritin concentrations in the samples were calculated against a recombinant pea ferritin protein standard curve (kind donation by Dr. Hurrell Institute of Food Science and Technology, Laboratory of Human Nutrition (Zurich, Switzerland)). Each sample was assayed in triplicate.

**Measurement of Iron, Zinc, and Phytate in Maize Seeds.** Dry $T_2$ and $T_3$ seeds of transgenic maize lines were digested by wet-ashing using a microwave. For the analysis of Fe content in $T_2$ generation seeds, 5 individual seeds were wet-ashed separately with 2 mL of 70% $HNO_3$ in a microwave at 250 W until the resulting liquid was clear. The samples were then diluted in deionized water to a final concentration of 14% $HNO_3$ and analyzed for Fe and Zinc (Zn) concentrations using an inductively coupled argon plasma (ICP) spectrometer. Fe and Zn concentrations in the $T_3$ seeds were measured in triplicate from pooled samples of 5 randomly selected seeds from each individual maize line. Phytate content in maize seeds was measured according to Gao et al.[46]

**Iron Bioavailability Measurements with Caco-2 Cells.** Maize samples were prepared as previously described.[47,48] In-vitro digestion was performed in duplicate from each transgenic line, and each sample was used in duplicate for cell uptake, thus giving a total of four different values for a single transgenic maize line. All reagents for cell culture work were from Sigma Aldrich (St. Louis, MO) or Gibco BRL (Grand Island, NY) unless otherwise mentioned. Caco-2 cells were obtained at passage 17 from American Type Culture Collection (Rockville, MD). The bioavailability assay was conducted with cell passages 23−26 as described previously.[47,49]

**RNA Isolation and qPCR.** Tissue samples (root, leaf, and seed) were collected from $T_2$ plants for each of the three transgenic maize lines analyzed and immediately frozen in liquid nitrogen until further use. Root and leaf samples were collected from 3 to 4 wk old plants, while seed samples were collected 22−23 days after pollination (DAP). Total RNA was isolated from frozen tissue samples using the TRIzol Reagent (GIBCO BRL, Rockville, MD). Two independent RNA preparations were made from pooled samples of each of the three maize tissues.

For quantitative real-time RT-PCR, first-strand cDNA was synthesized from DNase I-treated total RNA using the first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR was then performed with the synthesized cDNA's as described previously.[50] Primers used in these studies are as given in Table S1 (Supporting Information).

**Seed Germination Tests.** Determination of seed germination and viability was according to the Association of Official Seed Analysts of North America (AOSA) Rules for Testing Maize Seeds.[51,52] $T_3$ seeds of transformed and nontransgenic *lpa1-1* were placed in a row and germinated between moist rolled paper towels placed vertically in an incubator set at constant temperature between 20 and 30 °C. Seed germination and the emergence of normal shoot and root systems were evaluated at day 4 and at day 7. The experiment was repeated twice to confirm observations. Seed dry weight analysis was carried out with $T_3$ seeds; approximately 50 dry seeds from each ear were individually measured, and the calculated average weight of the seeds was compared to the nontransgenic seeds.

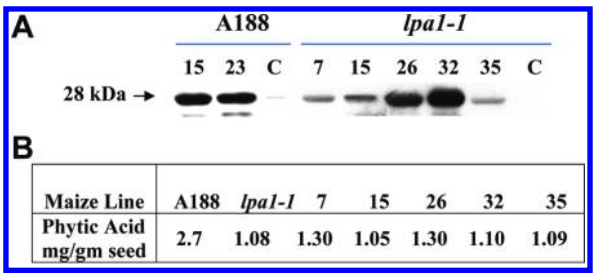
**Statistical Analyses.** Graphpad Prism software (version 4.00 for Windows, GraphPad Software, San Diego, CA) was used for all statistical analyses. Pearson correlations were performed to assess the relationship among ferritin, Fe, Zn, and Fe bioavailability. ANOVA with Tukey's multiple comparison test was used to compare Fe content and bioavailability among the four different maize lines. Multiple regression analysis was performed to determine the role of Fe, Zn, and phytate on Fe bioavailability. Mean differences were considered significant at $P \leq 0.05$.

## RESULTS

**Transgenic Maize Plants.** Two different sets of transgenic maize lines (*lpa1-1* (low phytate) and A188 (normal phytate)) were generated to assess the effect of soybean ferritin and phytate on Fe content and bioavailability. In this study, 15 transgenic *lpa1-1* plants and 13 transgenic A188 plants were regenerated from transformed calli positive for both *bar* and soybean ferritin gene. Of these, 10 transgenic *lpa1-1* plants and 3 transgenic A188 plants had good seed sets (>40 seeds/ear). The primary transformants ($T_1$ seeds) were initially screened by immunoblot analyses using a polyclonal antibody specific for soybean ferritin. Seven of the transgenic maize lines (5 *lpa1-1* and 2 A188 maize lines) that showed a 28 kDa protein band confirming soybean ferritin protein expression were chosen for further study (Figure 2A). $T_1$ progeny were obtained by pollinating regenerated $T_0$ plants (as females) with pollen from nontransgenic *lpa1-1* or A188 and were expected to segregate for the transgene. Since the total protein for immunoblot analysis was extracted from a randomly selected pool of $T_1$ seeds (containing both transgenic as well as nontransgenic seeds) from each individual line, the intensity of the ferritin band in Figure 2A is not an indication of ferritin protein concentrations in these transgenic maize lines.

The mean phytate content in nontransgenic *lpa1-1* maize (1.08 mg/g) is ~60% lower than the A188 seeds (2.7 mg/g), which is consistent with previous reports. The five transgenic *lpa1-1* maize lines also show a mean phytate concentration of 1.17 mg/g (ranging from 1.05 to 1.3 mg/g), which is similar to the nontransgenic parent line, thus validating their low phytate genetic background (Figure 2B).

**Iron and Zinc Accumulation in Transgenic Maize.** Individual $T_1$ seeds from each of the 7 transgenic maize lines (Figure 2A) were planted in the greenhouse, and the resulting $T_1$ plants were screened by genomic PCR to verify the presence of transgene. Approximately 50% of the plants analyzed from each transformation event (for example, 7, 15, 26, etc.) contained the soybean ferritin gene, suggesting a 1:1 segregation of transgene in the $T_1$ generation (data not shown). $T_2$ seeds resulting from 3 of the $T_1$ plants positive for the presence of soybean ferritin gene, for each individual transformation event (for example, lines 7−1, 7−3, and 7−5 from event 7) were analyzed for Fe content. Figure 3A shows that several transgenic *lpa1-1* lines (7−1, 7−3, 15−3, 26−5, 32−2, and 32−7) and A188 maize lines (15−1, 15−2, 23−2, 23−3, and 23−4) have enhanced Fe content, with Fe content in individual seeds ranging from 20−70 μg/g seed dry weight (DW) in *lpa1-1* transgenic plants and 23−43 μg/g DW in the A188 transgenic plants, respectively. Compared to the control nontransgenic plants, transgenic plants show a maximum increase of 2−3 fold Fe content in the seeds.




| Maize Line | A188 | *lpa1-1* | 7 | 15 | 26 | 32 | 35 |
|---|---|---|---|---|---|---|---|
| Phytic Acid mg/gm seed | 2.7 | 1.08 | 1.30 | 1.05 | 1.30 | 1.10 | 1.09 |

**Figure 2.** Screening of the primary maize transformants. (A) Expression of soybean ferritin protein. Approximately 50 μg of total protein extracts from $T_1$ seeds of transgenic *lpa1-1* and A188 lines were loaded onto a 12% SDS−PAGE and probed with an antisoybean ferritin polyclonal antibody. Five transgenic *lpa1-1* (lines 7, 15, 26, 32, and 35) and two A188 maize lines (lines 15 and 23) containing the 28 kDA ferritin protein band are shown. Nontransgenic *lpa1-1* and A188 (C) seeds had undetectable levels of the protein. (B) Phytate content in the $T_1$ seed of transgenic *lpa1-1* lines (7, 15, 26, 32, and 35) and nontransformed *lpa1-1* and A188. Seeds were analyzed for phytate content using the method described by Gao et al. 2007.[46]

Previous reports suggest a significant correlation between Fe and Zn accumulation in food crops.[9,53] The Zn content of individual transgenic *lpa1-1* seeds ranged from 32−72 μg/g DW, while transgenic A188 lines contained 30−56 μg/g DW, an improvement of 1.3-fold in some transgenic lines when compared to that of the nontransgenic control lines (Figure 3A). Furthermore, we found a positive correlation between Fe and Zn concentrations (Table 1) in both the transgenic *lpa1-1* ($R^2$ = 0.58, $P < 0.05$) and A188 ($R^2$ = 0.84, $P < 0.05$) lines.

Total ferritin concentrations in transgenic maize ranged from 5 μg−151 μg/g DW, resulting in a maximum increase of 4−5-fold in individual seeds of transgenic lines versus control (Figure 3B). Similar to the variability observed in Fe and Zn content (Figure 3A), there was a great variability in ferritin concentrations among the transgenic lines and within individual seeds. However, as expected, ferritin concentration and Fe content were correlated for both transgenic *lpa1-1* ($R^2$ = 0.63, $P < 0.05$) and A188 ($R^2$ = 0.74, $P < 0.05$) lines (Table 1). No significant correlation was found between Zn and ferritin concentrations.

**Effect of Low Phytate and High Seed Iron Content on Iron Bioavailability.** Based on of ferritin synthesis in Caco2 cells as an index of bioavailable Fe, transgenic *lpa1-1* maize lines clearly showed a significant increase in Fe bioavailability compared to both the A188 nontransgenic control (3-fold in some lines) and the A188 transgenic line (>1.5-fold) (Figure 3C). Furthermore, Fe ($R^2$ = 0.84; $P < 0.001$) and ferritin concentrations ($R^2$ = 0.56; $P < 0.05$) are significantly correlated with Fe bioavailability in transgenic *lpa1-1* seeds but not in transgenic

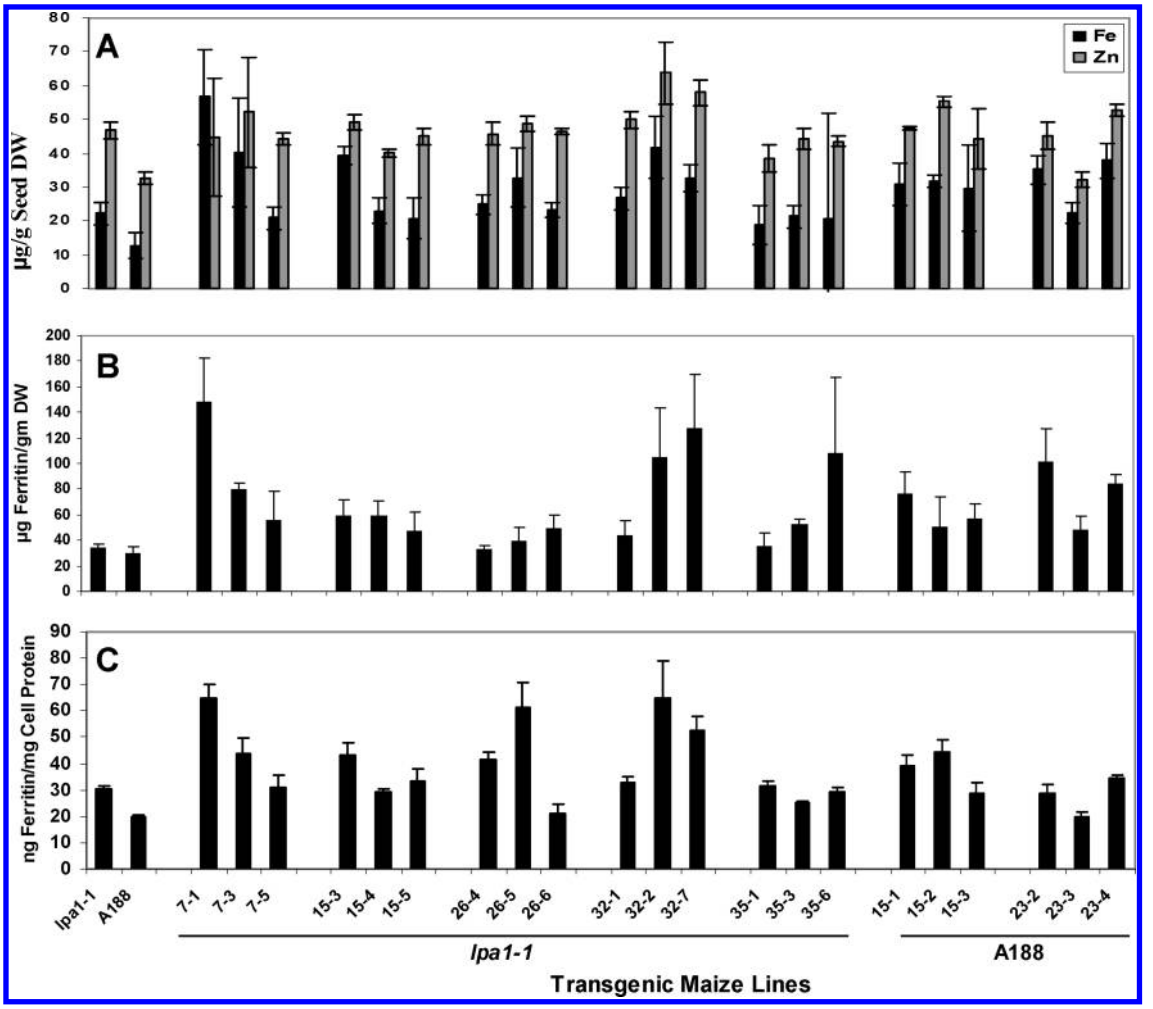



**Figure 3.** Analysis of $T_2$ seeds. (A) Total Fe and Zn contents of $T_2$ seeds of transgenic *lpa1-1* and A188 maize as determined by inductively coupled argon plasma emission spectrometry (ICP). Fe and Zn contents were measured from five different transformation events of *lpa1-1* (lines 7, 15, 26, 32, and 35) and two from A188 (lines 15 and 23). Three individual ears from each event were analyzed. Data represents the mean ± SD of five individual seeds from a single ear of each individual maize line. Nontransgenic A188 and *lpa1-1* are shown as controls. (B) Quantification of ferritin. Total ferritin concentrations in $T_2$ seeds of transgenic *lpa1-1* and A188 were measured by ELISA using an antiferritin polyclonal antibody.[45] Data represents the average ± SD of six individual seeds from a single ear of each individual maize line. The non transformed *lpa1-1* and A188 served as controls. (C) Measurement of Fe bioavailability from transgenic *lpa1-1* and A188 seeds using the in vitro Caco-2 cell model. The histogram illustrates ferritin synthesis in Caco-2 cells after the addition of digested maize seed samples (see Materials and Methods). Data represents the mean ± SD of 4 measurements each of 10 pooled seeds per individual maize line.

**Table 1. Intercorrelations among Zinc and Iron Contents, and Iron Bioavailability of *lpa1-1* and A188 Maize Lines[a]**

| variable | iron | zinc | bioavailability |
|---|---|---|---|
| ferritin | | | |
| *lpa1-1* | 0.63[b] | 0.41 | 0.56[b] |
| A188 | 0.74[b] | 0.26 | 0.35 |
| iron | | | |
| *lpa1-1* | | 0.58[b] | 0.84[c] |
| A188 | | 0.84[b] | 0.52 |
| zinc | | | |
| *lpa1-1* | | | 0.61[b] |
| A188 | | | 0.89[b] |

[a] $R^2$ values for the transgenic A188 lines ($n = 6$) and *lpa1-1* ($n = 15$). Correlations are Pearson's product−moment correlation coefficients. [b] $P \leq 0.05$. [c] $P \leq 0.001$.

A188 lines (Table 1). Consistent with this result, multiple regression analysis reveals that ~71% of the variability in Fe bioavailability is explained by Fe ($P < 0.0001$) and by maize line ($P < 0.02$) (Table 2), reflecting a difference in the phytate content of transgenic *lpa1-1* and A188. Because of the huge variation in Fe content and bioavailability of individual transgenic lines (Figure 3A and C), we compared the average Fe bioavailability of a pool of transgenic lines with enhanced seed Fe content versus their respective parent nontransgenic control (Figure 4). The nontransgenic *lpa1-1* seeds show a 1.5-fold increase in Fe bioavailability (31 ± 0.9 ng/mg protein) versus nontransgenic A188 (20 ± 0.5 ng/mg protein) (Figure 3C), but this difference was not found to be significant (Figure 4). Average Fe bioavailability of transgenic *lpa1-1* (52 ng/mg ranging from 26−65 ng/mg protein) is significantly higher than that of nontransgenic *lpa1-1* and transgenic as well as nontransgenic A188 maize (Figure 4).

**Inheritance of the High-Iron Trait in Transgenic Maize.** To test the inheritance of the high-Fe trait in transgenic maize plants, we measured the Fe content of $T_3$ generation seeds that showed enhanced Fe content in the $T_2$ generation (*lpa1-1* transgenic maize lines 7−1, 26−5, 32−2, and from the A188 line 23−4) (Figure 3A). Transgenic *lpa1-1* line 15−3 was not chosen because of paucity in the number of seeds available for further analysis. Total Fe content of the 3 individual maize lines from each of the aforementioned four $T_2$ transgenic maize lines (for example, 7−1−1, 7−1−2, and 7−1−3 from transgenic line 7−1) are shown in Figure 5. While progeny seeds from line 7−1 did not replicate the high-Fe trait, progeny from lines 26−5, 32−2, and 23−4 show ∼1.5−3-fold higher Fe content ranging from 24−58 μg/g for the transgenic *lpa1-1* maize and 26−29 μg/g for the A188 transgenic plants. The total zinc levels also change significantly for the transgenic *lpa1-1* and A188 plants. These results are consistent with Figure 3A and indicate stable inheritance of the high-Fe trait in some of the transgenic maize lines.

**Table 2. Multiple Regression Analysis to Determine Predictors of Fe Bioavailability in Transgenic Seed**[a]

| independent variable | parameter estimate | partial *R*-square | *P*-value |
|---|---|---|---|
| intercept | −0.66 | | |
| iron | 1.10 | 0.54 | <0.0001 |
| maize line | 9.49 | 0.11 | 0.016 |
| zinc | −0.37 | 0.06 | 0.09 |

[a] Model $R^2 = 0.709$; $F = 13.82$ ($P < 0.0001$); $n = 6$ for A188; and $n = 15$ for *lpa1-1* transgenic maize.

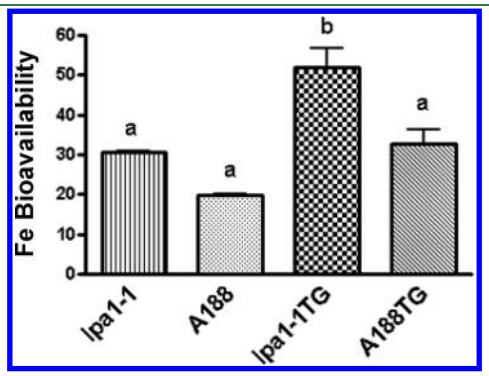



**Figure 4.** Mean bioavailability (ferritin ng/mg protein) of transgenic lines with enhanced Fe content compared to their respective nontransgenic maize lines. *lpa1-1* ($n = 5$) and A188 ($n = 5$) are the nontransgenic maize lines with low and normal phytic acid, respectively. *lpa1-1*TG ($n = 7$) and A188TG ($n = 6$) are the transgenic *lpa1-1* and A188 maize lines. Each data point represents the average of 4 replicates from each individual line. The differences in means were compared with ANOVA with Tukey's multiple comparison test. Means not sharing similar letters are significantly different ($P < 0.05$).

**Effect of Ferritin Overexpression on Iron-Regulated Genes in Transgenic Maize.** Plant iron homeostasis is an integrated process involving communication between different organs and multiple genes to balance the nutrient supply of the whole plant. To further understand the molecular basis for enhanced Fe accumulation in transgenic maize and changes in iron homeostatic mechanisms, we measured the expression of eight well-characterized genes of Fe homeostasis[7] in different tissues and seeds generated from transgenic *lpa1-1* (26−5 and 32−2) and A188 (23−4) that showed stable inheritance of the high-Fe trait. Overexpression of soybean ferritin in the maize endosperm resulted in differential expression of most of the genes analyzed. Expression of genes that mediate Fe acquisition from soil (nicotianamine synthase 2, *NAS2*; nicotianamine aminotransferase, *NAAT*; deoxymugineic acid synthase 1, *DMAS1*) was repressed in roots and leaves of the transgenic plants (Table 3). In contrast, nicotianamine synthase 3 (*NAS3*), which is negatively regulated by Fe,[54] and genes which mediate Fe transport (Fe-deficiency regulated protein 3, *FDR3*, and Yellow-stripe 1, *YS1*) showed induced expression in roots and/or in leaves; *YS1* expression was dramatically induced in leaves. Additionally, expression of genes encoding Fe storage proteins Ferritin 1 and 2 (*FM1* and *FM2*) was repressed in leaves but induced in the seeds. Together, these results suggest decreased uptake of Fe from soil and an increased Fe transport from leaves. Consistent with this hypothesis, leaf iron content was reduced by ∼40% when compared to that of nontransgenic leaves (data not shown).

**Impact of High Iron Accumulation on Seed Germination and Seedling Vigor.** To determine whether enhanced Fe accumulation in transgenic seeds can lead to improved seed function and plant growth, we analyzed germination rates and seed dry weights of transgenic *lpa1-1* lines from Figure 5. Seeds of transgenic *lpa1-1* lines 26−5−7 and 32−2-1 showed superior performance and germinated normally by day 7, with a frequency of 100% in filter-paper germination tests, whereas germination of the nontransgenic *lpa1-1* was delayed 1 day

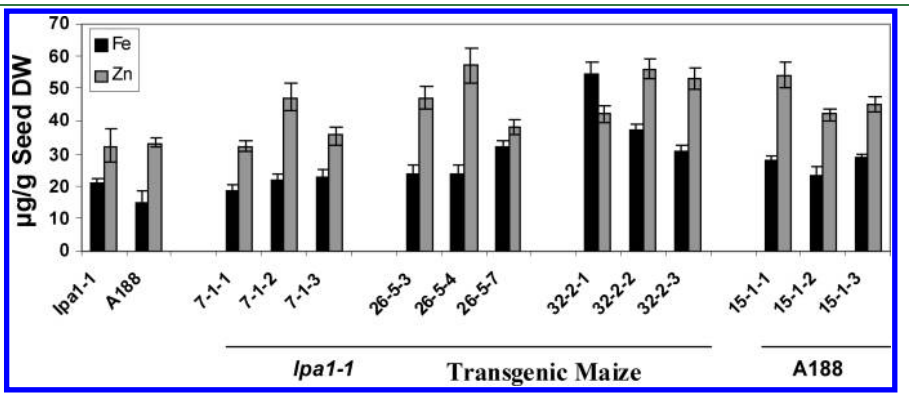



**Figure 5.** Analysis of $T_3$ seeds. Fe and Zn contents of transgenic *lpa1-1* maize plants 7−1, 26−5, 32−2, and A188 maize line 23−4 were measured by ICP. Three individual ears from each maize line were analyzed. Data represents the mean ± SD of triplicate samples from five bulked seeds from each line. Nontransgenic A188 and *lpa1-1* are shown as controls.

**Table 3. Expression Analysis of Representative Genes Mediating Plant Fe Homeostasis**[a]

| | root | | | leaf | | | seed | | |
|---|---|---|---|---|---|---|---|---|---|
| line # gene | 26 | 32 | 23 | 26 | 32 | 23 | 26 | 32 | 23 |
| *NAAT* | −2 | −2.3 | −4 | 2 | 2 | 2.5 | | | |
| *DMAS1* | −2.5 | −3 | −2.8 | −50 | −70 | −68 | | | |
| *NAS2* | 1 | 1 | 1 | −23 | −9 | −7.6 | | | |
| *NAS3* | 2.1 | 2.3 | 2.6 | 8.4 | 4 | 2 | | | |
| *FDR3* | −4.8 | −4.4 | −3.7 | 16 | 30 | 12 | | | |
| *YS1* | −2.2 | 1 | 1 | 51 | 53 | 58 | −5 | −6 | −25 |
| *FM1* | 1 | 1 | 1 | −6.6 | −7.5 | −8.9 | 4 | 1 | 1 |
| *FM2* | 1 | 1 | −2.5 | −3.2 | −5.8 | −5.1 | 8.1 | 3.6 | 1 |

[a] Total RNA was isolated from $T_3$ plants and seeds of transgenic *lpa1-1* (lines 26-5 and 32-2) and A188 (line 23-4) maize lines. Transcript levels were measured by qPCR using gene-specific primers, and the data were normalized to actin expression as a control. Values represent the mean relative expression levels of determinations from two separate experiments conducted with pooled samples. Numbers represent the induction or repression of genes in transgenic maize relative to their respective non-transgenic control maize line. *NAAT*, nicotianamine aminotransferase; *DMAS1*, deoxymugeneic acid synthase 1; *NAS2*, nicotianamine synthase 2; *NAS3*, nicotianamine synthase 3; *FDR3*, Fe-deficiency response 3; *YS1*, yellow stripe 1; *FM1*, ferritin 1; *FM2*, ferritin 2.

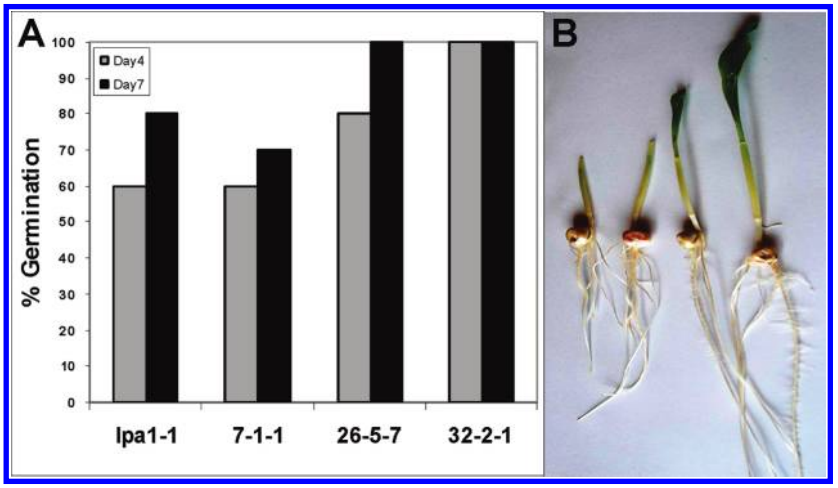



**Figure 6.** Seed germination test. (A) Germination of nontransgenic *lpa1-1* and $T_3$ seeds of transgenic *lpa1-1* maize 7−1-1, 26−5−7, and 32−2−1 using the filter-paper germination test method. Values represent the mean % germination of two experiments. (B) Representative seedlings of transgenic and nontransgenic *lpa1-1* at day 7. From left to right: nontransformed *lpa1-1*, 7−1-1, 26−5−7, and 32−2−1.

relative to the transgenic seeds, and the germination frequency reached a maximum of 80% by day 7 (Figure 6A). Furthermore, these transgenic plants showed increased seedling vigor with longer roots and early expanded leaves when compared to those of the nontransgenic plants (Figure 6B). In contrast, transgenic maize line 7−1−1 germinated at a rate similar to the control plants and did not have emerging and/or expanding leaves by day 7. The average seed dry weight was similar to the nontransgenic *lpa1-1* (data not shown).

## DISCUSSION

***Lpa1-1* Mutant of Maize as a Tool for Iron Biofortification.** Combating IDA with iron biofortification of staple food crops remains a major challenge due to the poor absorption of iron from cereal-based diets high in phytate. Bioavailability studies conducted with conventionally bred cultivars of maize and Fe-biofortified rice varieties showed that there was no significant correlation between Fe content and bioavailability.[13,14,55] Thus, increasing Fe content in plants either nonspecifically or through ferritin overexpression alone may not address the problem. Consistent with this notion, our studies show no significant correlation(s) between ferritin concentrations and bioavailability, and between Fe content and bioavailability in the A188 transgenic maize lines with high phytate content (Table 1). However, experiments conducted in rice to reduce phytate levels by overexpressing a heat-stable phytase were not successful, as the phytate levels were not reduced significantly, and the phytase was inactive at high temperatures.[19] To address the problem of thermotolerance of heterologous phytases in planta, Brinch-Pederson et al.[34] generated transgenic wheat with two different heat-stable phytases (*Aspergillus fumigatus* phytase and a synthetically generated phytase with high thermotolerance of 89.3 °C). However, both of these phytases were observed to be susceptible to high temperatures and showed significant reductions in activity with only 8−12% remaining after 20 min of boiling. Although transgenic maize expressing both ferritin and a fungal phytase[20] showed a significant reduction in seed phytate content and improved iron bioavailability, the phytase used in this study was not heat stable, and further food processing was necessary

for the enzyme to be active. Furthermore, these transgenic approaches in maize led to a maximum of only 1.7-fold improvement in Fe content (~30−35 μg/g).

We have taken advantage of the already existing homozygous *lpa1-1* mutants of maize to generate transgenic lines with increased Fe content and bioavailability, by overexpressing the soybean ferritin gene in an endosperm-specific manner using a modified and highly active γ-zein promoter.[40] This strategy not only overcomes difficulties associated with heterologous phytase expression and activity in transgenic plants (for example, heat stability) but also affords a parent line with a consistent reduction in seed phytate content. Although prior studies demonstrated a requirement for 90% reduction in seed phytate level for major improvements in Fe bioavailability,[56] studies conducted with *lpa1-1*[39] suggest that even a 60% reduction in seed phytate may have a significantly positive effect on Fe bioavailability. This level of reduction in seed phytate content could also represent a good compromise with respect to plant health, disease prevention,[14,57,58] and for combating iron deficiency in humans. In our study, both transgenic *lpa1-1* and A188 seeds showed increased Fe bioavailability in comparison to their respective nontransgenic controls, suggesting that ferritin-bound iron maybe a bioavailable form of iron (Figures 3C and 4). However, overall mean Fe bioavailability of transgenic *lpa1-1* seed was significantly higher than that of transgenic A188 and the nontransgenic seeds, indicating that both increased Fe content and reduced phytate levels are important for enhanced Fe bioavailability.

The level of seed iron content (~60−70 μg/g) achieved in this study greatly exceeds any thus far in maize (Figure 3), and these levels of iron were stable at least through the $T_3$ generation in some of the transgenic maize lines analyzed. The fact that bulked seeds were used for analyses of $T_3$ seeds suggests that Fe levels may have been higher than that observed in Figure 5. However, there was great variability in Fe content between individually transformed $T_2$ and $T_3$ lines as well as individual seeds from a single ear (Figures 3A and 5). Variation in transgenic maize is a common phenomenon[40,42] and maybe attributed to the germplasm used for transformation, transgene copy number, or to changes in the expression of genes (epigenetic effects). Other researchers have reported similar variation in transgenic maize and proceeded with the selection of lines that showed the highest levels of expression for subsequent generations.[42] Nevertheless, our studies indicate that the high-iron trait is heritable and can be maintained through generations. An exception to the stable inheritance of the high-Fe trait is the transgenic *lpa1-1* line 7−1, which reverted back to the phenotype of nontransgenic plants. Although we do not know the exact nature of this reversion, it is likely the result of gene deletion as genomic PCR did not reveal the presence of the soybean ferritin gene in $T_2$ plants of line 7−1 (data not shown).

Mendoza et al.[39] showed that ~2% of Fe is absorbed from maize with normal phytate content, whereas ~3% Fe is absorbed from *lpa1-1* maize. These results were corroborated in our studies (Figures 3C and 4). Taking into account the increased Fe content (~50−60 μg/g) of $T_3$ seeds of *lpa1-1* transgenic plants and assuming an average absorption of 3% from these maize seeds, an estimated 0.45−0.54 mg of Fe could be absorbed in humans with 300 g consumption of maize. Moreover, the 2-fold increase in the mean Fe bioavailability of transgenic *lpa1-1* compared to the nontransgenic *lpa1-1* seed (Figure 4) indicates that Fe absorption from the transgenic seeds may be as high as ~0.9−1.08 mg. Thus, two-thirds of the daily Fe requirement for women could be met by consuming 300 g of transgenic *lpa1-1* maize.

One long-standing concern in using the *lpa* mutants is poor seed quality and crop yield.[35−38] However, previous reports show that increasing seed mineral concentrations has a beneficial effect on crop productivity, seedling vigor, and viability.[13,14,59,60] This effect has been attributed to the production of more and longer roots in seedlings under micronutrient-deficient conditions. Although the seed germination tests we presented were performed with a small group of seeds and thus should be considered preliminary, our results are consistent with previous reports and showed that enhanced Fe accumulation in transgenic *lpa1-1* maize leads to enhanced seed germination rates and more vigorous seedling growth when compared to nontransgenic *lpa1-1* germplasm (Figure 6). Furthermore, the results observed for line 7−1−1 support our contention that seed Fe content is important to improved seed germination and seedling vigor. These findings largely mitigate the negative agronomic characteristics associated with the *lpa* mutants and improve their value to plant breeders.

***YS1* Expression Is Enhanced in Leaves of Transgenic Maize.** A previous study with transgenic rice expressing soybean ferritin gene in their endosperm suggests that Fe uptake by roots may be a limiting factor for proportional enhancement of Fe content in seeds of staple food crops.[61] This study also showed that transgenic rice leaves contained approximately half the normal levels of Fe. However, the underlying molecular basis for this long distance control by the shoot and/or interorgan signaling in transgenic crops was previously not explored. Our results (Table 3) suggest that a reduction in leaf Fe content may be due to enhanced expression of two important Fe-transport genes,[7,8] *YS1* and *FDR3*, in the leaves of transgenic maize, which perhaps results in an increase in Fe-export from leaves. Furthermore, the repression of Fe acquisition genes, for example, *DMAS1*, in roots of the transgenic plants is striking and suggests that enhanced accumulation of Fe in the seeds is not due to increased root Fe uptake, at least under the conditions tested. In support of this hypothesis, studies in transgenic rice showed that synergistic overexpression of *NAS* and soybean ferritin genes results in a 6−7-fold increase in Fe content,[21] which is significantly higher than that achieved in rice previously by ferritin overexpression alone.[19] In contrast to *NAS2* expression, *NAS3* is induced in roots and leaves of transgenic maize *lpa1-1* and A188 (Table 3). *NAS3* has been shown to be expressed under Fe-sufficient conditions and to synthesize nicotianamine (NA) to transport NA−Fe complexes.[54] Consistent with this notion, our studies show dramatic induction of the *YS1* gene, which functions to transport NA−metal complexes into and out of cells.[7,54] Taken together, these studies improve our understanding of plant iron homeostasis and the probable limiting factors that may be used for further enhancing seed iron concentrations in staple food crops.

Although more detailed human nutritional and field studies are necessary to corroborate the beneficial effects of high-Fe transgenic *lpa1-1* to nutritionists and plant breeders, the present study has provided proof-of concept of the considerable potential these plants have for enhancing the nutritional quality of maize and for improving agronomic traits of commercial value.

## ■ ASSOCIATED CONTENT

**Ⓢ Supporting Information.** Primers used for qPCR studies to determine the expression of the eight well-characterized genes

of Fe homeostasis in transgenic and nontransgenic maize. This material is available free of charge via the Internet at http://pubs.acs.org.

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