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Recurrent selection to alter seed phytic acid content and iron bioavailability in maize

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**Recurrent selection to alter seed phytic acid content
and iron bioavailability in maize**

by

Alyssa Beavers

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Molecular and Cellular Nutrition

Program of Study Committee:
Manju Reddy, Co-Major Professor
Paul Scott, Co-Major Professor
Kevin Schalinske

Iowa State University

Ames, Iowa

2014

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TABLE OF CONTENTS

	Page
LIST OF FIGURES	iv
LIST OF TABLES	v
ACKNOWLEDGEMENTS	vi
ABSTRACT	vii
CHAPTER 1 GENERAL INTRODUCTION	1
INTRODUCTON.....	1
THESIS ORGANIZATION.....	2
AUTHORS' ROLES.....	2
REFERENCES	3
CHAPTER 2. LITERATURE REVIEW	5
IMPORTANCE OF IRON IN HUMANS	5
Oxygen Transport and Storage	5
Iron Transport and Storage	5
Catalytic	6
IRON REQUIREMENTS	7
Absorbed Iron Requirements	7
Dietary Iron Requirements.....	9
IRON DEFICIENCY AND ANEMIA	11
Prevalence and Causes	11
Adverse Effects	13
IRON ABSORPTION	15
IRON HOMEOSTASIS	17
Systemic Iron Homeostasis.....	17
Cellular Iron Homeostasis.....	18
IRON BIOAVAILABILITY	19
Inhibitors	20
Enhancers	22
METHODS TO ASSESS IRON BIOAVAILABILITY	24
Human	24
Animal	25
Cell Culture.....	27
STRATEGIES TO IMPROVE IRON STATUS	27
Supplementation	27

Fortification.....	28
Biofortification.....	29
LPA MAIZE	34
Iron Bioavailability of <i>lpa</i> Maize.....	37
Recurrent Selection.....	37
SUMMARY	38
REFERENCES	39
CHAPTER 3. RECURRENT SELECTION TO ALTER GRAIN PHYTIC ACID CONCENTRATION AND IRON BIOAVAILABILITY	51
ABSTRACT.....	51
INTRODUCTION	52
MATERIALS AND METHODS.....	54
Breeding Strategy.....	54
Measurement of Phytic Acid and Available P for Recurrent Selection.....	55
Evaluation of Three Cycles of Selection for High or Low Phytic Acid Concentration.....	56
Seed Quality Evaluation: Standard Germination and Cold Tests.....	57
Iron Bioavailability	58
Phytic Acid Analysis of Bulked Maize Samples	61
Iron Analysis of Bulked Maize Samples	61
Data Analysis.....	62
RESULTS AND DISCUSSION.....	62
Relative Phytic Acid and Available P Concentrations for Recurrent Selection.....	62
Seed Quality.....	64
Iron Bioavailability, Phytate, and Iron of Bulked Maize Samples	65
CONCLUSIONS.....	66
REFERENCES	72
CHAPTER 4. SUMMARY AND CONCLUSIONS	75
SUMMARY	75
CONCLUSIONS.....	76
APPENDIX IRON BIOAVAILABILITY OF MAIZE HEMOGLOBIN IN A CACO-2 CELL CULTURE MODEL	77

LIST OF FIGURES

	Page
Figure 2.1 Dietary iron uptake	16
Figure 2.2 Iron homeostasis regulated by hepcidin	18
Figure 2.3 Regulation of transferrin receptor and ferritin expression by IRE's and IRP's.....	19
Figure 3.1 Iron bioavailability of bulks	71

LIST OF TABLES

	Page
Table 2.1 The recommended nutrient intakes (RNI's) for iron for different dietary iron bioavailabilities (mg/day).....	9
Table 2.2 Recommended dietary allowances (RDA's) for iron by population group	11
Table 2.3 Per capita consumption of meat from 1997-1999	12
Table 2.4 Anemia prevalence (%) in preschool-age children, pregnant women, and non-pregnant women in each WHO region	13
Table 2.5 Summary of <i>lpa</i> maize mutants	36
Table 3.1 Evaluation of relative standardized phytic acid	68
Table 3.2 Evaluation of seed quality	69
Table 3.3 Phytic Acid and Iron content of bulked maize samples.....	70

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ABSTRACT

Most of the phosphorus (P) in cereal grains is in the form of phytic acid, a potent inhibitor of iron absorption that cannot be digested by monogastric livestock or humans. High phytate content in staple crops contributes to the high incidence of iron deficiency in developing countries. Low phytic acid (*lpa*) maize mutants are seen as a potential strategy to improve iron bioavailability, but they have poor seed quality. Our objective was to develop both low and high phytic acid maize populations using recurrent selection, and to compare agronomic qualities and iron bioavailability of these two types of maize, as well as compare them with *lpa* mutant maize. Three cycles of selection were carried out in two broad-based synthetic populations, BS11 and BS31. Our research found that recurrent selection produced a significant difference in phytic acid content between the high and low BS11 populations ($P < 0.05$), but not in the BS31 populations ($P > 0.05$). The BS11LPA maize population had improved germination relative to *lpa* mutant inbred lines (13-16%, $P < 0.05$), but had similar iron bioavailability ($P > 0.05$).

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Anemia afflicts nearly a quarter of the world's population, 1.62 billion people, and it is estimated that half of anemia cases are due to iron deficiency (WHO, 2008). Iron deficiency is most prevalent in women of child-bearing age, pregnant women, infants, and children (WHO, 2008). Child development is impacted by iron deficiency due to its increased risk during the crucial periods of pregnancy and early childhood. Negative pregnancy outcomes are associated with maternal iron deficiency anemia (Klebanoff, et al., 1991, Scholl and Hediger, 1994, Steer, 2000), as well as brain development in iron-deficient babies (Grantham-McGregor and Ani, 2001, Lozoff, et al., 2006).

Dietary iron deficiency is not caused solely by a low quantity of iron in the diet, but also low iron bioavailability from the diet. Heme iron, found in animal foods, has a relatively high iron bioavailability when compared with nonheme iron, the only type of iron found in plant foods. Non-heme iron bioavailability is inhibited by various factors found in plants, but most notably phytate, which is abundant in cereal grains and legumes. Diets in developing countries are high in these staple crops and low in animal flesh foods, putting people in these countries at greatest risk of iron deficiency (WHO, 2001).

Because of phytate's negative effect on iron absorption, low phytic acid (*lpa*) mutants of many crops have been developed, including maize. These mutants have a large reduction in phytic acid content and have been shown to have improved iron bioavailability (Mendoza, et al.,

1998), but have poor agronomic qualities (Raboy, 2009). An alternative method to produce maize low in phytic acid would be through selective breeding. This could potentially allow for a reduction in seed phytic acid content, improved iron bioavailability, and no reduction in agronomic qualities. The aim of this thesis was to determine if recurrent selection, a method of selective breeding, could produce maize with a reduction in seed phytic acid content that has improved iron bioavailability, without harming seed quality.

Thesis Organization

This thesis consists of a literature review on the importance of iron, iron deficiency, iron bioavailability, biofortification, and *lpa* maize. Following the literature review is a paper entitled “Recurrent Selection to Alter Grain Phytic Acid Concentration and Iron Bioavailability.” This study examined the use of recurrent selection to alter phytic acid concentration in maize, and the comparison of seed quality and iron bioavailability of the resulting populations with *lpa* maize mutants. Following that is an overall conclusion of the thesis. The appendix contains a published manuscript that I am a co-author on.

Authors’ Roles

Throughout the duration of my master’s degree, I worked on a number of projects not included in this thesis. I performed iron bioavailability analysis on a fungal iron supplement, as well as assisted in conducting a human iron absorption study of this iron supplement. I also assisted in a human iron absorption study that examined adaptation of iron absorption to both high and low phytic acid diets.

Chapter 3 of this thesis was the focus of my master’s work, and was a collaborative effort that involved the work of many people. Dr. Susana Goggi performed the seed quality analysis for this project, wrote the methods for the seed quality analysis, and edited the manuscript. Dr.

Adrienne Moran-Lauter performed the phytic acid analysis and available phosphorus analysis. Dr. Paul Scott developed the breeding program, oversaw the project, and edited the manuscript. Dr. Manju Reddy oversaw the iron bioavailability portion of the project and edited the manuscript. My role in this project was performing the iron bioavailability work, analyzing iron and phytic acid content of the bulked samples, performing data analysis, and writing the manuscript.

My role in the manuscript found in the appendix of this thesis was performing the iron bioavailability work of the transformed corn and analyzing the data for this portion of the project.

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CHAPTER 2

LITERATURE REVIEW

Importance of Iron in Humans

Iron is essential for many processes within the human body. It is most commonly known for its role in the oxygen-carrying protein hemoglobin, but its functions reach far beyond oxygen transport. Iron is also a required co-factor in many enzymes in the body. Iron proteins can be classified in a number of ways, including by function of the iron atom (Crichton, 2009). Functionally, they may be divided into the categories of metal storage and transport, dioxygen (O_2) transport and storage, and catalytic (Crichton, 2009). The catalytic proteins can be subdivided by structure: heme, iron-sulfur, and nonheme/non-iron-sulfur.

Oxygen Transport and Storage

Iron transports and stores O_2 as part of the cofactor of the proteins hemoglobin and myoglobin. Nearly two-thirds of the body's iron is in hemoglobin (Pollycove and Mortimer, 1961), so named because it consists of a globin protein portion and heme, a protoporphyrin ring with a ferrous iron atom (Fe^{2+}) at the center. This red blood cell protein carries O_2 to all tissues of the body. In skeletal muscle, O_2 is transferred from hemoglobin to myoglobin, also a hemoprotein. Myoglobin temporarily stores O_2 , and shuttles it to the mitochondria to be utilized in the electron transport chain (Kamga, et al., 2012).

Iron Transport and Storage

Iron storage and transport are carried out by ferritin and transferrin, respectively. Transferrin is capable of binding two atoms of ferric iron, Fe^{3+} , and is responsible for carrying absorbed iron from the intestine to the liver via the blood stream. In the liver, excess iron is

stored in ferritin in its ferrous form, Fe^{2+} . The ferritin protein is essentially a hollow shell that can be filled with 2500-3000 atoms of Fe^{2+} . When this stored iron is needed by the body, iron is transferred from ferritin back to transferrin, which transfers it to its target location, again via the bloodstream (Jin and Crichton, 1987). These two proteins maintain iron homeostasis under direction of the peptide hormone hepcidin, the key regulator of iron status (Ganz, 2013). Iron homeostasis is described in detail in a later section.

Catalytic

The remaining iron proteins are enzymes in which iron plays a catalytic role. They can be subdivided by their structure into hemoenzymes, iron-sulfur enzymes, and nonheme/non-iron-sulfur enzymes.

Hemoenzymes

Enzymes containing a heme group are referred to as hemoenzymes (Crichton, 2009). Cytochrome oxidase is a well-known hemoenzyme because of its role in catalyzing the last step in the electron transport chain, the reduction of molecular oxygen to water. Other hemoenzymes include catalase, which converts hydrogen peroxide to water and oxygen; myeloperoxidase, in plasma and neutrophils and helps in the destruction of pathogens; and thyroperoxidase which is necessary for T3 and T4 production (Crichton, 2009). A number of cytochromes are also hemoproteins and are involved in the electron transport chain lipid metabolism, and detoxification of harmful substances (Crichton, 2009).

Iron-sulfur Proteins

In a number of enzymes, iron and sulfur form clusters which are used as co-factors to transfer electrons involved in chemical reactions (Netz, et al., 2013). These enzymes are involved in the TCA cycle, DNA replication and repair, amino acid metabolism, ribosome

function and tRNA modification, intracellular iron homeostasis and regulation, generation of the 5'-deoxyadenosyl radical from S-adenosyl methionine, and Ca²⁺ metabolism (Netz, et al., 2013).

Nonheme/Non-iron-sulfur

The last category of iron-containing catalytic proteins is nonheme/non-iron-sulfur containing proteins. This group is a catch-all for proteins that do not fit into the other groups, and thus is quite diverse. This group contains enzymes involved in amino acid metabolism, myelination of oligodendrocytes in the brain, and in neurotransmitter synthesis (Kwik-Urbe, et al., 2000, Yehuda and Youdim, 1989).

Iron Requirements

Absorbed Iron Requirements

Absorbed iron requirements vary depending on sex, age, and pregnancy/lactation status. In non-pregnant, non-lactating adults, iron requirements are equal to the amount of iron excreted. Iron is lost through urine and by sloughing of intestinal cells and skin, and these are considered basal losses, which are estimated at 14µg/kg body weight/day (Green, et al., 1968). In addition to basal losses, iron lost in menstrual blood must count toward iron needs in menstruating women, therefore their iron needs are much higher than those of men. Menstruating women require approximately 1.5-1.7 mg of absorbed iron daily (Hallberg and Rossander-Hulten, 1991, Hunt, et al., 2009), while men require approximately 1-1.2 mg (Green, et al., 1968, Hunt, et al., 2009) Requirements of post-menopausal women are similar to those of men (Hunt, et al., 2009).

Full-term infants are born with a sizable iron store and a high hemoglobin concentration. They mobilize these iron sources during the first six months of life, which provide nearly all iron needed during this time for exclusively breastfed infants. Breast milk provides a small amount of additional iron, 0.3 mg/100 mL, which is highly bioavailable and ample to meet iron needs

(Domellof, 2011). After the first six months, when they are no longer exclusively breastfed, an average baby will need an additional 200 mg of total body iron by the time they reach 12 months, which is equivalent to 0.1 mg/kg/day of absorbed iron (Domellof, 2011, Oski, 1993).

For children and adolescents, iron requirements are calculated by adding what is needed for growth to iron losses. The majority of the iron needed for growth goes to increases in hemoglobin associated with an increase in blood volume, but a small amount also goes to increases in tissue iron and, for children under 8, towards storage iron. Iron needs steadily increase with age until they reach a peak in the early teen years at approximately 30 $\mu\text{g}/\text{kg}$ body weight/day, twice that needed by adults. Iron needs then begin to decrease as growth slows, until they reach the adult iron requirement around age 18 (National Research Council, 2001).

During pregnancy, iron requirements must account for fetal and placental iron deposition and expanded blood volume of the mother. Little iron is passed into the fetus and placenta in the first trimester, and expansion of blood volume does not begin until the second trimester. During this time the iron requirement is estimated at 1.2 mg/day, which is actually lower than the requirement for non-pregnant women since menstruation ceases during pregnancy (National Research Council, 2001). However, during the second trimester iron needs increase drastically to 4.7 mg due to increased blood volume (National Research Council, 2001), and then even higher to 5.6 mg in the third trimester since iron is passed to the fetus during the last ten weeks of pregnancy (Cao and O'Brien, 2013). During lactation, iron needs take into account basal loss plus iron in breast milk, which is approximately 1.2 mg/day (National Research Council, 2001).

Dietary Iron Recommendations

Not all iron that is ingested is absorbed, which must be taken into account when setting dietary recommendations. In setting recommended dietary iron intakes, the WHO categorized diets into four iron bioavailabilities: 15%, 12%, 10%, and 5% (WHO/FAO, 2004):

- 15%: Western-type diet high in fruits and vegetables, and meat or fish
- 12%: typical Western-type diet moderate in fruits and vegetables, and meat or fish
- 10%: diets consisting mainly of starchy staples (cereals, roots, and tubers), with some meat, fish, and/or ascorbic acid-rich foods.
- 5%: diets consisting almost entirely of starchy staples, high in iron inhibitors, and negligible meat, fish, or ascorbic acid-rich foods

In **Table 2.1**, iron recommendations for each level of iron bioavailability are given, based on the 95th percentile of absorbed iron needs:

Table 2.1 The recommended nutrient intakes (RNIs) for iron for different dietary iron bioavailabilities (mg/day). From World Health Organization (2004).

Group	Age (years)	Mean body weight (kg)	Recommended nutrient intake (mg/day) for a dietary iron bioavailability of			
			15%	12%	10%	5%
Infants and children	0.5-1	9	6.2 ^a	7.7 ^a	9.3 ^a	18.6 ^a
	1-3	13	3.9	4.8	5.8	11.6
	4-6	19	4.2	5.3	6.3	12.6
	7-10	28	5.9	7.4	8.9	17.8
Males	11-14	45	9.7	12.2	14.6	29.2
	15-17	64	12.5	15.7	18.8	37.6
	18+	75	9.1	11.4	13.7	27.4
Females	11-14 ^b	46	9.3	11.7	14.0	28.0
	11-14	46	21.8	27.7	32.7	65.4
	15-17	56	20.7	25.8	31.0	62.0
	18+	62	19.6	24.5	29.4	58.8
Postmenopausal		62	7.5	9.4	11.3	22.6
Lactating		62	10.0	12.5	15.0	30.0

^aBioavailability of dietary iron during this period varies greatly.

^bPre-menarche

The United States also has their own set of dietary iron recommendations, called the recommended dietary allowances (RDA's), based on age, sex, and physiological state (pregnancy and lactation). The 97.5th percentile of absorbed iron needs are used and then multiplied by a bioavailability factor. For children over one year, adolescents, and non-pregnant adults, these recommendations assume an iron bioavailability of 18% (National Research Council, 2001). This is determined by first estimating the proportions of nonheme and heme iron in the American diet, 90% and 10% respectively (Raper, et al., 1984). Bioavailability of heme iron was conservatively estimated to be 25% (Hallberg and Rossander-Hulten, 1991). The bioavailability of nonheme iron was estimated by measuring nonheme iron bioavailability in the customary diets of Americans, and then correcting this value to a serum ferritin concentration of 15 µg/mL (Cook, et al., 1991). This resulted in an estimated nonheme iron bioavailability of 16.8%. This leads to an overall bioavailability of iron in the American diet of approximately 18%:

Overall iron absorption = (Fraction of nonheme iron [0.9] x proportion of nonheme iron absorption [0.168]) + (Fraction of heme iron [0.1] x proportion of heme iron absorption [0.25]) × 100 = 17.6 percent (National Research Council, 2001).

The RDA's for iron are included in **Table 2.2:**

Table 2.2 Recommended dietary allowances (RDA's) for iron by population group. From National Research Council (2001).

Age	RDA (mg/day)	
	Males	Females
0-6 months	.27 (AI)*	.27 (AI)
7-12 months	11	11
1-3 years	7	7
4-8 years	10	10
9-13 years	8	8
14-18 years	11	15
19-50 years	8	18
51+	8	8
Pregnancy		27
Lactation		9

*Adequate intake

Iron Deficiency and Anemia

Iron deficiency is defined by the WHO as “a condition in which there are no mobilizable iron stores and in which signs of a compromised supply of iron to tissues, including the erythron, are noted” (WHO, 2001). Iron deficiency may lead to anemia, but iron deficiency can occur before anemia is present. Negative consequences as a result of iron deficiency can be seen before clinical anemia. Anemia can have devastating consequences such as pregnancy complications, decreased cognitive development of children, and reduced work productivity in adults (WHO, 2001).

Prevalence and Causes

The worldwide prevalence of iron deficiency is difficult to measure, but it is known that it is the most prevalent nutrient deficiency in the world (WHO, 2001). Serum ferritin, an indicator of iron storage, is currently considered an accurate marker of iron deficiency in the absence of inflammation (serum ferritin can become elevated during inflammatory conditions, even when the subject is iron deficient) (Goddard, et al., 2011). However, serum ferritin is not routinely tested in developing countries because of its cost, so anemia is used as an indirect

measure of iron deficiency (WHO, 2001). Anemia is characterized by hemoglobin (Hb) concentration below a certain threshold, dependent on age and sex. The latest estimate of anemia prevalence is 1.62 billion people, or almost 25% of the world's population (WHO, 2008). Anemia prevalence in different population groups is 47.4% of preschool-age children, 41.8% of pregnant women, 30.2% of non-pregnant women, and 12.7% of men (WHO, 2008). Iron deficiency is responsible for half of anemia cases, while the other half is attributed to parasitic infections and deficiencies of other micronutrients (WHO, 2001).

The biggest risk factors for iron deficiency anemia are low intake of iron, high intake of inhibitors of iron absorption, and high iron needs during growth and pregnancy (WHO, 2008). The first and second of these risk factors are especially prevalent in developing countries where the rate of poverty is high. Income has a strong positive relationship with consumption of animal foods, particularly meat (WHO/FAO, 2003). The per capita meat consumption for different regions of the world is shown in **Table 2.3**. The poorest regions of the world, including Sub-Saharan Africa and South Asia, have much lower meat consumption than industrialized countries.

Table 2.3 Per capita consumption of meat from 1997-1999. Adapted from WHO/FAO (2003).

Region	Meat (kg/year)
World	36.4
Developing countries	25.5
Near East and North Africa	21.2
Sub-Saharan Africa ^a	9.4
Latin America and the Caribbean	53.8
East Asia	37.7
South Asia	5.3
Industrialized countries	88.2
Transition countries	46.2

^a Excludes South Africa

Meat is amongst the most iron-rich foods, so low meat intake typically implies a low total intake of iron. It is therefore not surprising that regions that consume the least meat also tend to have the highest prevalence of anemia. In **Table 2.4**, anemia prevalence in different regions of the world is given, subdivided into population groups that are most at-risk of anemia: preschool-age children, pregnant women, and non-pregnant women. It is evident that areas with the lowest meat consumption, Africa and South-East Asia, have the highest incidences of anemia.

Table 2.4 Anemia prevalence (%) in preschool-age children, pregnant women, and non-pregnant women in each WHO region. Adapted from WHO (2008).

WHO Region	Preschool-age children	Pregnant women	Non-pregnant women
Africa	67.6	57.1	47.5
Americas	29.3	24.1	17.8
South-East Asia	65.5	48.2	45.7
Europe	21.7	25.1	19.0
Eastern Mediterranean	46.7	44.2	32.4
Western Pacific	23.1	30.7	21.5
Global	47.4	41.8	30.2

In addition to having diets low in meat, people in developing countries consume over half their calories from cereals compared with only 30% in industrialized countries (WHO/FAO, 2003).

Cereal grains, including rice, wheat, and maize, have high levels of inhibitors of iron absorption.

Adverse Effects

Iron deficiency, both with and without anemia, has negative consequences on both physical and mental health. Since the risk of iron deficiency is highest amongst pregnant women and children, many of the known adverse health effects are related to maternal health and child development. However, non-pregnant adult women are susceptible to the effects of iron deficiency as well.

Iron deficiency during pregnancy is particularly dangerous to both the mother and the child. Maternal iron deficiency anemia is associated with inadequate gestational weight gain

(Scholl and Hediger, 1994), preterm delivery (Klebanoff, et al., 1991), and low birth weight babies (Steer, 2000). Prenatal iron supplementation has been shown to decrease these complications (see section on supplementation below).

For the child, brain development is particularly impacted by maternal iron deficiency. Brain development begins in utero, and remains quite active during the infant and toddler years. Iron deficiency in this period can have lasting effects, even if iron status is corrected later. Animal studies have elucidated some of the manners in which iron deficiency affects brain development. In rats, iron deficiency during pregnancy led to decreased brain iron in the fetuses, and disrupted prenatal oligodendrocyte genesis (Morath and Mayer-Proschel, 2002). Another study in rats suggests that maternal iron deficiency results in damage to dopaminergic neurons in the offspring. This led to behavioral impairments that lasted even after the rat pups had received iron supplementation and hemoglobin had normalized (Felt, et al., 2006). Studies in humans indicate that mental development in babies with iron deficiency anemia is lower than in iron replete babies (Lozoff, et al., 2006). They also indicate that this damage is permanent, because behavior and development is still altered after receiving iron therapy (Grantham-McGregor and Ani, 2001).

In adults, aerobic activity is negatively affected during iron deficiency. Anemic women's capacity to perform a set workout on a treadmill was lower than non-anemic women (Gardner, et al., 1977). Anemia has also been shown to reduce VO_{2max} (maximal oxygen consumption), a measure of aerobic capacity (Crouter, et al., 2012). This reduced aerobic capacity results in an overall reduction in physical activity in people with iron deficiency, even during light activity. A study of jute-factory workers (a non-strenuous job) found that anemic women were 5% less productive at work than their non-anemic counterparts, and performed 6.5 fewer hours of house

work per work (Scholz, et al., 1997). Iron deficient women also spend more sedentary time and less time in light activity than non-iron deficient women (Blanton, et al., 2013).

Cognitive and affective function are both impacted by iron deficiency in adults. Iron status in women was found to be correlated with measures of cognitive executive planning function (Blanton, et al., 2013) Serum ferritin was found to be lower in a group of depressed students than non-depressed students (Vahdat Shariatpanaahi, et al., 2007). This could potentially be due to iron's role in neurotransmitter synthesis, particularly dopamine (Bianco, et al., 2008)

Iron Absorption

Iron is absorbed in the duodenum of the small intestine (**Figure 2.1**). Heme iron, found only in animal foods, and nonheme iron, found in both animal and plant foods, are absorbed via different mechanisms. The majority of nonheme iron is present in the ferric form, but must be reduced to the ferrous form prior to uptake into the enterocyte (Raja, et al., 1992). This reduction is accomplished by an enzyme on the brush-border membrane of the enterocyte called duodenal cytochrome b (DcytB). Ferrous iron is transported into the enterocyte by the protein divalent metal transporter 1 (DMT1) (Gunshin, et al., 1997). This enzyme transports a number of other divalent metals into the enterocyte, including zinc and manganese.

Heme is transported into the enterocyte by endocytosis after binding to heme carrier protein 1 (HCP1). The location of HCP1 is regulated by iron status, with the protein being found primarily on the apical membrane in iron deficiency, and movement to the cytoplasm in iron sufficient conditions (Shayeghi, et al., 2005). Within the enterocyte, ferrous iron is cleaved from heme by the enzyme heme oxygenase, whose activity is increased in iron deficiency in order to increase the amount of iron absorbed into circulation (Raffin, et al., 1974).

Within the enterocyte, ferrous iron from heme and nonheme iron sources has three potential fates: it can be stored as ferritin, transported across the basolateral membrane, or used locally by the enterocyte. When iron status is adequate, much of it is bound to ferritin in the enterocyte, which prevents excess iron being absorbed (Bernier, et al., 1970). When the enterocytes are sloughed off, the iron in ferritin is excreted in the feces. Iron that is needed by the body is transported out of the enterocyte by the transport protein ferroportin1 (Donovan, et al., 2000, McKie, et al., 2000). It is then oxidized by hephaestin to Fe^{3+} before being transferred to transferrin in the blood (Vulpe, et al., 1999).

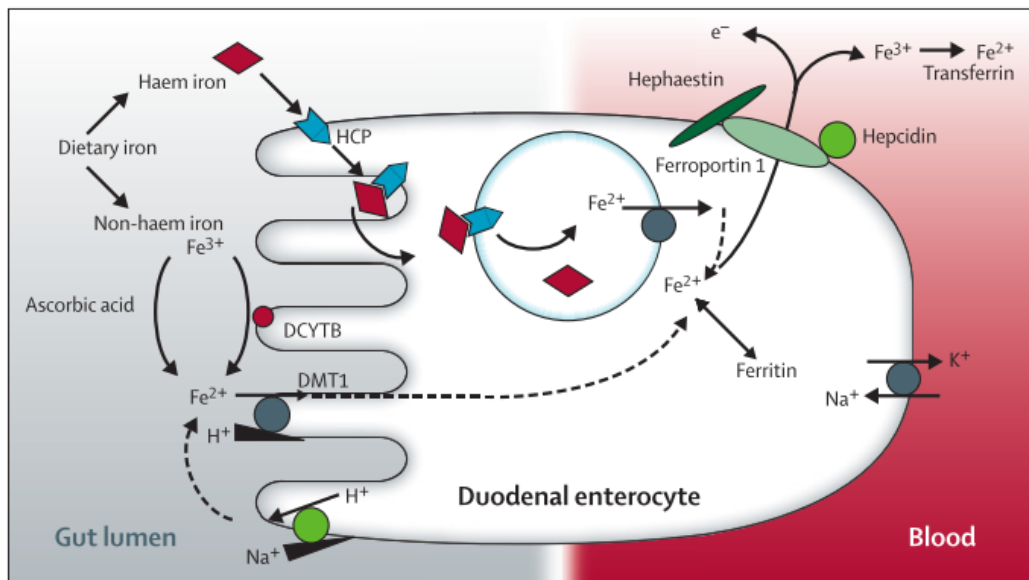


Figure 2.1 Dietary iron uptake. From Zimmerman and Hurrell (2007).

Both physiological and food-related factors affect iron absorption. The strongest predictor of iron absorption is serum ferritin. Serum ferritin is reflective of iron stores, with higher serum ferritin indicating higher iron stores. Lower serum ferritin leads to higher absorption of nonheme iron (Baynes, et al., 1987). Expression of the proteins DcytB and DMT1 are increased in iron deficiency (Gunshin, et al., 1997, Latunde-Dada, et al., 2002), which

increases the amount of iron that is absorbed. Food-related factors influencing dietary iron absorption are discussed later.

Iron Homeostasis

Systemic Iron Homeostasis

When iron is adequate in the diet, iron stores remain relatively constant (Ganz, 2013). To maintain iron homeostasis of the body, the small peptide hormone hepcidin is the key regulator (**Figure 2.2**). Hepcidin expression is regulated by both body iron stores and by plasma iron concentration, and its expression is increased in iron sufficient conditions (Feng, et al., 2012, Ganz, et al., 2008). Its mechanism of action is to bind to the iron export protein ferroportin, and cause its internalization and degradation, preventing iron export (Dao and Meydani, 2013). Ferroportin is expressed in three different types of cells: enterocytes, macrophages, and hepatocytes (Ganz, 2013). In enterocytes, the internalization of ferroportin leads to the inability of iron within the enterocyte to be taken into the bloodstream. The iron within the enterocyte will be excreted in the feces along with the enterocyte when it is sloughed off, thus hepcidin levels are inversely related to iron absorption (Young, et al., 2009). Likewise, in both macrophages and hepatocytes iron export will not occur when hepcidin levels are high and iron status is adequate. When iron status is low, hepcidin expression decreases and iron can exit cells via ferroportin. Absorption of iron into bloodstream will increase, ferritin stored in the liver can be used to supply the rest of the body, and iron can be released from macrophages (Ganz, 2013).

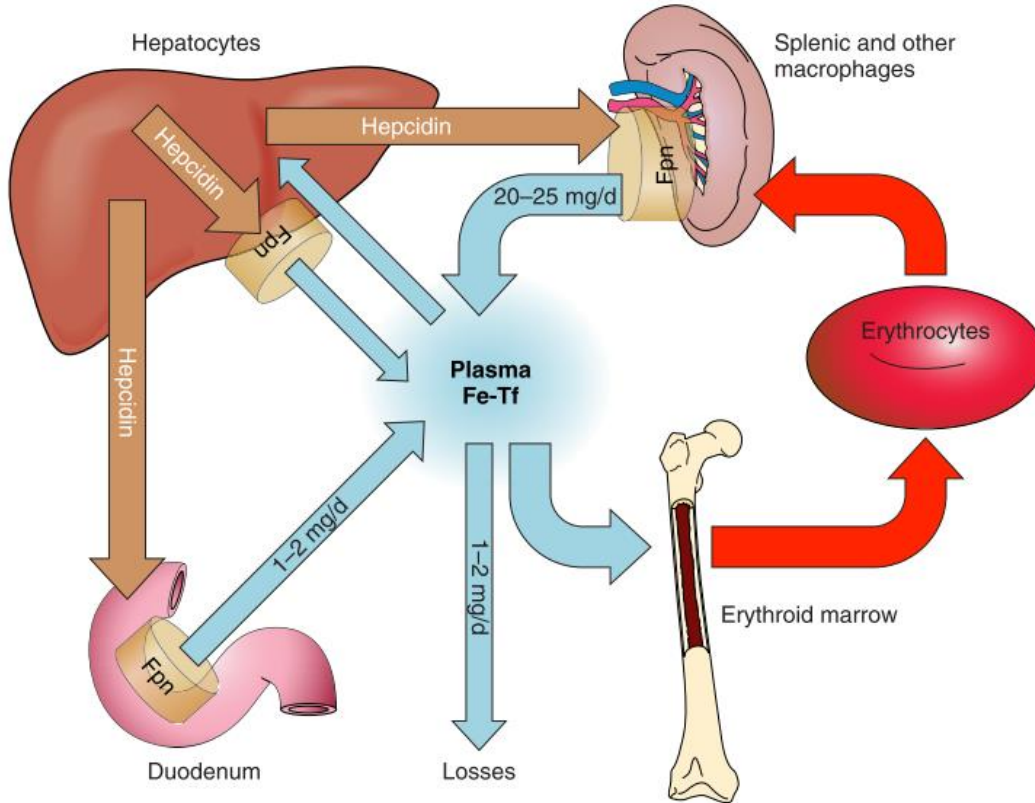


Figure 2.2 Iron homeostasis regulated by hepcidin. From Ganz (2013).

Cellular Iron Homeostasis

Although iron is necessary for life, it can create free radicals that damage cells. Because of this, iron status at the cellular level is tightly regulated. This is accomplished by iron regulatory proteins (IRPs), which recognize and bind iron responsive elements (IREs). IREs are hairpin loops of approximately 30 nucleotides found on the untranslated region (UTR) of mRNA for key proteins involved in iron metabolism, namely transferrin receptor and ferritin (Mullner and Kuhn, 1988, Rouault, et al., 1988). The conformation of IRPs is regulated by cellular iron status. When there is ample iron within the cells, iron forms iron-sulfur clusters on the IRPs, which inhibits it from binding to the IREs (Haile, et al., 1992). When the cell is iron depleted, IRPs are able to bind to the IREs which regulates the mRNA at the posttranscriptional level.

On the transferrin receptor mRNA there are multiple IREs at the 3' UTR. When iron conditions are low, the IRPs bind to the IREs leading to increased mRNA stabilization by preventing degradation of the mRNA by RNases, leading to increased translation of transferrin receptor (Pantopoulos, 2004). This results in cells being able to take up more transferrin-bound iron from circulation. In iron replete conditions, the IRPs do not bind to the IREs of the mRNA and it is degraded before transferrin receptor is translated.

On the mRNA for ferritin, there is one IRE on the 3' UTR. When iron conditions are low, IRP will bind to this IRE and prevent translation of ferritin, ensuring that iron will not be stored when it is needed by the cell. When the cell is iron replete, IRP will not bind the IRE and ferritin will be translated. Thus excess iron will be stored with ferritin, preventing free radical generation that can be caused by free intracellular iron (Pantopoulos, 2004). Figure 2.3 depicts the IRP/IRE system.

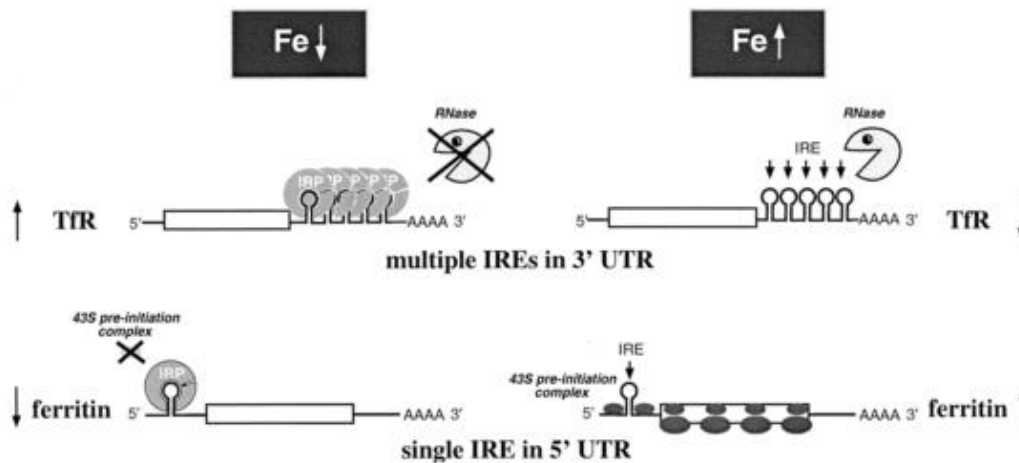


Figure 2.3 Regulation of transferrin receptor and ferritin expression by IREs and IRPs. From Pantopoulos (2004).

Iron Bioavailability

A commonly used definition of bioavailability is “the fraction of the ingested nutrient that is absorbed and subsequently used for normal physiological functions” (Fairweather-Tait

and Hurrell, 1996). Bioavailability is of utmost importance in iron nutrition because of the number and complexity of factors that influence its absorption. In the case of iron, bioavailability is further complicated because of there being two types of iron in the diet.

Dietary iron can be classified into two categories: heme and nonheme. Heme iron is found in animal tissue foods i.e. meat, fish, and poultry. Approximately 30-40% of iron in fish and pork and 50-60% in beef and chicken is heme iron, while the remainder is nonheme iron (Monsen, et al., 1978). In addition to animal tissue, nonheme iron is found in highest amounts in grains and legumes.

Heme iron and nonheme iron differ greatly in their bioavailability. Heme iron has a bioavailability of 25-35%, and is not influenced by dietary factors (Bjorn-Rasmussen, et al., 1974, Monsen, et al., 1978), whereas that of nonheme iron varies depending on the nutrient content of the whole meal. Numerous factors have been shown to affect nonheme iron bioavailability, both inhibitors and enhancers. Inhibitors include phytic acid, polyphenols, and calcium. Enhancers include ascorbic acid, other organic acids, and animal protein.

Inhibitors

The high presence of iron inhibitors, a form of antinutrients, in the diet is a large contributor to iron deficiency in developing countries. Populations that subsist primarily on legumes and cereals have a high prevalence of iron deficiency because antinutrients are found in high concentration in these foods.

Phytic Acid

Phytic acid is the most potent inhibitor of iron absorption. It is prevalent in cereal grains, legumes, nuts, and to a lesser extent in green vegetables. It chelates iron in the gut and passes through the body undigested (Sandberg and Andersson, 1988, Sharpe, et al., 1950). Multiple

studies have shown that phytic acid inhibits nonheme iron absorption in a dose-dependent manner. One study examined iron absorption from soy protein isolates, which are high in phytic acid (Hurrell, et al., 1992). To examine the effects of varying native phytic acid content on iron absorption, phytic acid content was reduced by two methods: acid-salt or enzymatic. Reducing the phytic acid content (by acid-salt) to 0.2 mg/g, 2% of the original, doubled iron absorption. Enzymatic phytate reduction reduced phytic acid content to virtually none (≤ 0.01 mg/g), and resulted in a fivefold increase in iron absorption. For both the acid-salt and enzyme-treated soy protein isolates, adding exogenous phytic acid back to the protein isolate reduced iron absorption back to the original amount (Hurrell, et al., 1992). In another study, extrinsic sodium phytate was added to phytic acid-free wheat rolls that were fortified with radiolabelled ferrous sulfate and fed to human subjects (Hallberg, et al., 1989). Addition of only a small amount, 2 mg phytate P, decreased iron absorption by 18%. Further increasing phytate P to 10 mg decreased iron absorption by 5%, and 250 mg phytate P decreased iron absorption by 82%. A more pronounced reduction in iron absorption was seen at the lower doses of phytic acid (Hallberg, et al., 1989), implying that even small amounts of phytic acid are very inhibitory to iron absorption.

Polyphenols

Polyphenols are a diverse class of compounds present in plant foods, including legumes, grains, and beverages. They inhibit iron absorption by forming insoluble complexes with iron in the GI tract (Brune, et al., 1989). Beverages that are high in polyphenols include coffee, herbal and black teas, and cocoa. They are all high in a particular class of polyphenols, tannins, and they reduce iron absorption by 50-94% (Hurrell, et al., 1999). In foods, many polyphenols are red or purple colored. This is why colored varieties of rice and beans have lower bioavailability than white varieties (Glahn, et al., 2002, Tako and Glahn, 2010). Since polyphenols are found in

the seed coat, seed coat removal can increase the iron bioavailability of legumes high in polyphenols (Ariza-Nieto, et al., 2007). One study shows that removing polyphenols from beans while maintaining phytic acid, and removing phytic acid while maintaining polyphenols showed no improvement in iron absorption. It was necessary to remove both polyphenols and phytic acid before iron absorption was improved (Petry, et al., 2010). This implies that polyphenols and phytic acid do not have an additive effect on the inhibition of iron absorption.

Calcium

Calcium is thought to inhibit nonheme iron bioavailability, but the results are conflicting. It is also is thought to be the only inhibitor of heme iron absorption (Hallberg, et al., 1993) When calcium and phosphate salts were given together, they decreased iron absorption in human subjects (Monsen and Cook, 1976). Another study also demonstrated that addition of calcium to a meal as calcium salts, milk, or cheese was also shown to inhibit iron absorption by approximately 50% (Hallberg, et al., 1991). However, it appears that these single-meal studies exaggerate the inhibitory effects of calcium on iron absorption. Calcium was not shown to have an inhibitory effect on iron absorption in a complete-diet study (Reddy and Cook, 1997), nor did calcium supplementation for one month decrease iron bioavailability (Rios-Castillo, et al., 2014)

Enhancers

While nonheme iron absorption is generally considered low, it can be markedly increased by the consumption of enhancers at the same time. This is seen in the WHO's classification of diets into different bioavailabilities, with overall diets assigned a higher bioavailability when they include enhancers of iron absorption.

Ascorbic Acid

Ascorbic acid (vitamin C) is the most effective enhancer of iron absorption (Cook and Monsen, 1977). It does this by two methods: it chelates iron, keeping it soluble in the intestinal lumen (Conrad and Schade, 1968), and also reduces Fe^{3+} to Fe^{2+} , the form that can be transported into the enterocyte by DMT1 (Han, et al., 1995). Ascorbic acid increases iron absorption in a dose-dependent manner (Cook and Monsen, 1977). The source of the ascorbic acid is not important, and it has been shown that fruit and fruit juices (Ballot, et al., 1987), vegetables (Hallberg, et al., 1986), and added ascorbic acid (Cook and Monsen, 1977) all enhance iron absorption similarly. However, ascorbic acid must be consumed at the same time as iron to improve its absorption (Cook and Reddy, 2001).

Ascorbic acid can also reverse the inhibition of iron bioavailability by phytate and polyphenols. A study by Hallberg and colleagues examined the effect of adding sodium phytate and ascorbic acid to a meal containing white bread and butter. The addition of 50 mg of ascorbic acid to a meal containing 0 mg, 25 mg, and 250 mg of phytate P increased iron absorption by 75%, 117%, and 184% respectively. Increasing the ascorbic acid to 100 mg further increased the iron absorption from meals containing 25 mg and 250 mg of phytic acid (256% and 243%, respectively) (Hallberg, et al., 1989). Another study examined the effect of adding varying amounts of ascorbic acid, 25 mg, 100 mg, and 500 mg, to a meal containing 420 mg of tannins. The iron absorption was increased 190%, 451%, and 611%, respectively, when compared to the meal containing tannins and no ascorbic acid (Siegenberg, et al., 1991)

Other Organic Acids

Organic acids other than ascorbic acid, namely citric, lactic, malic, and tartaric, also improve iron bioavailability. A study examined the iron absorption from a meal containing rice

and ascorbic, citric, malic, or tartaric acid, which are found in some vegetables and fruits. All acids increased the amount of iron absorbed, but ascorbic acid was found to be the most potent (Gillooly, et al., 1983). Lactic acid, present in some fermented foods, has also been found to improve iron bioavailability (Proulx and Reddy, 2007). There is some evidence that the effect of organic acids on iron bioavailability can be different if the iron is in the ferrous or ferric state (Salovaara, et al., 2002)

Meat, Fish, Poultry

Animal tissue foods are a good source of highly bioavailable iron. However, they also enhance nonheme iron absorption from other foods or fortification iron that are consumed at the same time. This effect is similar of all animal tissues, meat, fish, and poultry (Cook and Monsen, 1976). Like ascorbic acid, meat can reverse the inhibition on iron bioavailability caused by phytic acid in cell culture (Engle-Stone, et al., 2005) and in humans (Bach Kristensen, et al., 2005). The mechanism by which meat enhances nonheme iron absorption is still unknown. There is some evidence that glycosaminoglycans, oligosaccharides found in muscle tissue, may be responsible (Huh, et al., 2004). Another study points to peptides, particularly those high in histidine, may be important (Swain, et al., 2002).

Methods to Assess Iron Bioavailability

Human

Human iron absorption studies are done with radioisotopes, stable isotopes, and serum iron curves. In radioisotope studies, radiolabelled iron (Fe^{55} or Fe^{59}) is added to food through either intrinsic or extrinsic tagging. In intrinsic tagging, plants are grown hydroponically with radiolabelled iron added to the media, and the radiolabeled iron is incorporated into the plant (Layrisse, et al., 1969). In extrinsic tagging, a small amount of radioactive iron is added to a

meal (Cook, et al., 1972). It was found that isotopic exchange occurs between the radiolabelled iron and the iron present in food (Bjorn-Rasmussen, et al., 1972), which has resulted in reduced use of the intrinsic tagging technique. Stable isotopes, Fe⁵⁸ and Fe⁵⁷, can also be used in intrinsic tagging as alternatives to radioisotopes (Fairweather-Tait and Minski, 1986, Petry, et al., 2010). With these methods, total blood volume is estimated (Brown, et al., 1962) and isotope incorporation into erythrocytes is used as a measure of iron absorption (Hosain, et al., 1962). This method is highly accurate for measuring bioavailability. Disadvantages of stable isotope and radioisotope studies are that they are expensive to conduct and involve exposing subjects to radioisotopes.

Serum iron curves can also be used to estimate iron bioavailability in human subjects. In this method the subjects are fed a meal, and their blood is drawn at baseline and every half hour after consuming the meal for approximately four hours. Serum iron is measured, change in iron from baseline is plotted on a curve, and the area under the curve is calculated (Conway, et al., 2006). The benefits of this method are that there is no need for stable isotopes or radioisotopes, and measuring in humans is the most accurate way to measure bioavailability. The disadvantages are that, like all human studies, they are expensive to conduct, and that they involve repeated blood draws which are uncomfortable for the subject.

Animal

Numerous animal models have been used to model human iron absorption. Rats, pigs, and most recently chickens, are used in iron absorption experiments, but there are benefits and drawbacks to each one. Hemoglobin maintenance efficiency (HME) is generally used in assessing iron bioavailability in these animal models, and is determined using by the following equation:

$$\text{HME} = \frac{\text{Hb Fe, mg (final)} - \text{Hb Fe, mg (initial)} \times 100}{\text{Total Fe Intake, mg}}$$

Rats are the most economical to use for experiments, but they are much less sensitive to iron enhancers and inhibitors than humans. The increase in iron absorption when meat or ascorbic acid was added was 33 times and 12 times higher, respectively, in humans than in rats. Reduction in iron absorption with addition of wheat bran (high in phytic acid) was three times more in humans than in rats (Reddy and Cook, 1991). This is due to the presence of 30 times more phytase in the rat small intestine, than is present in human small intestine (Iqbal, et al., 1994). Furthermore, rats are also able to increase phytase activity when fed a diet high in phytic acid (Lopez, et al., 2000). This makes them a poor model to represent iron bioavailability from most foods (Reddy and Cook, 1991).

A chicken model of human iron bioavailability is also used. This model was chosen because of its short intestinal tract, sensitivity to mineral deficiency, and is fast-growing (Tako, et al., 2010). In addition to calculating HME, expression of duodenal iron transporters (DMT1, DcytB, and ferroportin), and liver ferritin are measured as markers of iron status (Tako, et al., 2011). Because of the newness of this model, the effects of inhibitors and enhancers of iron bioavailability have not been determined.

Pigs are also used as a model for studying iron bioavailability. Their digestive physiology is similar to humans, they have similar nutritional requirements, and are omnivores (Patterson, et al., 2008). Piglets are frequently used in iron bioavailability studies, and are made anemic by withholding the iron injection they typically receive at birth. Iron bioavailability can then be measured by hemoglobin repletion, described above (Perks and Miller, 1996, South, et al., 2000). Similar responses to iron enhancers and inhibitors have been shown in pigs. Nonheme iron bioavailability in pigs is increased by meat (South, et al., 2000), and inability to digest phytic

acid is evidenced by the common addition of phytase to the diets of commercial pigs. The primary disadvantage to using pigs for iron bioavailability is cost. Since they are relatively large animals, they require a lot of feed and area to house them in.

Cell Culture

Caco-2 cells are a fast and economical alternative to human iron absorption studies for studying iron bioavailability. These cells are human colon adenocarcinoma cells that spontaneously differentiate in cell culture to form a monolayer similar to epithelial cells. In iron uptake experiments, food first goes through in vitro digestion to simulate digestion in vivo, and the digesta is applied to the cells. The first uses of this model used extrinsic labelling of the food with radioactive iron prior to digestion (Alvarez-Hernandez, et al., 1991, Garcia, et al., 1996). Iron absorption was measured by determining the amount of radioisotope that crossed through the Caco-2 cells to the basolateral side. This method was later modified to eliminate the need for extrinsic labelling. Instead, iron bioavailability was measured by Caco-2 cell ferritin formation. It was found that Caco-2 cells form ferritin in response to the amount of iron that they absorb. Enhancers and inhibitors of iron absorption had the same effects in Caco-2 cells as in humans (Glahn, et al., 1998, Glahn, et al., 2002, Yun, et al., 2004), and correlates highly to human iron absorption studies (Au and Reddy, 2000)

Strategies to Improve Iron Status

Supplementation

Iron supplements are typically in pill form, contain a type of inorganic iron, and are sometimes combined with other nutrients that are important for the target population, such as folic acid for pregnant women. Supplementation is the most commonly used method to control iron deficiency in developing countries, and is used for both treatment and prevention (WHO,

2001). The WHO recommends supplementation for all pregnant women and low-birth weight infants, and for children and women of child-bearing age where the prevalence of anemia is greater than 40% (WHO, 2001). Iron supplementation has been shown to improve a number of the negative effects caused by iron deficiency.

Iron supplementation in anemic workers can increase work productivity. A study on anemic female tea-workers in Sri Lanka found that hemoglobin, activity level, and productivity increased for those receiving iron supplementation (30 mg of iron as FeSO₄ per day) compared with controls (Edgerton, et al., 1979). Another study, of cotton mill workers in China, iron supplementation for twelve weeks showed an increase in hemoglobin, serum ferritin, and a decrease in heart rate. Production efficiency, calculated as the ratio of heart rate to energy expenditure, also increased (Li, et al., 1994).

Iron supplementation is extremely common during pregnancy, and a recent meta-analysis examined its effectiveness. Iron supplementation was successful at reducing maternal iron deficiency and anemia, and reducing the risk of having low birth weight babies, but had no effect on pre-term birth or length at birth (Haider, et al., 2013). However, negative side effects can occur with iron supplementation, most notably gastrointestinal. Iron supplements can cause nausea, diarrhea, constipation, or black stools. This can result in poor adherence to the supplementation (WHO, 2008).

Fortification

Iron fortification programs exist in many countries, using a variety of foods as vehicles for fortification. Staple foods are frequently fortified, such as fortification of wheat flour in the U.S. and U.K, and fortification of rice in the Philippines (WHO, 2001). Condiments, particularly soy sauce, salt, and fish sauce are also been used (WHO/FAO, 2006). Two recent meta-analyses

of fortification programs indicate that they are quite successful at reducing iron deficiency. One specifically examined the effect of fortification on Hb concentration of children under 10, and found that fortification resulted in an increase of 4.74 g/L after 6-12 month fortification programs (Athe, et al., 2013). Another meta-analysis found a similar increase in Hb, 4.2 g/L, a 40% reduction in anemia risk, and a 50% reduction in iron deficiency. While fortification programs are quite successful, there are a number of challenges to implementing them, including collaboration of government and food industry, regulations, and choice of an iron fortificant that does not negatively affect sensory characteristics of the food (WHO/FAO, 2006). Furthermore, fortification programs generally do not reach rural areas where commercial food is not sold (WHO/FAO, 2006)

Biofortification

Biofortification is the improvement of nutritional traits of a staple crop with the aim of reducing nutrient deficiencies. This is done by altering the genetics of the plant through selective plant breeding, identification of genetic mutations, or insertion of transgenes. A number of crops have been targeted for iron biofortification: wheat, rice, barley, maize, millet, soy, and common bean (*Phaseolus vulgaris*). Biofortification to reduce iron deficiency has focused on three characteristics of crops: increase in iron concentration, increase in iron bioavailability, and decrease of antinutrients such as phytic acid and polyphenols (Murgia, et al., 2012).

Biofortification has benefits over supplementation and fortification. Once seeds are supplied to farmers, biofortification can benefit rural areas that do not have access to supplements or fortified foods, allowing more people access to high-iron foods. Farmers will be able to pass the seeds on to the next generation, creating a more self-sufficient and sustainable method of improving iron nutrition. There are drawbacks to biofortification however. Conventional plant

breeding can be a slow process, and changes in nutrient content of crops to levels needed is difficult. When transgenic methods are used, there is a low consumer acceptability.

High Iron Crops

Genetic variation in plants allows for a wide range of iron concentration within a given species. In cereals this variation can be as much as 4-fold, and in legumes more than 6-fold (White and Broadley, 2009). Through breeding programs, this inherent genetic variation is exploited to increase iron content in the commonly eaten varieties of crops. Quantitative trait locus (QTL analysis) is one of the methods used to discover the genes that control iron concentration in crops. This has been done in rice (Anuradha, et al., 2012), common bean (Blair and Izquierdo, 2012, Blair, et al., 2010), and wheat (Tiwari, et al., 2009).

Increased crop iron concentration has been successful at increasing absorbed iron from some plants, including pearl millet and rice. Iron-biofortified pearl millet, with approximately three times more iron than control millet, was fed to iron-deficient children in India. Using this millet to prepare porridge and flatbread resulted in approximately three times more iron absorbed over the course of the day than the control millet (Kodkany, et al., 2013). Another study of iron-biofortified pearl millet fed to adult women found that they absorbed twice as much iron over the day than control millet, but 25% less iron than fortified pearl millet (Cercamondi, et al., 2013). Consumption of iron-biofortified rice has shown some benefits with regards to iron status. A nine month feeding trial of women in the Philippines showed that consumption of rice with a nearly fivefold increase in iron content resulted in a modest increase in serum ferritin and body iron (Haas, et al., 2005).

High Iron Bioavailability Crops

Some biofortification has focused directly on improving iron bioavailability as a phenotype instead of on enhancing iron concentration or decreasing antinutrients. Iron bioavailability as assessed through Caco-2 cell culture was determined for a maize population, and used to identify quantitative trait loci (QTL), regions of DNA that are associated with a particular phenotype (Hoekenga, 2011). It was found that iron concentration in maize is not correlated with higher iron bioavailability, but that iron bioavailability is heritable in maize. Quantitative trait locus (QTL) analysis found 10 QTL for iron bioavailability, but phytic acid was not found to be different among lines (Lung'aho, et al., 2011). The QTL analysis was used to develop maize that lines that were genetically similar, but varied only in their bioavailability (high and low). This maize was used in a different study to measure its iron bioavailability in broiler chickens and in Caco-2 cells, and determined that the maize bred to have high bioavailability did in fact have higher iron bioavailability in vitro and in vivo (Tako, et al., 2013).

Transgenic Approaches

A number of transgenes other than phytase have been used in iron biofortification of staple crops. In rice, genes involved with iron homeostasis can be altered to increase iron concentration from anywhere between two and six-fold (Aung, et al., 2013, Johnson, et al., 2011, Masuda, et al., 2013).

A number of crops have been transformed by the incorporation of ferritin-containing transgenes. Ferritin is a protein that stores iron in plants, and is thought to a bioavailable source of iron (Lonnerdal, 2009). Varieties of rice with ferritin-containing transgenes result in a 2-3.4 increase in iron concentration (Aung, et al., 2013, Oliva, et al., 2014, Paul, et al., 2012), while bananas transformed with ferritin result in a 6-fold increase in iron concentration (Kumar, et al.,

2011). Crops have also been cotransformed with more than one transgene. One example is the transformation of rice with soybean ferritin, as well as genes involved in iron homeostasis, resulting in rice with up to six-fold or more increase in iron concentration (Masuda, et al., 2012, Masuda, et al., 2013, Wirth, et al., 2009).

Low Phytic Acid

As mentioned previously, inhibitors of iron absorption are a major contributor to iron deficiency. Reducing phytic acid content has been the major focus of biofortification to reduce the concentration of antinutrients in staple food crops. Seeds of many plants, including staple cereal grains and legumes, store excess phosphorus by synthesizing phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate or InsP₆). Approximately 75% of phosphorous in seeds is in phytic acid, while the remainder is inorganic phosphorous (Raboy, 2009). Phytic acid is found in plants as mixed salts of minerals, primarily K, Mg, Ca, Fe, and Zn. It is involved in many important processes in plants: regulation of ion flux (Lemtiri-Chlieh, et al., 2000), DNA repair, chromatin remodeling, RNA editing, and regulation of gene expression (Raboy, 2009).

Phytic acid is not just a contributor to micronutrient deficiencies in humans, but also poses problems in animal nutrition and to the environment. Phytic acid is not digested by monogastric animals, and is excreted into the feces, which can lead to water pollution in the form of eutrophication (Knowlton, et al., 2004). Furthermore, the phosphorus that is part of phytic acid is not available to these animals. The non-phytic acid phosphorus, available phosphorus, content of regular corn and soybeans is not sufficient to meet the demands of monogastric livestock, primarily poultry and swine. Therefore, diets of these animals is generally supplemented with phosphorus or treated with phytase, enzymes that release phosphate groups from phytic acid to form inorganic phosphorus and lower *myo*-inositols. Both phytase and

supplemental phosphorus add to production costs for the farmers. Low phytic acid crops have the potential to ameliorate both the environmental issues caused by phytic acid and evading the cost associated with these feed additives because they have a molar equivalent increase in available phosphorus that occurs with the decrease in phytic acid content (Raboy, 2009). Low phytic acid crops could prove to be versatile enough to use for both human consumption and livestock feed (Pfeiffer and McClafferty, 2007, Raboy, 2009).

Most low phytic acid crops are produced through mutations of genes involved in the phytic acid pathways in plants. Low phytic acid cultivars of wheat, rice, soybean, barley, common bean, and maize have been produced (Raboy, 2009). Low phytic acid crops typically have an increase in inorganic phosphorus that is molar-equivalent to the decrease in phytic acid (Raboy, 2009). The total amount of phosphorus in the seed remains roughly the same, but there is an increase in phosphorus that is digestible. This phosphorus is referred to as available P since it is available for digestion, and is particularly important in the nutrition of monogastric livestock.

Some low phytic acid crops do indeed have improved iron bioavailability, while others do not. In common bean, fractional iron absorption from *lpa* beans with a 90% reduction in phytic acid was 50% higher than the wild type parent (Petry, et al., 2013). Low phytic acid sorghum has improved zinc bioavailability, but not iron (Kruger, et al., 2013).

Transgenic approaches have also been used to produce low phytic acid crops. In these plants, genes for microbial phytases are inserted into the crop of interest, and they enzymatically reduce phytic acid content. This approach has been used in soybean (Bilyeu, et al., 2008) to produce a 90% reduction in phytic acid level. Transgenic maize over-expressing the phytase *phyA2* from *Aspergillus niger* (a fungus) was produced to reduce phytic acid content. Phytic

acid content was reduced by approximately 23%, and inorganic phosphorus content was increased by 300% in transgenic maize seeds when compared with their wild-type counterparts (Chen, et al., 2008). However, some phytases may become more active when they are in solution, which occurs during cooking. In another study, transgenic maize lines were produced, some that contained *Aspergillus niger* phytase (*phyA*), and others that contained soybean ferritin in addition to this phytase. The transgenic maize did not have any less phytic acid than the non-transgenic controls. However, after incubating a slurry of maize and water for two hours, 30-95% of the IP5+IP6 initially present was degraded, with the large variation dependent on maize line. Iron bioavailability studies were also performed on Caco-2 cell culture. Iron bioavailability was three times higher for the phytase transgenic maize than the non-transgenic control, but there was no further increase in iron bioavailability in the maize lines that also contained soybean ferritin despite an increase in iron content (Drakakaki, et al., 2005).

LPA maize

Thus far, mutations in three genes have been found that can contribute to a low phytic acid phenotype in maize. Therefore, low phytic acid mutants fall into three categories: *lpa 1*, *lpa 2*, and *lpa 3*, with each category affecting a different gene in the phytic acid pathway (Raboy 2009; Panzeri et al. 2011). A summary of characteristics of these mutants is given in **Table 2.5**. There is typically a reduction in germination in these mutants, which implies that phytic acid performs a beneficial role in plants, including fighting oxidative stress. In a study of one *lpa* mutant, it was found that mutants contained high incidence of oxidative stress: more free or weakly bound iron, higher free radical content, more carbonylated proteins, more damaged DNA, and less γ -tocopherol (an antioxidant) (Doria, et al., 2009). The following table gives an

overview of the currently known *lpa* mutants, their percentage reduction in phytic acid, change in P_i , total P, and germination.

Table 2.5 Summary of *lpa* maize mutants

Study	Gene affected	Mutant name	Reduction in PA	Inorganic P	Total P	Germination affected	Reduction in seed dry weight	Other Characteristics
Raboy, et al., 2009	Lpa 1	<i>lpa1-1</i>	66%	10-fold increase	unchanged	Reduced	8-23%	
Cerino Badone, et al., 2012	Lpa 1	<i>lpa1-7</i>	88%	10-fold increase	unchanged	Homozygous is lethal	N/D	
Pilu, et al., 2003 and 2009	Lpa 1	<i>lpa 1-241</i>	90%	10-fold increase	unchanged	30% decrease	N/D	
Shi, et al., 2007	Lpa 1	<i>lpa 1-mum1, -2, -3, -4</i>	93%	10-fold increase	unchanged	Homozygous is lethal	N/D	Knockout mutants
Raboy, et al., 2009; Shi, et al. 2003	Lpa 2	<i>lpa 2-1</i>	50%	4-fold increase	unchanged	Reduced	4-16%	Accumulation of IP4 and IP5
Shi, et al., 2003	Lpa 2	<i>lpa 2-2</i>	50%	4-fold increase	Unchanged	Reduced	N/D	
Shi, et al., 2003	Lpa 2	<i>Zmpk-mum1, -2, -3 -4</i>	30%	3-fold increase	unchanged	N/D	N/D	Knockout mutants
Shi, et al., 2005	Lpa 3	<i>lpa 3-1</i>	50%	6-fold increase	30% decrease	None observed	N/D	
Shi et al., 2005	Lpa 3	<i>lpa 3-2</i>	50%	6-fold increase	30% decrease	None observed	N/D	

Iron Bioavailability of *lpa* Maize

Iron absorption from *lpa1-1* maize was measured in humans. Tortillas were made with *lpa1-1* and wild type maize, and were labelled via extrinsic labelling. Blood was sampled two weeks after feeding, and incorporation of radiolabelled iron into red blood cells was measured. Iron absorption from tortillas made with *lpa* maize was 49% higher than tortillas made with wild type maize (Mendoza, et al., 1998). A similar study was performed to determine if *lpa* maize still had better iron bioavailability after the maize was fortified with ferrous sulfate or sodium iron EDTA. After fortification, there was no longer a difference in iron bioavailability (Mendoza, et al., 2001).

Iron bioavailability of *lpa* maize transformed with soybean ferritin was also measured, but in Caco-2 cells. When compared with wild-type maize, iron bioavailability of the non-transgenic *lpa* maize was increased by 50%, but this difference was not significant (Aluru, et al., 2011). The *lpa* maize transformed with soybean ferritin had higher iron bioavailability than regular *lpa* maize, wild-type maize, and wild-type maize transformed with ferritin (Aluru, et al., 2011).

Recurrent Selection

An alternative approach to inducing mutation to create a low phytic acid phenotype is to selectively breed for reduced phytic acid content. Recurrent selection uses the inherent quantitative genetic variation of a trait within a plant population as the starting point. The desired characteristic is bred for by screening plants for a desired phenotype, and then intermating plants with the desired phenotypes to obtain more extreme variation in phenotype. By carrying out multiple cycles of intermating, evaluating and selecting it is

possible to generate populations with extreme trait values. For example, researchers at the University of Illinois have been selecting for protein or oil content for more than 100 years and the resulting populations have compositions that are the most extreme in the entire species (Dudley and Lambert, 2010). It was found that a large variation in phytic acid content exists among different maize families, and the broad-sense heritability is high for phytic acid (Lorenz, et al., 2008). Recurrent selection has been successful at breeding for amino acid concentration (Scott, et al., 2008), but has not yet been attempted with phytic acid concentration. Using recurrent selection to breed for reduced phytic acid content could potentially produce a low phytic acid maize that ameliorates the problem of poor seed quality, and would facilitate its use in the real world setting.

Summary

Iron is a vital nutrient in human health for processes such as oxygen transport and brain function. However, a startlingly high number of people worldwide have iron deficiency, mainly children and women of childbearing age. Low dietary iron bioavailability is one of the leading contributors to iron deficiency. People in developing countries consume diets low in animal flesh foods, which provide highly bioavailable heme iron. They rely instead on plant sources of iron which only contain nonheme iron, which has a much lower iron bioavailability and can be influenced by inhibitors of iron absorption.

Phytic acid, the most potent inhibitor of iron absorption, is very high in the diets of people in the developing world because it is ubiquitous in legumes and cereal crops that make the basis of their diets. Reducing phytic acid content in staple crops is a potential solution to low dietary iron bioavailability in the developing world. It provides a more sustainable alternative to iron supplementation and fortification, both of which require

constant external inputs and oftentimes are not able to reach the people who most need them. Altering the phytic acid content of staple crops is more sustainable because the seeds need only be initially supplied to the farmers, and then they can be passed on to future generations

The current low phytic acid crops in maize are *lpa* mutants. They have a 30-90% reduction in phytic acid content and improved iron bioavailability. However, they have a reduction in seed quality which makes them impractical to farmers. Recurrent selection, selectively breeding for extremes of a given trait over many cycles, could be used as an alternative method to produce low phytic acid maize without diminishing seed quality.

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CHAPTER 3

RECURRENT SELECTION TO ALTER GRAIN PHYTIC ACID
CONCENTRATION AND IRON BIOAVAILABILITY

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Abstract

Most of the phosphorus (P) in cereal grains is in the form of phytic acid, a potent inhibitor of iron absorption that cannot be digested by monogastric livestock or humans. High phytate content in staple crops contributes to the high incidence of iron deficiency in developing countries. Low phytic acid (*lpa*) maize mutants are seen as a potential strategy to improve iron bioavailability, but they have poor seed quality. Our objective was to develop both low and high phytic acid maize populations using recurrent selection, and to determine if low phytic acid maize developed through this method has improved seed quality and iron bioavailability when compared to *lpa* mutant maize. Three cycles of selection were carried out in two broad-based synthetic populations, BS11 and BS31. Our research found that recurrent selection produced a significant difference in phytic acid content between the high and low BS11 populations ($P < 0.05$), but not in the BS31 populations ($P > 0.05$). The BS11LPA maize population had improved seed germination relative to *lpa* mutant inbred lines (13-16%, $P < 0.05$), and similar iron bioavailability ($P > 0.05$).

Introduction

Phytic acid, myo-inositol-1,2,3,4,5,6-hexakisphosphate or InsP6, is the most abundant phosphate in seeds (Bewley and Black, 1994). Its primary function in the seed is as a storage molecule for phosphorus (P); and greater than 75% of P in seeds is found as part of phytic acid (Raboy, 2009). Phytic acid in seeds is found as phytate, a mixed salt of phytic acid complexed with cations, including K, Mg, Ca, Zn, and Fe, and often associated with proteins.

Phytate cannot be digested by monogastric livestock species including swine and poultry, and as a result it passes undigested into the feces of these animals (Cromwell and Coffey, 1991). This causes environmental problems when run-off from livestock operations leads to eutrophication of waterways (Bouwman, et al., 2011). Phytate P is non-available to these animals, meaning it cannot be absorbed and utilized. The remainder of the P in these seeds, not bound to phytate, is referred to as available P because it has a relatively high bioavailability in livestock (National Research Council, 2012). The available P present in cereals and legumes is not enough for optimal animal growth, and thus P is either supplemented in the diets of these animals or phytase enzymes are added to the feed to break down phytic acid (Knowlton, et al., 2004). Both add to feed costs, and supplementing P in the diet does not reduce the environmental hazard of phytate run-off from animal feces.

Like other monogastric animals, humans do not produce significant quantities of phytase (Iqbal, et al., 1994). There is some evidence that the human gut microflora can produce phytases, but the evidence is not strong (Markiewicz, et al., 2013). While P deficiency is not of concern to humans, high phytate consumption contributes to deficiencies of microminerals, particularly iron. Iron absorption is strongly inhibited by phytate, which is one of the predominant reasons that iron deficiency is so prominent in developing countries

where diets consist primarily of staple cereal crops that are rich in phytic acid (World Health Organization, 2001).

Human iron absorption studies have examined the effects of adding varying amounts of phytate, as pure sodium phytate or as part of bran, on iron bioavailability. In one study, addition of even small amounts of sodium phytate (2 mg phytate P) added to a phytate-free bread meal significantly inhibited iron absorption. The inhibitory effect of increasing amounts of sodium phytate resulted in a dose-dependent decrease in iron absorption (Hallberg, et al., 1989). In another study, addition of phytate to bread as part of maize bran also showed a dose-dependent inhibition on iron absorption, with a less marked effect at low phytate levels (Siegenberg, et al., 1991). In both of these studies, the meals were fortified with inorganic iron. This may not be representative of how phytate affects iron naturally present in foods because of food-matrix effects.

Because of the nutritional and environmental problems caused by phytate, low phytic acid (*lpa*) mutants of cereal crops and legumes have been developed. In maize, these mutants have a 30-90% reduction in phytate content, and are generally accompanied by a molar equivalent increase in available P. These mutants have been shown to have beneficial effects on iron bioavailability, resulting in a 49% increase in iron absorption (Mendoza, et al., 1998), and also improved P utilization in pigs (Hill, et al., 2009). However, these mutants typically have a reduction in seed germination, yield, and/or dry weight, making them undesirable for farmers (Raboy, 2009).

An alternative approach to creation of maize with reduced phytate levels is through recurrent selection. This method of breeding involves selecting for extremes of a given trait or traits over multiple cycles. It results in progeny that have higher or lower levels of a trait

in a given phenotype, and has been successfully used to breed for grain composition (Dudley and Lambert, 2010) and grain amino acid content (Scott, et al., 2008). The objective of our research was to determine if recurrent selection can be used to select for both high and low phytic acid content. Our research also examined if the resulting maize populations had differing seed quality and iron bioavailability when compared to *lpa* mutants. Seed quality in this study is defined as the physiological seed characteristics that allow uniform and rapid seedling development (seed germination) and growth under a wide range of environmental conditions (seed vigor).

Materials and Methods

Breeding Strategy

Two broad-based synthetic maize populations were used in this study. BS31 is derived from FS8B and is largely tropical in origin (Horner, 1990), while BS11 is derived from the Pioneer two-ear composite, a corn belt population originally designed to be prolific (Hallauer, 1967). Individually harvested ears from both the BS11 and BS31 populations were screened for relative phytate concentration via the Wade method (described in detail below). The five ears with the highest and five ears with the lowest relative phytate concentrations from each population were chosen and made into balanced bulks, resulting in four categories each containing five ears each: BS11HPA (high phytic acid), BS11LPA (low phytic acid), BS31HPA, and BS31LPA (BS11HPA containing the ears with the highest phytate concentration from the BS11 population, BS31LPA containing the ears with the lowest phytate concentration from the BS31 population, etc.).

For the recurrent selection breeding, seeds from each bulk were planted in adjacent rows. The resulting plants were intermated in a chain-sib mating design. In this design, the

pollen from one plant (plant A) is used to pollinate the silks from another within the same population (plant B). The pollen from plant B is used to pollinate the silks on plant C. This pattern continues, and the pollen from the last plant is used to pollinate the silks on plant A. A total of 50 ears were made for each of the four populations. These ears were harvested individually and screened for relative phytate concentration. The five ears with the highest relative phytate concentration from the BS11HPA category were made into a balanced bulk, as were the five ears with the lowest phytate content from the BS11LPA category, and the same was done for BS31HPA and BS31LPA. These bulks are considered cycle 1 (C1). The following year, the C1 bulks were planted, intermated, screened for phytate content, and selections made in the same way as described above. All intermating was performed *within* a category, no intermating was done between categories. This method was continued for three cycles of recurrent selection.

Measurement of Phytic Acid and Available P for Recurrent Selection

Phytic acid and available P were measured using colorimetric assays modified from Vaintraub and Lapteva (1988) and Raboy et al. (2000) as described fully in Lorenz et al. (2007) using reagents obtained from Sigma-Aldrich (St. Louis, MO). A subsample of approximately 50 kernels from each ear was ground into fine flour with a Stein Mill grinder. Ten milligrams (± 2 mg) of flour from each sample were weighed into an assigned well in a 96-well plate. HCl, 0.65 M, was added to each well to extract both, the phytate and inorganic P; the plates were shaken overnight at room temperature, then centrifuged. The supernatant was transferred to each of two 96-well plates, one to measure phytate and the other to measure available P. To facilitate high throughput screening, and because ranking is sufficient for the purpose of selection, standards were not used for either assay.

For phytate analysis, Wade reagent (sulfosalicylic acid, $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, and deionized water) was added to the supernatant, and the absorbance at 490 nm was measured after incubating at room temperature. This assay is unusual amongst colorimetric assays in that *a lower phytic acid content is reflected by a higher absorbance*. The ears with the highest or lowest relative phytate concentration were chosen for HPA or LPA categories, respectively.

For available P analysis, deionized water and available P reagent (deionized water, ammonium molybdate, ascorbic acid, and sulfuric acid) were added to the supernatant. Absorbance was read at 820 nm after incubation at room temperature. Phytate and available P values were converted from absorbance to standardized values (mean 0, standard deviation 1) prior to data analysis to facilitate interpretation.

Evaluation of Three Cycles of Selection for High or Low Phytic Acid Concentration

To determine the effectiveness of recurrent selection on phytate concentration, seed from the individually harvested ears from C3 of recurrent selection was used. Four of the individually harvested ears from each of the four categories were chosen for use in the evaluation experiment. Each ear will henceforth be referred to as an entry. An additional four entries consisting of inbred *lpa1-1* mutants, which typically have a 66% reduction in phytate content compared to wild type maize from the same population (Raboy, et al., 2000), were also included in the experiment for comparison, two in the B73 genetic background and two in the Mo17 genetic background. The entries were planted in a randomized complete block design with environment as a blocking factor. Six environments were used in the study, three fields in 2010 and three fields in 2011. For each entry, three self-pollinations were made at each environment. At maturity, each ear was individually harvested and dried to approximately $150 \text{ g H}_2\text{O kg}^{-1}$ fresh weight.

Seed Quality Evaluation: Standard Germination and Cold Tests

Seed for these tests was harvested by hand at physiological maturity as determined by black layer formation according to Hunter et al. (1991). Ears were immediately placed in a single layer inside a small-scale experimental dryer (Navratil and Burris, 1982) and air-dried with forced air at temperatures below 35°C until the seed was at 120 g H₂O kg⁻¹ fresh weight. Ears were mechanically shelled individually and 100 seeds were counted from each ear for both the standard germination and cold tests.

Standard germination test

The standard germination test was used to evaluate seed viability under ideal growing conditions. The tests were conducted according to the Association of Official Seed Analysts Rules for Testing Seeds (2010). Each sample consisted of 100 seeds per ear, and 43-69 ears per maize type were tested. Samples were randomized within trays and germination carts, and four samples were planted in each tray. Seeds were planted on top of two sheets of crepe cellulose paper media (Kimberly Clark Corp., Neenah, WI) moistened with 800 mL of tap water on fiberglass trays (45 cm x 66cm x 2.54 cm), and lightly pressed into the media to create good seed-media contact. The trays were placed inside germination carts after planting, and the carts were placed inside a constant 25°C walk-in germination chamber with alternating 4-h of light and 4-h of darkness for a total of 12-h of light d⁻¹. Percentage of normal seedlings was evaluated at 7 d according to AOSA Rules for Testing Seeds (2010).

Cold test

A 7-d cold test (Association of Official Seed Analysts, 2009) was used to evaluate seed vigor. Samples, 45-69 per type of maize, were randomized within trays and germination carts, and four samples were planted in each tray. One hundred seeds per ear were planted on one sheet of Kimpak (Kimberly Clark Corp., Neenah, WI) watered with 1100 mL of water pre-chilled for 24-h at 10°C on fiberglass trays (45 cm x 66cm x 2.54 cm). One cm of dry 80% sand:20% soil mixture was used to cover the seed and media. The trays were placed inside enclosed germination carts after planting, and the carts were placed inside a dark walk-in chamber at constant 10°C for 7-d, and then moved to a constant 25°C walk-in germination chamber with alternating 4-h of light and 4-h of darkness for a total of 12-h of light d⁻¹. Normal seedlings (AOSA, 2010) were evaluated and recorded at 7-d after moving the tests to the constant 25°C walk-in germination chamber.

Iron Bioavailability

Iron bioavailability was assessed for BS11HPA, BS11HPA, and *lpa* mutants (the LPA and HPA categories from the BS31 population were not assessed because there was no statistical difference in phytate content). Balanced bulks from approximately 50 ears from the cycle three of each population were made from ground maize samples. Each bulk was then ground again in a Stein Mill grinder to ensure the bulks were thoroughly mixed. To measure iron bioavailability, samples were taken from the bulks and subjected to in vitro digestion, followed by iron uptake by Caco-2 cells. These cells produce ferritin in response to iron uptake, and thus iron bioavailability is expressed as ng ferritin/mg cell protein (Glahn, et al., 1998).

In vitro digestion

Samples and positive control were subjected to in vitro digestion as per Proulx and Reddy (2007) and modified as follows. All reagents used for in vitro digestion were purchased from Sigma-Aldrich. Ten mL of 0.01 M HCl was added to 1.75 g of maize flour along with 8.5 μ mole ascorbic acid in 0.01 M HCl to increase iron uptake response to detectable levels (Bodnar, et al., 2013). HCl was also added to a positive control consisting of 0.17 μ mole FeSO₄ in 0.01 M HCl. The pH was adjusted to 2.0 with 1 M HCl, and then 520 μ L of pepsin solution (0.2 g 1:60,000 porcine pepsin in 0.01 M HCl) was added to simulate gastric digestion and the samples were shaken in a water bath at 37° C for one hour. The pH was then increased to 6.0 with 1 M sodium bicarbonate, and 1.3 mL of pancreatin and bile solution (0.05 g of 4 \times USP porcine pancreatin and 0.3 g of bile extract in 25 mL of 0.1 M sodium bicarbonate) was added to simulate duodenal digestion. The samples were shaken again for 15 minutes at 37 ° C water bath. The digests were centrifuged at 5,000g, the resultant supernatant was collected into a new tube, and then heated at 100° C for four minutes to deactivate digestive enzymes.

Caco-2 Cell Iron Uptake

The iron uptake experiment was conducted with Caco-2 cells (American Type Culture Collection, Rockville, MD) as described fully in Bodnar et al. (2013). Reagents and other material were obtained from Sigma-Aldrich or Gibco BRL (Grand Island, NY) unless otherwise mentioned. Cells were grown in a cell culture flask, and were maintained with Dulbecco's modified Eagle's media (DMEM) with 1% v/v glutamine, 1% v/v antibiotic-antimycotic, and 10% fetal bovine serum (FBS). Cells were kept in an incubator at 37° C with 5% CO₂. Media was changed every other day, and cells were passaged into a new flask

when they reached 80-100% confluence. Prior to passaging, cells were rinsed with Earle's balanced salt solution (EBSS), and then treated with trypsin to detach cells from the flask. They were then centrifuged at 22.6g and transferred into a new flask. For the iron uptake experiments, cells were seeded at passage 30-32 on collagenized 12-well Costar cell culture plates at a density of 5×10^4 cells/cm². Media continued to be changed every other day.

At 15 days post-seeding, the cells were used for the iron uptake experiment. They were rinsed with EBSS, and were then treated with 0.5 mL serum free media (DMEM with 1% v/v glutamine, 1% v/v antibiotic-antimycotic solution, 0.4 mg/mL hydrocortisone, 0.5 µg/mL selenium, 3.4 µg/mL triiodothyronine (T3), 1 mmol/mL piperazine-N,N'-bis[2 ethanesulfonic acid] (PIPES), 2 µg/mL EGF (epidermal growth factor), and 1 mg/mL insulin) and 0.5 mL of the digest supernatant. Each digest was applied in duplicate in two separate wells on two separate cell culture plates (duplicates were averaged prior to analysis). Two hours later, the cells were treated with a second aliquot of 0.5 mL serum free media and then incubated at 37° C with 5% CO₂ for another 22 hours. At 24 hours after the first application of serum free media, the media was aspirated and the cells were rinsed with EBSS. Deionized water (0.5 mL) was added to each well, and the cells were then lysed for ten seconds at 1W with a probe-type sonic dismembrator. Cell lysate was transferred to microcentrifuge tubes and was lysed for an additional 10 seconds. Microcentrifuge tubes were centrifuged, and the supernatant was used for protein and ferritin determination. Protein was measured in duplicate with the Bradford Commassie Assay (Pierce Laboratories, Rockford, IL) and ferritin was measured via enzyme immunoassay (Ramco, Stafford, TX). Iron bioavailability was assumed to be as ng ferritin produced by cells in response to treatment per mg cell protein (ng ferritin/mg protein).

Phytic Acid Analysis of Bulked Maize Samples

Phytic acid and content of the bulked maize samples used for iron bioavailability was quantified as described in detail in Proulx and Reddy (2007), modified from Oberleas (1971). Each bulked maize sample (BS11LPA, BS11HPA, and *lpa* mutant) was extracted with 2.4% HCl for three hours. The samples were then centrifuged and filtered with Whatman's # 5 filter paper. One mL of supernatant was mixed with 0.5 mL 0.75 M NaOH and 0.5 mL 0.1 M Na₂EDTA. This mixture was run on a 1.5 x 30 cm ion-exchange column with AG1-X4, 100-200 mesh chloride form ion-exchange resin (Bio-Rad, Hercules, CA) and eluted with 0.7 M NaCl. The eluant was combined with H₂SO₄ and HNO₃ and heat digested until a precipitate formed, which was then dissolved in water. Phosphorus content was measured in a colorimetric assay with ammonium molybdate and sulfonic acid (4-amino-3-hydroxy-1-naphthalenedisulfonic acid in 0.15 mol/L Na₂SO₃ and 0.92 mol/L NaHSO₃) reagents. Phosphorus concentration was converted to phytic acid concentration based on the assumption that P content of phytic acid is 28.2%.

Iron Analysis of Bulked Maize Samples

Non-heme iron and non-protein bound iron were measured for the same bulked maize samples as described above. For total nonheme iron, trichloroacetic acid (TCA) in acid treatment was followed by a colorimetric assay (Torrance and Bothwell, 1968). Maize flour (0.5 g) was mixed with 2 mL 10% TCA/3 N HCl and incubated for 20 h at 65° C. The mixture was then centrifuged at 5,000g for 15 min, and the resultant supernatant was mixed with 0.02% ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt] in 2.05 M sodium acetate. The mixture was incubated for 10 min and abs. was measured at 563 nm. The protocol for non protein-bound iron was identical, except that the

digest was in water instead of HCl (Murray-Kolb, et al., 2003). Protein-bound iron was estimated by subtracting non protein-bound iron from total nonheme iron.

Data Analysis

To evaluate the self-pollinated ears that originated from the C3 selections, one-way ANOVA followed by Tukey-Kramer multiple comparisons test were performed separately for both populations (BS11 and BS31) comparing the HPA category, LPA category and *lpa* mutants. This was performed for phytic acid, available P, standard germination, and cold tests. For the iron bioavailability results, one-way ANOVA followed by Tukey-Kramer multiple comparisons test performed to compare the mean ng ferritin/mg protein amongst the three bulks.

Results and Discussion

Relative Phytic Aid and Available P Concentrations for Recurrent Selection

The LPA and HPA selections from the BS11 population had significantly different relative phytic acid content from each other and from the *lpa* mutants (Table 3.1, $P < 0.001$ for each pair). The *lpa* mutants were lowest, followed by the LPA group, and the HPA group had the highest relative phytic acid content. Analysis of phytic acid from the ears from the BS31 population found that the LPA group was slightly lower than the HPA group, but the difference was not significant. Both groups from this population had significantly higher phytic acid concentration than the *lpa* mutants (Table 3.1, $P < 0.0001$ for each pair). For both populations, the LPA and HPA categories had significantly lower relative available P than the *lpa* mutant, but were not significantly different from each other ($P < 0.05$, Table 3.1).

These results show that three cycles of recurrent selection were successful at significantly altering phytic acid in the BS11 population, but not in the BS31 population.

Although the results from this portion of the study were relative and not quantitative, it is evident that the phytic acid concentration of the BS11LPA was much higher than the phytic acid concentration of the *lpa* mutant. Recurrent selection is a slow process (Dudley and Lambert, 2010) and it will take many more cycles to determine if phytic acid concentration can be reduced close to the levels seen in *lpa* mutants.

Our results suggest that recurrent selection for reduced phytic acid concentration does not impact available P. Although in *lpa* mutants phosphorus is repartitioned from phytate-bound P to available P, this may not be true for non-mutant maize, as evidenced by Lorenz et al. (2007, 2008). However, in other maize families it has been shown that phytate and available P are typically highly positively correlated (Raboy, et al., 2001). Therefore, it is possible that the change in phytic acid was just not enough to have a significant impact on the available P concentration. More cycles of recurrent selection would be needed to determine if available P concentration will change, and in what direction, in response to a decrease in phytic acid content.

What happens to available P content through more cycles of recurrent selection would impact the suitability of this maize for use in animal feeds. While humans are generally at low risk for problems related to low phosphorus intake, the growth of livestock such as pigs can be limited if there is not adequate phosphorus in the diet (Zhai and Adeola, 2013). Therefore, if low phytate maize produced via recurrent selection does not result in increased available P, it would not mitigate the need for supplemental phosphorus.

Seed Quality Evaluation

For the standard germination test in the BS11 population, LPA and HPA had similar germination rates of 95% and 95%, respectively, which were significantly higher than the germination rate of the *lpa* mutants, 78% (Table 3.2, $P < 0.0001$). Similar results were found with BS31; both LPA and HPA had similar germination rates, 90%, which were significantly higher than that of the *lpa* mutants (Table 3.2, $P < 0.001$).

In the BS11 population, the cold germination of LPA was 89%, better than the *lpa* mutant's cold germination of 76% (Table 3.2, $P < 0.01$). The cold germination of HPA, 86% had similar cold germination as both LPA and *lpa* (Table 3.2, $P > 0.05$). For the BS31 population, LPA and HPA had better cold germination, 92%, than the *lpa* mutant (Table 3.2, $P < 0.0001$).

These results indicate that the recurrent selection process did not have a negative impact on seed quality, and that both the BS11 and BS31 LPA categories outperformed the *lpa* mutants. No other studies have examined the impact that naturally present phytate has on seed quality, but there is abundant evidence that *lpa* mutants of maize have a substantial negative impact on seed quality. Early studies on *lpa 1-1* did not show a reduction in seed germination, but did show a reduction in yield (Ertl, et al., 1998). However, later experiments demonstrated a large reduction in seed germination and stress tolerance for *lpa1-1* and *2-1* mutants (Raboy, 2009). There is also consistent evidence of a significant reduction in seed dry weight in both of these mutants, an undesirable characteristic (Raboy, et al., 2000, Shi, et al., 2007). Other *lpa* mutants also have demonstrated negative seed quality. The two mutants with the highest reduction in phytate, approximately 90%, have the greatest reduction in seed germination. The *lpa1-241* mutant is reported to have a 30%

reduction in germination (Pilu, et al., 2003) and the *lpa1-7* mutation is lethal when homozygous (Cerino Badone, et al., 2012). Shi et al. (2005) reported that the *lpa3-1*, with a 50% reduction in phytic acid, did not demonstrate reductions in emergence under field conditions, but germination tests were not conducted. The lack of an *lpa* mutant with good seed qualities gives impetus to determine if further cycles of recurrent selection can reduce phytate concentration without harming germination.

Iron Bioavailability, Phytate, and Iron of Bulked Maize Samples

After determining that the recurrent selection process resulted in significantly different phytate content between the HPA and LPA selections of BS11 but not BS31, only the BS11 population was used for the iron bioavailability experiments to compare with the *lpa* mutants. Phytate and iron (non-heme, non-protein-bound, and protein-bound) were also quantitatively measured on the two selections from BS11, as well as the *lpa* mutants. Phytic acid analysis of the bulked maize samples showed a 29% decrease in phytic acid content of the BS11LPA group when compared to the BS11HPA group, while the *lpa* mutant had 18% less phytic acid than the BS11LPA group (Table 3.3).

Iron bioavailability was significantly different among the three maize samples tested. The mean for BS11LPA was 38% higher than for BS11HPA, and the *lpa* mutant was 60% higher than BS11HPA ($P < 0.05$, Figure 3.1). There was no significant difference between BS11LPA and the *lpa* mutant ($P > 0.05$). These results indicate that even a small reduction in phytic acid content can lead to a significant increase in iron bioavailability in maize.

These results are promising for the future of low phytate crops. In contrast to previous studies that suggest that phytate must be almost completely removed before an increase in iron bioavailability occurs (Hurrell, et al., 1992), this study suggests that iron

bioavailability will significantly increase with only relatively minor reductions in phytate. This increases the feasibility of producing low phytate maize that will have improved iron absorption, because recurrent selection is unlikely to reduce phytate levels to those seen in the *lpa* mutants. More studies should be done to determine the optimal amount of phytic acid reduction in maize on its iron bioavailability.

The iron analyses (Table 3.3) indicate that there is a substantial difference in the distribution of iron between the two BS11 categories, and the *lpa* mutants. Approximately 45% of the iron in the mutants is protein-bound, compared with 30% and 24% in the LPA and HPA categories, respectively. Because of the toxicity of free iron in plants, iron is typically found bound to phytate or the protein ferritin (Morrissey and Guerinot, 2009). Therefore, in response to a reduction in phytate it would be necessary for the maize seed to produce more ferritin to limit the amount of free iron. The bioavailability of ferritin is reported between approximately half as much as ferrous sulfate (Murray-Kolb, et al., 2003) and similar to ferrous sulfate (Lonnerdal, et al., 2006) in humans. A study in which *lpa1-1* maize mutants were transformed to contain the soybean ferritin gene found that iron bioavailability in Caco-2 cells could be doubled by the addition of the ferritin transgene. This study also found that nontransgenic *lpa1-1* mutants have a 50% increase in ferritin when compared with non-mutant seeds (Aluru, et al., 2011). These findings suggest that ferritin in maize should be a bioavailable source of iron. If the increase in non-protein-bound iron found in the *lpa1-1* mutants is in fact ferritin-bound, we would have seen a much higher iron bioavailability in the *lpa1-1* mutants in the present study. More research should be conducted to determine if *lpa1-1* mutants are in fact higher in ferritin than the BS11LPA

maize, or other factors that would explain why *lpa1-1* did not have higher iron bioavailability.

Conclusions

This study concludes that recurrent selection can be successful at altering grain phytic acid concentration in maize without negatively affecting seed quality. Although progress will be slow, this process could potentially provide an alternative to *lpa* maize mutants that has improved germination and similar iron bioavailability. This study also demonstrates that even small decreases in phytic acid content can significantly improve iron bioavailability. More studies will be needed to determine the optimal level of naturally-present phytic acid in maize to best affect iron bioavailability.

Table 3.1 Evaluation of relative standardized† phytic acid (PA) concentration and standardized available P concentration for the high phytic acid (HPA) and low phytic acid (LPA) categories of populations derived from BS11 and BS31, and *lpa* mutants. For PA, a smaller value implies a higher PA content. Means in the same column followed by different letters are statistically different ($P < 0.05$).

	Standardized PA		Standardized Available P	
	BS 11	BS 31	BS 11	BS 31
HPA	-0.60 ^a	-0.21 ^a	-0.09 ^a	-0.10 ^a
LPA	0.04 ^b	-0.05 ^a	-0.08 ^a	-0.07 ^a
<i>lpa</i>	1.13 ^c	1.13 ^b	0.48 ^b	0.48 ^b

†For PA and available P, abs. at 490 and 820 nm, respectively, was converted to standardized data (mean of 0, standard deviation of 1). Units are standard deviation from mean.

Table 3.2 Mean seed standard germination and cold test of the high phytic acid (HPA) and low phytic acid (LPA) categories of populations derived from BS11 and BS31, and *lpa* mutants. The standard germination test measures germination under optimal conditions and cold test evaluates seed vigor under cold conditions. Values are given as percentage of normal seedlings, \pm SE.

	Std. Germ. Test		Cold Test	
	BS 11	BS 31	BS 11	BS 31
LPA	95 ^{a†} \pm 1	90 ^a \pm 2	89 ^a \pm 3	92 ^a \pm 2
HPA	95 ^a \pm 2	90 ^a \pm 2	86 ^{a,b} \pm 3	92 ^a \pm 3
<i>lpa</i>	78 ^b \pm 2	78 ^b \pm 2	76 ^b \pm 3	76 ^b \pm 2

[†]Means within a column followed by different letters are statistically different (P<0.05).

Table 3.3 Phytic Acid (PA, mg/g) and Iron ($\mu\text{g/g}$) content of bulked maize samples from the high phytic acid (HPA) and low phytic acid (LPA) categories derived from the BS11 population, and *lpa* mutants. Analyses were performed in duplicate.

	PA	Nonheme Iron	Non-protein -bound Iron	Protein-bound Iron
BS11LPA	7.8	16.1	11.2	4.9
BS11HPA	9.0	16.4	12.4	4.0
<i>lpa</i>	6.4	19.4	11.0	8.7

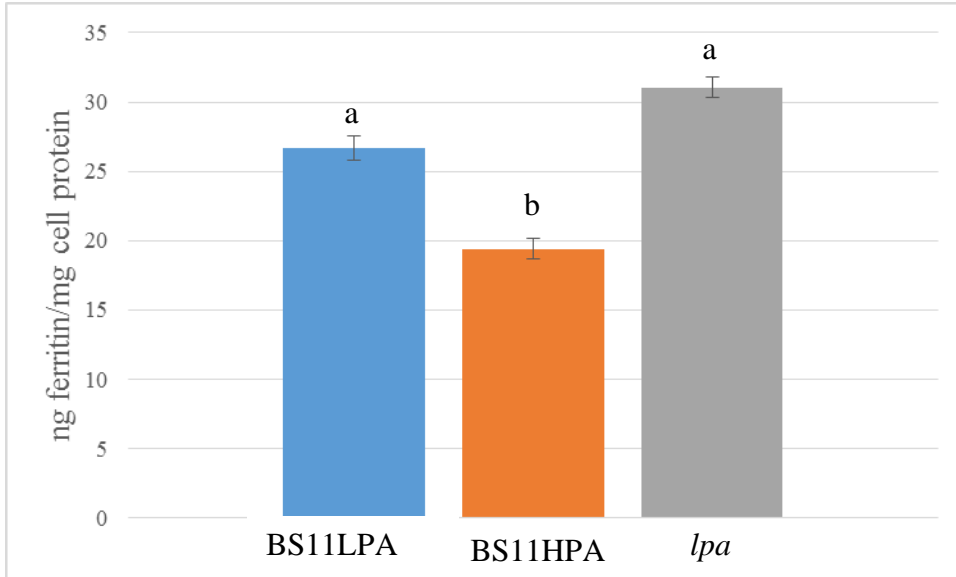


Figure 3.1 Iron bioavailability of bulks made from high phytic acid (HPA) and low phytic acid (LPA) categories of maize derived from the BS11 population, and *lpa* mutants expressed as ng ferritin/mg protein. Bars with different letters are significantly different ($P < 0.05$). Error bars are \pm SE. Four digestions were performed per sample, and each digest was applied to two wells (values from the two wells were averaged prior to analysis) ($n=4$).

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CHAPTER 4

CONCLUSIONS

Summary

One of the main contributors to iron deficiency worldwide is low dietary iron bioavailability. One of the strategies that has been employed to improve dietary iron bioavailability is through biofortification. Biofortification to enhance iron bioavailability can include both increasing nutrients, such as iron, and also by decreasing antinutrients, such as phytic acid. The current low phytic acid crops are predominantly mutants, which have a reduction in phytic acid and increase in iron bioavailability, but they also have poor seed quality making them unattractive to farmers. Recurrent selection, breeding for extremes of a trait over many cycles, could be used to reduce phytic acid content.

The study in chapter 3 of this thesis found that recurrent selection can successfully be used to alter seed phytic acid content in the BS11 maize population but not in the BS31 population, however the phytic acid content of the BS11LPA population was still much higher than that of the *lpa1-1* maize mutant populations. Seed quality was higher in the BS11LPA population when compared with the *lpa* mutants, and iron bioavailability was similar between the two.

In the appendix of this thesis, a transgenic approach was used to attempt to enhance iron bioavailability. Purified maize hemoglobin expressed in bacteria was found to have similar iron bioavailability to bovine hemoglobin, which suggests that if it were overexpressed in maize that this could enhance iron bioavailability. Overexpression of maize

hemoglobin was accomplished, however iron bioavailability was not improved when compared with untransformed maize.

Discussion

The results of the study in chapter 3 conclude that selective breeding is a viable alternative to genetic mutation for reduction of phytic acid content in maize. Furthermore, maize produced through recurrent selection can maintain a high seed quality because less drastic reductions in phytic acid are achieved. Even a small reduction in phytic acid content, as achieved by the breeding program in this study, was able to improve iron bioavailability. This suggests that phytic acid reductions may not need to be as drastic as seen in the *lpa* mutants to improve iron status in iron deficient populations.

The results of the appendix indicate that while maize hemoglobin is as bioavailable as bovine hemoglobin, overexpression of this protein in maize is not adequate to impart increased bioavailability. The results from this study suggest that although the protein portion of the hemoglobin was increased, iron was not incorporated into the protein. Since maize hemoglobin is predominantly found in the non-edible portions, the maize seed may not have the necessary proteins to incorporate the heme into the globin portion.

Future research should examine the effects of more cycles of recurrent selection on phytic acid content and seed quality in the BS11 maize population. If it is indeed possible to further reduce phytic acid content while maintaining seed quality, iron bioavailability should again be determined either through the use of an animal model such as pigs, or in humans. The studies in this thesis give hope that traditional breeding approaches may be able to avoid the disadvantages found in transgenic biofortification, and produce crops with higher iron bioavailability without risking the low consumer acceptance of transgenic crops.

APPENDIX**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. We hypothesize 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 gamma zein promoter. Iron bioavailability of maize hemoglobin produced in *E. 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 and that is significantly higher than unfortified flour. Transformed maize grain expressing maize globin was found to have similar iron bioavailability as 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 not be a successful 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 two billion people worldwide¹, and even mild deficiency has a wide range of negative health effects². 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 bioavailability³. 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 reduced⁸. Low phytate maize mutants⁹ can improve iron bioavailability^{10,11}, but have low yields and other negative effects^{12,13}. Although natural variability in maize iron bioavailability¹⁴ 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 ways⁷. Expression of soybean ferritin in rice resulted in bioavailability similar to ferrous sulfate fortified rice when evaluated in a

rat hemoglobin repletion model¹⁵, and human lactoferrin produced in rice had bioavailability similar to that of ferrous sulfate in young women¹⁶. Reduction of phytate has been achieved in maize through expression of *Aspergillus niger* phytase, without the undesired agronomic effects seen in low-phytate mutants¹⁷. Improved iron bioavailability was also achieved with co-expression of fungal phytase and soybean ferritin¹⁸ and by expression of soybean ferritin in low phytate maize¹⁹.

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 phenotype²⁰, may be more acceptable²¹, particularly if there is a nutritional benefit²². 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 like phytate²³. Symbiotic soybean leghemoglobin was found to be as bioavailable as bovine hemoglobin²⁴, 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 while the iron bioavailability of ferrous sulfate was significantly reduced by cooking²⁴, 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. While symbiotic plant globins are found only in legumes, all plants examined to date have genes coding for non-symbiotic globin proteins²⁵, and these globin genes in maize have been characterized²⁶. Non-symbiotic globins seem to

play a role in regulating hormone signal transduction²⁷. They are able to reversibly bind oxygen and they seem to be involved with stress response²⁸. 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 salt²⁹. When barley globin was overexpressed in maize tissue culture cells under low oxygen, nitrous oxide levels were reduced³⁰. To our knowledge, the iron bioavailability of non-symbiotic plant globins has not yet been determined.

Overexpression of maize hemoglobin in maize endosperm is a potential avenue for 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 non-symbiotic maize globin expressed in maize endosperm and of maize hemoglobin expressed in *E. coli*.

METHODS

Expression vector construction

A construct containing the *Zea mays* endosperm specific 27 kDa gamma zein promoter, a translational fusion of *Zea 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 pAct1IsGFP-1 plasmid³¹, containing the modified GFP gene sGFPs65T³² (Genbank accession ABB59985) and nos terminator sequence (modified from

Genbank accession V00087), was modified by the addition of the 27 kDa gamma 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 tissue³³. 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 SignalP³⁴, 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) from the ZmG cDNA. The primers were designed such that the PCR product included an NcoI site in conjunction with the start codon and completes an additional NcoI 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 CGCCCTTCCATGGCACTCGCGGAGGCC; reverse CCATGGCATCGGGCTTCATCTCCC; bold nucleotides are the NcoI sites and underlined nucleotides are not in the coding sequence of ZmG).

The PCR product was subcloned to pCR 2.1 Topo Vector (Invitrogen Corporation, Carlsbad, CA) for amplification in E. coli XL1 Blue (Stratagene, La Jolla, CA). The ZmG was restriction digested from pCR 2.1 by NcoI, and was inserted into the vector containing the 27 kDa gamma zein promoter and GFP at the NcoI 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 callus³⁵ was accomplished at the Plant Transformation Facility at Iowa State University. Callus was co-bombarded with the globin-GFP expression vector and with a plasmid containing the *bar* gene for bialaphos resistance³⁶. 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) (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 gamma 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 gamma 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. 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 non-transgenic 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 Shepherd³³ 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 based on 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 mass of positive and negative kernels was significantly different.

Maize hemoglobin and antibody production

Maize globin was expressed in *E. coli*, purified, and characterized as described by Smaghe 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 hemoglobin³⁷. 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, 10 mM Dithiothreitol and the FTIR spectrum was determined. Carbon monoxide was blown over the sample for about 10 seconds 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).

Transgenic kernel screening

Screening for transgene positive kernels consisted of visual screening (Figure 2) for GFP fluorescence using a Dark Reader hand lamp (Clare Chemical, Dolores, CO) 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) and measuring 4 points within each well. Comparisons between samples were made with a 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 uL of Lammeli buffer, boiled, centrifuged, and 15 uL of the supernatant was loaded on 15% SDS-PAGE gels. Separated proteins were then transferred to a nitrocellulose membrane, 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), or with anti-ZmHb polyclonal antibody (described above) with

1:25,000 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 1:50,000 in 2% milk in TBST. Immunoreactive bands were visualized by chemilluminescence.

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). The flour was fortified with one of the following fortificants: ZmHb produced in *E. coli*, bovine hemoglobin (Sigma Aldrich, St. Louis, MO), or ferrous sulfate (Sigma Aldrich, St. Louis, MO). 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 cooking³⁹, and since 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 1mM ascorbic acid in a glass pot until stiff (about 5 minutes). The porridge was then freeze-dried.

Each sample was digested in duplicate as described by Glahn⁴⁰ and modified by Proulx^{24,41}. The freeze-dried samples were mixed with 15 mL 0.1 M HCl and adjusted to pH 2.0 with 1 M HCl. Then, 1 mL of pepsin solution (1.2 g 1:10,000 porcine pepsin A in 10 mL 0.1 mol/L HCl) was added and the sample 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 NaHCO₃, and 5 mL of pancreatin and bile solution (0.05 g 4 x USP porcine pancreatin and 0.3 g bile extract in 35 mL of 0.1 mol/L NaHCO₃) 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 previously⁴¹, and centrifuged at 5000 x g for 10 min and frozen prior to use.

The iron uptake experiment was conducted as described by Proulx and Reddy⁴¹. 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 (American Type Culture Collection, Rockville, MD) 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% CO₂. Media was changed 3 times weekly. At 7 d, the cells were rinsed with Earle's Balanced Salt Solution (EBSS), trypsinised to dissociate the cells, and centrifuged at 22.6 x g. The cells were seeded at passage 29 at a density of 5x10⁴ cells/cm² on collagenized (Type 1 Rat tail collagen) 12-well Costar cell culture plates (Corning, Lowell, MA).

At 15 d post seeding, the cell monolayer was rinsed with EBSS. For cell uptake, 0.5 mL of serum free media 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 serum free media was then added, followed by further incubation for 22 h. Serum free media consisted of DMEM with 1% v/v nonessential amino acids, 1% v/v antibiotic-antimycotic solution, 0.4 mg/mL hydrocortisone, 0.5 ug/mL selenium, 3.4 µg/mL T3 (triiodothyronine), and 1 mmol/mL PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]), 2 µg/mL EGF (epidermal growth factor), and 1mg/mL insulin³⁹. After 24 h total incubation,

digests and media were removed by aspiration, and cells were rinsed with 1.5 mL EBSS. The cells were then lysed by addition of 0.5 mL deionized water to each well, and sonicated with a probe-type sonic dismembrator 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 Illinois), and ferritin was determined by radioimmunoassay (Fer-Iron II, Ramco Laboratories, Stafford TX) and measured with a Cobra-II gamma counter with SpectraWorks software (Packard BioSciences, Meriden CT). 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 a 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 μ moles of Fe with and without 34 μ moles of ascorbic acid (1:20 Fe:ascorbic acid), applied to cells, and iron bioavailability was assessed as described above. Based on 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 nonheme iron assay

described by Proux et al.⁴¹. FTIR spectrum was used to determine heme iron content of ZmHb-GFP as described above for maize hemoglobin produced in *E. coli*.

RESULTS

The bioavailability of iron in maize flour fortified with maize hemoglobin produced in *E. coli*:

In order to determine the utility of maize non-symbiotic 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 ZmHb protein was also confirmed as 18 kDa by Western blot (Figure 4), similar to the 18.3 kDa reported by Arechaga-Ocampo²⁶. 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. While these data don't support the hypothesis that hemoglobin iron is more bioavailable than non-chelated 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 and rearrangement of the transgene during bombardment or integration into the genome. Of these 15 events, 7 were selected for further experiments based on 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 mass of visually GFP positive and negative kernels from the same ear was not significantly different ($p = 0.9348$, $n = 100$ kernels each from 3 ears, data not shown), indicating that the transgene does not cause a change in mass. There were significant differences in kernel mass between ears ($p < 0.0001$) 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 7 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 their non-transformed negative controls (18.71 vs 16.25 $\mu\text{g/g}$) 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, 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% non-significant ($p=0.44$) difference was found when ascorbic was not added to the samples. Overall adding ascorbic acid improved iron bioavailability by 2.5-fold and 2.7-fold in ZmG-GFP positive and negative samples, but the relative differences were 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 response with the positive control and the negative controls were similar to 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 non-symbiotic 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 models¹⁶ and in humans¹⁷. It is now understood that ferritin is degraded during digestion and iron associated with the protein will enter the common non-heme iron pool, making it susceptible to phytate inhibition⁴³. Unlike ferritin, hemoglobin has a unique uptake pathway and transport mechanisms⁴⁴, and the heme pyrrole is protective against the inhibitory effects of phytate and other non-heme iron inhibitors²³.

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 development future plant 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 GFP⁴⁵. Therefore, ZmG-GFP lines could be used to evaluate iron bioavailability in future animal feeding studies with little concern with GFP. However, based on 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

suggest that 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 synthesis³⁶. 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 tissue²⁸, 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 14d seedlings when exposed to 48h 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 d seedlings but found that levels of globin were greatly decreased in 14 d seedlings²⁶.

Drakakaki et al. showed that localization of recombinant proteins contributes to protein functionality⁴⁷. While 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^{50,51,52} 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 nucleus⁵³, indicating that cytoplasmic accumulation of the protein would be sufficient for heme pyrrole insertion. Maize globin sequence is not predicted to have a signal sequence³⁴. 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. Further, 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-dependant metabolic processes such as photosynthesis and could lead to reductions in grain yield.

Since hemoglobin expressed in E-coli has similar bioavailability as 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 don't make sufficient amounts of pyrrole to meet the requirements of the over-expressed 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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Table 1. Iron bioavailability expressed in relative 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 ($\mu\text{g/g}$) 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. Both transformed and untransformed ZmG-GFP and GFP samples were tested with and without ascorbic acid. Statistical comparison was conducted with a Student's t-test.

		RBA (%)				Total iron ($\mu\text{g/g}$)			
		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					
ZmG-GFP without AA	Positive	4	5.00	0.39	0.44	2	16.25	0.80	
	Negative	4	5.51	0.50					
GFP with AA	Positive	4	8.92	1.70	0.32	2	15.49	0.36	
	Negative	4	6.67	1.16					
GFP without AA	Positive	4	3.70	0.43	0.23	2	14.09	0.68	
	Negative	4	3.05	0.23					

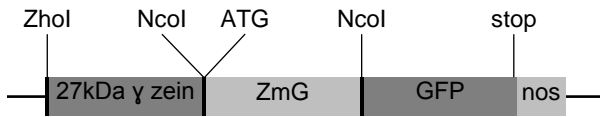


Figure 1. Gene construct used to create maize overexpressing maize globin. The construct contains the endosperm specific 27 kDa gamma zein promoter, a fusion of maize globin (ZmG) and green fluorescent protein (GFP) coding sequences, and the nos terminator. The position of the start codon ATG and the 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.



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 while non-fluorescing kernels appear dark or orange.

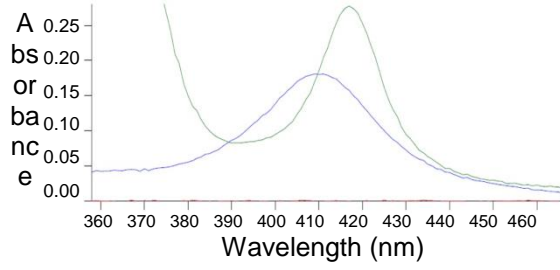


Figure 3. Heme iron in ZmHb produced in *E. coli* as detected with FTIR. Oxygenated ferric maize hemoglobin has a peak at 412 nm (abs 0.155) and deoxygenated ferric maize hemoglobin has a peak at 418 nm (abs 0.255).

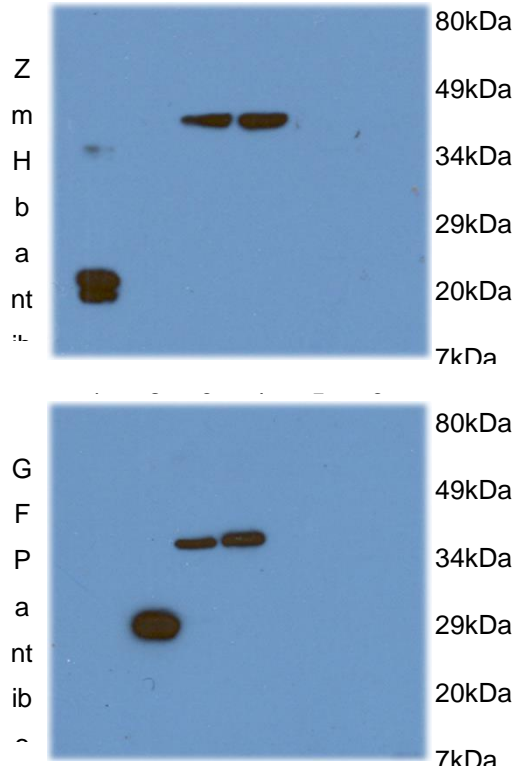


Figure 4. Western blots of maize endosperm using antibodies to GFP or maize hemoglobin (ZmHb). Lanes on both blots: 1 maize hemoglobin produced in *E. coli* (30 ng), 2 GFP standard (Clontech, Mountain View, CA) (20 ng), 3 transgenic event 26 F1, 4 transgenic event 65 F1, 5 untransformed A188, 6 untransformed 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.

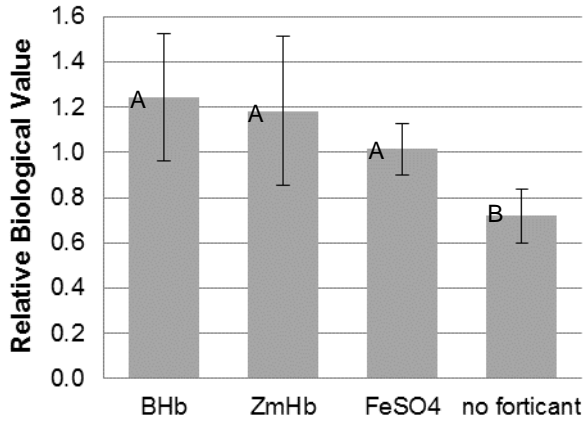


Figure 5. Iron bioavailability of fortified maize flour expressed as ferritin synthesis (ng ferritin / mg cell protein) relative to ferrous sulfate. The iron bioavailability for bovine hemoglobin (BHb), maize hemoglobin (ZmHb), and ferrous sulfate (FeSO₄) are not significantly different, but all three are significantly different from flour with no added fortificant ($p = 0.0005$, $n = 8$).

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