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Elizabeth C. Theil and Jean-Francois Briat

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Plant Ferritin and Non-Heme Iron Nutrition in Humans

*Elizabeth C. Theil and **Jean-Francois Briat

* CeBIC, Center for BioIron at CHORI (Children's Hospital Oakland Research Institute), Oakland, CA. USA
** Biochimie et Physiologie Moléculaire des Plantes. Centre National de la Recherche Scientifique, Institut National de la Recherche Agronomique, École Nationale Supérieur d'Agronomic. Université Montpellier 2.

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I | Introduction

Dietary iron deficiency is the most common and widespread nutritional disorder in the world (DeMaeyer et al. 1985), affecting more than two billion people (WHO 2001). This monograph discusses plant ferritin, an iron-rich, high molecular weight component of legumes that is one of the many dietary non-heme iron sources in human nutrition.

Iron in food from plants has more varied properties than iron in food from animals, where heme proteins are the most abundant form. The chemical form of non-heme iron in foods significantly affects dietary iron bioavailability, independent of other dietary components that can further alter bioavailability. The category "non-heme" iron must include the different chemical forms of iron (Table 1) besides just the non-heme source and the oxidation state, and should be expanded to include low molecular weight chelates such as citrate, phytate, and gluconate, and high molecular weight iron forms such as iron carbonyl ("rust particles"), iron-dextran (a polysaccharide with small clusters of ferric ions linked by oxygen atoms), and ferritin (a protein-iron complex with a protein cage and a mineral center of thousands of ferric ions linked by oxygen atoms). When known, the specific physiology of the different forms of non-heme dietary iron during absorption should be included in the expanded category of non-heme dietary forms of iron. When unknown, more research on the physiological behavior of the different forms of dietary iron is needed.

Because ferritin iron is separated from the iron-binding (chelating) components in food by its protein coat (Theil et al. 2001), it is less sensitive to chelators such as phytates, whose concentrations depend on soil and climatic conditions. How ferritin is absorbed during digestion is unknown, but its stability in solution (Theil et al. 2001) suggests that the protein and/or iron clusters survive digestion mostly, if not entirely, intact. Given the existence of a specific receptor for lactoferrin (Suzuki et al. 2001) and the large number of iron receptor genes in microorganisms, a specific ferritin receptor may exist. In addition to the possibility of a ferritin-specific receptor or iron incorporation mechanism in the gut, it is possible that there are multiple, specific receptor/absorption mechanisms that are matched to other chemical forms of iron in the gut other than the divalent cation transporter, DMT1. In addition, ferritin can be absorbed by the opening of specific pores (Liu et al. 2003), similar to the mechanism by which a number of viruses transfer their infectious components into cells. When the ferritin pores unfold, the ferritin becomes accessible to reduction and iron removal (Liu et al. 2003). Currently, only a few human genes controlling iron absorption are known (Miret et al. 2003), and the manner in

TABLE 1

Examples of the Complexity of Non-heme Food Iron

Organic/biochemical
Low molecular weight: ferric citrate, ferrous gluconate, ferrous fumarate
High molecular weight: ferric dextrans,* iron carbonyl,* ferritin

Inorganic
Low molecular weight: ferrous sulfate, ferrous carbonate, ferric chloride (pH < 2.5), ferric citrate, ferric EDTA
High molecular weight: ferric chloride (pH > 4)

* A protein cage or polysaccharide in a complex with an inorganic cluster or nanoparticle of hydrated ferric oxide. Except for ferritin, where the protein-mineral complex survives digestion mostly intact, iron in the other high molecular complexes is converted to low molecular weight forms during digestion.

which iron absorption genes are influenced by genetic variation has yet to be determined. Nevertheless, the combination of different iron absorption genetics along with differences in the content of iron and iron-binding chelators in foods and crops can easily explain the wide variations in the ability of different forms of non-heme iron to control iron deficiency (Casanueva and Viteri 2003).

When the human genes controlling iron digestion and absorption are more fully defined and the influence of genetic differences on iron absorption, especially in disease, is better understood, it will become possible to develop individualized diets to treat iron deficiency. In the meantime, bioavailable sources of natural food iron can help combat dietary iron deficiency in vulnerable populations.

II | Chemistry of Iron Compounds Found in Food

Each of the forms of non-heme food iron has a distinct chemical behavior that varies with the food source and is incompletely understood. Some predictions about food iron interactions during digestion can be made based on knowledge of the chemistry of dietary non-heme iron. Studies of food iron bioavailability appear to have conflicting results (see Table 2). These may be better understood by considering the behaviors of the different chemical forms of the non-heme iron used, such as ferric citrate or ferric chloride.

Each form has a different chemistry in the stomach or in the gut. For example, if the iron is present as ferric chloride, it will exist as single ferric ions in the stomach, and these will be fully chelated with phytate if it is present in sufficient amounts. If there is an excess of ferric chloride, at the neutral pH of the intestine, the iron will be polymerized into "rust" particles that may not be available for reduction to the ferrous form and subsequent absorption. On the other hand, if the iron is ferric citrate, there will be competition for the iron by phytate and citrate, and if there is excess citrate, such as in a meal rich in citrus fruits or juice, the iron will be complexed by citrate and will be more readily available for absorption. Finally, if the iron is present as ferrous sulfate, it will be stable as ferrous iron at the pH of the stomach and will not polymerize in the intestine unless it is oxidized to the ferric form.

Thus, the form of the non-heme-non-ferritin iron that can be absorbed from a meal depends on the amount of iron, the relative amounts of chelators such as phytate and citrate, and the amount of reducing compounds. Individual variations in human physiology, such as iron status and genetic differences in absorption mechanisms, create additional variation in the ability to absorb iron. Because ferritin iron exists within seed membranes as a solid mineral inside a very stable protein, it is chemically and physically different from other forms of non-heme iron and appears to survive digestion (Kelleher, Theil, and Lonnerdal, unpublished observations). A recent study of a group of young American women showed that the iron in pure ferritin is as well absorbed as animal (low phosphate) and plant (high phosphate) forms of mineralized ferrous sulfate when ingested as part of a light breakfast [Davila-Hicks, Theil and Lonnerdal, Am J Clin Nutr (2004), in press]. Analogous experiments with purified endogenously labeled soybean ferritin are essentially impossible because of the large amounts of radioactivity needed for isolation of a sufficient quantity of radioactive ferritin.

Ferrous iron has long been considered to be the main form of non-heme iron absorbed by humans. Absorption of ferrous iron is thought to occur *via* the receptor DMT1 (Fleming et al. 1997, Gunshin et al. 1997), and other forms of iron in the gut may be enzymatically converted to ferrous iron (Miret et al. 2003, McKie et al. 2001). However, DMT1 is not selective for iron, and it seems unlikely that a process as important as iron absorption would have only a single mechanism that is shared with several other ions. Indeed, other organisms such as bacteria have receptors specific for ferrous ions and ferric complexes, including ferric citrate, heme, and ferric siderophores (Braun et al. 1998, Zimmermann et al. 1984, Neilands 1991). Furthermore, in addition to DMT1, a developmentally

TABLE 2

Comparison of Whole Soy Iron Availability in Humans in Three Different Studies

	Sayers et al. 1973	Lynch et al.1984	Murray-Kolb et al. 2003
No. subjects	5	10	18
Gender	Female	Male	Female
Labeling	Intrinsic	Extrinsic	Intrinsic
Food iron form	Bean protein (Ferritin)	$FeCl_3$	Bean Protein(Ferritin)‡
Test iron form	$FeSo_4$	$FeSo_4$	$FeSo_4$
Form of meal	Biscuit	Soup	Soup/Muffin
% Uptake (soy meal)	20	1.6	25.9
% Uptake (reference dose)	72.8	16	58.9
% Uptake(meal/reference dose)	27.5	10.0	44.0
†Hct	--	0.47	0.40
†Hb (g/L)	108	--	131
†Serum ferritin (µg/L)	--	61	11.2

† Values on the day the soybean meal was consumed.
‡ Approximately 50% of the iron in the soybeans was in the ferritin form (see Murray-Kolb et al. 2003).

regulated receptor for lactoferrin has been identified and cloned in humans (Suzuki et al. 2001). Because useful mechanisms are often conserved between organisms, it is likely that a number of new human receptors for food iron remain to be identified. A discussion of strategies used for iron acquisition in different organisms can be found in (Kaplan 2002).

The iron oxidation state (ferric or ferrous), the size of the iron complex, and the type of complexing agents are all needed to describe the type of non-heme dietary iron (Table 1). Generally, the iron in food is in the oxidized ferric form. However, when iron complexes are added to food as ferrous salts, which are then oxidized by air, the addition of a reducing agent aids in absorption by reducing ferric iron back to the ferrous form. Often ascorbate is used to reduce ferric iron to ferrous iron, with the ascorbate acting as a reducing agent/antioxidant. However, when exposed to air, the combination of ascorbate and ferric iron can also produce free radical reactions. In this situation, ascorbate acts as a pro-oxidant rather than an antioxidant, and is converted to an ascorbate radical plus ferrous ions. The free radicals generated in the presence of air cause gastrointestinal disturbances and change the gut cell metabolism (Jourdheuil and Meddings 2001, Courtois et al. 2000). Thus, consideration should be given not only to the antioxidant properties of ascorbate in food mixtures, but also to its pro-oxidant properties.

Natural dietary iron sources in foods from plants vary depending on crop growing conditions, the specific food type, and the part of the plant consumed. In soybeans, for example, much of the ferritin is in the hulls (Ambe, 1994). Consequently, food made from whole soy such as soymilk or soy nuts contain much more ferritin iron than food from dehulled soybeans or processed foods such as tofu, where protein coagulation excludes much of the iron and immunoreactive ferritin (Harlow and Theil, unpublished observations). While the effects of total iron from plant crops in the diet have been studied, less is known about the effects of crop growing conditions on the different forms of iron in food from plants. Seed iron and chelator composition can be changed by drought, variations in temperature, pH, and soil composition. For example, the relative distribution between iron in phytate and

ferritin can change while the total iron content remains unchanged. Studies on the effects of crop growing conditions (Ambler and Brown 1974, Tang et al. 1990, Clarkson and Hanson 1980, Burton et al. 1998, Grusak 1995, Goto et al. 1999, Davila-Hicks et al. 2004) are often used only by plant nutritionists and are overlooked by human nutritionists. However, dietary bioavailability in humans can be altered significantly by crop growing conditions and consequent changes in crop composition. Evaluating the dietary consequences of plant variability in iron absorption is a multi-factorial, interdisciplinary problem requiring the application of contemporary computational methods.

Chemistry (Gut Absorption)

Low molecular weight forms of non-heme iron. Water-soluble forms of food iron with a low molecular weight are weak ferrous complexes and include ferrous gluconate and simple ferrous salts, such as ferrous sulfate. The chemistry of ferrous ions during digestion is similar to that of other required metal ions except ferric ions. Hydrated ferrous ions are mostly recognized by the DMT1 receptor. Except for the lactoferrin receptor, which is involved in early development (Suzuki et al. 2001, Miret et al. 2003), DMT1 is the only molecule currently known to take up iron from the mammalian gut. DMT1 also absorbs other divalent cations, including zinc and copper (Fleming et al. 1997, Gunshin et al. 1996). Ferrous ions are stable at the low pH of the stomach, but the rate of oxidation increases as the pH increases in the intestine. A ferri-reductase is known to enhance iron absorption in the gut (McKie et al. 2001). Whether its main role is to reduce ferric ions produced by the oxidation of ferrous salts in the food, or whether the enzyme converts all forms of food iron to ferrous iron (McKie et al., 2001) is not known. The alternative concept, that multiple gut receptors exist for different chemical forms of iron, is supported by the multiplicity of microbial iron receptors for both ferrous and ferric complexes (Braun et al. 1998) and the existence of the mammalian enterocyte lactoferrin receptor (Suzuki et al. 2001).

Ferric citrate and ferric-nitrilotriacetic acid (NTA), a chelator of ferric iron similar to ethylene diamine tetraacetic acid (EDTA), have specific three-dimensional structures that are relatively stable to pH changes during digestion, especially when citrate is in excess, such as in diets rich in citrus fruit. In bacteria, ferric citrate is a natural form of iron in the environment that is recognized by a specific receptor (Angerer and Braun 1998). Given the presence of a ferric citrate receptor in microorganisms, it is likely that there is also a human analogue. The question of whether ferric-EDTA or ferric NTA, which are non-biological iron complexes, use such a natural ferric receptor awaits further study. Ferric iron added to the diet (e.g., as ferric chloride) is stable as single, hydrated iron ions at the pH of the stomach. If phytate is present in the food, ferric phytate will form. However, at the pH of the gut, ferric ions that are not bound to chelators such as phytic acid will associate into clusters of hydrated ferric oxides or small "rust" particles. The enterocyte recognition system is likely to incorporate iron in this particle form by a phagocytosis-like process rather than by receptor-mediated endocytosis, which is used for protein-bound iron or iron chelates. Because receptor-mediated endocytosis triggers a set of intracellular trafficking reactions distinct from those triggered by phagocytosis, iron reaching the gut in the two different chemical forms may trigger distinct cell transport pathways. Indeed, the difference in tissue iron loading in rats fed equal molar amounts of ferrous sulfate or ferritin (Beard et al., 1996) may be a reflection of two different iron trafficking pathways in the gut enterocytes.

High molecular weight non-heme iron. The absorption mechanisms of high molecular weight iron forms, such as iron carbonyl or endogenous plant ferritin, are unknown. Iron dextran, a form of iron supplement that is usually administered parenterally, is a large complex and is likely taken into cells by phagocytic mechanisms (Henderson and Hillman 1969). It is often assumed that iron carbonyl and ferritin are subjected to acid hydrolysis during digestion, releasing the iron as single ferric ions that can be complexed by food chelators and reduced to the ferrous state at the enterocyte surface. However, there is insufficient experimental data to determine whether this hypothesis is valid, and, furthermore, as in plant ferritin, the high molecular

weight forms appear to be protected from digestion or are stable to digestion. If the iron clusters are solubilized more slowly and can transfer from the acidic pH of the stomach to the neutral pH of the gut, they may remain or reassociate into clusters at the gut enterocyte surface. How such iron in ferric-oxy aggregates crosses into the enterocyte is unknown. Direct experiments using models of digestion or cell culture are needed to understand the molecular basis for absorption of high molecular weight iron from food (Burton et al. 1998).

III | Ferritin and Dietary Availability of Ferritin Iron

Ferritin in food has mainly been studied in soybeans. All cells in plants and animals contain ferritin, but it is present in only small amounts and related gene activity maintains cellular iron homeostasis. Ferritin is not abundant in grains such as wheat (May et al. 1980), which contrasts with the high levels of iron and ferritin in legume seeds. The extra iron and ferritin in legume seeds may be due to recycling of the extra iron taken up during nitrogen fixation (Burton et al. 1998). A preliminary immunological analysis using an antibody to soy ferritin showed that lentils and chick peas have similar amounts of ferritin as soy (Liu, Yi and Theil, 2004, unpublished observations), but appropriate antibodies need to be developed for more accurate analyses of ferritins in other beans and legumes.

Dietary Studies with Ferritin and Soybeans

Conflicting results have been obtained from studies on the bioavailability of iron from ferritin or from soybeans (Sayers et al. 1973, Lynch et al. 1984, Murray-Kolb et al. 2003, Davila-Hicks et al. 2004, Layrisse et al. 1975, Beard et al. 1996). Differences in the results and conclusions are likely due to:

- the complexity of non-heme iron chemistry in food
- variations in the iron status of the human subjects (Sayers et al. 1973, Murray-Kolb et al. 2003)
- the unique physical and chemical properties of the non-heme iron in ferritin.

Early interpretations of iron incorporation data assumed that added non-heme iron, whatever the form, would equilibrate with the mineral iron in ferrritin (Layrisse, 1975) or with the different types of endogenous non-heme iron in foods (Lynch et al., 1984); however, subsequent research has shown that this is not always true (Beard et al. 1996, Murray-Kolb et al. 2003). Briefly, one main issue is that *in vivo* labeling does not produce uniformly labeled ferritin iron. This is particularly a problem when a single dose of radioactive iron is used under inflammatory conditions in animals to induce the synthesis of stress ferritin. In recent studies in humans, in contrast, soybeans were used from plants in which ferritin iron was uniformly labeled during normal plant maturation. In these experiments (Murray-Kolb et al. 2003), high iron absorption from soybean-based foods was observed even though the cultivation conditions produced soybeans with high phytate and, as a result, an increased percentage of iron in the form of ferric phytate. Fortunately, commercial conditions for growing beans vary much less than those available to the experimentalist and commercial beans have less variation in phytate content than beans used in experimental studies.

Extrinsic sources of non-heme iron salts do not equilibrate with the mineral iron inside ferritin in soy meal, and, when added to soy meal, the reaction with phytate depends on the iron salt. For example, compared to ferric citrate, where much of the iron remains associated with citrate and can be easily absorbed (Sayers et al. 1973), ferrous sulfate and ferric chloride readily bind to phytate and are poorly absorbed (Lynch et al. 1984). Table 2 shows a comparison of several studies using soybeans and ferritin to study iron absorption (Murray-Kolb et al. 2003). As in the study by Sayers et al. (1973), Murray-Kolb et al. (2003) showed that absorption of ferrous sulfate was high and that soy was effective as an iron source. In addition, the subjects had borderline iron deficiency, which would have been associated with heightened iron absorption, thereby increasing the sensitivity of the experiment. In the study by Lynch et al. (1984), the subjects had sufficient iron, low ferrous sulfate absorption, and low absorption of iron from an exogenous ferric chloride

salt added to the soy meal. Very likely, the low availability of iron measured in this study was due to the combination of the label in a non-heme iron salt with endogenous soy phytate and enhanced binding, together with the iron status of the subjects. Interestingly, in the 2003 study by Murray-Kolb et al., although the soybeans had an unusually high phytate content because of the high phosphate medium, the soybean iron was still well absorbed (26%) in the subjects with borderline iron deficiency, thus emphasizing both the physical separation of iron in ferritin and phytate complexes and the different chemical properties of iron in ferritin and iron in phytate complexes (Murray-Kolb et al. 2003).

A recent experiment using iron in pure ferritin, uniformly labeled by *in vitro* mineralization and eaten with a normal breakfast (bagel, cream cheese, and apple juice), showed directly that mean (and standard deviation) ferritin iron was absorbed to the same extent as ferrous sulfate (24.0 ± 14.6%), regardless of whether the ferritin mineral was of the plant type containing high phosphate (19.8 ± 19.2%) or the animal type containing low phosphate (23.9 ± 14.7%) (P. Davila-Hicks, E.C. Theil and B. Lonnerdal, Am J Clin Nut, 2004, in press). The subjects in the two recent iron absorption studies showing high iron absorption from ferritin, one using labeled iron in soybeans (Murray-Kolb et al, 2003) and the other using pure ferritin (P. Davila-Hicks, E.C. Theil and B. Lonnerdal, Am J Clin Nut, 2004, in press), all had borderline iron deficiency but were from different environments (California *vs.* Pennsylvania) and of different ethnic composition, emphasizing the generality of the absorption of soybean ferritin. Moreover, the earlier study using unlabelled iron in soy diets, which measured hemoglobin (Sayers et al.1973), achieved similar results in a population of women with borderline iron deficiency from India. One study did show poor absorption of iron added to soy; however, in that study the iron was added as a salt, which was unlikely to equilibrate with the iron in ferritin and likely to bind to soy phytate (Lynch et al., 1984). Moreover, the subjects were iron replete. Thus, to assess absorption of non-heme iron the variables that need to be assessed are :

- iron status of the subjects
- competing food components, such as phytate
- the chemical form of the non-heme iron including:
- size (single iron atoms vs. iron polymers/crystals)
- oxidation state (ferric/ferrous)
- presence of coordinating ligands/anions (e.g., citrate/phytate and tannins)
- presence of a stable protective coating, such as protein (ferritin) or polysaccharide (dextran)

Analyzing Ferritin in Foods

Little is known about the ferritin content and distribution in foods other than soybeans. Ferritin distribution has been studied by immunological methods (i.e., Western blotting of the ferritin protein component) in soluble aqueous extracts of freshly ground beans (Burton et al. 1998, Beard et al. 1996), by comparing trichloroacetic acid precipitates from whole beans for the distribution of iron between phytate and ferritin (Murray-Kolb et al. 2003), and by Mössbauer spectroscopy of the whole beans (Ambe et al. 1987, Ambe and Mossabauer 1994). Preliminary studies show that ferritin survives at high concentrations in processed foods, such as soy milk and soy bars made from whole soybeans, but is low in tofu and remains in the soluble fraction after coagulation (Theil, Harlow, and Yi, unpublished results).

A full understanding of the bioavailability of ferritin iron has only recently been achieved; hence the unique properties of the iron-protein complex have not yet been used to develop a selective assay for ferritin in foods. This may be possible by taking advantage of seed-specific immunological measurements combined with improvements in hull and seed extraction and the heat stability (stable for 10 min at 60°C) or large size (~700 kDa) of ferritin.

Ferritin Structure

Iron in ferritin is released slowly because it is a solid mineral inside a protein. The ferritin structure is unique among proteins. The structural conservation is limited to secondary, tertiary and quarternary structure (four helix bundles, assembled in a spherical protein cage with a large nanaocavity). However,

except in higher plants and animals where even the amino acid sequence is conserved, the amino acid sequence is highly variable, suggesting convergent evolution with selection for the higher order structure. Thousands of iron atoms can be concentrated inside a single ferritin iron-protein complex. All ferritins are made up of a spherical protein cage around a nanomineral of hydrated ferric oxide. Ferritins occur in two sizes: maxi-ferritins in plants, animals, and humans; and mini-ferritins, also known as *Dps* proteins, in bacteria. Whether mini-ferritins also occur in plants, animals, or humans is not yet known. Unlike many other proteins in which the linear amino acid sequence of the protein has common features in all organisms, it is the spherical protein/nanocavity structure of ferritins that is conserved, and it can, therefore, be formed by a variety of amino acid sequences.

Ferritin is a very stable protein (Theil 2001). The overall structure is stable to treatment with 6 M guanidine (Listowsky et al. 1972), heating to 60 to 85°C (Liu et al. 2003), and acidification to pH 2.5 (Santambrogio et al. 1992). However, the stability of ferritin in seeds or isolated from plants has been not been well studied.

Ferritin Function in Iron Mineral Utilization

The iron in ferritin is used when iron proteins are synthesized. Utilization of iron in ferritin is more readily observed in specialized organs that are iron-rich, such as nodules of legumes (Tang et al. 1990, Ragland et al. 1993), red blood cells (Peto et al. 1983, Vaisman et al. 1997), or liver under specialized conditions, such as repeated phlebotomy or hemorrhage (Bothwell et al. 1979, Theil 1987). In contrast to iron in the form of simple salts, the release of iron from cellular ferritin *in vivo* or upon digestion of food can be studied because of its resistance to acid and proteolytic degradation (Crichton 1969).

The structure of ferritin itself controls the flow of iron from the mineral (Liu et al. 2003). The slow release of iron from the mineral can be reversed by the opening or unfolding of ferritin-gated pores (Liu et al. 2003, Takagi et al. 1998, Jin et al. 2001). Ferritin pores connect the surface of the protein to the mineral in the protein nanocavity. When the ferritin protein assembles, and prior to mineral formation, the subunits of the polypeptide helix bundles (24 in maxi-ferritins or 12 in mini-ferritins) combine to form a large central space 5 to 8 nm in diameter. This central space is the site of iron mineralization (Theil 2001). Gated pores are formed at the junctions of three ferritin subunits. All of the amino acids that gate the pores are conserved in maxi-ferritins, but little is known about pores in the bacterial mini-ferritins. The cellular factors that recognize ferritin pores and control their opening and closing have not yet been determined. However, it is clear that the highly conserved structure of ferritin controls mineral dissolution, although it does not seem to be important for other functions. Thus, it appears that the pores have been selected to control iron mobilization. Whether the ferritin pores are opened during iron absorption is not known, and whether the ferritin is further protected from digestion by being inside plant plastids and often in seed hulls (Ambe 1994) remains to be determined. The high stability of ferritin protein in solution (Theil 1987, Crichton 1973, Harrison and Arosio 1996) suggests that plant ferritin is protected from digestion in the stomach and arrives in the intestine intact. This is also supported by the location of ferritin iron in a solid mineral, which, in turn, lies within the stable protein inside of seed cell plastids and is concentrated in seed hulls (Ambe 1994).

Resistance to Digestion

Ferritin is relatively resistant to proteolytic digestion (Crichton 1969), although, at least in animal ferritins, certain sites within the subunits are differentially sensitive to proteolysis. If the protein cage is destroyed, for example by very low pH (< 2.5), insoluble rust nanoparticles are produced. At low pH, these particles are eventually converted completely to ferric ions, although the amount of time required for this transformation in the stomach is unknown. Furthermore, even if all the iron remains dissolved in the stomach, the higher pH of the intestine promotes the conversion of unreduced or unchelated ferric ions to rust or polynuclear particles. What happens during the digestion of a ferritincontaining

meal, either seed or pure ferritin, is not yet known. However, soybean ferritin, even when there is a relatively high phytate content in soybeans, is absorbed from meal and converted to a form used by red blood cells (Murray-Kolb et al. 2003). The iron from ferritin in foods (e.g., legumes) clearly escapes any chemical traps, such as phytate chelators, and remains available during digestion for absorption and use.

In addition to developing a convenient method to analyze food for ferritin, further investigations need to address the following questions about the digestion of ferritin:

- What is the ferritin content of human foods other than soybeans?
- How do different growth conditions affect the ferritin iron content of beans and seeds?
- How stable are bean and seed ferritins to digestion *in vivo* and *in vitro*?
- What are the effects of other dietary components, such as phytate, tannins, and ascorbate, on the absorption of iron from ferritin in beans and seeds?
- How does the import and export of iron from ferritin by cultured enterocytes differ from other forms of iron, such as ferrous sulfate?
- What is the molecular mechanism of iron incorporation from ferritin in the gut?
- How do diseases or genetic variations in absorption affect the availability of iron from ferritin in beans and seeds?

Regardless of whether the answers to these questions are known, people have been eating and absorbing iron from ferritin and other high molecular weight forms of non-heme iron for at least as long as unhulled soybeans or other beans have been eaten.

IV | Biology of Iron Accumulation in Plant Tissues During Development

Plants are an essential component of the food chain because they are responsible for mineral acquisition from the soil and because they assimilate carbon, sulfur, and nitrogen into amino acids and vitamins. Plants also mine iron from the soil, facilitating the entry of iron into the biosphere. Iron is needed in the plants because iron-dependent proteins participate in carbon, sulfur, and nitrogen assimilation. Many of the iron-dependent reactions take place in specific subcellular organelles such as plastids, mitochondria, or the synergistic bacteroids of leguminous root nodules.

Iron uptake, transport, and storage is tightly regulated to prevent both iron deficiency and toxicity, thus ensuring optimal plant development. Various transporters are required to achieve these iron fluxes (Curie and Briat 2003). Plant iron storage takes place in the apoplasmic space, in the vacuoles (Briat and Lobreaux 1998), and the ferritins. Plant ferritins are located in the plastids and each ferritin particle can store up to 4,500 iron atoms (Harrison and Arosio 1996). Accumulation of iron in the various plant tissues during growth and development is a dynamic process resulting from an integrated regulation of genes encoding proteins for iron transport and storage. These processes depend on the plant genotype and are greatly influenced by environmental cues.

Iron Homeostasis in Vegetative Organs During the Plant Life Cycle

Leaves are a major organ for iron accumulation in plants, and 80% of this storage is in the chloroplasts. Most of the ferritin accumulates in non-green plastids, but a low level of this protein is found in mature chloroplasts where active photosynthesis occurs. Furthermore, this distribution of iron changes according to the stage of leaf development. Ferritin levels increase in developing leaves (Briat JF, Lobreaux 1997, Lobreaux and Briat 1991, Theil and Hase 1993), indicating that ferritin synthesis in leaves is developmentally controlled. This further suggests that ferritin is a source of iron for

the synthesis of iron-containing photosynthetic proteins at early stages of development. Ultrastructural (Seckback 1982) and molecular studies (Theil and Hase 1993, Buchanan-Wollaston and Ainsworth 1997) have also clearly established that ferritin accumulates in senescing tissues.

The regulatory mechanisms controlling ferritin synthesis during leaf development are still unknown. In fact, there is no direct correlation between the levels of ferritin subunit proteins and their mRNAs (Theil and Hase 1993, Ragland et al. 1990). Thus, posttranscriptional controls must be involved in the regulation of ferritin synthesis in leaves. More recently, *cis*-regulatory element(s) involved in activation of the *Arabidopsis AtFer1* ferritin gene during age-dependent senescence were found within its 1.4 kbp promoter sequence. These regulatory elements are distinct from the Iron-Dependent Regulatory Sequence (IDRS) box, which mediates iron-dependent expression of the *AtFer1* gene (Tarantino et al. 2003) as well as activation of *AtFer1* expression during dark-induced senescence.

The iron concentration in roots is much lower than in leaves. Nevertheless, ferritin is also present in the non-green plastids of this organ. In *Arabidopsis, AtFer1* is expressed close to the root tip in the endoderm cell layer as well as at the emergence of secondary roots (Tarantino et al. 2003). It is, therefore, plausible that ferritin in plastids buffers the endodermal cells after iron uptake by the epidermis and cortex.

Plants from the legume family fix nitrogen by developing a symbiotic relationship with some soil bacteria. This symbiotic process takes place in the nodules, specific root structures within the cortical cells where bacteria are transformed into bacteroids that can reduce atmospheric nitrogen into ammonia. This process requires some essential iron-containing proteins, such as nitrogenase and leghemoglobin (Tang et al. 1990). Iron uptake by the bacteroids has been well documented (Levier et al. 1996, Moreau et al. 1995, Wittenberg et al. 1996, Moreau et al. 1998, Kaiser et al. 2003), and plant ferritin has been shown to accumulate during the early stages of nodule development (Ko et al. 1987). Ferritin levels decrease with the appearance of nitrogenase and leghemoglobin, when the nodule becomes mature for nitrogen fixation (Bergersen 1963). In senescing nodules of *Lupinus luteus*, ferritin is resynthesized through the expression of two out of the three lupine ferritin genes (Strozycki et al. 2003). Ferritin is detected at many developmental stages when soybean plants are cultured in the presence of a mutant strain of *Bradyrhizobium* that is unable to develop functional nodules (Ko et al. 1987). These data support the concept that iron is transiently stored in ferritins and used for the accumulation of iron-containing proteins. However, there is no strict correlation between nodule ferritin subunit proteins and mRNA levels during leaf development (Ragland et al. 1993). Therefore, some posttranscriptional events must regulate the ferritin level during nodule development.

Iron Accumulation in Seeds

Study of the fate of iron during the course of vegetative organ growth and development has provided evidence for the dynamic nature of iron accumulation in seeds. The role of ferritin as a transient iron buffer has been well documented in these developmental processes. Seed formation studies have indicated that ferritins are also key proteins in long-term iron storage. A significant amount of iron is stored in pea seeds, and an increase in iron uptake by the roots occurs at early stages of seed development (Lobreaux and Briat 1991). Iron is also remobilized from vegetative organs to the seed. For example, leaf iron can account for 20% to 30% of the total seed iron content (Hocking and Pate 1978, Grusak 1994). In the soybean, however, it has been suggested that 40% to 60% of the seed iron may come from nodules (Burton et al. 1998). Indeed, root nodules of legumes have higher concentrations of iron than other vegetative organs. Therefore, legume seeds may have higher levels of iron than other vegetables because of active remobilization of nodule iron to the seed.

Seed iron is used during germination of plantlets. Ferritin subunits accumulate in the seed during maturation and remain in the dry seeds (Lobreaux and Briat 1991). This accumulation occurs in the embryo,

and ferritins are detected in the seed coat (Lobreaux and Briat 1991, Marentes and Grusak 1998). The amount of iron stored inside ferritins is estimated to be 92% of the total seed iron content (Marentes and Grusak 1998), suggesting that it is the major form of iron storage in seeds. During germination, ferritins are degraded, and iron is used for the growth of the seedling (Lobreaux and Briat 1991).

Regulation of Iron Accumulation in Plants

The dynamic fate of iron accumulation in various organs and tissues during the course of plant growth and development is an integrated process that results from the coordinated regulation of iron uptake from the rhizosphere, iron fluxes between various regions of the plant, and subcellular compartmentalization and storage of iron.

In response to deficiencies, grasses and non-grasses use different strategies to acquire iron from the soil. In *Arabidopsis*, iron deficiency induces synthesis of FRO2, a ferric-chelate reductase (Robinson et al. 1999). This leads to generation of ferrous iron, which is taken up across the root plasma membrane by specific transporter(s). IRT1 is the major root plasma membrane iron transporter (Eide et al. 1996, Vert et al. 2002). Regulation of the IRT1/FRO2 high-affinity iron uptake system in the root requires iron and an unknown shoot-borne signal (Vert et al. 2003). Our knowledge of the molecular regulation of IRT1/FRO2 comes from studies of the tomato chlorotic *fe,* a mutant that fails to activate iron deficiency responses. The tomato *IRT1* ortholog, *LeIRT1*, is down-regulated in the *fer* genetic background. Because the FER gene encodes a basic helix-loop-helix (bHLH) transcription factor, this appears to be due to altered regulation of *LeIRT1* transcription. Thus, FER appears to be the first identified regulator of iron nutrition in plants (Ling et al. 2002).

In contrast, iron deficiency in grasses induces the secretion of mugineic acids (MA) from the roots. MAs are synthesized from nicotianamine (NA), a structurally related precursor found in all plants (von Wiren et al. 1999). After synthesis, MA binds to soil ferric iron in the rhizosphere. The resulting complex is recognized and transported across the root plasma membrane by a ferric iron-MA uptake system. In maize, the *ys1* mutant carries a monogenic recessive mutation that causes a defect in the transport of ferric iron-MA through the root plasma membrane without affecting MA synthesis and secretion. Cloning of the maize *ys1* gene shows that it encodes a metal-MA transporter (Curie et al. 2001, Schaaf et al. 2004). An intriguing result of this work was the discovery that eight *Arabidopsis* genes share important sequence similarities with maize *ys1*, even though *Arabidopsis* does not produce MA.

Iron, once taken up by the root, is then loaded in the xylem sap and translocated to the plant aerial parts through the transpiration stream. Organic acids, especially citrate, are the main metal chelators in the xylem (Cataldo et al. 1988). This mechanism implies that active root transporters must load iron from the root cortex cells to the xylem; however, they are still uncharacterized at the molecular level. Once in the leaves, ferric citrate is likely to be the substrate of leaf ferric chelate reductase (Bruggemann et al. 1993).

Mobility of iron from source to acceptor tissues via the phloem sap is poorly documented. It is, nevertheless, well established that the phloem sap contains iron (Stephan et al. 1994) that arises from mobilization in source organs (Grusak 1995). NA is one of the molecules identified as a potential phloem metal-transporter. In addition, ITP, a phloem protein recently purified from *Ricinus communis*, has recently been shown to selectively complex ferric iron but not ferrous iron *in vivo*. Cloning of the ITP cDNA reveals that it encodes a protein belonging to the LEA (Late Embryogenesis Abundant) family (Krueger et al. 2002). Because there is a low but significant steady-state concentration of ferrous iron in the phloem (Maas et al. 1988), and because the bulk of iron in the phloem is chelated in the ferric form by ITP (Krueger et al. 2002), it is tempting to speculate that NA "it is tempting to speculate that NA may shuttle iron between ITP and the phloem.

The subcelluar distribution of iron in plants is poorly understood. There is also little information about

intracellular iron movement in plant cells. Members of the *ZIP, NRAMP,* and *YSL* gene families could be involved in iron transport into and/or out of the various plant cell organelles because some of them have been identified in the vacuole tonoplast (Curie and Briat 2003).

Indeed, plant vacuoles are likely to play a major role in handling excess iron. Upon iron overload, or in pea mutants that overaccumulate iron, the NA concentration increases (Pich et al. 2001). NA is observed in the cytoplasm under normal or deficient iron conditions (Pich et al. 2001), but when there is excess iron, the bulk of this iron chelator is moved to the vacuoles. Transporters of iron–NA complexes must therefore be present at the tonoplast. Also, studies in *Arabidopsis* show that remobilization of vacuolar iron stores could *occur via* AtNRAMP3 (Thomine et al. 2003).

The bulk of iron in leaves is found within the chloroplasts, and iron-ferritin represents more than 90% of the iron found in a pea embryo axis (Marentes and Grusak 1998). Iron transport into the plastids is therefore of primary importance in plant physiology, and, paradoxically, this subcellular iron transport activity is poorly documented. Iron uptake studies indicate that this process is light-dependent and requires ferric-chelate reductase activity (Bughio et al. 1997). In agreement with this result, an inward-directed transport of ferrous iron across the chloroplast inner membrane occurs by a potential-stimulated uniport mechanism (Shingles et al. 2002).

Plant and animal ferritins evolved from a common ancestor (Andrews et al. 1992). However, plant-specific ferritin regulatory pathways are suggested by differences plant specific gene organization, iron responses, mRNA structure differences in the concentration of ferritin mRNA and ferritin protein in the same tissues (Ragland and Theil 1993; Kimata and Theil 1994, Proudhon et al. 1996). During the regreening that occurs when plants recover from iron starvation, ferritin is synthesized and used as a safe iron buffer, transiently storing the iron required for the synthesis of iron-containing proteins. Ferritin mRNA can be detected in leaves as early as 3 hours after iron resupply to iron-deficient plants (Lobreaux et al. 1993). Iron resupply to iron-starved suspension cultures of soybean cells induces an accumulation of ferritin mRNA due to increased transcription (Lescure et al. 1991). For some maize ferritin genes, the increase in ferritin gene transcription is abscisic acid (ABA)-dependent in maize, whereas it is abscisic acid-independent for other ferritin genes, such as *ZmFer1* in maize and *AtFer1*, the *Arabidopsis* ortholog of *ZmFer* (Gaymard et al. 1988). The iron-dependent expression of *AtFer1* and *ZmFer1* is mediated by the IDRS *cis*-regulatory element (Petit et al. 2001). The IDRS sequence is also required for NO- or dark-induced senescence activation of *AtFer1* gene expression in *Arabidopsis* (Tarantino et al. 2003, Murgia et al. 2002). Another *cis*-regulatory element, named the Iron Responsive Element (FRE), was also identified in the promoter of a soybean ferritin gene (Wei and Theil 2000). To date, no *trans*-acting factors interacting with IDRS or FRE have been identified.

Deregulation of Iron Accumulation in Plants

Changes in iron homeostasis in plant mutants with altered iron signaling and in transgenic plants overexpressing ferritin demonstrate that there is genetic control of iron accumulation in various plant tissues, as is also observed during legume nodulation in plants that have not been engineered (Tang et al. 1990, Ragland and Theil 1993). The changes in iron homeostasis when ferritin is overexpressed by genetic manipulation, or when nodulation begins, include signals indicating iron deficiency and the need for enhanced iron uptake even though the plant iron content and iron environment are normal.

A number of mutants altered in iron status signaling have an abnormal iron-deficiency response in the roots, leading to modified iron accumulation. Among these, the pea mutants, *brz* and *dgl*, show bronze necrotic spotted leaves and brown degenerative leaves, respectively, due to hyperaccumulation of iron in the leaves. These two non-allelic mutants have a constitutive root ferric reductase activity and are incapable of turning off

root iron deficiency responses under iron-replete conditions (Grusak and Pezeshgi 1996, Kneen et al. 1990).

The tomato *chloronerva (chln)* mutant lacks the ability to synthesize NA due to a mutation in the NA synthase gene (Ling et al. 1999). Like *brz* and *dgl*, *chln* accumulates high levels of iron in its shoots and roots, regardless of the external iron status.

An *Arabidopsis frd3* mutant was isolated by virtue of its inability to turn off the root ferric reductase activity in conditions of iron sufficiency (Yi and Guerinot 1996). A variety of metals, including iron and manganese, accumulated in the mutant due to upregulation of the IRT1 metal transporter (Eide et al. 1996, Delhaize 1996). The FRD3 gene encodes a transmembrane protein belonging to the multidrug and toxic compound extrusion (MATE) family of efflux transporters (Rogers and Guerinot 2002), and is therefore likely to transport small organic molecules. FRD3 seems to be required for the shoot-to-root signaling of iron status, but, because FRD3 is expressed specifically in roots, it may mediate perception of a shoot-derived signal.

Knowledge of the role of ferritins in plant physiology is still very limited. Ferritin functions have been addressed by a molecular physiological approach using transgenic plants overexpressing ferritin in the plastids (Goto et al. 1999, Van Wuytswinkel et al. 1999, Deak et al. 1999). These studies have shown an unusual accumulation of ferritin in the leaves and seeds of the transgenic plants. A major consequence of this accumulation was a two- to three-fold increase in the concentration of iron in the leaf (Goto et al. 1999, Van Wuytswinkel et al. 1999). This accumulation also causes a concomitant elevation in both root ferric reductase and root H+-ATPase activities (Van Wuytswinkel et al. 1999, Vansuyt et al. 2000, 2003), two key determinants of iron uptake by dicotyledonous plants (Curie and Briat 2003). The change in iron accumulation can be attributed to the increased capacity for iron storage in the ferritin-transformed plants, driving leaf physiology towards an iron-deficient state.

Increasing Seed Iron Composition: The Biotechnological Approach

A biotechnological approach has been recently proposed to control human iron deficiency. Preliminary data show that transgenic plants can amplify the effect of environmental conditions on crop iron concentrations, resulting in both advantageous (Goto et al. 1999) and disadvantageous (Vansuyt et al. 2000) effects. Biotechnological manipulation is one of the many factors important in integrating variations in crop composition with dietary recommendations. For example, overexpression of the iron storage protein ferritin in transgenic plants can lead to a three-fold increase in leaf and seed iron content under some conditions (Goto et al. 1999). At the same time, studies in rats and humans indicate that soybean meal ferritin or transgenic rice ferritin are efficient supplemental sources of dietary iron (Murray-Kolb et al. 2003, Murray-Kolb et al. 2002). This has led to the proposal that iron-fortified ferritin-overexpressing transgenic plants could be safely used as a supplemental source of iron in human diets (Deak et al. 1999, Theil et al. 1997). However, because iron uptake by plant roots involves complex interactions between plant and soil within the rhizosphere (Lindsay 1995) and is independent of ferritin expression, the problem of engineering an increase in seed ferritin iron remains an active area of research. Furthermore, iron uptake in plants is connected to the uptake of other metals, some of which are potentially toxic for humans. For example, in grasses, uptake of phytosiderophores by the YS1 transporter results in zinc, copper, nickel, manganese, and cadmium loading (Schaaf et al. 2004). Recent studies have examined the influence of various soil conditions on the increase in leaf iron content of various tobacco plant genotypes. In one soil condition, ferritin-transformed plants were found to have a higher content than control plants of not only iron but also manganese, zinc, cadmium, and, to a much lesser extent, copper, nickel, and lead (Van Wuytswinkel et al. 1999, Vansuyt et al. 2000). Furthermore, the iron content of the leaves varied with the soil characteristics; leaf iron concentrations of control plants in some soils were as high as those of transgenic plants grown in other soils. In addition,

similar to the ferritin-transformed plants, an increased phosphorus concentration in two of the tested soils corresponded with an elevated leaf iron concentration in control plants. These findings indicate that the use of ferritin overexpression for the biofortification of leaf iron might be limited by the high dependence on the soil conditions. Much less is known about the effects of soil composition on the metal composition of seeds overexpressing ferritin (Goto 2001).

The tremendous advances over the last decade in our knowledge of the molecular mechanisms of iron accumulation indicate the possibility of a biotechnological solution to the problem of human iron deficiency (Theil et al. 1997, Lucca et al. 2002). A primary problem to be solved is the elucidation of the signaling networks involved in the regulation of iron accumulation in the various organs and at the various stages of development. Such information will be critical for avoiding the increased accumulation of toxic metals in seeds with enhanced iron uptake. Ensuring the appropriate improvement of seed iron and metal composition will require the overexpression of both iron-specific, seed-targeted iron transport systems (Curie and Briat 2003, Grusak and Pezeshgi 1996) and seed ferritin (Goto et al. 1999, Murray-Kolb et al. 2002, Goto 2001, Lucca et al. 2002) as well as studies integrating molecular plant physiology, animal and human nutrition, soil sciences, and variations in crop composition.

V | Perspective

The creation of diets that can effectively and safely control dietary iron deficiency must consider the following: the solid iron concentrated in ferritin of soy, especially whole soy, and possibly other beans; the distinctive chemistry of iron in ferritin; and the regulation of ferritin and seed iron in plants. The development of analytical tools for the routine monitoring of food ferritin iron and crop composition is an important area for future research because, currently, the only method available depends on immunoblotting, which is very laborious and limited in scope. Integration of dietary iron recommendations and variations in crop composition is a critical area of development for both natural and biotechnologically modified crops. Finally, problems of iron deficiency may be solved in the 21st century by matching dietary needs with individual variations in absorption and the use of ferritin in natural or modified crops.

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2033 K Street, NW, Washington, DC 20006-1002 USA
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