Distribution and Speciation of Iron and Zinc in Grain of Two Wheat Genotypes

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JANUARY 2014

Impact Factor: 2.91 · DOI: 10.1021/jf403331p · Source: PubMed

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Distribution and Speciation of Iron and Zinc in Grain of Two Wheat Genotypes

Tristan Eagling,[†] Andrew L. Neal,[†] Steve P. McGrath,[†] Susan Fairweather-Tait,[‡] Peter R. Shewry,[†] and Fang-Jie Zhao*[,§]

[†]Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, U.K.

[‡]Norwich Medical School, University of East Anglia, Norwich NR4 7TJ, U.K.

[§]State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China

Supporting Information

ABSTRACT: This study aimed to determine differences among wheat cultivars in the distribution and speciation of Fe and Zn in grain milling fractions. Cultivars with higher Fe and Zn concentrations in the wholemeal flour were found to contain higher concentrations in the white flour. Soluble Fe and Zn were extracted and analyzed by size exclusion−inductively coupled plasma mass spectrometry. Fe speciation varied between milling fractions with a low molecular weight (LMW) complex likely to be Fe−deoxymugenic acid/nicotianamine being the predominant extractable Fe species in white flour, accounting for approximately 85% of the extractable Fe. Bran fractions had a lower amount of LMW-Fe form but more as soluble Fe−phytate and an unidentified high molecular weight peak. In the white flour fraction soluble Zn was found to be present mainly as a LMW peak likely to be Zn−nicotianamine complex. Soluble Fe−phytate was found in the white flour fraction of a high-Fe cultivar but not in a low-Fe cultivar.

KEYWORDS: *wheat, iron, zinc, speciation, phytic acid, nicotianamine, deoxymugenic acid*

INTRODUCTION

Iron (Fe) and zinc (Zn) deficiencies are associated with increased mortality and morbidity. Populations in developing countries are at a disproportional risk, in part due to a greater reliance on less nutrient dense foods such as cereals.[1] One proposed approach to reducing Fe and Zn deficiencies is through biofortification, including the breeding of cereals with higher concentrations of Fe and Zn.[2,3] However, essential minerals are under tight homeostatic control within the grain as they are required at an adequate level for germination without causing toxicity.[4] Therefore, a greater understanding of how Fe and Zn are distributed within the grain is required.

The majority of wheat products consumed in the diet are produced after milling of the grain to yield white flour. This consists almost entirely of the starchy endosperm, while the bran, which contains the aleurone layer, embryo, and outer layers of the grain (pericarp and testa), is largely discarded.[5] However, the concentrations of Fe and Zn in the endosperm are much lower than those in the bran.[6] It is therefore important that biofortification strategies also consider the localization of minerals within the grain if the end products are to have increased nutritional benefits.

Variation in the concentrations of total Fe and Zn in whole grain of wheat genotypes has been widely reported. For example, a recent study of 130 cultivars grown on the same site showed that the concentrations of Fe and Zn ranged from 28 to 51 and from 13 to 35 mg/kg, respectively.[7] Other studies have shown even greater variation; analyses of lines grown in France showed variation of 19−58 and 14−35 mg/kg for Fe and Zn, respectively,[8] while in Asian wheat varieties the concentration of Fe ranged from 23 to 49 mg/kg [9] and of Zn from 25 to 56 mg/kg.[10] Variation from 4 to 16 mg/kg of Fe and from 5 to 16 mg/kg of Zn has also been reported for white flour of Chinese wheat cultivars.[11] Although this range of genotypic variation is encouraging from a plant breeding perspective, it is not clear whether the variation is consistent between different fractions of the grain and, in particular, whether the variation reported for whole grain is reflected in white flour from the same genotypes.

In addition to concentration and localization, the speciation of Fe and Zn is also an important factor in determining grain nutritional quality. Fe and Zn are present in plants as various chemical species, which may vary in their bioavailability. The nonprotein amino acid nicotianamine [2(*S*),3′(*S*),3″(*S*)-*N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]-azetidine-2-carboxylic acid, NA] complexes both Fe and Zn [12] and is involved in the transport of these minerals via the phloem and subsequent loading into the seed, as well as in the detoxification of free minerals.[13] Recently 2′-deoxymugineic acid (DMA), which is biosynthesized from NA, has also been shown to play a role in the phloem transport of Fe and is thought to be the predominant Fe chelator in the phloem sap of rice.[14,15] Complexes of Fe and Zn with NA and DMA are present in rice grain,[15] especially in the transgenic lines overexpressing NA synthase (NAS) genes,[16] indicating that

Received: July 30, 2013
Revised: December 24, 2013
Accepted: January 1, 2014
Published: January 1, 2014

they may also play a role in Fe and Zn storage. Elevated levels of NA in rice seed have also been shown to improve Fe and Zn bioavailability using both mouse and Caco-2 cell models.[12,17,18] However, to date there is no report of NA- or DMA-bound Fe or Zn in wheat grain.

Most of the Fe, Zn, and other minerals in cereal grain are complexed by phytic acid [inositol hexakisphosphate (IP_6)], resulting in salts known as phytates, which may be insoluble or soluble depending on the degree of metal bonding to phosphate groups.[19,20] However, it has recently been reported that Zn may not be complexed by phytate in barley embryos and is instead associated with S (as a proxy for thiol groups) and therefore likely to be bound to proteins.[21] There is also evidence that some of the soluble Fe in cereal grains is bound to inositol pentaphosphate (IP_5), a partially dephosphorylated soluble form of IP_6.[21,22] Since nonruminant animals (including humans) lack phytases, minerals complexed with IP_6 are unavailable for uptake.[23] Phytate has therefore been regarded as an "anti-nutrient". However, soluble forms of phytate-complexed Fe such as monoferric phytate (Fe−IP_6) and Fe−IP_5 have been suggested to have some degree of bioavailability.[19,22] By contrast, Zn bound to phytates is thought to be insoluble, being made up of heterometal salts and/or Zn_5IP_6 and therefore not bioavailable.[20]

Identifying and quantifying Fe and Zn species in cereals remains a technical challenge. Size-exclusion chromatography coupled with inductively coupled plasma mass spectrometry (SEC−ICP-MS) has been used to identify a soluble form of Fe complexed with phytate in barley embryos [21] and Fe and Zn complexed with NA and/or DMA in rice endosperm.[12,18] The presence of NA−Zn and DMA−Zn complexes in the low molecular weight (LMW) peak eluded from SEC was further confirmed by electrospray ionization mass spectrometry.[18] However, little is known about Fe and Zn speciation within wheat grain. The objective of this study was therefore to determine genotypic differences in the total concentrations and speciation of extractable Fe and Zn in different milling fractions of wheat, particularly white flour.

MATERIALS AND METHODS

Grain Materials. Analyses were initially performed on grain samples of six wheat cultivars selected on the basis of previous results which showed high Fe concentrations in three cultivars (Rialto, San Pastore, Tiger) and low Fe concentrations in the other three cultivars (Crousty, Rialto, Riband).[7] The six cultivars were grown at three European sites (Martonvásár, Hungary; Saxham, UK; Choryn, Poland) in 2007, as described by Shewry et al.[24] Climate and soil conditions of all three sites are reported by Shewry et al.[24] Grain samples from replicated plots at each site were pooled for each cultivar, giving rise to a total of 18 grain samples (6 cultivars grown at 3 sites). In addition, Rialto (a high-Fe cultivar) and Riband (a low-Fe cultivar) were grown in a field trial on a farm of Rothamsted Research, U.K., in 2010, in three replicated plots (1.8 × 10 m) in a completely randomized block design. Standard farm practices and fertilization (176 kg N/ha split into two applications) were followed. All grain samples were stored in a cold room at 4 °C prior to analysis.

Milling. Grain was dried at ambient temperature and the water content was raised to 15% before milling. Wholemeal samples were produced using a Perten Laboratory Mill 3100 (Des Plaines, IL) (with 0.5 mm sieve). White flour and bran fractions were prepared using a Brabender Quadrumat Junior Mill (Duisburg, Germany). The flour samples from the Brabender mill were sieved (150 μm mesh) to remove contaminating bran. Grain from the replicated field plots of Riband and Rialto was combined and 2.5 kg sample each was milled using a Bühler MLU-202 mill (Urzwil, Switzerland) to produce 10 milling fractions. These comprised six white flour fractions: three breaks (B1−B3) which are obtained sequentially and three reductions (R1−R3). These vary in purity, with the first break (B1) and reduction (R1) corresponding to the purest starchy endosperm tissue and the third break (B3) and reduction (R3) the least pure. The remaining four fractions are the bran fractions, which are then separated into offal flour, bran flour, coarse offal, and coarse bran.

Total Mineral Analysis. Mineral concentrations were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin-Elmer Optima 7500 DV, Waltham, MA). A 500 mg portion (oven-dried at 80 °C for 4 h) of each sample was digested in ultrapure HNO_3 and $HClO_4$ (87:13% v/v) in triplicate using digestion blocks (200 °C, 12 h) (Eurotherm MBB151, Durrington, UK). Working conditions for ICP-OES wereas follows: RF power 1.5 kW, auxilary gas (Ar) flow rate 0.2 L/min, carrier gas (Ar) flow 0.85 L/min. Blank and certified reference materials (CRM, BCR-189, wheat wholemeal flour and NIST1568a, rice flour) were included in each batch of analyses, and the values for the CRM were within <5% of the certified values for Fe and Zn. Signal drift was corrected by measuring a standard solution every 10−20 samples.

Extraction of Soluble Fe and Zn. All samples were extracted in a Tris-HCl buffer solution as described by Persson et al.[21] with some modifications. Preliminary experiments showed that grinding with quartz sand using a pestle and mortar, as reported by Persson et al.,[21] released contaminating Fe which bound to the samples; therefore, the grinding step was omitted in our study. To enhance extraction a longer extraction at an elevated temperature was used (18 h, 37 °C). This longer extraction was used because preliminary tests showed a higher extraction rate for Fe and Zn in endosperm compared with the 1 h extraction used by Persson et al.[21] However, it is possible that this longer extraction could result in a higher degree of digestion of phytate by phytase activity in the aleurone layer. Briefly, 140 mg of flour fractions or 70 mg of bran fractions was extracted with 7 mL of Tris-HCl buffer (50 mM, pH 7.5) in sterile vials with gentle shaking (120 rpm). Samples were then centrifuged (21 000*g*, 10 min) to remove the solid phase before being filtered through 0.2 μm sterile filters (Minisart). Total concentrations of Fe and Zn in the extracts were determined using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500ce, Agilent Technologies, Palo Alto, CA) with the following conditions: RF power 1.5 kW, carrier gas 0.85 L/min, nebulizer pump speed 0.2 L/min, H_2 gas 6 mL/min. All samples were measured with three technical replicates. A standard of 10 μg/L Fe and Zn and a blank were used to monitor signal stability.

Iron and Zinc Speciation. All analyses were performed on an HPLC (Agilent 1100 Series, Agilent Technologies, Waltham, MA) coupled to ICP-MS (Agilent 7500ce, Agilent Technologies, Waltham, MA). A Superdex 75 10/300 GL size exclusion column (glass, 10 × 300 mm, 13 μm cross-linked agarose/dextran, Amersham Biosciences) was used with an optimum separation range between 0.7 and 70 kDa. The mobile phase was Tris-HCl buffer (50 mM, pH 7.5), pumped through the column isocratically at 0.47 mL/min at a controlled temperature of 18 °C. The sample injection volume was 100 μL. After HPLC molecular size exclusion separation, the solution was measured sequentially by a UV detector at the wavelength of 214 nm and then by ICP-MS. ICP-MS was set up with the following conditions: RF power 1.5 kW, carrier gas 0.85 L/min, nebulizer pump speed 0.2 L/min, and collision/reaction gas H_2 3 mL/min and He 2 mL/min. Due to the low levels of Fe in the white flour samples the analyses were performed on ^{56}Fe, which is the most abundant isotope (91.8 atom %). H_2 and He were used as the collision/reaction gas as this prevented any polyatomic inference on this isotope. Molecular size calibration was achieved using a range of standard compounds varying in molecular size, including apoprotein (6.6 kDa), cytochrome *c* (12.4 kDa), vitamin B_{12} (1.35 kDa), triglycine (0.19 kDa), and glycine (0.075 kDa) (all from Sigma-Aldrich). The UV absorption was first measured. For cytochrome *c* and vitamin B_{12}, Fe and Co, respectively, were also measured by ICP-MS following UV detection. The delay in the retention time from the UV absorption peak to the ICP-MS signal peak was 0.6 min. A log−linear regression curve was derived from the plot of molecular size versus retention time (based on the ICP-MS

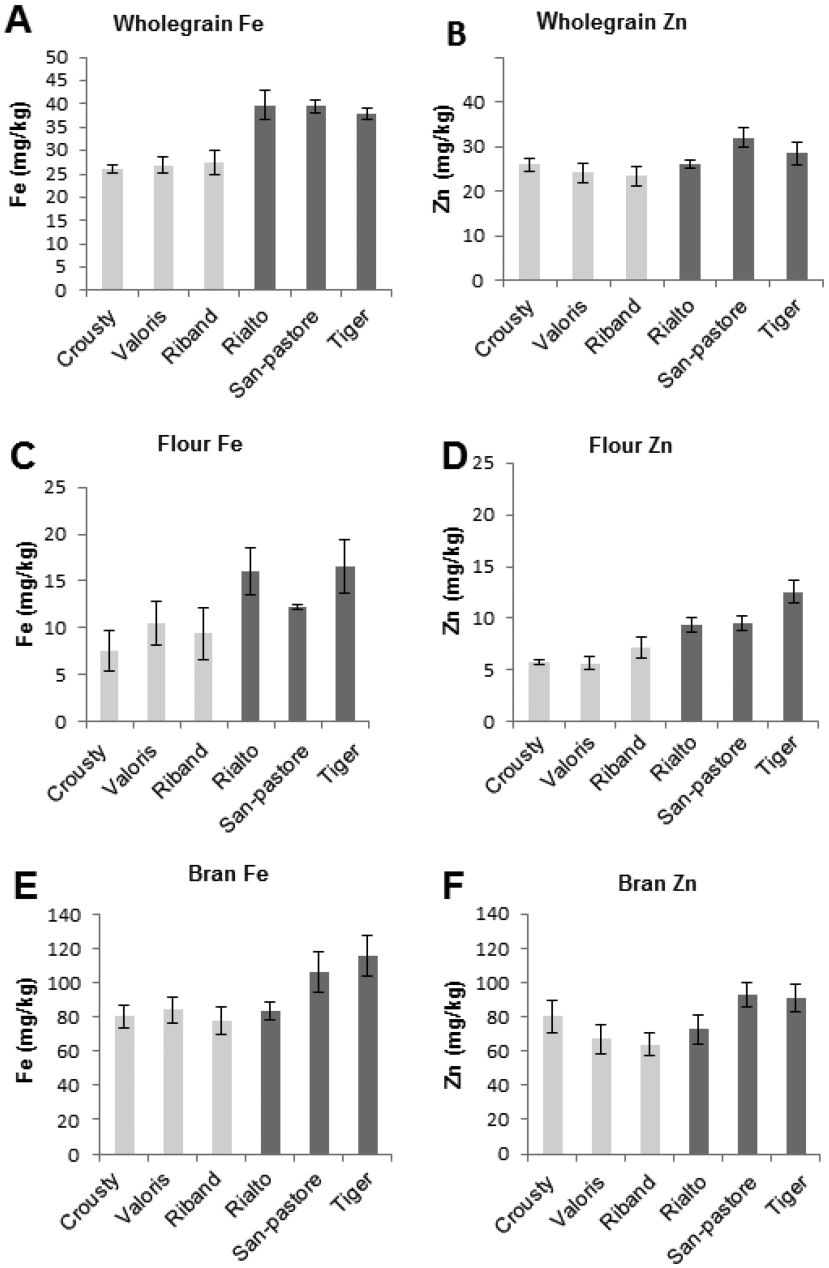

Figure 1. The concentrations of Fe and Zn in wholemeal, bran and white flour fractions of six wheat cultivars. Data are means (mg/kg dm ± SE) of materials grown in field trials at three sites (Hungary, Poland, UK) in 2007.

signal peak). A 100 μL aliquot of 20 mM EDTA/50 mM Tris-HCl buffer (pH 7.5) was injected between flour samples to remove residual metals adsorbed to the column. This wash was repeated twice after bran fractions due to the higher levels of Fe and Zn in the samples. A more thorough wash with 100 μL of pepsin (1 mg/mL), phytase (2 mg/mL), NaCl (0.5 M), and acetic acid (10%) (all from Sigma-Aldrich), at a flow rate of 0.2 mL/min at 36 °C, was performed between batches of samples. Finally, 10 mM EDTA/50 mM Tris-HCl buffer (pH 7.5) was run through the column for 40 min each day to remove any residual metals retained by the column. All speciation analysis was performed within 6 months of milling, as longer term storage may have an effect on phytate levels and therefore speciation.[23]

Ash Content. Ash contents of milling fractions were determined using the AACC-approved Method 08-12.[25]

Phytic Acid Content. Phytic acid analysis was performed using a commercially available kit (K-PHYT 12/12 Megazyme) as per the manufacturer's instructions.

Statistical Analysis. For the Rothamsted trial comparing the cultivars Rialto and Riband, one-way analysis of variance (ANOVA) was performed using GenStat (13th ed.; VSN, Hemel Hempstead, UK). For the multisite trials comparing six cultivars, ANOVA was not performed because replicated samples were bulked. Instead, correlation analyses between the Fe and Zn concentrations in the wholemeal and white flour samples were performed.

RESULTS

Concentrations of Fe and Zn in Whole Grain, Bran and Flour Fractions. Figure 1 shows the concentrations of Fe and Zn in the white flour and bran samples of six wheat cultivars grown on three European sites (in Hungary, Poland, UK). It was observed that cultivar differences were generally consistent across the three sites, and therefore, only the mean data (±SE across the three sites) are presented in Figure 1. Cultivars Rialto, San Pastore, and Tiger contained higher

dx.doi.org/10.1021/jf403331p | J. Agric. Food Chem. 2014, 62, 708−716

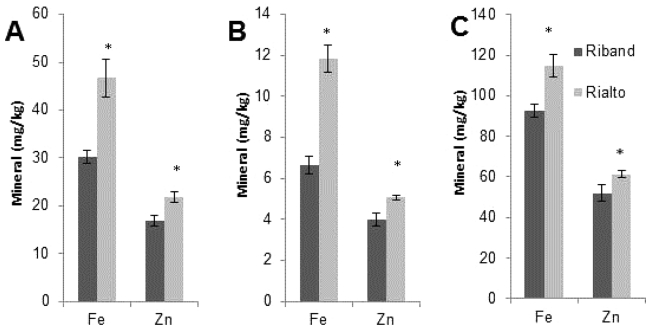

Figure 2. The concentrations of Fe and Zn in (A) wholemeal, (B) white flour, and (C) bran fractions of the cultivars Rialto and Riband grown at Rothamsted in 2010. Data are means ± SE of three biological replicates, and * represents significant difference at $p < 0.05$ according to ANOVA.

concentrations of Fe in the wholemeal than cultivars Crousty, Valoris, and Riband. This cultivar difference was generally maintained for the concentrations of Fe in both bran and white flour. Notably, the mean concentrations of Fe were 43% higher in the wholemeal samples and 43% higher in the white flour of the high-Fe cultivars when compared to the low-Fe cultivars. Similarly, the mean concentrations of Zn were 16% higher in the wholemeal samples and 58% higher in the white flour of the high-Fe cultivars when compared to the low-Fe cultivars. Among the six cultivars across the three sites, the concentrations of Fe and Zn of white flour were found to correlate significantly with those of the wholemeal ($r = 0.54$ and 0.48 for Fe and Zn, respectively; $p < 0.05$). There were also significant correlations between Fe and Zn concentrations in the wholemeal ($r = 0.60$, $p < 0.01$), bran ($r = 0.49$, $p < 0.05$), and white flour ($r = 0.61$, $p < 0.01$).

Figure 2 shows the concentrations of Fe and Zn in grain of Rialto and Riband grown at Rothamsted in 2010. Rialto had significantly ($p < 0.05$) higher concentrations of Fe and Zn than Riband, in wholemeal (by 54% and 29%, respectively), bran (by 24% and 18%), and white flour (by 80% and 26%). It is notable that the difference in the concentration of Fe was greatest in the white flour fraction. These differences were broadly similar to the data from the multisite trials (Figure 1).

Speciation of Fe and Zn in the Soluble Extracts from White Flour. Figures 3 and 4 show SEC−ICP-MS analyses of the Fe and Zn species in the Tris-HCl extractable fraction of white flour from the samples of Riband (low Fe) and Rialto (high Fe) grown at Rothamsted in 2010. The flour extracts of both cultivars showed two unidentified high molecular weight (HMW) forms of Fe (at 20 ± 0.4 and >70 kDa, respectively). A medium molecular weight (MMW, 5.0 ± 0.1 kDa) peak of Fe was identified in the extracts of Rialto, but not in Riband. Further analysis of this peak using He and O_2 as the collision/reaction gases and measuring P as ^{47}PO and Fe as ^{57}Fe showed that the 5 kDa ^{57}Fe peak coeluted with ^{47}PO (Supplementary

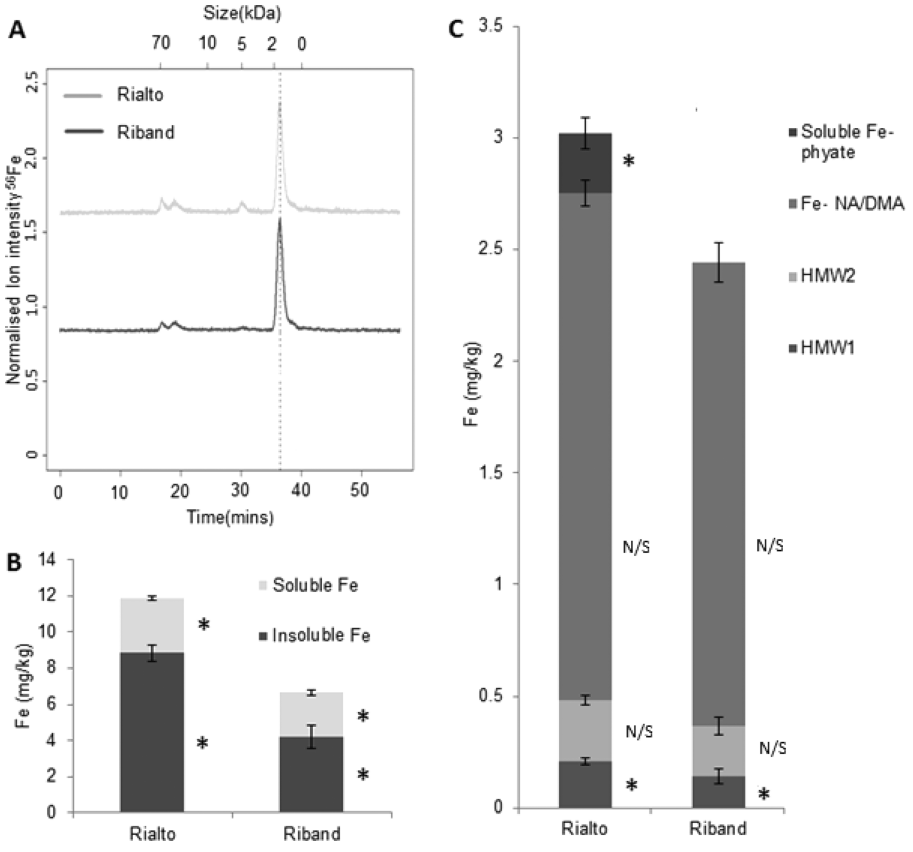

Figure 3. Determination of Fe speciation in white flour fractions of Rialto and Riband. (A) SEC−ICP-MS chromatograms (averages of three biological replicates). All samples were extracted in 50 mM Tris HCl at pH 7.5. (B) Soluble and insoluble Fe. (C) Quantification of soluble Fe species from relative peak areas on SEC−ICP-MS data and total soluble Fe by ICP-MS. Data in parts B and C represent means ± SE of three replicates; * indicates significant ($p < 0.05$) difference between the two cultivars for the individual Fe form and N/S connotes no significant difference.

dx.doi.org/10.1021/jf403331p | J. Agric. Food Chem. 2014, 62, 708−716

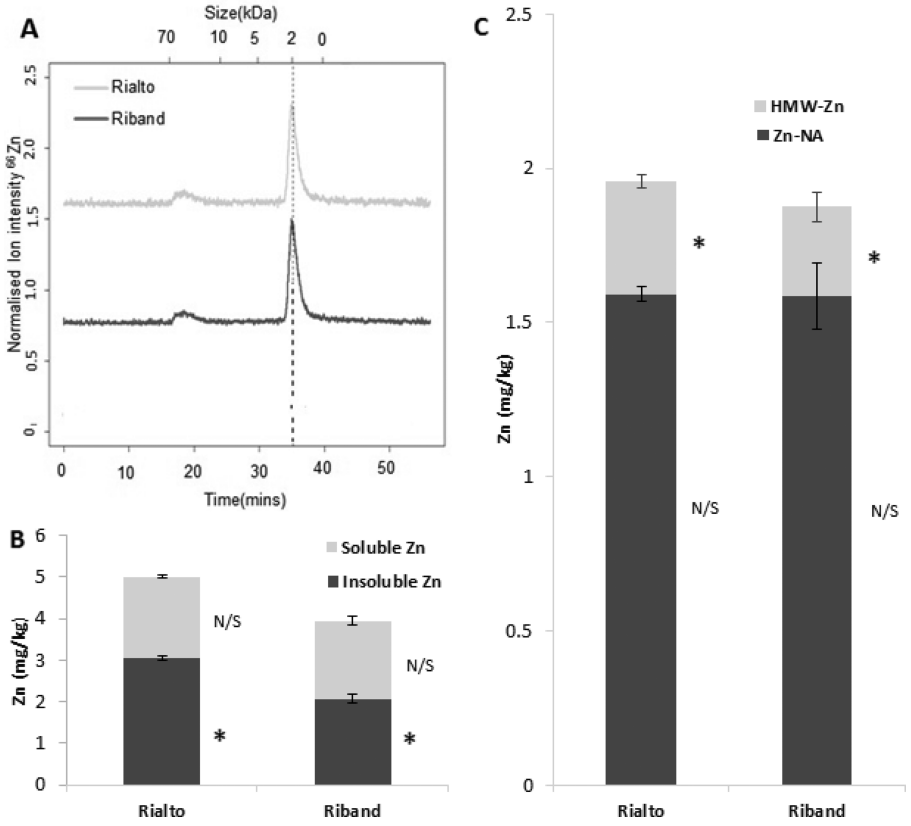

Figure 4. Determination of Zn speciation in white flour fractions of Rialto and Riband. (A) SEC−ICP-MS chromatograms (averages of three biological replicates). All samples were extracted in 50 mM Tris HCl at pH 7.5. (B) Soluble and insoluble Zn. (C) Quantification of soluble Zn species from relative peak areas on SEC−ICP-MS and total soluble Zn by ICP-MS. Data in parts B and C represent means ± SE of three replicates; * indicates significant ($p < 0.05$) difference between the two cultivars for the individual Zn form and N/S connotes no significant difference.

Figure 1, Supporting Information). We therefore tentatively consider this peak to be soluble Fe−phytate (possibly Fe−IP_6 and/or Fe−IP_5), as it has a similar composition to a peak identified as a soluble Fe−phytate oligomer in the extracts of barley embryo.[21]

The extracts of Riband and Rialto contained similar amounts of a low molecular weight (LMW, 1.5 ± 0.1 kDa) peak of Fe, which has no coelution of P (Supplementary Figure 1, Supporting Information). This LMW peak eluted at the same time as a standard of Fe−DMA; however, the retention time was also very similar to that of Fe−NA (Supplementary Figure 2, Supporting Information). Similarly, previous studies[12,18] using SEC−ICP-MS and ES-MS have shown that the LMW peak in the extract of rice endosperm contains both Fe−DMA and Fe−NA. For comparison, a rice endosperm sample was extracted and analyzed with the same method, and the LMW peak identical to that in wheat flour extracts was found (Supplementary Figure 2, Supporting Information). In contrast, Fe−citrate can be ruled out as the possible Fe−chelate form for the LMW peak, as it eluted at a much smaller size (Supplementary Figure 2, Supporting Information). On the basis of retention time matching and the similarity to previous studies on rice,[12,18] we tentatively consider the LMW peak to represent a mixture of Fe−DMA and Fe−NA.

SEC−ICP-MS also identified a LMW Zn complex (1.9 ± 0.2 kDa) in the extracts of white flour (Figure 4a) that was of a similar size to a peak identified in rice using SEC−ICP-MS.[18] On the basis of this report and on comparison with Zn−NA and Zn−DMA standards, which eluted at 1.9 and 2.5 kDa respectively (Supplementary Figure 3, Supporting Information), we tentatively identified the LMW peak as Zn−NA, although further investigation is needed to determine its exact identity.

The concentrations of soluble Fe differed significantly between the two cultivars ($p < 0.05$), with Rialto having 23% more soluble Fe than Riband (Figure 3B), but the total concentrations of soluble Zn (sum of both LMW Zn and Zn associated with HMW compounds) were not significantly different (Figure, 4B). Most of the soluble Fe and Zn were bound to LMW complexes and the total amounts of LMW Fe and LMW Zn did not differ significantly between the two cultivars (Figures 3C and 4C).

As a percentage, insoluble Fe made up the majority of the total Fe in the white flour, accounting for 74.6% in Rialto and 62.5% in Riband (Figure 3B). Fe−NA/DMA was the largest soluble fraction of Fe, making up 19.3% of the total Fe in Rialto and 32.1% in Riband. Soluble Fe−phytate accounted for 2% of the total Fe in Rialto, while HMW peaks accounted for 4.1% in Rialto and 5.8% in Riband. Insoluble Zn also made up the majority of the total Zn in the white flour, accounting for 60.7% in Rialto and 52.5% in Riband (Figure 4B). Zn−NA was the largest soluble fraction of Zn, accounting for 31.8% of the total Zn in Rialto and 40% in Riband, while HMW Zn peaks accounted for 7.3% in Rialto and 7.4% in Riband.

Fe Speciation in Milling Fractions. Figure 5 shows the speciation of Fe in soluble extracts of 10 milling fractions from Bühler mill for both Riband (low Fe) and Rialto (high Fe) grown in the Rothamsted trial. The break fractions B1−B3 and

dx.doi.org/10.1021/jf403331p | J. Agric. Food Chem. 2014, 62, 708−716

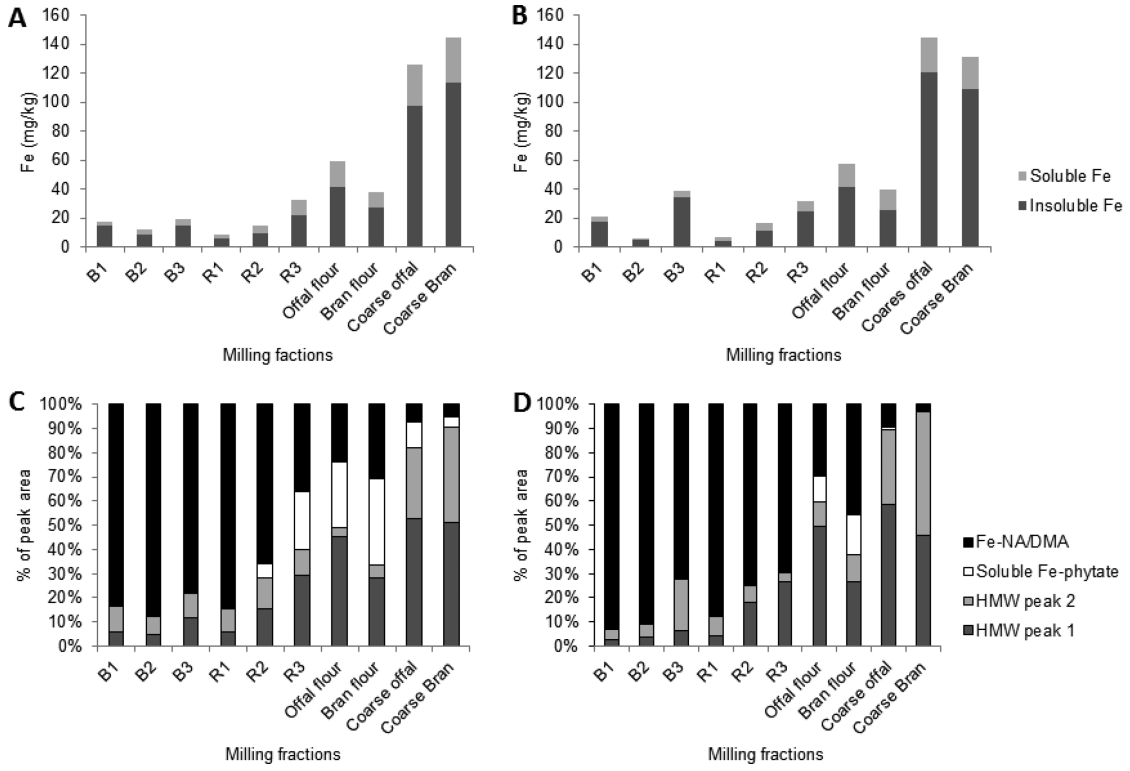

Figure 5. Quantification of the Fe forms in different milling fractions of Rialto (A, C) and Riband (B, D) from the Rothamsted trial. Parts A and B show the concentrations of different Fe forms and parts C and D the relative proportions of soluble Fe species in the total soluble Fe. Results are means of two technical replicates. The standard deviation of the method was less than 0.5% by analysis of five replicate samples of bran flour from Rialto.

Table 1. Extraction Yield, Ash Content, and Phytic Acid Content of 10 Milling Fractions from Bühler Mill

	B1	B2	B3	R1	R2	R3	offal flour	bran flour	coarse offal	coarse bran
					Extraction Yield (%)					
Rialto	6.1	9.1	1.5	47.6	5.9	1.8	2.1	2.5	7.2	16.3
Riband	8.1	9.4	1.8	42.1	9.2	3.0	2.1	3.1	7.1	14.0
					Ash Content (%)[a]					
Rialto	0.2	0.4	0.7	0.4	0.7	1.3	1.3	1.5	6.1	4.6
Riband	0.3	0.5	1.8	0.4	0.7	1.1	1.3	1.7	6.3	4.9
					Phytic Acid (g/100 g of dry matter)[a]					
Rialto	0.10	0.07	0.02	0.08	0.23	0.76	0.29	0.45	2.83	2.04
Riband	0.03	0.014	0.08	0.03	0.13	0.36	0.42	0.36	2.31	2.05

[a]Mean of three replicates.

the reduction fractions R1−R3 correspond to white flour, with R1 accounting for 47.6% and 42.1% of the total mass in Rialto and Riband, respectively (Table 1). The R1 and B1 fractions have very low ash and phytate contents (Table 1) and are therefore considered to represent the purest starchy endosperm fraction derived from the central part of the grain.[26] Ash content, an indicator of bran contamination, decreases closer to the center of the grain. A similar effect is observed with phytic acid content (Table 1).

The total concentrations of Fe (Figure 5A,B) varied greatly between the six flour fractions (R1, R2, R3, B1, B2, and B3), from 9 to 32 mg/kg in Rialto and 5 to 35 mg/kg in Riband, respectively. The differences were even greater between the six flour and four bran/offal (offal flour, bran flour, coarse offal and coarse bran) fractions, with a 26-fold difference in Fe concentration between the R1 and coarse offal fractions from Riband. The speciation of Fe in soluble extracts also differed between the flour fractions and bran/offal fractions, with LMW complexes being the predominant soluble form of Fe in flour (Figure 5C,D). By contrast, Fe bound to HMW components was the predominant form in bran/offal.

The main difference observed between the milling fractions from the two cultivars was in the amount of soluble Fe−phytate (Figures 5C,D). These results agree with the data in Figure 3A, showing that soluble Fe−phytate was present in the white flour extracts of Rialto but not of Riband. In addition, more soluble Fe−phytate was present in the bran fractions, especially the offal fractions, of Rialto compared to Riband.

The calculated amounts of soluble Fe species in whole grains of Rialto and Riband are given in Table 2, based on the yields of the milling fractions and the SEC−ICP-MS analyses. This shows that the concentration of soluble Fe−phytate was over 5-fold higher in Rialto than in Riband, although this form still represents only a small percentage of the total grain Fe (1.6%). The contents of LMW Fe (likely to be Fe−NA and/or Fe−DMA) were similar in the two cultivars but it represented a

dx.doi.org/10.1021/jf403331p | J. Agric. Food Chem. 2014, 62, 708−716

Table 2. Quantification of Forms of Fe in Grain of Rialto and Riband, Based on Analyses of Milling Fractions and Whole Grain

	Fe species in whole grain[a]						
	Fe-HMW1	Fe-HMW2	soluble Fe−phytate	Fe−NA/DMA	insoluble Fe	sum	total Fe determined by ICP-OES[b]
Rialto (mg/kg)	4.1	2.9	0.7	2.3	33.1	43.1	46.7
%	9.6	6.7	1.6	5.3	76.7		
Riband (mg/kg)	3.8	3.3	0.12	2.5	24.2	33.8	30.3
%	11.2	9.6	0.38	7.3	71.4		
technical error (%)[c]	0.5	0.5	0.2	0.4			

[a]The total concentrations and percentages of each form of Fe were calculated on the basis of the peak relative area on SEC−ICP-MS and total soluble Fe determined by ICP-MS for each milling fraction and the proportions of the milling fractions (Table 1). [b]Total Fe determined by ICP-OES of whole grain. [c]Technical error was determined by analyzing five replicate samples of Rialto bran.

higher percentage of the total Fe in Riband. The concentration of insoluble Fe in Rialto was 36% higher than that in Riband (Table 2).

DISCUSSION

The initial aim of our study was to establish if there was a relationship between the total concentrations of Fe and Zn in whole grain and in white flour of wheat. There is evidence that the loading of minerals, such as Fe and Zn, into the starchy endosperm is highly regulated and occurs independently of loading into the outer layers that constitute the bran.[27,28] Consequently, the concentrations of minerals in the white flour may not reflect those in whole grain, which is dominated by the minerals stored in the outer layers (especially the aleurone) and the embryo. Our results (Figures 1 and 2) show that cultivars with higher concentrations of Fe and Zn in whole grain also tend to have higher concentrations of these minerals in the white flour, although the concentrations in the white flour are much lower than those in the whole grain. Therefore, for biofortification purposes, the rankings of wholegrain and white flour Fe and Zn concentrations among wheat cultivars are likely to be reasonably consistent.

The second aim of our study was to determine Fe and Zn speciation in the soluble extracts of wheat flour and to investigate if they differ between a high-Fe (Rialto) and a low-Fe (Riband) cultivar. Tris-HCl buffer-soluble Fe accounted for 25−37% and 23−29%, respectively, of the total Fe in white flour (Figure 3) and whole grain (Table 2). The high-Fe cultivar (Rialto) had a larger concentration of soluble Fe than the low-Fe cultivar Riband; however, when this concentration was expressed as a percentage of the total Fe, the cultivar difference was the reverse. Compared to Fe, Zn appeared to be more soluble in white flour, accounting for 39−48% of the total Zn (Figure 4).

SEC−ICP-MS analyses showed the presence of HMW, MMW, and LMW peaks of Fe and Zn (Figures 3 and 4). The LMW Fe in wheat is likely to be a mixture of Fe−NA and Fe−DMA, whereas Zn−NA appears to be the only form of soluble Zn in the endosperm in both rice and wheat (Supplementary Figures 2 and 3, Supporting Information). This is in agreement with other studies which have shown that Zn binds more favorably with NA [29] and Zn−NA was the only Zn−chelate in the phloem sap of rice.[14] Proportionally, LMW Fe increased from the bran to the endosperm fraction and was quantitatively the most important species in the soluble extracts of white flour, suggesting that NA/DMA may also play an important role in the storage of Fe in the endosperm of wheat. However, there was no significant difference in the proportion of this Fe form between the two cultivars. Zn−NA was also the most important soluble Zn species in the white flour extracts, with no significant difference between the two cultivars being observed.

The most notable difference between the two cultivars was the presence of Fe bound to soluble phytate, which appeared as MMW complexes in the white flour of the high-Fe cultivar (Rialto) but not of the low-Fe cultivar (Riband) (Figure 3). The highest concentrations of soluble Fe−phytate were found in the offal and bran flour fractions, with Rialto having higher concentrations than Riband (Figure 5). One explanation for the difference between the two cultivars could be premature sprouting of Rialto, which is known to be prone to prematurity amylase production. This is supported by the determination of the Hagberg falling number (HFN), which was lower for Rialto than for Riband (112.3 ± 10.0 compared to 217.0 ± 23.3, $p < 0.05$). Although HFN measures the activity of amylase on starch, the synthesis of phytase also occurs during germination.[30] Increased phytase activity could therefore be responsible for the higher soluble phytate in Rialto. We found no evidence of soluble forms of phytate−Zn in white flour (Figure 4) or other milling fractions (data not shown), which is in agreement with analyses of barley embryos.[21] Another factor which needs to be considered is how each cultivar responds to shear forces during the milling process, as milling of hard (e.g., Rialto) and soft (e.g. Riband) wheat may result in different amounts of aleurone tissue being present in the white flour fractions, especially the late break and reduction fractions (i.e., B3, R3). In particular, white flour from hard wheat cultivars contains more aleurone material, especially the break fractions (B1−B3).[26] The degree to which this would affect our data is not known, but the observation that the R2 and R3 fractions of the hard wheat Rialto contained more ash, phytate, and soluble Fe−phytate (Table 1, Figure 5) is consistent with the presence of aleurone tissue.

In addition to the complexes with soluble phytate and NA/DMA, Fe and Zn are also bound to HMW compounds (Figures 3−5, Table 2). The identification of these peaks is challenging. HMW1 is excluded from the size exclusion column, suggesting that it is in excess of 70 kDa in size and could represent any complexes above this size. HMW2 also elutes very close to HMW1. The Fe storage protein ferritin may contribute to these peaks as it is a soluble complex of subunits of mass 40−44 kDa.[31] However, ferritin is present in low abundance in cereals (1−3% of total Fe).[32,33] Therefore, it is possible that these peaks are also made up of other large Fe-containing complexes (e.g., soluble proteins). Previous studies using high resolution secondary ion mass spectroscopy (NanoSIMS) have shown the presence of Fe in the cytoplasm surrounding the starch granules in starchy endosperm cells of wheat grain, suggesting a possible association of Fe with proteins.[34] Our results also show that these HMW forms are present in higher concentrations in bran

fractions than in the white flour; the former are more protein rich than the latter.[26]

Very little is known about the insoluble forms of Fe and Zn in wheat, which in the case of Fe make up 76.7% in Rialto and 71.4% in Riband of the total amount under our extraction conditions. In the outer fractions at least part of this is probably bound to insoluble phytate,[35] while another proportion may be bound to tannic acid, which is present in the outer layers of the grain and forms both soluble and insoluble complexes with Fe.[19]

The results from this study demonstrate the usefulness of SEC−ICP-MS in the speciation of Fe and Zn in wheat flour. This information is likely to be important for understanding the factors that influence the bioavailability of the minerals and will allow for more targeted breeding strategies for improved mineral content and speciation in wheat grain.

■ ASSOCIATED CONTENT

Supporting Information

Chromatogram demonstrating coelution between Fe and P in the soluble phytate peak (Supplementary Figure 1), chromatogram providing evidence comparing LMW peaks from wheat and rice extracts to NA and DMA standards for Fe (Supplementary Figure 2) and Zn (Supplementary Figure 3), and distribution of Zn in the different milling fractions of Buhler mill in Riband and Rialto (Supplementary Figure 4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: Fangjie.zhao@njau.edu.cn. Phone: +86 (0) 2584396509. Fax: +86 (0)2584399551.

Funding

T.E. is supported by a Ph.D. studentship from the Biotechnology and Biological Sciences Research Council of the UK and by HarvestPlus Project 8055 "Optimising the quality, species and availability of Fe in modern bread wheat cultivars in order to prevent global Fe deficiencies". Rothamsted Research receives strategic funding from the Biotechnological and Biological Sciences Research Council (BBSRC).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Zoltan Bedo and Br Mariann Rakszegi of the Centre for Agricultural Research, Agricultural Institute, Hungarian Academy of Sciences (Martonvasar, Hungary) for providing wheat samples produced under the EU FP6 HEALTHGRAIN project and Prof. Jian Feng Ma (Okayama University, Japan) for providing nicotianamine.

■ REFERENCES

(1) Allen, L.; de Benoist, B.; Dary, O.; Hurrell, R. *Guidelines on Food Fortification with Micronutrients*; World Health Organization: Geneva, Switzerland, 2006; p 375.

(2) Cakmak, I. Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? *Plant Soil* **2008**, *302*, 1−17.

(3) Zhao, F. J.; Shewry, P. R. Recent developments in modifying crops and agronomic practice to improve human health. *Food Policy* **2011**, *36*, S94−S101.

(4) Morrissey, J.; Guerinot, M. L. Iron uptake and transport in plants: The good, the dad, and the ionome. *Chem. Rev.* **2009**, *109*, 4553−4567.

(5) Cakmak, I.; Kalayci, M.; Kaya, Y.; Torun, A. A.; Aydin, N.; Wang, Y.; Arisoy, Z.; Erdem, H.; Yazici, A.; Gokmen, O.; Ozturk, L.; Horst, W. J. Biofortification and localization of zinc in wheat grain. *J. Agric. Food Chem.* **2010**, *58*, 9092−9102.

(6) Zhang, Y. Q.; Shi, R. L.; Rezaul, K. M.; Zhang, F. S.; Zou, C. Q. Iron and zinc concentrations in grain and flour of winter wheat as affected by foliar application. *J. Agric. Food Chem.* **2010**, *58*, 12268−12274.

(7) Zhao, F. J.; Su, Y. H.; Dunham, S. J.; Rakszegi, M.; Bedo, Z.; McGrath, S. P.; Shewry, P. R. Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. *J. Cereal Sci.* **2009**, *49*, 290−295.

(8) Oury, F. X.; Leenhardt, F.; Remesy, C.; Chanliaud, E.; Duperrier, B.; Balfourier, F.; Charmet, G. Genetic variability and stability of grain magnesium, zinc and iron concentrations in bread wheat. *Eur. J. Agron.* **2006**, *25*, 177−185.

(9) Ihsanullah; Qureshi, I. M. J.; Khattak, T. N.; Akhtar, J. Determination of iron in different types of wheat flours. *Inter. J. Agric. Biol.* **2002**, *4*, 297−299.

(10) Morgounov, A.; Gomez-Becerra, H. F.; Abugalieva, A.; Dzhunusova, M.; Yessimbekova, M.; Muminjanov, H.; Zelenskiy, Y.; Ozturk, L.; Cakmak, I. Iron and zinc grain density in common wheat grown in Central Asia. *Euphytica* **2007**, *155*, 193−203.

(11) Tang, J. W.; Zou, C. Q.; He, Z. H.; Shi, R. L.; Ortiz-Monasterio, I.; Qu, Y. Y.; Zhang, Y. Mineral element distributions in milling fractions of Chinese wheats. *J. Cereal Sci.* **2008**, *48*, 821−828.

(12) Lee, S.; Jeon, U. S.; Lee, S. J.; Kim, Y. K.; Persson, D. P.; Husted, S.; Schjorring, J. K.; Kakei, Y.; Masuda, H.; Nishizawa, N. K.; An, G. Iron fortification of rice seeds through activation of the nicotianamine synthase gene. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 22014−22019.

(13) Colangelo, E. P.; Guerinot, M. L. Put the metal to the petal: Metal uptake and transport throughout plants. *Curr. Opin. Plant Biol.* **2006**, *9*, 322−330.

(14) Nishiyama, R.; Kato, M.; Nagata, S.; Yanagisawa, S.; Yoneyama, T. Identification of Zn−nicotianamine and Fe−2′-deoxymugineic acid in the phloem sap from rice plants (*Oryza sativa* L.). *Plant Cell Physiol.* **2012**, *53*, 381−390.

(15) Bashir, K.; Ishimaru, Y.; Nishizawa, N. K. Iron uptake and loading into rice grains. *Rice* **2010**, *3*, 122−130.

(16) Masuda, H.; Ishimaru, Y.; Aung, M. S.; Kobayashi, T.; Kakei, Y.; Takahashi, M.; Higuchi, K.; Nakanishi, H.; Nishizawa, N. K. Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Sci. Rep.* **2012**, *2*.

(17) Zheng, L. Q.; Cheng, Z. Q.; Ai, C. X.; Jiang, X. H.; Bei, X. S.; Zheng, Y.; Glahn, R. P.; Welch, R. M.; Miller, D. D.; Lei, X. G.; Shou, H. X., Nicotianamine, a novel enhancer of rice iron bioavailability to humans. *PLoS One* **2010**, *5*.

(18) Lee, S.; Persson, D. P.; Hansen, T. H.; Husted, S.; Schjoerring, J. K.; Kim, Y. S.; Jeon, U. S.; Kim, Y. K.; Kakei, Y.; Masuda, H.; Nishizawa, N. K.; An, G. Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. *Plant Biotechnol. J.* **2011**, *9*, 865−873.

(19) Engle-Stone, R.; Yeung, A.; Welch, R.; Glahn, R. Meat and ascorbic acid can promote Fe availability from Fe−phytate but not from Fe−tannic acid complexes. *J. Agric. Food Chem.* **2005**, *53*, 10276−10284.

(20) Simpson, C. J.; Wise, A. Binding of zinc and calcium to inositol phosphates (phytate) in vitro. *Br. J. Nutr.* **1990**, *64*, 225−232.

(21) Persson, D. P.; Hansen, T. H.; Laursen, K. H.; Schjoerring, J. K.; Husted, S. Simultaneous iron, zinc, sulfur and phosphorus speciation analysis of barley grain tissues using SEC−ICP-MS and IP−ICP-MS. *Metallomics* **2009**, *1*, 418−426.

(22) Sandberg, A. S.; Brune, M.; Carlsson, N. G.; Hallberg, L.; Skoglund, E.; Rossander-Hulthen, L. Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *Am. J. Clin. Nutr.* **1999**, *70*, 240−246.

(23) Schlemmer, U.; Frolich, W.; Prieto, R. M.; Grases, F. Phytate in foods and significance for humans: Food sources, intake, processing, bioavailability, protective role and analysis. *Mol. Nutr. Food Res.* **2009**, *53*, S330−S375.
(24) Shewry, P. R.; Piironen, V.; Lampi, A. M.; Edelmann, M.; Kariluoto, S.; Nurmi, T.; Fernandez-Orozco, R.; Ravel, C.; Charmet, G.; Andersson, A. A. M.; Aman, P.; Boros, D.; Gebruers, K.; Dornez, E.; Courtin, C. M.; Delcour, J. A.; Rakszegi, M.; Bedo, Z.; Ward, J. L. The HEALTHGRAIN wheat diversity screen: Effects of genotype and environment on phytochemicals and dietary fiber components. *J. Agric. Food Chem.* **2010**, *58*, 9291−9298.
(25) AACC. *Approved Methods of the AACC*, 10th ed.. American Association of Cereal Chemists: St. Paul, MN, 2000.
(26) Greffeuille, V.; Abecassis, J.; Bar L'Helgouach, C.; Lullien-Pellerin, V. Differences in the aleurone layer fate between hard and soft common wheats at grain milling. *Cereal Chem.* **2005**, *82*, 138−143.
(27) Borg, S.; Brinch-Pedersen, H.; Tauris, B.; Holm, P. B. Iron transport, deposition and bioavailability in the wheat and barley grain. *Plant Soil* **2009**, *325*, 15−24.
(28) Stomph, T. J.; Choi, E. Y.; Stangoulis, J. C. R. Temporal dynamics in wheat grain zinc distribution: Is sink limitation the key? *Ann. Bot.* **2011**, *107*, 927−937.
(29) von Wiren, N.; Klair, S.; Bansal, S.; Briat, J. F.; Khodr, H.; Shioiri, T.; Leigh, R. A.; Hider, R. C. Nicotianamine chelates both Fe-III and Fe-II. Implications for metal transport in plants. *Plant Physiol.* **1999**, *119*, 1107−1114.
(30) Greiner, R.; Konietzny, U. Phytase for food application. *Food Technol. Biotechnol.* **2006**, *44*, 125−140.
(31) Lukac, R. J.; Aluru, M. R.; Reddy, M. B. Quantification of ferritin from staple food crops. *J. Agric. Food Chem.* **2009**, *57*, 2155−2161.
(32) Reddy, M. B.; Lukac, R.; Maneesha, A. The quantitation of ferritin in the seeds of staple crops: ELISA method development. *FASEB J.* **2008**, *22*.
(33) Hoppler, M.; Zeder, C.; Walczyk, T. Quantification of ferritin-bound iron in plant samples by isotope tagging and species-specific isotope dilution mass spectrometry. *Anal. Chem.* **2009**, *81*, 7368−7372.
(34) Moore, K. L.; Zhao, F. J.; Gritsch, C. S.; Tosi, P.; Hawkesford, M. J.; McGrath, S. P.; Shewry, P. R.; Grovenor, C. R. M. Localisation of iron in wheat grain using high resolution secondary ion mass spectrometry. *J. Cereal Sci.* **2012**, *55*, 183−187.
(35) Bohn, L.; Josefsen, L.; Meyer, A. S.; Rasmussen, S. K. Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. *J. Agric. Food Chem.* **2007**, *55*, 7547−7552.