Effects of Nitrogen on the Distribution and Chemical Speciation of Iron and Zinc in Pearling Fractions of Wheat Grain

ARTICLE · JANUARY 2014

CITATIONS
4

READS
57

7 AUTHORS, INCLUDING:

Yanfang Xue
Shandong Academy of Agricultural Sciences, J…
6 PUBLICATIONS **37** CITATIONS
SEE PROFILE

Tristan Jake Eagling
Rothamsted Research
6 PUBLICATIONS **46** CITATIONS
SEE PROFILE

Xinsheng Wang
Nanjing University
119 PUBLICATIONS **7,449** CITATIONS
SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Effects of Nitrogen on the Distribution and Chemical Speciation of Iron and Zinc in Pearling Fractions of Wheat Grain

Yan-Fang Xue,[†,‡] Tristan Eagling,[‡] Jibin He,[‡] Chun-Qin Zou,[†] Steve P. McGrath,[‡] Peter R. Shewry,[‡,§] and Fang-Jie Zhao*[,∥]

[†]Key Laboratory of Plant-Soil Interactions, Ministry of Education, Center for Resources, Environment and Food Security, China Agricultural University, Beijing 100193, China

[‡]Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

[§]School of Agriculture, Policy and Development, University of Reading, P.O. Box 237, Reading RG6 6AR, United Kingdom

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

Supporting Information

ABSTRACT: Increasing nitrogen supply can increase Fe and Zn concentrations in wheat grain, but the underlying mechanisms remain unclear. Size-exclusion chromatography coupled with inductively coupled plasma mass spectrometry was used to determine Fe and Zn speciation in the soluble extracts of grain pearling fractions of two wheat cultivars grown at two N rates (100 and 350 kg of N ha^{-1}). Increasing N supply increased the concentrations of total Fe and Zn and the portions of Fe and Zn unextractable with a Tris−HCl buffer and decreased the concentrations of Tris−HCl-extractable (soluble) Fe and Zn. Within the soluble fraction, Fe and Zn bound to low molecular weight compounds, likely to be Fe−nicotianamine and Fe−deoxymugineic acid or Zn−nicotianamine, were decreased by 5−12% and 4−37%, respectively, by the high N treatment, whereas Fe and Zn bound to soluble high molecular weight or soluble phytate fractions were less affected. The positive effect of N on grain Fe and Zn concentrations was attributed to an increased sink in the grain, probably in the form of water-insoluble proteins.

KEYWORDS: *wheat, iron, zinc, speciation, distribution*

INTRODUCTION

Iron and zinc deficiencies are widespread nutritional disorders, affecting over two billion people in the world.[1,2] Insufficient dietary intakes of Fe and Zn and limited dietary diversity are thought to be responsible for human micronutrient deficiencies, especially in developing countries, where high proportions of cereal grains with inherently low concentrations of Fe and Zn, such as wheat and rice, are consumed as staple foods.[1,3] The bioavailability of Fe and Zn in cereal grains is also relatively low due to the presence of antinutritional compounds such as phytic acid and phenolic compounds.[1] Additionally, milling of wheat grain into white flour further results in reduced concentrations of Fe and Zn, because they are enriched in the outer parts of the grains, consisting mainly of the aleurone layer, embryo, pericarp, and testa.[4−6] Therefore, increasing Fe and Zn concentrations and/or their bioavailability in white flour is desirable for tackling the problem of micronutrient malnutrition.

Recent studies have shown that nitrogen supply is an important factor affecting the concentrations of Fe and Zn in wheat grain. For example, under both field and glasshouse conditions, increasing N supply generally enhances Fe and Zn concentrations in wheat grain.[5,7−9] It has been reported that N increases Zn uptake by roots, Zn translocation from roots to shoots and Zn remobilization from leaves to grain in wheat,[4,10] but the mechanisms are not well understood.

Nicotianamine (NA), a nonprotein amino acid, is an important nitrogenous compound involved in Fe and Zn transport via the phloem and subsequent loading into seeds.[11−15] Increasing NA concentration in the rice seed by overexpression of the rice NA synthase genes (*OsNAS*) was found to enhance not only Fe and Zn concentrations in the seed but also their bioavailability.[16−18] The increased Fe and Zn concentrations in the endosperm of the transgenic rice seed were mainly present as complexes with NA and deoxymugineic acid (DMA).[12,16] DMA is synthesized from NA and is involved not only in the acquisition of Fe from the rhizosphere but also in the phloem transport of Fe.[19,20] There is evidence that DMA is the major Fe chelate in rice phloem sap.[21] Therefore, it is possible that the beneficial effect of N results from an increased synthesis of NA and/or DMA facilitating the transport of Fe and Zn to the grain via the phloem. Another possible explanation is that increased N supply may create a larger sink in the grain for Fe and Zn, as there is evidence of positive correlations between protein and Fe and Zn concentrations among diverse wheat cultivars.[22−24]

The bioavailability of a given element is determined by both its total concentration and its distribution among various chemical species;[25] both the concentration and speciation may vary among different tissues or milling fractions of cereal grains. The effects of N on the total concentrations of Fe and Zn in

Received: January 16, 2014
Revised: May 5, 2014
Accepted: May 7, 2014
Published: May 7, 2014

different milling fractions of wheat grain have been studied, with the results showing generally positive effects in different grain fractions such as in the embryo, endosperm, and bran.[5,8,26] However, little is known about the effect of the N supply on the chemical speciation of Fe and Zn in different wheat grain fractions. This information could provide an important clue to understanding the effect of N on Fe and Zn distribution in the grain. Recently, size-exclusion chromatography coupled with inductively coupled plasma mass spectrometry (SEC−ICP-MS) has been used to identify the soluble forms of Fe complexed with soluble phytate and Zn bound to peptides in barley embryos,[27] Fe and Zn bound to NA and DMA in rice endosperm,[12,16] and Fe and Zn bound to NA/DMA, soluble phytate, and high molecular weight compounds in wheat flour.[28] If increasing N supply increases the transport of NA- or DMA-chelated forms of Fe and Zn to the grain, one may expect increased levels of these complexes in wheat grain.

The objective of the present study was therefore to investigate how the N supply affects the chemical speciation of Fe and Zn in the soluble extracts of whole wheat grain and fractions prepared by sequential pearling of the grain.

MATERIALS AND METHODS

Field Experiment. Two wheat cultivars (*Triticum aestivum* cv. Hereward and Paragon) were grown in the Wheat Genetic Improvement Network (WGIN) trial at Rothamsted, United Kingdom, in October 2008. Hereward is a winter wheat, and Paragon is a spring wheat, both being hard wheat with good bread-making properties.[29] The information about the experimental site, climate, and crop husbandry for the experiment has been previously described.[30] The soil was a clay loam with a pH of 8.1, a total Zn concentration of 88.8 mg kg^{-1}, and a DTPA-extractable Zn concentration of 1.6 mg kg^{-1}. Each cultivar was sown in three replicate plots (each 3 × 10 m) with two N application rates (100 and 350 kg of N ha^{-1}, recorded as N100 and N350, respectively). A completely randomized block design was used for the experiment. Nitrogen fertilizer, as ammonium nitrate, was applied in split top dressings in March (nominal growth stage (GS) 24), April (GS 31), and May (GS 32) 2009.[31] In addition, 145 kg of P_2O_5 ha^{-1} and 120 kg of K_2O ha^{-1} as potassium dihydrogen phosphate were applied in March 2009. All plots received 111 kg of sulfate ha^{-1} as kieserite in March 2009.

Pearling of Wheat Grain. The grain was harvested at crop maturity and air-dried to 12% moisture content before pearling. Wholemeal was produced using a ball mill (Glen Creston, Stanmore, England). Pearling was carried out as described by Tosi et al.[32] using a Streckel and Schrader (Hamburg, Germany) pearling mill. This gave fractions 1−6 corresponding to 7%, 6%, 7%, 10%, 10%, and 10% of the grain weight. Previous studies have shown that these fractions are enriched in the pericarp tissue (bran), the aleurone layer, the subaleurone layer, and three progressively more central areas of the starchy endosperm, respectively.[32] The grain remaining after pearling, representing the core endosperm and accounting for about 50% of the total grain weight, was ground in a ball mill and called fraction 7. Between 95% and 97% of the initial mass was recovered in the seven fractions after pearling.

Chemical Analysis. Whole grain (WG) and grain pearling fractions (oven-dried at 50 °C for 10 h) were digested with HNO_3/$HClO_4$ (87/13, v/v) in a heating block.[33] Total Fe, Zn, and P concentrations in digested solutions were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; PerkinElmer Optima 7500 DV, Waltham, MA). Two blanks and a certified reference material (NIST1567a, wheat flour) were included in each batch of digestion to ensure analytical quality. Repeated analysis (n = 7) of NIST 1567a gave mean values (±SD) of 14.1 ± 1.7 mg of Fe kg^{-1}, 11.7 ± 0.5 mg of Zn kg^{-1}, and 1.33 ± 0.03 g of P kg^{-1}, which are in good agreement with the certified values of 14.1 ± 0.5 mg of Fe kg^{-1}, 11.6 ± 0.4 mg of Zn kg^{-1}, and 1.34 ± 0.06 g of P kg^{-1}, respectively. The nitrogen concentration in the samples was determined with the American Society for Testing and Materials (ASTM) standard protocol E1019 using a LECO combustion analysis system based on the Dumas method.[31] Phytate P in the samples was determined according to the method of Haug and Lantzsch.[34]

Wholemeal and three selected grain pearling fractions (fractions 3, 5, and 7, corresponding to the subaleurone layer, central endosperm, and core endosperm, respectively) of both cultivars with two N treatments were extracted according to Eagling et al.[28] Approximately 70 mg (subaleurone layer), 100 mg (whole grain flour), and 140 mg (endosperm) of the selected fractions were extracted with 7 mL of 50 mM Tris−HCl buffer (pH 7.5) in sterile vials. The extracts were incubated at 37 °C for 18 h with shaking at 120 rpm. At the end of the incubation period, the extracts were centrifuged (21000g, 13 °C, 10 min) and the supernatant was filtered through a 0.2 μm filter (Minisart). Total Fe and Zn concentrations in the soluble extracts were determined using ICP-MS (Agilent 7500ce, Agilent Technologies, Palo Alto, CA). Fe and Zn speciation in the soluble extracts was analyzed using SEC−ICP-MS. A Superdex 75 10/300 GL size-exclusion column (glass, 10 × 300 mm, 13 μm cross-linked agarose/dextran, Amersham Biosciences, United States) was used to obtain an optimum separation range between 0.7 and 70 kDa. Tris−HCl buffer (50 mM, pH 7.5) was used as the mobile phase with a flow rate of 0.47 mL min^{-1} at a controlled temperature of 18 °C. The volume of the sample injected was 100 μL. SEC−ICP-MS analyses were performed on a high-performance liquid chromatograph (Agilent 1100 series, Agilent Technologies, Waltham, MA) coupled to an ICP mass spectrometer with the following conditions: rf power, 1.5 kW; carrier gas flow rate, 0.85 L min^{-1}; nebulizer pump speed, 0.2 L min^{-1}; collision/reaction gas, H_2 (3 mL min^{-1}) and He (2 mL min^{-1}). A mixture of He and H_2 was used as the collision/reaction gas to minimize polyatomic interferences on the isotope signals.

Molecular size calibration was performed using a UV detector (Agilent Technologies, Palo Alto, CA) at 214 nm and the standards apoprotein (6.6 kDa), cytochrome c (12.4 kDa), vitamin B12 (1.35 kDa), triglycine (0.19 kDa), and glycine (0.075 kDa) (all from Sigma-Aldrich). A log-linear regression curve was constructed from molecular size versus retention time. A 100 μL volume 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. The column was rinsed twice after bran samples due to the higher levels of Fe and Zn. To remove any residual metals retained by the column, it was cleaned rigorously between batches with 100 μL of pepsin (1 mg mL^{-1}), phytase (2 mg mL^{-1}), NaCl (0.5 M), and acetic acid (10%) (all from Sigma-Aldrich) at a flow rate of 0.2 mL min^{-1} at 36 °C. EDTA (10 mM)/Tris−HCl (50 mM) buffer (pH 7.5) was injected through the column for 1 h each day.

As Tris−HCl provided a pH buffer only, Tris−HCl-extractable Fe and Zn can be considered to represent the water-soluble fraction. Insoluble Fe and Zn concentrations were estimated by the difference between the total and soluble concentrations. The concentrations of Fe and Zn bound to the individual peaks in the Tris--HCl-extractable fractions were estimated by multiplying the relative peak areas by the soluble concentrations of these elements in the extracts.

Statistical Analysis. The significance of the effects of N application rates, cultivars, and pearling fractions and their interactions on Fe and Zn concentrations was evaluated by analysis of variance (ANOVA). Significant differences between treatment means were determined using Tukey's honest significant difference (HSD) test at the 5% level (P < 0.05) using GenStat, 14th ed. (VSN International, Hemel Hempstead, U.K.).

RESULTS

Grain Yields. Increasing the N dose from 100 to 350 kg ha^{-1} increased the grain yield by 33% and 27% in the cultivars Hereward and Paragon, respectively (P < 0.001; Supplementary Figure 1, Supporting Information). There was no significant

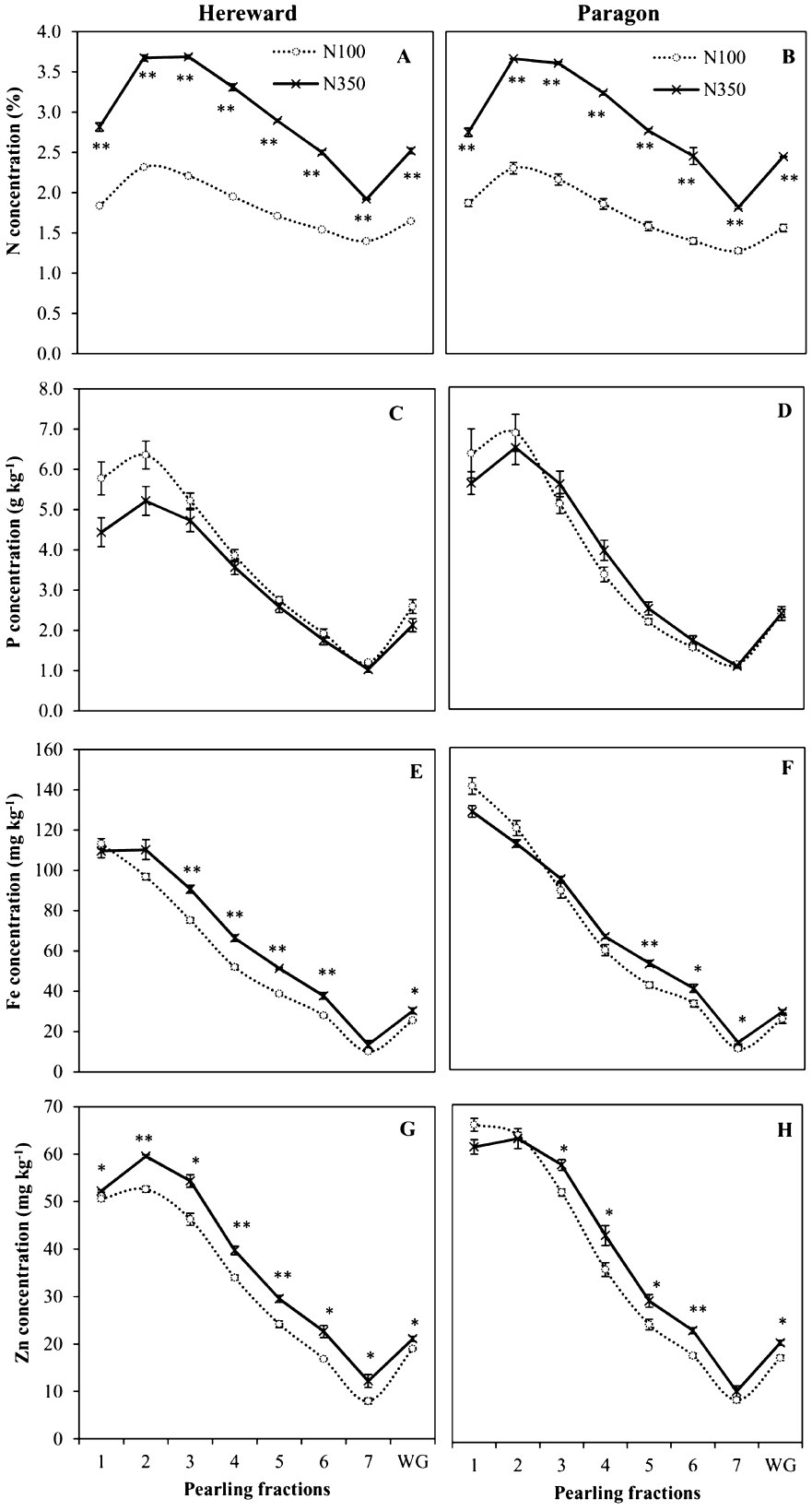

Figure 1. Effects of N treatments (low N, 100 kg of N ha^{-1}; high N, 350 kg of N ha^{-1}) on the N, P, Fe, and Zn concentrations in the pearling fractions and whole grain of the wheat cultivars Hereward and Paragon. Data are means ± SE of three independent replications. One and two asterisks indicate significant differences between the two N treatments for the same pearling fraction at $P < 0.05$ and $P < 0.01$, respectively. Key: fraction 1, bran; fraction 2, aleurone layer; fraction 3, subaleurone layer; fractions 4–6, three progressively more central areas of the starchy endosperm; fraction 7, core endosperm; WG, whole grain.

dx.doi.org/10.1021/jf500273x | J. Agric. Food Chem. 2014, 62, 4738–4746

difference between the two cultivars in grain yield at either level of N treatment.

Distributions of Fe and Zn in Different Pearling Fractions. Figure 1 shows the effects of the N supply on the total concentrations of N, P, Fe, and Zn in WG and pearling fractions of the two wheat cultivars. Two-way ANOVA revealed significant effects of the N supply on the N, P, Fe, and Zn concentrations and significant effects of the cultivar on the N and Zn concentrations of WG (Supplementary Table 1, Supporting Information). The interactions between N supply and cultivar were also significant for the P and phytate P concentrations of WG. Three-way ANOVA showed significant effects of the interactions of N supply, cultivar, and pearling fraction on the Fe and Zn concentrations with the exception of the nonsignificant effect of the interaction between N and cultivar on the Zn concentration (Supplementary Table 1). The high N treatment (350 kg of N ha^{-1}) significantly increased the N concentrations in all pearling fractions and WG of both cultivars, the effect being more pronounced in the outer layers (pearling fractions 2 and 3) than in the core endosperm (Figure 1A,B). There was no significant difference in the P concentration of WG or pearling fractions between the two N treatments (Figure 1C,D). The high N treatment significantly decreased the phytate P concentration in the core endosperm (fraction 7) in Hereward, but had no significant effects in Paragon (Figure 2). The high N treatment significantly increased the total Zn concentration in WG of both cultivars and the total Fe concentration in Hereward (Figure 1E–H). There were also significant and positive effects of the N supply on the Fe and Zn concentrations in most of the pearling fractions of Hereward (Figure 1E–G). In Paragon N decreased the Fe and Zn concentrations in the bran and/or aleurone layer but increased their concentrations in the inner fractions (Figure 1F,H). In general, the effect of N was larger and more consistent in Hereward than in Paragon.

There was a progressive decrease in the concentrations of N, P, Fe, and Zn from the outer to the inner part of the grain (Figure 1). For example, the highest concentrations of N, P, Fe, and Zn in the outermost bran or aleurone layer (fraction 1 or 2) were 0.7-, 4.3-, 10.4-, and 5.6-fold higher than those in the inner core endosperm (fraction 7) in the low N treatment, while the corresponding values were 0.9-, 4.1-, 7.2-, and 3.9-fold higher in the high N treatment, respectively, in Hereward. Similar results were also found in Paragon.

Supplementary Table 2 (Supporting Information) presents the effects of the two N treatments on the distributions of Fe and Zn in different pearling fractions. In Hereward, the outermost three layers (bran, aleurone layer, and subaleurone layer) constituted about 20% of the grain mass, but accounted for 54% and 47%, respectively, of the total Fe and Zn contents in the low N treatment. In the high N treatment, these outer layers accounted for slightly less of the grain Fe (48%) and Zn (41%) than in the low N treatment. By contrast, the remaining core endosperm, constituting about 50% of the grain mass, contained only 14–16% and 19–24%, respectively, of the total Fe and Zn contents (Supplementary Tables 2 and 3, Supporting Information). Similar results were also found in Paragon.

Fe and Zn Speciation in Grain Pearling Fractions. Figures 3 and 4 show SEC–ICP-MS analysis of the Fe and Zn species in the Tris–HCl extracts of wholemeal (Figures 3A,B and 4A,B) and pearling fractions 3 (Figures 3C,D and 4C,D), 5 (Figures 3E,F and 4E,F), and 7 (Figures 3G,H and 4G,H). Due to the long run time for each sample, the samples of the two cultivars were analyzed in two separate batches. For this reason, the absolute peak heights or peak areas were not comparable between the two cultivars.

For Fe speciation, the extracts of all samples contained a low molecular weight (LMW; 1.5 ± 0.1 kDa) complex which eluted at ~36 min. Our previous study[28] showed that this LMW peak probably contains a mixture of Fe–DMA and Fe–NA. Similarly, the LMW peak has been shown to contain a mixture of Fe–DMA and Fe–NA in rice extracts.[12,16] The wheat extracts also contained one or two high molecular weight (HMW) forms of Fe at retention times of ~16 and 18 min (corresponding to >70 ± 0.4 and 20 ± 0.4 kDa, respectively). The identities of these HMW peaks are unknown, but could be soluble proteins.[28] Additionally, the extracts from some fractions, such as the subaleurone layer and the central endosperm of Hereward and the subaleurone layer of Paragon, showed the presence of a medium molecular weight (MMW) complex at a retention time of ~29 min (corresponding to 5.0 ± 0.1 kDa) (Figure 3C–E). The study by Eagling et al.[28] showed that this peak coeluted with P, suggesting that it is a soluble iron phytate. Persson et al.[27] identified a similar MMW peak in the extracts of barley embryo as a soluble iron phytate oligomer.

For Zn speciation, the extracts of all samples contained an HMW complex (>70 kDa) at a retention time of ~16 min and an LMW complex at a retention time of ~35 min (1.9 ± 0.2 kDa) (Figure 4); the latter has been identified as a mixture of Zn–NA and Zn–DMA complexes in rice endosperm.[12] A

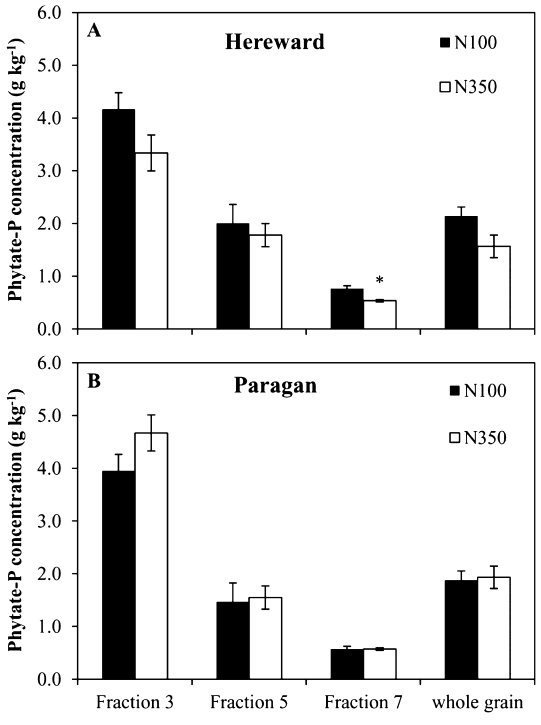

Figure 2. Effects of N treatments (low N, 100 kg of N ha^{-1}; high N, 350 kg of N ha^{-1}) on the phytate P concentrations in the selected pearling fractions and wholemeal flour of the wheat cultivars Hereward and Paragon. Data are means ± SE of three independent replications. An asterisk indicates a significant difference between the two N treatments for the same pearling fraction at $P < 0.05$. Key: fraction 3, subaleurone layer; fraction 5, central endosperm; fraction 7, core endosperm.

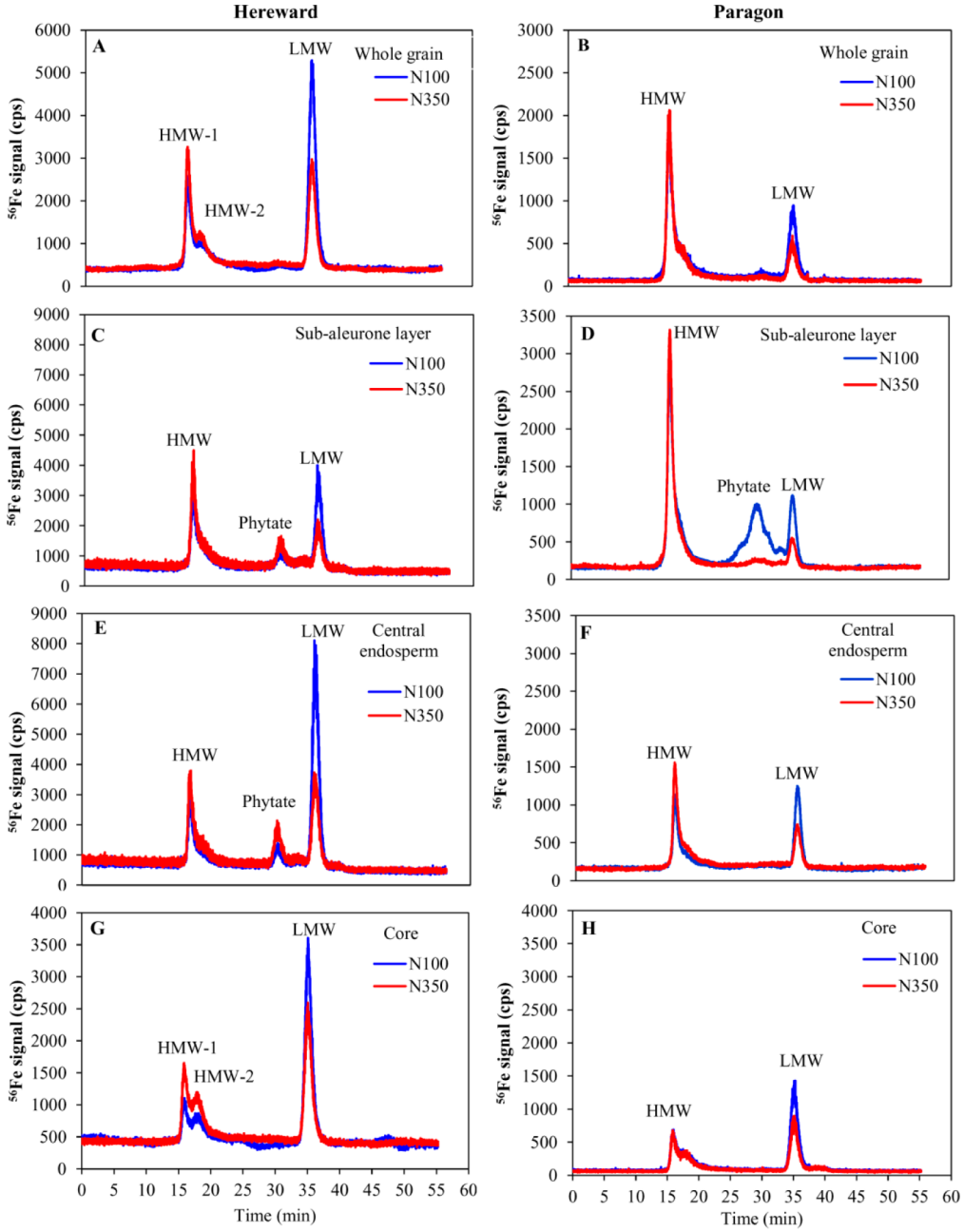

Figure 3. Chromatograms of Fe speciation in the 50 mM Tris−HCl buffer (pH 7.5) extracts of wheat fractions (wholemeal flour, subaleurone layer, central endosperm, and core endosperm) as determined by SEC−ICP-MS.

comparison with the Zn−NA and Zn−DMA standards suggests that the LMW peak in wheat extract is more likely to be only Zn−NA.[28]

The nitrogen supply had a distinct effect on Fe species in the soluble extracts. The high N treatment significantly enhanced the peak area of HMW Fe, but decreased that of LMW Fe in both cultivars, as well as that of the soluble iron phytate (MMW) in the subaluerone layer of Paragon (Figure 3). Generally, the effect of N on Zn species was similar to that for Fe species, although less pronounced (Figure 4).

Tables 1 and 2 present the concentrations and percentage distributions of Fe and Zn species, respectively, in the selected samples of both cultivars. The nitrogen supply had opposite effects on the soluble (extracted in 50 mM Tris−HCl buffer, pH 7.5) and insoluble fractions of Fe and Zn. In the wholemeal and all pearling fractions except the core endosperm of Hereward, the concentrations of soluble Fe were significantly lower in the high N than in the low N treatment. Similar but less pronounced results were also found for the soluble Zn concentration. By contrast, the insoluble fractions of Fe and Zn were significantly increased by the high N treatment. In the wholemeal of Hereward, soluble Fe represented 33.5% and 21.6% of the total Fe concentrations in the low and high N treatments, respectively, with the remainder being the insoluble fractions. For Zn, the soluble fraction accounted for 46.2% and 35.3%, respectively, in the low and high N treatments. There was a general trend of increasing percentage of soluble Fe and Zn from the subaleurone/central endosperm layer to the inner core endosperm. For Paragon, the effects of N on insoluble and soluble Fe and Zn concentrations were similar to those

dx.doi.org/10.1021/jf500273x | J. Agric. Food Chem. 2014, 62, 4738−4746

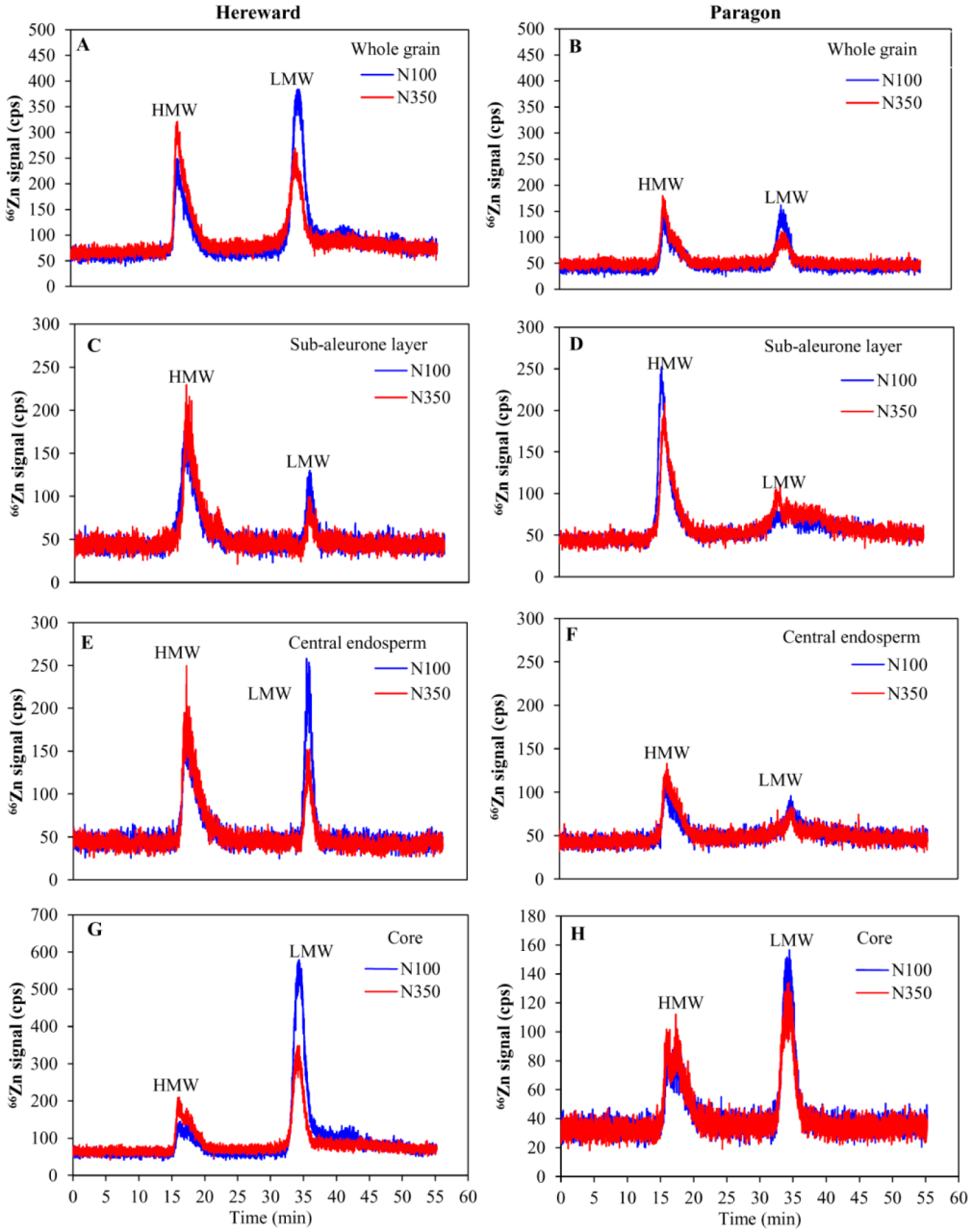

Figure 4. Chromatograms of Zn speciation in the 50 mM Tris−HCl buffer (pH 7.5) extracts of wheat fractions (wholemeal flour, subaleurone layer, central endosperm, and core endosperm) as determined by SEC−ICP-MS.

observed in Hereward with the exception of the wholemeal, where the soluble Zn concentration was significantly increased by the high N treatment. In general, the effects of N were more significant and consistent in Hereward than in Paragon.

Among the soluble species, the concentrations of LMW Fe (likely Fe−NA and Fe−DMA complexes) and LMW Zn (likely Zn−NA) were lower in the high N than in the low N treatment, the differences being significant in most of the pearling fractions and the wholemeals of both cultivars (Tables 1 and 2). Among all pearling fractions of the two cultivars, soluble LMW Fe accounted for 1.5−24.8% of the total Fe concentration; this percentage was the highest in the core endosperm and was significantly decreased by the high N treatment in most pearling fractions. Soluble LMW Zn accounted for 4.8−58.4% of the total Zn concentration, which was also significantly decreased by the high N treatment in most pearling fractions. The effects of N on other soluble species of Fe or Zn (i.e., those associated with HMW and soluble phytate) were not significant.

DISCUSSION

The present study has shown that increasing the N application rate from 100 to 350 kg ha^{-1} generally increased the concentrations of Fe and Zn in wheat grain, although there were some subtle differences between the two wheat cultivars and between the different pearling fractions (Figure 1). The positive effect of N is generally consistent with other studies,[5,8] although the extent of increase was relatively small in the present study possibly due to a dilution effect of increased yield and limited availability of Zn and Fe in the soil with a high pH. Similar to previous studies,[26,35] both Fe and Zn concentrations were found to decrease from the outer layer of the grain to the

dx.doi.org/10.1021/jf500273x | J. Agric. Food Chem. 2014, 62, 4738−4746

Table 1. Insoluble Fe Concentration, Soluble Fe Concentration, Soluble Fe Chemical Speciation Concentrations, and Fe Species Content (%) in Different Wheat Grain Pearling Fractions of the Two Cultivars As Affected by N Treatments[a]

					soluble Fe chemical speciation concn (mg kg^{-1})				Fe species content (%)				
cultivar	fraction	N level (kg ha^{-1})	insoluble Fe concn (mg kg^{-1})	soluble Fe concn (mg kg^{-1})	HMW-1	HMW-2	iron phytate	LMW	insoluble Fe	HMW-1	HMW-2	iron phytate	LMW
Hereward	whole grain	100	16.9 e	8.5 bc	2.0 bc	1.0 a	–	5.5 a	66.5 c	7.8 ab	4.0 a	–	21.7 a
		350	24.3 d	6.7 c	2.9 b	0.9 a	–	2.9 b	78.4 b	9.1 a	3.1 a	–	9.4 c
	subaleurone layer	100	60.2 b	13.7 a	6.7 a	–	1.2 ab	5.8 a	81.5 ab	9.1 a	–	1.6 a	7.9 cd
		350	81.7 a	10.8 b	7.1 a	–	1.4 a	2.3 bc	88.3 a	7.6 ab	–	1.5 a	2.5 e
	central endosperm	100	29.6 d	8.4 bc	2.2 bc	–	0.6 b	5.6 a	77.9 b	5.9 ab	–	1.5 a	14.7 b
		350	45.2 c	6.3 c	2.9 b	–	1.1 ab	2.3 bc	87.7 a	5.7 ab	–	2.2 a	4.4 de
	core endosperm	100	6.8 f	3.3 d	0.4 c	0.4 a	–	2.5 bc	67.1 c	4.2 b	3.8 a	–	24.8 a
		350	11.5 ef	3.5 d	0.9 bc	0.7 a	–	1.8 c	76.8 b	5.8 ab	4.9 a	–	12.5 bc
Paragon	whole grain	100	19.6 de	6.7 c	4.8 b	–	–	1.9 bc	74.0 de	18.6 a	–	–	7.4 bc
		350	23.6 d	5.0 dc	4.1 b	–	–	0.9 c	82.4 abcd	14.3 ab	–	–	3.3 dc
	subaleurone layer	100	64.0 b	27.4 a	15.0 a	–	8.7	3.7 a	70.0 e	16.4 a	–	9.5	4.0 dc
		350	80.0 a	14.6 b	13.2 a	–	–	1.4 c	84.6 abc	13.9 abc	–	–	1.5 d
	central endosperm	100	36.9 c	6.5 c	3.2 bc	–	–	3.3 ab	85.0 ab	7.4 bcd	–	–	7.6 bc
		350	48.6 c	5.1 dc	3.8 b	–	–	1.2 c	90.6 a	7.1 bcd	–	–	2.3 d
	core endosperm	100	8.3 e	2.9 d	0.7 d	0.4 a	–	1.8 c	74.4 cde	6.3 dc	3.6 a	–	15.6 a
		350	10.8 e	2.8 d	0.9 dc	0.5 a	–	1.4 c	79.3 bcde	6.3 d	4.1 a	–	10.3 b

[a]A dash means not detected. Means in a column followed by different letters are significantly different among different pearling fractions and N treatments ($P < 0.05$).

Table 2. Insoluble Zn Concentration, Soluble Zn Concentration, Soluble Zn Chemical Speciation Concentrations, and Zn Species Content (%) in Different Wheat Grain Pearling Fractions of the Two Cultivars As Affected by N Treatments[a]

					soluble Zn chemical speciation concn (mg kg^{-1})		Zn species content (%)		
cultivar	fraction	N level (kg ha^{-1})	insoluble Zn concn (mg kg^{-1})	soluble Zn concn (mg kg^{-1})	HMW Zn	LMW Zn	insoluble Zn	HMW Zn	LMW Zn
Hereward	whole grain	100	10.2 de	8.8 b	3.3 bcd	5.5 a	53.8 ab	17.4 ab	28.8 b
		350	14.0 de	7.6 bc	4.3 bc	3.3 dc	64.7 a	20.0 ab	15.3 bcd
	subaleurone layer	100	30.8 b	14.2 a	10.3 a	3.8 bc	68.5 a	23.0 a	8.5 dc
		350	40.6 a	12.9 a	10.4 a	2.6 de	75.8 a	19.4 ab	4.8 d
	central endosperm	100	16.1 d	7.6 bc	4.2 bc	3.5 bcd	67.9 a	17.6 ab	14.5 bcd
		350	22.3 c	7.1 bcd	5.4 b	1.7 e	75.9 a	18.2 ab	5.9 d
	core	100	2.4 f	5.1 dc	0.7 d	4.3 b	31.6 b	10.0 b	58.4 a
		350	8.2 ef	4.9 d	2.1 dc	2.8 d	62.5 a	15.8 ab	21.7 bc
Paragon	whole grain	100	7.2 b	10.9 d	6.5 bcd	4.4 ab	39.7 bc	35.9 bc	24.4 abc
		350	5.4 b	15.6 c	8.8 bc	6.8 a	25.5 dc	42.4 bc	32.1 ab
	subaleurone layer	100	6.8 b	45.5 a	39.1 a	6.3 ab	12.9 d	75.0 a	12.1 c
		350	19.1 a	39.5 b	33.4 a	6.1 ab	32.6 bc	56.9 ab	10.4 c
	central endosperm	100	14.9 a	9.8 d	6.5 bcd	3.2 ab	60.5 a	26.5 c	13.0 bc
		350	19.0 a	11.6 d	9.9 b	1.7 b	62.1 a	32.5 c	5.5 c
	core	100	3.8 b	5.5 e	1.9 d	3.6 ab	41.2 bc	19.8 c	39.0 a
		350	5.5 b	5.9 e	3.2 dc	2.7 ab	47.8 ab	27.8 c	24.4 abc

[a]Means in a column followed by different letters are significantly different among different pearling fractions and N treatments ($P < 0.05$).

central endosperm, a pattern that reflects the localization of the phytate granules in the aleurone layer and their role in sequestering Fe and Zn.[36] It should be noted that, due to the presence of a crease in the wheat grain, the pearling method used may not completely remove the bran and the aleurone layer from the central endosperm fraction, thus explaining the presence of some soluble phytate P in this fraction (Figure 3E). However, the positive effect of the N supply on the Fe and Zn concentrations was not related to phytate, because its concentration was unaffected or even slightly decreased by

dx.doi.org/10.1021/jf500273x | J. Agric. Food Chem. 2014, 62, 4738−4746

the high N treatment (Figure 2). Small concentrations of soluble iron phytate complexes were found in some of the pearling fractions, but they were not significantly affected by N treatments.

It has been shown that Fe and Zn are likely to be complexed with NA and/or DMA during their transport in the phloem, an important process for the translocation of these minerals from leaves to grains.[13,15,19,20] A high N supply may enhance NA and/or DMA biosynthesis and therefore its availability for the phloem transport of Fe and Zn.[4,10,37] However, contrary to this hypothesis, we found that the concentrations of LMW Fe (likely Fe−NA/Fe−DMA) and LMW Zn (likely Zn−NA) in the different pearling fractions and in the whole grain were significantly decreased by the high N treatment (Figures 3 and 4, Tables 1 and 2). Mainly due to this negative effect, N also significantly decreased the concentrations and percentages of soluble Fe and Zn. These results suggest that enhanced NA and/or DMA availability is not the reason for the positive effect of N on Fe and Zn accumulation in wheat grain. Alternatively, it is possible that elevated NA and/or DMA are responsible for the Fe and Zn transport into the grain but that Fe and Zn are then sequestered in other forms.

Interestingly, insoluble forms of Fe and Zn were significantly increased by the high N treatment, especially in Hereward (Tables 1 and 2). This effect was much larger than the negative effect of N on the soluble Fe and Zn, resulting in an overall increase in the total concentrations (Figure 1). This N effect may be explained by an increased content of protein (Figure 1A,B), which may bind Fe and Zn in the grain. A recent study using high-resolution secondary ion mass spectrometry (NanoSIMS) showed that Fe in the wheat endosperm is associated with the protein matrix surrounding the starch granules and the protein bodies.[38] The identities of the proteins that bind Fe and Zn in the endosperm are not known, but the lack of Fe signals inside the protein bodies in the NanoSIMS images suggests that they are not storage proteins. Ferritins are Fe storage proteins, but they are soluble[39] and represent only a small proportion of the total Fe in cereals such as wheat.[40,41] Other compounds such as tannins and certain types of insoluble fiber may also interact with Fe and Zn,[42] but may not explain the effect of N. Taken together, the results of the present study suggest that a high N supply provides a larger protein sink for the storage of Fe and Zn in the wheat grain. This larger sink may also sequester Fe and Zn at the expense of the soluble forms, including Fe and Zn complexes with NA/DMA, resulting in lower concentrations of the soluble Fe and Zn.

The effect of N in influencing the distribution of Fe and Zn between the soluble species and the insoluble fraction on their bioavailability to humans remains to be investigated. Previous studies have shown that the Fe−NA and Zn−NA complexes in rice are bioavailable using an in vitro digestion/Caco-2 cell culture model and mouse feeding experiments.[12,16,18] Therefore, decreased NA−Fe and NA−Zn may have negative effects on Fe and Zn bioavailability. However, Fe and Zn bound to insoluble proteins may become bioavailable during gastrointestinal digestion processes. Additionally, the positive effect of N was not associated with an increased phytate level, which may be favorable to Fe and Zn bioavailability.

■ ASSOCIATED CONTENT

Supporting Information

Grain yield of two wheat cultivars as affected by N treatments (Supplementary Figure 1), two-way and three-way ANOVA) of the effects of the N supply, cultivars, and pearling fractions on the concentrations of N, P, Fe, and Zn in the whole grain and pearling fractions of wheat (Supplementary Table 1), effects of N treatments on the distributions of Fe and Zn in different pearling fractions of the two wheat cultivars (Supplementary Table 2), and relative weight percentage of each grain tissue after pearling (Supplementary Table 3). 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)25 84396509. Fax: +86 (0)25 84399551.

Funding

This research was funded by HarvestPlus Project 8055, "Optimizing the quality, species and availability of Fe in modern bread wheat cultivars in order to prevent global Fe deficiencies". Y.-F.X. acknowledges funding by the China Scholarship Council. Grain samples were from field trials at Rothamsted Research supported by the Defra-funded Wheat Genetic Improvement Network (WGIN) project. 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 Adrian Crosland for assistance in sample analysis and Rodger White for statistical advice.

■ REFERENCES

(1) Welch, R. M.; Graham, R. D. Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* **2004**, *55*, 353−364.

(2) White, P. J.; Broadley, M. R. Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* **2009**, *182*, 49−84.

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

(4) Cakmak, I.; Pfeiffer, W. H.; McClafferty, B. Biofortification of durum wheat with zinc and iron. *Cereal Chem.* **2010**, *87*, 10−20.

(5) Kutman, U. B.; Yildiz, B.; Cakmak, I. Improved nitrogen status enhances zinc and iron concentrations both in the whole grain and the endosperm fraction of wheat. *J. Cereal Sci.* **2011**, *53*, 118−125.

(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) Gooding, M. J.; Fan, M. S.; McGrath, S. P.; Shewry, P. R.; Zhao, F. J. Contrasting effects of dwarfing alleles and nitrogen availability on mineral concentrations in wheat grain. *Plant Soil* **2012**, *360*, 93−107.

(8) Shi, R. L.; Zhang, Y. Q.; Chen, X. P.; Sun, Q. P.; Zhang, F. S.; Römheld, V.; Zou, C. Q. Influence of long-term nitrogen fertilization on micronutrient density in grain of winter wheat (*Triticum aestivum* L.). *J. Cereal Sci.* **2010**, *51*, 165−170.

(9) Xue, Y. F.; Yue, S. C.; Zhang, Y. Q.; Cui, Z. L.; Chen, X. P.; Yang, F. C.; Cakmak, I.; McGrath, S. P.; Zhang, F. S.; Zou, C. Q. Grain and shoot zinc accumulation in winter wheat affected by nitrogen management. *Plant Soil* **2012**, *361*, 153−163.

(10) Erenoglu, E. B.; Kutman, U. B.; Ceylan, Y.; Yildiz, B.; Cakmak, I. Improved nitrogen nutrition enhances root uptake, root-to-shoot translocation and remobilization of zinc (^{65}Zn) in wheat. *New Phytol.* **2011**, *189*, 438−448.

(11) 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.
(12) 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.
(13) Curie, C.; Cassin, G.; Couch, D.; Divol, F.; Higuchi, K.; Le Jean, M.; Misson, J.; Schikora, A.; Czernic, P.; Mari, S. Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann. Bot.* **2009**, *103*, 1−11.
(14) Tauris, B.; Borg, S.; Gregersen, P. L.; Holm, P. B. A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. *J. Exp. Bot.* **2009**, *60*, 1333−1347.
(15) Trampczynska, A.; Kuepper, H.; Meyer-Klaucke, W.; Schmidt, H.; Clemens, S. Nicotianamine forms complexes with Zn(II) in vivo. *Metallomics* **2010**, *2*, 57−66.
(16) 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.
(17) Johnson, A. A. T.; Kyriacou, B.; Callahan, D. L.; Carruthers, L.; Stangoulis, J.; Lombi, E.; Tester, M. Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS One* **2011**, *6*.
(18) 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*.
(19) Bashir, K.; Ishimaru, Y.; Nishizawa, N. K. Iron uptake and loading into rice grains. *Rice* **2010**, *3*, 122−130.
(20) Inoue, H.; Kobayashi, T.; Nozoye, T.; Takahashi, M.; Kakei, Y.; Suzuki, K.; Nakazono, M.; Nakanishi, H.; Mori, S.; Nishizawa, N. K. Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *J. Biol. Chem.* **2009**, *284*, 3470−3479.
(21) 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.
(22) 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.
(23) Peleg, Z.; Saranga, Y.; Yazici, A.; Fahima, T.; Ozturk, L.; Cakmak, I. Grain zinc, iron and protein concentrations and zinc-efficiency in wild emmer wheat under contrasting irrigation regimes. *Plant Soil* **2008**, *306*, 57−67.
(24) 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.
(25) Theil, E. C. Iron, ferritin, and nutrition. *Annu. Rev. Nutr.* **2004**, *24*, 327−343.
(26) 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.
(27) 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.
(28) Eagling, T.; Neal, A. L.; McGrath, S. P.; Fairweather-Tait, S.; Shewry, P. R.; Zhao, F. J. Distribution and speciation of iron and zinc in grain of two wheat genotypes. *J. Agric. Food Chem.* **2014**, *62*, 708−716.
(29) National Association of British and Irish Millers Wheat Guide. www.nabim.org.uk (accessed on May 2, 2014).
(30) Barraclough, P. B.; Howarth, J. R.; Jones, J.; Lopez-Bellido, R.; Parmar, S.; Shepherd, C. E.; Hawkesford, M. J. Nitrogen efficiency of wheat: genotypic and environmental variation and prospects for improvement. *Eur. J. Agron.* **2010**, *33*, 1−11.
(31) He, J.; Penson, S.; Powers, S. J.; Hawes, C.; Shewry, P. R.; Tosi, P. Spatial patterns of gluten protein and polymer distribution in wheat grain. *J. Agric. Food Chem.* **2013**, *61*, 6207−6215.
(32) Tosi, P.; Gritsch, C. S.; He, J.; Shewry, P. R. Distribution of gluten proteins in bread wheat (*Triticum aestivum*) grain. *Ann. Bot.* **2011**, *108*, 23−35.
(33) Zhao, F. J.; McGrath, S. P.; Crosland, A. R. Comparison of three wet digestion methods for the determination of plant sulphur by inductively coupled plasma atomic emission spectroscopy (ICP-AES). *Commun. Soil Sci. Plant Anal.* **1994**, *25*, 407−418.
(34) Haug, W.; Lantzsch, H. J. Sensitive method for the rapid determination of phytate in cereals and cereal products. *J. Sci. Food Agric.* **1983**, *34*, 1423−1426.
(35) Liu, Z. H.; Wang, H. Y.; Wang, X. E.; Zhang, G. P.; Chen, P. D.; Liu, D. J. Phytase activity, phytate, iron, and zinc contents in wheat pearling fractions and their variation across production locations. *J. Cereal Sci.* **2007**, *45*, 319−326.
(36) Brinch-Pedersen, H.; Borg, S.; Tauris, B.; Holm, P. B. Molecular genetic approaches to increasing mineral availability and vitamin content of cereals. *J. Cereal Sci.* **2007**, *46*, 308−326.
(37) Aciksoz, S. B.; Ozturk, L.; Gokmen, O. O.; Roemheld, V.; Cakmak, I. Effect of nitrogen on root release of phytosiderophores and root uptake of Fe(III)-phytosiderophore in Fe-deficient wheat plants. *Physiol. Plant.* **2011**, *142*, 287−296.
(38) 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.
(39) Theil, E. C. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu. Rev. Biochem.* **1987**, *56*, 289−315.
(40) Lukac, R. J.; Aluru, M. R.; Reddy, M. B. Quantification of ferritin from staple food crops. *J. Agric. Food Chem.* **2009**, *57*, 2155−2161.
(41) May, L.; Morris, E. R.; Ellis, R. Chemical identity of iron in wheat by Moessbauer spectroscopy. *J. Agric. Food Chem.* **1980**, *28*, 1004−1006.
(42) Lestienne, I.; Caporiccio, B.; Bresancon, P.; Rochette, I.; Trechet, S. Relative contribution of phytates, fibers, and tannins to low iron and zinc in vitro solubility in pearl millet (*Pennisetum glaucum*) flour and grain fractions. *J. Agric. Food Chem.* **2005**, *53*, 8342−8348.